Doctoral Dissertation

Distribution of avenolide-type hormones among actinomycetes and their roles as communication signals

エバノライド型放線菌ホルモンの分布及びシグナル伝達物質としての役割

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April 2018

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CHAPTER 1

General introduction

1.1 The genera of actinomycetes

The genera of actinomycetes include a large number of aerobic, Gram-positive bacteria that have high G+C content in their genomes.[1] Actinomycetes are widely present in the ecosystems, especially in soil, where they have very important roles in biodegradation, since they produce many extracellular enzymes to decompose complex mixtures of polymers in dead plants and in, fungal and animal material.[2] Based on an array of taxonomic tools, especially partial sequence analysis of 16S ribosomal RNA, actinomycetes have been divided into a large number of genera, among which the genus *Streptomyces* is the most abundant and well characterized. Other actinomycetes not belonging to the *Streptomyces* genus are considered as rare actinomycetes; these include strains from the genera *Nocardiopsis*, *Micromonospora*, *Tsukamurella* and *Nocardia*.[1]

Two important characteristics of actinomycetes are their unusually complex life cycle and their ability to produce a wide range of secondary metabolites.

1.1.1 The life cycle of actinomycetes

Most actinomycetes develop mycelia at one or more stages in their life cycles. Many of them, especially those that belong to the genus *Streptomyces*, later develop spores to aid in the spread and existence of the species.[1]

On solid media, the life cycle of an individual actinomycete begins with the germination spores, which leads to the production of hyphae. The hyphae then grow and branch to form vegetative mycelia. In the exponential growth phase, the vegetative mycelia branch and their tips grow to form a complex mycelia network.[3]

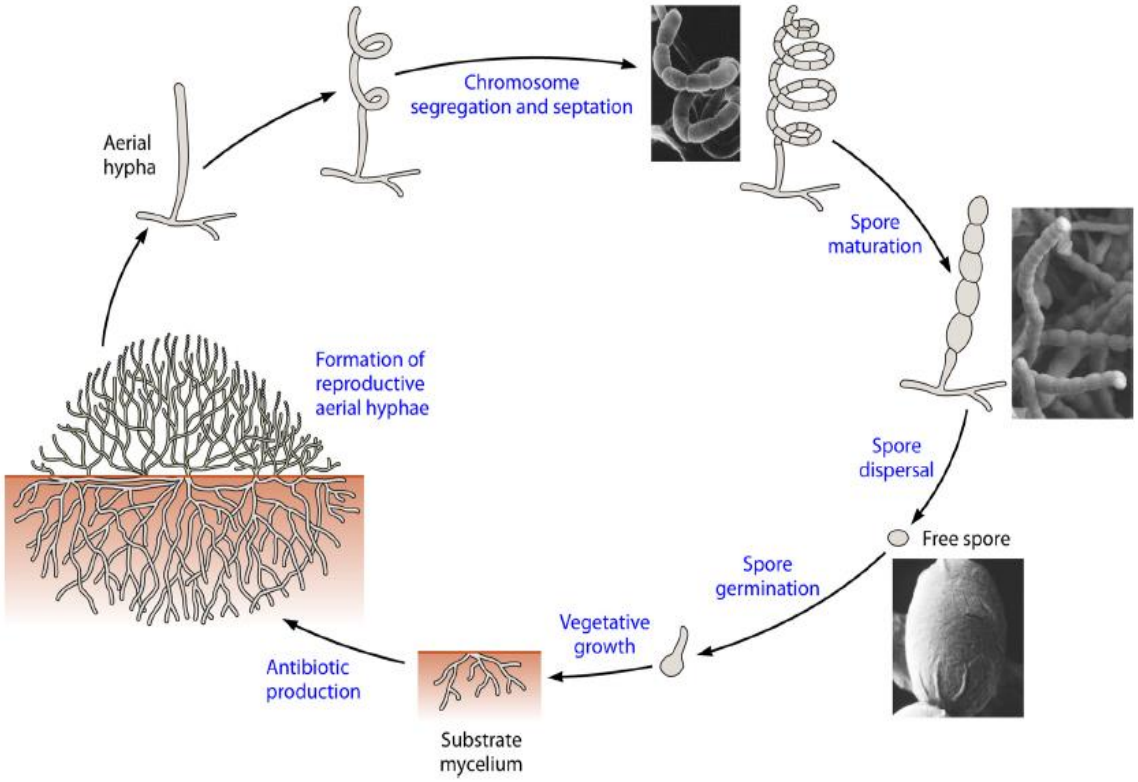

Figure 1.1 The life cycle of sporulating actinomycetes. [4]

According to a new study on the life cycle of actinomycetes, there are two kinds of mycelia, the compartmentalized mycelia (MI) and multinucleated mycelia (MII).[5] As actinomycetes colonies develop further, an additional kind of mycelium is produced. This is the aerial mycelium, which is actually an MII mycelium with a hydrophobic coating.[5] The hydrophobic hyphae then break through the moist surface of the media and project into

the air. This is the beginning of the reproductive phase, triggered by nutrient depletion and other types of physiological stress. The substrates necessary for aerial hyphae production are derived from the vegetative mycelia, which undergo programming cell death to release material such as nucleic acids, proteins, and storage compounds. Therefore, at this stage of development, actinomycetes produce many bioactive compounds such as antibiotics to protect their nutrient sources from the scavenging activity of other microorganisms. Eventually, sporulation-programmed hyphae are formed, producing chains of mono-nucleoid spores, which are released after a poorly understood maturation process.[3]

1.1.2 Actinomycetes as a source of useful secondary metabolites

Different from primary metabolites, secondary metabolites are not directly involved in the growth of organisms but can impair their long-term survivability. Secondary metabolites have diverse chemical structures and different secondary metabolites are produced by specific groups of organisms.[6] Actinomycetes are well known as producers of a wide range of secondary metabolites with diverse biological activities, including anti-microbial, anti-fungal, and anti-tumor activities.[4] As a result, thousands of bioactive compounds from actinomycetes have been isolated and characterized, many of which have been developed into pharmaceutical drugs to treat a wide range of diseases in humans, plants and animals.[7]

The best-known of the secondary metabolites produced by actinomycetes are antibiotics. In fact, actinomycetes produce the majority of naturally occurring antibiotics. In the Golden Age of antibiotics (the 50s to 70s), 60% of new antibiotics were isolated from

actinomycetes, and most of these were from the genus *Streptomyces*.[8] Examples of antibiotics produced by actinomycetes include streptomycin from *Streptomyces griseus*, actinomycin from *Streptomyces antibioticus* and streptothricin from *Streptomyces lavendulae*. In addition to their production of antibiotics, actinomycetes also produce many other secondary metabolites that can be used as herbicides, antifungal agents, anthelmintic agents, antitumor drugs or immunosuppressant drugs.[4]

In recent years, thanks to the availability of inexpensive DNA sequencing technologies, an increasing number of actinomycetes genomes have been sequenced. The study of these genomes has revealed that actinomycetes might have the capacity to produce many more secondary metabolites than we expected. For example, the well-studied *Streptomyces coelicolor* A3(2) was previously thought to produce only four secondary metabolites. However, sequencing of the *Streptomyces coelicolor* A3(2) genome revealed an additional 18 biosynthetic gene clusters.[9] In fact, each actinomycete strain appears to have the genetic potential to produce 10–20 secondary metabolites.[7] Therefore, for some years into the future, actinomycetes will likely continue to be the most potent source for the production of secondary metabolites, antibiotics, and other bioactive compounds.

Table 1.1 Examples of secondary metabolites produced by actinomycetes [4]

Activity	Producing strains	Secondary metabolites
Antibacterial	*Streptomyces aureofaciens* *Streptomyces venezuelae* *Streptomyces rimosus* *Streptomyces ambofaciens* *Streptomyces lydicus* *Streptomyces kanamyceticus* *Streptomyces niveus* *Micromonospora purpurea* *Nocardia lurida* *Amycolatopsis orientalis*	Tetracycline Cloramphenicol Oxytetracycline Spiramycin Streptolydigin Kanamycin Novobiocin Gentamycin Ristocetin Vancomycin
Antifungal	*Streptomyces nodosus* *Streptomyces venezuelae* *Streptomyces natalensis* *Streptomyces hygroscopius* *Streptomyces tendae* *Streptomyces kasugaensis* *Streptomyces galbus* *Nocardia transvalensis*	Amphotericin B Jadomycin Natamycin Validamycin Nikkomycin Kasugamycin Galbonolides Transvalencin
Antiparasitic	*Streptomyces avermitilis* *Streptomyces coelicolor* *Streptomyces bottropensis*	Avermectins Prodiginine Trioxacarcin
Antitumor	*Norcadia asteroides* *Salinispora tropica* *Streptomyces peuceticus*	Asterobactine Salinosporamide Doxorubixin
Antiparasitic	*Streptomyces avermitilis* *Streptomyces coelicolor*	Avermectins Prodiginine
Immunosuppressive	*Norcardia brasiliensis* *Streptomyces filipinensis*	Brasilicardin Pentalenolactone

1.2 Regulation of secondary metabolite production in actinomycetes

The majority of bioactive secondary metabolites produced by actinomycetes are cytotoxic. Moreover, it is metabolically costly to produce many secondary metabolites at the same time. For these reasons, the production of secondary metabolites in actinomycetes is regulated in a tightly controlled manner to assure the rigorous timing and quantity of production. In liquid cultures, the production of these metabolites begins as the culture enters the stationary phase, while in agar cultures, it often coincides with the onset of morphological differentiation.[1]

The expression of genes encoding secondary metabolites in actinomycetes is controlled by complex regulatory mechanisms that are not readily explained in a simple unifying model.[1] However, it has been proposed that the regulatory network for secondary metabolite productions is often organized in a hierarchical manner that involves both pleiotropic regulators and pathway-specific regulators. Pleiotropic regulators are global regulators that may control several secondary metabolite biosynthesis gene clusters and are not linked to any specific biosynthesis pathway.[10] These regulators are influenced by various stress signals from the surrounding environment, such as phosphate and nitrogen starvation, the presence of chitin and N-acetylglucosamine in the culture medium or various physiological stresses such as heat shock and cell wall damage.[1,10]

Genes for pathway-specific regulators are often clustered together with genes for the biosynthesis of target secondary metabolites. Most of the pathway-specific regulators are activators belonging to the *Streptomyces* antibiotic regulatory protein (SARP) family.

They contain a DNA-binding domain in the vicinity of the N-terminus and a bacterial transcriptional domain.[11] Deletion of the SARP-encoding genes leads to the impairment or complete loss of the production of the target secondary metabolites.[10] Examples of SARPs include StrR, which controls the streptomycin production in *Streptomyces griseus* or RedD, which controls undecylprodigiosin in *Streptomyces coelicolor*.[12,13] In addition to SARPs, proteins belonging to other families such as the LysR-type regulator family and large regulators of the LAL family also function as pathway-specific regulators for the biosynthesis of secondary metabolites.[10]

A pyramidal control system was discovered in various actinomycetes, in which pleiotropic regulators modulate the expression of pathway-specific regulators, which in turn control the expression of secondary metabolite biosynthesis gene clusters. A well-known representative of this type of pyramidal control system is the control of antibiotics by γ-butyrolactone.[14] In addition to this system, the phosphate control of *Streptomyces* metabolism by the Phor-PhoP two-component system and the nitrogen-source regulation mediated by the orphan response regulator GlnR are also important systems that regulate secondary metabolite production in actinomycetes.[15,16]

The fact that the production secondary metabolites are tightly controlled has hindered their isolation and structure elucidation. Therefore, in order to increase the yield and facilitate the discovery of new secondary metabolites, it will be necessary to elucidate the regulatory mechanism controlling their production. Recently, several research groups have successfully increased the yield of secondary metabolites by manipulating their

regulatory mechanism,[15,16] highlighting the potential of regulatory-network manipulation as a useful tool to explore the rich sources of secondary metabolites encoded in the genome of actinomycetes.

1.3 *Streptomyces* hormones

In actinomycetes, the most intensively studied pleiotropic regulators are those belonging to a group of hormone-like autoregulators. These autoregulators are diffusible signaling molecules that can elicit antibiotics production and/or trigger morphological differentiation at nanomolar concentrations, which are therefore considered *Streptomyces* hormones.[14] The largest class of *Streptomyces* hormones are the γ-butyrolactone-type *Streptomyces* hormones, with 13 compounds having been discovered so far. Hormones belonging to this class share a core 2,3-disubstituted γ-butyrolactone skeleton but differ in length, branching and stereochemistry of their fatty acid side chains.[14] Another major class of *Streptomyces* hormones is the butenolide class, which was discovered less than 5 years ago. In this class, the butenolide group is the core structure instead of γ-butyrolactone. Recently, five methylenomycin furans from *S. coelicolor* have also been considered to make up a furan class of *Streptomyces* hormones, although the minimum concentration at which these furans induce secondary metabolite production was not reported.[17]

The effects of *Streptomyces* hormones are transmitted via specific receptor proteins that usually belong to the tetR family of transcriptional regulators, a widely distributed group of regulators among bacteria.[18,19] Most of these receptor proteins act as repressors and are highly specific to their cognate *Streptomyces* hormones.[14] The binding of the

receptor proteins to their cognate *Streptomyces* hormones induces the expression of the target genes. Targets of the *Streptomyces* hormone receptor proteins can be genes encoding for pleiotropic regulators at a lower hierarchical level or pathway-specific regulators that directly control the expression of specific secondary metabolites (Figure 1.2).

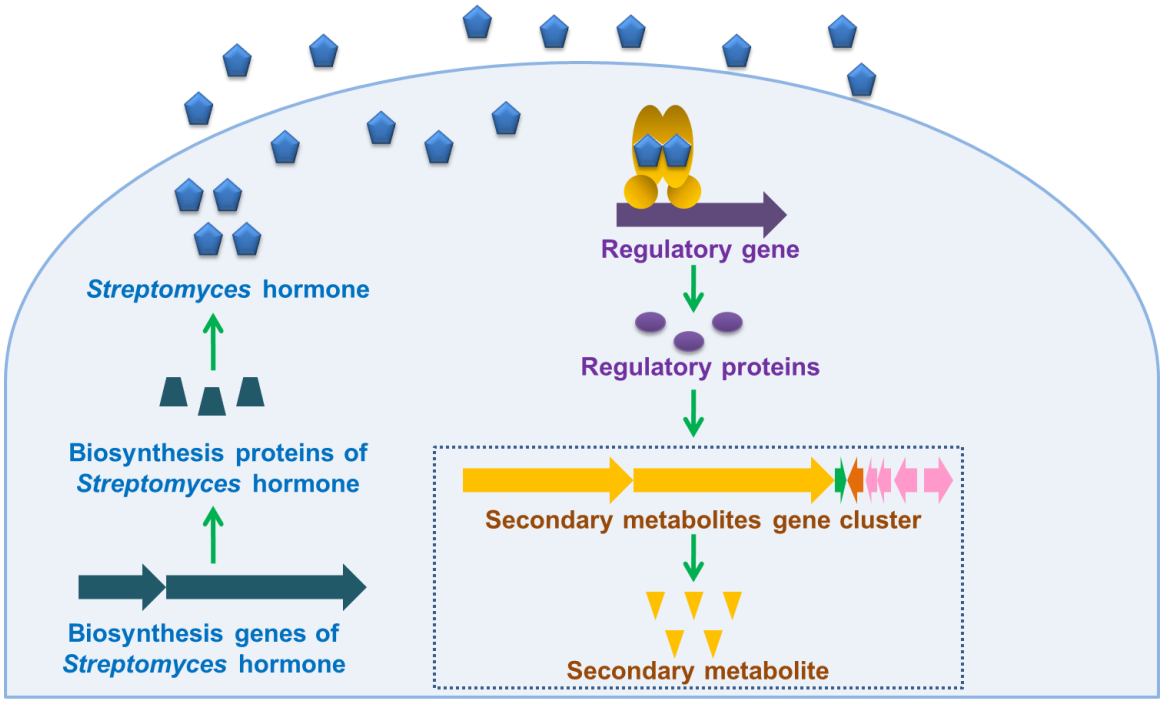

Figure 1.2 The signaling cascade of a *Streptomyces* hormone.

1.3.1 The γ-butyrolactone class of *Streptomyces* hormones

γ-Butyrolactone is the best-characterized class of *Streptomyces* hormones so far. A large percentage of actinomycetes (64.1%) produce this class of *Streptomyces* hormones.[20] They can be further categorized into three smaller subclasses: the A-factor-type, virginiae butenolide (VB)-type and IM-2-type based on the differences in the stereochemistry of their

fatty acid side chains. A-factor hormones have a keto group while VB hormones have an α-hydroxyl group and IM-2 hormones have a ß-hydroxyl group at C-1'.

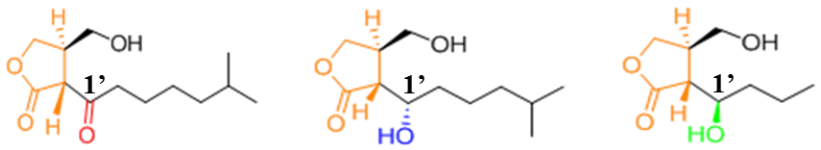

Figure 1.3 Representatives of 3 subclasses of γ-butyrolactone *Streptomyces* hormones.

The A-factor subclass includes only A-factor from *Streptomyces griseus*.[21,22] On the other hand, the VB subclass includes 5 VBs (VB-A, VB-B, VB-C, VB-D and VB-E) from *Streptomyces virginiae*[23] and 3 Grafe's factors from *Streptomyces bikinensis* and *Streptomyces cyaneofuscatus*.[24] The IM-2 subclass contains IM-2 from *Streptomyces lavendulae*,[25] factor I from *Streptomyces viridochomogenes*,[26] 3 SCBs (SCB1-3) from *Streptomyces coelicolor*[27,28] and SVB1, which is identical to SCB3, from *Streptomyces venezuelae*.[29] All of the γ-butyrolactone *Streptomyces* hormones play important roles in controlling secondary metabolism (Table 1.2).

