

**CHEMICAL MANIPULATION OF *STREPTOMYCES*'
*SECONDARY METABOLISM***

**USING SMALL MOLECULES TO ALTER SECONDARY
METABOLISM IN *STREPTOMYCES***

By SALMAN AHMED, B.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial
Fulfilment of the Requirements for the Degree of Master of Science

McMaster University © Copyright by Salman Ahmed, July 2012

McMaster University MASTER OF SCIENCE (2012) Hamilton,
Ontario (Biochemistry & Biomedical Sciences)

TITLE: Using Small Molecules to Alter Secondary Metabolism in
Streptomyces

AUTHOR: Salman Ahmed, B.Sc. (McMaster University)

SUPERVISOR: Dr. Justin R. Nodwell

NUMBER OF PAGES: x, 62

ABSTRACT

Secondary metabolites produced by bacterial species serve many clinically useful purposes such as anti-bacterial, anti-cancer, and immunosuppressive agents.

Actinobacteria, particularly the genus *Streptomyces*, have been an abundant source of such metabolites for the past half century. The production of secondary metabolites is controlled through vast regulatory cascades, but the activation and control of these pathways is still poorly understood. This leads to the inability to isolate all of the secondary metabolites that *Streptomyces* are capable of producing. This study focuses on the comparison of synthetic small molecules, which were found to alter the production of secondary metabolites in *S. coelicolor*. A comparative analysis of two of these molecules, ARC2 and ARC6, shows they modulate secondary metabolites in different ways. In a separate study, ARC2 was shown to achieve this phenotype through the inhibition of a target in fatty acid biosynthesis. The results of this study suggest that ARC6 does not have the same target, although it may target the same metabolic system. Furthermore, these two molecules also have opposite effects on *S. coelicolor* development. The cumulative results of this study suggest that ARC2 and ARC6 can act as separate chemical tools in enhancing the understanding of secondary metabolism.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor, Dr. Justin Nodwell, for allowing me to be a part of his lab as an undergraduate and graduate student. Your wisdom, patience, and optimism motivated me throughout my project. Above all, I appreciated your invaluable advice and open door policy when it came to discussing science- and non-science-related matters. It was a privilege to work with you over the past two and a half years.

I would also like to thank my committee members, Dr. Nathan Magarvey and Dr. Michael Surette, for their suggestions and advice during my committee meetings. I was truly astonished at how each of our meetings managed to completely change my perspective on aspects of my project. Your feedback helped shape the course of my project and for that I am extremely grateful.

My sincerest thanks also goes out to my lab mates, past and present. Each and every one of you has affected me and my project in some shape or form. In particular, I would like to acknowledge Arryn Craney for her support from the day I stepped foot into the Nodwell Lab as an undergraduate project student. I could always count on you to lend an ear or piece of advice whenever I managed to get myself into a scientific bind. You even looked out for me when I did not realize that I was doing something wrong – I could not have asked for a better mentor. I would also like to thank Dr. Sheila Pimentel-Elardo for all her advice and patience with me when it came to LC-MS analysis. Your organized and methodical process inspired me to pick up my game.

Finally, I would like to thank my family for their unconditional support. I could always count on your support even when I got lost in my work and would not check in for weeks. My graduate school experience would not have been so pleasant if I was not coming home to each one of you.

TABLE OF CONTENTS

Abstract	iii
Acknowledgements	iv
Table of contents	v
List of Figures	vii
List of Tables	viii
Abbreviations	ix
Declaration of Academic Achievement	x

Chapter 1: Introduction

1.1 <i>Streptomyces coelicolor</i> as a Model for Secondary Metabolism	1
1.2 Secondary Metabolite Interactions	3
1.3 Regulation of Secondary Metabolism in <i>Streptomyces coelicolor</i>	5
1.4 Chemical Manipulation of Secondary Metabolism	10

Chapter 2: Background

2.1 Chemical Screen for Antibiotic Remodelling Compounds	12
--	----

Chapter 3: Materials and Methods

3.1 Actinorhodin and Undecylprodigiosin Assays	17
3.2 Metabolite Profiling	19
3.3 Developmental Gene Expression	21
3.4 Fatty Acid Analysis	21
3.5 Scanning Electron Microscopy	23
3.6 Media Recipes	23
3.7 Strains	24

Chapter 4: Results

4.1 Effects of ARCs on <i>S. coelicolor</i>	25
4.2 Understanding the Structure of ARC6	32
4.3 Does ARC6 Have the Same Target as ARC2?	37
4.4 Effects of ARC2 and ARC6 on <i>S. coelicolor</i>	40

Chapter 5: Discussion

Discussion	51
Conclusion	54
References	56

LIST OF FIGURES

1.1	Life cycle of <i>S. coelicolor</i>	3
1.2	Regulatory pathways influencing actinorhodin production in <i>S. coelicolor</i>	8
2.1	10 of the ARCs selected for further screening	13
2.2	Target of ARC2 in fatty acid biosynthesis of <i>Streptomyces</i>	14
3.1	Schematic representation of typical ACT and RED assays with liquid cultures	19
4.1	ACT and RED assays on the 10 ARCs	27
4.2	Effect of the ARCs on ACT induction in <i>S. lividans</i>	27
4.3	LC-MS traces showing levels of germicidin A and germicidins B/C	28
4.4	Luminescence-based assays for 10 ARCs	30
4.5	Effects of SAR study molecules on ACT production	34
4.6	ACT and RED assays to determine potency of the SAR molecules	35
4.7	Summary of structural determinants of ARC6	36
4.8	Concentration-dependent effect of ARC6 on branched- versus straight-chain FAs	39
4.9	Effect of ARC2 and ARC6 on ACT, RED, and CDA	43
4.10	SEM images of <i>S. coelicolor</i> grown in the presence of DMSO, ARC2, and ARC6	45
4.11	Assays for the effects of ARC2 and ARC6 on developmental genes	46
4.12	Examples of LC-MS traces corresponding to metabolites screened with ARC6	49

LIST OF TABLES

3.1	Strains used in this study	24
4.1	Summary of ARC effects on secondary metabolites and sporulation	31
4.2	Fatty acid composition of <i>S. coelicolor</i> grown with ARC2 and ARC6	38

ABBREVIATIONS

ACN – Acetonitrile

ACT – Actinorhodin

ARC – Antibiotic Remodelling Compound

CCC – Canadian Compound Collection

CDA – Calcium-dependent Antibiotic

DMAE – Dimethylaminoethanol

DMSO – Dimethyl Sulfoxide

FA – Fatty Acid

FAME – Fatty Acid Methyl Ester

FAS – Fatty Acid Synthesis

LC-MS – Liquid Chromatography-Mass Spectrometry

MIC – Minimum Inhibitory Concentration

PPTase – phosphopantetheinyl transferase

RED – Undecylprodigiosin

SAR – Structure-Activity Relationship

SARP – *Streptomyces* Antibiotic Regulatory Protein

SAM – S-adenosylmethionine

SEM – Scanning Electron Microscopy

SPE – Solid Phase Extraction

WAC – Wright Actinomycete Collection

DECLARATION OF ACADEMIC ACHIEVEMENT

All work in this thesis was completed by me except the following: Strains for luminescence-based assays were provided by Arryn Craney from the Nodwell lab. Wild isolate (WAC) strains of *Streptomyces* were collected by the Wright lab. Extraction and quantification of fatty acids was conducted by Sussex Research Laboratories, 100 Sussex Drive, Suite 1120G, Ottawa, ON, Canada.

CHAPTER 1: INTRODUCTION

1.1 *Streptomyces coelicolor* as a Model for Secondary Metabolism

Secondary metabolites are features of living organisms that have been observed since the 19th century when Julius Sachs described them as by-products of primary metabolism which are not used in the formation of new cells¹. Sachs was one of the pioneers in the field of phytochemistry, the study of the chemicals and secondary metabolites produced by plants¹. While phytochemicals were the earliest understood examples of secondary metabolites, the discovery of the antibiotic penicillin from the *Penicillium* genus of fungi shifted scientific interest towards new sources². Selman Waksman's discovery of the antibiotic streptomycin brought focus on the *Streptomyces* genus of Actinobacteria³. Following this crucial scientific finding, *Streptomyces* has become a great source of antibiotics, producing more than half of the ones used in clinics today⁴.

One of the key traits of *Streptomyces* is their relatively complex life cycle (**Figure 1.1**), which starts with a spore⁴. The spore undergoes germination, growing out from the spore by tip elongation and forming a network of filaments called substrate mycelium, where cell division is rare creating cells with multiple chromosomes⁴. Once nutrients or space for growth has become scarce, the colony begins to project extensions upwards known as aerial hyphae which subsequently compartmentalize into spores, thus completing the growth cycle⁴. The aerial hyphae stage of the cell cycle is particularly useful in the laboratory because it coincides with the onset of secondary metabolites

production⁴. The initiation of this stage is dependent on the production of surfactant proteins, SapB or the chaplins, which enable the aerial hyphae to break the surface tension at the colony-air interface and rise into the air⁵. The production of the surfactants relies on a number of *bld* genes, the disruption of which prevents the formation of aerial hyphae and gives *Streptomyces* colonies a “bald” appearance⁶. The final steps of the *bld* signaling cascade involve the expression of *bldM* (dependent on the σ -factor *bldN*) which activates *whiG*, the major aerial hyphae σ -factor⁷. This leads to the subsequent expression of genes involved in compartmentalization and DNA condensation (*whiA*, *whiB*, *whiH*, *whiI*, *ftsZ*), as well as spore maturation (*whiD*, *sigF*, *whiE*)⁷.

In the model organism *S. coelicolor*, the aerial hyphae stage brings about the production of four known antibiotics: actinorhodin (ACT), undecylprodigiosin (RED), calcium-dependant antibiotic (CDA), and methylenomycin⁸. Of these antibiotics, actinorhodin and undecylprodigiosin are particularly useful in laboratory experiments because they produce distinct blue and red pigmentation, respectively⁹. The onset of colour allows for visual observation of ACT and RED production, as well as providing a means to quantify these products spectroscopically.

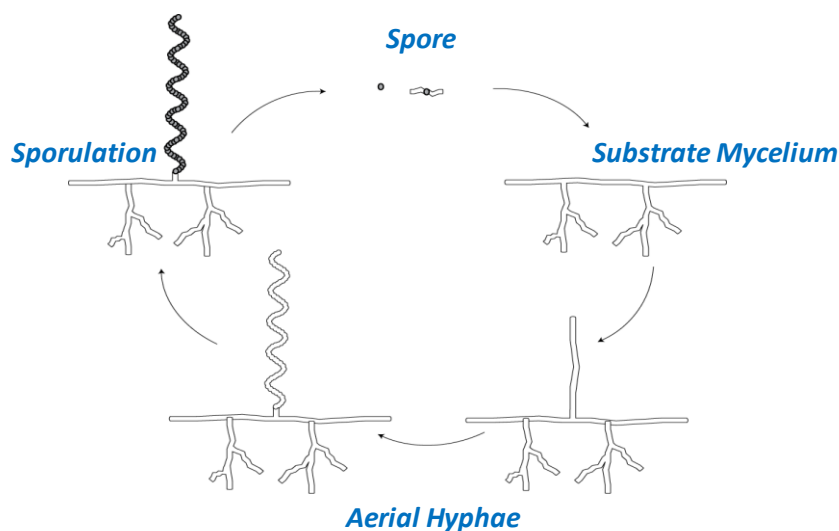


Figure 1.1 – Life cycle of *S. coelicolor*. A colony begins as spore which germinates to form branched mycelium into the growth surface. Upon nutrient depletion, aerial hyphae are extended into the air, subsequently forming into unigenomic compartments. These will eventually mature into spores, completing the cycle. (Image adapted from Leslie Cuthbertson)

1.2 Secondary Metabolite Interactions

While secondary metabolites often fall under categories such as antibiotics, signalling molecules, or pigments, these subdivisions are merely human-assigned labels based on observable effects and features of the compounds¹⁰⁻¹³. The lack of extensive studies of the effects of well-characterized antibiotics below their MIC levels have likely contributed to the poor understanding of the true roles they serve for the producing organisms¹⁴. Goh and co-workers showed quite convincingly that the antibiotics erythromycin and rifampicin, when delivered at sub-inhibitory concentrations, caused significant changes in the gene expression of *Salmonella typhimurium*¹⁵. This study highlighted the potential of some antibiotics to cause gene modulation effects that require further investigation. As such, follow up studies of various antibiotic classes at sub-inhibitory concentrations

showed interesting effects. A study by Hoffman and co-workers showed that sub-MIC levels of the aminoglycoside antibiotic, tobramycin, induced the formation of *Pseudomonas aeruginosa* biofilms that was independent of an increase in the production of alginate – a major extracellular component of the biofilms¹⁶. Furthermore, at sub-MIC concentration, the β -lactam antibiotic imipenem was shown to induce the expression of alginate biosynthesis genes, correlated with increased biofilm volume¹⁷. Such effects caused by antibiotics at the genetic-level highlights the complexity of the role these small molecules play in their natural environments.

Another example of a metabolite serving two seemingly different roles would be hormaomycin, produced by *Streptomyces griseoflavus*¹⁸. At nanomolar concentrations, the molecule has shown to stimulate growth and antibiotic production in various actinomycetes. At similar concentrations, hormaomycin acts a potent narrow-spectrum antibiotic against coryneform actinomycetes¹⁸. An attempt to understand the network of interactions that occurs between bacteria in isolates of soil grains was recently undertaken by Kishony and co-workers¹⁹. While the study does not truly reflect the extent of interactions that occurs in natural environments, nor does it claim to, it gives an excellent account for the influence one microorganism in the environment has on another. This monumental task was carried out by first isolating *Streptomyces* strains from individual grains of soil. Each individual strain was grown on *conditioned* medium which contained excreted metabolites from another strain. Each bacterial isolate acted as a “sender” (the strain that conditioned the medium), as well as the “receiver” (the strain that was grown on the medium). It should be noted that an *interaction* in the Kishony study is assessed as an effect on growth, either positive or negative, caused by

one bacterial strain on another. Therefore, a host of other interactions, which are not manifested as growth alterations, are likely being ignored. The results of the study showed a remarkable diversity in interaction profiles – of 64 isolates tested, at least 42 distinct profiles were observed. Furthermore, interaction profile similarity largely lacked correlation with phylogenetic similarity, assessed through 16S rRNA analysis. The Kishony study provides evidence towards dynamic and complex metabolite interactions in *Streptomyces* communities.

1.3 Regulation of Secondary Metabolism in *Streptomyces coelicolor*

A major driving force behind the discovery of new secondary metabolites from *Streptomyces* has been the sequencing of entire genomes. “Mining” genomes for clusters associated with the biosynthetic machinery required for the production of these metabolites has become a powerful tool in the discovery of novel compounds²⁰⁻²¹. At the time the *S. coelicolor* genome was sequenced, the genome cluster was only known for 5 secondary metabolites – ACT, RED, CDA, hopene, and the gray spore pigment²²⁻²³. In a matter of a decade, the availability of the *S. coelicolor* genome along with more sensitive analytical techniques led to the characterization of at least 14 more²³⁻²⁴. Interestingly, even in a well-studied organism like *S. coelicolor* this only represents about half of the secondary metabolites predicted by the genome sequence²³.

