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Biosynthesis of Marineosin, a Spiroaminal Undecylprodiginine Natural Product

by

Shaimaa Mohamed Salem

A dissertation submitted in partial fulfillment of the
requirements of the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
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Portland State University
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ABSTRACT

Marineosins A and B are two spiroaminal-ring containing tripyrrole compounds isolated from the marine actinomycete, *Streptomyces* CNQ-617, and were found to possess potent and selective cytotoxic activity against leukemia and melanoma. Marineosins belong to the prodiginines class of natural products, examples of which are undecylprodiginine and streptorubin B. Unlike marineosins, prodiginines structures are characterized by the presence of fully conjugated tripyrrole nucleus linked to an alkyl chain (that lacks any oxygen). Cyclic prodiginines arise from an oxidative cyclization of the alkyl chain onto the tripyrrole, a step catalyzed by Rieske-oxygenase like enzymes such as RedG. The biosynthesis of prodiginines is directed via the *red* gene cluster. The unique structural differences between marineosin and other prodiginines spurred the proposal of a number of hypotheses for its biosynthesis, none of which have been experimentally tested. A *red* gene cluster homolog which has only one extra dehydratase-encoding gene; *marA* has been identified from the genomic library of *Streptomyces* CNQ-617, and the identified cluster was proposed to direct the biosynthesis of marineosin. In this study, the identified putative gene cluster was expressed in the heterologous host, *S. venezuelae*, and marineosin production in the new strain; JND2 was confirmed via LC/MS and ¹H-NMR. The new engineered strain also produces a myriad of marineosin related shunt metabolites and pathway intermediates. This study hence presents the first identified gene cluster proved to direct the biosynthesis of marineosin; the *mar*

gene cluster and proves that the cloned cluster encodes most, if not all the enzymes required to direct the biosynthesis of marineosin.

Deletion of the Rieske-oxygenase encoding gene; *marG* (a RedG homolog) from the *mar* gene cluster led to the accumulation of 2-hydroxyundecylprodiginine; G410 with an m/z 410.28 and molecular formula $C_{25}H_{35}O_2N_3$. This data proves that MarG is not responsible for the introduction of the spiroaminal ring oxygen on the alkyl chain, but is required for catalyzing macrocyclic ring formation between C-8 and C-9 of G410. Undecylprodiginine production in *marG* deletion mutant was not observed which indicates that undecylprodiginine is likely not an intermediate along the pathway for marineosin biosynthesis, and indicates that the spiroaminal ring oxygen is introduced early in the pathway, possibly due to the incorporation of a 3-hydroxy-butyric acid starter unit.

Deletion of the dehydratase-encoding gene; *marA*, from the *mar* gene cluster led to the accumulation of compounds JN408 and JN422 with m/z 408.26 and 422.24 and molecular formulae $C_{25}H_{33}O_2N_3$, and $C_{25}H_{31}O_3N_3$, respectively. Purification and structure elucidation of JN408 proves it to be an oxidized marineosin analog which has fully aromatic tripyrrole rings while; purification and structure elucidation of JN422 proves it to be a 9-keto-JN408 derivative. Both JN408 and JN422 compounds have a spiroaminal ring which indicates that MarA does not catalyze spiroaminal ring formation but catalyzes the reduction of pyrrole ring B of JN408 to yield marineosin. Therefore, we are proposing that MarA acts as a dehydrogenase, rather than a dehydratase. We are proposing

that the intramolecular spiroaminal ring formation is catalyzed by either MarG or occurs non-enzymatically. JN422 is a shunt metabolite produced due to promiscuous activity of either MarG or an unidentified oxidase in the *mar* cluster, possibly MarT.

From the data generated in this study, we present the first experimentally supported pathway for the biosynthesis of marineosin and the opportunity to generate novel compounds with potentially useful biological activities.

To the memory of my parents,

Without their love and support, nothing in my life would have been possible

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
CID	Collision induced dissociation
CoA	Coenzyme A
COSY	Correlation spectroscopy
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
HMBC	Heteronuclear multiple-bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
IR	Infra-red spectroscopy
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
O.D	Optical density
PCR	polymerase chain reaction
PKS	Polyketide synthases
TOCSY	Total correlation spectroscopy
UV	Ultraviolet

1. INTRODUCTION

1.1 NATURAL PRODUCTS AND *STREPTOMYCES*

Secondary metabolites or natural products are small organic molecules that are naturally produced by plants, fungi, algae, soft corals, sponges and bacteria. Although natural products do not play an explicit role in the vegetative growth of the producing organism, many scientists believe that secondary metabolites play a role in increasing the survivability and the adaptation of the producer organism in its complex environment by eliminating competing organisms [1], abstracting necessary metals from the environment [2], or mediating cell to cell contact [3]. Natural products possess a wide variety of clinical and pharmacological applications where they serve as antibiotics such as erythromycin A (1), antifungals such as amphotericin B (2), immunosuppressant such as rapamycin (3), hypolipidemics such as lovastatin (4) and anticancer drugs such as doxorubicin (5) compounds (Figure 1). Natural products account for about 47% of the drugs approved by the FDA for treatment of cancer [4] and natural products isolated from the genus; *Streptomyces* accounts for about two thirds of the world's antibiotics [5].

Streptomyces is a Gram-positive actinobacteria that can be found in terrestrial or marine environments and is characterized by high G+C percent DNA as compared to other Gram-positive bacteria such as *Bacillus* and *Streptococcus* [6].

Figure 1: Examples of Natural Products

Unlike other bacteria, *Streptomyces* colonies consist of differentiated cells and the life cycle [6] begins when a spore germinates one or more germ tubes that develop vegetative or substrate mycelia. Due to nutrition requirements, the vegetative mycelia branches out to form aerial mycelia that will be later compartmentalized via sporulation septa to form new spores [6].

Figure 2: Life Cycle of *Streptomyces lividans*. Scanning electron micrographs showing a. vegetative mycelia, b. vegetative mycelia branching into aerial mycelia, c. sporulation septa is formed in the aerial hyphae, d. spore chains. The figure is adapted from reference 6.

The importance of *Actinomycetes* to biomedical research and drug discovery comes from the ability of these bacteria to produce enormous numbers of

structurally diverse antibiotics that by 2002, it was estimated that 8700 antibiotics were isolated from terrestrial *Streptomyces* versus 7800 antibiotics from all other bacteria and fungi combined [7].

1.2 BIOSYNTHESIS OF NATURAL PRODUCTS

Natural products are biosynthesized via complex dedicated enzymatic machinery encoded by genes adjacent to each other in the organism's genome to form a biosynthetic gene cluster. The dedicated biosynthetic machinery utilizes common primary metabolites such as dicarboxylic acid thioesters, fatty acids, sugars and amino acids. The structural diversity and complexity of natural products is best manifested in two classes of natural products: polyketides and non-ribosomal peptides synthesized by polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), respectively. A type I PKS (Figure 3) is a megasynthase or a multifunctional multienzyme complex that catalyzes multiple head to tail decarboxylative condensations or Claisen condensation of dicarboxylic acid thioesters to form a polyketide in a manner analogous to fatty acid biosynthesis. There are four main domains (Figure 3) required for the biosynthesis of a polyketide: acyltransferase (AT), acyl carrier protein (ACP), ketide synthase (KS), and a thioesterase (TE) domain. The biosynthesis of a polyketide usually starts with the loading of a starter unit such as acetyl, propionyl, or butyryl-CoA onto the N-terminal ACP domain via the adjacent AT domain. The chain is then elongated by 2 carbons via a decarboxylative condensation between the tethered acyl-ACP

and one dicarboxylic acid thioester extender unit molecule such as malonyl-CoA in a reaction catalyzed by the KS domain. After several cycles of elongation, the polyketide is released from the PKS megasynthase by the TE domain to yield either a linear or cyclic product (Figure 3).

Figure 3: The Modular PKS of Pikromycin, an Example of Type I PKS

In type I PKS systems, an additional ketoreductase (KR) domain (module 1, Figure 3) can reduce a keto group into a hydroxyl group and then a dehydratase

(DH) domain (module 2, Figure 3) can catalyze double bond formation via the elimination of water from the polyketide. An enoyl-reductase (ER) domain (module 4, Figure 3) can then fully reduce the growing polyketide [8], [9]. It should be noted that for the ACP domain to be functional, it has to be post-translationally modified from the inactive *apo* form to the active *holo* form by a separate enzyme called phosphopantetheinyltransferase (PPTase). PPTase transfers a 4'-phosphopantetheine group from Coenzyme A (CoA) to a highly conserved serine residue in the ACP domain. The long and flexible phosphopantetheine arm provides a terminal sulfhydryl group that carries the growing polyketide chain via a thioester bond and transfers the tethered polyketide chain among the PKS domains. The biosynthetic gene clusters usually encode other enzymes that do not participate in the biosynthesis but rather play a regulatory role and/or confer resistance to the natural product in question to protect the producing organism [1], [10]. Examples of polyketides biosynthesized via type I PKS are: pikromycin (Figure 3), erythromycin (1) (Figure 1) antibiotics and the cholesterol lowering agent; lovastatin (4) (Figure 1).

1.3 STUDY OF NATURAL PRODUCTS BIOSYNTHESIS

The proven clinical and economic value of natural products has fueled the search for tools to aid in understanding their biosynthesis as well as enabling the biosynthetic engineering of analogs of better pharmacological activity, improved

bioavailability or reduced toxicity. Some of the tools to study the biosynthesis of a natural product of microbial origin are outlined below:

i) Isotope tracer studies:

As mentioned in section 1.2, natural products are made of building blocks that can be provided through primary metabolism such as fatty acids and amino acids. Therefore, feeding the growing microorganism with stable isotope-labeled precursors can be used to identify the general biosynthetic origin of a natural product since the incorporation of the isotope-labeled precursor in the natural product skeleton results in unique coupling and labeling patterns [11]. Such incorporation patterns can suggest the involvement of a particular pathway in the biosynthesis of the secondary metabolite such as the shikimic acid pathway involved in the biosynthesis of hygromycin from *Streptomyces hygroscopicus* [12] and phoslactomycin from *Streptomyces* sp. strain HK803 [13]. Stable isotope-labeling can even lead to the discovery of new pathways such as the discovery of the non-mevalonate pathway involved in the biosynthesis of isoprenoids after feeding [$^{13}\text{C}_1$]-glucose isotopomers and [^{13}C]-acetate isotopomers to eubacteria producing the isoprenoids, hopanoids [14].

ii) The construction, characterization and screening of genomic libraries:

A genomic library for a microorganism can be generated from its total or genomic DNA (gDNA) using cosmid, fosmid, or BAC vectors depending on the insert size and whether heterologous expression of a particular gene cluster is required [15],

[16]. The genomic libraries can then be screened for the gene cluster presumed to be responsible for the biosynthesis of a particular secondary metabolite using specific probes. The probes are usually short radio-labeled DNA sequences homologous to gene(s) specific to the cluster required and are amplified from the organism's gDNA using degenerate primers using PCR. For example; the use of the *chcA* gene probe for the screening of the genomic library of *Streptomyces* sp. strain HK803 to identify the gene cluster encoding the biosynthesis of phoslactomycin [13], [17]. More recent screening techniques include the use of non-radioactive probes, antibodies, and PCR methods [18].

iii) The heterologous expression of gene clusters:

Heterologous expression of a biosynthetic gene cluster seeks the production of the natural product in question in an organism other than the original producer by introducing the genes presumed to be involved in the biosynthesis to the new organism. When successful, heterologous expression provides unequivocal evidence of the responsibility of the gene cluster expressed in the biosynthesis of the natural product in question. It also indicates that all the genes required for the biosynthesis are present in the cluster as well as any regulatory and resistance-conferring genes. Choosing *E. coli* or *Streptomyces* species as heterologous hosts allow rapid genetic manipulations of the gene cluster via Red/ET-mediated recombination [19], [20], to further study the biosynthesis of the compound and facilitates engineering of other analogs. Heterologous expression can also

facilitate the over-expression of pathway-specific regulatory genes and therefore enhance the production of the secondary metabolite in question [21], [22]. One of the techniques employed in the heterologous expression of a particular gene cluster in *Streptomyces* involves modifying the BAC or cosmid vector carrying the cluster by including phage Φ C31 integration functions to allow for site-specific integration of the pathway in the *Streptomyces* genome [23]. The presence of *oriT*, an origin of transfer in the cosmid carrying the gene cluster facilitates DNA mobilization between *E.coli* and *Streptomyces* via conjugation [24] and the presence of an antibiotic resistance-conferring gene or an antibiotic resistance marker such as *tet*, which encodes resistance to tetracycline, would allow for selection of exconjugants actively expressing the gene cluster [25]. The use of *E. coli*-*Streptomyces* Artificial Chromosomes (ESAC) allows for the integration of DNA fragments > 100 kb in *Streptomyces* [26], [27].

iv) Gene deletion and/or replacement studies:

The deletion or replacement of individual genes along a biosynthetic pathway can be used to probe the role of the deleted genes in the biosynthesis *in vivo*. In this process, a particular gene can be completely deleted or replaced with another gene that confers resistance to a certain antibiotic such as ampicillin via PCR-targeted recombination or Red/ET-mediated recombination [16], [19], [20]. Gene deletion or replacement generates a mutant inefficient or totally incapable of producing the natural product in question. Comparing the metabolite pool of

the resultant mutant with the wild type organism pinpoints any intermediates, precursors or shunt metabolites accumulated due to the gene deletion and thus proposes a role for that gene in the biosynthesis of the natural product. Gene deletion experiments can be followed by complementation of the mutant strain with either the needed precursor or a precursor analog (chemical complementation); or with an expression plasmid carrying the deleted gene or another gene homolog (genetic complementation) to either rescue the production of the natural product itself or produce an engineered analog.

v) Protein expression of individual genes:

In order to assess substrate specificity *in vitro* and to provide a detailed kinetic analysis of individual enzymes participating in the biosynthesis of a certain natural product, their respective genes can be expressed in a heterologous host such as *E.coli*, followed by enzyme purification. A variety of high-level expression vectors are now available to allow gene expression under the influence of the bacteriophage T7 polymerase promoter [28]. The development of the HisTag technology allows the fusion of 6 histidine amino acid residues to either the N- or C-terminal end of the expressed protein which facilitates the purification of the recombinant protein by affinity chromatography using metal chelation resins [29]. After purification, the proteins can be used in a number of assays depending on the reactions they catalyze to monitor the substrate specificity as well as the rate by which they catalyze their reactions. The *in vitro* assays using the expressed

enzymes provide greater and more valuable insight into the mechanistic role of the individual enzyme in the biosynthetic process as a whole.

1.4 PRODIGININES FAMILY OF NATURAL PRODUCTS

1.4.1 History and Significance

Prodiginines (also known as prodigiosins) are tripyrrole red-colored compounds (Figure 4) isolated from *Serratia marcescens*, *Streptomyces coelicolor* A3(2), *Hahella chejuensis* and many other terrestrial and marine microorganisms [30]. These compounds are thought to be responsible for a number of prodigious miracles linked to the appearance of blood on bread and other starchy foods in the middle ages [31]. Prodiginines can either be linear, like prodigiosin (6) (Figure 4) and undecylprodiginine (7) (Figure 4), or cyclic like streptorubin B (8) (Figure 4), metacycloprodigiosin (9) (Figure 4) and marineosins (10 and 11) (Figure 4). Prodiginines have excellent cytotoxic activity against a wide variety of cancer cell lines via inducing apoptosis [32], facilitating oxidative DNA cleavage by intercalating Cu (II) and DNA [33], and by other mechanisms. Due to their unique and selective mode of action as anticancer compounds, GX15-070 or obatoclax (12) (Figure 4); a synthetic prodiginine analog is in phase 1 and 2 clinical trials by Gemin-X pharmaceuticals [34]. Prodiginines also possess potent immunosuppressant activity via inhibition of T lymphocytes-mediated antibody response [35–37] which spurred the synthesis of many prodiginine analogs of which; PNU-156804 (13) (Figure 4) was found to be the most potent with unique

immunosuppressant activity, minimal cytotoxicity and improved therapeutic index [36], [37]. Prodiginines also possess strong antibacterial and antimalarial activities [38].

Figure 4: Example of Prodiginines

1.4.2 Biosynthesis:

The biosynthesis of undecylprodigine (7) (Figure 4), a linear prodigine isolated from *S. coelicolor* A3(2), was initially probed using isotope labeling studies in the early 1970s. It has now been shown that undecylprodigine (7) (Figure 4) is biosynthesized via a bifurcated pathway (Figure 5) that involves the condensation of 2 subunits; 2-undecylpyrrole (2-UP) (Figure 4, 10) and 4-methoxy-[2, 2'-bipyrrole]-5-carboxaldehyde (MBP) (Figure 4, 9) subunits [39–42]. Feeding of stable isotope-labeled precursors showed that the 2-UP subunit (Figure 4, 10) is derived from seven acetate molecules and one L-glycine while the MBP subunit (Figure 4, 9) is derived from one molecule of acetate, one L-proline, one L-serine and a methyl group derived from L-methionine amino acid.

Figure 5: The bifurcated pathway leading to the biosynthesis of undecylprodigine and streptorubin B in *S. coelicolor* A(3)2

The gene cluster responsible for the biosynthesis of undecylprodigine and streptorubin B (Figure 4, 2 and 3 respectively) in *S. coelicolor* A(3)2, known as the *red* cluster was cloned and expressed in the heterologous host *Streptomyces parvulus* in 1990 [43], [44]. The complete sequencing of the *red* cluster was achieved in 2002 as part of the genome sequencing project of *S. coelicolor* A(3)2 [45]. The *red* cluster (Figure 6) is arranged in four transcription units consisting of 23 genes two of which are regulatory; *redD* and *redZ* (Figure 6). Most of the genes involved in the biosynthesis of undecylprodigine and streptorubin B were characterized via gene deletion and feeding experiments, as well as some enzymatic studies. The *red* genes were assigned as either involved in the biosynthesis of the MBP subunit (Figure 4, 9) or the 2-UP subunit (Figure 4, 10). The genes involved in the biosynthesis of the MBP subunit (9) were found to be *redM*, *O*, *W*, *X*, *N*, *V*, *U* and *redI* while the genes involved the 2-UP subunit (10) biosynthesis are *redP*, *R*, *Q*, *J*, *L*, *J* and *redK*. The condensation of the two subunits to form undecylprodigine is catalyzed by *redH*, while the cyclization of undecylprodigine into streptorubin B is catalyzed by *redG* [46–51].

Figure 6: The *red* gene cluster responsible for the biosynthesis of undecylprodigine and streptorubin B in *S. coelicolor* A(3)2. Red color is for regulatory proteins, green, genes involved in the biosynthesis of the MBP subunit, dark blue, genes involved in the biosynthesis of the 2-UP subunit, light blue, condensation of the MBP and 2-UP subunits, orange, genes involved in oxidative cyclization, grey, genes encoding membrane proteins, black, genes encoding hypothetical proteins.