Table 1.2 *Streptomyces* hormones belonging to the γ-butyrolactone class and the bioactive compounds that they regulate

Subclass	*Streptomyces* hormone	Regulated compound	Strain
A-factor	A-factor	Streptomycin	*S. griseus*
VB	VBs	Virginiamycins	*S. virginiae*
	Grafe's factors	Anthracycline	*S. bikinensis* *S. cyaneofuscatus*
IM-2	IM-2	D-cycloserine Blue pigment Showdomycin	*S. lavendulae*
	Factor I	Anthracycline	*S. viridochomogenes*
	SCB1, SCB2 SCB3	Actinorhodin Undecylprodigiosin	*S. coelicolor* *S. coelicolor*
	SVB1	Jadomycin	*S. venezuelae*

1.3.1.1 The A-factor subclass

The *Streptomyces* hormone A-factor is well known as an important autoregulatory factor that controls streptomycin production and cellular differentiation in *S. griseus*.[22] A-factor was first identified in 1967 by a group of Russian scientists,[30] and the discovery of AsfA, a key enzyme for A-factor biosynthesis, was made in 1983.[31] In 1995, Horinouchi

and co-workers identified the A-factor receptor protein.[32] Based on these and other studies, the A-factor regulatory pathway was already well-characterized.[33] This signaling cascade is a good example of a hierarchical regulatory model, in which A-factor and its cognate receptor ArpA are located at the highest position of the signaling cascade.

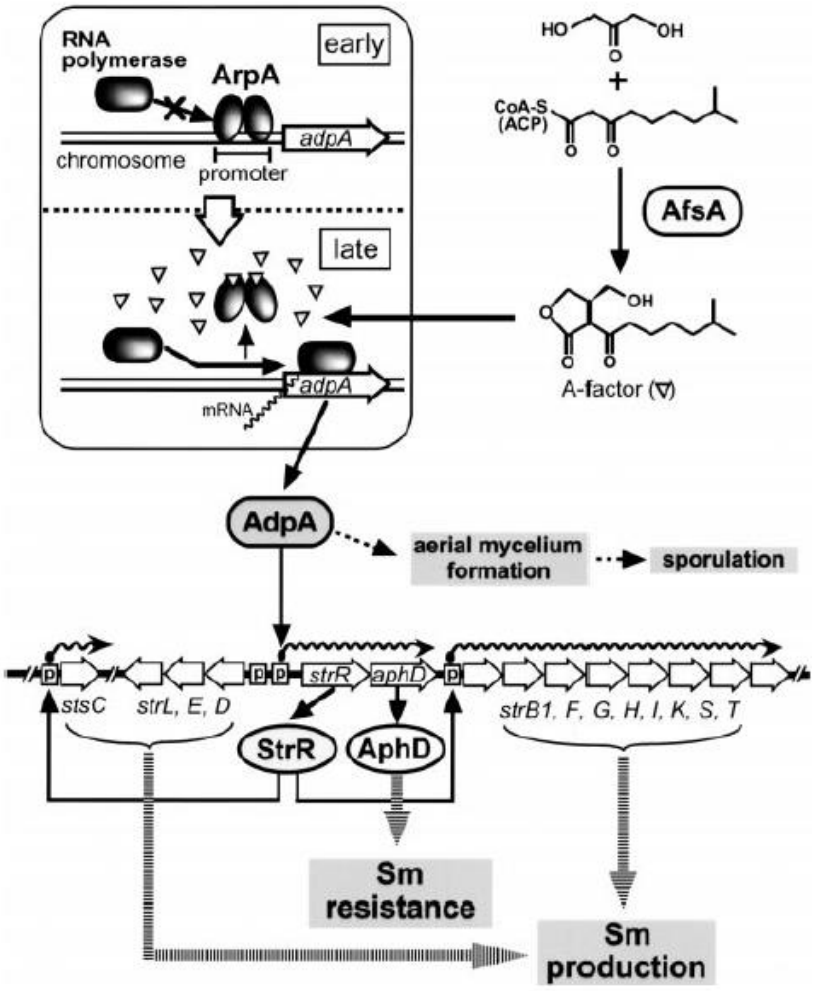

Figure 1.4 The A-factor signaling cascade in *S. griseus*.[33]

When A-factor is absent, the repressor-type receptor ArpA binds to a specific sequence in the upstream region of adpA, a pleiotropic regulatory gene. When A-factor is

produced and accumulated to a threshold concentration, which is extremely low, it binds to ArpA and dissociates it from the upstream region of *adpA*.[22] As a result, the transcription of *adpA* is activated. Since AdpA is a pleiotropic regulator, it initiates the transcription of many genes required for morphological differentiation and secondary metabolism. They include *strR*, the pathway-specific regulator for the streptomycin production,[33] and *amfR*, an essential gene for aerial mycelium formation.[34]

1.3.1.2 The VB subclass

VBs are the *Streptomyces* hormones that trigger the production of two streptogramin antibiotics, M and virginiamycin S, which were discovered in *S. virginiae* in 1986, 10 years after the discovery of A-factor.[23] In *S. virginiae*, two genes, *barS1* and *barS2* are directly involved in the biosynthesis of VBs. The gene encoding for the receptor of VBs, *barA*, was also located in the same locus with *barS1* and *barS2*.[35,36] BarA acts as a pleiotropic regulator in virginiamycin M and virginiamycin S biosynthesis. The mutant strain, in which *barA* was inactivated, produced virginiamycins 7 h earlier than the wild-type strain, indicating that the *barA* protein negatively control virginiamycins biosynthesis.[37] A gene encoding for a positive regulator of VM biosynthesis, *VmsR*, is situated 4.7 kbp upstream of the *barA* gene.[36] VmsR functions as an activator of virginiamycins biosynthesis by activating the transcription of two pathway-specific regulatory genes, *vmsS* and *vmsT*.[38] VmsS, which belongs to the SARP family, is a pathway-specific regulator for both virginiamycin M and virginiamycin S biosynthesis. On the other hand, vmsT is a pathway-specific regulator for virginiamycin M biosynthesis alone.[38] vmsR also contributes to the expression of virginiamycin biosynthetic genes, independent of vmsS and vmsT.[38] In summary,

virginiamycin production is controlled by both pleiotropic regulators and three pathway-specific regulators, which regulate the expression of the biosynthetic gene cluster in a hierarchically manner.

1.3.1.3 The IM-2 subclass

IM-2 was discovered as a signaling molecule that could induce the biosynthesis of the blue pigment indigoidine in *S. lavendulae* FRI-5 in 1988.[25] Later, it was observed that IM-2 also induced the production of two nucleoside antibiotics, showdomycin and minimycin, but repressed the production of D-cycloserine.[39] This finding demonstrated the unique feature of IM-2 as the only *Streptomyces* hormone that can both switch on and switch off the production of antibiotics.

The IM-2 receptor protein was designated as FarA. In vitro studies of FarA indicated that it was a dimeric DNA binding protein that can recognizes and binds to specific DNA sequences located in the promoter region of a target gene when IM-2 is absent.[40,41] Binding of IM-2 to FarA results in the dissociation of FarA from the DNA, which initiates the transcription of the target gene. In order to confirm that FarA is actually involved in the IM-2 signaling cascade, a *farA* deletion mutant of *S. lavendulae* was constructed.[42] The result of this study demonstrated that FarA plays role as a negative regulator in the production of nucleoside antibiotics and indigoidine. Moreover, it was revealed that the presence of both IM-2 and FarA is required for the suppression of D-cycloserine production, indicating the regulatory roles of the IM-2/FarA complex. FarA also functions as a transcriptional repressor of *farA* itself (Figure 1.5).

FarX, the biosynthesis gene of IM-2, was found adjacent to *farA*.[43] In this gene cluster, two putative regulatory genes encode for SARP family proteins (*farR3* and *farR4*) and two putative transcriptional regulatory genes (*farR1* and *farR2*) are also present.[43] FarR3 is located in the downstream level of the IM-2/FarA signaling cascade and has been shown to positively control the biosynthesis of indigoidine.[44] In the same study, loss of FarR4 led to the early production of IM-2 by up-regulating *farX* transcription, suggesting that FarR4 controls the biosynthesis of IM-2 in a negative manner.[44] In addition, FarR2 disruption led to the delayed production of indigoidine.[45] These results demonstrated the complicated nature of the IM-2 signaling cascade.

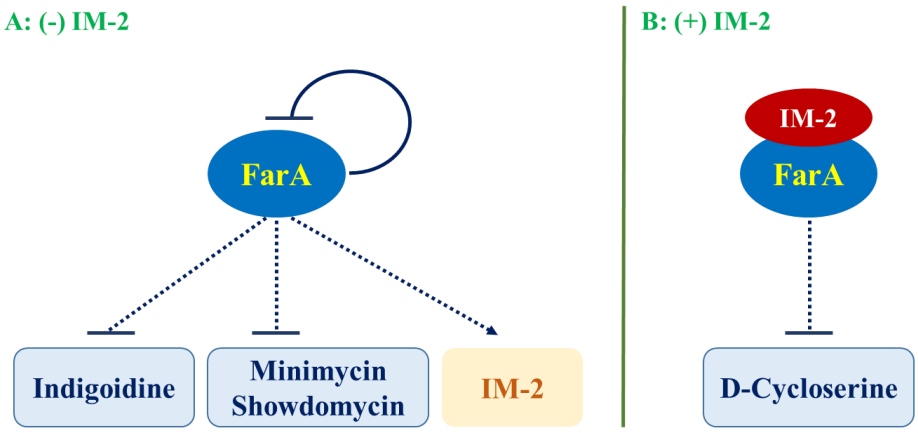

Figure 1.5 Model of IM-2 regulatory cascade in the absence (A) or presence (B) of IM-2. Solid line and dashed lines represent direct or indirect regulation, respectively. Horizontal lines and arrows indicate repression and activation of the regulation, respectively.[42]

SCB1 is another well-studied member of the IM-2 subclass. SCB1 can induce the production of the pigmented antibiotics undecylprodigiosin and actinorhodin in *S. coelicolor*.[27] ScbA and ScbR are the biosynthesis protein and the cognate receptor of SCB1,

respectively.[46] Genes encoding for these two proteins lie adjacent to each other in the genome of *S. coelicolor*.[46] ScbR controls antibiotic production via the transcription repression of its own gene and the transcription repression of *cpkO*, which is a pathway-specific regulatory gene for a cryptic type I polyketide antibiotic biosynthesis gene cluster.[47] In a subsequent study, SCB2 and SCB3, which are two additional signaling molecules, were also identified in *S. coelicolor*.[28] Like SCB1, these molecules belong to the IM-2-type *Streptomyces* hormone family and can trigger precocious antibiotic production in an *S. coelicolor* bioassay. SCB3 showed the highest affinity toward ScbR, followed by SCB1 and SCB2, respectively. The biosynthesis of SCB2 and SCB3 also required the activity of ScbA.[28]

1.3.2 The butenolide class of *Streptomyces* hormones

Different from the γ-butyrolactone class, the butenolide class of *Streptomyces* hormones was discovered in recent years and is distinguished from the former class by the butenolide group in the core structure. Avenolide was the first member of this class and was discovered in *S. avermitilis* in 2011,[48] followed by the discovery of 2 SRBs from *S. rochei* in 2012.[49] Unlike members of the γ-butyrolactone class, which only differ in the length and the stereochemistry of their fatty acid side chains, avenolide and SRBs show differences in the butenolide core structure, the length of the fatty acid side chains and the position at which the side chain is attached to the butenolide group (Figure 1.6). Since the discovery of SRBs in 2012, no additional members of this class have been identified. Therefore, whether butenolide class *Streptomyces* hormones are widely distributed among actinomycetes remains to be clarified.

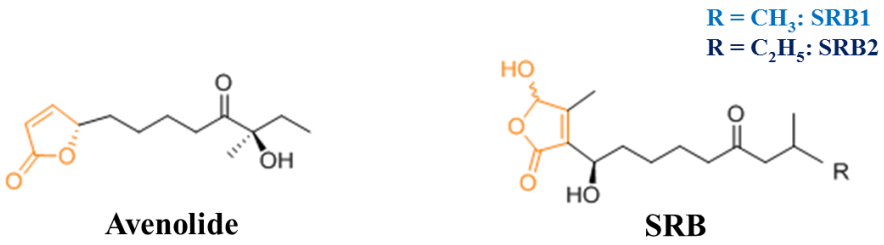

Figure 1.6 Members of the butenolide class of *Streptomyces* hormones.

1.3.2.1 Avenolide

Avenolide has been shown to control the production of avermectin, an important anthelmintic agent, in *S. avermitilis*.[48] In a later study, avermectin was chemically modified to a more effective derivative called ivermectin.[50] Ivermectin plays a crucial role in the treatment of onchocerciasis (river blindness) and lymphatic filariasis.[51,52] Onchocerciasis and lymphatic filariasis are two diseases caused by parasitic worms and negatively affect the life of millions of people, especially in the world's poorest populations. Ivermectin also shows efficacy against an increasing number of other parasitic diseases.[53] In 2015, in recognition of the contribution of avermectin to the lives of large numbers of people, William C. Campbell and Satoshi Ōmura were awarded the Nobel Prize in Physiology or Medicine for its discovery.

Avermectin was discovered in 1984, but it was not until 2011 that avenolide was identified as the signaling molecule controlling avermectin production.[48] Avenolide is only produced in extremely small amounts (0.6 ng ml^{-1}); indeed, 2000 L of *S. avermitilis* culture was needed for its isolation and structure identification.[48]

In the genome of *S. avermitilis*, the *avaR* cluster plays important roles in avenolide biosynthesis and avermectin production. This locus includes *aco*, *cyp17* and three homologs of γ-butyrolactone-autoregulator receptors (*avaR1-3*) (Figure 1.7).[48]

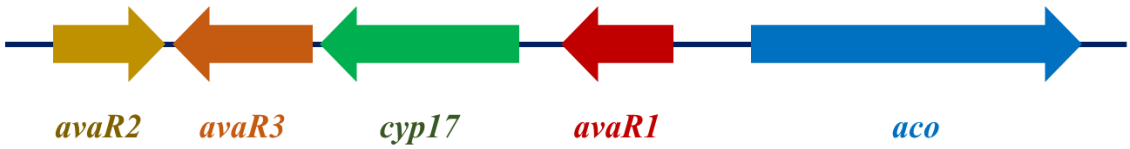

Figure 1.7 Organization of the *avaR* cluster in *S. avermitilis*.[48]

Aco and *cyp17* encode for a putative acyl-CoA oxidase and for a cytochrome P450 hydroxylase involved in avenolide biosynthesis.[48] Aco has been suggested to introduce a double bond between the C-2 and C-3 positions of avenolide.[48] When the *aco* gene was disrupted, the mutant strain lost the ability to produced avenolide. As a result, the level of avermectin production in the *aco* disruption mutant was significantly reduced to only 6% of the level in the wild-type.[48]

Through gene disruption analysis, it was clear that AvaR3 plays role as a pleiotropic regulator that controls avermectin production and cell morphology.[54] AvaR3 might function as a transcriptional activator for the production of avermectin by indirectly regulating the expression of *aveR*, an in-cluster pathway-specific regulatory gene for avermectin.[54] AveR positively regulates the expression of both polyketide biosynthetic genes and postpolyketide modification genes belonging to avermectin biosynthesis gene cluster.[55] However, it remains to be clarified whether avaR3 is the receptor of avenolide. On the other hand, avaR1 was shown to bind specifically to avenolide in a gel-shift assay experiment.[48]

A study performed by Sultan *et al.* revealed that avaR1 acts as a transcriptional repressor for the biosynthesis of avenolide but does not show any effect on avermectin production.[56] However, in a recent study by Zhu *et al.*, the authors demonstrated that in the absence of avenolide, avaR1 inhibits avermectin production through the direct repression of *aveR*.[57] The disagreement between the studies might have been due to the different experimental strains and growth conditions used. Zhu *et al.* also demonstrated that Avar2 has avenolide-binding activity and controls avermectin production by directly repressing the transcription of *aco* and *aveR*.[58]

Until recently, *S. avermitilis* was the only strain known to use avenolide to control its secondary metabolism. However, gene clusters that contain *aco*, *cyp17*, and *avaR1* homologs in the same arrangements were found in the genome of some actinomycetes such as *Streptomyces fradiae*, *Streptomyces griseoauranticus* M045 and *Streptomyces ghanaensis* ATCC 14672, indicating that these strains also use avenolide as a signaling molecule to control their secondary metabolisms (Figure 1.8).[48] Further studies on the distribution of avenolide will thus be needed to clarify whether avenolide constitutes a minor group of *Streptomyces* hormone or is a commonly used signal among other actinomycetes.

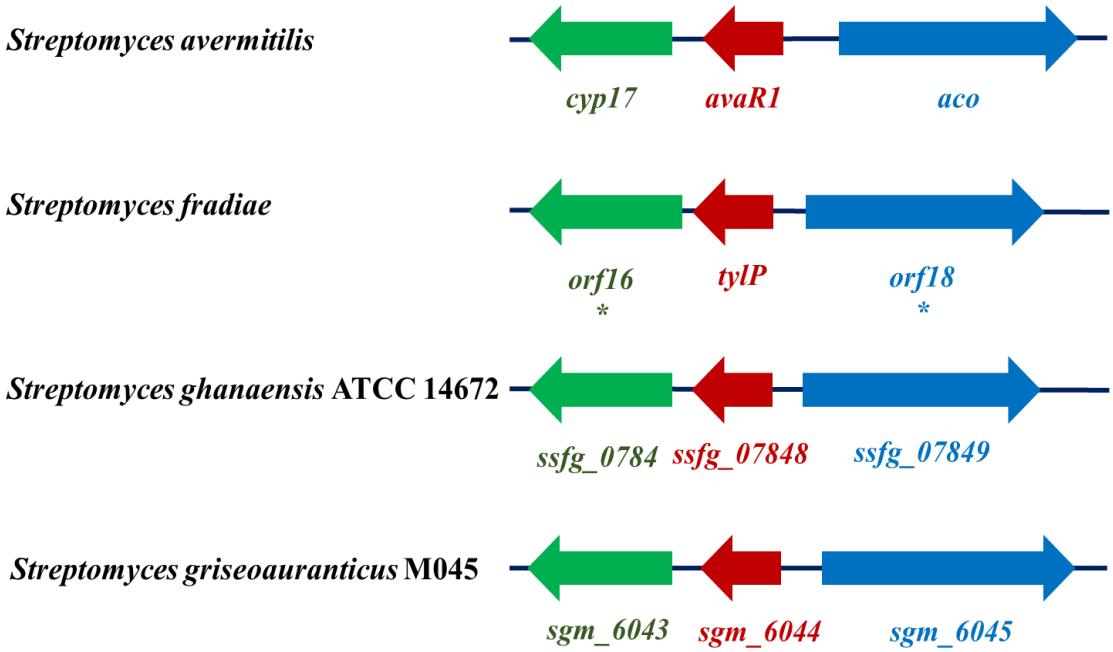

Figure 1.8 Actinomycetes that have homolog of *cyp17*/*avaR1*/*aco* cluster in their genomes.[48]

1.3.2.2 SRBs

SRB1 and SRB2 were identified in 2012 as signaling molecules that trigger lankamycin and lankacidin production in *S. rochei*.[49] The minimum concentrations of SRB1 and SRB2 required to induce antibiotic production in *S. rochei* revealed to be 42 nM and 40 nM, respectively, indicating that they have roughly equivalent antibiotic-inducing activity.[49]

Similar to asfA, which is involved in IM-2 biosynthesis in *S. griseus*, an asfA homolog (srrX) has been suggested to play important role in the biosynthesis of SRB1 and SRB2 in *S. rochei*.[49] A gamma-butyrolactone receptor gene homologs (srrA) might be the common receptor of SRB1 and SRB2.[59] Through transcriptional and extensive mutational

analyses, *srrY*, which belongs to the SARP gene family, was revealed to be the target of SrrA.[60] Therefore, it can be hypothesized that the SRB signaling pathway goes from *srrX* through *srrA* to *srrY*, leading to lankamycin and lankacidin production.