The genomic clusters representing secondary metabolites which are not produced to an observable degree in a laboratory setting have been described as “silent”²⁵. The complexity of the regulatory pathways that control secondary metabolism provides a

challenge in efforts to reliably express these silent clusters. **Figure 1.2** shows the plethora of pathways that exert an effect on expression of ACT in *S. coelicolor*²⁶⁻⁴⁹. The expression of the secondary metabolite clusters is dependent on the *Streptomyces* antibiotic regulatory proteins (SARPs) – metabolite specific activators of the biosynthetic genes²⁸. The SARP responsible for ACT production is ActII-ORF4²⁸, while the SARP for RED is RedD²⁸. These SARPs are under the influence of pleiotropic regulators, which have the ability to alter multiple biosynthetic clusters²⁸. While most of these regulators have been observed to exert an effect on the expression of the ACT, RED, or CDA clusters, there is a significant lack of understanding in the interactions between these regulatory pathways. The activation signals for most of these pathways are also unknown.

One of the better characterized regulatory pathways involves the AfsK/R/S signaling cascade. AfsK is a membrane-bound serine-threonine sensor kinase which interacts with the response-regulator AfsR²⁶. The autophosphorylation of AfsK has been shown to be dependent on its binding to KbpA²⁶. While AfsK acts as the primary kinase in the phosphorylation of AfsR, its phosphorylation can also be mediated by the AfsL and PkaG²⁹. Phosphorylation of AfsR enhances its DNA-binding activity to the promoter of *afsS*, thereby activating the expression of AfsS, which in turn binds and enhances expression of *actII-ORF4* and *redD*²⁶. More recently, this system has been shown to be interconnected with the phosphate-dependent PhoR-PhoP cascade²⁷. This pair is part of a two-component system that enhances the production of ACT and RED in response to a low inorganic phosphate concentration²⁷. The binding site of the response regulator PhoP was found to be located in the promoter region of *afsS*, indicating that PhoP does

not directly enhance *actII-ORF4* or *redD*, but rather, it feeds into the AfsK/R/S system²⁷. This system is an excellent example of the complexity of the regulation of secondary metabolites and shows how many regulatory cascades, that were previously thought to be separate, may in fact be interconnected.

Another well-characterized regulatory pathway for the control of secondary metabolites in *S. coelicolor* is the two-component system AbsA1-AbsA2. Unlike the AfsK/R/S system, the AbsA system negatively regulates its targeted metabolites – ACT, RED, and CDA²⁸. The phosphorylated response regulator, AbsA2, was shown to bind the promoters of the SARPs *actII-ORF4* and *cdaR*²⁸. Furthermore, AbsA2 also represses RED production by blocking the expression of *redZ*, which encodes a response-regulator-type protein involved in the activation of the SARP, *redD*²⁸. While the targets of AbsA2 have been established, the signals acting upon AbsA1 are presently unknown.

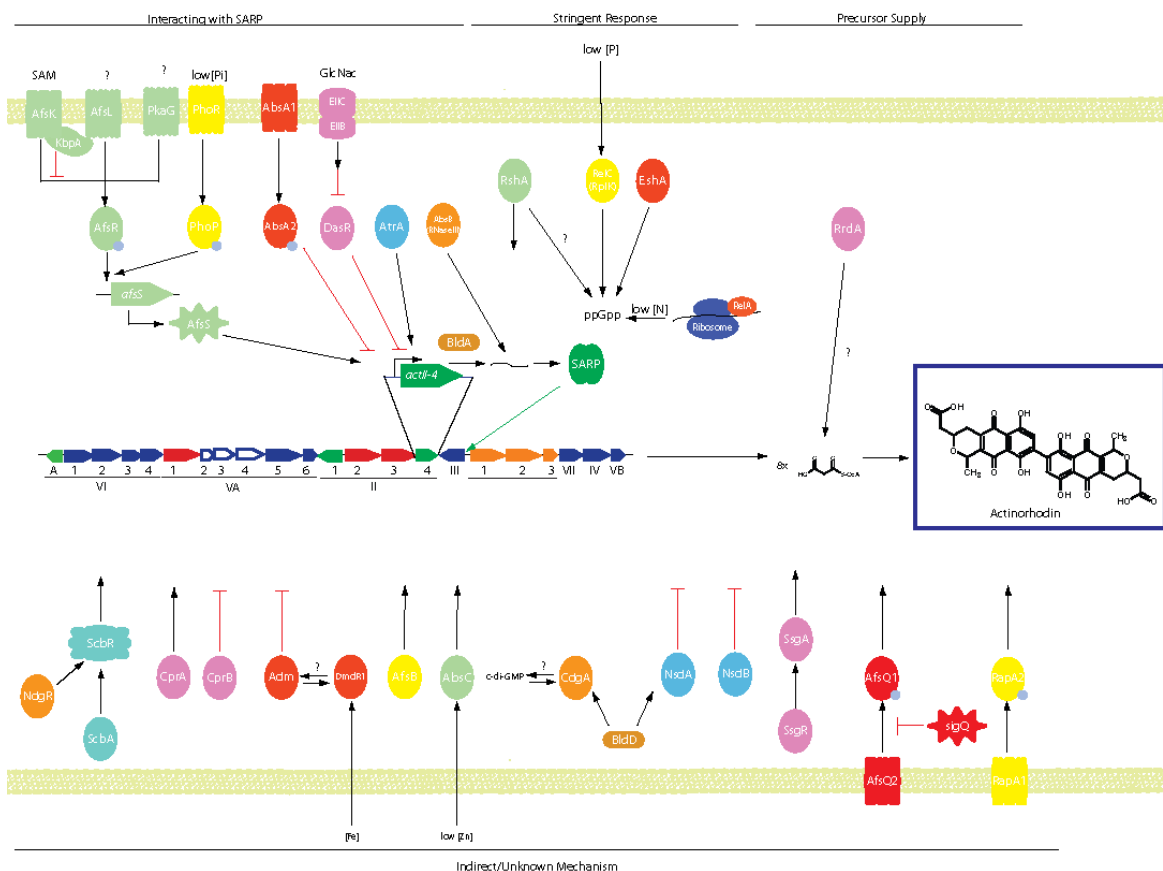


Figure 1.2 – Regulatory pathways influencing actinorhodin production in *S. coelicolor*. So far over 20 distinct regulatory pathways have been implicated in the control of ACT production. While certain pathways have been shown to interact with one another (AfsK/R/S and PhoR/P systems), cross-interaction between most pathways is largely a mystery. Furthermore, the signals that activate most of the regulatory cascades are unknown. ACT serves as an excellent example for the fact that significant probing and investigation is necessary in order to understand the complex nature of the regulation of secondary metabolites. Black arrows indicate activation while red arrows indicate inhibition.

In fact, we have very few clues about the signals that activate secondary metabolism. We know certain nutritional factors tend to affect secondary metabolism in *Streptomyces*. For example, both nitrogen and phosphate have been shown to decrease the production of certain metabolites⁴⁹. However, only a few signalling molecules have been identified that are involved in the regulation of secondary metabolites. The first identified group of these molecules are the 2,3-disubstituted gamma-butyrolactones⁴⁸. One of the better characterized examples of this group is the autoregulatory factor (A-factor) from *S. griseus* which is involved in the regulation of streptomycin. Briefly, A-factor, synthesized by *AfsA*, has been shown to bind the A-factor Receptor Protein (*ArpA*), relieving its repression of the *adpA* gene⁵⁰. *AdpA* is a transcriptional activator of various genes, including *strR* – the pathway-specific activator for streptomycin biosynthesis⁵⁰. A number of genes homologous to *afsA* have been identified in other *Streptomyces*, including *scbA* in *S. coelicolor*⁴⁸. *ScbA* is involved in the synthesis of *Scb1*, a gamma-butyrolactone involved in signalling the increase of ACT and RED⁴⁸. The specific interaction between the gamma-butyrolactones and their receptor protein is unclear thus far, since a crystal structure of the complex does not exist⁴⁸.

Another example of a molecule that has been shown to influence secondary metabolism is S-adenosylmethionine (SAM), which typically acts as a methyl donor in many biological reactions⁵¹. The *metK* gene, which encodes a SAM synthetase in *S. spectabilis*, was found to increase ACT production when introduced into *S. lividans* TK23⁵¹. Follow up studies found that *metK* had similar results in other *Streptomyces*, including increased RED and CDA in *S.coelicolor*, as well as increased pristinamycin IIb in *S. pristinaespiralis*⁵¹. Based on these results, SAM has been proposed as a ligand in the activation of multiple biosynthetic genes⁵¹. It is, however, unclear whether SAM

directly binds regulators in specific secondary metabolite pathways or whether it affects a more general cellular system that leads to a downstream effect on secondary metabolism⁵¹.

The relatively poor understanding of the regulation of secondary metabolism in *Streptomyces* is further complicated when media effects are taken into consideration. For example, *Shu et al.* demonstrated the varying effect of the two-component regulators AfsQ1-AfsQ2 when *S. coelicolor* is grown on rich medium versus minimal medium⁴⁶. *S. coelicolor* AfsQ1-AfsQ2 has been previously shown to induce ACT and RED production in *S. lividans*⁴⁶. In *S. coelicolor*, the disruption of this system caused no effect on ACT, RED, or CDA production on rich medium but caused an array of changes on minimal medium, including decreased production of these antibiotics⁴⁶. It is of course entirely possible that some of the regulatory pathways are intimately linked to primary metabolism, which is heavily affected by the nutrients available in growth medium⁵². An example of this effect can be found in the study by *Capstick et al.* showing the varying importance of the chaplin proteins in *S. coelicolor* development, which are critical proteins on soy flour mannitol medium, but not essential on rich medium⁵. The link between primary and secondary metabolism in *Streptomyces* is scarcely studied and is an interesting avenue for future investigations.

1.4 Chemical Manipulation of Secondary Metabolism

As mentioned previously, the expression of silent genes in *Streptomyces* genomes has proven to be a challenging venture. Recently, *Foley et al.* observed the enhancement of actinorhodin production by *S. coelicolor* using a synthetic small molecule⁵³. During the

course of the study, the lead compound was found to increase actinorhodin levels in culture by a remarkable 800%. The molecule itself was initially selected by the researchers to inhibit the phosphopantetheinyl transferase (PPTase) enzymes of *Bacillus subtilis*, which are responsible for converting the *apo*-form of acyl carrier proteins into the *holo*-form. This disruption was required to improve efficiency of a previous protocol developed by the researchers to label carrier protein domains⁵³. Presumably, the increased level of actinorhodin production caused by the molecule is due to the disruption of specific PPTases of *S. coelicolor*⁵³. This type of approach of changing the secondary metabolite profiles of *Streptomyces* through synthetic chemical means has not been explored and represents an exciting avenue of research.

The interest of the pharmaceutical industry in *Streptomyces* as a source of new antibiotics and secondary metabolites has fallen greatly over the past few decades⁵⁴. However, the advancement of sequencing technology and the subsequent publication of full species genomes has shown that this genus offers many more natural product discoveries²³. The challenge now is to develop novel ways in which to express these secondary metabolites – either through ways which directly enhance their production, or through the better-understanding and manipulation of regulatory networks.

CHAPTER 2: BACKGROUND

2.1 Chemical Screen for Antibiotic Remodelling Compounds

One way of manipulating a cell's regulatory network is through the use of exogenous small molecules. While this method has been shown to have a great impact on ACT production, it has never been utilized as a strategy to enhance secondary metabolism. Perturbing the regulatory networks of *Streptomyces* species offers two benefits: not only would it give the means to probe individual, interconnected sections of the regulatory network, but it may also enhance the expression of otherwise silent or lowly-expressed secondary metabolites. This hypothesis was the driving force behind a chemical screen conducted in this lab by another student, Arryn Craney⁵⁴. The screen consisted of a library of 30 989 molecules from the Canadian Compound Collection (CCC), which were selected because they had been shown to be bioactive or they had drug-like properties; essentially they followed Lipinski's Rule of 5. This rule, proposed by Christopher Lipinski in 1997, describes parameters for molecules that have a tendency to be clinically useful drugs, largely due to their solubility properties⁵⁵. For the screen, colonies of *S. coelicolor* were grown on media plates containing molecules from the CCC. Colonies were grown for several days and the resulting phenotypes were monitored. Several of the molecules were observed to cause an alteration in secondary metabolite production. Of these molecules, 19 were confirmed to increase ACT production and were referred to as antibiotic remodeling compounds (ARC). Upon preliminary follow-up experiments, a number of molecules appeared interesting for several different reasons. Firstly, a group of 4 molecules – the “ARC2 Series” – had relatively similar structures; a core of two

benzene rings joined by an ether group, an electron withdrawing group on one side of the molecule and a tail on the other (**Figure 2.1**). The most potent molecule, however, did not belong to the ARC2 Series and instead was the molecule ARC6. Another smaller cluster of two molecules, ARC15 and ARC17, also had similar structures. Furthermore, many molecules displayed the ability to increase ACT production on multiple media types (R5M and MS media). 10 of these molecules were selected for follow-up experiments as part of this study (**Figure 2.1**).

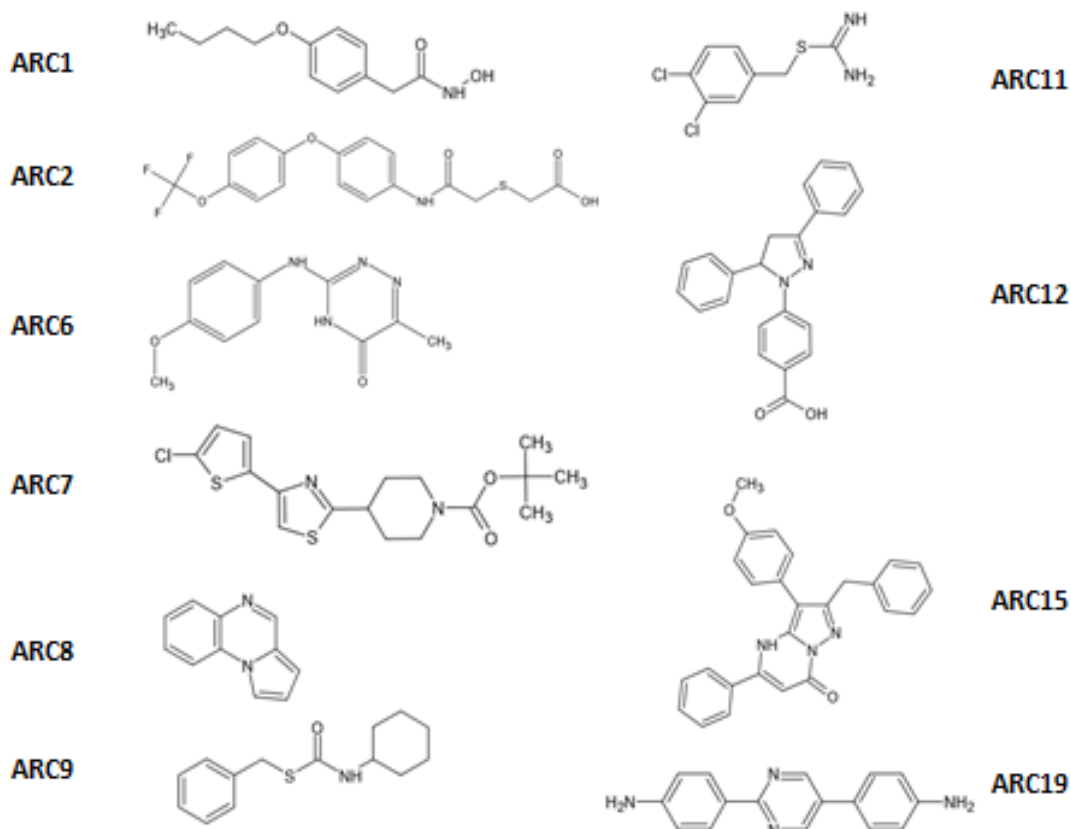


Figure 2.1 – 10 of the ARCs selected for further screening. These molecules were selected from the 19 ARCs in order to isolate a particular molecule for follow-up testing. All of these molecules had either strong ACT induction effects on a particular media, or were found to induce ACT in several different media types.