An outline of the biosynthesis is summarized below:

The biosynthesis of the MBP subunit (9): The biosynthesis of the MBP subunit (Figure 5) starts with the activation of L-proline amino acid as an adenylate intermediate by RedM which is then passed over to the PCP (peptidyl carrier protein); RedO. The tethered L-proline is then oxidized by RedW to form pyrrolyl-2-carboxyl-S-RedO which is extended by 2 carbons via a decarboxylative condensation of malonyl-CoA with pyrrolyl-2-carboxyl-S-RedO catalyzed by RedX; a ketosynthase (KS) (Figure 5). The resultant pyrrolyl- β -ketothioester is condensed with L-serine amino acid residue with the release of CO₂ in a reaction catalyzed by the PLP-dependent α -oxoamine synthase (OAS) domain of RedN to give 4-hydroxy-2,2'-bipyrrole-4-methanol (HBM). Methylation of HBM is catalyzed by RedI; an S-adenosylmethionine (SAM)-dependent methyl transferase. The post-translational phosphopantetheinylation of RedO and the two ACP domains of RedN (to convert them from the *apo*- inactive form to the *holo*- active form) is proposed to be catalyzed RedU [30], [48].

b. The biosynthesis of the 2-UP subunit (10): the biosynthesis of the dodecanoic acid moiety (Figure 5) of the 2-UP subunit (10) of prodiginines portrays a great level of interplay between fatty acid biosynthesis; a primary metabolic process and secondary metabolism. As mentioned above, the 2-UP subunit (10) consists of one L-glycine amino acid residue and seven acetate molecules arranged head to tail in a manner analogous to fatty acid biosynthesis catalyzed by type II fatty acid synthases (Fas/Fab enzymes). It is proposed that the biosynthesis of 2-UP

(Figure 5) is initiated by RedP (a FabH homolog) which catalyzes the decarboxylative condensation of one molecule of acetyl-CoA with malonyl-RedQ (or malonyl-ACP) to yield 3-keto-butyryl-RedQ that will be fully reduced into butyryl-RedQ via the FAS enzymes, since homologs of such enzymes are completely absent from the *red* cluster. The resultant 3-keto-butyryl-RedQ is then elongated by six malonyl-CoA extender units in a reaction catalyzed by RedR (a FabF homolog). In each round of elongation, it is proposed that the acyl chain is fully reduced by the aid of FAS enzymes to yield dodecanoyl-RedQ (Figure 5). RedJ (a type II thioesterase) has been shown to hydrolyze the acyl chain of RedQ to produce free dodecanoic acid which is the substrate for the multi-functional protein; RedL (Figure 5). RedL catalyzes one more round of acyl chain elongation with malonyl-CoA to yield 3-ketomyristoyl-ACP which is then condensed with L-glycine amino acid residue with the elimination of CO₂ to produce 4-keto-2-undecylpyrroline. Finally, RedK reduces 4-keto-2-undecylpyrroline into 4-hydroxyl-2-undecylpyrroline which spontaneously loses H₂O to yield the 2-UP subunit (Figure 5).

The two subunits, MBP (14) and 2-UP (15), are then condensed to yield undecylprodigine via RedH, which is proposed to do so by catalyzing an ATP-dependent phosphorylation of the aldehyde oxygen of the MBP subunit (14), which facilitates a nucleophilic attack by C5 of 2-UP (15) to yield undecylprodigine (7) [51]. The conversion of undecylprodigine into the cyclic

analog; streptorubin B (8) is mediated by RedG; a Fe₂S₂ Rieske oxygenase-like enzyme [52].

1.5 MARINEOSINS, NEW UNDECYLPRODIGININE ANALOGS

1.5.1 Structure and discovery

Marineosins A and B (Figure 4) (10 and 11), are natural prodiginine analogs isolated from the marine *Streptomyces* related strain; CNQ-617. Marineosin A was found to have a potent cytotoxic activity against human colon cancer cell line, HCT-116 with an IC₅₀ of 0.5 µm. In the NCI-60 anticancer drug screening, marineosin A had a broad anticancer activity with great selectivity against leukemia and melanoma [53]. Structurally, marineosins are very distinct from other prodiginines due to the presence of an unusual spiroaminal ring and a reduced pyrrole ring. The biosynthetic origin of these structural differences is not readily explained by the current understanding of the biosynthesis of prodiginines (Figure 5) and the enzymes involved have not yet been identified.

1.5.2 Biosynthesis hypotheses

The potent anticancer activity of marineosin and its intriguing structure spurred the proposal of a number of hypotheses to explain its biosynthesis. The first hypothesis (Figure 7) was proposed by Fenical and co-workers in 2008 where, they proposed that the MBP subunit normally biosynthesized in most prodiginines is condensed with a 2-UP subunit analog; 2-keto-undec-3-enylpyrrole to yield an enone-derivative of undecylprodiginine. A hetero-Diels-

Alder cyclization of the enone-undecylprodiginine intermediate would form the tetrahydropyran and spiroaminal ring concomitantly [53] (Figure 7).

Figure 7: Fenical's Hypothesis for the Biosynthesis of Marineosin. Fenical and co-workers postulates the formation of enone-undecylprodiginine intermediate [53].

The above hypothesis was put to the test in the beginning of 2010 when Lindsay and co-workers tried to synthesize marineosin using Fenical's proposal [54]. Lindsay's group synthesized the enone-undecylprodiginine intermediate and attempted to produce marineosin through the hetero- Diels-Alder cyclization as proposed by Fenical and co-workers but they were not successful [54]. Molecular modeling studies by Lindsay and co-workers further revealed that the intramolecular Diel-Alder reaction is energetically unfavorable and cannot be

accomplished synthetically, however, they did not rule out the possibility that such cyclization could be accomplished via enzymatic catalysis [54]. In late 2010, another hypothesis for the biosynthesis of marineosin was postulated by Snider and co-workers [55]. Snider's hypothesis involves the formation of a macrocyclic free radical (Figure 8) in a reaction catalyzed by a RedG-analog. The macrocyclic free radical will be hydroxylated by the same proposed RedG-analog to form a macrocyclic alcohol intermediate which undergoes a number of hydride shifts to yield marineosins A and B [55].

It should be noted that there is no experimental evidence supporting either of the hypotheses proposed by either Fenical's group or Snider's group. The enone-undecylprodiginine intermediate postulated by Fenical's group was never isolated and the enzymes required for its biosynthesis cannot be determined from our current understanding for the biosynthesis of prodiginines. Though appealing, Snider's group's hypothesis is not well supported by the current theories of action of RedG homologs in the cyclization of prodiginines [52]. Therefore, the identification of the gene cluster involved in the biosynthesis of marineosin is necessary to provide an experimentally supported proposal for the biosynthesis of marineosin.

Figure 8: Snider's Hypothesis for the Biosynthesis of Marineosin. Snider and co-workers postulates the formation of a macrocyclic free radical that undergoes a number of hydride shifts mediated by a RedG homolog [55].

1.5.3 The identification of a hypothetical gene cluster

A SuperCos1 genomic library of *Streptomyces* CNQ-617 (marineosins-producing strain) was constructed in *E.coli* by Reynolds and co-workers (unpublished data). Screening of the genomic library for a *red* cluster homolog was performed using a ^{32}P -labelled probe generated by PCR amplification of a *redW* homolog named *marW* using degenerate primers. RedW (Figure 5) starts the biosynthesis of prodiginines by catalyzing the oxidation of L-proline into pyrroline, a committed step in the biosynthesis of the MBP subunit (Figure 4) (14) of prodiginines [48]. A RedW homolog is proposed to be essential for the biosynthesis of marineosin. Cosmids positive for *marW* were further screened using a ^{32}P -labelled probe generated by PCR amplification of a *redG* homolog named *marG* using degenerate primers. RedG (Figure 5) is a Rieske-oxygenase that culminates the biosynthesis of streptorubin B (Figure 4) (8), a cyclic prodiginine analog by catalyzing the cyclization of the acyl chain of undecylprodiginine (Figure 4) (7) [52]. A RedG homolog is also proposed to be essential for the biosynthesis of marineosin. The screening of the genomic cosmid library of *Streptomyces* CNQ-617 using *redW* and *redG* homologs led to the identification of the cosmid 8A7 that carries the entire gene cluster proposed to be involved in the biosynthesis of marineosin; a putative *mar* cluster together with about 6 kbp of DNA, apparently encoding primary metabolic genes (unpublished data). Shotgun sequencing of the putative *mar* cluster revealed a gene assembly almost identical to that of the *red* cluster (Figure 9) with the presence of only one extra gene; *marA*. The MarA

enzyme shares ~ 56% identity to a monoamine oxidase C (Mao C) like dehydratase from *Streptomyces hygroscopicus* and was therefore annotated as a dehydratase.

Figure 9: A comparison between the *red* gene cluster and the putative *mar* cluster. The *mar* cluster (bottom panel) compared to the *red* gene cluster (top panel). Red color is for regulatory proteins, green, involved in the biosynthesis of the MBP subunit, dark blue, involved in the biosynthesis of the 2-UP subunit, light blue, condensation of the MBP and 2-UP subunits, orange, oxidative cyclization, yellow, dehydratase, grey, membrane protein, black, hypothetical proteins.

The *marA* gene is proposed to be involved in the biosynthesis of the tetrahydropyran ring while another gene, *marG* is proposed to catalyze the reductive cyclization of the acyl chain of marineosin. MarG shares a 65% identity to its *red* cluster counterpart RedG. The striking degree of homology among the putative *mar* genes and their *red* genes counterparts (Table 1) together with the abundance of the *red* cluster in several terrestrial and marine organisms raises two major concerns. The first is whether or not the identified gene cluster is truly responsible for the biosynthesis of marineosin and not another prodiginine analog. The second is whether or not an extra tailoring enzyme is required for the biosynthesis of marineosin which is not included in the 8A7 cosmid.

Table 1: A comparison between the genes of the putative *mar* cluster and its *red* counterparts:

MarD	RedD, a TetR family transcriptional regulator, <i>Streptomyces griseus</i> . Identities = 150/264 (56%), Positives = 181/264 (68%)
MarX	RedX, <i>Streptomyces coelicolor</i> A3(2), a polyketide synthase. Identities = 79/987 (58%), Positives = 675/987 (68%)
MarW	RedW, <i>Streptomyces coelicolor</i> A3(2), an acyl-CoA dehydrogenase. Identities = 304/388 (78%), Positives = 332/388 (85%)
MarY	RedY, <i>Streptomyces coelicolor</i> A3(2), unknown function. Identities = 60/104 (57%), Positives = 73/104 (70%)
MarZ	RedZ, <i>Streptomyces coelicolor</i> A3(2), a response regulator. Identities = 111/206 (53%), Positives = 149/206 (72%)
MarV	RedV, <i>Streptomyces coelicolor</i> A3(2), unknown function. Identities = 192/350 (54%), Positives = 230/350 (65%)
MarU	RedU, <i>Streptomyces coelicolor</i> A3(2), hypothetical protein. Identities = 146/246 (59%), Positives = 169/246 (68%)
MarT	RedT, <i>Streptomyces coelicolor</i> A3(2), hypothetical protein. Identities = 181/278 (65%), Positives = 211/278 (75%)
MarR	RedR, <i>Streptomyces coelicolor</i> A3(2), 3-oxoacyl-[acyl-carrier-protein] synthase II. Identities = 181/278 (65%), Positives = 211/278 (75%)

MarQ	RedQ, <i>Streptomyces coelicolor</i> A3(2), acyl carrier protein. Identities = 55/80 (68%), Positives = 62/80 (77%)
MarP	RedP, <i>Streptomyces coelicolor</i> A3(2), 3-oxoacyl-(acyl carrier protein) synthase III. Identities = 212/286 (74%), Positives = 239/286 (83%)
MarO	RedO, <i>Streptomyces coelicolor</i> A3(2), putative peptidyl carrier protein. Identities = 63/87 (72%), Positives = 71/87 (81%)
MarN	RedN, <i>Streptomyces coelicolor</i> A3(2), 8-amino-7-oxononanoate synthase. Identities = 465/641 (72%), Positives = 515/641 (80%),
MarM	RedM, <i>Streptomyces coelicolor</i> A3(2), peptide synthase. Identities = 368/529 (69%), Positives = 408/529 (77%)
MarL	RedL, <i>Streptomyces coelicolor</i> A3(2), polyketide synthase. Identities = 1095/1822 (60%), Positives = 1258/1822 (69%)
MarK	RedK, <i>Streptomyces coelicolor</i> A3(2), oxidoreductase. Identities = 243/346 (70%), Positives = 280/346 (80%)
MarJ	RedJ, <i>Streptomyces coelicolor</i> A3(2), thioesterase. Identities = 164/252 (65%), Positives = 180/252 (71%)
MarI	RedI, <i>Streptomyces coelicolor</i> A3(2), methyltransferase. Identities = 248/346 (71%), Positives = 291/346 (84%)

MarH	RedH, <i>Streptomyces coelicolor</i> A3(2), phosphoenolpyruvate-utilizing enzyme. Identities = 642/916 (70%), Positives = 736/916 (80%)
MarG	RedG, <i>Streptomyces coelicolor</i> A3(2), oxidase. Identities = 218/334 (65%), Positives = 269/334 (80%)
MarE	RedE, <i>Streptomyces coelicolor</i> A3(2), a hypothetical protein. Identities = 221/284 (77%), Positives = 248/284 (87%)
MarA	Monoamine oxidase C (MaoC) like dehydratase, <i>Streptomyces hygroscopicus</i> . Identities = 85/151 (56%), Positives = 111/151 (73%)

1.6 SPECIFIC AIMS OF CURRENT RESEARCH

Up to date, the total chemical synthesis of marineosins is still incomplete [54], [55]. There is no experimental evidence supporting the biosynthetic hypotheses provided by either Fenical's or Lindsley's group and there is also no experimental evidence that the gene cluster identified by Reynolds and co-workers is indeed responsible for the biosynthesis of marineosin. The objective of the present research is to investigate the key steps leading to the biosynthesis of marineosin and to provide an experimentally supported proposal for its biosynthesis which paves the road for the directed biosynthesis of marineosin analogs.

This objective will be achieved through the following specific aims:

- 1) Determination of the role played by the putative gene cluster identified by Reynolds and co-workers in the biosynthesis of marineosin or other prodiginine analogs via expression in a heterologous host.
- 2) Probing the role of *marG*, encoding a Rieske oxygenase in the biosynthesis of marineosin, via gene replacement experiments followed by characterization and structure elucidation of the accumulated intermediates/shunt metabolites accumulated using mass spectrometry and NMR.
- 3) Probing the role of *marA*, encoding a dehydratase potentially involved in the biosynthesis of marineosin, via gene replacement experiments followed by characterization and structure elucidation of the intermediates/shunt metabolites accumulated using mass spectrometry and NMR.
- 4) Provide a proposal for the biosynthesis of marineosin that is experimentally supported by the genetic replacement experiments and analysis of the metabolite pool of the mutants generated in aims 2 and 3.

2. THE IDENTIFICATION OF THE GENE CLUSTER RESPONSIBLE FOR THE BIOSYNTHESIS OF MARINEOSIN: THE MAR CLUSTER.

2.1 SUMMARY

The screening of a SuperCos1 genomic library of *Streptomyces* CNQ-617 – the marineosin producer, led to the identification of a cosmid clone that carries a gene cluster that may direct the biosynthesis of marineosin. Gene annotation of the putative *mar* cluster revealed a gene assembly similar to that of the *red* cluster responsible for the biosynthesis of undecylprodigine and streptorubin B in *S. coelicolor* A3(2) (Figure 9) (Reynolds KA and co-workers, unpublished work). Whether or not the identified gene cluster is indeed involved in the biosynthesis of marineosin and whether it contains all the genes required for the biosynthesis was never shown.

In this research, the SuperCos1 cosmid; 8A7 carrying the putative *mar* cluster was expressed in the heterologous host; *Streptomyces venezuelae* and the production of marineosin was confirmed via LC/UV, LC/UV-MS, Tandem mass spectrometry and ¹H-NMR. This study is the first direct evidence that the gene cluster identified by Reynolds KA and co-workers is the one responsible for the biosynthesis of marineosin and therefore, paves the road for a thorough evaluation of the biosynthesis of marineosin and the engineering of marineosin analogs via genetic and chemical complementation experiments. The newly engineered strain *Streptomyces venezuelae* JND2 produces ~ 2 mg/L of marineosin. The engineered strain also produces a number of marineosin

analogs named JN408, JN422 and JN424 characterized with λ_{max} 320 nm and have $[\text{M}+\text{H}]^+$ of m/z 408.28, 422.26, and 424.28 corresponding to molecular formulae of $\text{C}_{25}\text{H}_{34}\text{O}_2\text{N}_3$, $\text{C}_{25}\text{H}_{32}\text{O}_3\text{N}_3$, and $\text{C}_{25}\text{H}_{34}\text{O}_3\text{N}_3$, respectively. These compounds can be either pathway intermediates or shunt metabolites formed due to accumulation of intermediates along the pathway.

In addition, other red-colored compounds with a λ_{max} of 530 nm, were also observed in the engineered strain. No tri-oxygenated red-colored compounds were observed in *S. venezuelae* JND2 which suggests that additional oxidation steps can occur later in the biosynthetic pathway, after loss of tripyrrole conjugation. All observed compounds were absent from the control *S. venezuelae* acetone extract.

Based on the results of our studies, we propose four hypotheses that can explain the biosynthetic origin of the spiroaminal moiety of marineosin.

2.2 INTRODUCTION

As described in section 1.5, marineosins A and B are two prodiginine analogs isolated from a true marine *Streptomyces* with a strong and selective cytotoxic activity against colon cancer and leukemia [53]. Marineosins are characterized by the presence of an intriguing spiroaminal ring and a reduced pyrrole ring that cannot be explained based on the bifurcated pathway established for the biosynthesis of other prodiginines (Figure 5). Spiroaminal ring containing natural

products are characterized by numerous biological activities such as the immunosuppressant sanglifehrin (Figure 10), (16) [56] the antiviral crambescidin

Figure 10: Examples of spiroaminal compounds

alkaloids (Figure 10) (17 and 18) with activity against herpes simplex virus type-1 (HSV-1) and human immunodeficiency virus (HIV) [57], [58] in addition to the herbicidal hydantocidin (Figure 10) (19) [59]. Although the total synthesis of spiroaminals was reported several times [55], [57], [59], [60], [61], [62], the biosynthesis of this important class of natural products has been generally neglected. The intriguing structure of marineosin spurred the proposal of numerous hypotheses discussing its biosynthesis but no experimental data was ever provided. A putative gene cluster similar to that of the *red* cluster

responsible for the biosynthesis of undecylprodiginine and streptorubin B was identified by Reynolds and co-workers after construction of a SuperCos1 genomic library of *Streptomyces CNQ-617* however; its role in the biosynthesis of marineosin was not established experimentally.

2.3 MATERIALS AND METHODS

2.3.1 Antibiotics, enzymes and chemicals

All antibiotics, chemicals and HPLC-grade solvents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Primers for PCR were purchased from Integrated DNA Technologies (Coralville, IA). Marineosin standard was kindly provided by Dr. William Fenical (Scripps Institute of Oceanography, San Diego, CA). Phusion high-fidelity DNA polymerase was purchased from New England Biolabs (Ipswich, MA). Platinum High Fidelity polymerase supermix was purchased from Invitrogen.

2.3.2 Bacterial strains and culture conditions

The *E. coli* strain harboring the λ -RED plasmid known as BW25113/pIJ790 and the methylation deficient *E. coli* donor strain ET12567/pUZ8002 were provided by the John Innes Institute (Norwich, UK) and have been described previously in literature [19].

E. coli BW25113/pIJ790 was grown at 30°C in Lysogeny Broth (LB) media [63] and super optimal broth (SOB) media [64], each containing 25 μ g/ml of

chloramphenicol while *E. coli* ET12567/pUZ8002 was grown at 37°C in LB media containing 25 µg/ml kanamycin and 25 µg/ml chloramphenicol. *Streptomyces venezuelae* was propagated on sporulation agar (SPA) plates [65] at 30°C while SCM media was used for seed and large scale cultures.

