

AN ABSTRACT OF THE THESIS OF

Florina Alexandra Vulpanovici for the degree of Doctor of Philosophy in
Biochemistry and Biophysics presented on July 23 2003.

Title: Biosynthesis, Production and Structural Studies of Secondary Metabolites in
Cultured Marine Cyanobacteria

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Abstract approved: _____

William H. Gerwick

This thesis details investigations of marine cyanobacterial secondary metabolism, with emphasis on a strain of *Phormidium* sp. collected in Indonesia. These studies assessed the effects of nineteen putative elicitor compounds on the growth and metabolite production of five species of marine cyanobacteria, biosynthetic investigation of an intriguing secondary metabolite, phormidolide, and the discovery of one novel halogenated peptide, phormidamide.

The growth, biomass production and the ratio of the components of the extract were affected by some of the elicitors in most of the cyanobacterial species tested. However, production of a novel secondary metabolite or a significant change in the bioactivity of the extracts was not observed.

Biosynthetic investigations of a brominated brine shrimp toxic polyketide, phormidolide, were conducted on a cultured *Phormidium* sp. strain originally isolated from Indonesia. Stable isotope feeding experiments confirmed its

polyketide nature and established a new example of a general trend in cyanobacterial metabolism where both S-adenosyl methionine and C2 of acetate contribute to the biogenesis of pendant methyl groups. At the same time, feedings with deuterated acetate provided insight into the HMG-CoA synthase - like mechanism by which addition of pendant methyl groups from C2 of acetate takes place.

Studies of phormidolide production in bromine-depleted medium showed that two analogs are produced, debromophormidolide with a terminal olefin in place of the vinyl bromide, and iodophormidolide, introducing iodine in place of bromine from the trace amounts present in the medium. Supplementation of the bromine-depleted culture medium with iodine resulted in a 10-fold increase of iodophormidolide production, while bromine supplementation resulted in a more moderate (2.5 fold) enhancement in phormidolide yield.

A novel halogenated cytotoxic peptide, phormidamide, was isolated and a planar structure is proposed, pending confirmation by X-ray crystallographic analysis. Phormidamide contains a unique bromophenylalanine functionality, three chlorine atoms, and a very high number of quaternary carbon atoms which have hindered structural elucidation efforts through spectroscopic methods.

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Biosynthesis, Production and Structural Studies of Secondary Metabolites in
Cultured Marine Cyanobacteria

by

Florina Alexandra Vulpanovici

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Dean of the Graduate School

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Florina Alexandra Vulpanovici, Author

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Finally, I would like to express my special thanks to my roommates Corina, Diana and Dragos, to all the friends made in Corvallis and my family for their company and moral support throughout my graduate studies at OSU.

CONTRIBUTION OF AUTHORS

Dr. Kerry McPhail ran some of the extensive 2D NMR experiments discussed in chapter five and consulted on the structural elucidation of phormidamide, described in the same chapter. Brian Arbogast, Jeff Morre and Lilo Barofsky ran all mass spectra.

Mary Roberts and Mirjam Musafija-Girt assisted in the culturing of the cyanobacteria for all the experiments described in this thesis. Dr. Tatsufumi Okino performed the neuroblastoma assay used in chapter five. Dr. Doug Goeger also conducted this assay for establishing the LC_{50} reported in chapter five.

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I wish to dedicate this thesis to my husband Nicu, who would have been very happy to see it completed and helped me enormously along the way.

BIOSYNTHESIS, PRODUCTION AND STRUCTURAL STUDIES OF SECONDARY METABOLITES IN CULTURED MARINE CYANOBACTERIA

CHAPTER ONE

GENERAL INTRODUCTION

HIGHLIGHTS OF BIOACTIVE MARINE NATURAL PRODUCTS

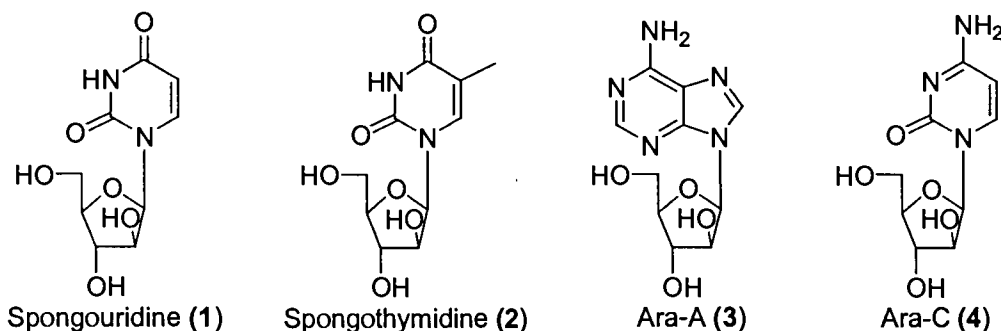
Humans have used natural products for treatment of various ailments since prehistoric times, but only in the past 100 years has scientific investigation been applied to the discovery, isolation and purification of individual active compounds for use as therapeutics. It has been estimated that approximately 45% of all current drugs are either a natural product, natural product derivative, or inspired by natural products chemistry, while 37% of all pharmaceutical sales are from natural product derived medicines (Frommann and Jas, 2002). In the particular field of cancer treatment, approximately 60% of the agents in clinical trials owe their origin to natural products (Cragg and Newman, 2000).

This intense drug discovery effort has been focused traditionally on terrestrial plants and microorganisms, with remarkable results, in particular in cancer chemotherapy (vinca alkaloids, taxol, bleomycin, camptothecin), pain control (morphine, codeine), or as cholinergics and anticholinergics (physostigmine, atropine, scopolamine). In the past 50 years, the marine habitat has also been recognized as a vast reservoir of biological and structural diversity,

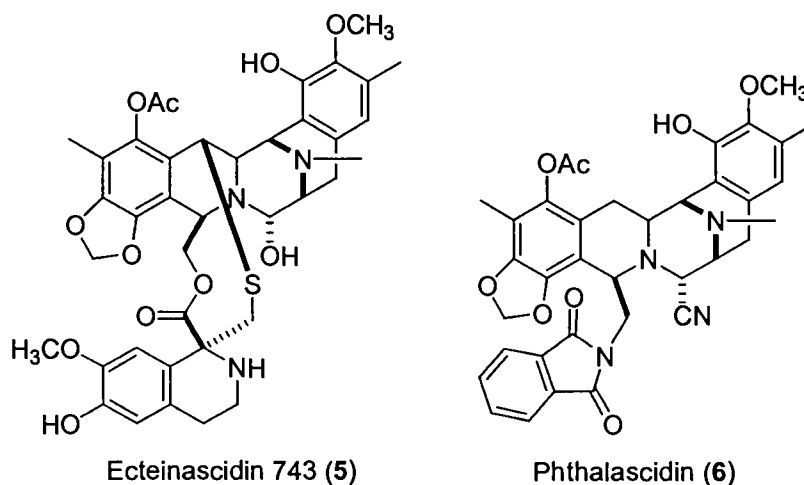
and thus serious attempts to tap the vast potential of marine organisms as sources of bioactive metabolites were started.

A number of promising compounds have been identified from marine sources that are already at advanced stages of clinical trials (most of them in the treatment of cancer) or have been selected as promising candidates for extended preclinical evaluation (Faulkner, 2000). Interestingly, the majority of marine natural products currently in clinical trials or under preclinical evaluation are produced by invertebrates such as sponges, tunicates, molluscs or bryozoans and not by algae. This greatly differs from the situation present in terrestrial natural products where plants by far exceed animals with regard to the production of bioactive metabolites (Proksch *et al.*, 2002).