A separate study from our lab characterized the effects and target of ARC2 – the representative molecule from the ARC2 Series⁵⁴. Briefly, the molecule was found to have an effect on the fatty acid synthesis (FAS) system of *S. coelicolor*, which shares precursor molecules with the polyketide synthesis system. ARC2 weakly inhibits the function of the enoyl reductase, FabI, during fatty acid chain elongation (**Figure 2.2**), resulting in the accumulation of the acetyl-CoA and malonyl-CoA precursor molecules. The increase in the precursor pool forces the molecules to be diverted toward polyketide synthesis, thus resulting in the increased production of polyketides such as ACT. Furthermore, the effects of ARC2 are not limited to *S. coelicolor* as the molecule was found to increase the levels of several polyketides in other *Streptomyces* species, consistent with the conservation of the fatty acid machinery in bacteria.

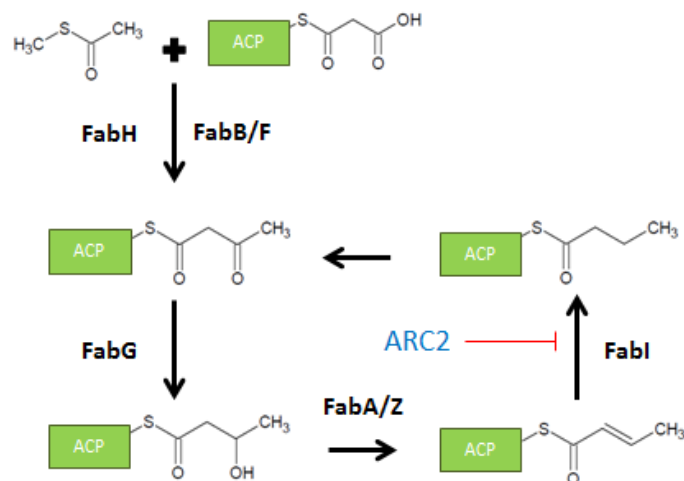


Figure 2.2 – Target of ARC2 in fatty acid biosynthesis of *Streptomyces*. ARC2 has been shown to hinder the function of FabI, an enoyl reductase responsible for reduction of unsaturated bonds during extension stage of fatty acid biosynthesis.

The body of work with ARC2 led to several outcomes that showed the utility of using chemicals to probe secondary metabolism regulation. Firstly, it provided evidence toward the links between primary and secondary metabolism – a concept that remains vague in the *Streptomyces* field. Furthermore, ARC2 was established to be a chemical tool in enhancing the levels of low-expressing polyketides in several *Streptomyces*. Despite the many conclusions that were drawn from the work on ARC2, the molecule is only one of several interesting ARCs that were derived from the chemical screen. The work described in this thesis is based on analyzing the other molecules that increased ACT production in *S. coelicolor*, and characterizing the effect of one in detail, ARC6. In particular, the molecule ARC6 was chosen for detailed follow-up comparisons with ARC2 in order to assess its mode of action as it had the greatest ability to stimulate ACT yields in follow up studies. The current study looked to answer several questions:

1. What are the other effects of the ARCs on *S. coelicolor*, and do any warrant further characterization?
2. What are the structural requirements for ARC6 activation of ACT and how do they compare to those of ARC2?
3. Do ARC2 and ARC6 have the same target pathways in *S. coelicolor*?
4. Can ARC6 alter secondary metabolism in other *Streptomyces* species?

Through these questions, this study is aimed at further chemically probing the secondary metabolite regulatory network of *S. coelicolor*. Furthermore, the detailed analysis of another ARC will establish whether the chemical screen provided more tools for exploring secondary metabolism. This includes gaining a better understanding of

regulation in *S. coelicolor*, as well as potentially using these chemicals in the discovery of novel compounds.

CHAPTER 3: MATERIAL AND METHODS

3.1 Actinorhodin and Undecylprodigiosin Assays

Liquid Culture Preparation

The first step of sample preparation involved the inoculation of R5M media with *S. coelicolor* M145 spores (**Figure 3.1**). In liquid media, M145 is more useful for this type of experiment since it produces ACT and RED faster than *S. coelicolor* J1501. Generally, growth of *S. coelicolor* was observed to be slower, and thus the faster production of ACT and RED in M145 produced a more convenient time frame for these experiments. The large inoculated culture was incubated for 18 hours at 30°C with shaking. After the overnight incubation, the culture was split into nine smaller subcultures. Three of these cultures received 25 µM ARC2, while another three received 25 µM ARC6. DMSO was added to the final three subcultures to represent the negative controls. The subcultures were then incubated for 120 hours at 30°C with shaking, with a sample being collected every 24 hours. These samples were subjected to Total ACT, intracellular ACT, extracellular ACT, and/or RED assays.

Total and Intracellular ACT

In order to measure the total actinorhodin present in the samples, the cells were lysed using 1M KOH. The debris was removed by centrifugation and the absorbance was read at 640nm. Similarly, 1M KOH was used for the intracellular ACT assays. Prior to the

lysis, however, the supernatant was removed in order to remove any extracellular ACT that was present.

Extracellular ACT

The supernatant from the culture samples was used for these assays. The supernatant was first acidified using 1M HCl, which was followed by a chloroform-methanol extraction. The chloroform phase was collected and the absorbance was measured at 542 nm.

Undecylprodigiosin

The pellet from the culture sample was collected and resuspended in acidic methanol. The samples were shaken overnight and the absorbance of the supernatant was measured at 530 nm.

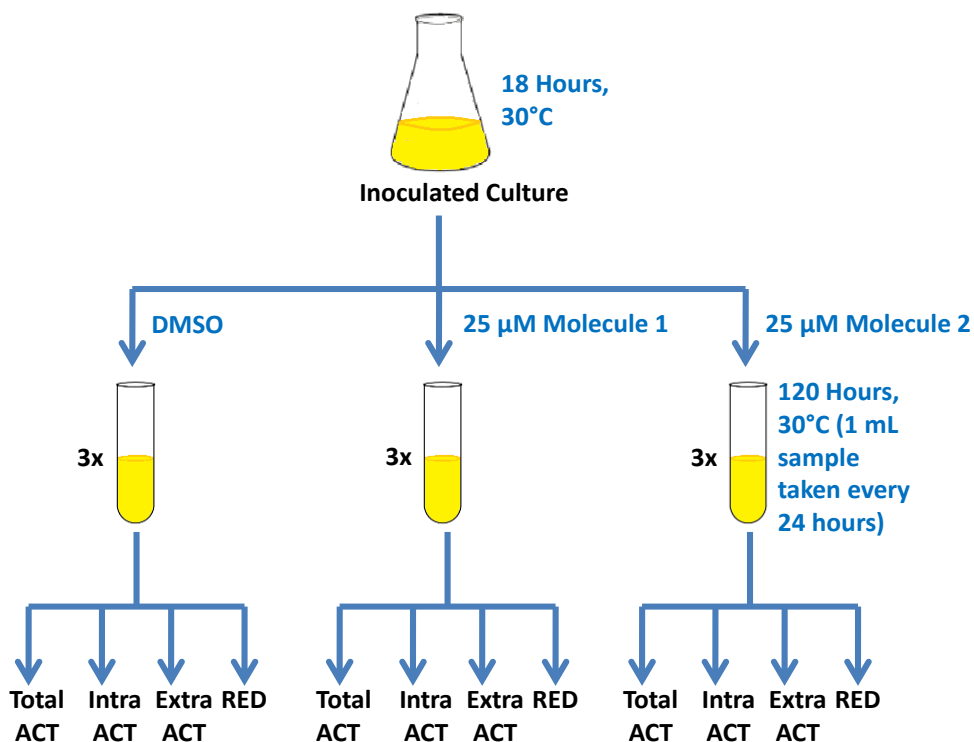


Figure 3.1 – Schematic representation of typical ACT and RED assays with liquid cultures. A flask of media was inoculated with M145 spores and incubated for 18 hours at 30°C while shaking. After the incubation, the culture was split and 25 µM of molecules were added into triplicate subcultures. These subcultures were again incubated at 30°C, this time for a total of 120 hours, with 1 mL samples being collected every 24 hours. Total ACT, intracellular ACT, extracellular ACT, and RED were extracted from these samples, and absorbance readings were taken for quantification.

3.2 Metabolite Profiling

LC-MS Specifications

For all LC-MS experiments performed during this study (with the exception of the fatty acid analysis), the same instruments and specifications were used. The instrument used was the Agilent 1200 “RR” series Liquid Chromatography System connected to the

Bruker microTOF II Mass Spectrometer with ESI ionization. The column used for the study was the Phenomenex Kinetix C-18 2.1x50mm, 2.6 μ M. The LC program itself ran for 15 minutes starting at 5% of solvent A (0.1% formic acid in ACN) and 95% solvent B (0.1% formic acid in water), and running through a gradient to a final mixture of 95% solvent A and 5% solvent B. The flow rate of the solvent was held at 0.2mL/min, while being heated at 40°C. The MS conditions were as follows: capillary voltage = 4.5kV for positive mode; nebulizing gas pressure (N_2) = 3 bar; dry gas flow rate (N_2) = 6mL/min; temperature = 200°C; scan rate = 1 Hz.

Preparation of Cultures and Extractions

Strains were inoculated on 60 mm petri dishes containing solid R5M medium. DMSO, 10 μ M ARC2, or 50 μ M ARC6 was added to these plates during the inoculation. The plates were incubated at 30°C for a specific number of days (typically 3 and 7), following which they were extracted overnight with butanol. After extraction, the solvent was filtered and evaporated *in vacuo*. The pellet was then resuspended in 1:1 ACN:water.

Preparation of CDA Extracts

CDA extracts were prepared using the protocol by *Hojati et al. (2002)*⁵⁶. Briefly, *S. coelicolor* M145 was inoculated on petri dishes and incubated at 30°C for 7 days. The dishes were then frozen at -20°C overnight, after which they were allowed to thaw. The exuding liquid was collected and run over a StrataX Reversed Phase C-18 SPE column. The column was washed with water and eluted with MeOH. The eluant was evaporated *in vacuo*, and resuspended in 1:1 ACN:water.

3.3 Developmental Gene Expression

Luminescent Strains

The developmental gene expression assay was performed using *S. coelicolor* M145 strains containing an integrated *luxCDABE* cassette (**Table 3.1**). The strains were obtained from Arryn Craney.

Gene Expression Assay

The developmental gene expression assay was performed in a 96-well plate format. Each replicate well contained either 25 μ M ARC2, 25 μ M ARC6, or DMSO. An equivalent number of spores from a particular gene-*lux* fusion strain were added to each well. The plates were incubated at 30°C and luminescence readings were taken throughout the day. The readings were taken using the Perkin Elmer Victor X Plate Reader.

3.4 Fatty Acid Analysis

After sample preparation, the fatty acid extraction and analysis steps were conducted by Sussex Research Laboratories, 100 Sussex Drive, Suite 1120G, Ottawa, ON, Canada.

Sample Preparation

Samples were prepared by inoculating a 300 mL starter culture of *S. coelicolor* M145 in R5M media. The culture was split into 50 mL subcultures after 18 hours of incubation (30°C with shaking) and 25µM ARC2, ARC6, or DMSO was added. Cultures were incubated for a further 72 hours, following which cells were pelleted for fatty acid extraction.

Hydrolysis, Extraction, and Derivatization

100µL of ISWS-1 (25 µg/mL C16:0-d31 in ACN) was added to approximately 20-40 mg of sample pellet. The solvent was evaporated under a stream of nitrogen, following which 0.5 mL of 50:50 1.2 N NaOH:MeOH was added and incubated at 105°C for 45 minutes. After cooling, 0.5 mL of 2M phosphoric acid was added and the samples were extracted twice with 1 mL of hexane. The hexane layer was collected and evaporated to dryness under a stream of nitrogen. In order to derivatize the fatty acids into fatty acid methyl esters (FAME), 20µL of CMP/TEA (1/1.14 mg/mL in ACN) was then added to the dried samples. 40µL of dimethylaminoethanol (DMAE) was added to the samples, which were then incubated at 60°C for 90 minutes. Calibration standards and blanks underwent the same hydrolysis, extraction, and derivatization procedure.

LC-MS/MS Analysis

Quantification of FAMEs was conducted using an LC-MS/MS system utilizing a Phenomenex Kinetix C8, 100 x 2.1, 2.6µ HPLC column attached to an API-2000 MS/MS

system. The mobile phases were composed of Milli-Q water, ammonium acetate, acetonitrile, and formic acid. The calibration curve range was 0.1 to 5.0 µg total mass for each fatty acid.

3.5 Scanning Electron Microscopy

In preparation for SEM imaging, *S. coelicolor* M145 colonies were grown on solid MS media containing DMSO, 25 µM ARC2, or 25 µM ARC6 for 5 days. After incubation, colonies were submerged in fixative (2.5% gluteraldehyde) overnight. Upon fixation, the surfaces of the colonies were coated in a film of gold and viewed using a Vega||LSU scanning electron microscope at 6000x magnification.

3.6 Media Recipes

R5-Maltose (R5M):

Maltose	100 g
K ₂ SO ₄	0.25 g
MgCl ₂ · 6H ₂ O	10.12 g
Difco Casamino Acids	0.10 g
Difco (Bacto) Yeast Extract	5.00 g
TES	5.73 g
Distilled Water	1.00 L

Added after autoclaving:

Trace Elements	2 mL
0.5% KH ₂ PO ₄	10 mL
5M CaCl ₂	2 mL
20% L-Proline	15 mL
1M NaOH	4 mL (adjusted for final pH of 7)

Mannitol Soy Flour (MS)

Soy Flour	20 g
Mannitol	20 g
Tap Water	1.00 L

3.7 Strains**Table 3.1 – Strains used in this study**

Strain	Notes
ACT and RED Assays	
<i>S. coelicolor</i> J1501	SCP1 ⁻ , SCP2 ⁻ , <i>hisA1</i> , <i>uraA1</i> , <i>strA1</i> , Pgl ⁻
<i>S. coelicolor</i> M145	SCP1 ⁻ , SCP2 ⁻
<i>S. lividans</i> 1326	-
Luminescence Assays	
<i>S. coelicolor</i> M145 pMU1Flux- <i>hrdBp</i>	Derived from <i>Craney et. al (2007)</i> ⁵⁷ ; <i>luxCDABE</i> operon in opposite direction
<i>S. coelicolor</i> M145 pMU1Flux- <i>cpkp</i>	Derived from <i>Craney et. al (2007)</i> ⁵⁷ ; <i>luxCDABE</i> operon in opposite direction
<i>S. coelicolor</i> M145 pMU1Flux- <i>blbNp</i>	Derived from <i>Craney et. al (2007)</i> ⁵⁷ ; <i>luxCDABE</i> operon in opposite direction
<i>S. coelicolor</i> M145 pMU1Flux- <i>whiHp</i>	Derived from <i>Craney et. al (2007)</i> ⁵⁷ ; <i>luxCDABE</i> operon in opposite direction
<i>S. coelicolor</i> M145 pMU1Flux- <i>whiEp</i>	Derived from <i>Craney et. al (2007)</i> ⁵⁷ ; <i>luxCDABE</i> operon in opposite direction
Metabolite Profile Screen	
<i>Streptomyces pristinaespiralis</i>	Pristinamycin IA, IIA producer
<i>Streptomyces aureofaciens</i>	Chlorotetracycline producer
<i>Streptomyces avermitilis</i>	Avermectin, oligomycin producer
<i>Saccharopolyspora erythraea</i>	Erythromycin producer
<i>Streptomyces griseus</i>	Streptomycin producer
<i>Streptomyces peucetius</i>	Daunorubicin, doxorubicin producer
<i>Streptomyces roseosporus</i> NRRL 15998	Daptomycin producer
<i>Streptomyces</i> sp. SPB74	Mycangimycin producer
<i>Streptomyces viridochromogenes</i>	Various characterized secondary metabolites (avilamycin)
<i>Streptomyces</i> sp. Wat1	Wild Isolate (Wright Actinomycete Collection)
<i>Streptomyces</i> sp. Ja2B	Wild Isolate (Wright Actinomycete Collection)

CHAPTER 4: RESULTS

4.1 Effects of ARCs on *S. coelicolor*

Based on a preliminary screen of the ARCs, 10 molecules were selected for a follow-up survey (**Figure 2.1**). These molecules were selected based on the initial screen during which they were either capable of inducing ACT on multiple different media, or they strongly induced ACT on one particular media. Furthermore, ARC15 was selected because it belonged to a cluster of two analogues amongst the 19 ARCs. The purpose of this follow-up was to characterize the variety of effects these molecules have on secondary metabolism, as well as to select a particular molecule for a comparison with ARC2. The survey consisted of testing the effects of the 10 ARCs on a number of *S. coelicolor* secondary metabolites: ACT, RED, the germicidins, as well as the gene expressions of *cpk* (encoding a polyketide associated with a yellow pigment) and *whiE* (encoding the grey spore pigment).