2.3.3 DNA manipulation and construction of pMAR cosmid

The pSET1520 plasmid and 8A7 cosmid DNA were isolated from *E. coli* cells using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). DNA sequencing was performed at the MMI core facility and at The Primate Center, Oregon Health and Sciences University (Portland, OR). Primer design and analysis of DNA sequences was performed using MacVector (MacVector Inc., Cary, NC) and Accelrys DS gene (Accelrys Inc., San Diego, CA) software.

In order to allow the integration of the 8A7 cosmid carrying the putative *mar* cluster into the genome of *Streptomyces*, the 8A7 cosmid was first transformed into *E. coli* BW25113/pIJ790 (recombination strain) via electroporation according to standard protocol [19]. Then, the *bla* gene encoding resistance to ampicillin antibiotic in the 8A7 cosmid was replaced with three genes using PCR targeted recombination (Figure 11): *oriT* which is an origin of transfer, $\Phi C31$ integrase gene which allows for integration of the cosmid at the *attB* site in *Streptomyces* genome, and *aac(3)/IV* gene which allows for selection of exconjugants expressing the *mar* cluster by conferring resistance to apramycin antibiotic (Figure 11).

Figure 11: Recombination strategy employed to generate an integrative *mar* gene cluster. pSET 1520 plasmid was used as a template for PCR amplification of *oriT*, phiC31 integrase and *aac(3)IV*. The PCR product was used to replace the *bla* gene in cosmid 8A7 via PCR targeted recombination followed by site-specific integration of the modified 8A7 cosmid into *S. venezuelae*. *bla* = β -lactamase; *aac(3)IV* = apramycin resistance gene; *oriT* = origin of transfer; *attP/aatB* = phiC31 attachment site of phage/bacteria; *int* = phiC31 integrase.

The three genes *aac(3)IV*, *oriT*, and $\Phi C31int$ were amplified from pSET1520 plasmid template using 5'- **TGA TAA ATG CTT CAA TAA TAT TGA AAA AGG** **AAG AGT** ATG GTG CAA TAC GAA TGG CGA AA -3' as the forward primer and 5'- **AAT CTA AAG TAT ATA TGA GTA AAC TTG GTC TGA CAG** TTA CTA CGC CGC TAC GTC TTC C -3' as the reverse primer with the bases in bold italic homologous to the *bla* gene of SuperCos1. Amplification of the three-gene 3966 bp long PCR product, was achieved using GeneAmp PCR system 2700 (Applied Biosystems) according to the following protocol: initial denaturation at 98°C for 30 seconds followed by 30 cycles of amplification (denaturation at 98°C for 10

seconds, and annealing at 72°C for 1 minute) followed by a final extension step for 10 minutes at 72°C. The PCR product was purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA) and the PCR was eluted using 10 µl of EB buffer. A poly-A tail was added to the purified blunt-ended PCR product by incubating it with Platinum PCR High Fidelity Supermix (Invitrogen) at 72°C for 10 minutes. The PCR product was cloned into pCR8/GW/TOPO vector (Invitrogen) to generate TOPO-pS1 that was transformed into Mach1 *E. coli* (Invitrogen). After confirmation of the TOPO-pS1 plasmid DNA sequence, the plasmid was digested with *EcoRI* and loaded onto a 1% agarose gel and subjected to gel electrophoresis. The fragment corresponding to ~ 4 kbp three-gene was excised from the gel and purified using QIAquick Gel Purification kit (Qiagen). The linear purified *aac(3)/IV*, *oriT* , and $\phi C31int$ tri-gene was used in PCR targeted recombination of 8A7 cosmid according to standard Red/ET recombination protocol [19] to generate the pMAR cosmid which has the ability to integrate at the *attB* site in the *Streptomyces* genome (Figure 11). The recombination of pMAR cosmid was confirmed by PCR amplification of *aac(3)/IV*, *oriT*, and $\phi C31int$ tri-gene using the same primers used above. The pMAR cosmid was transformed into *E. coli* ET12537/pUZ8002 (donor strain) to generate ET/pMAR donor strain which is apramycin resistant and ampicillin sensitive and capable of conjugation with *Streptomyces venezuelae* (receiving strain).

2.3.4 Conjugation of *E.coli* ET/pMAR and *S. venezuelae*

The conjugation between the *E.coli* ET/pMAR and *S. venezuelae* was done according to standard protocol with minor modifications [65]. *S. venezuelae* was propagated on SPA agar and incubated at 30°C for 7 days. Fresh spores were used to inoculate 25 ml of SGGP media [66] and was allowed to grow for 3 days at 30°C. On day 3, 1 ml of the SGGP culture was used to inoculate 9 ml of TSB media and was incubated at 30°C for 18 hours. At the same time, *E. coli* ET/pMAR was streaked on LB-agar plates supplied with 50 µg/ml of apramycin and 25 µg/ml of chloramphenicol and was incubated at 37°C for 18 hours. The next day, 2 ml of the overnight *S. venezuelae* TSB culture was used to inoculate 18 ml of fresh TSB media and incubated at 30°C for 18 hours while a single colony from the *E. coli* ET/pMAR LB agar plate was used to inoculate 2 ml of TSB media containing 50 µg/ml of apramycin and 25 µg/ml of chloramphenicol. The next day, 1 ml of the overnight *S. venezuelae* TSB culture was used to inoculate 9 ml of fresh TSB media and incubated at 30°C for 3 hours while 100 µl of the overnight *E. coli* ET/pMAR LB culture was used to inoculate 10 ml of TSB media supplied with 50 µg/ml of apramycin and 25 µg/ml of chloramphenicol. *E. coli* ET/pMAR was allowed to grow at 37°C until O.D₆₀₀ ~ 0.4 was reached. Both *E. coli* ET/pMAR and *S. venezuelae* cultures were centrifuged at 4°C and 5000 rpm for 5 minutes to recover the cells. The recovered cells were washed twice with fresh TSB media to remove any inhibiting antibiotics and cells were collected by centrifuge again. The cells of *E. coli* ET/pMAR (donor) and *S. venezuelae*

(recipient) were finally suspended in 2 ml of TSB and mixed together in 1:1, 1:9 and 9:1 donor: receiver ratios and 100 µl of each mixture was spread onto pre-dried AS1 media containing 10 mM MgCl₂. After overnight incubation at 30°C, each plate was overlaid with 1 ml of LB media containing 500 µg/ml nalidixic acid, 500 µg/ml of apramycin and 500 µg/ml of kanamycin and plates were returned to incubation at 30°C for an additional 7-14 days [65]. Colonies were visible after approximately 3-5 days. The exconjugants expressing the *mar* cluster were characterized by a distinctive red color and were propagated on SPA plates supplied with 50 µg/ml apramycin. The new strain was named JND2.

2.3.5 LC/MS and MS/MS analysis of JND2 crude extract

To prepare extracts for MS analysis, fresh spores of *S. venezuelae* (wild type) and *S. venezuelae* JND2 strain was used to inoculate 10 ml of SCM media. After 72 hrs, the cells were harvested and the cells' organic constituents were extracted with acetone. The organic content of the supernatant obtained after cell harvest was extracted by a solid-phase extraction method using Amberlite XAD-7 resin where the supernatant was stirred with the resin for 2 hours followed by filtration to collect the resin. The resin was washed with water and then the organic content of the resin was obtained by washing with acetone. The acetone extract was dried under vacuum and stored at -20°C till further analysis. The dry extracts from wild type *S. venezuelae* and JND2 were dissolved in MS-grade methanol and were injected directly into the mass spectrometer. Direct MS and

Tandem MS experiments were conducted using a ThermoElectron LTQ-Orbitrap high resolution mass spectrometer equipped with Accela HPLC system (ThermoFinnigan) (Mass Spectrometry Facility, Portland State University). Data was analyzed using Xcalibur software. For LC/MS analysis, the crude extract from JND2 strain was dissolved in methanol and 20 µl of 0.5 mg/ml extract was injected into MicroToF-Q mass spectrometer (Bruker, Billerica, MA) equipped with an Agilent 1100 series HPLC (Agilent technologies, Santa Clara, CA) with MWD detector (Mass Spectrometry Facility, Portland State University) using 2.1 x 250 mm, 5µm, reverse phase Supelco ¹⁸C-column (Supelco, St. Louis, MO) and using a 50 min gradient from 5% ACN/H₂O to 100 % ACN containing 0.05% formic acid at flow rate of 200 µl/min.

2.3.6 Purification and characterization of marineosin from S. venezuelae JND2

Fresh spores of *S. venezuelae JND2* were used to inoculate 10 ml of SCM media containing 50 µg/ml of apramycin to generate a seed culture that was incubated at 30°C, 220 rpm for 48 hours. 1% of the seed culture was used to inoculate larger scale fermentations that were grown for an additional 7 days at 30°C, and 220 rpm.

The JND2 cells were harvested by centrifugation at 10000 rpm, 6° C for 10 minutes. The harvested cells were disrupted by the addition of acetone followed by sonication for 5 minutes. After filtration using cotton wool, the acetone extract was dried under vacuum and stored at -20°C till further analysis. The crude cell

extract from JND2 strain was dissolved in methanol and purified using a Waters HPLC equipped with a DWD detector using 20.0 x 250.0 mm, 10 µm, reverse phase Ascentis ¹⁸C-column (Supelco, St. Louis, MO) using buffer A: water/acetonitrile/methanol (5:1:4) and buffer B: 100% methanol using the gradient specified in Table 2.

Time (min)	Flow (ml/min)	% A	% B
0	10.0	90.0	10.0
2.0	10.0	90.0	10.0
40.0	10.0	0.0	100.0
50.0	10.0	0.0	100.0
51.0	10.0	90.0	10.0
60.0	10.0	90.0	10.0

Table 2: HPLC gradient for first purification of marineosin

The peak eluting at 45 minutes was collected, dried under vacuum and subjected to another round of purification using an Agilent HPLC equipped with a MWD detector using 4.6 x 150.0 mm, 3.5 µm, reverse phase Zorbax SB-¹⁸C-column (Agilent technologies, Santa Clara, CA) using buffer A: water acidified with 0.05 % formic acid and buffer B: acetonitrile acidified with 0.05 % formic acid using the gradient specified in Table 3. The peak with retention time of 12 minutes was collected and dried under vacuum to yield pure marineosin.

Time (min)	Flow (ml/min)	% A	% B
0.0	1.0	75.0	25.0
7.5	1.0	75.0	25.0
8.0	1.0	50.0	50.0
13.0	1.0	50.0	50.0
13.5	1.0	25.0	75.0
21.0	1.0	25.0	75.0
21.5	1.0	0.0	100.0
25.0	1.0	0.0	100.0
25.5	1.0	75.0	25.0
30.0	1.0	75.0	25.0

Table 3: HPLC gradient used in second purification of marineosin

The dried marineosin was dissolved in acetone- d_6 for characterization by ^1H -NMR. ^1H -NMR spectra was generated using a Bruker AMX-400 MHz spectrometer (Bruker Daltonics), calibrated using TMS as an internal standard.

2.4 RESULTS AND DISCUSSION

2.4.1 Identification of the gene cluster responsible for the biosynthesis of marineosin: The *mar* cluster

Heterologous expression of the putative *mar* gene cluster was pursued to verify the role of the presumed cluster in the biosynthesis of marineosin. The 8A7 cosmid carrying the putative cluster was genetically engineered into the *pMAR* cosmid which has the ability to integrate in the genome of *S. venezuelae*. Conjugation was used to deliver *pMAR* cosmid into *S. venezuelae* to generate the JND2 strain. Unlike the wild type *S. venezuelae* which is grey in color (Figure

12), the JND2 strain is characterized by a distinctive red color. This change in phenotype indicates that the expression of the gene cluster in question leads to the accumulation of red-colored metabolites, although marineosin itself is not red [53]. The red-colored metabolites could be undecylprodiginine (7) (Figure 4) or other prodiginine analogs. *S. venezuelae* JND2 is also apramycin resistant unlike the wild type, which is apramycin sensitive.

Figure 12: SPA agar plates showing change in phenotype of wild type *S. venezuelae* (left panel, grey) as a result of expression of the *mar* gene cluster to yield *S. venezuelae* JND2 (right panel, red).

The MS profile of acetone extracts from wild type *S. venezuelae* shows the expected $[M+H]^+$ of m/z 470.31 and 526.34 corresponding to methymycin and pikromycin antibiotics respectively, naturally produced by the strain [67] (Figure 13). However, the MS profile of *S. venezuelae* JND2 strain indicates the presence of multiple new compounds in addition to methymycin and pikromycin

that are absent from the wild type strain (Figure 13). Among the new compounds detected in JND2 extract MS spectrum, is a compound with $[M+H]^+$ of m/z 410.28 corresponding to molecular formula of $C_{25}H_{36}O_2N_3$ which matches the published mass and molecular formula of marineosin [53].

S-venz_120112094728 #6-227 RT: 0.07-1.99 AV: 222 NL: 1.35E7
T: FTMS + p ESI Full ms [200.0]

JND2_120112095025 #7-230 RT: 0.07-2.00 AV: 224 NL: 1.13E7
T: FTMS + p ESI Full ms [200.0]

Figure 13: A comparison between the total ion spectrum of wild type *S. venezuelae* (top panel) and *S. venezuelae* JND2 (bottom panel). The MS profile of the wild type *S. venezuelae* (top panel) shows the presence of methymycin and pikromycin antibiotics with $[M+H]^+$ of m/z 470.31 and 526.34 respectively, while the MS profile of *S. venezuelae* JND2 shows the putative presence of marineosin with $[M+H]^+$ of m/z 410.28 in addition to methymycin, pikromycin and other new compounds.

Comparing the MS/MS profile of the m/z 410.28 in the JND2 extract (Figure 14, bottom panel) to that of a standard marineosin (Figure 14, top panel), reveals that both compounds have exactly the same MS/MS profile where both compounds have daughter fragments m/z 378.26 and 230.19 corresponding to structures 20 and 21 (Figure 15), respectively.

Figure 14: A comparison between the MS/MS profile of marineosin standard (top panel) and the compound with m/z 410.28 in *S. venezuelae* JND2 extract (bottom panel). Compounds in both top and bottom panels show the presence of m/z 230.19, m/z 302.21 and m/z 378.25.

Figure 15: Fragment ions observed in the MS/MS profile of marineosin. Figure is adapted from reference 53.

Moreover, LC/MS analysis (Figure 16) of the JND2 extract and a standard marineosin sample shows that both marineosin standard (Figure 16, top panel) and *m/z* 410.28 compound in the JND2 extract (Figure 16, bottom panel) have almost the same retention time with only 0.2 minute difference.

Figure 16: An LC/MS comparison between marineosin standard and JND2 extract. An extracted ion chromatogram (EIC) of m/z 410.28 for marineosin standard (top panel) and JND2 extract (bottom panel).

The compound with $[M+H]^+$ m/z 410.28 produced by *S. venezuelae* JND2, was purified from the acetone extract via 2 rounds of HPLC purifications (Figure 17).

^1H -NMR spectral data (400 MHz, acetone- d_6) of the purified compound (Figure 18) was found to be:

δ 10.99 (br, 1-NH), δ 8.26 (br, 1-NH), δ 6.98 (dd, J = 1.5 Hz and 2.5 Hz, 1H), δ 6.38 (dd, J = 1.5 Hz and 3.5 Hz, 1H), δ 6.1 (dd, J = 2.5 Hz and 3.5 Hz, 1H), δ 5.69 (t, J = 2.5 Hz, 1H), δ 5.45 (t, J = 3 Hz, 1H), δ 4.24 (m, J = 6.5 Hz, 1H), δ

3.86 (t, $J = 8.5$ Hz, 1H), δ 3.41 (s, 3H), δ 2.91 (d, $J = 12$ Hz, 1H), δ 2.88 (dd, $J = 8.5$ Hz and 16 Hz, 2H), δ 2.32 (m, 1H), δ 2.23 (m, 2H), δ 1.88 (dd, $J = 8.5$ Hz and 16 Hz, 2H), δ 1.77 (m, $J = 12$ Hz, 2H), δ 1.61 (td, $J = 6.5$ Hz and 12 Hz, 2H), δ 1.51 (d, $J = 7$ Hz, 3H), δ 1.64 (m, 2H), δ 1.52 (m, 2H), δ 1.37 (m, 2H), δ 1.35 (m, 2H), δ 1.34 (m, 2H), δ 1.28 (m, 2H), δ 1.18 (m, 2H), δ 1.02 (m, 2H), δ 0.88 (m, 2H), δ 0.88 (m, 2H), δ 0.69 (m, 2H), δ 0.52 (m, 2H).

This data is consistent with the published data for marineosin [53] which proves the identity of the $[M+H]^+$ m/z 410.28 compound produced in *S. venezuelae* JND2 as marineosin. *Streptomyces venezuelae* JND2 strain produces 2 mg/L of marineosin vs. 0.4 mg/L from the original producer *Streptomyces* CNQ-617 [53]. Comparing the MS profile of JND2 cell extract to that of JND2 XAD-extract reveals that marineosin is primarily located inside the cell and is not secreted into the media.

Figure 17: HPLC purification of marineosin from *S. venezuelae* JND2. Marineosin was purified from JND2 extract in 2 HPLC purification steps. The peak at 45 min from the first purification (top panel) was collected and subjected into another round of HPLC purification (bottom panel) to yield marineosin.

Figure 18: ^1H -NMR of marineosin isolated from *S. venezuelae* JND2.

The successful production of marineosin in the heterologous host, *S. venezuelae* provides the first experimental evidence that marineosin is biosynthesized via a *red* gene cluster homolog which raises interesting questions about the origin of the spiroaminal ring oxygen of marineosin. To the best of our knowledge, the *mar* cluster is also the first identified gene cluster to be involved in the biosynthesis of a spiroaminal ring containing compound which will provide a wealth of information on the biosynthesis of this unique and important class of natural products. Having the *mar* cluster expressed in a heterologous host such as *S. venezuelae* facilitates the engineering of marineosin analogs and allows for yield

improvements of marineosin using methods such as over-expression of regulatory genes in the *mar* gene cluster.

2.4.2 Hypotheses explaining the origin of the spiroaminal ring oxygen

As mentioned earlier in section 1.5.3, the *mar* gene cluster shows striking homology and displays the same architecture of the *red* gene cluster responsible for the biosynthesis of undecylprodigine and streptorubin B in *S. coelicolor* A(3)2, with the presence of only one extra dehydratase-encoding gene named, *marA* (Figure 9). Interestingly, the results of all the studies presented in this section indicate that the expression of the *mar* gene cluster in the heterologous host, *S. venezuelae*, was successful and that the new strain, *S. venezuelae* JND2, is fully capable of producing marineosin with three times higher yield than the original producer. Therefore, the cloned *mar* gene cluster encodes most if not all of the genes required to direct the biosynthesis of marineosin. We hypothesize that the intramolecular cyclization and spiroaminal ring formation is catalyzed by the dehydratase, MarA. However, the *mar* gene cluster lacks any dedicated oxidases or p450 homologs that can explain the origin of the hydroxyl group in the acyl chain of marineosin which is required for the biosynthesis of the spiroaminal moiety (Figure 4) (10 and 11). There are four hypotheses (Figure 19) that can explain the origin of that hydroxyl group:

1. The *marG* gene, which encodes the Rieske-oxygenase MarG, plays a dual role in the biosynthesis of marineosin. In this case, MarG catalyzes the oxidative

cyclization of the acyl chain of undecylprodiginine, like it normally does in the biosynthesis of streptorubin B (Figure 5) [52] and also introduces the second hydroxyl group required to initiate the biosynthesis of the spiroaminal moiety of marineosin. If this hypothesis is correct, a deletion of *marG* gene from the *mar* gene cluster should lead to the accumulation of undecylprodiginine as an intermediate (Hypothesis no. 1, Figure 19).