1.4 Overview and objective of this study

As described in the previous sections, *Streptomyces* hormones belonging to the γ-butyrolactone class are commonly found in actinomycetes and their distribution were thoroughly investigated. It is estimated that approximately 64% of actinomycetes produce this class of *Streptomyces* hormones. On the other hand, the butenolide class, of which avenolide is a member, was only discovered in recent years and there is a lack of information regarding its distribution. Until recently, *S. avermitilis* is the only strain known to use avenolide to control its secondary metabolite production. Therefore, whether avenolide-type *Streptomyces* hormones are commonly found in other actinomycetes has attracted my interest. In this study, I aimed to investigate whether other actinomycetes also produce avenolide-active compounds. The present experiments should thus contribute to our knowledge regarding the distribution of both the avenolide subclass and the butenolide class of *Streptomyces* hormones. *S. avermitilis* uses avenolide to trigger the production of the widely used drug avermectin. Therefore, I considered that it would also be worth investigating the role of avenolide-active compounds in other actinomycetes. An overview of the study is as follows.

Chapter 2 describes the screening of actinomycetes producing avenolide-type hormones. *Streptomyces* hormones are often present only at nanomolar concentrations in

culture, which hinders their detection. However, the cognate host can respond to the presence of specific types of hormones even at extremely low concentrations. Therefore, a bioassay method was selected for the screening process. To identify actinomycetes producing avenolide-type hormones, *S. avermitilis* Δ*aco*, a disruptant of an essential avenolide biosynthesis gene, was used as an indicator strain in the bioassay. Since avenolide is not synthesized, avermectin is also not produced in this strain. Ethyl acetate extracts from 51 strains (40 *Streptomyces* and 11 endophytic actinomycetes strains) were tested in the bioassay for their ability to restore avermectin production in the indicator strain (avenolide activity). The result showed that the extracts from 12 out of 51 strains (24%) had avenolide activity, indicating that these strains might produce avenolide or avenolide derivatives. In particular, 5 of the 12 strains had very high avenolide activity ($\geq$ 500 units). In *Streptomyces albus* J1074 (a strain producing antimycins, candicidins and paulomycins), avenolide activity was as high as 1,000 units. Next, therefore, I sought to identify the compounds responsible for the avenolide activity of *S. albus* J1074 and to investigate their roles.

Chapter 3 describes the characterization of avenolide-active compounds produced by *Streptomyces albus* J1074. After screening the genome information of *S. albus* J1074, only one copy of the *aco* homolog was identified. By disrupting this *aco* homolog and comparing the secondary metabolite profiles between the wild-type and disruptant, the *aco*-dependent compounds were identified. The results showed that *aco* homolog is directly involved in the biosynthesis of 4 butenolides, which share a similar backbone structure to that of avenolide. All 4 butenolides showed avenolide activity when tested separately in an avenolide assay, although the units of activity differed among them. However, no other

compound besides these 4 butenolides disappeared in the secondary metabolite profile of *S. albus* J1074 after the *aco* homolog was disrupted, indicating that these 4 butenolides do not play any role in controlling the production of secondary metabolites in *S. albus* J1074. On the other hand, when co-cultivating *S. albus* J1074 with *S. avermitilis* Δ*aco*, it was observed that avermectin production was restored in *S. avermitilis* Δ*aco*, suggesting the role of the 4 butenolides as communication signals between these two strains.

Lastly, the general summary and conclusions are noted in Chapter 4.

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CHAPTER 2

Screening of actinomycetes producing avenolide-type hormones

2.1 Introduction

Actinomycetes are Gram-positive bacteria that has high-GC-content in the genome. They can produce a wide range of secondary metabolites, many of which were developed into anti-bacterial, anti-cancer, and anti-fungal medicines. Production of these compounds in actinomycetes is controlled by many environmental and physiological factors, including autoregulators.[61] Even though these autoregulators are produced at extremely low concentrations, they play crucial roles in regulating secondary metabolism and in some cases, morphological differentiation in actinomycetes.[14] Consequently, they are considered '*Streptomyces* hormones'. A-factor, which was identified in 1967 as a signal controlling streptomycin production in *Streptomyces griseus*;[33] was the first *Streptomyces* hormones studied so far. Following the discovery of A-factor, VB was identified in 1987, which regulates virginiamycin M_1 and virginiamycin S production in *Streptomyces virginiae*.[23] In 1989, IM-2, which induces the production of the blue pigment indigoidine as well as several nucleoside antibiotics in *Streptomyces lavendulae*, was also discovered. A-factor, VB and IM-2 belong to the γ-butyrolactone class of *Streptomyces* hormone and their distribution among actinomycetes was studied intensively.[21,62,63] On the other hand, avenolide, a member of butenolide class of *Streptomyces* hormone, was discovered recently and there has been no study about its distribution among actinomycetes.[48]

The distribution of avenolide is of my interest because it will provide clues to evaluate the significance of avenolide as controlling tool of secondary metabolite production in the genus actinomycetes. However, the distribution study of avenolide is hindered by the fact that it is produced only in very small amounts at approximately 0.6 ng/ml in liquid culture medium.[48] This hinders the detection, characterization and quantification procedures by chemical (colorimetric or enzymatic) or physical (GC or HPLC) methods. On the other hand, avenolide can initiate the production of it cognate secondary metabolite even at nanomolar concentrations, indicating the bioassay with the responsive *Streptomyces* strains can be the most sensitive and the most high through-put method.[62]

In the distribution study of A-factor, Hara and Beppu used bioassay approach with A-factor deficient strains as the indicator strain.[21] This mutant was obtained by UV-irradiation and has lost the ability to produce both A-factor and streptomycin.[21] If an actinomycetes strain produces A-factor, it is capable of restoring streptomycin production in the A-factor-deficient *S. griseus*.

During the process of clarifying the avenolide regulatory cascades in actinomycetes, avenolide-deficient strain was successfully constructed in our laboratory by disrupting essential gene in avenolide biosynthetic pathway.[48] Therefore, similar to the study of A-factor distribution, in the present study, the avenolide-deficient strain was used as an indicator strain to investigate the avenolide distribution for the first time.

2.2 Materials and methods

2.2.1 Bacterial strains and cultivation conditions

The 51 actinomycetes strains used in this study are listed in Table 2.1 and Table 2.2. Among the 40 *Streptomyces* strains listed in Table 2.1, 38 strains were obtained from the Biological Resource Center, NITE, Tokyo, Japan. *Streptomyces fradiae* C373.1D and *Streptomyces albus* J1074 were provided by Professor Eric Cundliffe (University of Leicester, England) and Professor Mervyn Bibb FRS (John Innes Centre, Norwich), respectively. 11 actinomycetes listed in Table 2.2 were isolated form leaves samples collected in Izu Peninsula, Japan. f-medium (containing [in grams per liter] bacto casitone [Becton, Dickinson and Company, Franklin Lakes, NJ, USA], 7.5; yeast extract [Wako Pure Chemical, Osaka, Japan], 7.5; glycerol [Wako Pure Chemical, Osaka, Japan], 15; NaCl [Wako Pure Chemical, Osaka, Japan], 2.5) was used for cultivation.[23] To prepare seed cultures, 20 ml of f-medium in a 100-ml Erlenmeyer flask was inoculated with spores and incubated on a reciprocating shaker (120 spm) at 28^{o}C for 3 days. The main cultivation was prepared by inoculating 2.4 ml of the seed culture into 80 ml of f-medium in a 500-ml baffled flask, followed by incubation for 3 days on a reciprocating shaker (120 spm) at 28^{o}C for 3 days.

Table 2.1 *Streptomyces* strains used in this study

Strain	**Strain**
S. aburaviensis NBRC 12830	*S. lividus* NBRC 13787
S. albidoflavus NBRC 12790	*S. longwoodensis* NBRC 14251
S. albus J1074	*S. matensis* NBRC 12889
S. ambofaciens NBRC 12836	*S. murinus* NBRC 14802
S. antibioticus NBRC 3126	*S. narbonensis* NBRC 12801
S. aureofaciens NBRC 12843	*S. niveus* NBRC 12804
S. bambergiensis NBRC 13479	*S. niveus* NBRC 12917
S. bikiniensis NBRC 14598	*S. nodosus* NBRC 12895
S. cirratus NBRC 13398	*S. ostreogriseus* NBRC 13423
S. cyaneofuscatus NBRC 13190	*S. rameus* NBRC 13399
S. erythraeus NBRC 13426	*S. ramulosus* NBRC 15798
S. eurythermus NBRC 12764	*S. sahachiroi* NBRC 13928
S. fradiae C373.1D	*S. sclerogranulatus* NBRC 14301
S. ghanaensis NBRC 15414	*S. sioyaensis* NBRC 12820
S. griseocarneus NBRC 13428	*S. varsoviensis* NBRC 13093
S. griseochromogene NBRC 13413	*S. venezuelae* NBRC 13096
S. griseus NBRC 13189	*S. vinaceus* NBRC 13425
S. griseus NBRC 13350	*S. violaceoruber* NBRC 13385
S. hawaiiensis NBRC 12784	*S. viridifaciens* NBRC 13352
S. jumonjinensis NBRC 13869	*S. xanthochromogenes* NBRC 12828

Table 2.2 Endophytic actinomycetes used in this study and their 16S ribosomal RNA accession number

Strain	DDBJ Accession number
Streptomyces sp. HN5	LC260149
Streptomyces sp. HN25	LC260159
Streptomyces sp. HN35	LC260158
Streptomyces sp. HN38	LC260157
Pseudonorcadia sp. HN40	LC260156
Streptomyces sp. HN46	LC260155
Streptomyces sp. HN51	LC260150
Streptomyces sp. HN65	LC260154
Streptomyces sp. HN66	LC260153
Streptomyces sp. HN70	LC260152
Streptomyces sp. HN78	LC260151

2.2.2 Preparation of ethyl acetate extract

The main culture (60 ml) of each strain was adjusted to pH 3.0 with 3M HCl and was extracted three times with 60 ml of ethyl acetate. Then, the solvent layer was

evaporated to dryness and the residue re-dissolved in 600 µl of ethyl acetate. The dissolved samples were then assayed for avenolide activities.

2.2.3 Assay of avenolide activity

Avenolide activity was assayed in solid culture of the *aco* disruptant *S. avermitilis* strain (*S. avermitilis* Δ*aco*), which was previously constructed in our laboratory,[48] by measuring the avenolide-dependent production of avermectin. Because *aco* is one of the essential biosynthetic genes of avenolide, *S. avermitilis* Δ*aco* lost the ability to produce endogenous avenolide.[48] Spores (1.0 x10^5 CFU) of *S. avermitilis* Δ*aco* were spread onto 2 ml YMS-MC medium pre-mixed with an ethyl acetate extract of each strain in a 16-well-plate, followed by incubation at 28^{o}C for 8 days.[54] The agar culture was diced and extracted with an equal volume of methanol for 2 hours. The methanol extract was collected by centrifugation and the present of avermectin in the extract was analyzed by high-pressure liquid chromatography (HPLC).[55]

2.2.4 Quantification of avenolide activity

One unit of avenolide was defined as the minimum amount of avenolide required to trigger the production of avermectin. One unit corresponds to 8 nM of synthetic avenolide. Various amounts of ethyl acetate extract of each strain were tested on avenolide assay. The minimal amount of ethyl acetate extract that triggered the production of avermectin was considered to contain one unit per milliliter of avenolide. Authentic avenolide was chemically synthesized as described previously.[64]

2.3 Results

2.3.1 Specificity of avenolide assay

In order to confirm that avenolide assay can discriminate avenolide with other *Streptomyces* hormones belonging to the γ-butyrolactone class, I investigated the specificity of the avenolide assay. Synthetic *Streptomyces* hormones at different concentrations were added to avenolide assay and their avenolide activities were measured. The results of the specificity test are shown in Table 2.3.

Table 2.3 Specificity of avenolide assay

	***Streptomyces* hormones**			
	Avenolide	A-factor	IM-2-C_5	VB-C_7
Concentration (nM)	8	20,000	20,000	20,000
Avenolide activity	+	−	−	−

+: avermectin production in *S. avermitilis* Δ*aco* could be restored
-: avermectin production in *S. avermitilis* Δ*aco* could not be restored

As we could see, avenolide showed inducing effect at as low as 8 nM. On the other hand, A-factor, IM-2-C_5 and VB-C_7 could not trigger avermectin production even at 20 μM, indicating that *S. avermitilis* Δ*aco* could discriminate avenolide at least 2,500-fold more specifically from A-factor, IM-2 or VB. This result confirmed the specificity of avenolide assay toward avenolide-type hormones.

2.3.2 Distribution of avenolide in 40 strains of *Streptomyces*

To investigate the distribution of avenolide in *Streptomyces* species, I selected 40 *Streptomyces* strains available in my laboratory for this study (Table 2.1).

Avenolide was discovered for the first time in *S. avermitilis* in 2011 and was considered as a new class of *Streptomyces* hormones.[48] However, until recently, no other strains were found to have an avenolide signaling pathway. From the result of my study, it was revealed that by using avenolide bioassay system, 9 among 40 strains might produce avenolide-active compounds (Table 2.4).

Table 2.4 *Streptomyces* strains producing avenolide-active compounds

***Streptomyces* strain**	**Secondary metabolite**	**Avenolide activity (units)**
S. albidoflavus NBRC 12790	Limocrocin*	500
S. albus J1074	Antimycins, Candicidins, Paulomycins, 6-*epi*-alteramides[65]	1000
S. bambergiensis NBRC 13479	Moenomycin[66]	500
S. cyaneofuscatus NBRC 13190		4
S. eurythermus NBRC 12764	Angolamycin*	10
S. griseus NBRC 13189		67
S. griseus NBRC 13350	Streptomycin*, Grisemycin*	7
S. rameus NBRC 13399	Streptomycin*	20
S. sahachiroi NBRC 13928	Carzinophilin*, Azinomycin*	7

* Secondary metabolites as shown in online catalog of NITE Biological Resource Center (NBRC) (Japan)

Comparing to avenolide original producer *S. avermitilis*, which produced approximately 33 units of avenolide, *Streptomyces albidoflavus* NBRC 12790, *Streptomyces albus* J1074, and *Streptomyces bambergiensis* NBRC 13479 showed considerably high avenolide activity (≥ 500 units). On the other hand, the avenolide activity of *Streptomyces cyaneofuscatus* NBRC 13190, *Streptomyces griseus* NBRC 13350, and *Streptomyces sahachiroi* NBRC 13928 are very low (< 10 units).

The *aco/cyp17* cluster was identified in *S. avermitilis* as the essential gene cluster for avenolide biosynthesis.[48] In *S. fradiae* and *Streptomyces ghanaensis*, genes clusters that have high homology and share same gene arrangements to that of the *aco/cyp17* cluster was identified,[48] indicating that these two species might also employ avenolide signaling cascade to control secondary metabolites production. However, result of avenolide assay revealed that neither of them produces avenolide in f-medium. In MM-1 medium (tylosin production medium for *S. fradiae*)[67] and TSB medium (moenomycin production medium for *S. ghanaensis*)[68], avenolide production was also not detected, indicating that the *aco/cyp17* homolog in the genomes of *S. fradiae* and *S. ghanaensis* might not function as avenolide biosynthetic gene.

2.3.3 Distribution of avenolide in endophytic actinomycetes

In recent years, endophytic actinomycetes have proved to be a promising source of bioactive secondary metabolites.[69] Therefore, whether secondary metabolites produced by endophytic actinomycetes are regulated by *Streptomyces* hormones is also worth investigating. In order to answer this question and to have a broader picture about the

distribution of *Streptomyces* hormones in actinomycetes, I selected 11 strains with different morphologies and 16S DNA sequence from my laboratory in-house library of endophytic actinomycetes (Table 2.2) and tested their ability to produce avenolide-active compounds. Among them, three strains showed avenolide activity (Table 2.5). Especially, *Streptomyces* sp. HN70 and *Streptomyces* sp. HN5 had high avenolide activity of 333 units and 500 units, respectively, suggesting that they might be producers of avenolide-type hormones.

Table 2.5 Endophytic actinomycetes producing avenolide-active compounds

Endophytic actinomycetes	**Antimicrobial activity against**	**Avenolide activity (units)**
Streptomyces sp. HN5	*C. albicans, S. cerevisiae*	500
Streptomyces sp. HN46	*B. subtilis , S. cerevisiae*	20
Streptomyces sp. HN70	*B. subtilis*	333

2.4 Summary and discussion

So far, in actinomycetes, the signaling cascade that involves γ-butyrolactone autoregulators (A-factor-type, VB-type, and IM-2-type autoregulators) is one of the most thoroughly studied regulation cascade of secondary metabolite production. A-factor, VB, and IM-2 were first identified in *S. griseus*, *S. virginiae*, and *S. lavendulae* FRI-5 in 1967, 1987, and 1989, respectively, as low-molecular-weight signaling molecules that induce the production of streptomycin, virginiamycin, and blue pigment, respectively, resulting in the general term '*Streptomyces* hormones'.[23,25,30]

The distribution studies of *Streptomyces* hormones are very complicated because they are usually produced only at nanomolar concentrations. Consequently, chemical (colorimetric or enzymatic) or physical (GC or HPLC) means of detection are not effective to detect and quantify the *Streptomyces* hormones in actinomycetes. On the other hand, *Streptomyces* hormones can induce secondary metabolites production in the cognate host at extremely low concentrations and have high host specificity. Therefore, bioassay is the most efficient approach to detect the presence of *Streptomyces* hormones in actinomycetes.