4.1.1 Effects of 10 ARCs on secondary metabolites

The effect of the molecules on ACT production was assessed visually on solid media (**Figure 4.1A**). The strain chosen for this experiment was *S. coelicolor* J1501. In prior experiments, this strain grew at the optimal rate for visual observation of the blue pigmentation caused by ACT production. J1501 colonies were grown in the presence of increasing concentrations the molecules – 0, 1, 2.5, 5, 10, and 25 μM – for a total of 96 hours. All 10 of the molecules were observed to induce ACT levels, as expected since they were all hits from the initial screen. On the other hand, ARC2 and ARC7 (to a

lesser degree) were the only two molecules that induced ACT production on MS media. The effect of the molecules on the regulation of ACT was also tested in *S. lividans* (**Figure 4.2**). This species, while possessing the ACT gene cluster, does not normally produce the metabolite in laboratory conditions. Therefore, an induction of the pigmented antibiotic implies a change in its regulation. After 5 days of growth, only ARC2 induced the production of ACT to an observable degree (**Figure 4.2**). After several more days, ARC7 caused a slight induction as well, but the effects were very minute. RED production in the presence of these molecules was tested in *S. coelicolor*. The assay was conducted using liquid cultures and RED was measured spectroscopically (**Figure 4.1B**). When compared to the DMSO control, ARC2, 7, 8, and 19 all decreased RED production, while only ARC6 and ARC11 induced it.

The levels of germicidins were assessed using LC-MS. *S. coelicolor* spores were plated on R5M media and grown for 3 and 7 days. Following the incubation, the plates were extracted with butanol overnight and the extracts were analyzed using LC-MS. Peaks corresponding to germicidin A and germicidin B/C were monitored using their m/z ratio – 197 [M+H]⁺ and 183 [M+H]⁺, respectively (**Figure 4.3**). Overall, ARC2, 6, and 7 were found to increase levels of the germicidins that were monitored, while only ARC12 decreased them. The remainder of the molecules had no significant effect on germicidin levels.

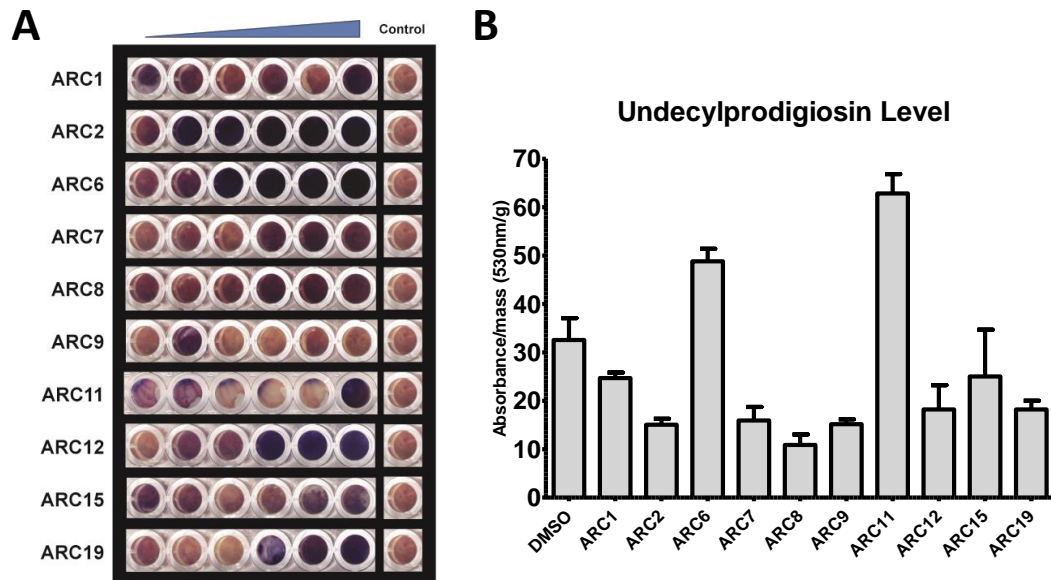


Figure 4.1 – ACT and RED assays on the 10 ARCs. (A) Assays to assess ACT production in presence of the ARCs were performed on solid media. *S. coelicolor* J150 colonies were grown for 96 hour in the presence of an increasing concentration of each ARC. The highest concentration was 25 μ M and was serially diluted by two in each subsequent well. (B) RED production was measured in liquid cultures of *S. coelicolor* M145 grown for 72 hours in the presence of 25 μ M of each ARC molecule. The level of metabolite was quantified through absorption at 530 nm.

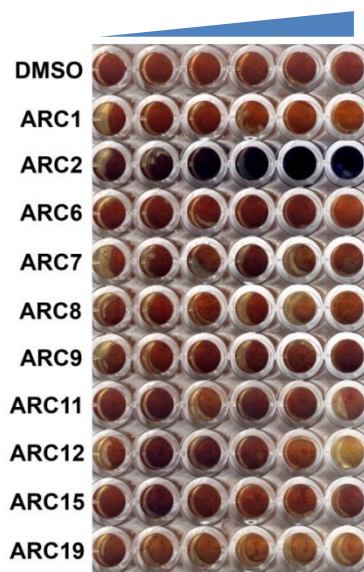


Figure 4.2 – Effect of the ARCs on ACT induction in *S. lividans*. Stimulation of ACT production in *S. lividans* was monitored on solid media in the presence of increasing concentration of ARCs. The ACT cluster in this species is normally silent.

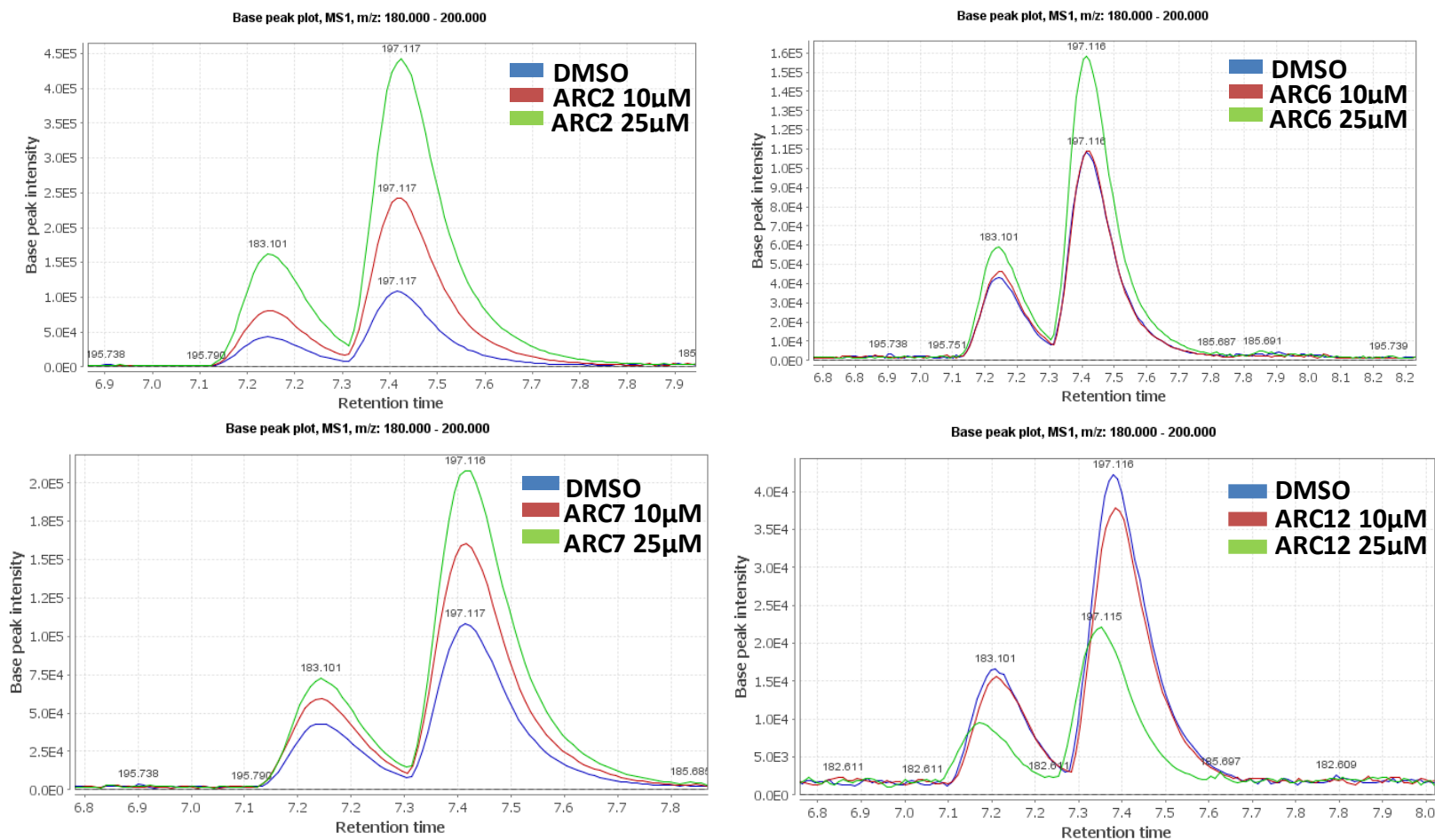


Figure 4.3 – LC-MS traces showing levels of germicidin A (197 [M+H]⁺) and germicidins B/C (183 [M+H]⁺) in the presence of ARCs. Butanol extracts of *S. coelicolor* grown in the presence of ARCs were analyzed using LC-MS. Peaks corresponding to the germicidins were identified based on known masses.

4.1.2 Effects on gene expression levels

The effects of the 10 ARCs on expression of the *cpk* and *whiE* genes were measured using a luminescence-based reporter system. This system utilizes *S. coelicolor* M145 strains with an integrated promoter-*luxCDABE* fusion corresponding to the gene of interest. The activation of a desired promoter is monitored through the production of light. The assay for *cpk* expression was conducted in a 96-well plate format using liquid cultures grown in the presence of each ARC at a concentration of 25 μ M. Initially, the molecules ARC15 and ARC19 were measured to increase the expression of *cpk*, while ARC2, 7, 9, and 12 decreased the expression (**Figure 4.4A**). However, after looking at the expression of the positive control, *hrdB*, it became evident that ARC7, 9, 12, and 19 were significantly affecting the growth of the *S. coelicolor* colonies in their early stages. Therefore, ARC2 and ARC15 were the only molecules that significantly affected *cpk* expression, independent of growth. The assay for *whiE* expression was conducted on solid media since the expression of *whiE* requires the *S. coelicolor* colonies to sporulate. ARC6, 8, and 19 were found to increase *whiE* expression, while ARC2 and ARC7 decreased it (**Figure 4.4B**).

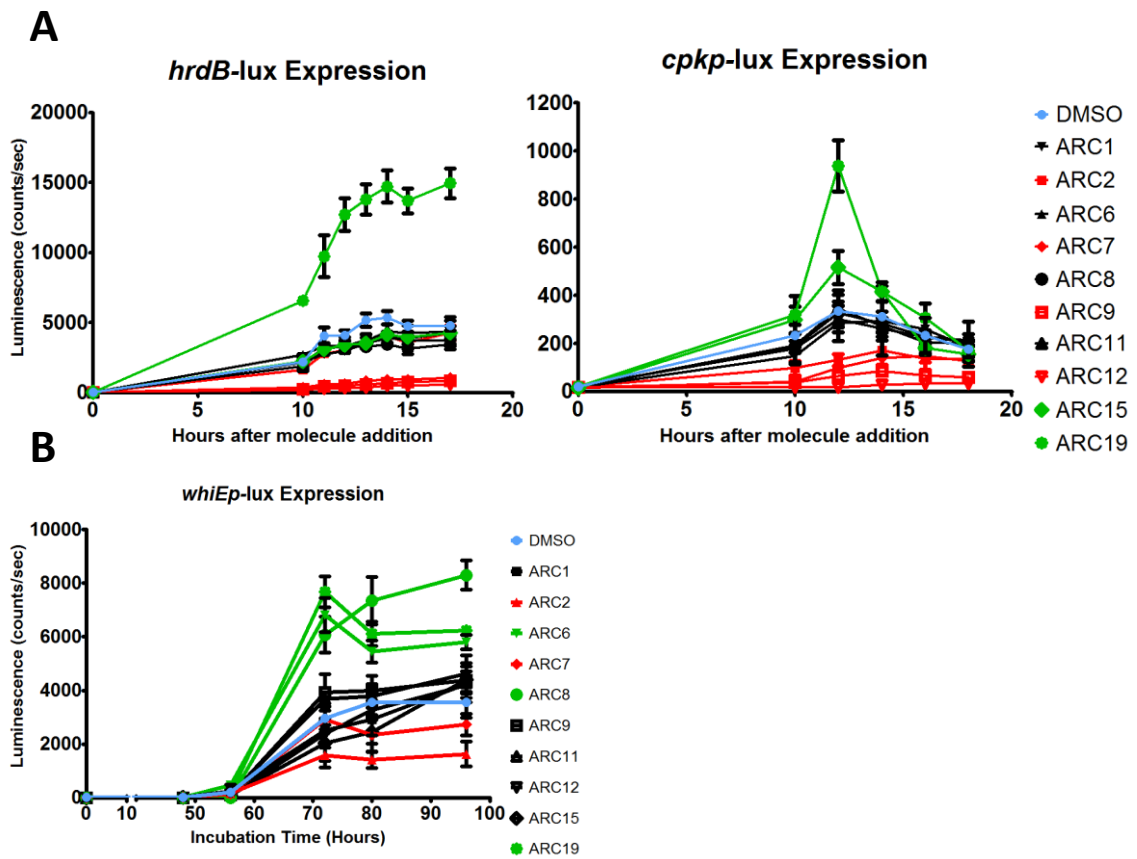


Figure 4.4 – Luminescence-based assays for 10 ARCs. (A) Gene expression of *hrdB* and *cpk* was measured in liquid cultures of *S. coelicolor* grown in the presence of 25 μ M of each ARC. **(B)** *whiE* expression in the presence of 25 μ M of survey molecules was measured on solid media. In all of the graphs, green lines represent molecules that caused an increase in expression, while red lines represent a decrease. DMSO control is shown in blue.