2. An unidentified Mar enzyme catalyzes the required hydroxylation. The *mar* gene cluster, as well as its *red* gene cluster homolog, encodes two genes of unknown function named *marT* and *marE* and therefore were annotated as hypothetical proteins. Deletion of one or both genes can be used to probe their roles in the biosynthesis of marineosin (hypothesis no. 2, Figure 19).

3. Marineosin production in the original producer, *Streptomyces* CNQ-617 requires the presence of an extra tailoring oxidase enzyme which is not clustered with the *mar* gene cluster. In this case, the biosynthesis of marineosin in *S. venezuelae* can be explained by the presence of a promiscuous p450 oxidase capable of hydroxylating the acyl chain of undecylprodiginine (7) to yield 2-hydroxy-undecylprodiginine (25) and thus rescue marineosin production (hypothesis no. 3, Figure 19). However, there is no evidence that *S. venezuelae* harbors such an enzyme.

4) Different Starter Unit

Figure 19: Hypotheses explaining the biosynthetic origin of the spiroaminal oxygen of marineosin. For details, please refer to text.

4. The hydroxyl group is incorporated early in the biosynthetic pathway due to the incorporation of a 3-hydroxy-butyric acid starter unit (22) by the RedP homolog, MarP, which is a 3-ketoacyl-ACP-synthase, to yield 2-hydroxy-dodecanoic acid (23). 2-hydroxy-dodecanoic acid (23) becomes a substrate for the polyketide

synthase, MarL to yield a 2-hydroxy-undecylpyrrole (2-hydroxy-UP) subunit (24). MarH then catalyzes the condensation of the 2-hydroxy-UP subunit (24) with the MBP subunit (14) (Figure 4), which leads to the biosynthesis of 2-hydroxy-undecylprodiginine (25) rather than undecylprodiginine (7) (hypothesis no. 4, Figure 19). In this case, all enzymes involved in the biosynthesis of 2-hydroxy-UP subunit (24) as well as MarH and MarG must accept hydroxylated substrates unlike their Red enzymes counterparts. If this hypothesis is correct, deletion of *marG* gene from the *mar* gene cluster will lead to the accumulation of 2-hydroxy-undecylprodiginine (25).

2.4.3 New compounds observed in *S. venezuelae* JND2

The prodiginine family of compounds is characterized by having a maximum UV absorbance (λ_{max}) of 530 nm due to aromatic ring system conjugation, and thus has a distinctive red color. The λ_{max} of marineosin standard is 320 nm due to the reduction of one of the pyrrole rings (ring B, Figure 4) and loss of conjugation. The extract of *S. venezuelae* JND2 was analyzed via MicroToF-Q mass spectrometer using reverse phase HPLC equipped with MWD detector. The LC-UV analysis of the extract indicates the presence of two groups of compounds: a group of non-conjugated compounds with a λ_{max} of 320 nm and a group of conjugated red-colored compounds with a λ_{max} of 530 nm. The non-conjugated compounds include marineosin in addition to three other compounds named JN422, JN424, and JN408 with $[M+H]^+$ of m/z 422.26, 424.28, and 408.26 and

molecular formulae $C_{25}H_{32}O_3N_3$, $C_{25}H_{34}O_3N_3$, and $C_{25}H_{34}O_2N_3$, respectively. The conjugated, red-colored compounds include $[M+H]^+$ m/z 406.25, 408.27, and 410.28 with respective molecular formulae of $C_{25}H_{32}O_2N_3$, $C_{25}H_{34}O_2N_3$, and $C_{25}H_{36}O_2N_3$ in addition to a mixture of other red compounds (Figure 20).

Figure 20: The LC-UV/MS profile *S. venezuelae* JND2. The blue chromatogram represents absorption at 530 nm and the orange chromatogram represents absorption at 320 nm.

Based on our knowledge of prodiginines biosynthesis and the fact that marineosin is non-conjugated, the accumulation of conjugated red-colored compounds in *S. venezuelae* JND2 suggests that the reduction of the pyrrole ring B of marineosin occurs later in the pathway. The detection of JN422 and JN424 compounds via LC/MS, which have extra oxygen in their molecular formulae, is very intriguing as it suggests that these compounds have a second hydroxyl or keto group in their structure. The fact that di-hydroxylated red compounds were not detected suggests that the second hydroxylation occurs later in the pathway,

due to the activity of either a Mar enzyme or a promiscuous p450 oxidase in *S. venezuelae*. In the latter case, JN422 and JN424 would be artifacts of the heterologous expression of the *mar* gene cluster and not true intermediates. Undecylprodiginine (7) (Figure 4, Figure 19) production was not observed in this strain which can be explained by two possibilities: either that undecylprodiginine (7) is not an intermediate in the biosynthesis of marineosin altogether, consistent with either hypothesis number 2 or 4 (Figure 19) or undecylprodiginine (7) is produced but is converted to marineosin very quickly by the action of a Mar enzyme or an additional p450 oxidase in *S. venezuelae*, which is consistent with the other two hypotheses (Figure 19).

2.4.3 Comparison between the fragmentation patterns of undecylprodiginine, marineosin, and JND2 compounds

The electrospray tandem mass spectrometry ES-MS/MS profile of the prodiginines family of natural products is characterized by the neutral loss of m/z 15 which corresponds to the loss of a methyl radical from the pyrrole ring B to generate odd electron fragments [68]. Upon ESI-CID fragmentation of undecylprodiginine, protonation first occurs at the nitrogen atom of pyrrole ring B which leads to electron delocalization and the subsequent formation of a pseudo-seven-membered ring which is stabilized by conjugation. Therefore, the loss of a methyl radical provides the most stable ground state $[M+H]^+$ (Figure 21, top panel). This fragmentation pattern was used to determine the structure of a

prodiginine analog present in low concentration in *Hahella chejuensis* extract without prior purification [68]. The same experiment was attempted using undecylprodiginine and marineosin in order to understand the differences between the two compounds when subjected to ESI-CID. This knowledge can then be used to gain preliminary insight into the structure of other compounds accumulated in *S. venezuelae* JND2 and other engineered strains, which will be discussed in more detail in chapter 3.

In case of undecylprodiginine (Figure 21, top panel), a daughter fragment of m/z 379.26 corresponding to the neutral loss of m/z 15 (NL=15) was observed as expected (Figure 21, top panel). However, in case of marineosin, a daughter fragment of m/z 378.26 corresponding to the neutral loss of m/z 32 (NL=32) rather than 15 was observed (Figure 21, bottom panel). In case of marineosin (Figure 21, bottom panel), pyrrole ring B is not conjugated and therefore, protonation of the nitrogen in ring B does not lead to charge delocalization and subsequently, the pseudo-seven-membered ring, if formed, will not be stabilized by conjugation as in the case of undecylprodiginine. Therefore, marineosin favors the loss of a methanol molecule, NL=32 from ring B which probably creates the most stable resonance-stabilized ground state $[M+H]^+$ pyrrole ring and thus, the generation of even electron fragments in ES-MS/MS (Figure 21).

Figure 21: Mechanism and comparison between the MS/MS profile of undecylprodiginine standard (top panel) and marineosin (bottom panel). A) The mechanism and MS/MS profile of undecylprodiginine (top panel) show a daughter fragment of m/z 379.26 corresponding to the neutral loss of m/z 15 (NL=15). The parent ion of undecylprodiginine, m/z 394.29 is not shown. B) The mechanism and MS/MS profile of marineosin (bottom panel) show daughter fragments of m/z 378.26 corresponding to the neutral loss of m/z 32 (NL=32).

2.5 CONCLUSION

A genomic library of *Streptomyces* CNQ-617 was generated by Reynolds KA and co-workers and a *red* gene cluster homolog was identified and proposed to be responsible for the biosynthesis of marineosin but that assumption was not tested experimentally. The putative *mar* gene cluster has only one extra gene named *marA*, encoding a dehydratase. We hypothesize that MarA is involved in the biosynthesis of the spiroaminal ring of marineosin. The putative gene cluster was expressed in the heterologous host *S. venezuelae* and the production of marineosin was confirmed via tandem mass spectrometry, LC/MS and ¹H-NMR of marineosin purified from the engineered *S. venezuelae* JND2 strain. *S. venezuelae* provides 3 times higher yield of marineosin than the original producer, *Streptomyces* CNQ-617.

Four hypotheses were proposed to explain the origin of the hydroxyl group required for the biosynthesis of the spiroaminal ring of marineosin. Deletion of *marG* gene, encoding the Rieske oxygenase MarG, is necessary to provide evidence supporting any of the proposed hypotheses and deletion of *marA* gene, encoding the dehydratase MarA, is necessary to probe its role in the biosynthesis of the spiroaminal ring.

A number of pathway intermediates and possibly some shunt metabolites were observed in the acetone extract of *S. venezuelae* JND2. The intermediates were either conjugated with bright red color or non-conjugated compounds.

2.6 SIGNIFICANCE

We are reporting the first gene cluster experimentally proved responsible for the biosynthesis of marineosin and the first gene cluster involved in the biosynthesis of a spiroaminal natural product. The identification of the *mar* gene cluster and its expression in a heterologous host will facilitate studying the biosynthesis of this intriguing class of secondary metabolites by probing the role of individual genes along the pathway. Engineering of gene deletion and/or replacement mutants and analysis of the accumulating metabolites can provide analogs of marineosin.

3. Probing the role of the oxidase, *marG* and the dehydratase, *marA* in the biosynthesis of marineosin

3.1 SUMMARY

The role of MarG and MarA in the biosynthesis of marineosin was investigated via gene replacement experiments, and the major metabolites accumulated in the resultant mutant strains were purified and identified. The deletion of MarG, a non-heme iron Rieske oxygenase led to the accumulation of 2-hydroxyundecylprodiginine (26). This data disproves the hypothesis that MarG catalyzes the introduction of the hydroxyl group required to initiate the biosynthesis of the spiroaminal ring of marineosin, and suggests that the origin of such –OH group is either a 3-hydroxybutyric acid starter unit recruited via MarP or an unidentified hydroxylase potentially in the *mar* gene cluster. The deletion of MarA, a putative dehydratase led to the accumulation of 2 marineosin analogs named JN408 (31) and JN422 (32) with molecular formulae $C_{25}H_{33}O_2N_3$ and $C_{25}H_{31}O_3N_3$, respectively. JN408 (31) is an oxidized marineosin, where ring B is a pyrrole ring rather than dihydropyrrole, and JN422 (32) is a 9-keto derivative of JN408. This suggested that MarA is not involved in the biosynthesis of the spiroaminal ring of marineosin but is rather involved in the reduction of pyrrole ring B. We propose that spiroaminal ring formation happens via a concerted non-enzymatic mechanism. Marineosin production was not observed in either *marG*

or *marA* deletion mutants which suggest that MarG and MarA are crucial for the biosynthesis of marineosin. From the above data, an experimentally-supported scheme for the biosynthesis of marineosin was proposed.

3.2 INTRODUCTION

From our work detailed in Chapter 2, the gene cluster directing the biosynthesis of marineosin; the *mar* gene cluster was expressed in the heterologous host *S. venezuelae* and marineosin production was confirmed via mass spectrometry and ¹H-NMR of purified marineosin. The identified *mar* gene cluster has a high degree of homology to the *red* gene cluster directing the biosynthesis of undecylprodiginine (7) and streptorubin B (8) in *S. coelicolor* A(3)2. The current knowledge of prodiginines biosynthesis does not explain the origin of the spiroaminal ring or the enzymes involved in the reduction of pyrrole ring B of marineosin. We proposed four hypotheses (Figure 19) to explain the biosynthesis of marineosin in which MarG and MarA were proposed to have important roles in the biosynthesis of marineosin. Additional experiments were needed to test these hypotheses. In this study, we sought the deletion of *marG* and *marA* genes to probe the role of their respective enzymes in the biosynthesis of marineosin as well as propose a scheme for marineosin biosynthesis.

3.3 MATERIALS AND METHODS

3.3.1 Antibiotics, enzymes and bacterial strains

All antibiotics, chemicals and HPLC-grade solvents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. HPLC-purified DNA-primers were purchased from Integrated DNA technologies (Corvallis, IA). Phusion high-fidelity polymerase was purchased from New England Biolabs (Ipswich, MA). Information regarding bacterial strains used in recombination was detailed in section 2.3.2.

3.3.2 Targeted gene disruption of *marG* and *marA* genes

The 1150 bp *aadA* gene and its promoter were amplified from pIJ788 spectinomycin resistance cassette [19], [20] using Phusion polymerase and primers specified in Table 4 and according to the protocol in Table 5.

Primers for amplification of <i>aadA</i> gene	
<i>aadA</i> _FWD	5'- <u>GTATAC</u> GGCTGACGCCGTTGGATA -3'
<i>aadA</i> _REV	5'- <u>GTATACT</u> TATTTGCCGACTACCTTGGT -3'

Table 4: Primers used for PCR amplification of *aadA* gene from pIJ788. Bold, italicized and underlined font represents restriction endonuclease site for Bstz17I.

	30 X				
98 °C	98 °C	64 °C	72 °C	72 °C	4 °C
30 sec	10 sec	15 sec	17 sec	10 min	∞

Table 5: Protocol used in PCR amplification of *aadA* gene

The PCR product was purified using QIAquick PCR Purification kit (Qiagen, Valencia, CA) and was eluted using 10 µl of EB buffer. The PCR product was directly used as a template for another PCR reaction using primers specified in Table 6 and the protocol specified in Table 7.

Primers used for <i>marG</i> disruption	
<i>marG_KO_FWD</i>	5'- <u>GTGCTCGGCGGGAACTCGAAAGAGAGGGACTG</u> <u>CATG</u> <i>GTATACGGCTGACGCCGTTG</i> -3'
<i>marG_KO_REV</i>	5'- <u>GCCGTACCATGCGTCTTCGTCCGGGACGCCGCCGC</u> <u>GTCAG</u> <i>TATACTTATTTGCCGACT</i> -3'
Primers used for <i>marA</i> disruption	
<i>marA_KO_FWD</i>	5'- <u>TTGTACGCCCACGCTCCGGTGCACAGAATCGTGGCC</u> <u>ATG</u> <i>GTATACGGCTGACGCCGTTG</i> -3'
<i>marA_KO_REV</i>	5'- <u>AGAGGCGACGGAGATCATGACGGGATACTTCTGGC</u> <u>GTCAG</u> <i>TATACTTATTTGCCGACT</i> -3'

Table 6: Primers used for disruption of *marG* and *marA* genes. Homology to the nucleotide sequence flanking *marG* or *marA* is indicated by bold underlined font while the italicized font, indicates homology to *aadA* gene.

	30 X				
98 °C	98 °C	64 °C	72 °C	72 °C	4 °C
30 sec	30 sec	19 sec	10 sec	10 min	∞

Table 7: PCR protocol used in targeted disruption of *marG* and *marA* genes

The new generated PCR product was used, according to protocol, in the Red/ET mediated targeted gene disruption [20] of either *marG* or *marA* genes in pMAR

cosmid where the genes in question were replaced by *aadA* gene, a spectinomycin resistance marker (Figure 22).

Figure 22: Recombination strategy used to generate *S. venezuelae* JND2ΔG (top panel) and *S. venezuelae* JND2ΔA (bottom panel). pIJ788 plasmid was used as a template for PCR amplification of *aadA* gene, conferring resistance to spectinomycin antibiotic. The PCR product was used to replace *marG* gene (top panel) and *marA* (bottom panel) in pMAR cosmid via PCR targeted recombination. The mutated cosmids were transferred to *S. venezuelae* by either protoplast transformation (*marG*, top panel) or by conjugation (*marA*, bottom panel) to generate *S. venezuelae* JND2ΔG and *S. venezuelae* JND2ΔA respectively. *aadA* = adenytransferase gene.

marG and *marA* gene replacements were confirmed by PCR using outer primers that bind ~ 200 bp upstream and ~ 200 bp downstream of the amplified gene (Table 8). The mutant cosmids were named pMAR Δ G and pMAR Δ A.

Outer primers used to verify gene deletion	
<i>marG</i> _Out_FWD	5'- ACACCGACGCGACATGGACC -3'
<i>marG</i> _Out_REV	5'- CGGGCAGCTCCTCGTCCT -3'
<i>marA</i> _Out_FWD	5'- GTCGGCCGGTGACGGTGC -3'
<i>marA</i> _Out_REV	5'- GGAACGCGACGACAGCTAT -3'

Table 8: Primers used to verify *marG* and *marA* gene deletions

3.3.3 Generation of *S. venezuelae* JND2 Δ G and *S. venezuelae* JND2 Δ A mutants

3.3.3.1 Generation of *S. venezuelae* JND2 Δ G mutant

To generate *S. venezuelae* JND2 Δ G mutant, the pMAR Δ G cosmid was transformed into *dam*⁻/*dcm*⁻ methylation deficient *E. coli* (New England Biolabs, Ipswich, MA) and the *dam*⁻/*dcm*⁻ cosmid was purified. Protoplasts of wild type *S. venezuelae* were prepared according to the following protocol modified from reference [69]:

S. venezuelae spores were propagated onto SPA agar and incubated at 30 °C for 7 days. Fresh spores were used to inoculate 30 ml of SGGP media, in 300 ml baffled flask, and the culture was allowed to grow overnight at 30 °C. Mycelia were harvested by centrifugation at room temperature. The harvested mycelia

were suspended in 8 ml P-buffer (sucrose, K_2SO_4 , $MgCl_2 \cdot 6H_2O$, and trace elements) containing 1 mg/ml of lysozyme and then was incubated for 45 min at 30 °C. The mycelia suspension was mixed by pipetting up and down for 3 times using a 5 ml pipette then returned for 30 °C incubation for an additional 15 min. 5 ml of fresh P-buffer were added followed by pipetting up and down for 3 times using a 5 ml pipette followed by filtration through sterile cotton wool. The filtrate was centrifuged for 7 min at room temperature at 3000 rpm and the precipitated protoplasts were washed twice with fresh P-buffer. The protoplasts were finally suspended in 5 ml P-buffer and aliquoted as 250 µl volumes. For transformation of *dam⁻/dcm⁻* pMARΔG cosmid, the following protocol, modified from reference [69], was used:

1 ml of sterile PEG 8000 was melted in microwave for 20 sec and then mixed with 3 ml of fresh P-buffer to form a 25% PEG 8000 solution. Quick-thawed protoplasts were centrifuged at room temperature for 2 min at 3000 rpm, decanted and the precipitated cells were suspended in the 100 µl left. 1 µg of *dam⁻/dcm⁻* pMARΔG cosmid were added to the protoplasts immediately followed by the addition of 600 µl of 25% PEG 8000 solution. 50 µl transformation mixture was spread onto pre-dried R2YE plates and incubated overnight at room temperature. Plates were overlaid with 1 ml LB broth containing 500 µg/ml spectinomycin and 500 µg/ml of apramycin and plates were incubated at 30 °C for 5-7 days. Transformants were red in color and was propagated onto SPA agar plates supplied with 50 µg/ml spectinomycin and 50 µg/ml apramycin.

3.3.3.2 Generation of *S. venezuelae* JND2ΔA mutant

To generate *S. venezuelae* JND2ΔA mutant, pMARΔA cosmid was transformed into *E. coli* ET12537/pUZ8002 (donor strain) which was later used in conjugation with wild type *S. venezuelae* as specified in section 2.3.4 with the addition of 50 µg/ml of spectinomycin and 50 µg/ml of apramycin.