Disruption strains of essential biosynthetic genes for *Streptomyces* hormones are often employed in the distribution study of *Streptomyces* hormones. In these strains, endogenous *Streptomyces* hormones are not produced, enabling them to respond solely to the exogenously added *Streptomyces* hormones. Hence, they are regarded as suitable bioassay hosts. The distribution of A-factor with A-factor-deficient strains of *S. griseus* was previously carried out by Hara and Beppu and by Eritt *et al*., revealing that 14.8% and 24.1% of actinomycetes had the ability to produce A-factor-active compounds.[21,70] A study on IM-2 distribution using *S. lavendulae* FRI-5 (*ΔfarX*) and VB distribution using *S. virginiae* (*ΔbarX*) also revealed that 20% of actinomycetes can produce IM-2 and another 20% can produce VB.[63] A combination of these results (20% for IM-2, 20% for VB, and 24.1% for A-factor) suggests that approximately 64.1% of actinomycetes have the capacity to produce at least one type of *Streptomyces* hormones belonging to the γ-butyrolactone class, confirming that γ-butyrolactone is the most abundant type of *Streptomyces* hormones in actinomycetes.

Considering the remaining 35.9% of actinomycetes that produce neither A-factor, VB, nor IM-2, whether they produce different kinds of *Streptomyces* hormones or do not employ any signaling compounds to control secondary metabolites production remained to be clarified. In the process of addressing this question in *S. avermitilis*, a representative non producer of the γ-butyrolactone *Streptomyces* hormones, avenolide as a novel member of butenolide-type *Streptomyces* hormones was identified.[48] This result suggested that the butenolide-type *Streptomyces* hormones (including SRBs from *S. rochei*) might act as signaling molecules in the remaining 35.9% of actinomycetes. The bioassay result in this chapter revealed that 12 of the 51 strains had the capacity to produce avenolide-active compounds. In addition, among those 12 strains, Southern blot analysis revealed that homologs of the essential biosynthetic gene *aco* were detected in the genome of 11 strains (data not shown), indicating that these strains are likely to produce avenolide-like compounds. This, in turn, implies that approximately 24% (12 out of 51 strains) of actinomycetes might produce butenolide-type *Streptomyces* hormones. Taken these results together, I conclude that most actinomycetes possibly use either γ-butyrolactone *Streptomyces* hormones or butenolide *Streptomyces* hormones to regulate their secondary metabolites production; γ-butyrolactone *Streptomyces* hormones in 64.1% of actinomycetes and butenolide *Streptomyces* hormones in 24% of actinomycetes. The remaining 11.9% of actinomycetes might not employ *Streptomyces* hormones to control secondary metabolism, or they might use other kinds of *Streptomyces* hormones that have not yet been identified.

Among the strains being investigated in this study, five had avenolide activity higher than 200 units. Especially, in *S. albus* J1074 (producer of antimycins, candicidins, and

paulomycins), the highest avenolide activity was detected at 1,000 units. In *S. bambergiensis* (the producer of moenomycin) and *Streptomyces* sp. HN5 (producing antimicrobial compounds against *Candida albicans* and *Saccharomyces cerevisiae*, Table 2.5), high avenolide activity (500 units) were also measured. More experiments is required to identify the avenolide-like compounds produced by these strains and to investigate whether the avenolide-like compounds regulate the secondary metabolism in these strains.

CHAPTER 3

Characterization of avenolide-active compounds produced by *Streptomyces albus* J1074

3.1 Introduction

Members of the genus *Streptomyces* are famous for their capacity to produce a wide variety of secondary metabolites, which have anti-microbial, anti-fungal, anti-parasitic and anti-cancer activities. In *Streptomyces*, secondary metabolism starts only at a certain time point in their life cycles, which has prompted many researchers to investigate the mechanism by which the production of secondary metabolites is initiated. Regulation of secondary metabolism is a complex process controlled by various factors, including external and internal signaling molecules.[61] Antibiotics produced by other *Streptomyces* species or mycolic acids in the cell wall of neighboring bacteria are typical examples of external signals. The angucycline antibiotic jadomycin B from *Streptomyces venezuelae* induces the production of the pigmented antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2),[71] and mycolic acid-containing bacteria, such as *Tsukamurella pulmonis* TP-B0596, trigger the cryptic antibiotics undecylprodigiosin production in *Streptomyces lividans* TK23 by co-cultivation.[72] On the other hand, autoregulators are representatives of internal signals that control secondary metabolism in the same host.[14] The autoregulators have been referred to as *Streptomyces* hormones, because they have activity at considerably

low concentrations (~nM) and induce secondary metabolite production by binding to specific receptor proteins.

Streptomyces hormones are commonly categorized into five groups based on the difference in their chemical structures.[18] Avenolide (**1**) (Figure 3.2A), which was discovered in 2011, is a representative of butenolide-type *Streptomyces* hormone, and can induce the production of the anthelmintic compound avermectin at 4 nM in *Streptomyces avermitilis*.[48] The biosynthesis of avenolide requires the enzymatic functions of Aco (a putative acyl-CoA oxidase) and Cyp17 (a putative cytochrome P450 monooxygenase),[48] and is regulated by the avenolide receptor AvaR1.[56] In Chapter 2, I investigated the distribution of avenolide using an *S. avermitilis aco* disruptant (*S avermitilis* Δ*aco*) as a biosensor to detect avenolide activity. Results in Chapter 2 revealed that 24% (12 out of 51 strains) of actinomycetes have avenolide activity, suggesting that, similar to the γ-butyrolactone-type *Streptomyces* hormones, the butenolide-type *Streptomyces* hormones are commonly produced by actinomycetes as well. These results also indicate that the strains showing avenolide activity may produce avenolide or avenolide-like compounds to control their secondary metabolism.

Streptomyces albus J1074 is a derivative of the *S. albus* strain G and is commonly used as heterologous expression host for biosynthetic genes of secondary metabolites.[73] As previously described in Chapter 2, *S. albus* J1074 had the highest avenolide activity among 51 strains that were investigated. In *S. albus* J1074, many of the identified secondary metabolites such as: albaflavenone,[74] desferrioxamine,[75] isorenieratene,[76] antimycins, candicidins, 6-epi-alteramides, 5-hydroxyectoine, indigoidine, and paulomycin[65] are

unlikely to have avenolide activity. Therefore, in this chapter, I would like to report the isolation of compounds showing avenolide activity in *S. albus* J1074 through gene disruption of the *aco* homolog, and investigate the structure-activity relationship of avenolide. I will also discuss the physiological role of the avenolide-active compounds in the interspecies interaction between *S. albus* J1074 and *S. avermitilis*.

3.2 Materials and methods

3.2.1 Bacterial trains, primers, plasmids and cultivation conditions

S. albus J1074 was grown at 28°C on medium A consisting of 2.1% MOPS (morpholinepropanesulfonic acid) (Nacalai Tesque, Kyoto, Japan), 0.5% glucose (Wako Pure Chemical, Osaka, Japan), 0.05% yeast extract (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.05% meat extract, and 0.1% casamino acids (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with pH 7.0 adjusted by KOH for spore formation.[77] *S avermitilis* Δ*aco*[48] was grown at 28°C on YMS-MC (yeast extract-malt extract-soluble starch medium supplemented with 10 mM MgCl2 and 10 mM CaCl2 medium) for spore formation.[54] *Escherichia coli* DH5α was used for general DNA manipulation, and the DNA methylation-deficient *E. coli* ET12567 (John Innes Centre) was used for *E. coli*/*Streptomyces* conjugation.[78] *E. coli* ET12567 containing pUZ8002 plasmid (*E. coli* ET12567/ pUZ8002), which is derived from the antibiotic multi-resistant plasmid RP4 and can assist in the mobilization of plasmid from *E. coli* to *Streptomyces*. The homologous recombination plasmid, pKC1132, containing *oriT* of RK2 plasmid and an apramycin resistance gene for selection in actinomycetes and *E. coli* was used for gene

disruption.[79] The integration plasmid, pLT129, containing hygromycin resistance gene for selection in actinomycetes and *E. coli* was used for gene complementation.[80] All the primers are listed in Table 3.1.

Table 3.1 Primers used in this study

Primer	**Sequence (5'-3')**
For construction of *S. albus aco* disruptant	
aco-Fw	CCGGATCGGCACGTTCCTGT
aco-Re	CTCCACGTCCGCCATCAGGT
aco-tFw	GAGGAGTTCGGCCACCGGGACTT
aco-tRe	ATGTCGCGTCCGGCTCGCTCT
apr-Fw	CCCCGGCGGTGTGCTG
apr-Re	GACGTCGCGGTGAGTTCAGGC
For genetic complementation of *S. albus aco* disruptant	
aco-comp-nFw	CCGGAATTCTGATCCCCTTCCGCTTTTCGC
aco-comp-nRe	GCTCTAGACGGAGGACGAGAGACGCGAGGA
aco-comp-eFw	CGTGCCGGTTGGTAGGGAGATATGACACATGGTCAGT
aco-comp-eRe	CTTTAGATTCTAGAGCCTCAGCCCGTCATGTCGCGTC
hyg-Fw	CTACGCGGAGCCTGCGGAACGAC
hyg-Re	GAGCAGCGCGGCCAGGATCTCGC

3.2.2. Construction of the *S. albus aco* disruptant

An internal fragment (1,002 bp) of the *aco* homolog gene from *S. albus* J1074 was amplified by PCR with the primer pair aco-Fw/aco-Re, and then cloned into the *Eco*RV site of pKC1132, resulting in pLT166 plasmid, which is used for *aco* disruption in *S. albus* J1074. The pLT166 plasmid was transferred from *E.coli* ET12567/pUZ8002 to *S. albus* J1074 by intergeneric conjugation. The donor *E. coli* ET12567/pUZ8002 harboring pLT166 plasmid was grown in 2xYT medium (containing [in grams per liter] bacto trypton, 16; yeast extract, 10; NaCl, 2.5) to an optical density at 600 nm (OD_{600}) of 0.4-0.6. The cells harvested were washed twice with 2xYT and resuspended in a 2 ml volume of 2xYT. Spore of *S. albus* J1074 was centrifuged to removed glycerol, washed 1 times with 2xYT and resuspended in 500 μl of 2xYT. After incubating at 40^{o}C for 10 minute, 500 μl of the spores was mixed with the *E. coli* donor cells in a ratio of 1:1 (v/v). After incubation at 30^{o}C for 30 min, the mixture was spread on Difco^{TM} ISP medium 2 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 10 mM $MgCl_2$. After incubation at 28^{o}C for 18 h, the plates were overlaid with water containing 1 mg of apramycin and 0.5 mg of nalidixic acid (Wako Pure Chemical, Osaka, Japan) and the incubation was continued at 28^{o}C for 3-4 days. In the *S. albus* J1074 cells where pLT166 plasmid was transferred successfully, the *aco* homolog gene could be inactivated through single-crossover homologous recombination (Figure 3.1). *S. albus* J1074 *aco* disruptant strain (*S. albus* Δ*aco*) was screened by apramycin resistance test and genome PCR analysis. *S. albus* Δ*aco* shown to be stable even after three rounds of sporulation in the absence of apramycin as selection pressure.

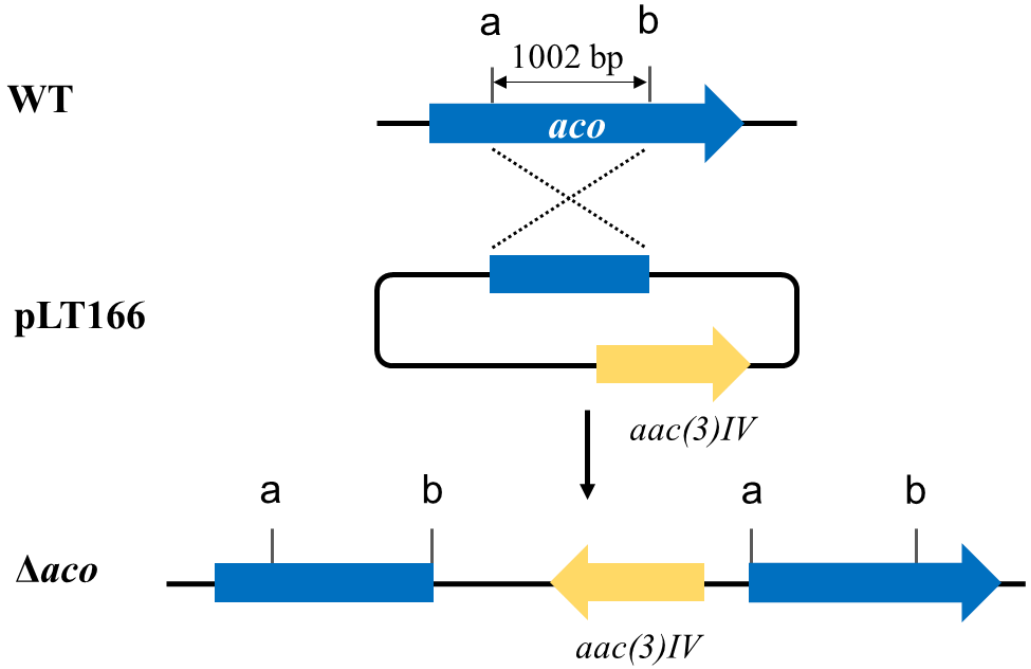


Figure 3.1 Schematic representation of the strategy for *aco* homolog gene disruption in *S. albus* J1074. WT: wild-type strain; Δ*aco*: *aco* disruptant. The *aac(3)IV* genes confer resistance for apramycin.

3.2.3 Genetic complementation of the *S. albus aco* disruptant

For genetic complementation, the integration plasmid pLT129 was used. This plasmid contains an origin of replication, a hygromycin resistance gene for selection, a constitutive and strong promoter *ermEp**, a phiC31 integrase gene and an attP site for integration in the genome of *Streptomyces*. The entire *aco* gene homolog with its 122 bp upstream region was PCR-amplified by the primer pair aco-comp-nFw/aco-comp-nRe. The amplified fragment was digested with *Eco*RI and *Xba*I, and inserted into the *Eco*RI/*Xba*I site of pLT129 to generate pLT167 plasmid. When pLT129 was treated with *Eco*RI/*Xba*I,

the promoter *ermEp** was removed from the plasmid. As a result, in plasmid pLT167, *aco* homolog gene was under the control of its native promoter. To place the *aco* homolog gene under the control of the promoter *ermEp**, the *aco* homolog gene was PCR-amplified by the primer pair aco-comp-eFw/aco-comp-eRe, and then cloned into the *Bam*HI site of pLT129 using a GeneArt Seamless Cloning and Assembly Kit (Life Technologies, Carlsbad, CA) to generate pLT168 plasmid. Each plasmid was introduced into the *S. albus* Δ*aco* strain by intergeneric conjugation and integration. Integration of the plasmid was confirmed by hygromycin resistance and PCR analysis.

3.2.4 Detection of the avermectin-inducing activity of *S. albus* strains

Spores (6.0×10^8 CFU) of the *S. albus* strains were inoculated into 20 ml of f-medium (Chapter 2) in a 100-ml Erlenmeyer flask. Mycelia were harvested after incubation of the flask on a reciprocal shaker (120 spm) at 28°C for 3 days. The seed culture was inoculated into 80 ml of A-3M medium[81] in a 500-ml baffled flask on a reciprocal shaker (120 spm) at 28°C, followed by incubation for 3 days. The culture broth was extracted twice with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated and dissolved in 0.8 ml of methanol. The avermectin-inducing activity (avenolide activity) of each samples was measured by using the indicator strain *S. avermitilis* Δ*aco* as described previously in Chapter 2. Statistical analysis was performed using a Student's *t*-test. A p value < 0.05 was considered significant.

3.2.5 HPLC analysis of metabolite profiles from *S. albus* strains

The ethyl acetate extract for the detection of avermectin-inducing activity was dissolved in 1.6 ml of dimethyl sulfoxide (DMSO), and analyzed by using a high-pressure liquid chromatography (HPLC) system (Agilent Technologies, USA) 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 (15% for 0 to 3 min; 15% to 40% for 3 to 13 min; 40% to 50% for 13 to 33 min; 50% to 90% for 33 to 73 min) containing 0.1% HCOOH (flow rate, 1.2 ml/min; UV detection, 210 nm).

3.2.6 Isolation and structure elucidation of butenolides produced by *S. albus*

The 8-liter culture broth of *S. albus* J1074 was extracted with 2 volumes of ethyl acetate, and the ethyl acetate layer was evaporated to dryness. The crude extract (5.0 g) was subjected to silica gel column chromatography with a step-wise gradient of hexane/ethyl acetate (Kanto Chemical Co., Inc., Japan) at 1:0, 9:1, 8:2, 7:3, 6:4 and 5:5 v/v ratio. Compounds **2** and **3** were eluted in fractions 4 and 5, compound **4** in fraction 3, and compound **5** in fraction 2. Final purifications of compounds **2**, **3** and **4** were achieved by preparative C_{18} HPLC using an XTerra RP_{18} column (5 μm; 10 by 150 mm; Waters, Milford, MA) with 30% CH_3CN/0.1% HCOOH at 3.5 ml/min and detection at 210 nm to yield 2.0, 1.4 and 1.3 mg of purified compounds, respectively. Final purification of compound **5** was also achieved by the same process except that the preparative C-18 HPLC condition was changed to 60% CH_3CN/0.1% HCOOH at a flow rate of 3.5 ml/min and detection at 210 nm to yield 6.0 mg of purified compound **5** from 12 liters of culture broth. High-resolution

fast atom bombardment mass spectrometry (HR-FAB-MS) of purified compounds were 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 ([MeOH]-d_4: δ_C 49.1, δ_H 3.31).