Table 4.1 - Summary of ARC effects on secondary metabolites and sporulation

ARC	RED	ACT in <i>S. lividans</i> ?	Germicidins	<i>cpk</i>	<i>whiE</i>
1	-	No	-	-	-
2	Decrease	Yes	Increase	Decrease	Decrease
6	Increase	No	Increase	-	Increase
7	Decrease	Yes	Increase	-	Decrease
8	Decrease	No	-	-	Increase
9	Decrease	No	-	-	-
11	Increase	No	-	-	-
12	-	No	Decrease	-	-
15	-	No	-	Increase	-
19	Decrease	No	-	-	Increase

“-” indicates no change

4.1.3 Summary of the effects of 10 ARC on secondary metabolism and sporulation

In general, each of the ARCs had a distinct overall profile when all of the assays were taken into consideration. The only exceptions to this result were ARC2 and ARC7 which behaved similarly in all experiments, albeit ARC7 generally had a weaker effect. While many molecules decreased levels of RED, only two molecules, ARC6 and ARC11, caused an increase in levels of the metabolite. Furthermore, only ARC2 and ARC15 caused an enhancement of *cpk* gene expression. Based on the results of this survey (**Table 4.1**), ARC6 was selected as the candidate for follow-up studies. Initially found as the most potent inducer of ACT, ARC6 was one of the only 2 molecules that increased RED as well. The molecule also increased levels of germicidins and expression of *whiE*, thereby making it a molecule that stood out for follow-up analysis. ARC6 was also a strong candidate for a comparison with ARC2 since the effects of the two molecules were generally different. With the exception of the effects on ACT and the germicidins,

the two molecules behaved differently in all other experiments. This suggests that the ARC2 and ARC6 are likely affecting different pathways. Since the overall goal of this study was to better understand secondary metabolism through chemical manipulation, comparing molecules that perturb different aspects of metabolite regulation will presumably be a more informative undertaking.

4.2 Understanding the Structure of ARC6

4.2.1 Structure-activity relationship (SAR) study

It was important to understand what parts of the molecule was essential for it to exert its phenotypic effect, and whether ARC6 had any other analogues that were more potent. A structure-activity relationship (SAR) study was conducted in order to perform this analysis. This type of study uses chemical analogues in order to observe the effect of structural changes on a particular biological activity – stimulating ACT production in this case. The major components of ARC6 structure consist of a benzene ring and a 1,2,4-triazine connected through an amine group (**Figure 2.1**). Decorations around this core structure include carbonyl and methyl groups on the triazine, as well as a methoxy group on the benzene. Furthermore, a comparison of how much change the structures of ARC2 and ARC6 can accept would also shed light on the similarity of their core activating structures. A total of 16 molecules were selected for this study along with ARC6. These molecules shared a structure similar to that of ARC6 but had key modifications, which were separated into three groups – changes to the benzene ring, changes to the triazine ring, and changes to the linker (**Figure 4.5**).

When assayed on solid media, four of the ARC6 analogues were found to exert an effect on ACT production – molecules 2, 3, 5, and 8 (**Figure 4.5**). However, when these molecules were tested on liquid cultures, molecule 8 did not cause an effect and was removed from subsequent analysis (data not shown). In order to determine which of the molecules was the most potent, ACT and RED assays were repeated using lower concentrations of the molecules – 1,5, and 10 μM (**Figure 4.6**). For comparative purposes, ARC4 was included in the experiment to represent the ARC2 Series molecules. The change from ARC2 to ARC4 for this particular experiment was due to the unavailability of ARC2 at the time. Once the molecule was available again, the phenotypic effects of ARC2 and ARC4 were compared and were found to be similar. The results from the ACT assay showed that molecule 3 was the most potent, exerting a significant effect at the 1 μM concentration. Molecule 2 appeared to be the least effective at increasing ACT. In terms of RED production, however, ARC6 along with molecules 3 and 5 appeared to be roughly equivalent in their effects.

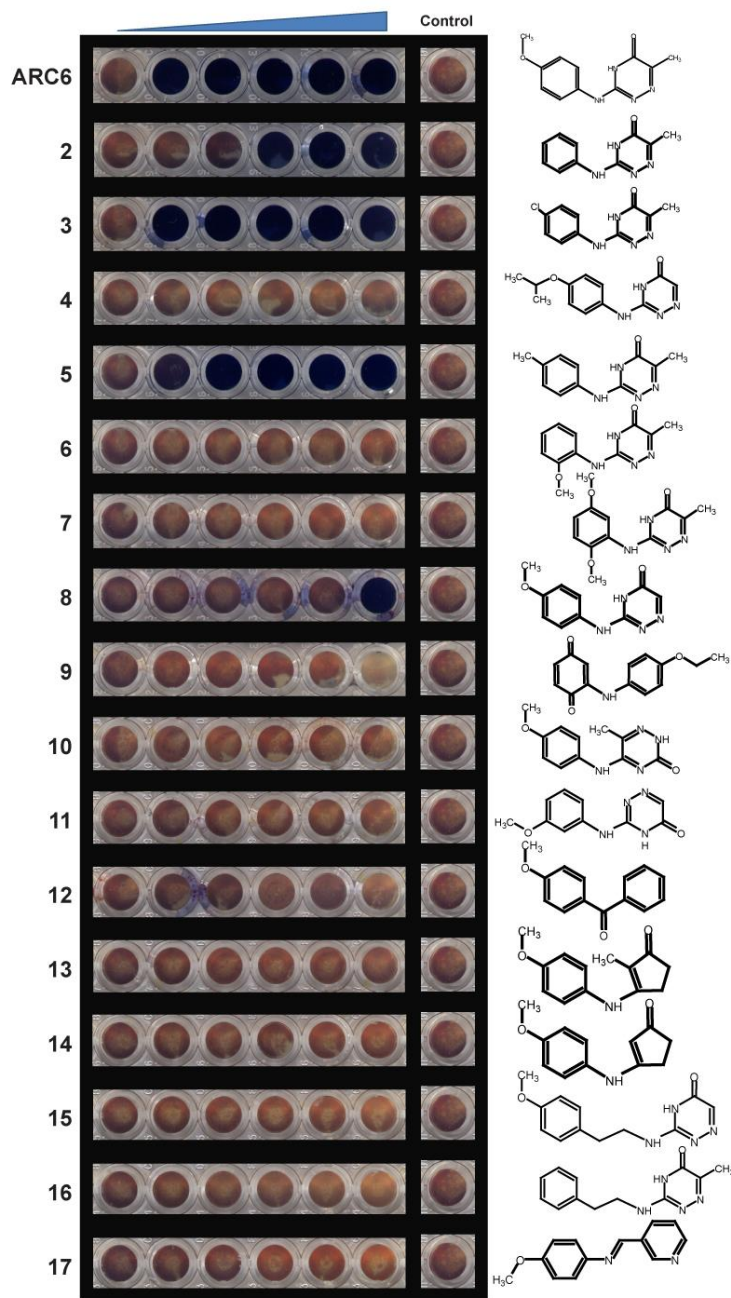
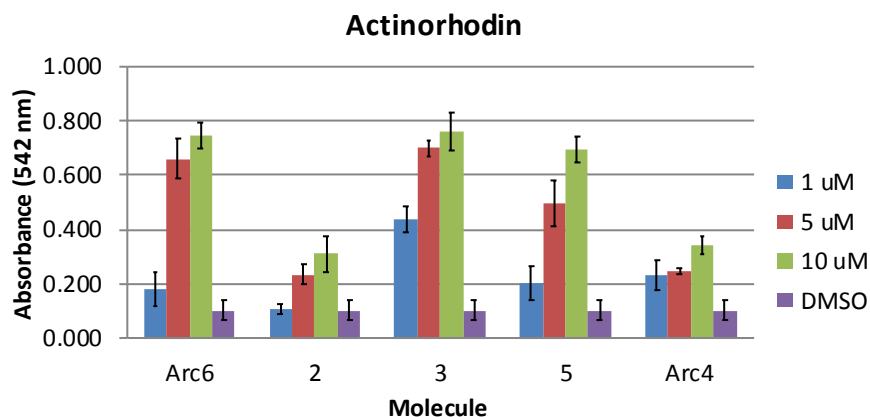


Figure 4.5 – Effects of SAR study molecules on ACT production. The molecules were selected to modify key features of the ARC6 molecules: the benzene ring (molecules 2-7), the triazine ring (molecules 8-14), and the linker (molecules 15-17). *S. coelicolor* colonies were grown with increasing concentration of each molecule to observe the effect on ACT production.

A



B

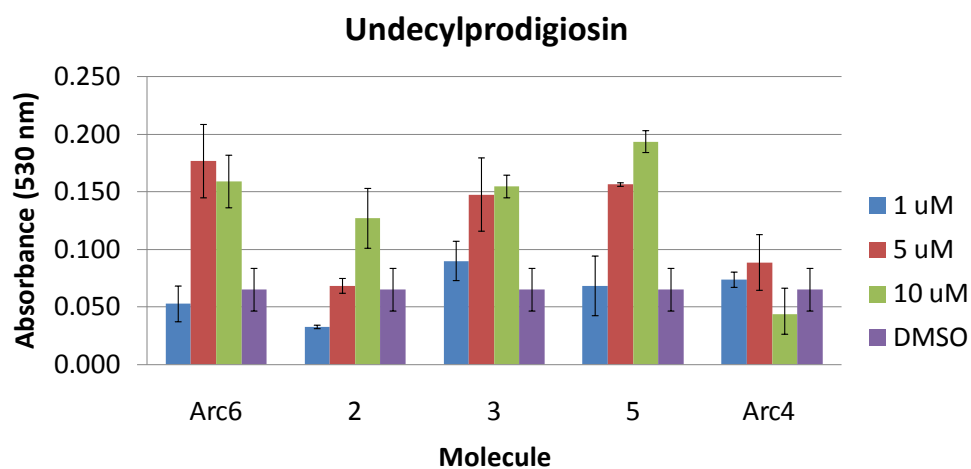


Figure 4.6 – ACT and RED assays to determine potency of SAR molecules. Cultures were grown in varying concentrations of selected molecules from the SAR study. ARC4 was included in the experiment to test the relative potency of the ARC2 Series at concentrations lower than typically used in most experiments. ACT (**A**) and RED levels (**B**) were measured spectroscopically.

4.2.2 Structural determinants of ARC6 and comparison to the ARC2 Series

When analyzing the ARC6 analogues' ability to affect ACT and RED production, some trends became apparent (summarized in **Figure 4.7**). Firstly, any changes made to the triazine ring were found to be detrimental to the phenotypic effects. Even the removal of the methyl group completely eliminated the effects, as can be seen when comparing ARC6 to molecule 8. Furthermore, the length of the linker between the two rings also appeared critical in order for the molecules to exert an effect on ACT and RED (molecule 15). Modifying the methoxy group on the benzene ring, however, did not hinder the phenotypic effects to the same degree. Eliminating the group altogether merely decreased the potency of the molecules as was seen with molecule 2. The placement of a highly electronegative group at that location, such as the halogen in molecule 3, may serve to increase the potency of the molecule. Upon the completion of the SAR study, molecule 3 was determined to be slightly more potent than ARC6. However, since there was not a major difference between these two molecules, ARC6 was used for further experiments.

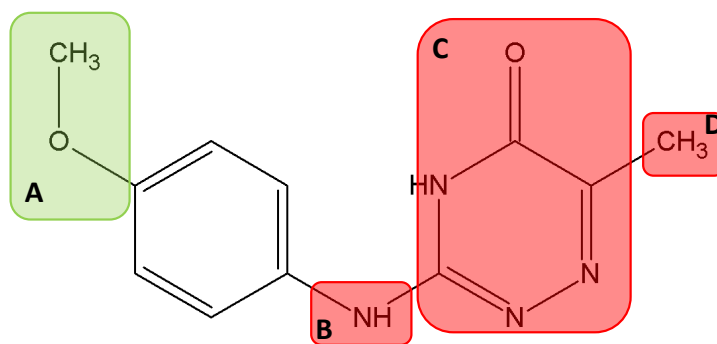


Figure 4.7 – Summary of the structural determinants of ARC6. An SAR study of ARC6 revealed that only one functional group (**A**) of the molecule could be modified while leaving its phenotypic effects intact. Any modification of the linker chain (**B**), the triazine ring (**C**), or the methyl group (**D**) eliminated ACT and RED induction by the molecule.

4.3 Does ARC6 Have the Same Target as ARC2?

4.3.1 Effects of ARC2 and ARC6 on Fatty Acid Composition

As mentioned previously, the target of ARC2 was identified in a separate study and lies within the fatty acid (FA) biosynthesis system. Therefore, the most direct means to test whether ARC2 and ARC6 have the same target is to analyze the effect of each on the FA composition of *S. coelicolor*. As such, a quantitative LC-MS/MS technique was employed in order to measure the composition of FA in the presence of ARC2 and ARC6.

For the analysis, liquid cultures of *S. coelicolor* M145 were grown for 3 days in media containing 25 μ M ARC2, 25 μ M ARC6, or DMSO. The pellets from these cultures were hydrolyzed using base, followed by extraction of the free FAs using hexane. The FAs were subsequently derivatized using DMAE and analyzed using LC-MS/MS. The results showed that both molecules had a degree of effect on FA composition (**Table 4.2**), although the specific changes were different. ARC2 caused a slight shift towards unsaturated FAs (approximately 5% change of total FA). This result fits with the theory that ARC2 hinders the function of an enoyl reductase – an enzyme responsible for reducing unsaturated bonds during FA biosynthesis. Conversely, ARC6 caused a slight shift towards saturated FAs, albeit, smaller in magnitude than the change caused by ARC2. Perhaps more significant, however, is a shift towards branched-chain FAs caused by ARC6 – a change of nearly 10% of total FA content (**Table 4.2**). A dose-dependence of this effect by ARC6 was also observed (**Figure 4.8**). At a concentration of 10 μ M ARC6, the level of branched-chain FAs was seen to be increased by

approximately 7%, while with 25µM ARC6, an increase of 10% was measured once again.