3.3.4 LC/MS and MS/MS analysis of *S. venezuelae* JND2ΔG and *S. venezuelae* JND2ΔA

S. venezuelae JND2ΔG and *S. venezuelae* JND2ΔA strains were propagated on SPA agar containing 50 µg/ml of apramycin and spectinomycin antibiotics. Fresh spores were used to inoculate 10 ml of SCM media containing the same antibiotics. The mycelia were harvested after 72 hours by centrifugation at 5000 rpm, 4 °C, for 10 minutes. The cells were lysed by the addition of acetone followed by sonication. The acetone extract was concentrated under vacuum and the dried acetone extract was dissolved in methanol prior to MS analysis. The acetone extract of *S. venezuelae* JND2ΔG was analyzed using ThermoElectron LTQ-Orbitrap mass spectrometer equipped with an Agilent MWD HPLC using buffer A: water/acetonitrile/methanol (5:1:4) and buffer B: 100 % methanol according to protocol specified in Table 9. The acetone extract of *S. venezuelae* JND2ΔA was analyzed using MicroToF-Q mass spectrometer equipped with an Agilent 1100 series HPLC using buffer A: water with 0.1 % formic acid and buffer B: acetonitrile with 0.1% formic acid and according to protocol specified in Table

10. The LC/MS analysis of both strains was done using 2.1 x 250 mm, 5 μ m, reverse phase Supelco 18C-column (Supelco, St. Louis, MO).

Time (min)	Flow (ml/min)	% A	% B
0	0.2	90.0	10.0
5.0	0.2	90.0	10.0
40.0	0.2	0.0	100.0
50.0	0.2	0.0	100.0
51.0	0.2	90.0	10.0
60.0	0.2	90.0	10.0

Table 9: HPLC conditions used in the LC/MS analysis of *S. venezuelae* JND2ΔG

Time (min)	Flow (ml/min)	% A	% B
0	0.2	95.0	5.0
2.0	0.2	95.0	5.0
25.0	0.2	0.0	100.0
30.0	0.2	0.0	100.0
31.0	0.2	95.0	5.0
36.0	0.2	95.0	5.0

Table 10: HPLC conditions used in the LC/MS analysis of *S. venezuelae* JND2ΔA

3.3.5 Purification and structure elucidation of compounds isolated from *S. venezuelae* JND2ΔG and *S. venezuelae* JND2ΔA

3.3.5.1 Purification and structure elucidation of G410 from S. venezuelae JND2ΔG

Spores of *S. venezuelae* JND2ΔG were propagated onto SPA agar plates supplied with 50 µg/ml of apramycin and 50 µg/ml of spectinomycin antibiotics. The plates were incubated at 30 °C for 7 days and fresh spores were used to inoculate 10 ml of SCM media, in 50 ml flasks, supplied with 50 µg/ml of apramycin and 50 µg/ml of spectinomycin antibiotics to form a seed culture. Once the seed culture started to produce a red color, approximately 48-72 hours after incubation, it was used to inoculate large scale fermentations of SCM media, 1L each in a 4L baffled flasks, at 1% v/v. Large fermentations were also supplied with the same antibiotics, at the same concentrations, and were incubated at 30 °C for 6 days and 220 rpms. The mycelia were harvested by centrifugation at 10000 rpm, 6 °C for 10 min. The harvested cells were disrupted by the addition of acetone followed by sonication 2 times, 5 min each. The acetone extract was dried under vacuum and the residual water was partitioned 3x with ethyl acetate. The ethyl acetate fraction was evaporated under vacuum and the dried extract was stored at -20 °C until further use.

The crude extract was dissolved in methanol to yield 10 mg/ml solution and was purified using a Waters HPLC equipped with a DWD detector using 20.0 x 250.0 mm, 10 µm, reverse phase Ascentis ¹⁸C-column (Supelco, St. Louis, MO) using

buffer A: water/acetonitrile/methanol (5:1:4) and buffer B: 100% methanol using the gradient specified in Table 11.

Time (min)	Flow (ml/min)	% A	% B
0	10.0	90.0	10.0
2.0	10.0	90.0	10.0
40.0	10.0	0.0	100.0
50.0	10.0	0.0	100.0
51.0	10.0	90.0	10.0
60.0	10.0	90.0	10.0

Table 11: HPLC conditions for first round of G410 purification

The peak eluting at retention time 25 min (Figure 23) was collected, dried under vacuum and the residual water was removed by lyophilization. The 25 min fraction was dissolved in chloroform and loaded onto 20 x 20 cm preparative silica gel 60A TLC plates (Whatman). The TLC plate was developed using 50 % ethyl acetate/hexane mixture. The bottom layer was excised from the TLC plate and extracted with acetone (Figure 24).

Figure 23: First HPLC purification of G410. Peak at retention time 25 minutes was collected, concentrated and subjected to preparative TLC.

Figure 24: Preparative TLC purification of G410. The arrow show the layer that was later excised from the TLC plate and subjected to another round of HPLC purification.

The semi-pure G410 was subjected to a final round of HPLC purification using an Agilent HPLC equipped with a MWD detector using 10.0 x 250.0 mm, 5 μ m, and reverse phase 18 C-phenomenex column using method specified in Table 12.

Time (min)	Flow (ml/min)	% A	% B
0	4.0	95.0	5.0
25.0	4.0	0.0	100.0
30.0	4.0	0.0	100.0
31.0	4.0	95.0	5.0
40.0	4.0	95.0	5.0

Table 12: HPLC method for final purification of G410

Peak eluting at retention time 14 min was collected, dried under vacuum and the residual water was eliminated via lyophilization to yield 1 mg of pure G410.

Figure 25: Final HPLC purification of G410. The peak eluting at 14 min was collected and evaporated to yield pure G410.

The 98 % pure G410 was dissolved in acetone- d_6 for NMR characterization. NMR spectra were obtained using a Bruker AMX - 600 MHz spectrometer (Bruker Daltonics) calibrated using residual acetone peak at 2.05 ppm.

3.3.5.2 Purification and structure elucidation of JN422 and JN408 from *S. venezuelae* JND2ΔA

Spores of *S. venezuelae* JND2ΔA were propagated onto SPA agar plates supplied with 50 µg/ml of apramycin and 50 µg/ml of spectinomycin antibiotics. For large scale purification, plates were incubated at 30 °C for 7 days and fresh spores were directly used to inoculate 60-100 ml of SCM media, in 300-500 ml flasks, supplied with 50 µg/ml of apramycin and 50 µg/ml of spectinomycin antibiotics. Large scale production from seed culture or using bigger flasks was unsuccessful for this strain. Flasks were incubated at 30 °C for 6 days and 220 rpm. The mycelia were harvested by centrifugation at 10000 rpm, 6 °C for 10 min. The harvested cells were disrupted by the addition of acetone followed by sonication 2 times, 5 min each. The acetone extract was dried under vacuum and the residual water was removed by lyophilization. The crude acetone extract was stored at -20 °C until further use. At time of purification, the extract was dissolved in methanol and the extract was partially purified using Agilent HPLC equipped with a MWD detector using 10.0 x 250.0 mm, 5 µm, and reverse phase ^{18}C -phenomenex column using the method specified in Table 13.

Time (min)	Flow (ml/min)	% A	% B
0	4.0	95.0	5.0
30.0	4.0	15.0	85.0
35.0	4.0	15.0	85.0
36.0	4.0	95.0	5.0
46.0	4.0	95.0	5.0

Table 13: HPLC method used in the purification of JN422 and JN408 from *S. venezuelae* JND2ΔA.

The peaks with retention time 15 min and 21 min (Figure 26); corresponding to JN422 and JN408 respectively, were collected and concentrated under vacuum. The residual water was removed via lyophilization and the semi-pure compounds were each subjected to another round of HPLC purification using the same conditions as specified in Table 13 (Figure 27).

Figure 26: First HPLC purification of JN422 and JN408 from *S. venezuelae* JND2ΔA. The peak with retention time of 15 minutes corresponds to JN422 while, the peak with retention time of 21 minutes corresponds to JN408. Both peaks were collected and subjected to another round of HPLC purification.

Figure 27: Second HPLC purification of JN422 (top panel) and JN408 (bottom panel). The peak with retention time of 15 minutes (top panel) corresponds to JN422. The peak with retention time of 22 minutes (bottom panel) corresponds to JN408.

The peak with retention time 15 min (top chromatogram, Figure 27) was collected and dried under vacuum. Residual water was removed via lyophilization to yield 1 mg of 90 % pure JN422 (Figure 28) while collection of peak with retention time of 21 min (bottom chromatogram, Figure 27) and lyophilization yielded 0.5 mg of 95 % pure JN408 (Figure 29). Purity estimations were based on HPLC, MS and NMR data.

Figure 28: HPLC chromatogram of pure JN422

Figure 29: HPLC chromatogram of pure JN408

Pure dried JN422 and JN408 were each dissolved in acetone- d_6 for NMR characterization. NMR spectra were obtained using a Bruker AMX - 600 MHz spectrometer (Bruker Daltonics) calibrated using residual acetone peak at 2.05 ppm.

3.4 RESULTS AND DISCUSSION

3.4.1 The role of MarG and MarA in the biosynthesis of marineosin

3.4.1.1 The role of MarG in the biosynthesis of marineosin

3.4.1.1.1 Sequence analysis of *marG*

The *marG* gene is 1356 bp long with a predicted protein sequence consists of 450 amino acids, corresponding to a calculated molecular weight of 50.96 kDa. MarG shares a relatively lower degree of annotated identity (65% identity) to its *red* gene cluster counterpart, RedG when compared to homologies among other *red* and *mar* homologs (Table 1). Both enzymes belong to the family of non-heme iron Rieske oxygenases.

Typically, non-heme iron Rieske-oxygenases are multi-component enzyme systems consisting of a reductase and an oxygenase component. The oxygenase component of the Rieske-oxygenases is characterized by the presence of highly conserved CXH, CXXH and DXHX₄H motifs. The histidine amino acid residues in the CXH and CXXH motifs are required to coordinate one iron of the [2Fe-2S] cluster while the cysteine amino acid residues are responsible for the coordination of the second iron atom [70] , [71]. It should be noted that the histidine-liganded iron is the one undergoing reduction from Fe³⁺ to Fe²⁺ after the gain of an electron [72–75]. The histidine amino acid residue in the DXHX₄H motif is required to coordinate a mononuclear non-heme iron center, which performs the catalysis. The Rieske center transfers electrons from reductive component to the mononuclear iron center via the aspartate amino acid

residue in the DXHX₄H motif [52]. The mononuclear iron forms a 2-His-1-carboxylate facial triad that allows the iron center to bind three exogenous ligands, one of which is molecular oxygen [76]. The activated metal-oxygen intermediate can catalyze numerous kinds of reactions such as mono- and dihydroxylations of aromatic rings and oxidative ring cyclizations [77]. It has been proposed from numerous structural analyses of this class of enzymes, that the identity of the ligand *trans* to the oxygen dictates the type of reaction this class of enzymes catalyze. While both MarG and RedG have conserved CXH and CXXH motifs at their N-terminal end, both enzymes have EXHX₄H motif at their C-terminal end rather than DXHX₄H motif characteristic to other Rieske-oxygenases (Figure 30). Comparison of MarG and RedG with RphG and McpG, another two Rieske oxygenases proposed to be involved in the biosynthesis of roseophilin (26) and metacycloprodiginine (9) (Figure 31), both cyclic prodiginine analogs, indicates that all four enzymes have the same aspartate to glutamate modification. Although both amino acids have acidic nature and should catalyze the same types of reactions, an aspartate to glutamate mutation of naphthalene dioxygenase Rieske enzyme yielded an inactive enzyme [78]. Based on that study, we are proposing that the EXHX₄H motif at the C-terminal end of MarG and other Rieske oxygenases involved in carbon-carbon ring closures may be crucial for catalysis.

3.4.1.1.2 Targeted gene replacement of *marG* and the generation of *S. venezuelae* JND2 Δ G: A *marG* knockout strain

In Section 2.4.2, we proposed that undecylprodiginine (7) is a possible intermediate along the pathway that leads to the biosynthesis of marineosin. We also hypothesized that MarG enzyme, a Rieske oxygenase, could be involved in the introduction a hydroxyl group to the acyl chain of undecylprodiginine (7) intermediate. This hydroxylation is essential for the biosynthesis of the spiroaminal moiety of marineosin in hypothesis No. 1 (Figure 19). To test this hypothesis, *marG* gene encoding the Rieske-oxygenase MarG in the pMAR cosmid, was replaced with a spectinomycin resistance marker gene called *aadA*. The gene replacement was confirmed via PCR using primers designed to amplify the region flanking the *marG* gene. The PCR of pMAR cosmid using these primers led to the amplification of a fragment of ~ 1800 bp corresponding to *marG* gene (1356 bp) plus the flanking 400 bp (lane C, Figure 32). When the PCR done using the same primers using *marG* replacement mutant cosmid, *pMAR Δ G* led to the amplification of ~ 1500 bp fragment corresponding to *aadA* gene (1150 bp) plus the flanking 400 bp (lane Δ G, Figure 32).

Figure 32: Confirmation of *marG* replacement, from pMAR cosmid, with *aadA* gene conferring resistance to spectinomycin antibiotic. Lane L = DNA ladder, lane C = PCR control performed using pMAR cosmid, lane ΔG = PCR performed using pMAR ΔG cosmid.

The mutant cosmid, *pMAR ΔG* was delivered to wild type *S. venezuelae* via protoplast transformation and the new strain was named *S. venezuelae* JND2 ΔG . The new mutant strain is apramycin and spectinomycin resistant and is bright red in color.

3.4.1.1.3 MS, LC/MS and LC-UV/MS analysis of *S. venezuelae* JND2 ΔG

In order to determine the main metabolites accumulated as a result of *marG* replacement, the MS profile of the crude acetone extract of *S. venezuelae* JND2 ΔG was determined using ThermoElectron LTQ-Orbitrap high resolution mass analyzer. The analysis was run side by side with crude acetone extracts of

both, wild type *S. venezuelae* and *S. venezuelae* JND2, marineosin producer strain (Figure 33).

Figure 33: Comparison of the MS profiles of *S. venezuelae* JND2ΔG (top panel), JND2 strain (middle) and wild type *S. venezuelae* (bottom). JND2ΔG (top panel) and JND2 (middle panel) strains have the same MS profile where both strains show a compound of m/z 410.28 in addition to the metabolites normally produced by the wild type *S. venezuelae* (bottom) such as methymycin and pikromycin, m/z 470.31 and 526.34 respectively.

The MS profile of both JND2ΔG and JND2 strains was found to be different from that of wild type *S. venezuelae*. In addition to methymycin and pikromycin antibiotics, both strains share the accumulation of a compound of $[M+H]^+$ m/z 410.28 and corresponding molecular formula $C_{25}H_{36}O_2N_3$, which is the same as that reported for marineosin [53]. Since production of marineosin by JND2 strain

was already demonstrated in section 2.4.1, this preliminary data suggested that JND2 Δ G mutant strain also produces marineosin and therefore, MarG is not required for the biosynthesis of marineosin. However, analysis of the same acetone extract using LC/MS indicated that both marineosin in JND2 strain and m/z 410.28 compound in JND2 Δ G mutant strain have different retention times (Figure 34) and that the compounds are different.

Figure 34: EIC of m/z 410.28 in *S. venezuelae* JND2 Δ G (top panel) and *S. venezuelae* JND2 (bottom panel). The extracted ion chromatogram (EIC) of m/z 410.28 from the LC/MS of both *marG* replacement mutant (top panel) and JND2 strains (bottom).

As described in section 2.4.3, LC/UV analysis of the crude acetone extract of *S. venezuelae* JND2 indicates the presence of two groups of compounds: a group with a UV λ_{max} of 320 nm and a group of λ_{max} of 530 nm. The LC-UV/MS chromatogram of *S. venezuelae* JND2 Δ G on the other hand, has only one group of red-colored compounds characterized with λ_{max} 530 nm which indicates that deletion of *marG* abolishes the production of marineosin and other non-conjugated compounds observed in JND2 strain (Figure 35). These results demonstrate that *marG* is essential for the biosynthesis of marineosin and supports the hypothesis that the loss of conjugation and hence; the red color, occurs at a later step of the biosynthetic pathway.

Figure 35: The LC-UV/MS profile of *S. venezuelae* JND2 Δ G. The red chromatogram represents absorption at 530 nm while blue chromatogram represents absorption at 320 nm.

Undecylprodiginine (7) production was not observed in this strain which indicates that MarG is not responsible for the introduction of the hydroxyl group required to initiate the biosynthesis of the spiroaminal ring of marineosin. This observation excludes hypothesis no. 1 proposed in section 2.4.2 (Figure 19). It should be noted that some of the non-conjugated compounds were di-hydroxylated, which may indicate that MarG is responsible for the introduction of that additional hydroxyl group.

3.4.1.1.4 2-hydroxyundecylprodiginine is the main metabolite accumulating in *S. venezuelae* JND2ΔG

The main compound produced by *S. venezuelae* JND2ΔG; a *marG* mutant, was named G410 (27) (Figure 36). The compound was isolated from the crude acetone extract of *S. venezuelae* JND2ΔG using ethyl acetate extraction, HPLC, preparative TLC and a final HPLC purification step to yield ~ 0.5 mg of 95% pure compound (Figure 37). Detailed analysis of the MS, UV, IR and NMR spectral data of G410 compound indicated its identity to be 2-hydroxyundecylprodiginine (27) (Figure 36).

Figure 36: Structure of 2-hydroxyundecylprodiginine, G410

Figure 37: HPLC chromatogram of 95% pure G410. The red chromatogram represents absorption at 530 nm while blue chromatogram represents absorption at 320 nm.

The UV spectrum of G410 (27) (Figure 38) has a prominent λ_{max} of 530 nm, characteristic to prodiginines family of natural products due to their distinct red color. This observation suggested a fully conjugated tripyrrole skeleton for G410 (27) similar to that of undecylprodiginine (7).

Figure 38: UV spectrum of G410

HR-ESI-MS analysis of G410 (27) exhibits $[M+H]^+$ of m/z 410.2807 (Figure 39). The acquired mass was analyzed for molecular formula $C_{25}H_{36}O_2N_3$ or, $C_{25}H_{35}O_2N_3$ without the H-adduct. The difference between the mass acquired for G410 and the mass of the predicted molecular formula is only m/z 0.0005 (Figure 40). The theoretical isotope pattern of the predicted molecular formula was found to be identical to the isotope pattern acquired for G410 (27) (Figure 40). This molecular formula indicates an index of hydrogen deficiency equivalent to ten or the presence of ten degrees of unsaturation.

G410-pure #10-238 RT: 0.10-2.00 AV: 114 NL: 7.00E6
T: FTMS + p ESI Full ms [200.00-1207.88]

Figure 39: HR-ESI-MS of G410

Figure 40: Comparison between the acquired (top panel) mass and isotope pattern of G410 and predicted mass and isotope pattern for molecular formula, $C_{25}H_{36}O_2N_3$ (bottom panel). There is a difference of only m/z 0.0005 between the acquired and the predicted formula.

The MS/MS fragmentation profile of G410 (27) has daughter fragments of m/z 395.33, 392.33, and 378.33 equivalent to the loss of m/z 18, 15 and 32 respectively (Figure 41). This fragmentation is consistent with the loss of water, a methyl radical and/or methanol, respectively (Figure 42). As described in section 2.4.3, The loss of a methyl radical or m/z 15 is characteristic to prodiginines family of natural products such as undecylprodiginine (7) but was not observed for marineosin (Figure 21) [68].

Figure 41: The MS/MS profile of G410. Daughter fragments of m/z 395.33, 392.33, and 378.33 equivalent to the loss of m/z 18, 15 and 32 respectively. The parent ion, m/z 410.28 is not shown.