3.2.7 Dual culture assay of *S. albus* strains with *S. avermitilis* Δ*aco*

Spores (3.6×10^9 CFU) of *S. albus* strains were plated on one half of a petri plate (∅60 mm x 15 mm) containing A-3M agar medium, and an equal quantity of spores of *S. avermitilis* Δ*aco* were plated on the other half of the petri plate. After incubating for 8 days at 28^{o}C, the agar culture containing mycelia of *S. avermitilis* Δ*aco* was diced and extracted with an equal volume of methanol. Later, the methanol layer was separated by centrifugation. Avermectin levels were analyzed by using a reversed-phase C_{18}-HPLC system as described previously.[55]

3.2.8 Sample preparation for MALDI-IMS

S. albus J1074 and *S. avermitilis* Δ*aco* were cultivated on cellophane film over the solid medium, as described in the dual culture assay in the previous section. A piece of conductive, double-sided adhesive tape (3M, St. Paul, MN) was used to fix the film dissected with a microtome blade (Leica, Nussloch, Germany) onto the indium-tin-oxide-coated glass slide (100 Ω/sq without MAS coating) (Matsunami Glass, Osaka, Japan). After mounting, the film was coated with 9-aminoacridine (9-AA) (Tokyo Chemical Industry, Tokyo) using a vacuum sublimation system (iMLayer; Shimadzu, Kyoto, Japan) in preparation for matrix-assisted laser desorption/ionization (MALDI). The vacuum pressure

in the chamber was maintained at 10^{-3} Pa during the deposition. Subsequently, the 9-AA was heated to 220°C, and the vapor was deposited on the specimen surface. During sublimation, the thickness of the 9-AA was monitored with transmittance of laser light. When the thickness reached 0.5 μm, the matrix coating was stopped, and the pressure inside the vacuum chamber was released to restore the chamber to atmospheric pressure.[82]

3.2.9 MALDI-IMS using an iMScope

MALDI-IMS was performed with an iMScope (Shimadzu, Kyoto, Japan), which is a scope specially designed for IMS.[83] The instrument could take optical pictures under microscopic views and ion distribution images within the same system. The laser spot size was approximately 12.5 μm (full-width half maximum), and data step intervals of 150 μm in the x-direction and 75 μm in the y-direction were used in this study. A laser irradiated the tissue surface with 80 shots (repetition rate; 1 kHz) for each pixel. Mass spectra were acquired in the negative-ion detection mode for visualization of avermectin B1a with an external calibration method using polyethylene glycol 600 sulfate (Tokyo Chemical Industry, Tokyo). The voltage of the detector was maintained constant at 2.1 kV. The laser power was set at 47 (in arbitrary iMScope units) to maximize the peak intensity derived from avermectin B1a. After obtaining the mass spectra, the peak intensity maps were reconstructed with imaging MS solution (Shimadzu, Kyoto, Japan). The maximum value of the peak intensity map was set as 1000 (in arbitrary units) for the comparison of samples.

3.3 Results

3.3.1 Effect of *aco* gene disruption on avenolide activity in *S. albus* J1074

In Chapter 2, I demonstrated that the ethyl acetate extract of *S. albus* J1074 has the capacity to induce avermectin production in the avenolide-deficient *S. avermitilis* Δ*aco* strain. Moreover, the avenolide activity of this strain (1,000 units) was found to be the highest among all the actinomycetes strains investigated. This result indicates that *S. albus* J1074 has the potential to produce avenolide or other compounds that imitate avenolide activity.

The *aco* gene of *S. avermitilis* is one of the crucial genes for avenolide biosynthesis. It encodes a putative acyl-CoA oxidase that might introduce a double bond between the C-2 and C-3 positions in the structure of avenolide.[48] Besides *aco* gene, the *cyp17* gene encoding for the cytochrome P450 hydroxylase CYP105B2 in *S. avermitilis* also plays important role in the generation of the C-10 hydroxy group in avenolide.[48] After analyzing the genomic data of *S. albus* J1074[84] and searching for sequence homology, one copy of the Aco homolog (XNR_2339) (49% identity and 60% similarity) and one copy of the Cyp17 homolog (XNR_2340) (34% identity and 45% similarity) were found to be encoded in the same locus in the genome of this strain (Figure 3.2B).

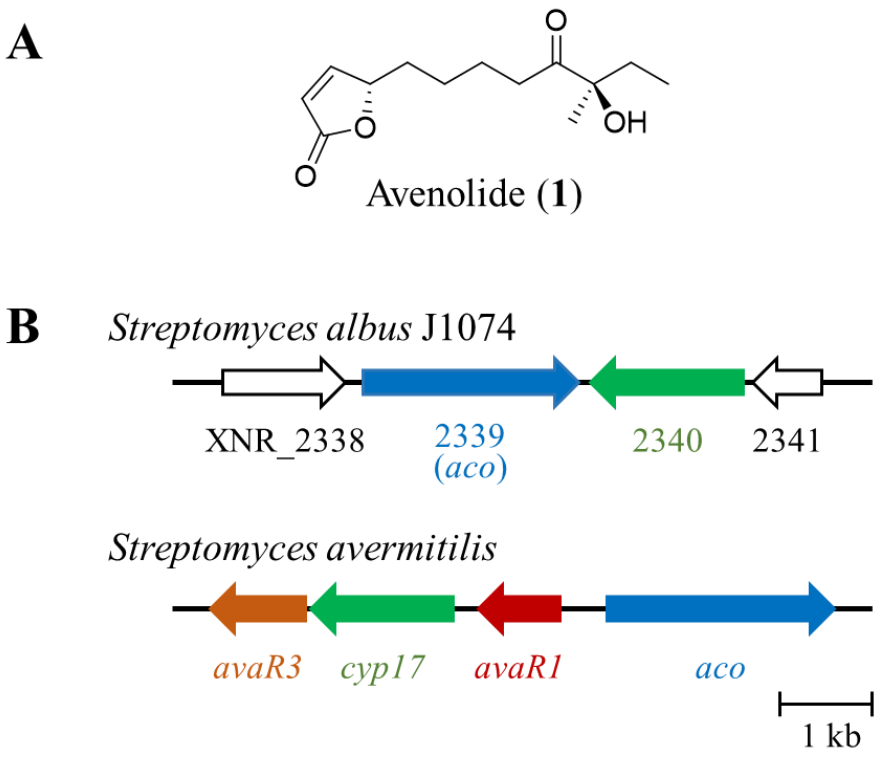


Figure 3.2 Chemical structures of butenolide-type *Streptomyces* hormones avenolide (A) and organization of biosynthetic genes for *Streptomyces* hormones in *S. albus* J1074 and *S. avermitilis* (B). (A) Structure of avenolide (**1**) from *S. avermitilis*. (B) The genes are indicated by arrows. Blue arrows indicate genes encoding an acyl-CoA oxidase, and green arrows indicate genes encoding a cytochrome P450 monooxygenase. *XNR_2338* encodes a putative YihY/virulence factor BrkB family protein, and *XNR_2341* encodes a putative ATP/GTP-binding protein. The *avaR1* gene encodes an avenolide receptor, and the *avaR3* gene encodes a transcriptional regulator for antibiotic production and morphological development.

To investigate the function of *XNR_2339* (designated *aco* homolog in *S. albus* J1074) in the avenolide activity of this strain, the *aco* gene homolog was disrupted by insertional inactivation via a single crossover, resulting in an *S. albus aco* disruptant (*S. albus* Δ*aco*) (Figure 3.3).

To ensure that the change in *S. albus* Δ*aco* was due solely to the *aco* homolog disruption, the genome-integrating plasmid pLT167 containing the *aco* homolog gene with its 122 bp upstream region or pLT168 containing the *aco* homolog gene under the control of the strong constitutive *ermEp** promoter was reintroduced into the *S. albus* Δ*aco* strain, respectively.

The genomes of *S. albus* Δ*aco* and two complement strains were confirmed by PCR analysis (Figure 3.3).

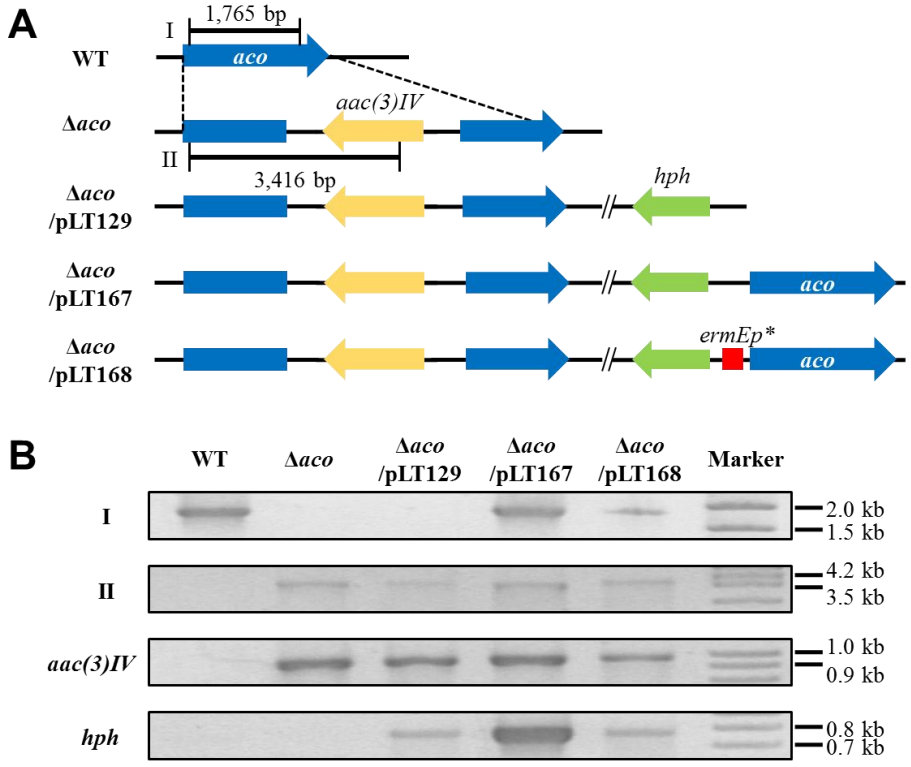


Figure 3.3 Disruption of the *aco* homolog (*XNR_2339*) gene in *S. albus* J1074 and genetic complementation of the *aco* homolog disruptant. WT: wild-type strain; Δ*aco*: *S. albus* J1074 *aco* disruptant; Δ*aco*/pLT129: Δ*aco* strain carrying pLT129; Δ*aco*/pLT167: Δ*aco* strain carrying pLT167, containing the *aco* homolog gene with its upstream region; Δ*aco*/pLT168: Δ*aco* strain carrying pLT168, containing the *aco* homolog gene under the control of the *ermEp** promoter. The *aac(3)IV* and *hph* genes confer resistance for apramycin and hygromycin, respectively. (A) Schematic representation of the genome of the *aco* homolog disruptant and the complementation strains. (B) PCR analysis to confirm the genotype of the *aco* homolog disruptant and the complementation strains. A 1.8 kb internal fragment (I) of the *aco* gene was detected by using the primer pair aco-tFw/aco-tRe. With the primer pair aco-tFw/apr-Fw, a 3.4 kb fragment (II) containing a part of the *aco* gene and pKC1132 was amplified with PCR. The *aac(3)IV* gene (971 bp) was amplified using the primer pair apr-Fw/apr-Re, and the *hph* gene (782 bp) was amplified using the primer pair hyg-Fw/hyg-Re.

Avenolide activity of the *S. albus* Δ*aco* strain was assessed by evaluating the effect of its ethyl acetate extract to the avermectin producing capacity (avenolide activity) of the avenolide-deficient *S. avermitilis* Δ*aco* strain. The *S. albus* Δ*aco* strain did not exhibit any avenolide activity, suggesting that the *aco* homolog gene is involved in the production of avenolide activity (Figure 3.4).

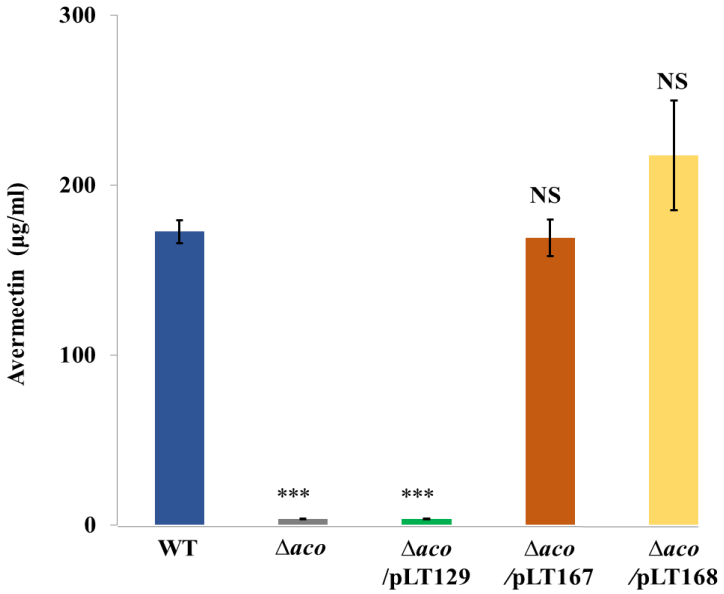


Figure 3.4 Restoration of avermectin production in *S. avermitilis* Δ*aco* with the ethyl acetate extracts from *S. albus* J1074 strains. Larger amounts of avermectin were correlated with higher avenolide activity. Error bars indicate standard deviations from three separate measurements. NS: not significant; ***: $p < 0.001$ for comparison with avermectin production in the wild-type strain. WT: extract of *S. albus* J1074 wild-type strain; Δ*aco*: extract of *S. albus* Δ*aco*; Δ*aco*/pLT129: extract of *S. albus* Δ*aco* strain carrying the control pLT129 plasmid; Δ*aco*/pLT167: extract of *S. albus* Δ*aco* strain carrying pLT167 plasmid, containing an *aco* homolog gene with its upstream region; Δ*aco*/pLT168: extract of *S. albus* Δ*aco* strain carrying pLT168, containing the *aco* homolog gene under the control of the *ermEp** promoter.

To ensure that the abolished avenolide activity was due solely to the *aco* homolog disruption, the avenolide activity of two complement strains Δ*aco*/pLT167 and Δ*aco*/pLT168 was also measured. Results showed that these two strains have avenolide activity level similar to that of the *S. albus* J1074 wild-type strain (Figure 3.4). All of these results clearly suggested that Aco homolog is essential for exerting avenolide activity in *S. albus* J1074.

3.3.2 Metabolite profiling in *S. albus* Δ*aco* strain

Avenolide acts as a signaling molecule to induce avermectin production in *S. avermitilis*. Therefore, I expected that avenolide activity of *S. albus* J1074 would be correlated with the production of secondary metabolites in this strain. To evaluate this hypothesis, I compared the HPLC profiles of the ethyl acetate extract from the *S. albus* J1074 wild-type strain and the *S. albus* Δ*aco* strain. After 3 days of cultivation in liquid culture, several peaks [elution times of 17.1 min (compound **2**), 17.6 min (compound **3**), 18.8 min (compound **4**), and 47.0 min (compound **5**)] of the *S. albus* J1074 wild-type strain had disappeared in the HPLC chromatogram from the *S. albus* Δ*aco* strain (Figure 3.5). The four peaks were also detected in the HPLC chromatogram from the Δ*aco*/pLT168 complementation strain, indicating that Aco is involved in the production of these compounds as well as the production of avenolide or compounds showing avenolide activity in *S. albus* J1074.

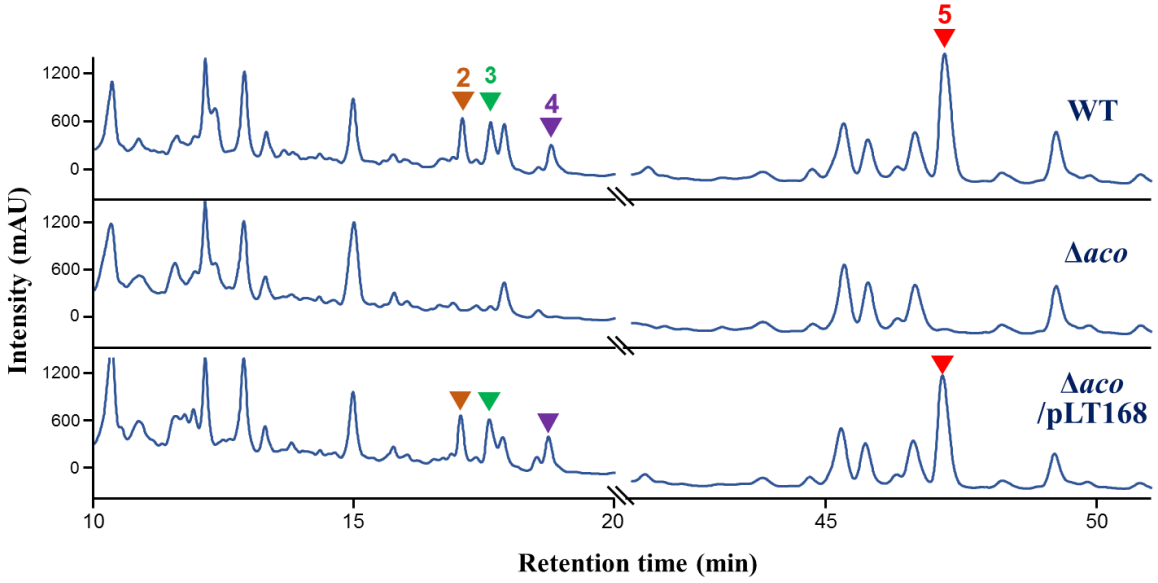


Figure 3.5 HPLC chromatograms of the ethyl acetate extract from *S. albus* strains. WT: wild-type strain; Δ*aco*: *aco* disruptant; Δ*aco*/pLT168: Δ*aco* strain carrying pLT168 plasmid. mAU: milliabsorbance units at 210 nm. Compounds **2**, **3**, **4**, and **5** were detected at the retention times of 17.1, 17.6, 18.8 and 47.0 min, respectively, and are represented by inverted triangles.

These results prompted me to investigate whether the loss of avenolide activity is responsible for the abolished production of compounds **2**-**5**. In order to check this hypothesis, I added the ethyl acetate extract from the culture broth of the *S. albus* J1074 wild-type strain into the culture of *S. albus* Δ*aco* strain. However, the defect in the production of the four compounds was not restored (data not shown), suggesting that the change in the metabolite production of *S. albus* Δ*aco* strain is not due to the impaired

signaling pathways that are correlated to avenolide activity. These results indicated that one or more of compounds **2**-**5** are likely to be chemical entities showing avenolide activity.

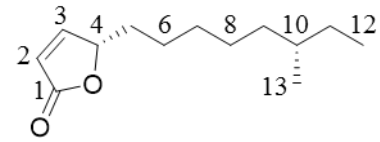
3.3.3 Isolation and structural elucidation of compounds 2-5

To clarify the chemical structure of compounds **2**-**5** that disappeared in the *S. albus* Δ*aco* strain, I purified these compounds from the *S. albus* J1074 wild-type strain and elucidated their structures.