The first steps toward obtaining therapeutic agents from the marine environment were performed in the 50's by Bergmann and Feeney through their discovery of the nucleosides spongouridine (1) and spongothymidine (2) from *Tethya crypta* (Bergmann, W. and Feeney, R. J., 1951). Subsequent development of synthetic analogues has provided the clinically relevant agents arabinosyl adenine (Ara-A, 3), an anti-viral (Lopez and Giner-Sorolla, 1977) and arabinosyl cytosine (Ara-C, 4), an anti-cancer agent for the treatment of acute myelocytic leukemia and non-Hodgkin's lymphoma (Bodey *et al.*, 1969).



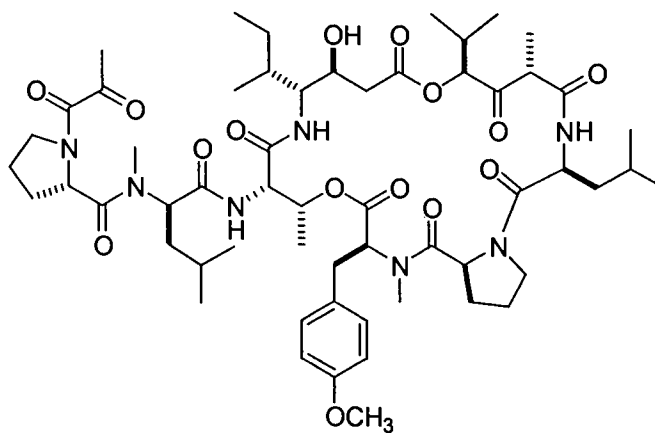
One of the most promising agents currently in clinical trials is the alkaloid ecteinascidin 743 (ET-743, 5) from the marine tunicate *Ecteinascidia turbinata* (Verschraegen and Glover, 2001). This drug has a broad-spectrum anti-tumor activity and is especially effective against solid tumors such as sarcomas and ovarian cancer (Valoti *et al.*, 1998). ET-743 has a unique mechanism of action by forming covalent guanine adducts at specific sites in the DNA minor groove and poisoning transcription-coupled nucleotide excision repair. It also causes inhibition of MDR1 gene transcription (Jin *et al.*, 2000) and thus induces apparent reversal of multi drug resistance, which makes it potentially very effective in combination with other chemotherapy drugs. Although total synthesis of ET-743 has been achieved, its structural complexity has prompted the design of less complicated analogues of equal potency and greater stability, such as phthalascidin (6, Martinez *et al.*, 1999; Martinez *et al.*, 2001).



Another family of anti-tumor compounds isolated from ascidians (tunicates) are the didemnins. The didemnins are a novel family of cyclic depsipeptides isolated from *Trididemnum solidum* that exhibit a remarkable array of antitumor, antiviral and immunosuppressive activities (Sakai *et al.*, 1996). The

anti-tumor activity is due to interference with protein synthesis (Ahuja *et al.*, 2000). Didemnin B received the most attention and proceeded to phase II clinical trials, but its development as a drug was recently cancelled due to hepatotoxic side effects. An analogue of didemnin B, aplidine (7, dehydrodidemnin B) isolated from the Mediterranean tunicate *Aplidium albicans*, could prove to be a favorable substitute for didemnin B. Aplidine (7) exhibits enhanced activity and less toxicity (Geldof *et al.*, 1999); it has completed phase I clinical trials and is scheduled for phase II trials (Proksch *et al.*, 2002).

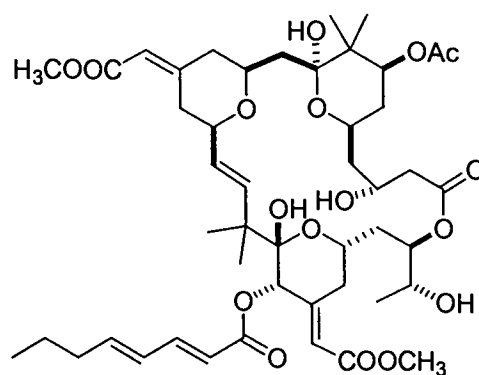
The bryostatins are a family of cytotoxic macrocyclic lactones isolated from the bryozoan *Bugula neritina* (Pettit *et al.*, 1982). They have exhibited exceptional activity against various cancers including non-Hodgkin's lymphoma, melanoma and renal carcinoma, with bryostatin 1 (8) currently in phase II clinical trials (Clamp and Jayson, 2002).



Aplidine (7)

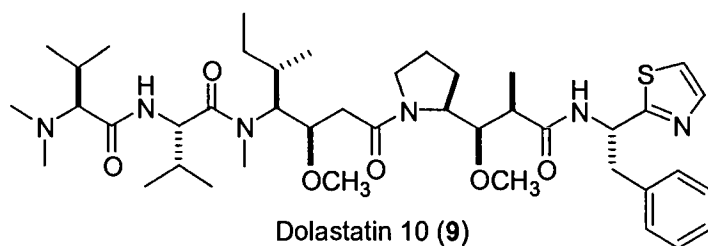
The mechanism of action is largely unknown, but they seem to act as inhibitors of the PKC family kinases by binding strongly to PKC isozymes without acting as tumor promoters (Hale *et al.*, 2002). Recent evidence suggests a bacterial

origin for these macrocyclic lactones. The uncultivated proteobacterial symbiont "*Candidatus Endobugula sertula*" (Haygood and Davidson, 1997) is probably involved in the biosynthesis of the bryostatins, since laboratory colonies of *Bugula neritina* showed reduced numbers of the symbiont when treated with antibiotics concomitant with reduced bryostatin content (Davidson *et al.*, 2001).

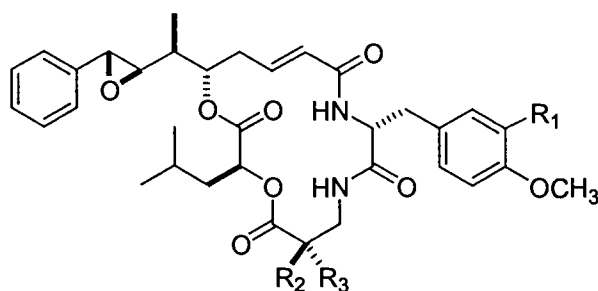


Bryostatin 1 (8)

The sea hare *Dolabella auricularia*, an herbivorous mollusc from the Indian Ocean, has been the source of more than 20 cytotoxic peptides, collectively referred to as the dolastatins, from which dolastatin 10 (9, Pettit *et al.*, 1987) is the most active. Originally isolated in only minute amounts from *D. auricularia* (approximately 1 mg of 9 per 100 kg of mollusc), subsequent re-isolation of 9 from the marine cyanobacterium *Symploca* sp. has unequivocally demonstrated the true origin of this bioactive metabolite (Luesch *et al.*, 2001). Dolastatin 10 passed phase I clinical trials, but performed poorly in several phase II clinical studies against advanced renal carcinoma, indolent lymphoma and chronic lymphocytic leukemia.