Figure 42: Fragment ions observed in the MS/MS profile of G410

The IR spectrum of G410 (27) (Figure 43) provided additional evidence supporting the presence of a hydroxyl group as the spectrum has a broad peak at ν_{max} 3354 cm^{-1} and 1710 cm^{-1} which is characteristic for O-H stretch and C=O stretch, respectively. Other peaks were observed at ν_{max} 2921, 2852, 1630, 1605, 1550, 1464, 1367, 1264, 1185, 1041, 959, and 797 cm^{-1} .

Figure 43: IR spectrum of G410

All of these spectral analyses suggested that G410 is an undecylprodiginine analog with a -OH substituent, and therefore, further NMR spectral analyses were done side by side with undecylprodiginine standard (7) (Table 14), to facilitate structure comparison. The ^1H -NMR spectrum (Figure 44) of G410 (27) has 7 peaks with chemical shifts δ 6 -7 ppm which indicates the presence of 7 aromatic protons at δ 6.02 (*d*, J = 5.16 Hz), δ 6.15 (*s*), δ 6.24(*t*, J = 4.87 and 8.89

Hz), δ 6.58 (*d*, J = 5.2 Hz), δ 6.74 (*s*), δ 6.78 (*d*, J = 5.78 Hz), and δ 7.02 (*br*). This is similar to the 7 aromatic protons present in ^1H -NMR (Figure 45) of undecylprodiginine (7). ^1H - ^1H 2D TOCSY correlations among these aromatic protons (Figure 46), and comparing it to the ^1H - ^1H 2D TOCSY correlations of undecylprodiginine (Figure 47), illustrated that the 7 aromatic protons were components of three pyrrole rings.

Name G410-2-acetone
 PROTON Acetone {C:\Bruker\TOPSPIN} Reynolds-Shaimaa 4

Figure 44: ^1H -NMR spectrum of **G410**. Structure shown above.

Figure 45: ^1H -NMR spectrum of the HCl salt of undecylprodiginine standard. Structure shown above.

Figure 46: ^1H - ^1H 2D TOCSY correlations among aromatic protons of G410 and UP

Figure 47: ^1H - ^1H 2D TOCSY NMR spectrum of G410 (top panel) and UP (bottom panel) - aromatic region expanded.

Comparison of the ^1H -NMR of the aliphatic region of both compounds, revealed that G410 (27) has an extra proton with chemical shift δ 3.67 ppm (*m*), consistent with a $-\text{CH-OH}$ (Figure 44). The characteristic methyl protons at C-24 in G410 (26) are slightly shifted downfield (δ 1.09) relative to the same protons in UP (7) (δ 0.86) (Figure 48) suggesting their proximity to a heteroatom. Also, the protons at C-24 in G410 show a prominent doublet splitting ($J = 9.18$ Hz) while the same protons in UP, show a triplet splitting ($J = 7.17$ Hz). This splitting pattern suggested that the methyl group at position 24 of G410 is adjacent to a $-\text{CH}$ group rather than a $-\text{CH}_2$ group (Figure 48) which is consistent with the presence of $-\text{CH-OH}$ at C-23 of G410 (27).

Figure 48: ^1H -NMR spectrum showing comparison between the splitting pattern of methyl protons at C-24 in undecylprodiginine (red, top) and G410 (blue, bottom)

^1H - ^1H 2D COSY (Figure 49) and ^1H - ^1H 2D TOCSY NMR (Figure 50) correlations between protons at C-24 and C-23 in G410, confirmed the position of $-\text{CH-OH}$ group to be adjacent to the terminal methyl at C-24 (Figure 49), (Figure 50). Such correlations are absent from the ^1H - ^1H 2D TOCSY NMR spectrum of undecylprodiginine (7) (Figure 51).

Figure 49: ^1H - ^1H 2D COSY NMR correlations between C-22, C-23, and C-24 of G410. Structure shown above.

Figure 50: ^1H - ^1H 2D TOCSY correlations among C-22, C-23 and C-24 of G410. Structure is shown above.

Figure 51: ^1H - ^1H 2D TOCSY correlations of the aliphatic region of undecylprodiginine

All the above experiments as well as a detailed comparison between the chemical shifts of both undecylprodiginine (7) and G410 (27) (Table 14), confirmed the identity of G410 as 2-hydroxyundecylprodiginine. It should be noted that the chemical shifts reported for undecylprodiginine (7) protons are slightly shifted downfield as the compound is in the HCl salt form. The full ^1H - ^1H 2D TOCSY spectra of G410 (27) and undecylprodiginine (7) is provided in Figure 52 and Figure 53, respectively.

Table 14: NMR Spectral data for G410 (600 MHz, Acetone-d6) and side by side comparison of G410 and UP

Position*	G410 δ_H^*	UP δ_H^{**}
1	7.02	7.38
2	6.24	6.41
3	6.78	7.24
4		
5		
6	6.74	7.15
7		
8		
9	6.15	6.65
10		
11	6.58	7.07
12	6.02	6.31
13		
14	2.70	2.92
15	1.69	1.79
16	1.33	1.38
17	1.33	1.38
18	1.33	1.38
19	1.33	1.38
20	1.33	1.38
21	1.33	1.38
22	1.34	1.38
23	3.67	1.28
24	1.09	0.86
25	3.91	4.09

*Assignments are for protons bonded to the specified positions using 1D (^1H), and 2D (COSY, TOCSY) NMR spectroscopy in comparison to UP. ** The chemical shifts reported are for the HCl salt of UP.

G410-MLEVPHPR

Figure 52: Full ^1H - ^1H 2D TOCSY correlations and spectrum of G410. The bold bonds indicate 16H integrated into one large peak at 1.3 ppm.

Figure 53: Full ^1H - ^1H 2D TOCSY correlations and spectrum of undecylprodiginine. The bold bonds indicate 16H integrated into one large peak.

The production of 2-hydroxyundecylprodiginine (26), a straight chain undecylprodiginine analog by *S. venezuelae* JND2ΔG as a result of *marG* deletion, indicates that MarG is required for the cyclization of the acyl chain of 2-hydroxyundecylprodiginine with the reduction of the double bond at the methine bridge connecting C-8 and C-9 (Figure 54). The absence of undecylprodiginine (7) in this strain is inconsistent with the hypothesis (Figure 19), that MarG is responsible for the introduction of the spiroaminal ring oxygen of marineosin. The production of 2-hydroxyundecylprodiginine (26) suggests other possibilities including (Figure 54):

- A different substrate specificity of MarP, that leads to the incorporation of a hydroxy-butyric acid starter unit in the acyl chain of 2-undecylpyrroline subunit. Or,
- An unknown hydroxylase enzyme, such as MarT or MarE (both annotated as hypothetical proteins), is involved in the introduction of the hydroxyl group to undecylprodiginine structure.

Figure 54: Hypotheses explaining the biosynthesis of G410

3.4.1.2 The role of MarA in the biosynthesis of marineosin

3.4.1.2.1 Sequence analysis of *marA*

The *marA* gene is 468 bp, whose predicted protein sequence consists of 155 amino acids, corresponding to a calculated molecular weight of 16.07 kDa. An NCBI BLAST search of MarA protein indicates that MarA has 56% identity to MaoC-like dehydratase and 45% identity to enoyl-CoA hydratase, and accordingly, MarA was provisioned as a putative dehydratase (Table 1). A conserved domain data search of MarA primary protein sequence reveals that MarA belongs to the HotDog superfamily of proteins which comprises a wide range of enzymes with distinct catalytic activities such as dehydratases,

thioesterases, dehydrogenases and acyltransferases. The primary protein sequence of MarA suggests that it specifically belongs to the MaoC dehydratase-like HotDog subfamily which also includes the human β -hydroxysteroid dehydrogenase and NodN, an enzyme involved in cell signaling of *Rhizobium* roots [79].

Figure 55: Graphical summary of conserved domain hits obtained after NCBI CDD search. The figure indicates that MarA belong to the HotDog superfamily of enzymes.

3.4.1.2.2 Targeted gene replacement of *marA* and the generation of *S. venezuelae* JND2ΔA: A *marA* knockout strain

In Section 2.4.2, we proposed that the putative dehydratase, MarA catalyzes the final spiroaminal ring formation of 2-hydroxyundecylprodiginine intermediate (Figure 19). To test our hypothesis, *marA* gene in pMAR cosmid, was replaced with a spectinomycin resistance marker gene, *aadA*. Gene replacement was confirmed via PCR using primers designed to amplify the region flanking the *marA* gene. The PCR of the mutant cosmid *pMAR*ΔA, a *marA* replacement

mutant cosmid led to the amplification of ~ 1500 bp fragment corresponding to *aadA* gene (1150 bp) plus the flanking 400 bp (lane ΔA , Figure 56). A PCR done using the same primers but using pMAR cosmid, as a control led to the amplification of a fragment of ~ 1000 bp, corresponding to *marA* gene (468 bp) plus the flanking 400 bp (lane C, Figure 56). The mutant cosmid, *pMAR ΔA* was delivered to wild type *S. venezuelae* via conjugation and the new strain was named *S. venezuelae* JND2 ΔA . The new strain is both apramycin and spectinomycin resistant and is characterized by a faint red color when compared to *S. venezuelae* JND2 ΔG mutant strain.

Figure 56: Confirmation of *marA* replacement, from pMAR cosmid, with *aadA* gene conferring resistance to spectinomycin antibiotic. Lane L = DNA ladder, lane C = a control PCR reaction performed using pMAR cosmid, lane ΔA = PCR reaction performed using pMAR ΔA cosmid.

3.4.1.2.3 MS, LC/MS and LC-UV/MS analysis of *S. venezuelae* JND2ΔA

The MS profile of *S. venezuelae* JND2ΔA strain was monitored via ThermoElectron LTQ-Orbitrap high resolution mass analyzer to determine the main metabolites accumulated as a result of *marA* gene replacement. The MS profile of the crude acetone extract of *S. venezuelae* JND2ΔA was compared to that of the crude acetone extracts of *S. venezuelae* JND2 strain, marineosin producer (Figure 57).

Figure 57: Comparison between the MS profiles of *S. venezuelae* JND2ΔA (top panel), and JND2 strain (bottom panel). MS profile of JNDΔA strain (top panel) shows two main compounds with *m/z* 408.26 and 422.24 while the MS profile of JND2 strain shows the same two compounds, to a much lower extent, and mainly marineosin, *m/z* 410.28.

The MS profile of *S. venezuelae* JND2ΔA crude acetone extract is characterized by the presence of two main signals with $[M+H]^+$ m/z 408.27 and 422.24 that were named JN408 and JN422, respectively. JN408 and JN422 were also found in *S. venezuelae* JND2 control but to a much lower extent.

As observed for other JND2 engineered strains, the LC-UV/MS analysis of the acetone extract of JND2ΔA indicates two groups of compounds (Figure 58). One group has a $\lambda_{\max}$ 320 nm which indicates non-conjugated, non-red compounds and the other group has a $\lambda_{\max}$ 530 nm which characterizes red-colored prodiginine analogs. Marineosin production was not observed in this strain which suggested that MarA is essential for the biosynthesis of marineosin. Undecylprodiginine (7) production was also not observed in this strain which suggests that undecylprodiginine (7) is either not an intermediate along the pathway for the biosynthesis of marineosin, or does not accumulate in the mutant because it gets hydroxylated very quickly by an unidentified oxidase.

Figure 58: LC-UV/MS analysis of *S. venezuelae* JND2ΔA. Two groups of compounds exist: a group with $\lambda_{\max}$ 320 nm (green color) and a group with $\lambda_{\max}$ 530 nm (magenta color).

3.4.1.2.4 JN408 and JN422 are two marineosin analogs produced by *S. venezuelae* JND2ΔA

As mentioned earlier, the deletion of *marA* from the marineosin gene cluster and the generation of *S. venezuelae* JND2ΔA led to the accumulation of two major compounds named JN408 (31) and JN422 (32) (Figure 59). Both compounds were isolated from the acetone extract of *S. venezuelae* JND2ΔA after two rounds of HPLC purifications to yield ~ 0.4 mg of JN408 and ~ 1 mg of JN422 (Figure 60). Detailed analysis of the MS, UV, IR and NMR spectral data of JN408 (31) indicated it to be an oxidized marineosin analog while analysis of JN422 (32) data indicated it to be a 9-keto-derivative of JN408 (Figure 59).

Figure 59: Structures of JN408 and JN422, produced by *S. venezuelae* JND2ΔA

Figure 60: HPLC chromatogram of purified JN408 (top panel) and purified JN422 (bottom panel). The red trace represents absorption at λ_{max} 530 nm and blue trace represents absorption at λ_{max} 320 nm.

The UV spectrum of both JN408 (31) and JN422 (32) is characterized by λ_{max} 320 nm similar to the UV spectrum of marineosin (Figure 61). This preliminary data suggested that both compounds have a non-conjugated tri-pyrrole ring system similar to marineosin.

Figure 61: UV spectrum of JN408 (top panel) and JN422 (bottom panel)

HR-ESI-MS analysis of JN408 (31) exhibits $[M+H]^+$ of m/z 408.2660 (Figure 62). The acquired mass was analyzed for molecular formula $C_{25}H_{34}O_2N_3$ or $C_{25}H_{33}O_2N_3$ without the H-adduct. The difference between the mass acquired for JN408 (31), and the mass of the predicted molecular formula is only m/z 0.0014 (Figure 63). The theoretical isotope pattern of the predicted molecular formula was found to be identical to the isotope pattern acquired for JN408 (31). The molecular formula $C_{25}H_{34}O_2N_3$ corresponds to an index of hydrogen deficiency of 11.

Figure 62: HR-ESI-MS of JN408

Figure 63: Comparison between the acquired mass and isotope pattern (top panel) and the predicted mass (bottom panel) for JN408. There is a difference of m/z 0.0014 between the acquired and predicted mass for JN408.

The MS/MS of JN408 (31) is characterized by the presence of daughter fragment ions m/z 391.33 (33), and 376.33 (34) corresponding to the loss of m/z 17 and 32, respectively (Figure 64) (Figure 65). This profile is consistent with the loss of a hydroxyl radical to generate fragment ion (33) followed by the loss of a methyl radical to generate fragment ion (34) (Figure 65). The generation of m/z 230.25 (21) fragment ion was observed previously in the MS/MS profile of marineosin (Figure 14) (Figure 15) which indicated that JN408 and marineosin share similar structures. The neutral loss of m/z 15 characteristic for conjugated red-colored prodiginines such as undecylprodiginine (7) (Figure 21) or G410 (27) (Figure 41), was not observed in the MS/MS profile of JN408 which further corroborated a structure similar to marineosin rather than undecylprodiginine (7) or G410 (27).

JN408-2 #6-363 RT: 0.04-1.99 AV: 179 NL: 6.44E5
F: ITMS + p ESI Full ms2 408.26@cid20.00 [110.00-

Figure 64: ESI-CID-MSⁿ profile of JN408. The MS/MS profile of JN408 is characterized by the presence of m/z 230.25, 376.33 and 391.33 daughter ion fragments. Parent ion, m/z 408.26 is not shown. Please refer to text for more details.

Figure 65: Fragment ions observed in the MS/MS profile of JN408. The daughter fragment m/z 230.19 was observed before in the MS/MS profile of marineosin which suggested that JN408 is a marineosin analog. The loss of m/z 17 from JN408, corresponding to the loss of a hydroxyl radical leads to the generation of fragment (33). The consequent loss of a methyl radical from fragment ion (33) leads to the generation of fragment ion (34). It should be noted that the masses recorded in this figure are for the calculated values and are slightly different from the experimentally acquired values shown in Figure 64.

The HR-ESI-MS analysis of JN422 (32) exhibits $[M+H]^+$ of m/z 422.2450 (Figure 66). The acquired mass was analyzed for molecular formula $C_{25}H_{32}O_3N_3$ or $C_{25}H_{31}O_3N_3$ without the H-adduct. This corresponds to an index of hydrogen

deficiency of 12. The difference between the acquired mass of JN422 and the theoretical mass of the predicted molecular formula is only m/z 0.0012 (Figure 67). From the molecular formula, it was proposed that JN422 is a keto-derivative of JN408, but the position of that keto group was unclear.

Figure 66: HR-ESI-MS of JN422

Figure 67: Comparison between the acquired mass and isotope pattern (top panel) and the predicted mass (bottom panel) for JN422. There is a difference of m/z 0.0012 between the acquired and predicted mass for JN422.

The MS/MS of JN422 (32) is characterized by the presence of daughter fragment ions m/z 405.25 (36), and 390.33 (37) corresponding to the loss of m/z 17 and 32, respectively (Figure 68) (Figure 69). This profile is consistent with the loss of a hydroxyl radical from JN422 to generate fragment ion (36) followed by the loss of a methyl radical to generate fragment ion (37) (Figure 69). A daughter fragment ion m/z 244.25 (35) is also observed in the MS/MS profile of JN422 which corresponds to the keto-derivative of fragment ion (21) observed in the MS/MS profile of JN408 and marineosin (Figure 65) (Figure 15). From fragment ion (35), we concluded that a keto group is present in the acyl chain of JN422. As observed in JN408 (31), the MS/MS profile of JN422 (32) lacks any daughter fragments representing a neutral loss of m/z 15 characteristic for conjugated prodiginines and therefore, JN422 was proposed to be a keto-derivative of JN408 rather than undecylprodiginine (7).

Figure 68: ESI-CID-MSⁿ profile of JN422. Parent ion, m/z 422.24 is not shown. Please refer to text for more details.

Figure 69: Fragment ions observed in the MS/MS profile of JN422. The daughter fragment m/z 244.17 (34) is the keto-derivative of fragment ion (21) observed in the MS/MS profile of both marineosin and JN408. The loss of m/z 17 from JN422, corresponding to the loss of a hydroxyl radical leads to the generation of fragment 35. The consequent loss of a methyl radical from fragment ion 35 leads to the generation of fragment ion 36. It should be noted that the masses recorded in this figure are for the calculated values and are slightly different from the experimentally acquired values shown in Figure 68.

The IR spectrum of JN408 (31) compound (Figure 70) has IR $\nu_{\max}$ 2925, 2855, 1617, 1427, 1363, 1349, 1270, 1222, 1119, 1059, 1031, 956, 771 and 712 cm^{-1} . JN422 (32) was found to have the same IR $\nu_{\max}$ (Figure 70) peaks as JN408 with only minor differences in the $\nu_{\max}$ values of individual peaks. However, the IR spectrum of JN422 is characterized by the presence of an additional characteristic IR $\nu_{\max}$ peak at 1705 cm^{-1} corresponding to C=O stretch of a keto group. This comparison between the IR spectra of JN408 and JN422 provided additional evidence that both compounds are related and that the predominant difference is the presence of a keto group in JN422.

All UV-profile, HR-ESI-MS, MS/MS profile and IR spectra of JN408 (31) and JN422 (32) indicated that both compounds are marineosin analogs and that are both compounds probably have the same structure except for the presence of an extra keto group in the acyl chain of JN422 (32). Therefore, further NMR spectral analyses of both compounds were done side by side. The structure of JN422 (32) was elucidated using ^1H -NMR, ^1H - ^1H 2D COSY, ^1H - ^1H 2D TOCSY, 2D HSQC and 2D HMBC experiments. Due to limited quantity of JN408 (31), some cross peak correlations in the 2D HSQC and 2D HMBC spectra were not determined and therefore, the missing correlations were deduced by comparison to JN422 2D HSQC and 2D HMBC spectra.

Figure 70: The IR spectrum of JN408 (top panel) and JN422 (bottom panel)

^1H -NMR spectra of both JN408 and JN422 (Figure 71) (Figure 72) have 6 peaks with chemical shifts, δ 5.5 - 7.5 ppm which indicates the presence of 6 aromatic protons.

Figure 71: ^1H -NMR spectrum of JN408. Key protons peaks are assigned.