After purification, I obtained 2.0 mg of compound **2**, 1.4 mg of compound **3**, 1.3 mg of compound **4**, and 6.0 mg of compound **5**. They are in the form of colorless oils, and showed an absorption maximum at 210 nm. The molecular formula of **5** was deduced to be $C_{13}H_{22}O_2$ by the HR-FAB-MS analysis (positive ion mode) {*m*/*z* 211.1716 $[M + H]^+$ (calculated exact mass for $C_{13}H_{23}O_2$, 211.1698)}.

The planar structure of compound **5** was established as 4-hydroxy-10-methyl-dodec-2-en-1,4-olide by interpreting the ^1H and ^{13}C NMR spectroscopic data (Table 3.2). Compound **5** was isolated previously from marine-derived *Streptomyces* strains and shown to have a peroxisome proliferator-activated receptor α (PPARα) agonistic activity and an anti-adenoviral activity.[85,86]

Table 3.2 ^{13}C-NMR and ^{1}H-NMR spectroscopic data of compound **5** in comparison with 4-hydroxy-10-methyl-dodec-2-en-1,4-olide

Position	**Compound 5**		 **4-hydroxy-10-methyl-dodec-2-en-1,4-olide**[86]	
	δ_C	δ_H, H, mult (*J* in Hz)	δ_C	δ_H, H, mult (*J* in Hz)
1	175.9		173.2	
2	121.7	6.12, 1H, dd (5.7, 2.0)	121.5	6.11, dd (5.7, 1.9)
3	159.7	7.71, 1H, dd (5.8, 1.5)	156.3	7.45, dd (5.7, 1.5)
4	85.7	5.14, 1H, m	83.4	5.06-5.01, 1H, m
5	34.2	1.85-1.78, 1H, m 1.67-1.59, 1H, m	33.2	1.82-1.62, 2H, m
6	26.2	1.51-1.40, 2H, m 1.40-1.24, 7H, m 1.20-1.07, 2H, m	25.0	1.51-1.38, 2H, m 1.38-1.21, 7H, m 1.18-1.03, 2H, m
7	30.9		29.6	
8	28.1		26.8	
9	37.7		36.4	
10	35.8		34.3	
11	30.7		29.4	
12	11.8	0.92-0.81, 6H, m	11.4	0.87-0.82, 6H, m
13	19.7		19.2	

The molecular formula of compounds **2** and **3** was deduced as $C_{13}H_{22}O_3$ from HR-FAB-MS {*m*/*z* 227.1646 $[M + H]^+$ and *m*/*z* 227.1653 $[M + H]^+$, respectively (calculated exact mass for $C_{13}H_{23}O_3$, 227.1647)}, and the molecular formula of compound **4** as $C_{13}H_{20}O_3$ {*m*/*z* 225.1488 $[M + H]^+$ (calculated exact mass for $C_{13}H_{21}O_3$, 225.1491)}.

A comparison of the HR-FAB-MS and the ^1H NMR data among compounds **2**-**4** and the butenolide compounds identified previously (Table 3.3, 3.4, and 3.5) strongly suggested that the planar structures of compounds **2**, **3**, and **4** were 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide, 4,11-dihydroxy-10-methyl-dodec-2-en-1,4-olide, and 4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide, respectively.

Similar to compound **5**, compounds **2, 3** and **4** were identified from marine-derived *Streptomyces* strains previously.[86–88] In addition, compound **4** was recently isolated in a genetically modified *S. albus* strain.[89] However, compounds **2**, **3**, and **5** have never been isolated from *S. albus* J1074.

Table 3.3 ^{1}H-NMR spectroscopic data of compound **2** in comparison with 4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide

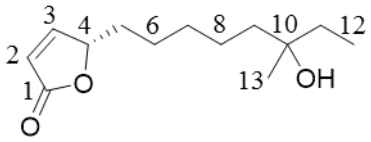
Position	**Compound 2**	 **4,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide**[86]
	δ_H, H, mult (*J* in Hz)	**δ_H, H, mult (*J* in Hz)**
1		
2	6.12, 1H, dd (5.7, 2.0)	6.11, 1H, dd (5.8, 2.0)
3	7.71, 1H, dd (5.7, 1.4)	7.44, 1H, dd (5.8, 1.5)
4	5.14, 1H, m	5.05-5.00, 1H, m
5	1.86-1.78, 1H, m 1.67-1.61, 1H, m	1.84-1.72, 1H, m 1.72-1.61, 1H, m
6	1.53-1.40, 6H, m 1.40-1.34, 4H, m	1.53-1.40, 6H, m 1.40-1.31, 4H, m
7		
8		
9		
10		
11		
12	0.88, 3H, t (7.5)	0.89, 3H, t (7.5)
13	1.11, 3H, s	1.14, 3H, s

Table 3.4 ^{13}C-NMR and ^{1}H-NMR spectroscopic data of compound **3** in comparison with 4,11-dihydroxy-10-methyl-dodec-2-en-1,4-olide

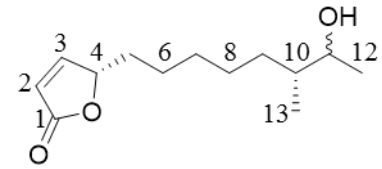
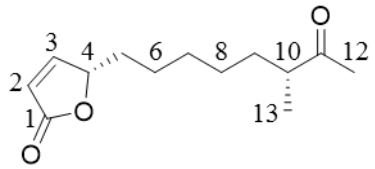
Position	**Compound 3**		 **4,11-dihydroxy-10-methyl-dodec-2-en-1,4-olide**[86]	
	δ_C	**δ_H, H, mult (*J* in Hz)**	**δ_C**	**δ_H, H, mult (*J* in Hz)**
1	176.35		173.12	
2	122.06	6.12, 1H, dd (5.7, 2.0)	121.57	6.11, dd, 1H (5.7, 1.9)
3	160.14	7.71, 1H, dd (5.7, 1.3)	156.20	7.44, dd, 1H (5.7, 1.4)
4	86.11	5.14, 1H, m	83.36	5.06-5.01, 1H, m
5	34.58	1.85-1.78, 1H, m 1.67-1.59, 1H, m	33.16	1.82-1.72, 1H, m 1.72-1.61, 1H, m
6	26.54	3.60-3.55, 1H, m 1.54-1.21, 9H, m	24.94	3.68-3.61, 1H, m 1.59-1.17, 9H, m
7	31.24		29.60	
8	28.69		26.97	
9	34.21		32.33	
10	41.59		39.98	
11	72.72		71.71	
12	19.73	1.09, 3H, d (6.36)	19.51	1.13, 3H, d (6.34)
13	15.42	0.86, 3H, d (6.73)	14.58	0.86, 3H, d (6.69)

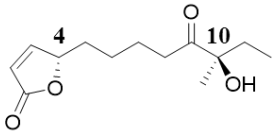
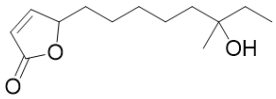
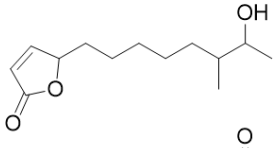
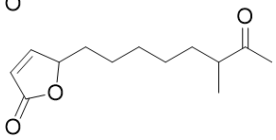
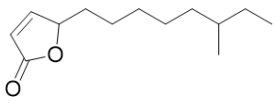
Table 3.5 [1]H-NMR spectroscopic data of compound **4** in comparison with 4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide

Position	**Compound 4**	 **4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide**[86]
	δ_H, H, mult (*J* in Hz)	**δ_H, H, mult (*J* in Hz)**
1		
2	6.12, 1H, dd (5.7, 1.9)	6.11, 1H, dd (5.7, 2.0)
3	7.71, 1H, dd (5.7, 1.3)	7.44, 1H, dd (5.7, 1.4)
4	5.13, 1H, m	5.05-5.00, 1H, m
5	1.85-1.77, 1H, m 1.70-1.58, 2H, m 1.50-1.40, 2H, m 1.40-1.22, 5H, m	1.82-1.72, 1H, m 1.70-1.58, 2H, m 1.51-1.38, 2H, m 1.39-1.21, 5H, m
6		
7		
8		
9		
10	2.57, 1H, m	2.53-2.44, 1H, m
11		
12	2.14, 3H, s	2.13, 3H, s
13	1.07, 3H, d (7.0)	1.08, 3H, d (7.4)

3.3.4 Avenolide activity of compounds 2-5

The chemical structure of compounds **2-5** strongly resembles that of avenolide, indicating that the avenolide activity of *S. albus* J1074 is probably attributable to these compounds. To evaluate the avenolide activity of compounds **2**-**5**, I measured the lowest concentration of each compound (minimum effective concentration) that can induce avermectin production in *S. avermitilis* Δ*aco* strain. All the butenolide compounds (**2** to **5**) showed the ability to induce avermectin production (Table 3.6).

Table 3.6 Minimum effective concentration of avenolide (**1**) and compounds **2-5** in the avenolide assay

Compound		Minimum effective concentration (nM)
Avenolide (**1**)		8
2		6
3		1,000
4		2,000
5		3,000

However, only compound **2** can induce avermectin production in *S. avermitilis* Δ*aco* at nanomolar concentrations (6 nM), which is comparable to the minimum effective concentration of avenolide (8 nM),[48] whereas other butenolide compounds (**3** to **5**) were effective at micromolar concentrations (1-3 μM). Taken together with the fact that both the avenolide activity and the production of compounds **2-5** are simultaneously abolished in *S. albus* Δ*aco* strain, these results suggested that compound **2** is mainly responsible for the avenolide activity in *S. albus* J1074 and indicated that the enzymatic function of Aco is necessary for the biosynthesis of compounds **2-5**.

3.3.5 Avermectin production elicited by side-by-side cultivation with *S. albus* J1074

Compounds **2-5** exhibit avenolide activity to trigger avermectin production in *S. avermitilis*, which implied that these compounds might play roles as communication signals between these two strains. To confirm this hypothesis, I performed dual culture assays of *S. albus* strains with *S. avermitilis* Δ*aco* strain. When the *S. albus* J1074 wild-type strain or *S. albus* Δ*aco* strain carrying pLT168 plasmid (complementation strain) was cultivated on solid medium side-by-side with *S. avermitilis* Δ*aco* strain, avermectin production could be detected (Figure 3.6). In contrast, side-by-side cultivation of the *S. albus* Δ*aco* strain showed no effect on avermectin production in *S. avermitilis* Δ*aco*. Therefore, I concluded that compounds **2-5** synthesized by *S. albus* J1074 wild-type strain are diffused in the medium to elicit avermectin production in *S. avermitilis*.

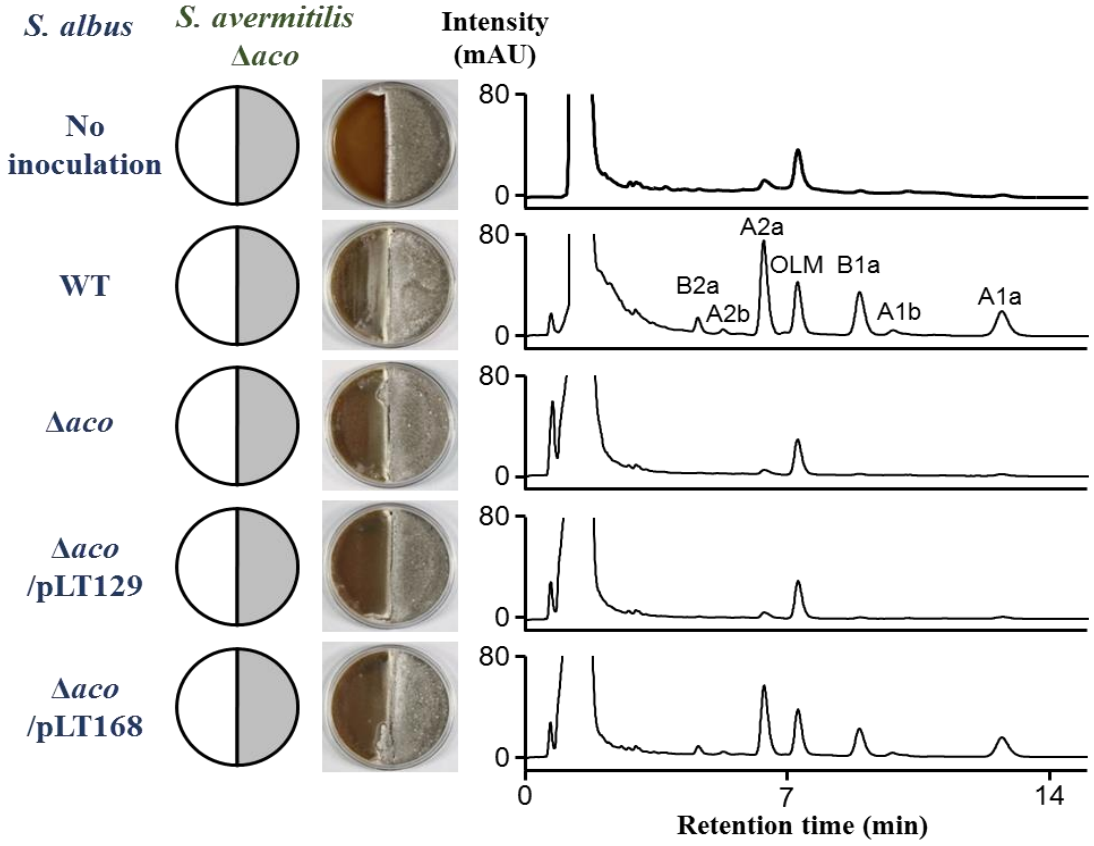


Figure 3.6 Dual culture assay of *S. albus* strains with *S. avermitilis* Δ*aco* strain. Each of the *S. albus* strains was inoculated on the left side of the medium, and *S. avermitilis* Δ*aco* strain was inoculated on the right side of the medium. WT: *S. albus* J1074 wild-type strain; Δ*aco*: *albus* Δ*aco* strain; Δ*aco*/pLT129: *S. albus* Δ*aco* strain carrying the control pLT129 plasmid; Δ*aco*/pLT168: *S. albus* Δ*aco* strain carrying pLT168 plasmid. Photos of plates were taken from above after 8 days of cultivation. HPLC chromatograms for avermectin production in the dual culture assay are shown in the right panels. Individual avermectins were identified using authentic avermectin standards. The peaks eluted at 4.6 min, 5.3 min, 6.4 min, 8.9 min, 9.8 min, and 12.7 min were assigned to the following avermectin derivatives: avermectin B2a, avermectin A2b, avermectin A2a, avermectin B1a, avermectin A1b and avermectin A1a, respectively. The peak eluted at 7.3 min is oligomycin A (OLM).

Next, MALDI-IMS was employed to follow the production of compounds **2-5** and avermectin in the dual culture assay. However, none of the compounds **2-5** was detected by MALDI-IMS (data not shown), presumably because these compounds are produced at very low levels and have low ionization efficiency. In contrast to compounds **2-5**, avermectin B1a could be detected by MALDI-IMS after 6 days of cultivation (Figure 3.7).

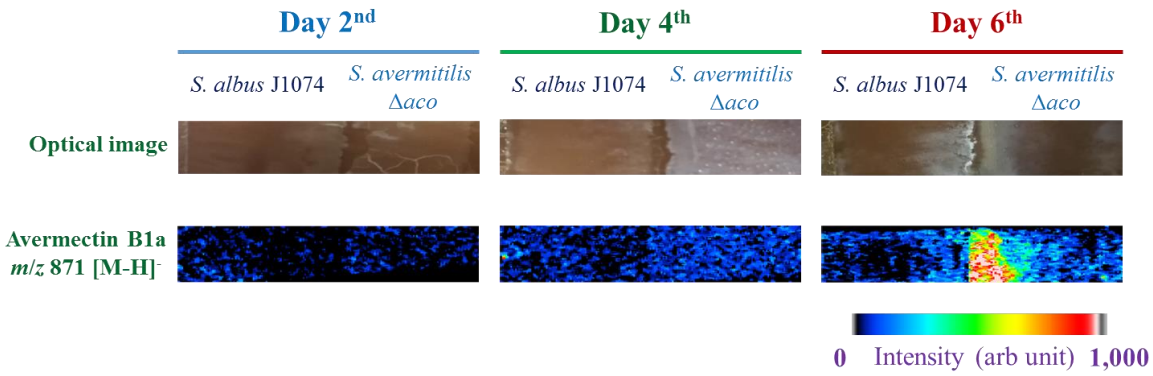


Figure 3.7 MALDI-IMS of avermectin B1a in interspecies interaction. *S. albus* J1074 wild-type strain was cultivated side-by-side with *S. avermitilis* Δ*aco* strain for 2 days (left), 4 days (middle), or 6 days (right). Optical images are shown in the upper panel. Ion abundance of avermectin B1a (*m*/*z* 871.48, $[M - H]^-$) is visualized as a heat map and is shown in the lower panel.

Avermectin B1a produced by *S. avermitilis* Δ*aco* strain was accumulated only at the area closest to *S. albus* J1074. In addition, the concentration of avermectin B1a was dependent on the distance from the edge of the area of *S. albus* J1074 as observed after 6 days of cultivation (Figure 3.7), and increased in a time-dependent manner (Figure 3.8).

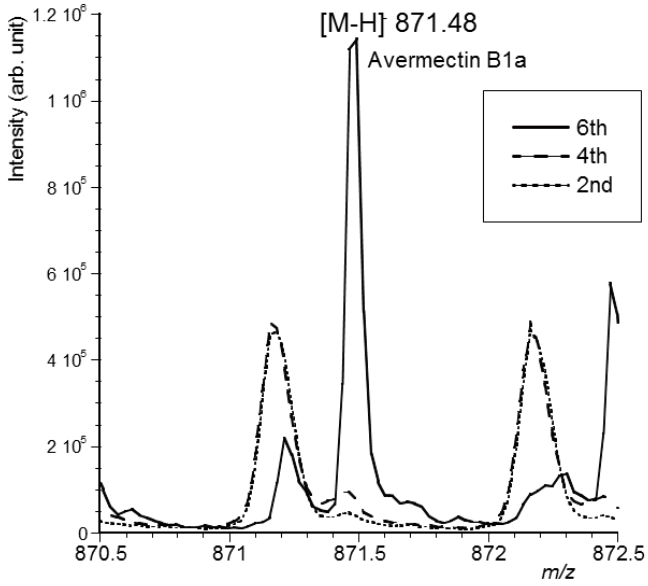


Figure 3.8 Comparison of the ion intensity of avermectin B1a (*m*/*z* 871.48, $[M - H]^-$) in the dual culture assay. Samples were analyzed by MALDI-IMS after 2 days (dotted line), 4 days (dashed line), and 6 days (straight line) of cultivation.