Fatty Acid	% of Total Fatty Acid Content		
	DMSO Control	ARC2 (25µM)	ARC6(25µM)
Iso-C13:0	0.51	0.42	0.27
Ante-Iso-C13:0	0.12	0.12	0.10
C13:0	0.03	0.02	0.01
Iso-C14:0	3.55	3.33	3.51
C14:0	0.76	0.71	0.33
Iso-C15:0	20.38	21.89	20.18
Ante-Iso-C15:0	8.43	10.30	8.87
C15:0	1.27	0.86	0.91
Iso-C16:1	5.03	7.65	4.75
C16:1	6.46	8.87	1.79
Iso-C16:0	26.75	23.80	32.10
C16:0	9.58	7.47	5.43
Iso-C17:0	11.74	10.35	13.03
Ante-Iso-C17:0	3.81	3.11	5.79
C17:0	0.36	0.14	0.17
Iso-C18:1	0.92	0.78	2.48
C18:1	0.13	0.11	0.05
Iso-C18:0	0.07	0.01	0.14
C18:0	0.08	0.05	0.11
Total branched-chain	81.32	81.77	91.21
Total straight-chain	18.68	18.23	8.79
Total saturated	87.46	82.59	90.94
Total unsaturated	12.54	17.41	9.06

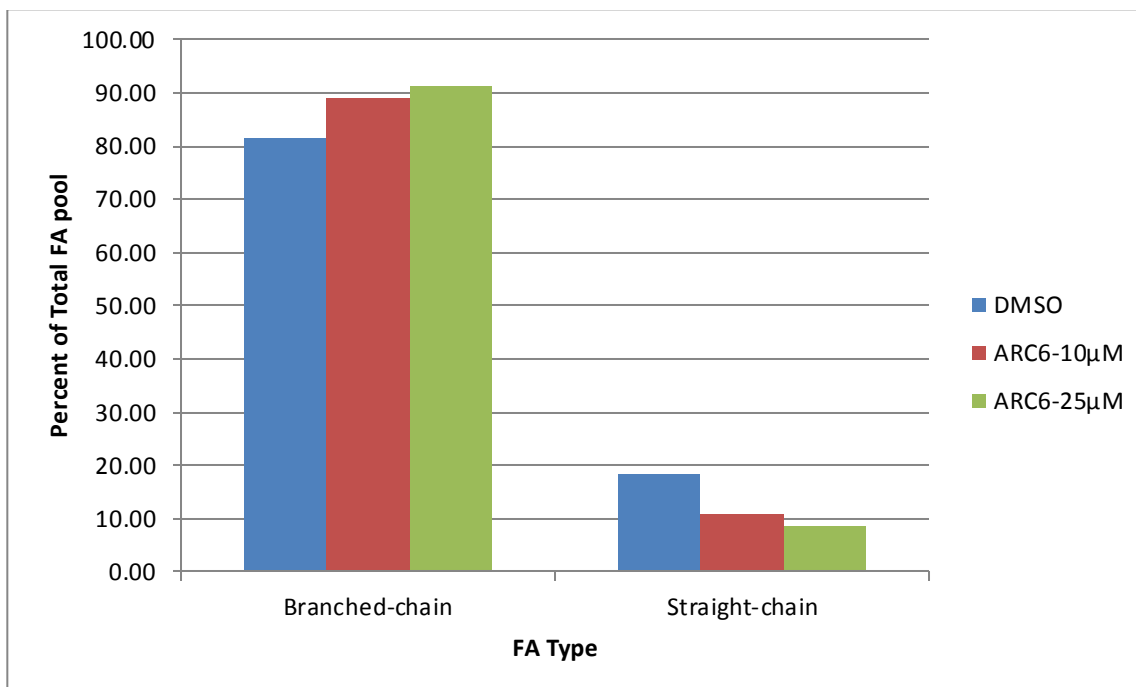


Figure 4.8 – Concentration dependent effect of ARC6 on branched- versus straight-chain FAs in *S. coelicolor*. The effect of different concentrations of ARC6 on FA composition was measured. *S. coelicolor* liquid cultures were grown with 10 µM ARC6, 25 µM ARC6, or DMSO

4.3.2 ARC2 and ARC6 may affect fatty acid synthesis by different mechanisms

Based on the previous experiments, the expectation was that ARC2 would cause a change in FA composition, while ARC6 would not. This hypothesis was based on the fact that ARC2 caused a change in every assay of the ARC screen, while ARC6 was only effective at modulating a few of the secondary metabolite levels. The scope of changes caused by ARC2 implied that it was affecting a more central pathway and thus its effect was more global than that of ARC6. Surprisingly, upon testing the influence of ARC2 and ARC6 on FA biosynthesis, ARC6 was also found to modulate this critical system. While both of the molecules affect this metabolic system, the changes caused

by the two molecules are substantially different. The effect of ARC2 appears to be on the saturation of FAs, while the major change caused by ARC6 appears to be on FA branching. Although ARC6 causes alterations in FA biosynthesis, the particular target of the molecule remains unclear. The enzyme responsible for determination of branching in FA biosynthesis is FabH⁵⁸⁻⁵⁹, and may be an interesting avenue for further exploration. It should, however, be noted that this effect by ARC6 on FAs may be a side effect of other changes in the cells, and therefore, this system may not be the primary target at all. Small molecules, such as antibiotics, have been previously shown to cause vast changes on gene expression levels¹⁵, and may well be the case here.

4.4 Effects of ARC2 and ARC6 on *S. coelicolor*

One of the purposes of conducting the chemical screen on *S. coelicolor* was to identify molecules that could alter secondary metabolism. The means by which most of the ARCs exert their effect presumably varies since they cause different phenotypic changes on the secondary metabolites of *S. coelicolor*. ARC2 has been demonstrated to affect a primary metabolic pathway and, therefore, it has caused some degree of effect in all assays conducted in the initial ARC screen. Since this critical pathway is conserved throughout most bacteria, ARC2 altered secondary metabolism in many of the *Streptomyces* that were tested. While ARC6 also altered FA content, it is unclear whether it has the same molecular target as ARC2. Thus, a detailed comparison of the effects of ARC2 and ARC6 was set up in order to determine if they share a largely conserved phenotype. Similar phenotypes would suggest a similar mechanism of action. It is also reasonable to assume that ARC6 may also have wide spread effects on

secondary metabolites in *Streptomyces* due to its change in FA content. This was determined on a panel of *Streptomyces*.

4.4.1 Comparison of the timing and effect of ARC2 and ARC6 on actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic

Solid media assays were first used in order to identify the timing of the effect of ARC2 and ARC6. The onset of pigmentation in the colonies was visible for both molecules after 72 hours of incubation with the molecules, although it was clear that ARC2 was having a much stronger effect at this time (**Figure 4.9A**). After 96 hours, however, both molecules were causing a significant increase in ACT production. Due to the fact that the blue pigmentation caused by ACT was much stronger than the red pigmentation, it was difficult to assess the effect on RED by the 96 hour mark. In order to further establish the timing effect of ARC2 and ARC6 on both ACT and RED, assays of liquid cultures were conducted. A number of interesting results came out of these assays (**Figure 4.9B**). Firstly, ARC6 was the better inducer of ACT over the 96-hour period, but ARC2 also significantly increased production. However, it appeared that ARC2 and ARC6 were working in different ways – ARC6 had a delay in its induction of ACT (between 48 and 72 hours), whereas ARC2 had an effect from the first time point. ARC2 and ARC6 also differed significantly in their effect on RED. While ARC6 increased RED production over the 96-hours compared to the control, ARC2 reduced it.

One of the other known antibiotics produced by *S. coelicolor* is the calcium-dependent antibiotic (CDA). Using the protocol by *Hojati et al. (2002)*⁵⁹, the CDA4a variant was extracted and detected by LC-MS (**Figure 4.9C**). This protocol involves collecting the

freeze-thaw exudate from *S. coelicolor* plates incubated for 7 days, followed by SPE column cleanup. The expected m/z ratio of approximately 1495 $[M+H]^+$ was detected successfully. In repeated experiments, the same pattern emerged for CDA – ARC2 had a significant negative effect on CDA production. The effect of ARC6 was also negative, but not to the extent of ARC2.

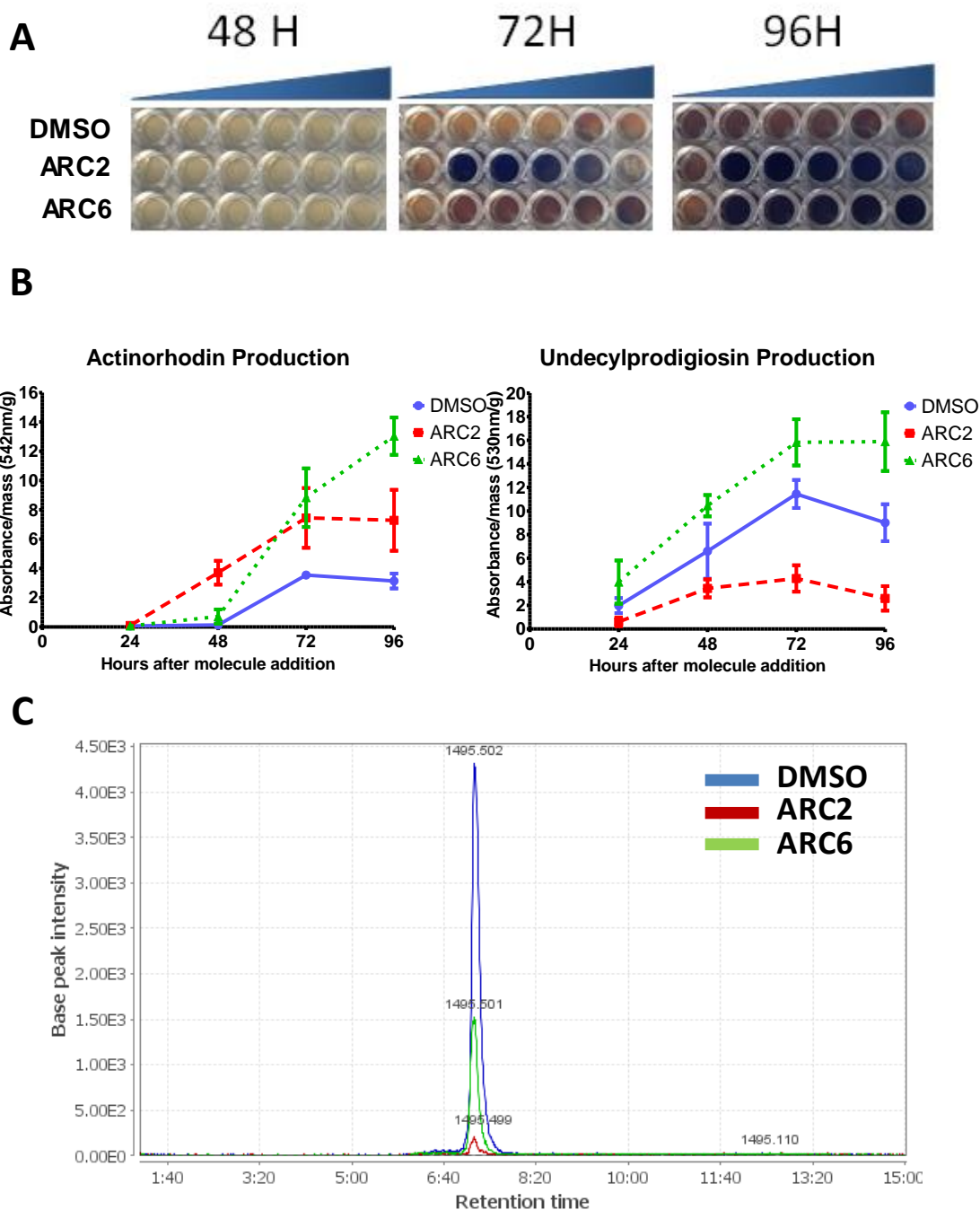


Figure 4.9 – Effect of ARC2 and ARC6 on ACT, RED, and CDA. (A) Solid medium assays for the effects of ARC2 and ARC6 on ACT at increasing concentrations. The highest concentration used was 25 μ M and was serially diluted by 2 in each subsequent well. **(B)** The timing effect of ARC2 and ARC6 on ACT and RED levels was measured spectroscopically. Red and green lines represent ARC2 and ARC6, respectively, while blue lines represent the DMSO control. **(C)** The effect of ARC2 and ARC6 on CDA levels, as represented by levels of CDA4A ($m/z = 1495 [M+H]^+$), was assessed through LC-MS analysis.

4.4.2 Effect of ARC2 and ARC6 on developmental genes

The effect of ARC2 and ARC6 on sporulation was also characterized since visual analysis of growing colonies suggested that ARC2 reduced spore formation while ARC6 caused precocious sporulation. To confirm this observation, scanning electron microscopy (SEM) was performed on *S. coelicolor* M145 colonies grown for 5 days in the presence of DMSO, ARC2, or ARC6. The SEM images confirmed that ARC2 decreased sporulation as very few septated spores were visible (**Figure 4.10**). Largely, long filaments were observed corresponding to undifferentiated aerial hyphae – consistent with the observed “whiter” phenotype. The SEM images of colonies grown with ARC6 showed a slightly greater abundance of spores when compared to the DMSO control, although the change was less discernible when compared to ARC2 (**Figure 4.10**). In order to definitively measure the effects of the two molecules on sporulation (particularly ARC6), a luminescence reporter system was utilized. The particular genes used for the experiment were the early response genes *blaN* and *whiH*, the late response gene *whiE*, as well as *hrdB* – a housekeeping σ -factor used as the positive control. Each strain was grown in the presence of DMSO, 25 μ M ARC2, or 25 μ M ARC6 and luminescence was observed over time (**Figure 4.11**). All of the sporulation-related genes that were tested showed a similar pattern – ARC6 caused higher expression, while ARC2 caused lower expression when compared to the control. Generally, the expression of each gene began at roughly the same time point, regardless of the presence of a particular molecule. Furthermore, the positive control, *hrdB*, showed expression levels independent of the presence of ARC2 or ARC6. Although one time point for the ARC2

set showed a slight decrease in expression, overall the levels of *hrdB* expression remained consistent with the DMSO control.

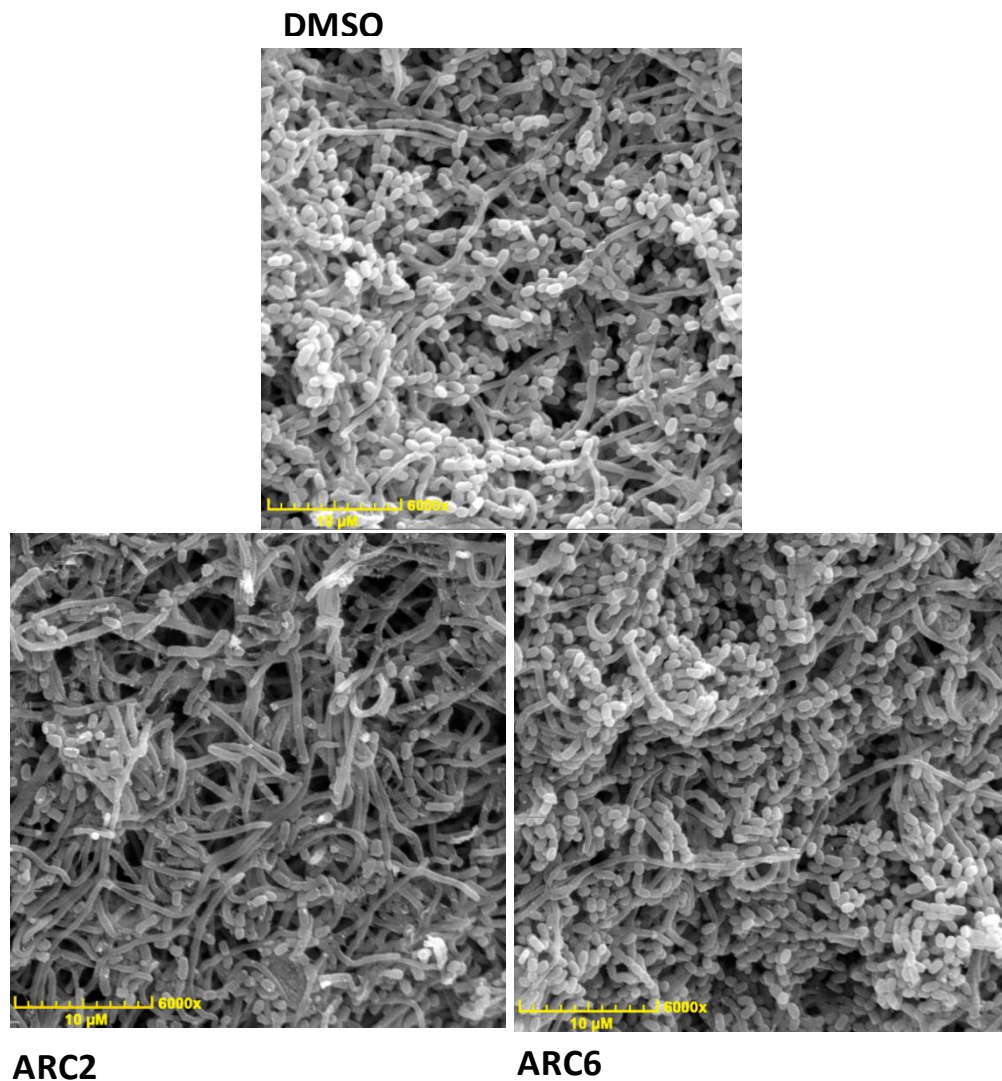


Figure 4.10 – SEM images of *S. coelicolor* colonies grown for 5 days in the presence of DMSO, ARC2, or ARC6. The effect of ARC2 and ARC6 on spore formation was first assessed qualitatively through visual analysis using SEM.