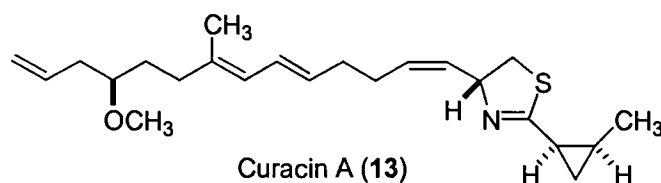
Cyanobacteria, the most ancient of the microalgae, have also proved to be rich producers of novel and bioactive secondary metabolites (Gerwick *et al.*, 2001). The bioactive peptide cryptophycin A (10) was originally isolated from a cyanobacterium *Nostoc* sp. for its antifungal activity, but is now well known for its



Cryptophycin A (10)	R ₁ = Cl	R ₂ = CH ₃	R ₃ = H
Arenastatin A (11)	R ₁ = H	R ₂ = H	R ₃ = H
Cryptophycin 52 (12)	R ₁ = Cl	R ₂ = CH ₃	R ₃ = CH ₃

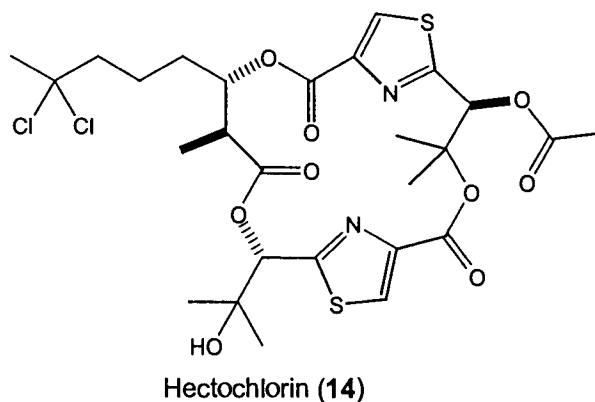
potential application in the treatment of cancer (Smith *et al.*, 1994). Interestingly, the cytotoxin arenastatin A (11, Kobayashi *et al.*, 1994) isolated from the Okinawan sponge *Dysidea arenaria* was found to be structurally identical to cryptophycin 24. Several synthetic analogues have been prepared, including cryptophycin 52 (12, Panda *et al.*, 1998), currently in phase II trials against a variety of solid tumors. The cryptophycins display anticancer activity by their ability to destabilize microtubules through binding at or near the vinca-alkaloid binding site inducing phosphorylation of bcl-2, leading to apoptosis.

In our laboratory, Curacin A (**13**), a novel thiazole-containing lipid, isolated from the marine cyanobacterium *Lyngbya majuscula*, was initially discovered through its toxicity to brine shrimp (Gerwick *et al.*, 1994).



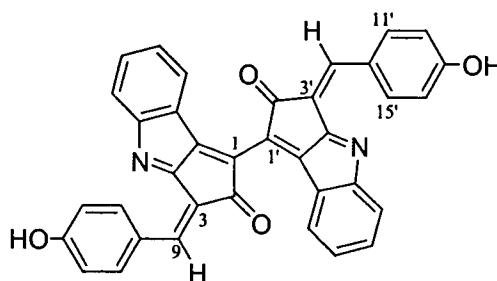
Curacin A and its structural analogues have shown exceptional toxicity to cancer cells in preclinical studies by inhibiting microtubule polymerization through interaction at the colchicine binding site (Blokhin *et al.*, 1995; Verdier-Pinard *et al.*, 1999). Recent efforts have mainly focused on development of synthetic curacins with greater stability and water solubility (Wipf *et al.*, 2002).

A different *Lyngbya majuscula* strain initially collected from Hector Bay, Jamaica and propagated in culture at OSU yielded hectochlorin (**14**, Marquez *et al.*, 2002). Hectochlorin is a potent antifungal agent which causes hyperpolymerization of actin and had greatest potency in the colon, melanoma, ovarian and renal panels in the *in vitro* 60-cell line screen at the National Cancer Institute (overall average GI₅₀ = 5.1 μM).



Interestingly, hectochlorin was also isolated from a red *L. majuscula* strain collected from Bocas del Toro, Panama (Marquez *et al.*, 2002).

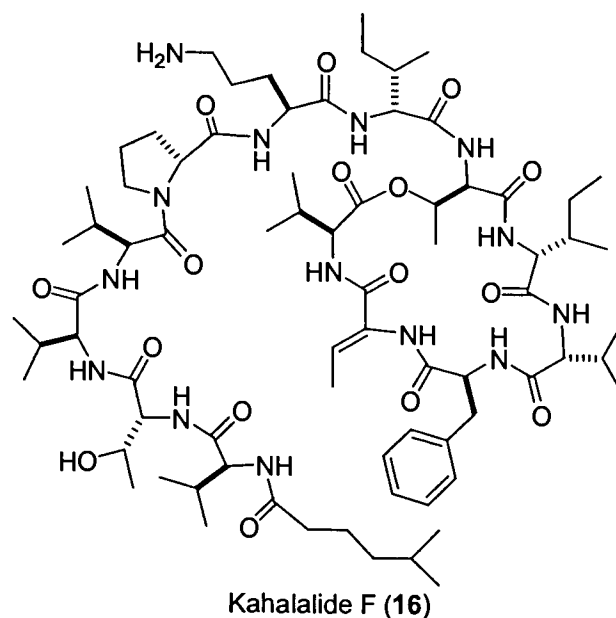
Another promising compound isolated in our laboratory, scytonemin (15, Proteau *et al.*, 1993), is a UV-protective pigment found in marine and freshwater cyanobacteria.



Scytonemin (15)

(15) is the first described small molecule inhibitor of human polo-like kinase, a serine/threonine kinase that plays an integral role in regulating the G(2)/M transition in the cell cycle (Stevenson *et al.*, 2002). Scytonemin has potential in treating both inflammatory as well as abnormal proliferative diseases.

The kahalalides are a collection of cytotoxic peptides initially isolated from a Hawaiian mollusc, *Elysia rufescens* (Hamann and Scheuer, 1993). However, during collection of this herbivorous mollusc for chemical evaluation, *E. rufescens* was observed feeding on the green alga *Bryopsis* sp. Following collection of the alga and purification of its active constituents, the *Bryopsis* sp. yielded identical chemistry to that isolated from *E. rufescens*, including kahalalide F (Hamann *et al.*, 1996). Currently undergoing preclinical evaluation, kahalalide F (16) shows selectivity against various solid tumor cell lines (Nuijen *et al.*, 2001); phase I trials are expected to be initiated shortly.

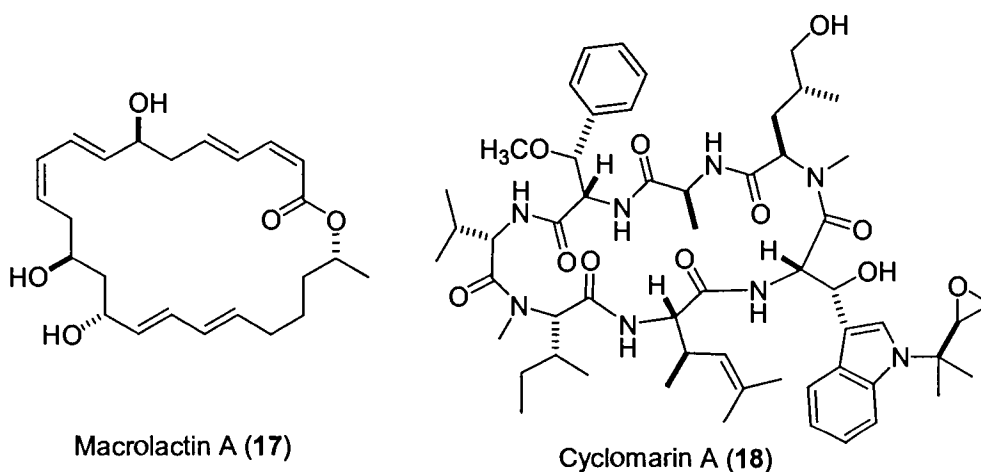


Clearly, the stronghold of marine natural products with clinical applications is in the area of cancer chemotherapy, possibly due to more intensive screening through NCI's 60-cell line assay. However, there are promising compounds that might lead to new drugs in other important indications such as inflammation or pain.