These results suggested that the butenolide compounds **2-5** function as interspecies signaling molecules from *S. albus* J1074 to *S. avermitilis* to affect secondary metabolism of the recipient.

3.4 Discussion

γ-Butyrolactone-type *Streptomyces* hormones are widely distributed among streptomycetes and are regarded as the major signaling molecules that regulate secondary metabolite production and/or morphological development.[61] In recent years, my laboratory discovered that avenolide is a signaling molecule that trigger avermectin production in *S. avermitilis* and belongs to a new class of *Streptomyces* hormones (called butenolide-type *Streptomyces* hormones).[48] In Chapter 2, I found that butenolide-type *Streptomyces*

hormones are also common among actinomycetes, including *S. albus* J1074. In this chapter, I have shown that *S. albus* J1074 produces butenolide compounds (**2-5**) that have different levels of avenolide activity, and that these compounds diffuse in the medium as interspecies signals to elicit avermectin production in *S. avermitilis* cultivated side-by-side with *S. albus* J1074 wild-type strain.

It was reported in a few previous studies that different streptomycetes might employ the same extracellular signals to regulate secondary metabolite production. For example, in *S. coelicolor* A3(2), the γ-butyrolactone-type *Streptomyces* hormone SCB3 induces the production of actinorhodin and undecylprodigiosin.[28] Interestingly, SCB3 also acts as a signaling molecule to trigger jadomycin production in *S. venezuelae*, and combined-culture of the jadomycin-deficient *S. venezuelae* strain and *S. coelicolor* A3(2) leads to the restoration of jadomycin production.[29] Among the butenolide compounds isolated from *S. albus* J1074, compound **2** shows the highest avenolide activity to restore avermectin production in the avenolide-deficient *S. avermitilis* Δ*aco* strain. Compound **4** was isolated by Ahmed *et al* in a mutant *S. albus* strain, in which gene encoding for a putative transcriptional regulator (XNR_3174) was disrupted, and probably induce the production of tetramate macrolactams, candicidins and antimycins A.[89] However, the *in vivo* function of compounds **2-5** in the original producer *S. albus* J1074 remains to be clarified, because no metabolite changes, other than those of compounds **2-5**, were observed in *S. albus* Δ*aco* strain (Figure 3.5). These findings indicate that compound **2** and other butenolide compounds **3-5** are new members of butenolide-type *Streptomyces* hormones, and are likely

to be “one-way” interspecies signaling molecules from *S. albus* J1074 to *S. avermitilis*, unlike in the case of SCB3.

Similar to the extracellular signaling molecules, secondary metabolites themselves can also act as signals to control the growth or secondary metabolism of other streptomycetes. For example, the angucycline antibiotic Jadomycin B produced by *S. venezuelae* positively regulates both morphological development and undecylprodigiosin production in *S. coelicolor* A3(2),[71] and the siderophore desferrioxamine E produced by *Streptomyces griseus* stimulates the growth and morphological development of *Streptomyces tanashiensis*.[90] Recently, Xu *et al*. showed that addition of ivermectin B1a (22,23-dihydroavermectin B1a) to the culture of *S. albus* J1074 induced the production of cryptic secondary metabolites,[91] suggesting that avermectin or avermectin derivatives might function as interspecies signals to awaken the production of cryptic secondary metabolites in *S. albus* J1074. On the other hand, the butenolides **2-5** produced by *S. albus* J1074 may function as interspecies signals to induce avermectin production in *S. avermitilis* (Figure 3.9).

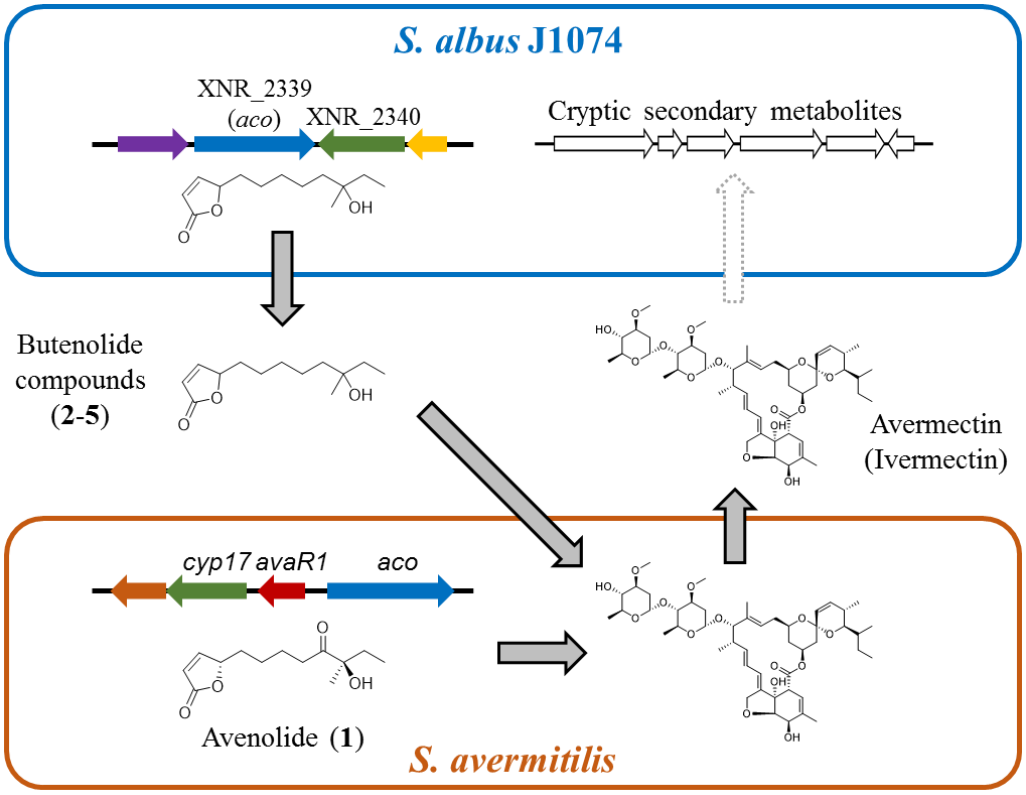


Figure 3.9 The hypothetical metabolite network between *S. albus* J1074 and *S. avermitilis*. *S. albus* J1074 produces butenolide compounds **2-5** through the function of XNR_2339 (Aco homolog) and possibly XNR_2340. These compounds act as external signals to stimulate avermectin production in *S. avermitilis*. Avermectin production is also regulated by avenolide, which is generated by Aco and Cyp17 of *S. avermitilis* and acts as an internal signal. Avermectin or avermectin derivatives might awaken the production of cryptic secondary metabolites in *S. albus* J1074.

The spatial/temporal chemical profiling in microbial colonies could provide valuable information about the interspecies interaction between different microorganisms. In Chapter 3, a mass spectrometry technique, MALDI-IMS, was employed to profile the chemical output from *S. albus* J1074 in an interaction with *S. avermitilis*. The high-

resolution profiles of these metabolites could not be revealed by the usual metabolite analysis. This technique enabled me to observe the secondary metabolites directly from the bacterial colonies. MALDI-IMS analysis revealed that, in side-by-side cultivation, avermectin B1a was accumulated at the edge of the *S. avermitilis* mycelium adjacent to the *S. albus* mycelium (Figure 3.7). The distal mycelium of *S. avermitilis* did not produce avermectin B1a, suggesting that the concentration of the diffused compounds **2**-**5** from *S. albus* J1074 in that area did not reach the threshold to induce avermectin production. In addition, a high level of avermectin B1a production was observed after 6 days of cultivation (Figure 3.7, 3.8). These MALDI-IMS results illustrate that the chemical landscape by the interspecies interaction between *S. albus* J1074 and *S. avermitilis* is dynamic.

Compounds **2**-**5** were isolated in various actinomycetes besides *S. albus* J1074,[86–88] and show a PPARα agonist activity and an anti-adenoviral activity.[85,86] In addition to these bioactivities, in this chapter, I demonstrated the avenolide activity of these compounds with minimum effective concentrations ranging from nanomolar (compound **2**) to micromolar (compounds **3**-**5**). Regarding the structure-activity relationship of these compounds, the presence of the C-10 hydroxy group was suggested to be important for the binding activity of avenolide to the AvaR1 receptor in a previous study.[48] This suggestion agreed well with the result of this chapter that compound **2** containing the C-10 hydroxy group has avenolide activity comparable to that of avenolide, but compounds **3**-**5** lacking the C-10 hydroxy group exhibit very low activity (Table 3.6). This finding confirmed that the C-10 hydroxy group is crucial for the high avenolide activity of compound **2** to induce avermectin

production at nanomolar concentration. On the other hand, the C-9 keto group is unlikely to be involved in the avenolide activity.

In a previous study, *aco* gene homolog in *S. albus* was found to be involved in the biosynthesis of compound 4.[89] Result from this chapter showed that the disruption of the *aco* gene homolog abolishes the production of compounds **2**-**5** in *S. albus* J1074. In addition, my preliminary study in *Streptomyces bambergiensis* NBRC 13479 (which exhibits high avenolide activity of 500 units as described in Chapter 2) revealed that disruption of the *aco* homolog also resulted in complete loss of avenolide activity in *S. bambergiensis* (data not shown), suggesting that the *aco* homologs are commonly involved in the biosynthesis of avenolide-type compounds in streptomycetes. Together with Cyp17, which is involved in the generation of the C-10 hydroxy group, the pair of the *aco* and *cyp17* genes seems to be an important component for the biosynthesis of avenolide-type compounds. The *aco* and *cyp17* homologs are widely spread among streptomycetes, and they are commonly localized in the same locus,[89] suggesting that butenolide-type *Streptomyces* hormones function as a general class of signaling molecules to regulate secondary metabolite production in *Streptomyces* and also to communicate with other *Streptomyces* strains.

In conclusion, in this chapter, compounds **2**-**5** showing avenolide activity were identified in *S. albus* J1074. In addition, compounds **2**-**5** represent signaling molecules in the interspecies interaction between *S. albus* J1074 and *S. avermitilis*. These results contribute to our understanding of the complex chemical interaction among streptomycetes.

CHAPTER 4

General conclusion

Actinomycetes are famous for their ability to produce a large number of useful secondary metabolites, including many antibiotic, antifungal, antiparasitic and antitumor compounds. However, actinomycetes only produce these secondary metabolites at certain time points in the life cycle and/or under certain conditions. This can be explained by the fact that secondary metabolite production in actinomycetes is tightly regulated by complicated mechanisms that have not been fully characterized. One of them is the control by small-molecule autoregulators, called *Streptomyces* hormones. The study of *Streptomyces* hormones will help clarify the complex regulatory mechanism of secondary metabolism in actinomycetes. These finding might also be employed to enhance the yields of secondary metabolite production, which in turn could facilitate the secondary metabolites discovery process.

So far, all of the well-studied *Streptomyces* hormones belonged to the γ-butyrolactones class, which is widely distributed in approximately 64% of actinomycetes. However, in a recent study, our laboratory identified avenolide, a novel *Streptomyces* hormone that has a butenolide backbone, in *S. avermitilis*. *S. avermitilis* uses avenolide to induce the production of avermectin, an important anthelmintic agent that shows efficacy against a wide range of parasitic diseases.

Until recently, *S. avermitilis* was the only known strain that uses avenolide to control its secondary metabolite production. Therefore, whether avenolide-type *Streptomyces* hormones are commonly found in other actinomycetes has attracted my interest. In order to address this question, my study aimed to identify actinomycetes that produce avenolide-type hormones and to investigate the roles of avenolide-type hormones in those actinomycetes.

In Chapter 1, I provided the prerequisite background knowledge, including a summary of the avenolide signaling system, to facilitate the understanding of my work.

In Chapter 2, I summarized my experiments on the distribution of avenolide-type hormones among actinomycetes.

The detection of *Streptomyces* hormones, including avenolide, is often hindered by the fact that they are present only at nanomolar concentrations in culture. However, the cognate host strain can respond to the presence of specific types of hormones at extremely low concentrations. Therefore, a bioassay was the most suitable approach to detect the presence of *Streptomyces* hormones or compounds that mimic their activity.

To identify actinomycetes that produce avenolide-type hormones, an avenolide bioassay system was employed. In this bioassay, a disruptant strain that had lost the ability to produce avenolide and avermectin (*S. avermitilis* Δ*aco*) was used as an indicator strain. This bioassay showed high sensitivity and specificity toward avenolide-type hormones and did not respond to *Streptomyces* hormones belonging to other classes.

Ethyl acetate extracts from 51 strains (40 *Streptomyces* and 11 endophytic actinomycetes strains) were investigated using the avenolide assay. The results showed that 12 of the 51 extracts (24%) contained compounds that can mimic avenolide activity and restore avermectin production in the indicator strain, indicating that 12 strains can produce avenolide-type hormones.

This is the first study on the distribution of avenolide-type hormones. From the results of this study, it appears that not only *S. avermitilis* but also approximately 24% of actinomycetes might produce avenolide-type hormones. Combined with the previous finding that about 64% of actinomycetes produced *Streptomyces* hormones belonging to the γ-butyrolactones class (A-factor, VB and IM-2), the present results provide a clearer picture of the distribution of *Streptomyce*s hormones among actinomycetes.

The avenolide activity detected in my study varied among strains. However, 5 of the 12 strains exhibited very high avenolide activity ($\geq$ 500 units). The avenolide activity was particularly high in *S. albus* J1074 (1,000 units). *S. albus* J1074 is a well-studied strain with accessible genome information and well-established genetic manipulation techniques. Moreover, *S. albus* J1074 can produce many secondary metabolites, such as antimycins, candicidins and paulomycins. Therefore, it is interesting to identify the compounds that are responsible for avenolide activity of *S. albus* J1074 and investigate the roles of those compounds.

In Chapter 3, I characterized the avenolide-active compounds produced by *S. albus* J1074.

Because in *S. avermitilis*, an *aco* gene encoding for an acyl-CoA oxidase is directly involved in avenolide biosynthesis, homologs of *aco* are likely to be present in the genome of *S. albus* J1074. After analyzing the genome information of *S. albus* J1074, I identified one copy of an *aco* homolog. By disrupting this *aco* homolog and comparing the secondary metabolite profiles between the wild-type and disruptant, the *aco* homolog-dependent compounds were identified in *S. albus* J1074.

The result showed that the *aco* homolog is directly involved in the biosynthesis of 4 butenolides, which share a backbone structure similar to that of avenolide. All 4 butenolides showed avenolide activity when tested separately in an avenolide assay, although the units of avenolide activity differed among them. However, no other compound besides these 4 butenolides disappeared in the secondary metabolite profile of *S. albus* J1074 after the *aco* homolog was disrupted, indicating that these 4 butenolides do not play any role in controlling the production of secondary metabolites in *S. albus* J1074.

On the other hand, when *S. albus* J1074 was cultivated side-by-side with *S. avermitilis* Δ*aco* in agar media, it was observed that avermectin production was restored in *S. avermitilis* Δ*aco*, indicating that the 4 butenolides can diffuse in the medium to elicit avermectin production in *S. avermitilis*. Moreover, avermectin B1a was accumulated only in the area closest to *S. albus* 1074, as demonstrated by MALDI-IMS results, suggesting that the 4 butenolides function as interspecies signals from *S. albus* J1074 to *S. avermitilis* to affect secondary metabolism of the recipient. The roles of butenolides compounds as signaling molecules in interspecies interaction were proposed for the first time in my study, providing insights into the complex interaction between different *Streptomyces* species.

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List of publications

1. Nguyen, T. B., Kitani, S., Nitta, H., Tomioka, T. & Nihira, T. Discovering potential *Streptomyces* hormone producers by using disruptants of essential biosynthetic genes as indicator strains. *J. Antibiot.* **70,** 1004–1008 (2017).

2. Nguyen, T. B., Kitani, S., Shimma, S. & Nihira, T. Butenolides from *Streptomyces albus* J1074 act as external signals to stimulate avermectin production in *Streptomyces avermitilis*. *Appl Environ Microbiol.* **84,** e02791–17 (2018).

Acknowledgements

First of all, I would like to express my deepest gratitude to Professor Takuya Nihira for giving me the opportunity to carry out research in Nihira Laboratory and for his valuable support and guidance throughout my research. I also would like to express my sincere appreciation to Associate Professor Shigeru Kitani for patiently guiding me through my Doctor course, giving valuable advices for my experiments and assisting me in writing papers. I also would like to thank Assistant Professor Hiroshi Kinosita for all useful suggestions and fruitful discussions.

I would like to thank my collaborators, Professor Haruo Ikeda from Kitasato Institute for Life Science for providing *Streptomyces avermitilis* KA320, Professor Tohru Nagamitsu from Kitasato University for providing synthetic avenolide, Professor Mervyn Bibb from John Innes Centre for providing *Streptomyces albus* J1074 and Associate Professor Shuichi Shimma for helping me with MALDI-IMS analysis.