JN422-64scans-nonspin

Figure 72: ^1H -NMR spectrum of JN422. Key protons are assigned.

The ^{13}C -chemical shifts of JN408 (31) and JN422 (32) were indirectly determined from the 2D HSQC (Figure 73) and 2D HSQCED spectra (Figure 74) of both

molecules. The 2D HSQCED spectra of JN408 (31) and JN422 (32) confirmed that the 6 protons with chemical shifts δ 5.5 – 7.5 ppm are attached to carbons with ^{13}C -chemical shifts δ 93 – 131 ppm which is usually observed for carbons part of an aromatic ring system. Detailed ^1H - and ^{13}C -chemical shifts of JN408 and JN422 is presented in Table 15.

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Figure 73: 2D HSQC spectrum of JN408

Figure 74: 2D HSQCED spectrum of JN408 (top panel) and JN422 (bottom panel). Key peak assignments are indicated on spectra.

Table 15: NMR Spectral data for JN408 and JN422 (600 MHz, Acetone-d₆)

Position	JN408 $\delta_{\text{H}}^{\text{a}}$	JN422 δ_{H}	JN408 $\delta_{\text{C}}^{\text{a}}$	JN422 δ_{C}
1	7.37	7.45	131.3	131.5
2	6.37	6.39	111.6	112.4
3	7.08	7.20	127.5	125.9
4			N.D.	N.D.
5			N.D.	N.D.
6	5.93	6.01	93.1	93.1
7			N.D.	180.6
8			N.D.	97.4
9	2.89	2.90	46.1	46.3
10			N.D.	125.7
11	5.64	5.61	109.0	108.3
12	5.46	5.48	104.5	105.5
13			N.D.	130.1
14	2.24, 2.37	2.37, 2.73	28.0	23.6
15	1.37	2.16, 3.05	26.5	39.9
16	1.34, 1.37		29.3	211.3
17	0.54, 0.80	1.71, 2.21	24.7	43.6
18	1.42, 1.86	1.29, 1.31	24.4	29.4
19	1.05, 1.20	0.94, 1.33	24.3	22.1
20	1.00, 1.37	1.12, 1.26	31.9	24.7
21	2.71	2.80	28.1	27.3
22	1.67, 1.91	1.67, 1.82	37.8	37.9
23	4.36	4.36	70.1	70.0
24	1.50	1.50	20.9	20.6
25	3.99	4.02	59.1	59.4

^a Assignment by 1D (¹H), and 2D (COSY, TOCSY, HSQC) NMR spectroscopy; N.D.: not determined.

^1H - ^1H 2D TOCSY (Figure 75) aromatic correlations among the 6 aromatic protons observed in both JN408 (31) and JN422 (32) confirms that these protons constitute the tripyrrole ring skeleton characteristic to prodiginines family of natural products. The presence of 6 aromatic protons in JN408 and JN422 vs. only 5 aromatic protons in marineosin suggested that the pyrrole ring B in JN408 (31) and JN422 (32) is oxidized and fully conjugated. It should be noted from Table 15 and ^1H - ^1H 2D TOCSY spectra (Figure 75) (Figure 76) of JN408 (30) and JN422 (31), that both compounds are identical in their aromatic regions represented by C1, C2, C3, C6, C11 and C12. Similar 2D TOCSY correlations were observed for G410 (27) and undecylprodiginine (7) (Figure 46) which again confirms that all three pyrrole rings of JN408 (31) and JN422 (32) are non-reduced, unlike that observed in marineosin.

Figure 75: ^1H - ^1H 2D TOCSY spectrum of JN408. Top panel: JN408 showing selected ^1H - ^1H 2D TOCSY correlations among aromatic protons only, shown in bold. Bottom panel: full ^1H - ^1H 2D TOCSY spectrum of JN408 with the aromatic region correlations boxed and key correlations assigned.

Figure 76: ^1H - ^1H 2D TOCSY spectrum of JN422 showing correlations between aromatic protons. Top panel: selected ^1H - ^1H 2D TOCSY correlations of JN422 among aromatic protons, in bold. Bottom panel: full ^1H - ^1H 2D TOCSY spectrum of JN422 with the aromatic region correlations boxed and key correlations assigned.

The 2D HSQCED of JN408 (31) indicated the presence of 8 methylene groups (Figure 77) while that of JN422 (32) indicated the presence of only 7 methylene groups (Figure 78).

Figure 77: 2D HSQCED spectrum of JN408 - aliphatic region expanded. Carbons bonding even number of protons are shown in blue while carbons bonding odd number of protons are shown in green. Aliphatic – CH₂ groups are assigned and boxed. A total of 8 –CH₂ groups are present.

Figure 78: 2D HSQCED spectrum of JN422 - aliphatic region expanded. Carbons bonding even number of protons are shown in green while carbons bonding odd number of protons are shown in blue. Aliphatic – CH₂ groups are assigned and boxed. A total of 7 –CH₂ groups are present. A cross peak at (F1=1.12 ppm, F2=24.7 ppm), corresponding to the second proton attached to C-20 is not shown in this spectrum.

In JN422, ^1H - ^1H 2D TOCSY correlations were found between protons at C9 and C21 (Figure 79) which suggested that a covalent bond exists between C9 and C21 to form a macrocyclic ring.

Figure 79: Selected ^1H - ^1H 2D TOCSY correlations (top panel) and spectrum (bottom) of JN422 - aliphatic region expanded. Correlations between protons at C9 and C21 and neighboring $-\text{CH}_2-$ groups are represented by bold bonds in JN422 structure (top panel). The same correlations are shown and boxed in the TOCSY spectrum (bottom panel).

These data were confirmed by 2D HMBC correlations between protons at C9 and C7, C8, C10, C11, C20, C21 and C22 (Figure 80).

Figure 80: Selected 2D HMBC (^1H - ^{13}C) correlation (top panel) and spectrum (bottom panel) of JN422 - aliphatic region expanded. Correlations between proton at C9 and neighboring carbons are represented by bold bonds in JN422 structure (top panel). Correlations between protons at C-17 and the carbonyl carbon at C-16 are also in bold. The same correlations are boxed and assigned in the 2D HMBC spectrum (bottom panel).

The same clear correlations between proton at C9 and proton at C21 were observed in the ^1H - ^1H 2D TOCSY spectrum of JN408 (Figure 81).

Figure 81: Selected ^1H - ^1H 2D TOCSY correlations (top panel) and spectrum (bottom) of JN408- aliphatic region expanded. Correlations between protons at C9 and C21 and neighboring $-\text{CH}_2$ groups are represented by bold bonds in JN408 structure (top panel). The same correlations are boxed in the TOCSY spectrum (bottom panel). Key correlations for C9 and C21 are indicated.

Unfortunately, due to limited quantity of JN408 (31), these correlations could not be confirmed by 2D HMBC data. However, the TOCSY data was sufficient to conclude that JN408 has a macrocyclic ring similar to that present in JN422 (32) and marineosin.

In JN422, weak (^1H - ^{13}C) HMBC correlations (Figure 80) between a carbonyl carbon at δ 211 ppm and protons at C17 suggested that the carbonyl group observed in the IR spectrum of JN422 exists in the vicinity of C17. ^1H - ^1H 2D TOCSY spectrum of JN422 shows strong correlations between protons at C14 and C15 but these correlations are not extended to any other proton in the acyl chain of JN422, suggesting that C15 is next to either a heteroatom or a quaternary carbon. Also, protons at C15 and C17 in JN422 (32) are slightly shifted downfield relative to their counterparts in JN408 (31) (Table 15). Therefore, we assigned the position of the keto group of JN422 (32) at C16.

Since all protons were accounted for except the protons at C8 and C23, we concluded that a spiroaminal ring exists between C8 and C23 in both JN408 (31) and JN422 (32).

Full spectral data and correlations of JN408 (31) and JN422 (32) are presented in Figure 83, Figure 84, Figure 85, Figure 86, Figure 87, Table 16, Table 17, and Table 18.

Figure 82: ^1H - ^1H 2D TOCSY spectrum of JN422 showing correlations between C14 and C15. Correlations between protons at C14 and C15 are indicated. The correlations are not extended to neighboring $-\text{CH}_2$ groups in the TOCSY spectrum indicating that both groups are either adjacent to a heteroatom or a quaternary carbon.

A)

B)

Figure 83: A) ^1H - ^1H 2D COSY correlations of JN408. B) 2D COSY NMR spectrum of JN422. Selected COSY correlations in JN408 are drawn in bold.

A)

B)

Figure 84: A) Selected COSY (H↔H; bold lines) and TOCSY (H↔H; double arrows) NMR correlations for JN408. B) 2D TOCSY NMR spectrum of JN408.

Position	δ_{H}	COSY	TOCSY
1	7.37	2, 3	2, 3
2	6.37	1, 3	1, 3
3	7.08	1, 2	1, 2
4			
5			
6	5.93		
7			
8			
9	2.89	21	20, 21, 22, 23, 24
10			
11	5.64	12	12
12	5.46	11	11
13			
14	2.24, 2.37	15	15, 16, 17
15	1.37	14, 17	14, 16, 17, 18
16	1.34, 1.37	15, 18	14, 15, 17, 18, 19
17	0.54, 0.80	16, 18	15, 16, 18, 19, 20
18	1.42, 1.86	17, 19	15, 16, 17, 19, 20
19	1.05, 1.20	18, 20	16, 17, 18, 20, 21
20	1.00, 1.37	19, 21	9, 17, 18, 19, 21, 22
21	2.71	9, 20, 22	9, 19, 20, 22, 23, 24
22	1.67, 1.91	21, 23	9, 20, 21, 23, 24
23	4.36	22, 24	9, 20, 21, 22, 24
24	1.50	23	9, 21, 22, 23
25	3.99		

Table 16: Selected COSY ($\text{H} \leftrightarrow \text{H}$) and TOCSY ($\text{H} \leftrightarrow \text{H}$) NMR correlations for JN408

A)

B)

Figure 85: A) ^1H - ^1H 2D COSY correlations of JN422. B) 2D COSY NMR spectrum of JN422. Selected COSY correlations in JN422 are drawn in bold.

A)

B)

Figure 86: A) Selected COSY ($H \leftrightarrow H$; bold lines) and TOCSY ($H \leftrightarrow H$; double arrows) NMR correlations for JN422. B) 2D TOCSY NMR spectrum of JN422.

A)

B)

Figure 87: A) Selected HMBC (¹H→¹³C) NMR correlations for JN422. B) 2D HMBC spectrum of JN422.

Position	δ_H	COSY	TOCSY
1	7.45	2, 3	2, 3
2	6.39	1, 3	1, 3
3	7.20	1, 2	1, 2
4			
5			
6	6.01		
7			
8			
9	2.90	21	20, 21, 22, 23, 24
10			
11	5.61	12	12
12	5.48	11	11
13			
14	2.37, 2.73	15	15
15	2.16, 3.05	14	14
16			
17	1.71, 2.21	18	18, 19, 20
18	1.29, 1.31	17, 19	17, 19, 20
19	0.94, 1.33	18, 20	17, 18, 20, 21
20	1.12, 1.26	19, 21	9, 17, 18, 19, 21, 22
21	2.80	9, 20, 22	9, 19, 20, 22, 23, 24
22	1.67, 1.82	21, 23	9, 20, 21, 23, 24
23	4.36	22, 24	9, 20, 21, 22, 24
24	1.50	23	9, 21, 22, 23
25	4.02		

Table 17: Selected COSY (H $\leftrightarrow$ H) and TOCSY (H $\leftrightarrow$ H) NMR correlations for JN422

Position	$\delta_{\text{H}}^{\text{a}}$	$\delta_{\text{C}}^{\text{a}}$		Selected HMBC correlations
1	7.45	131.5	CH	
2	6.39	112.4	CH	
3	7.20	125.9	CH	
4		N.D.	C	
5		N.D.	C	
6	6.01	93.1	CH	
7		180.6	C	
8		97.4	C	
9	2.90	46.3	CH	7, 8, 10, 11, 20, 21, 22
10		125.7	C	
11	5.61	108.3	CH	10, 12, 13
12	5.48	105.5	CH	10, 13
13		130.1	C	
14	2.37, 2.73	23.6	CH ₂	12, 13, 15, 16
15	2.16, 3.05	39.9	CH ₂	
16		211.3	C=O	
17	1.71, 2.21	43.6	CH ₂	16, 19
18	1.29, 1.31	29.4	CH ₂	19, 20
19	0.94, 1.33	22.1	CH ₂	18
20	1.12, 1.26	34.7	CH ₂	9
21	2.80	27.3	CH	
22	1.67, 1.82	37.9	CH ₂	
23	4.36	70.0	CH	
24	1.50	20.6	CH ₃	22, 23
25	4.02	59.4	CH ₃	7

Table 18: NMR Spectral data for JN422 (600 MHz, Acetone-d₆) and Selected HMBC correlations.

3.5 CONCLUSIONS

From the structures of JN408 (31) and JN422 (32), it is evident that MarA does not catalyze spiroaminal ring formation because its deletion led to the accumulation of two spiroaminal marineosin analogs. Although an NADH/NADPH binding site could not be identified from the primary protein sequence of MarA, our data suggests that MarA acts as a dehydrogenase/reductase that is involved in the reduction of pyrrole ring B of JN408 (31) to yield marineosins. We are proposing that spiroaminal ring formation is either catalyzed by MarG or occurs non-enzymatically via a concerted mechanism. In the latter case, the lone pair of electrons of the –OH group of 2-hydroxyundecylprodiginine, G410 (27) undergoes a nucleophilic attack on C-8, with the subsequent reduction of the methine bridge between C-8 and C-9 (Figure 88). The –OH group of G410 (27) can be introduced either early in the pathway due to the hydroxylation of 2-UP subunit by an unidentified oxidase (route 1, Figure 88), or the recruitment of hydroxy-butyric acid starter unit by MarP (route 2, Figure 88). The hydroxyl group can be also introduced later in the pathway, due to the presence of an unidentified Mar protein that catalyzes the hydroxylation of undecylprodiginine (7) such as MarT (route 3, Figure 88). The fact that undecylprodiginine (7) intermediate was never detected in any of the strains generated in this study suggests that route 1 or route 2 of our hypothesis are the most likely ones to be correct. Primary protein sequence analysis of MarT and other hypothetical proteins encoded by the *mar* gene cluster does not show any similarity to known

p450 oxidases, or non-heme iron Rieske oxygenases. Moreover, homologs of MarT and other hypothetical Mar proteins are also encoded by the *red* gene cluster but no hydroxylated undecylprodiginine was ever reported before, which makes route 3 of our hypothesis less plausible. We are proposing that JN422 (32) is a shunt metabolite generated from the introduction of a keto group to either JN408 (31), or very early in the pathway due to incorporation of a keto-butyric acid starter unit, or a modified specificity of the fatty acid synthases involved in the biosynthesis of the acyl side chain of marineosin (Figure 88). To the best of our knowledge, JN422 (32) is the first isolated cyclic prodiginine analog with a keto group in the *ansa*-bridged macrocycle and studying the biological activity of this compound would be very interesting.

Figure 88: Scheme for the biosynthesis of marineosin. Isolated metabolites are boxed.

4. Conclusions and future work recommendations

The work presented in this dissertation describes the biosynthesis of marineosin, a novel prodiginine analog and how it differs from the biosynthesis of other prodiginines compounds directed by the *red* gene cluster. It also provides valuable insights into the *in vivo* role of two key enzymes involved in the biosynthesis of marineosin and proposes an experimentally-supported scheme describing the key steps en route to marineosin.

The present study paves the way to the targeted biosynthesis of a valuable combinatorial library of marineosin and prodiginine analogs and opens up a treasure of research opportunities.

Conclusion 1: Marineosin biosynthesis is directed by a *red* gene cluster homolog named, *mar* gene cluster; with the presence of only one extra gene encoding a putative dehydrogenase/reductase named MarA. No additional tailoring enzymes are involved in the biosynthesis of marineosin.

Conclusion 2: MarG, a non-heme iron Rieske-oxygenase catalyzes the oxidative cyclization of the acyl chain of 2-hydroxyundecylprodiginine (27) with the subsequent formation of an *ansa*-bridged 12-membered carbocyclic ring. MarG does not catalyze the introduction of the –OH group required to initiate the biosynthesis of the spiroaminal ring of marineosin. Undecylprodiginine (7) is not a

detectable intermediate along the biosynthetic route to marineosin. The reduction of pyrrole ring B happens later in the pathway, after MarG catalysis step.

Conclusion 3: MarA, a putative dehydrogenase/reductase catalyzes the reduction of pyrrole ring B of JN408 (31) intermediate to generate marineosin. MarA is not involved in neither the introduction of the –OH group of 2-hydroxyundecylprodiginine (27) nor the biosynthesis of the spiroaminal ring. Deletion of *marA* gene leads to the accumulation of a novel marineosin analog.

Conclusion 4: The origin of the spiroaminal ring oxygen is probably due to a different substrate specificity of MarP; the first enzyme involved in the biosynthesis of the acyl chain of marineosin or, an unidentified oxidase that hydroxylates the acyl chain of undecylprodiginine (7).

Conclusion 5: The first experimentally-supported scheme describing the biosynthesis of marineosin was presented.

Future work:

1. The substrate specificity and mechanism of action of MarG enzyme can be studied after its expression and purification. MarG belongs to a class of Rieske oxygenases whose function is still not fully understood and its characterization could provide a wealth of information on C-H bond activation.

2. The exact function of MarA enzyme can be only be verified through detailed kinetic analysis following its expression as a recombinant protein. The substrate specificity of MarA can be determined by testing its reductase activity with several natural and synthetic, straight chain and cyclic prodiginine analogs.

3. The exact origin of the spiroaminal ring oxygen can be pinned down through the feeding of isotope-labeled hydroxyl-butyric acid starter unit to *S. venezuelae* JND2ΔG followed by purification and characterization of isotope labeling patterns of the generated 2-hydroxyundecylprodiginine. Also, the deletion of MarT-encoding gene from the *mar* gene cluster can be used to study whether or not it plays a critical role in the biosynthesis of marineosin.

References

- [1] R. A. Maplestone, M. J. Stone, and D. H. Williams, "The evolutionary role of secondary metabolites—a review", *Gene*, vol. 115, no. 1–2, pp. 151–157, 1992.
- [2] B. M. Hopkinson and F. M. M. Morel, "The role of siderophores in iron acquisition by photosynthetic marine microorganisms", *Biometals*, vol. 22, no. 4, pp. 659–669, 2009.
- [3] L. C. Vining, "Functions of secondary metabolites", *Annu. Rev. Microbiol.*, vol. 44, pp. 395–427, 1990.
- [4] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs over the last 25 years", *J. Nat. Prod.*, vol. 70, no. 3, pp. 461–477, Mar. 2007.
- [5] A. T. Keatinge-Clay, D. A. Maltby, K. F. Medzihradzsky, C. Khosla, and R. M. Stroud, "An antibiotic factory caught in action", *Nat Struct Mol Biol.*, vol. 11, no. 9, pp. 888–893, 2004.
- [6] D. A. Hopwood, "Soil to genomics: the *Streptomyces* chromosome", *Annu. Rev. Genet.*, vol. 40, pp. 1–23, 2006.
- [7] J. Bérdy, "Bioactive microbial metabolites", *J. Antibiot.*, vol. 58, no. 1, pp. 1–26, Jan. 2005.
- [8] S. Smith and S.-C. Tsai, "The type I fatty acid and polyketide synthases: a tale of two megasynthases", *Nat. Prod. Rep.*, vol. 24, no. 5, p. 1041, 2007.
- [9] J. L. Meier and M. D. Burkart, "The chemical biology of modular biosynthetic enzymes", *Chem. Soc. Rev.*, vol. 38, no. 7, p. 2012, 2009.
- [10] D. A. Hopwood, "The Leeuwenhoek Lecture, 1987: Towards an Understanding of Gene Switching in *Streptomyces*, the Basis of Sporulation and Antibiotic Production", *P. R. Soc. London., Series B, Biological Sciences*, vol. 235, no. 1279, pp. 121–138, 1988.
- [11] T. Mahmud, "Isotope tracer investigations of natural products biosynthesis: the discovery of novel metabolic pathways", *J. Label. Compd. Radiopharm.*, vol. 50, no. 11–12, pp. 1039–1051, Oct. 2007.