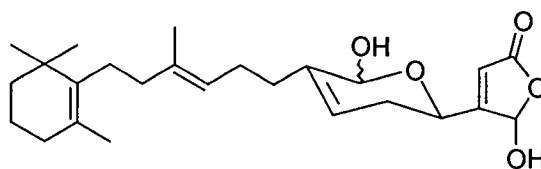
A success story is the pain-killing marine natural product ω -conotoxin MVIIA (SNX-111), which has successfully completed phase III clinical trials for two therapeutic applications: to alleviate pain associated with malignant diseases and as an analgesic for nonmalignant neuropathic pain (Olivera, 2000). The compound is currently on the market in the US for pain treatment via the intrathecal route, under the generic name ziconotide. Ziconotide is a 25-amino-acid linear peptide found along with other similar peptides in the venom of the predatory Indo-Pacific marine mollusc *Conus magus*, a fish-hunting mollusc that uses its venom to paralyze prey. The remarkable analgesic activity of ziconotide (the compound proved to be 1,000 times more active than morphine in animal

models of nociceptive pain) is due to the blockage of N-type, neuron-specific calcium channels that are believed to regulate synaptic transmission in nociceptive neurons.

Marine bacteria are also producers of potential medicines, such as the macrolactins, isolated from a liquid culture of an unidentified Gram-positive bacterium collected from deep-sea sediment. The major metabolite macrolactin A (17) displays both antiviral and antineoplastic *in vitro* activities (Gustafson *et al.*, 1989). More recently, cyclomarin A (18, Renner *et al.*, 1999) was reported as a novel anti-inflammatory cyclic peptide from a marine *Streptomyces* sp. Cyclomarin acts as anti-inflammatory agent both when applied to skin or taken orally and was licensed for development to Phytera Inc.

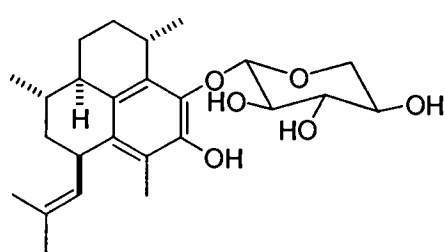


Another anti-inflammatory marine compound is manoalide (19, DeSilva and Scheuer, 1980), isolated from the sponge *Luffariella variabilis*, which acts as a phospholipase A₂ inhibitor and has become a standard drug in inflammation research.

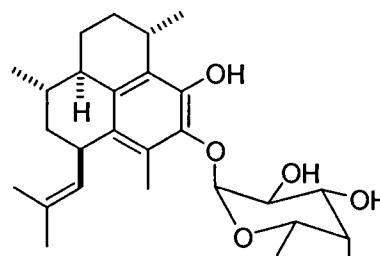


Manoalide (19)

Other phospholipase A₂ inhibitors currently in use, although in another field of application not directly concerned with drug discovery, are the diterpene glycosides pseudopterosins (20, 21) from the Caribbean gorgonian *Pseudopteroorgia elisabethae* (Look *et al.*, 1986). Extracts of *P. elisabethae* show anti-inflammatory activity and are currently used as an ingredient for cosmetic skin care products. A simple derivative of pseudopterosin E is currently in phase I clinical trials as a potential new anti-inflammatory agent (Proksch *et al.*, 2002).



Pseudopterosin A (20)



Pseudopterosin E (21)

FUNCTIONS OF BIOACTIVE METABOLITES IN THE PRODUCING ORGANISMS

The function of secondary metabolites has been a subject of debate since the first isolation and characterization of these often times structurally complex chemical compounds. By definition, secondary metabolites are naturally produced compounds that are not essential for the survival of the producing organism, as opposed to primary metabolites. Bioassays have revealed a wide range of biological activities, such as cytotoxicity, ichthyotoxicity, antimicrobial and antifeedant activity, antifoulant and anti-cancer activities. However, some of these activities are manifested against organisms that do not represent logical targets, since they would never be encountered by the producing organism in their natural habitat (for instance, anti-cancer chemicals).

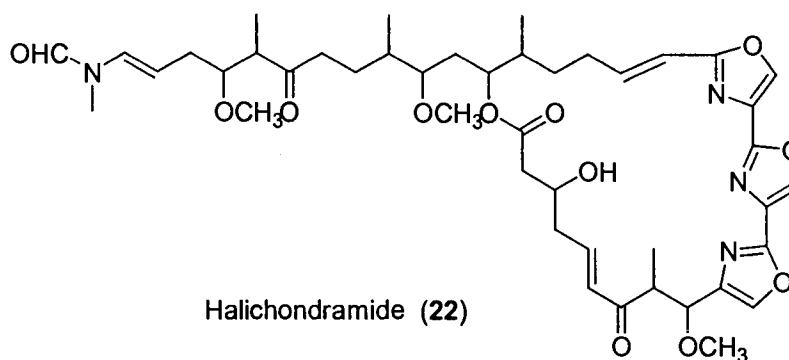
This apparent paradox has generated two conflicting views on the origin and rationale of production of secondary metabolites. The first hypothesis contends that secondary metabolites are redundant, 'waste' products that no longer play a role in enhancing the fitness of an organism (Davies, 1990). The second view postulates that they have definite biological activities that increase the fitness and survival of the producer, or at least have done so at a recent point in evolution (Stone and Williams, 1992).

Common sense suggests that the energy expended for producing and processing this dazzling variety of chemical structures and motifs is too great for organisms to keep useless pathways active. Chemical compound biosyntheses are costly because they utilize resources that could have been allocated to growth and reproduction (Herms and Mattson, 1992). Thus, secondary metabolism must serve a purpose in the general economy of the producing organism. The most obvious use for these compounds consists in acting as defense mechanisms against predators or competing species. This fundamental question has prompted intense

research in the field of chemical ecology, and nowadays the second hypothesis is widely accepted.

These considerations apply in the field of marine chemical ecology as well. It has been repeatedly shown that chemical defense through accumulation of toxic or distasteful natural products is an effective strategy to fight off potential predators, such as fish and sea urchins or to suppress competitors, pathogens and fouling (Proksch, 1999). It follows that the wealth of bioactive metabolites isolated from soft-bodied, sessile or slow-moving marine invertebrates that usually lack morphological defense structures such as spines or a protective shell reflects the ecological importance of these constituents for the respective invertebrates. Furthermore, it has been shown that frequently the source of the invertebrates' chemical defenses can be traced to their diet, and thus the bioactive compounds are collected and sequestered from their prey rather than produced 'de novo' (Proksch, 1994).

One well-studied example is represented by the nudibranch mollusc *Hexabranhus sanguineus* - the 'Spanish dancer'. *H. sanguineus* is protected from predation by reef fish or hermit crabs due to macrocyclic oxazole alkaloids, such as halichondramide (22), which act as feeding deterrents (Pawlik *et al.*, 1988). These anti-feedant alkaloids are concentrated in the most vulnerable parts of the mollusc, the dorsal mantle and the egg ribbons and their origin was determined as dietary and traced to sponges of the genus *Halichondria*.



Another such example is the opisthobranch sea hare *Stylocheilus longicauda*, a reef grazer that feeds specifically on *Lyngbya majuscula* and also sequesters cyanobacterial metabolites, conceivably for defense.