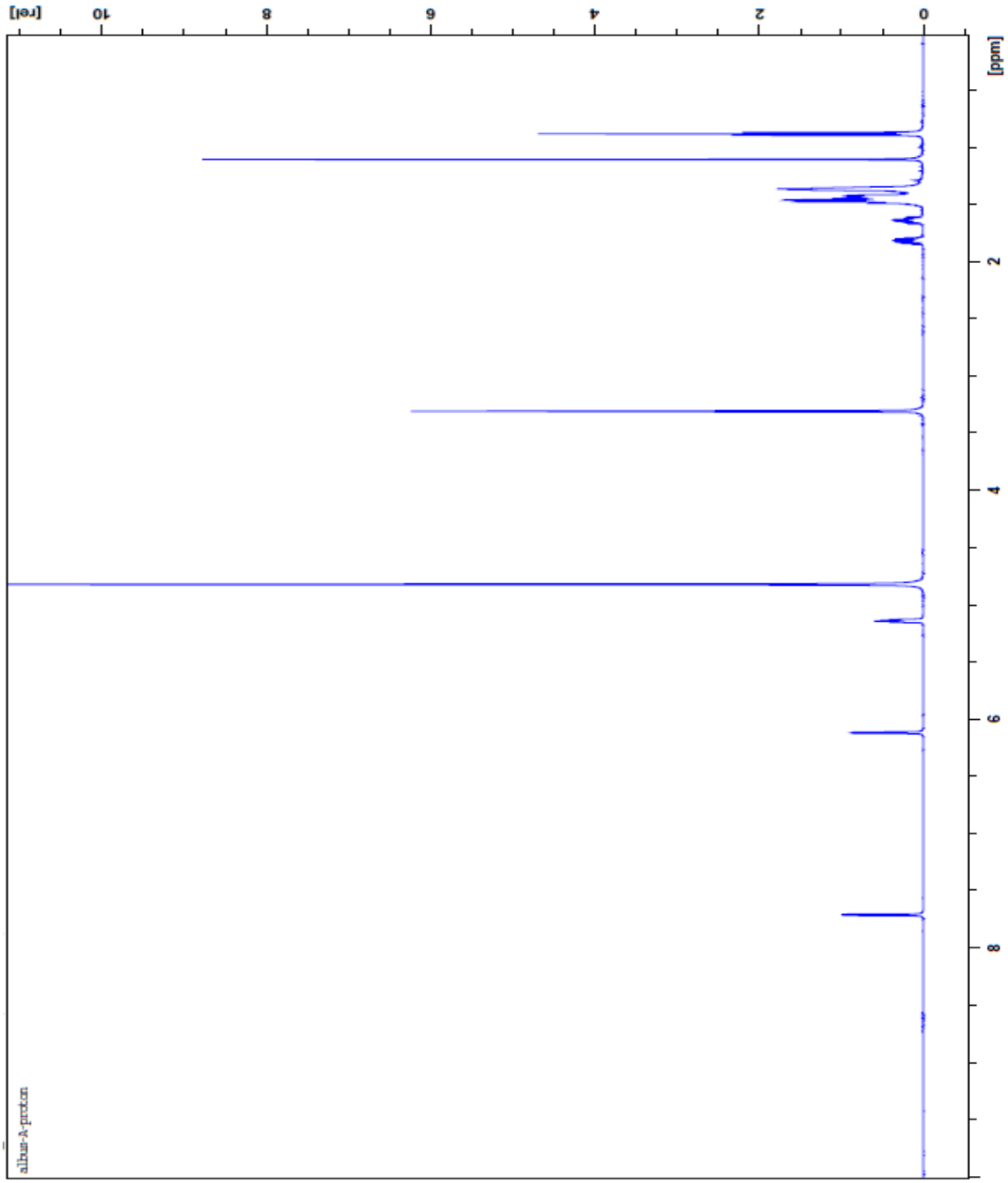
I also would like to express my appreciation to my committee members, Professor Toshiya Muranaka and Professor Hajime Watanabe for their valuable suggestions and insightful comments on my thesis.

I also want to thank my lab mates for teaching me experimental techniques and for sharing valuable knowledge and experiences. I really enjoyed the time that we spent travelling, partying, eating together and appreciate our friendship.

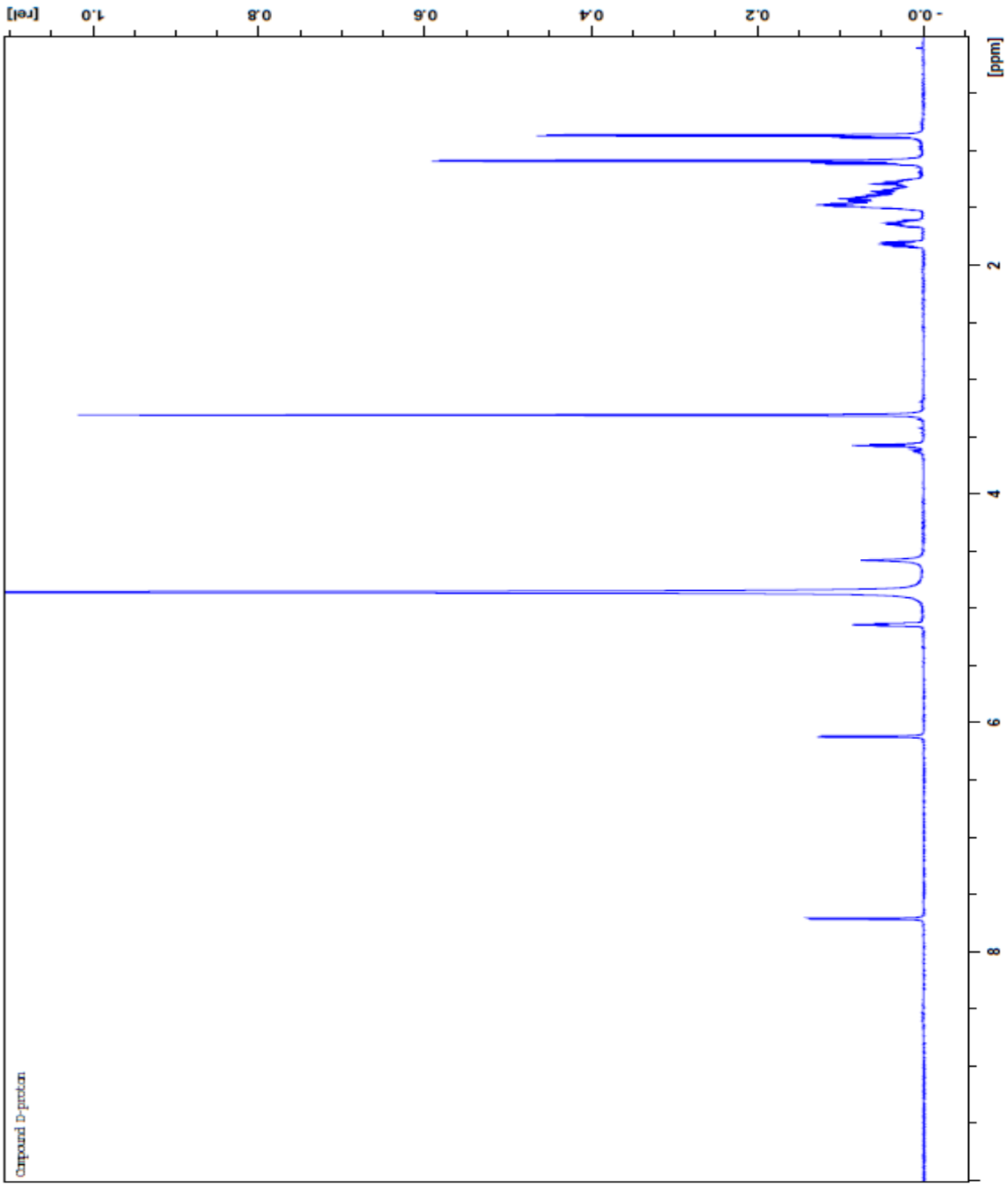
Last but not least, I would like to express my deepest gratitude to my close friends and beloved family who are always besides me, love and support me in every step of the way. I would not have been to where I am now without your support. I also want to thank MEXT Japan for the financial aid so that I could have the opportunity to study at Osaka University.

Appendices

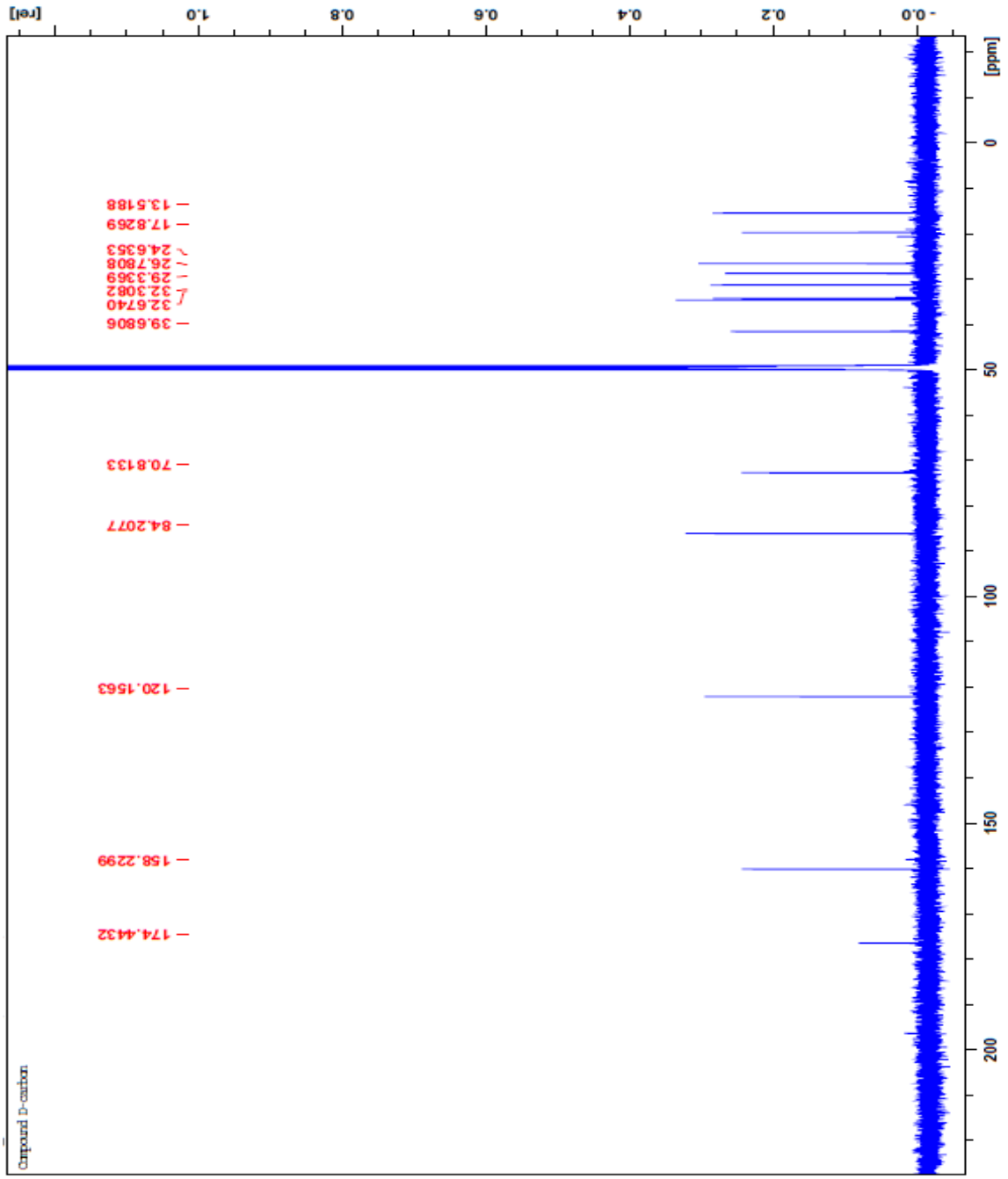
Appendix 1 ^{1}H-NMR spectrum (600 MHz, CD_3OD) of compound **2**



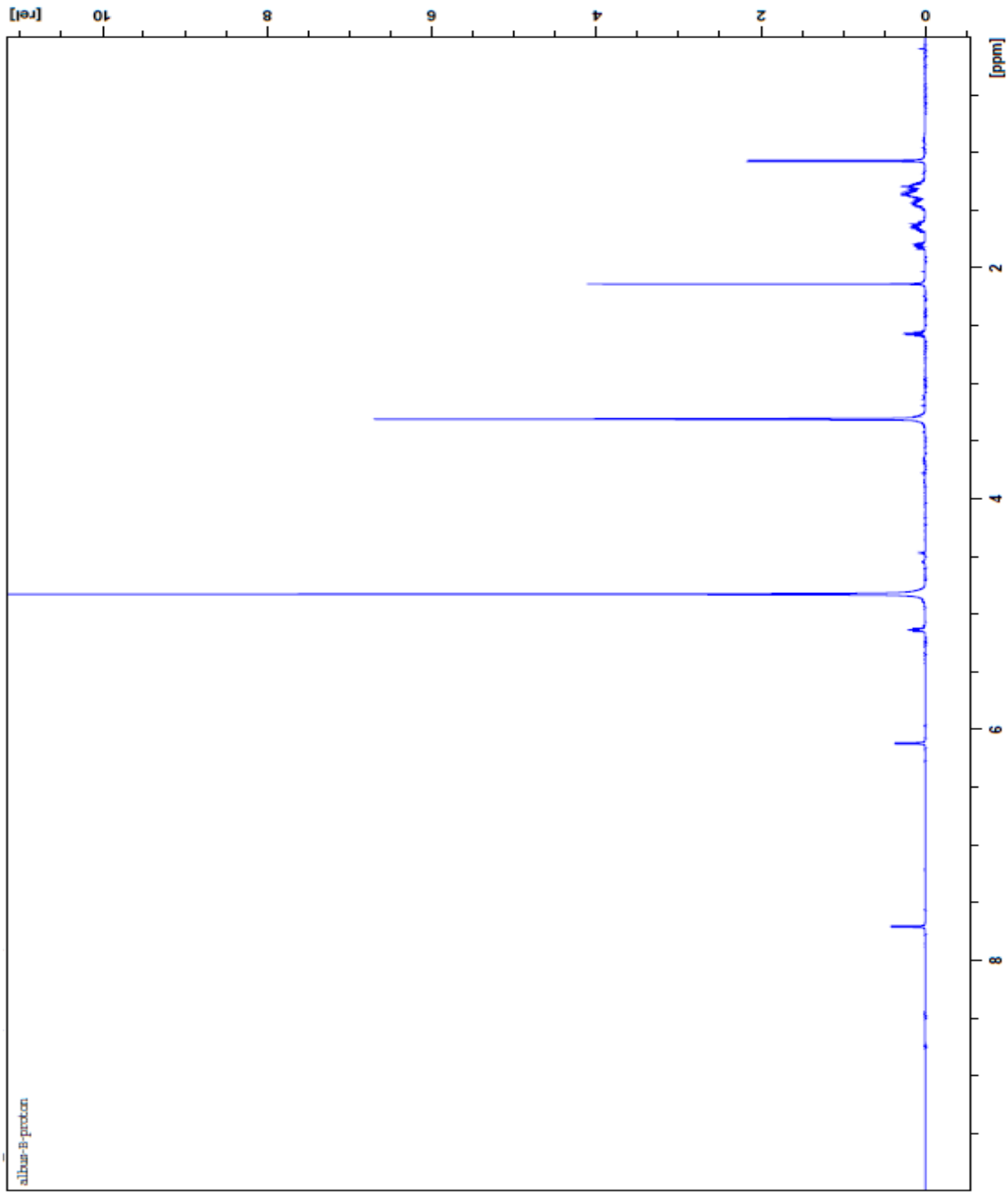
Appendix 2 ^{1}H-NMR spectrum (600 MHz, CD_3OD) of compound **3**



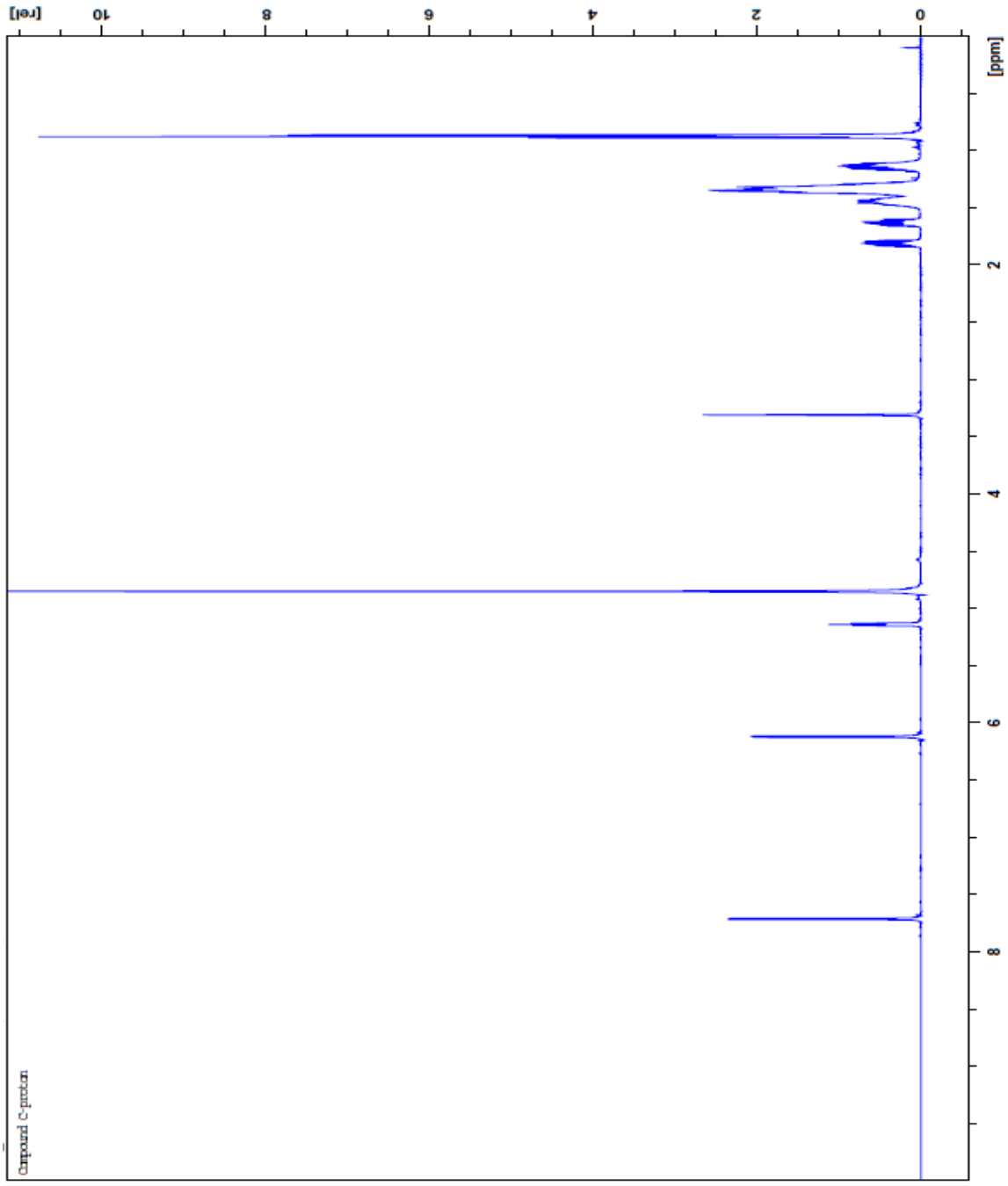
Appendix 3 ^{13}C-NMR spectrum (150 MHz, CD_3OD) of compound **3**



Appendix 4 ^{1}H-NMR spectrum (600 MHz, CD_3OD) of compound **4**



Appendix 5 ^{1}H-NMR spectrum (600 MHz, CD_3OD) of compound **5**



Appendix 6 ^{13}C-NMR spectrum (150 MHz, CD_3OD) of compound **5**