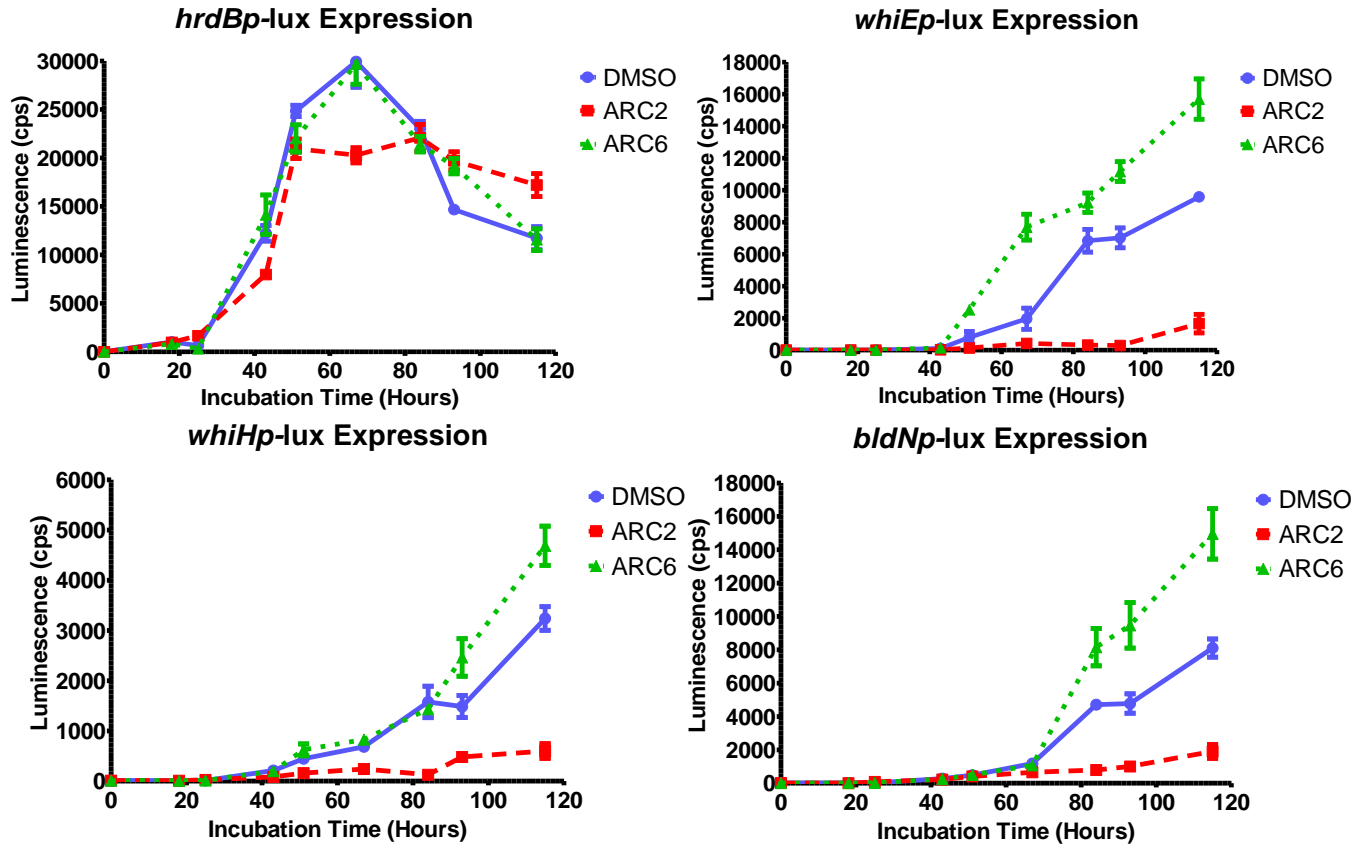


Figure 4.11 – Luminescence-based assay for the effect of ARC2 and ARC6 on developmental genes. The expression of the genes *bldN*, *whiH*, and *whiE* was measured in order to test the effect of ARC2 and ARC6 on sporulation. *hrdB* represented the positive control. ARC2 and ARC6 are depicted by the red and green lines, respectively. DMSO control is shown in blue.

4.4.2 Does ARC6 affect other Actinomycetes?

One of the major aspects of this study was to test whether ARC2 and ARC6 can be used as tools for the discovery of previously unobserved secondary metabolites from various Actinomycetes. The separate study of ARC2 revealed that the molecule can modulate levels of several secondary metabolites in a range of *Streptomyces*. In this study, the effects of ARC6 on secondary metabolite profiles of various *Streptomyces* (and one non-*Streptomyces* Actinomycete) were tested using LC-MS. The protocol involved growth of the particular strain on solid R5M media for 3 and 7 days, followed by a butanol extraction. A detailed analysis of 5 strains was conducted preliminarily. This involved a thorough analysis of all reproducible changes in the overall LC-MS profile of a species. The strains selected for this first stage were *S. pristinaespiralis*, *S. sp.* SPB74, *Saccharopolyspora erythraea*, *S. sp.* Wat1, and *S. sp.* Ja2B. The first 3 strains were selected since their genome has been sequenced by the Broad Institute, while the latter 2 are wild isolates from the Wright Actinomycete Collection. In general, ARC6 did not cause many changes in the secondary metabolite profiles of these 5 strains when compared to ARC2. Some minor changes in *S. erythraea* were observed but ARC6 did not modulate levels of the antibiotic erythromycin, which is the major known compound produced by the strain. Any other metabolic changes by ARC6 in the remaining species tested were either too minor or not reproducible. Based on this result, it was important to test ARC6 with other strains that produce previously characterized compounds. This test was implanted in order to provide more conclusive evidence whether ARC6 affects other *Streptomyces* or whether its effect is specific to *S. coelicolor*. Since the exact mass for a previously characterized compound is known, this test gave a more reliable association between a peak on the LC-MS trace and the particular compound being affected.

Moreover, the retention time for some of these compounds could be deduced since many of them are available commercially for purchase.

The expansion of the screen involved the inclusion of 6 more strains – *S. griseus*, *S. roseosporus*, *S. avermitilis*, *S. aureofaciens*, *S. peucetius*, and *S. viridochromogenes*. Each of these strains produces at least one well characterized secondary metabolite (**Table 3.1**). Upon testing the effect of ARC6 on these strains, none of the metabolites were significantly affected by inclusion of the molecule in the growth media (selected examples are shown in **Figure 4.12**). Combining both stages of the screen, the effect of ARC6 was tested on a total of 12 strains and results showed it only affects secondary metabolism in *S. coelicolor*. This result is starkly different than ARC2. While this effectively eliminates the utility of ARC6 as a direct inducer of unexpressed metabolites outside of *S. coelicolor*, it makes ARC6 a powerful probe of secondary metabolite regulation in this species.

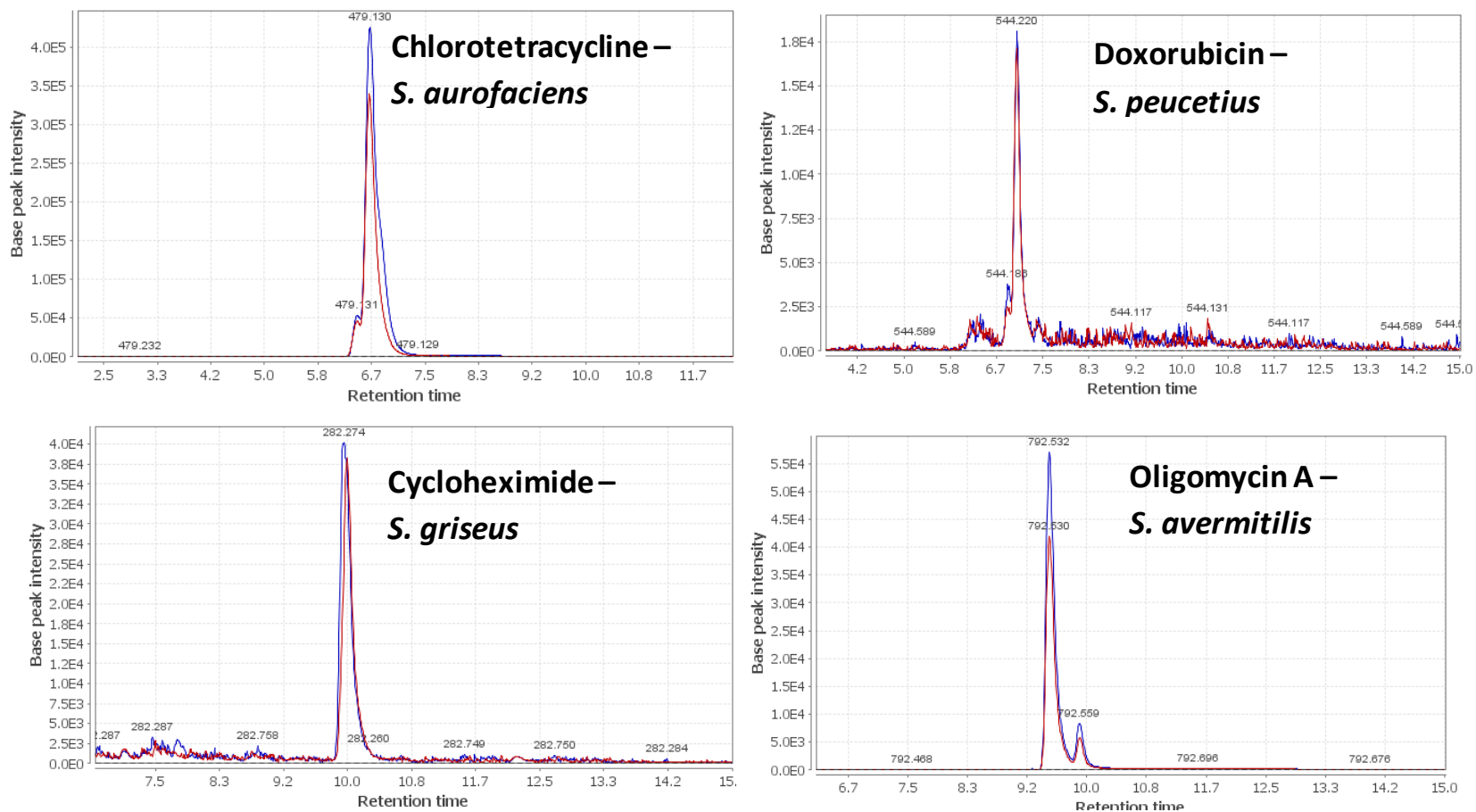


Figure 4.12 – Examples of LC-MS traces corresponding to particular metabolites that were screened with ARC6. Butanol extracts from the 11 strains tested were analyzed using LC-MS. 4 metabolites from the 11 strains tested are shown as an example. Red traces represent extract of colonies grown in the presence of 25µMARC6, while blue traces correspond to the DMSO control.

4.4.3 ARC6 as a probe for *S. coelicolor* secondary metabolism

After testing the effects of ARC2 and ARC6 on secondary metabolism, a few conclusions become apparent. Firstly, evidence suggests that the direct target of ARC2 and ARC6 is different. The influence of each molecule on ACT, RED, and CDA is significantly varied. Furthermore, ARC2 appears to be acting significantly earlier than ARC6, which supports the idea of separate targets. Perhaps the most astounding variation, however, is the effect of the two molecules on other *Streptomyces*. Even though ARC6 also seems to influence fatty acid biosynthesis (albeit in a different way), it has no effect outside of *S. coelicolor*. This could be due to two possible reasons: either the target of ARC6 is unique to *S. coelicolor*, or the species lacks the ability to break down ARC6 unlike other *Streptomyces*.

While the differences between the effects of ARC2 and ARC6 on secondary metabolism had already been established, their differing effects on developmental genes added another avenue to distinguish the two molecules. Along with the evidence that these ARCs influence both primary and secondary metabolism, the link to developmental genes suggests that the regulation of cell processes may be more interconnected and complex than previously believed.

CHAPTER 4: DISCUSSION

The overall goal of this project was to characterize the effects of synthetic chemicals that have been shown to modulate secondary metabolism in *Streptomyces coelicolor*. These molecules can possibly act as a means to better understand the regulation of secondary metabolites, as well as aid in the discovery of novel ones. To begin, detailed analysis of 10 ARCs was carried out in order to test their ability to alter secondary metabolism in *S. coelicolor*. This analysis was performed to assess the variation in chemical probes that came out of the screen. Largely, the molecules that were selected for further testing behaved differently when considering all of the assays that were conducted, suggesting that all 10 may exert their effects through different mechanisms. Only two molecules, ARC2 and ARC7, appeared to show similar results through the assays, although the effects of ARC7 were significantly weaker. This result suggests that ARC2 and ARC7 may influence the same target pathway. Furthermore, ARC6 and ARC11 were the only molecules that increased RED production, with ARC11 having a more potent effect. Only two molecules, ARC2 and ARC15, affected the expression of *cpk*, while several of the molecules modulated levels of germicidins and *whiE* expression. The results of these assays suggested that the ARCs can act as several unique probes for secondary metabolism in *S. coelicolor*.

Based on the amalgamated results of the ARC survey, ARC6 was selected for further characterization, since it had the greatest stimulatory effect on ACT and was one of 2 molecules to enhance RED as well. As only ARC2 had a characterized mechanism of action, ARC6 was compared with this molecule in detail to determine whether they

affected similar processes. While both molecules were found to alter the fatty acid content of *S. coelicolor*, the changes observed in the presence of the two molecules were largely different. In terms of fatty acid composition, ARC2 caused a shift towards unsaturated FAs, while ARC6 caused a shift towards branched-chain FAs. However, it is uncertain at this point if the shift in FA composition caused by ARC6 is due to a direct targeting of FA biosynthesis, or whether it is merely a side-effect of other changes in the cells.

Differences in effect were also evident when considering secondary metabolism. With the exception of ACT, all of the metabolites tested in this study were influenced in different ways by ARC2 and ARC6. Even with ACT, there was a significant difference in the timing of the effect where ARC2 caused stimulation early, whereas ARC6 caused an effect significantly later. Conversely, both molecules caused a decrease in CDA levels, although ARC2 was significantly more potent in this effect. Furthermore, ARC2 and ARC6 caused opposite effects on RED production. Continuing the theme of differences, ARC2 and ARC6 caused opposite effects on the expression of developmental genes as well. Each of the sporulation-related genes tested (*bldN*, *whiH*, *whiE*), showed the same pattern: ARC2 lowered their expression, while ARC6 increased them. The analysis of the structures of the two molecules revealed that ARC6 is less accepting of changes when compared to ARC2⁵⁴. The structure of ARC6 only allowed for the modification of the methoxy group on the benzene while still retaining the phenotypic effects. However, the removal of this group led to decreased potency, thus suggesting that an electron-withdrawing group at this location aids in affecting the target. This is in contrast to ARC2, which is accepting of most changes. The molecule was found to be largely dependent on the core ring structure as well as an electron withdrawing group at one

end of the molecule⁵⁴. The majority of other decorations on the ARC2 core structure could be modified without the loss of its phenotypic effects.

Through the comparison of ARC2 and ARC6, it is evident that they work in distinct ways and likely affect different regulatory pathways. This conclusion suggests that further characterization of the targets could provide information on separate regulatory cascades. This would yield a broader probe into secondary metabolism and how it connects with primary metabolism and cell growth. The next step in achieving this task would be to definitively identify the primary target of ARC6. Although evidence suggests that ARC6 may work through by altering fatty acid biosynthesis, it remains unclear whether the system is the primary target of ARC6, or whether the changes are merely a side-effect. Further exploration of this pathway is warranted and may reveal the target of ARC6.