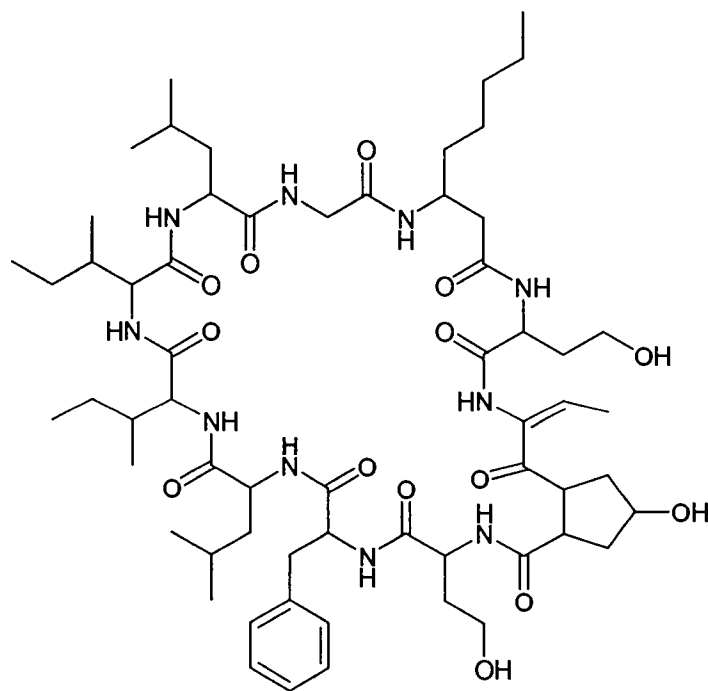
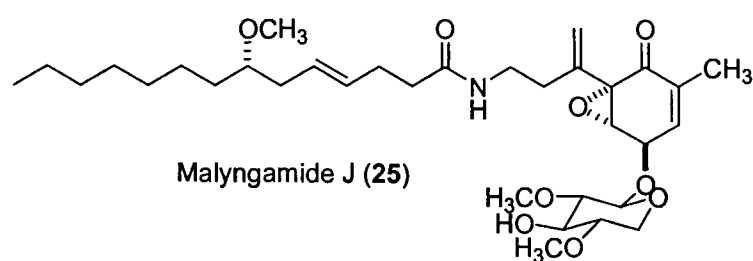
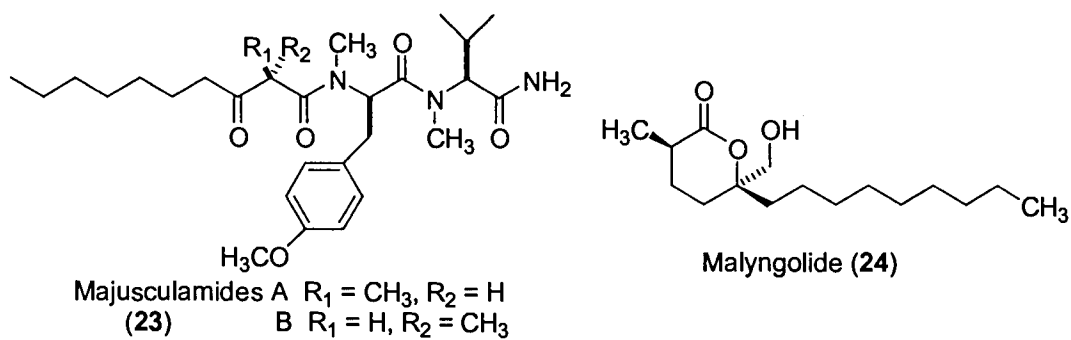
Marine algae are another class of organisms rich in secondary metabolites proven to serve multiple roles through field and laboratory bioassays: defense against consumers, antifouling, antimicrobial and allelopathic effects (Hay, 1996). The majority of natural products isolated from marine macroalgae are terpenoids, polyketides or aromatic compounds (Paul, 1992). Nitrogenous compounds, while frequently encountered in marine invertebrates, are very rare in macroalgae. The contents of bioactive metabolites in macrophytes are known to vary widely, depending on geographic location, environmental conditions and the induced or basal state of the alga (Hay, 1996). Studies aimed at investigating the presence of inducible production of metabolites in a manner similar to terrestrial plants have been very scarce.

However, one study reported that grazing by the amphipod *Ampithoe longimana* induced an increased accumulation of dictyol-type terpenes in the marine brown alga *Dictyota menstrualis* (Cronin and Hay, 1996). These terpenes also reduced the palatability of *D. menstrualis* to the amphipod. Another experiment showed that water-borne cues from actively feeding herbivorous gastropods can serve as external signals to induce production of defense chemicals

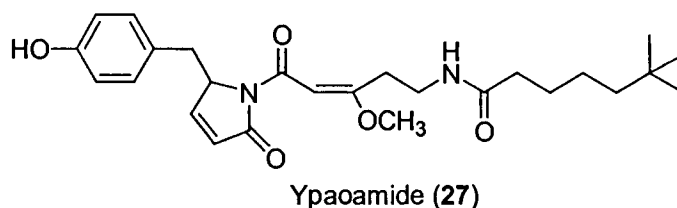
(phlorotannins) in unharmed individuals of the brown seaweed, knotted wrack (*Ascophyllum nodosum*). Furthermore, the increased levels of defense chemicals deterred further feeding by periwinkles (*Littorina obtusata*) (Toth and Pavia, 2000).

Marine cyanobacteria are known as very rich producers of toxic and bioactive secondary metabolites with practical applications, while the biological and ecological roles of these metabolites are relatively under-investigated. Most of the studies on the ecological significance of cyanobacterial metabolites were performed in the past ten years, after recognizing the importance of cyanobacteria as producer organisms for many compounds that were initially isolated from other sources.

It is relatively well-established that toxic secondary metabolites in marine cyanobacteria have chemical defense roles, through studies on their activity as anti-feedants. For example, a recent report found that the organic extracts of either *Lyngbya majuscula*, or mixed assemblages of *Lyngbya* sp., *Schizotrix calcicola* and *Microcoleus* sp. all deterred feeding by parrotfish (*Scarus schlegeli*), but to varied extent. Further chemical characterization of the crude extracts brought forth the existence of distinct 'chemotypes', with characteristic secondary metabolite composition and proportion, even between monoculture samples of the same species (Nagle and Paul, 1999). Purified secondary metabolites isolated from *L. majuscula* (Thacker *et al.*, 1997), *H. enteromorphoides* (Pennings *et al.*, 1997) and mixed assemblages (Nagle and Paul, 1998) were tested in similar experiments and proved to be effective feeding deterrents to juvenile rabbitfish and parrotfish. Among the most active were: majusculamides (23), malyngolide (24), malingamides (25) and laxaphycin (26).



Ypaoamide (27) is a recently isolated broadly acting feeding deterrent produced by a bloom of *L. majuscula* with *S. calcicola* (Nagle and Paul, 1998). Ypaoamide (27) is a lipopeptide and deters feeding by herbivorous reef fishes as well as sea urchins.



Further studies on the biology and ecology of marine secondary metabolites are necessary, especially in many cases where the toxins produced by marine cyanobacteria and dinoflagellates have negative effects on human health and the fishing industry through increasingly frequent harmful algal blooms. Knowledge accumulated in this field could serve for the prediction, monitoring and/or prevention of future outbreaks.

BIOSYNTHETIC STUDIES OF MARINE CYANOBACTERIAL METABOLITES

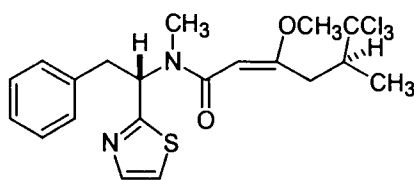
The field of biosynthetic investigations has been tremendously expanding as a result of the isolation of increasing numbers of structurally novel and biomedically promising natural products from marine sources. The current understanding of biosynthetic pathways in marine organisms is lagging behind the level attained in terrestrial organisms due to the relatively recent development of marine natural product chemistry relative to its terrestrial counterpart. At the same time, biosynthetic studies in marine organisms pose difficult experimental problems, since labeled precursor feeding experiments necessitate the development of novel methods, adequate for use in an aqueous environment.

Bioactive natural products generally face two major hurdles on the road to development into a commercial product (therapeutic or agrichemical). On one hand, generation of a sufficient supply of the compound for the initial characterization, activity and safety studies, as well as for commercialization is often problematic for natural products, typically isolated in minute quantities from the producing organism. On the other hand, bioactive natural products are rarely used in their natural form; chemical derivatization is needed most times to improve their specificity and activity levels or to reduce toxicity. This second consideration is also hindered by a lack of sufficient material for the optimization studies.