- [12] E.-S. E. Habib, J. N. Scarsdale, and K. A. Reynolds, "Biosynthetic origin of hygromycin A", *Antimicrob. Agents Chemother.*, vol. 47, no. 7, pp. 2065–2071, Jul. 2003.
- [13] M. Ghatge, N. Palaniappan, S. Das Choudhuri, and K. Reynolds, "Genetic manipulation of the biosynthetic process leading to phoslactomycins, potent protein phosphatase 2A inhibitors", *J. Ind. Microbiol. Biotechnol.*, vol. 33, no. 7, pp. 589–599, Jul. 2006.
- [14] M. Rohmer, M. Knani, P. Simonin, B. Sutter, and H. Sahm, "Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate", *Biochem. J*, vol. 295 (Pt 2), pp. 517–524, Oct. 1993.
- [15] L. Frangeul, K. E. Nelson, C. Buchrieser, A. Danchin, P. Glaser, and F. Kunst, "Cloning and assembly strategies in microbial genome projects", *Microbiology (Reading, Engl.)*, vol. 145 (Pt 10), pp. 2625–2634, Oct. 1999.
- [16] B. Gust, "Chapter 7 Cloning and Analysis of Natural Product Pathways", in *Complex Enzymes in Microbial Natural Product Biosynthesis, Part A: Overview Articles and Peptides*, vol. Volume 458, Academic Press, 2009, pp. 159–180.
- [17] I. A. Darby and T. D. Hewitson, *In situ hybridization protocols*. Humana Press, 2006.
- [18] T. N. Campbell and F. Y. M. Choy, "Approaches to library screening", *J. Mol. Microbiol. Biotechnol*, vol. 4, no. 6, pp. 551–554, Nov. 2002.
- [19] B. Gust, G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater, "PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin", *Proc. Natl. Acad. Sci. U.S.A*, vol. 100, no. 4, pp. 1541–1546, Feb. 2003.
- [20] B. Gust, G. Chandra, D. Jakimowicz, T. Yuqing, C. J. Bruton, and K. F. Chater, "Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*", *Adv. Appl. Microbiol*, vol. 54, pp. 107–128, 2004.

- [21] E. Takano, H. C. Gramajo, E. Strauch, N. Andres, J. White, and M. J. Bibb, "Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2)", *Mol. Microbiol*, vol. 6, no. 19, pp. 2797–2804, Oct. 1992.
- [22] H. C. Gramajo, E. Takano, and M. J. Bibb, "Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated", *Mol. Microbiol*, vol. 7, no. 6, pp. 837–845, Mar. 1993.
- [23] H. M. Thorpe and M. C. Smith, "In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family", *Proc. Natl. Acad. Sci. U.S.A*, vol. 95, no. 10, pp. 5505–5510, May 1998.
- [24] M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner, "Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp", *Gene*, vol. 116, no. 1, pp. 43–49, Jul. 1992.
- [25] A. S. Eustáquio, B. Gust, U. Galm, S.-M. Li, K. F. Chater, and L. Heide, "Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters", *Appl. Environ. Microbiol*, vol. 71, no. 5, pp. 2452–2459, May 2005.
- [26] M. Sosio, F. Giusino, C. Cappellano, E. Bossi, A. M. Puglia, and S. Donadio, "Artificial chromosomes for antibiotic-producing actinomycetes", *Nat. Biotechnol.*, vol. 18, no. 3, pp. 343–345, Mar. 2000.
- [27] M. Sosio, E. Bossi, and S. Donadio, "Assembly of large genomic segments in artificial chromosomes by homologous recombination in *Escherichia coli*", *Nucleic Acids Res.*, vol. 29, no. 7, p. E37, Apr. 2001.
- [28] F. W. Studier and B. A. Moffatt, "Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes", *J. Mol. Biol*, vol. 189, no. 1, pp. 113–130, May 1986.

- [29] E. Hochuli, W. Bannwarth, H. Dobeli, R. Gentz, and D. Stuber, "Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent", *Nat. Biotech.*, vol. 6, no. 11, pp. 1321–1325, Nov. 1988.
- [30] N. R. Williamson, P. C. Fineran, F. J. Leeper, and G. P. C. Salmond, "The biosynthesis and regulation of bacterial prodiginines", *Nat. Rev. Micro.*, vol. 4, no. 12, pp. 887–899, Dec. 2006.
- [31] J. W. Bennett and R. Bentley, "Seeing red: the story of prodigiosin", *Adv. Appl. Microbiol.*, vol. 47, pp. 1–32, 2000.
- [32] R. Pérez-Tomás, B. Montaner, E. Llagostera, and V. Soto-Cerrato, "The prodigiosins, proapoptotic drugs with anticancer properties", *Biochem. Pharmacol.*, vol. 66, no. 8, pp. 1447–1452, 2003.
- [33] R. A. Manderville, "Synthesis, proton-affinity and anti-cancer properties of the prodigiosin-group natural products", *Curr. Med. Chem. Anticancer Agents*, vol. 1, no. 2, pp. 195–218, Aug. 2001.
- [34] M. Nguyen, R. C. Marcellus, A. Roulston, M. Watson, L. Serfass, S. R. Murthy Madiraju, D. Goulet, J. Viallet, L. Bélec, X. Billot, and others, "Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis", *P. Natl. Acad. Sci.*, vol. 104, no. 49, p. 19512, 2007.
- [35] T. Azuma, N. Watanabe, H. Yagisawa, H. Hirata, M. Iwamura, and Y. Kobayashi, "Induction of apoptosis of activated murine splenic T cells by cycloprodigiosin hydrochloride, a novel immunosuppressant", *Immunopharmacology*, vol. 46, no. 1, pp. 29–37, Jan. 2000.
- [36] R. D'Alessio, A. Bargiotti, O. Carlini, F. Colotta, M. Ferrari, P. Gnocchi, A. Isetta, N. Mongelli, P. Motta, A. Rossi, M. Rossi, M. Tibolla, and E. Vanotti, "Synthesis and Immunosuppressive Activity of Novel Prodigiosin Derivatives", *J. Med. Chem.*, vol. 43, no. 13, pp. 2557–2565, Jun. 2000.

- [37] A. Fürstner, "Chemistry and biology of roseophilin and the prodigiosin alkaloids: a survey of the last 2500 years", *Angew. Chem. Int. Edit.*, vol. 42, no. 31, pp. 3582–3603, 2003.
- [38] K. Papireddy, M. Smilkstein, J. X. Kelly, Shweta, S. M. Salem, M. Alhamadsheh, S. W. Haynes, G. L. Challis, and K. A. Reynolds, "Antimalarial Activity of Natural and Synthetic Prodiginines", *J. Med. Chem.*, Jul. 2011.
- [39] H. H. Wasserman, R. J. Sykes, P. Peverada, C. K. Shaw, R. J. Cushley, and S. R. Lipsky, "Biosynthesis of prodigiosin. Incorporation patterns of carbon-13-labeled alanine, proline, glycine, and serine elucidated by Fourier transform nuclear magnetic resonance", *J. Am. Chem. Soc.*, vol. 95, no. 20, pp. 6874–6875, Oct. 1973.
- [40] N. N. Gerber, A. G. McInnes, D. G. Smith, J. A. Walter, J. L. C. Wright, and L. C. Vining, "Biosynthesis of prodiginines. ^{13}C resonance assignments and enrichment patterns in nonyl-, cyclononyl-, methylcyclodecyl-, and butylcycloheptylprodiginine produced by actinomycete cultures supplemented with ^{13}C -labeled acetate and ^{15}N -labeled nitrate", *Can. J. Chem.*, vol. 56, no. 9, pp. 1155–1163, 1978.
- [41] W. K. Tanaka, L. B. de Medina, and W. R. Hearn, "Labeling patterns in prodigiosin biosynthesis", *Biochem. Biophys. Res. Commun.*, vol. 46, no. 2, pp. 731–737, Jan. 1972.
- [42] N. N. Gerber, "Prodigiosin-Like Pigments", *Cr. Rev. Microbiol.*, vol. 3, no. 4, pp. 469–485, Jan. 1975.
- [43] J. S. Feitelson, F. Malpartida, and D. A. Hopwood, "Genetic and Biochemical Characterization of the red Gene Cluster of *Streptomyces coelicolor* A3(2)", *J. Gen. Microbiol.*, vol. 131, no. 9, pp. 2431–2441, Sep. 1985.
- [44] F. Malpartida, J. Niemi, R. Navarrete, and D. A. Hopwood, "Cloning and expression in a heterologous host of the complete set of genes for

- biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin”, *Gene*, vol. 93, no. 1, pp. 91–99, Sep. 1990.
- [45] S. D. Bentley, K. F. Chater, A.-M. Cerdeño-Tárraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C.-H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O’Neil, E. Rabbinowitsch, M.-A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood, “Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)”, *Nature*, vol. 417, no. 6885, pp. 141–147, May 2002.
- [46] A. M. Cerdeno, M. J. Bibb, and G. L. Challis, “Analysis of the prodiginine biosynthesis gene cluster of *Streptomyces coelicolor* A3 (2): new mechanisms for chain initiation and termination in modular multienzymes”, *Chem. Biol.*, vol. 8, no. 8, pp. 817–829, 2001.
- [47] M. G. Thomas, M. D. Burkart, and C. T. Walsh, “Conversion of L-proline to pyrrolyl-2-carboxyl-S-PCP during undecylprodigiosin and pyoluteorin biosynthesis”, *Chem. Biol.*, vol. 9, no. 2, pp. 171–184, 2002.
- [48] A. E. Stanley, L. J. Walton, M. Kourdi Zerikly, C. Corre, and G. L. Challis, “Elucidation of the *Streptomyces coelicolor* pathway to 4-methoxy-2,2?-bipyrrole-5-carboxaldehyde, an intermediate in prodiginine biosynthesis”, *Chem. Commun.*, no. 38, p. 3981, 2006.
- [49] S. Mo, B. S. Kim, and K. A. Reynolds, “Production of Branched-Chain Alkylprodiginines in *S. coelicolor* by Replacement of the 3-Ketoacyl ACP Synthase III Initiation Enzyme, RedP”, *Chem. Biol.*, vol. 12, no. 2, pp. 191–200, Feb. 2005.
- [50] S. Mo, P. K. Sydor, C. Corre, M. M. Alhamadsheh, A. E. Stanley, S. W. Haynes, L. Song, K. A. Reynolds, and G. L. Challis, “Elucidation of the

- Streptomyces coelicolor* Pathway to 2-Undecylpyrrole, a Key Intermediate in Undecylprodiginine and Streptorubin B Biosynthesis”, *Chem. Biol.*, vol. 15, no. 2, pp. 137–148, Feb. 2008.
- [51] S. W. Haynes, P. K. Sydor, A. E. Stanley, L. Song, and G. L. Challis, “Role and substrate specificity of the *Streptomyces coelicolor* RedH enzyme in undecylprodiginine biosynthesis”, *Chem. Commun.*, no. 16, p. 1865, 2008.
- [52] P. K. Sydor, S. M. Barry, O. M. Odulate, F. Barona-Gomez, S. W. Haynes, C. Corre, L. Song, and G. L. Challis, “Regio- and stereodivergent antibiotic oxidative carbocyclizations catalysed by Rieske oxygenase-like enzymes”, *Nat. Chem.*, vol. 3, no. 5, pp. 388–392, May 2011.
- [53] C. Boonlarpadab, C. A. Kauffman, P. R. Jensen, and W. Fenical, “Marineosins A and B, cytotoxic spiroaminals from a marine-derived actinomycete”, *Org. Lett.*, vol. 10, no. 24, pp. 5505–5508, Dec. 2008.
- [54] L. N. Aldrich, E. S. Dawson, and C. W. Lindsley, “Evaluation of the biosynthetic proposal for the synthesis of marineosins A and B”, *Org. Lett.*, vol. 12, no. 5, pp. 1048–1051, Mar. 2010.
- [55] X.-C. Cai, X. Wu, and B. B. Snider, “Synthesis of the spiroiminal moiety of marineosins A and B”, *Org. Lett.*, vol. 12, no. 7, pp. 1600–1603, Apr. 2010.
- [56] J. J. Sanglier, V. Quesniaux, T. Fehr, H. Hofmann, M. Mahnke, K. Memmert, W. Schuler, G. Zenke, L. Gschwind, C. Maurer, and W. Schilling, “Sanglifehrins A, B, C and D, novel cyclophilin-binding compounds isolated from *Streptomyces* sp. A92-308110. I. Taxonomy, fermentation, isolation and biological activity”, *J. Antibiot.*, vol. 52, no. 5, pp. 466–473, May 1999.
- [57] L. E. Overman and Y. H. Rhee, “Total synthesis of (-)-crambidine and definition of the relative configuration of its unique tetracyclic guanidinium core”, *J. Am. Chem. Soc.*, vol. 127, no. 44, pp. 15652–15658, Nov. 2005.
- [58] A. Olszewski, K. Sato, Z. D. Aron, F. Cohen, A. Harris, B. R. McDougall, W. E. Robinson Jr, L. E. Overman, and G. A. Weiss, “Guanidine alkaloid

- analogs as inhibitors of HIV-1 Nef interactions with p53, actin, and p56lck", *P. Natl. Acad. Sci. U.S.A.*, vol. 101, no. 39, pp. 14079–14084, Sep. 2004.
- [59] A. P. J. Pal, P. Kadigachalam, A. Mallick, V. R. Doddi, and Y. D. Vankar, "Synthesis of sugar-derived spiroaminals via lactamization and metathesis reactions", *Org. Biomol. Chem.*, vol. 9, no. 3, pp. 809–819, Jan. 2011.
- [60] M. Sinibaldi, I. Canet, M. Sinibaldi, and I. Canet, "Synthetic Approaches to Spiroaminals, Synthetic Approaches to Spiroaminals", *Eur. J. Org. Chem.*, vol. 2008, 2008, no. 26, 26, pp. 4391, 4391–4399, 4399, Sep. 2008.
- [61] C. J. Forsyth, "Asymmetric Synthesis of Cyclic Ketal and Spiroaminal-Containing Natural Products", *ChemInform.*, vol. 39, no. 2, p. i, Jan. 2008.
- [62] S. Nguyen, J. Xu, and C. J. Forsyth, "Facile biomimetic syntheses of the azaspiracid spiroaminal", *Tetrahedron*, vol. 62, no. 22, pp. 5338–5346, May 2006.
- [63] G. Bertani, "Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*", *J. Bacteriol.*, vol. 62, no. 3, pp. 293–300, Sep. 1951.
- [64] D. Hanahan, "Studies on transformation of *Escherichia coli* with plasmids", *J. Mol. Biol.*, vol. 166, no. 4, pp. 557–580, Jun. 1983.
- [65] L. Zhao, N. J. Beyer, S. A. Borisova, and H. Liu, "Beta-glucosylation as a part of self-resistance mechanism in methymycin/pikromycin producing strain *Streptomyces venezuelae*", *Biochemistry*, vol. 42, no. 50, pp. 14794–14804, Dec. 2003.
- [66] D. P. Brown, S. J. Chiang, J. S. Tuan, and L. Katz, "Site-specific integration in *Saccharopolyspora erythraea* and multisite integration in *Streptomyces lividans* of actinomycete plasmid pSE101", *J. Bacteriol.*, vol. 170, no. 5, pp. 2287–2295, May 1988.
- [67] J. Yan, S. Gupta, D. H. Sherman, and K. A. Reynolds, "Functional dissection of a multimodular polypeptide of the pikromycin polyketide

- synthase into monomodules by using a matched pair of heterologous docking domains”, *Chembiochem*, vol. 10, no. 9, pp. 1537–1543, Jun. 2009.
- [68] K. Chen, N. S. Rannulu, Y. Cai, P. Lane, A. L. Liebl, B. B. Rees, C. Corre, G. L. Challis, and R. B. Cole, “Unusual odd-electron fragments from even-electron protonated prodiginine precursors using positive-ion electrospray tandem mass spectrometry”, *J. Am. Soc. Mass Spectrom.*, vol. 19, no. 12, pp. 1856–1866, 2008.
- [69] Practical *Streptomyces* genetics. John Innes Foundation, 2000.
- [70] J. S. Rieske, R. E. Hansen, and W. S. Zaugg, “Studies on the electron transfer system properties of a new oxidation-reduction component of the respiratory chain as studied by electron paramagnetic resonance spectroscopy”, *J. Biol. Chem.*, vol. 239, pp. 3017–3022, Sep. 1964.
- [71] D. J. Ferraro, L. Gakhar, and S. Ramaswamy, “Rieske business: structure-function of Rieske non-heme oxygenases”, *Biochem. Biophys. Res. Commun.*, vol. 338, no. 1, pp. 175–190, Dec. 2005.
- [72] J. F. Cline, B. M. Hoffman, W. B. Mims, E. LaHaie, D. P. Ballou, and J. A. Fee, “Evidence for N coordination to Fe in the [2Fe-2S] clusters of *Thermus* Rieske protein and phthalate dioxygenase from *Pseudomonas*”, *J. Biol. Chem.*, vol. 260, no. 6, pp. 3251–3254, Mar. 1985.
- [73] J. A. Fee, K. L. Findling, T. Yoshida, R. Hille, G. E. Tarr, D. O. Hearshen, W. R. Dunham, E. P. Day, T. A. Kent, and E. Münck, “Purification and characterization of the Rieske iron-sulfur protein from *Thermus thermophilus*. Evidence for a [2Fe-2S] cluster having non-cysteine ligands”, *J. Biol. Chem.*, vol. 259, no. 1, pp. 124–133, Jan. 1984.
- [74] D. Kuila and J. A. Fee, “Evidence for a redox-linked ionizable group associated with the [2Fe-2S] cluster of *Thermus* Rieske protein”, *J. Biol. Chem.*, vol. 261, no. 6, pp. 2768–2771, Feb. 1986.
- [75] Y. Zu, M. M.-J. Couture, D. R. J. Kolling, A. R. Crofts, L. D. Eltis, J. A. Fee, and J. Hirst, “Reduction potentials of Rieske clusters: importance of the

- coupling between oxidation state and histidine protonation state”, *Biochemistry*, vol. 42, no. 42, pp. 12400–12408, Oct. 2003.
- [76] E. L. Hegg and L. Que Jr, “The 2-His-1-carboxylate facial triad--an emerging structural motif in mononuclear non-heme iron(II) enzymes”, *Eur. J. Biochem.*, vol. 250, no. 3, pp. 625–629, Dec. 1997.
- [77] L. Que, “One motif — many different reactions”, *Nat. Struct. Mol. Biol.*, vol. 7, no. 3, pp. 182–184, Mar. 2000.
- [78] R. E. Parales, J. V. Parales, and D. T. Gibson, “Aspartate 205 in the Catalytic Domain of Naphthalene Dioxygenase Is Essential for Activity”, *J. Bacteriol.*, vol. 181, no. 6, pp. 1831–1837, Mar. 1999.
- [79] S. C. Dillon and A. Bateman, “The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases”, *BMC Bioinformatics*, vol. 5, p. 109, Aug. 2004.