The fact that ARC6 only alters secondary metabolism in *S. coelicolor* is a fascinating result. This suggests that the target of the molecule is either only present in this species, or the other *Streptomyces* that were tested have a way to break down the molecule before it exerts an effect. Bioinformatic comparison of the *S. coelicolor* genome to that of other *Streptomyces* may provide clues as to which of the two theories is correct. In particular, *S. coelicolor* and *S. lividans* have nearly identical genomes but ARC6 is ineffectual in the latter. Analysis of these two genomes together may provide the most informative clues about the target of ARC6. Aside from this approach, measuring global transcription levels through microarray or RNA sequencing may also reveal the genes affected by ARC6. This approach may be a better indicator of the scope of the

molecule's effect, because it would reveal genes from all of the cellular pathways that are affected by ARC6.

Conclusion

The abundance of unexpressed secondary metabolites indicated by the analysis of *Streptomyces* genomes suggests a vast potential for novel therapeutic agents. However, expressing these silent gene clusters in a laboratory setting has proven to be a challenge. Chemical perturbation of the regulatory networks that control secondary metabolism is a technique with great potential when it comes to overcoming this hurdle. Traditional approaches for probing regulatory pathways typically involve genetic manipulation, such as gene knockouts. Using small molecules for these investigations allows one to bypass the limitations of genetic techniques, such as working with species that are difficult to genetically manipulate. Furthermore, the effects of small molecules can be modulated with dosage since they are heavily dependent on the concentration of available molecule. Not only can small molecules, such as ARC2, be used to study regulation, they can also be used to enhance levels of lowly expressed metabolites. The ARCs can act as an array of tools in the aid of achieving both of these goals. Each of the ARCs that was tested showed a distinct profile of effects, suggesting that each can be used as a distinct tool to probe secondary metabolism. Characterizing these molecules further is important because it would vastly improve the arsenal for investigating the regulation of secondary metabolites. This understanding may provide reasons as to why only about half of *S. coelicolor* secondary metabolites are expressed. Additionally, testing each of the ARCs for its effects in other *Streptomyces* may also provide a means of inducing silent or poorly-expressed metabolite cluster. With the

decline in efficacy of the current arsenal of antibiotics due to emerging resistance, finding new molecules with antibacterial activities is becoming a high priority. Chemically inducing the expression of novel secondary metabolites, through the use of the ARCs, offers a promising avenue to combat this growing clinical problem.

REFERENCES

1. Hartmann, T. (2007). From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry* 68, 2831-2846
2. Fox, E.M., and Howlett, B.J. (2008). Secondary metabolism: regulation and role in fungal biology. *Current Opinion in Microbiology* 11, 481-487.
3. Baltz, R.H. (2006). Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? *Journal of Industrial Microbiology and Biotechnology* 33, 507-513.
4. Kieser, T., Bibb, J., Buttner, M.J., Chater, K.F., Hopwood, D.A. (2000). *Practical Streptomyces Genetics*. (John Innes Foundation, Norwich, UK, ed.1)
5. Capstick, D.S., Willey, J.M., Buttner, M.J., and Elliot, M.A. (2007). SapB and the chaplins: connections between morphogenetic proteins in *Streptomyces coelicolor*. *Molecular Microbiology* 64, 602-613.
6. Kelemen, G.H., and Buttner, M.J. (1998). Initiation of aerial mycelium formation in *Streptomyces*. *Current Opinion in Microbiology* 1, 656-662.
7. Chater, K.F. (2001). Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? *Current Opinion in Microbiology* 4, 667-673.
8. Bibb, M. (2005). Regulation of secondary metabolism in streptomycetes. *Current Opinion in Microbiology* 8, 208-215.
9. Tahlan, K., Ahn, S.K., Sing, A., Bodnaruk, T.D., Willems, A.R., Davidson, A.R., and Nodwell, J.R. (2007). Initiation of actinorhodin export in *Streptomyces coelicolor*. *Molecular Microbiology* 63, 951-961.
10. Wright, L. F. and D. A. Hopwood (1976). Actinorhodin is a chromosomally-determined antibiotic in *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* 96, 289-297.
11. Bystrykh, L.V., Fernandez-Moreno, M.A., Herrema, J.K., Malpartida, F., Hopwood, D.A., and Dijkhuizen, L. (1996). Production of Actinorhodin-related "Blue Pigments" by *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 178, 2238-2244.

12. Arias, P., Fernandez-Moreno, M.A., and Malpartida, F. (1999). Characterization of the Pathway-Specific Positive Transcriptional Regulator for Actinorhodin Biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-Binding Protein. *Journal of Bacteriology* 181, 6958-6968.
13. Beltran-Alvarez, P., Arthur, C.J., Cox, R.J., Crosby, J., Crump, M.P., and Simpson, T.J. (2009). Preliminary kinetic analysis of acyl carrier protein–ketoacylsynthase interactions in the actinorhodin minimal polyketide synthase. *Molecular BioSystems* 5, 511.
14. Murakami, K. and Tomasz, A. (1989) Involvement of multiple genetic determinants in high-level methicillin resistance in *Staphylococcus aureus*. *Journal of Bacteriology* 171(2), 874-879.
15. Goh, E.B., Yim, G., Tsui, W., McClure, J., Surette, M., and Davies, J. (2002). Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences* 99, 17025-17030.
16. Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., and Miller, S.I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436, 1171-1175.
17. Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E.P., and Hoiby, N. (2004). *Pseudomonas aeruginosa* Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and -Lactamase and Alginate Production. *Antimicrobial Agents and Chemotherapy* 48, 1175-1187.
18. Höfer, I., Crüsemann, M., Radzom, M., Geers, B., Flachshaar, D., Cai, X., Zeeck, A., and Piel, J. (2011). Insights into the Biosynthesis of Hormaomycin, An Exceptionally Complex Bacterial Signaling Metabolite. *Chemistry & Biology* 18, 381-391.
19. Eisen, J.A., Vetsigian, K., Jajoo, R., and Kishony, R. (2011). Structure and Evolution of *Streptomyces* Interaction Networks in Soil and In Silico. *PLoS Biology* 9, e1001184.
20. Zerikly, M., and Challis, G.L. (2009). Strategies for the Discovery of New Natural Products by Genome Mining. *ChemBioChem* 10, 625-633.

21. Wyatt, M.A., Wang, W., Roux, C.M., Beasley, F.C., Heinrichs, D.E., Dunman, P.M., and Magarvey, N.A. (2010). Staphylococcus aureus Nonribosomal Peptide Secondary Metabolites Regulate Virulence. *Science* 329, 294-296.
22. Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Parkhill, J., and Hopwood, D.A. (2002). Complete Genome Sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417, 141-147.
23. Nett, M., Ikeda, H., and Moore, B.S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Natural Product Reports* 26, 1362.
24. Pawlik, K., Kotowska, M., and Kolesinski, P. (2010). *Streptomyces coelicolor* A3(2) produces a new yellow pigment associated with the polyketide synthase Cpk. *Journal of Molecular Microbiology and Biotechnology* 19, 147-151.
25. Van Wezel, G.P., McKenzie, N.L., Nodwell, J.R. (2009) Chapter 5 Applying the genetics of secondary metabolism in model actinomycetes to the discovery of new antibiotics. *Methods in Enzymology* 458, 117-141.
26. Horinouchi, S. (2003). AfsR as an integrator of signals that are sensed by multiple serine/threonine kinases in *Streptomyces coelicolor* A3(2). *Journal of Industrial Microbiology and Biotechnology* 30, 462-467.
27. Santos-Beneit, F., Rodríguez-García, A., Sola-Landa, A., and Martín, J.F. (2009). Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. *Molecular Microbiology* 72, 53-68.
28. McKenzie, N.L., and Nodwell, J.R. (2007). Phosphorylated AbsA2 Negatively Regulates Antibiotic Production in *Streptomyces coelicolor* through Interactions with Pathway-Specific Regulatory Gene Promoters. *Journal of Bacteriology* 189, 5284-5292.
29. Rigali, S., Titgemeyer, F., Barends, S., Mulder, S., Thomae, A.W., Hopwood, D.A., and van Wezel, G.P. (2008). Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO reports* 9, 670-675.
30. Uguru, G.C., Stephens, K.E., Stead, J.A., Towle, J.E., Baumberg, S., and McDowall, K.J. (2005). Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in *Streptomyces coelicolor*. *Molecular Microbiology* 58, 131-150.

31. Price, B., Adamidis, T., Kong, R., and Champness, W. (1999). A *Streptomyces coelicolor* antibiotic regulatory gene, *absB*, encodes an RNase III homolog. *Journal of Bacteriology* *181*, 6142-6151.
32. Saito, N., Xu, J., Hosaka, T., Okamoto, S., Aoki, H., Bibb, M.J., and Ochi, K. (2006). EshA accentuates ppGpp accumulation and is conditionally required for antibiotic production in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* *188*, 4952-4961.
33. Chakraborty, R., and Bibb, M. (1997). The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *Journal of Bacteriology* *179*, 5854-5861.
34. Ochi, K., Zhang, D., Kawamoto, S., and Hesketh, A. (1997). Molecular and functional analysis of the ribosomal L11 and S12 protein genes (*rplK* and *rpsL*) of *Streptomyces coelicolor* A3(2). *Molecular and General Genetics* *256*, 488-498.
35. Yang, Y.-H., Song, E., Kim, E.-J., Lee, K., Kim, W.-S., Park, S.-S., Hahn, J.-S., and Kim, B.-G. (2008). NdgR, an lclR-like regulator involved in amino-acid-dependent growth, quorum sensing, and antibiotic production in *Streptomyces coelicolor*. *Applied Microbiology and Biotechnology* *82*, 501-511.
36. Onaka, H., Nakagawa, T., and Horinouchi, S. (1998). Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. *Molecular Microbiology* *28*, 743-753.
37. Tunca, S., Barreiro, C., Coque, J.-J.R., and Martín, J.F. (2009). Two overlapping antiparallel genes encoding the iron regulator DmdR1 and the Adm proteins control sidephore and antibiotic biosynthesis in *Streptomyces*
38. Horinouchi, S., Malpartida, F., Hopwood, D.A., and Beppu, T. (1989). *afsB* stimulates transcription of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. *Molecular and General Genetics* *215*, 355-357.
39. Hesketh, A., Kock, H., Mootien, S., and Bibb, M. (2009). The role of *absC*, a novel regulatory gene for secondary metabolism, in zinc-dependent antibiotic production in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* *74*, 1427-1444.

40. Den Hengst, C.D., Tran, N.T., Bibb, M.J., Chandra, G., Leskiw, B.K., and Buttner, M.J. (2010). Genes essential for morphological development and antibiotic production in *Streptomyces coelicolor* are targets of BldD during vegetative growth. *Molecular Microbiology* 78, 361-379.
41. Li, W., Ying, X., Guo, Y., Yu, Z., Zhou, X., Deng, Z., Kieser, H., Chater, K.F., and Tao, M. (2006). Identification of a Gene Negatively Affecting Antibiotic Production and Morphological Differentiation in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 188, 8368-8375.
42. Zhang, L., Li, W.C., Zhao, C.H., Chater, K.F., Tao, M.F. (2007) NsdB, a TPR-like-domain-containing protein negatively affecting production of antibiotics in *Streptomyces coelicolor* A3(2). *Wei Sheng Wu Xue Bao* 47(5), 849-854.
43. Ou, X., Zhang, B., Zhang, L., Zhao, G., and Ding, X. (2009). Characterization of *rrdA*, a TetR Family Protein Gene Involved in the Regulation of Secondary Metabolism in *Streptomyces coelicolor*. *Applied and Environmental Microbiology* 75, 2158-2165.
44. van Wezel, G.P., van der Meulen, J., Kawamoto, S., Luiten, R.G.M., Koerten, H.K., and Kraal, B. (2000). *ssgA* is Essential for Sporulation of *Streptomyces coelicolor* A3(2) and Affects Hyphal Development by Stimulating Septum Formation. *Journal of Bacteriology* 182, 5653-5662.
45. Traag, B.A., Kelemen, G.H., and Van Wezel, G.P. (2004). Transcription of the sporulation gene *ssgA* is activated by the lclR-type regulator SsgR in a whi-independent manner in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* 53, 985-1000.
46. Shu, D., Chen, L., Wang, W., Yu, Z., Ren, C., Zhang, W., Yang, S., Lu, Y., and Jiang, W. (2008). *afsQ1-Q2-sigQ* is a pleiotropic but conditionally required signal transduction system for both secondary metabolism and morphological development in *Streptomyces coelicolor*. *Applied Microbiology and Biotechnology* 81, 1149-1160.
47. Lu, Y., Wang, W., Shu, D., Zhang, W., Chen, L., Qin, Z., Yang, S., and Jiang, W. (2007). Characterization of a novel two-component regulatory system involved in the regulation of both actinorhodin and a type I polyketide in *Streptomyces coelicolor*. *Applied Microbiology and Biotechnology* 77, 625-635.

48. Takano, E. (2006) γ -Butyrolactones: Streptomyces signalling molecules regulating antibiotic production and differentiation. *Current Opinion in Microbiology* 9(3), 287-294.
49. Sola-Landa, A. (2003). The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. *Proceedings of the National Academy of Sciences* 100, 6133-6138.
50. Natsume, R., Ohnishi, Y., Senda, T., and Horinouchi, S. (2004). Crystal structure of a γ -Butyrolactone autoregulator receptor protein in *Streptomyces coelicolor* A3(2). *Journal of Molecular Biology* 336, 409-419.
51. Huh, J.-H., Kim, D.-j., Zhao, X.-Q., Li, M., Jo, Y.-Y., Yoon, T.-M., Shin, S.-K., Yong, J.-H., Ryu, Y.-W., Yang, Y.-Y., *et al.* (2004). Widespread activation of antibiotic biosynthesis by S-adenosylmethionine in streptomycetes. *FEMS Microbiology Letters* 240, 245-245.
52. Jakeman, D.L., Graham, C.L., Young, W., Vining, L.C. (2006). Culture conditions improving the production of jadomycin B. *Journal for Industrial Microbiology & Biotechnology*. 33 (9), 767-772.
53. Foley, T.L., Young, B.S., and Burkart, M.D. (2009). Phosphopantetheinyl transferase inhibition and secondary metabolism. *FEBS Journal* 276, 7134-7145.
54. Craney, A., Ozimuk, C., Pimentel-Elardo, S.M., Capretta, A., Nodwell, J.R. (2012) Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. *Chemistry & Biology*. *Manuscript accepted*.
55. Lipinski, C.A., Lombardo, F., Dominy, B.W., and Feeney, P.J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* 23, 3-25.
56. Hojati, Z., Milne, C., Harvey, B., Gordon, L., Borg, M., Flett, F., Wilkinson, B., Sidebottom, P.J., Rudd, B.A.M., Hayes, M.A., *et al.* (2002). Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. *Chemistry & Biology* 9, 1175-1187.
57. Craney, A., Hohenauer, T., Xu, Y., Navani, N.K., Li, Y., and Nodwell, J. (2007). A synthetic luxCDABE gene cluster optimized for expression in high-GC bacteria. *Nucleic Acids Research* 35, e46

58. Reville, W.P., Bibb, M.J., Scheu, A.K., Kieser, H.J., and Hopwood, D.A. (2001). -Ketoacyl Acyl Carrier Protein Synthase III (FabH) Is Essential for Fatty Acid Biosynthesis in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 183, 3526-3530.
59. Florova, G., Kazanina, G., and Reynolds, K.A. (2002). Enzymes involved in fatty acid and polyketide biosynthesis in *Streptomyces glaucescens*: Role of FabH and FabD and their acyl carrier protein specificity. *Biochemistry* 41, 10462-10471.