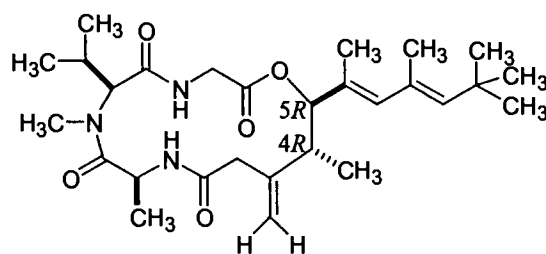
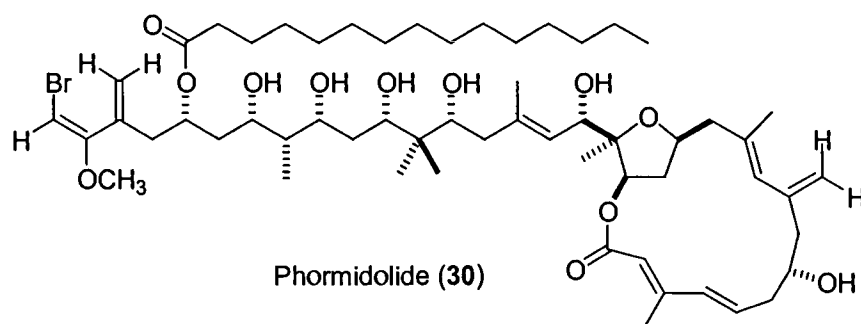
In many cases, synthetic approaches are not feasible or economically viable due to the complex structures of natural products leading to numerous steps with low yields. Biological methods, such as aquaculture, cell culture or *in vitro* enzymatic transformation of more abundant precursors have been successfully employed in some of these cases, thus permitting the commercial development of the natural product in question.

Identification of the biosynthetic building blocks and intermediates can suggest compounds that might increase the production of the natural product when supplied in excess in the diet or the nutrient broth of an organism or culture (Kerr, 2000). Understanding the details of the biosynthesis also facilitates identification of the enzymes responsible for the individual steps and provides assays for their isolation or information for designing probes for the cloning of the entire biosynthetic pathway. Then, through recombinant DNA technologies, the pathway could be expressed in a heterologous host and virtually unlimited quantities of natural product become accessible, as well as opportunities for combinatorial biosynthesis to obtain analogs - 'unnatural' natural products (Khosla, 2000).

Marine cyanobacteria produce a vast array of bioactive and structurally unique secondary metabolites, as shown in the first part of this introductory chapter. The most predominant theme in marine cyanobacterial biosynthesis is the combination and integration of peptide and polyketide biosynthetic pathways, giving rise to the 'mixed NRPS/PKS' metabolites. This can occur in 2 ways: 1) the linking of polyketides to amino acids through amide or ester linkages, and 2) the utilization of amino acids as starter units for polyketide extension. The polyketides found in ester or amide linkage with amino acids are generally of small size (diketides to tetraketides), while ketide extended amino acids vary from a single acetate unit (e.g. barbamide, **28**) to as many as 15 acetate or propionate units (e.g. scytophycin B, **29**) (Gerwick *et al.*, 2001).

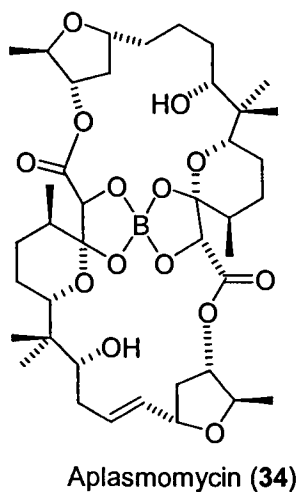
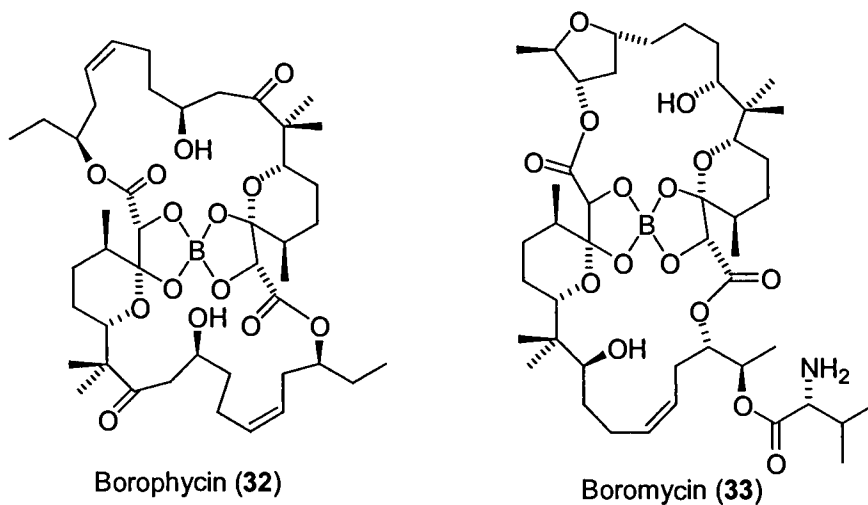


Barbamide (**28**)



As a general observation, the pendant methyl branches present on the carbon backbone of marine (and freshwater) cyanobacterial polyketides are derived from S-adenosyl methionine or, less frequently, from C2 of acetate, as shown in the case of virginiamycin, oncorhincolide and jamaicamides (Kingston *et al.*, 1993; Needham *et al.*, 1992; Nogle, 2002). Interestingly, this trend applies even to starter unit biogenesis in the case of borophycin (32), a cytotoxic boron-containing polyketide from *Nostoc linckia*. Borophycin biosynthesis proceeds from a three carbon starter unit derived by methylation of acetate by methionine instead of the expected propionate (Hemscheidt *et al.*, 1994). Furthermore, studies on the biosynthesis of two closely related boron-containing antibiotics from *Streptomyces*, boromycin (33) from a terrestrial strain and aplasmomycin (34) from a marine species indicate that the analog three carbon starter unit is derived from phosphoglycerate or phosphoenolpyruvate while the pendant methyl groups originate from methionine as in the cyanobacterial metabolite (Moore, 1999; Kerr, 2000). Thus, extreme care

should be taken in the attempt to extrapolate biosynthetic pathway characteristics between genera, since biogenesis can differ significantly even in the case of closely related structures.



Despite the number of structurally interesting and bioactive natural products reported from marine cyanobacteria to date, biosynthetic investigations have been scarce, due to the scarcity of cyanobacterial strains established in culture.

GENERAL THESIS CONTENTS

The research presented within this PhD. thesis focuses on the natural products chemistry, bioactivity, secondary metabolism and its regulation in marine cyanobacteria, especially that of the genus *Phormidium*. These organisms have been collected from diverse locations and successfully cultured in our laboratory, making projects described in the subsequent chapters possible.

The first chapter consists of a general introduction to marine natural products, a discussion of the roles they might play in the producing organisms and the biosynthetic origin and metabolism of the rich diversity of chemical structures found in marine cyanobacteria. Next, chapter two details the exploration of the effects of several putative 'elicitor' treatments on five species of cultured marine cyanobacteria. The results entail biological, physiological and chemical parameters such as growth, biomass production, chemical composition and bioactivity of the organic extracts of these microalgae.

The third chapter describes the biosynthetic investigations of phormidolide, a brine shrimp toxic macrolide isolated from a cultured *Phormidium* sp. collected in 1995 from Indonesia. Stable isotope feeding experiments were utilized in this work to gain insight into the biosynthetic building blocks involved in this complex metabolite's assembly. The fourth chapter discusses the effects of altered culture conditions on phormidolide production by this cyanobacterium, which give insight into the possible mechanisms and enzymes at work in the process of biological halogenation in this organism.

Chapter five then describes the challenging structure determination of phormidamide, a cytotoxic brominated cyclic peptide isolated from the same cultured *Phormidium* sp. This complex molecule required detailed analyses of NMR spectroscopy and mass spectral data and posed difficult problems in its

structure characterization due to the high level of unsaturation, halogenation and other derivatization of the component amino acids.

The final chapter, six, concludes this thesis with a brief summary of the projects described within the text, as well as general thoughts on the future direction of the pursuit of the marine environment as a viable source for novel chemistry of medicinal value.

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CHAPTER TWO

EFFECTS OF PUTATIVE "ELICITOR" TREATMENTS ON THE GROWTH
AND METABOLITE PRODUCTION IN CULTURED MARINE
CYANOBACTERIA

ABSTRACT

Marine cyanobacteria are extremely rich in structurally diverse secondary metabolites possessing unique biological and pharmacological properties. Nevertheless, a disparity between the chemical diversity and biological activity of in situ collected and laboratory-cultured algae was observed in empirical studies in our laboratory. Based on the phenomenon of elicitation in bacteria and plants, we designed a set of experiments to explore the possible elicitation effects of an array of 19 compounds on five species of cultured marine cyanobacteria. The cultures were obtained from collections of cyanobacteria that were present in nature only in small biomass. The elicitor treated cultures were extracted and subjected to chemical and biological evaluation by thin layer chromatography, gradient high performance liquid chromatography, antimicrobial bioassay and brine shrimp toxicity assay. The growth, biomass production and the ratio of the components of the extract were affected by some of the elicitors in most of the cyanobacterial species tested. However, production of a novel secondary metabolite or a significant change in the bioactivity of the extracts was not observed.

INTRODUCTION

The phenomenon of elicitation of production of secondary metabolites has received increasing attention over the last 10 years due to the practical applications for enhancement of the yield of economically valuable compounds (Radman *et al.*, 2003). These studies, combined with the investigation and elucidation of the molecular mechanisms evolved for elicitation, have provided further insight into the more general questions of biological regulation (Hahn, 1996; Ebel and Mithofer, 1998) and the functions that secondary metabolites may possess in the producing organism.

Pioneering studies on elicitation were done in the 1970's in plants in relation to their defense mechanisms against microbial attack. Plant defenses include synthesis of secondary metabolites as pathogenic growth inhibitors (Darvill and Albersheim, 1984), physiological changes, such as deposition of lignin for strengthening the cell wall (Kogel *et al.*, 1988), production of lytic enzymes such as chitinase and glucanase (Ham *et al.*, 1991; Koga *et al.*, 1992), production of reactive oxygen species at the site of infection (Levine *et al.*, 1994) and apoptosis and necrosis in the vicinity of the attack (Ricci *et al.*, 1989). These chemical and physiological changes are collectively known as the hypersensitive response. The hypersensitive response is triggered by small molecules called elicitors which are usually produced by plant pathogens.

Since then, a multitude of study systems have been investigated, from soybean (*Glycine max*) (Keen, 1975) to tomato (*Lycopersicon esculentum*) (Picard *et al.*, 2000; Ebel and Mithofer, 1998), tobacco (*Nicotiana tabacum*) (Ebel and Mithofer, 1998), *Catharanthus roseus* (Namdeo, 2002; Rijhwani and Shanks, 1998), rice (*Oryza sativa*) (Koga *et al.*, 1998), and recently *Taxus brevifolia*, the yew tree, for enhancement of taxol production (Hezari *et al.*, 1995; Ketchum *et al.*, 1999; Wu *et*

al., 2001). The main categories of compounds found to act as elicitors include carbohydrates (alginate, pectin, chitin, chitosan, glucuronate), proteins (cellulase, elicitors) and lipids (lipopolysaccharides and volatile fatty acids such as methyl jasmonate) (Radman *et al.*, 2003).

An extensive study on the effect of elicitors on plant secondary metabolism was recently published, showing that chemical elicitors selectively and reproducibly induced the production of bioactive metabolites and dramatically increased the hit rate in bioassays. The roots of 588 species of hydroponically grown plants were exposed to acetate, chitosan, methyl salicylate or methyl jasmonate and the result was a doubling of the number of extracts active *in vitro* against bacteria, fungi or cancer. For example, out of 119 species active against at least one cancer cell line, 64% (76) were active only after elicitation, leaving only 43 species active in the nonelicited state. As an interesting corollary, 11 species were active only in the nonelicited treatment, while the elicited samples were inactive and 2 had activity against a different cancer cell line after elicitation than initially (Poulev *et al.*, 2003).

Fungi have also been investigated and carbohydrates have been found to be the main class of active elicitors. For example, mannuronate and guluronate oligosaccharides obtained from hydrolysis of alginate induce increases in the penicillin G yield of *Penicillium chrysogenum* (Ariyo *et al.*, 1997).

The effect of elicitors on bacteria is largely unknown. One report indicates that depolymerized alginate promotes cell growth upon addition to *Bifidobacteria* cultures. This is consistent with an elicitation process since *Bifidobacteria* do not use alginates as a carbon source for growth (Akiyama *et al.*, 1992). However, the phenomenon of quorum sensing can be seen as a 'self-elicitation' process, since it regulates production of secondary metabolites in the later (post-exponential) growth stages of the bacterial culture. The first studies were done on two

luminous marine bacteria, *Vibrio fischeri* and *Vibrio harveyi* (Nealson and Hastings, 1979). Quorum sensing has been studied intensely in the past 10 years, and was shown to regulate a vast array of physiological activities: symbiosis, virulence, competence, conjugation, motility, sporulation, antibiotic production, and biofilm formation. In general, Gram-positive bacteria use processed oligo-peptides as elicitors, while in Gram-negative bacteria, acylated homoserine lactones perform the role of chemical communication. (Miller and Bassler, 2001).

One prime example of an elicitation study in cyanobacteria is the work on tolytoxin production by *Scytonema ocellatum* (Patterson and Bolis, 1997). Tolytoxin is highly toxic to fungi and mammalian cells at nanomolar concentrations by disrupting microfilament organization (Patterson and Carmeli, 1992). The toxin production was increased after treatment with fungal cell walls, chitin and chitin oligomers, while biomass production was slightly inhibited. This finding provided support for a physiological role for tolytoxin as a defense chemical against fungal invasion.

The working hypothesis for this study was that many, if not all, species of marine cyanobacteria possess the genetic capacity to produce unique secondary metabolites, but do not express them in culture because they may lack the conditions or the chemical signals necessary to activate the respective biosynthetic pathways.

RESULTS AND DISCUSSION

In order to investigate the possibility of inducing the production of new secondary metabolites in marine cyanobacteria by treatment with putative elicitor compounds, a panel of five marine cyanobacteria was selected from our culture collection. The 19 putative elicitor compounds were chosen from literature surveys of chemicals that manifested elicitor effects in plants or were involved in quorum sensing in bacteria, since cyanobacteria are photosynthetic prokaryotes.

Thus, jasmonic acid and a precursor fatty acid, linolenic acid, were used due to the involvement of methyl jasmonate in the hypersensitive response in plants. Chitin and laminarin were introduced as mimics of a fungal or insect attack, while phenazine methosulfate (PMS) and menadione sodium bisulfite (MSB) were known promoters of oxidative stress. N-acyl-homoserine lactones (HSL) with various acyl chain lengths and analogs represent quorum sensing molecules in bacteria. Ten different homoserine lactones and analogs were grouped into two treatments of five HSLs mixed in each, as depicted in Figure II.1. One treatment used a culture of the bacterium associated with the curacin-producing strain 19L of *Lyngbya majuscula*, which may possibly contribute to the high production of active compounds in this strain. Therefore, nine different putative elicitor treatments were tested on each of the five cyanobacteria species. Experiments were performed in triplicate, together with a control and a solvent control (also in triplicate).

The cyanobacterial species selected comprised two *Lyngbya* sp., two *Phormidium* sp. and one *Synechocystis* sp., all isolated from marine systems. Criteria for selection included: purity of the culture in our repository, relatively rapid growth rate to allow for sufficient biomass production in a reasonable time frame -

ca. 1 month, and either previously demonstrated production of bioactive secondary metabolites, or intriguing observations on that species' physiology.

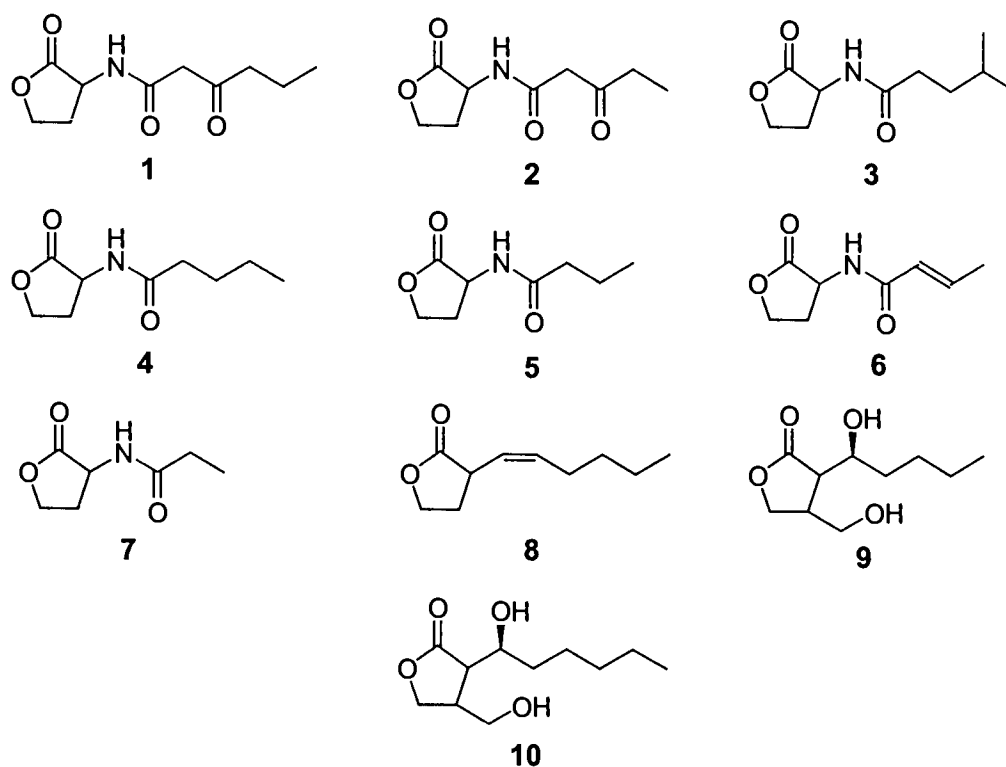


Figure II.1. Structures of the homoserine lactone analogs used for elicitor treatment mixtures HSL #1 (combined 1-5) and HSL #2 (combined 6-10).

Thus, the first species - 'Black Band' *Phormidium* (*Phormidium corallyticum*) is found on coral reefs, and is one of the members of a pathogenic microbial consortium called 'black band disease' of corals which causes the eventual death of the coral (Richardson and Kuta, 2003), potentially due to the cyanobacterium's physical presence or secondary metabolites. No bioactive secondary metabolites have been reported from this species to date.

The two strains of *Lyngbya majuscula* were both previously known to produce bioactive metabolites from studies in our laboratory. 'Curacin' *Lyngbya* 19L is a strain isolated in our laboratory from a field collection that produces the

tubulin inhibitor curacin A (11, Gerwick *et al.*, 1994) and the snail toxin barbamide (12, Orjala and Gerwick, 1996), shown in Figure II.2. The 'Jamaican' *Lyngbya* produces the actin polymerization promoter hectochlorin (13, Marquez *et al.*, 2002) and the jamaicamides A-C (14, Edwards *et al.*, in prep.) (Figure II.2).



Figure II.2. Structures of bioactive cyanobacterial metabolites present in the species under study.

The other *Phormidium* species has been investigated in our laboratory and two bioactive compounds have been isolated as a result: phormidolide (15) (figure II.2), a brine shrimp toxic macrolide (Williamson *et al.*, 2002), and a chlorophyll A derivative with Ras-Raf inhibitory activity (Singh *et al.*, in prep.).

Last, the *Synechocystis* selected was not known to produce any bioactive compounds, but was chosen based on the extensive body of knowledge accumulated on the metabolism and genetics of this cyanobacterial genus. Furthermore, one occurrence of active metabolites was reported by our laboratory from a collection of a marine *Synechocystis* species (Nagle and Gerwick, 1995).

The elicitors were introduced in cultures at day 4 post-inoculation, the biomass was harvested at day 30 and organic extracts were prepared from each replicate. Assessment of the results was done in 3 areas: growth characteristics, recorded by biomass wet and dry weights and organic extract weights, chemistry profiling by 2 D TLC and gradient PDA-detected reverse phase HPLC chromatography, and biological activity as measured by brine shrimp toxicity and antimicrobial assay of the extracts.

Effects on Growth Characteristics and Biomass Production

The effects of the elicitors on growth were quantified by recording the biomass wet weight (measured after harvest), the dry weight of the ground materials remaining after extraction, and the weight of the organic extract obtained for each replicate. As a general observation, the two *Phormidium* strains were the fastest-growing species, followed, in order, by the 'Jamaican' *Lyngbya*, the 'Curacin' *Lyngbya* and the *Synechocystis* strain. A good correlation was observed between the biomass weights and the extract weights, with the most reproducible biomass weight being the wet weight, possibly due to losses during extraction and concentration of salts from the sea water associated with the specimens after extraction and drying.

Results are depicted in Figure II.3, where biomass dry weights and organic extract weights are plotted for all the elicitor treatments and controls. The growth of 'Black Band' *Phormidium* and 'Curacin' *Lyngbya* was inhibited by menadioneSB

and phenazineMS treatments. 'Curacin' *Lyngbya* also showed growth inhibition by linolenic acid and chitin and an apparent stimulation by the HSL mixes, particularly mix I. The *Synechocystis* sp. showed an apparent growth stimulation by HSL mix I, but this did not achieve statistical significance. Interestingly, the growth stimulation by HSL treatment was not mirrored by an increase in organic extract weight in either of the two species in which it was present. The other two species under study did not demonstrate any effect of the treatments on the biomass wet or dry weight, partly due to large variations between the triplicate experiments.

The organic extract weights mirrored the behavior of the biomass weights in those cyanobacteria and treatments where an inhibitory effect was observed. Furthermore, in the 'Black Band' *Phormidium* the linolenic acid treatment caused a significant decrease in organic extract weight, while chitin and the HSL mix I had the opposite effect, significantly increasing extract weights. Opposing trends were observed in different cyanobacteria: for example, chitin caused a significant increase in extract weight in 'Phormidolide' *Phormidium* and a decrease in the 'Curacin' producer, while the HSL mixes had exactly opposite effects - they caused a decrease in extract weights in 'Phormidolide' *Phormidium* and an increase in 'Curacin' *Lyngbya*. The treatments had no significant effects on the growth and organic extract yield of the 'Jamaican' *Lyngbya*.

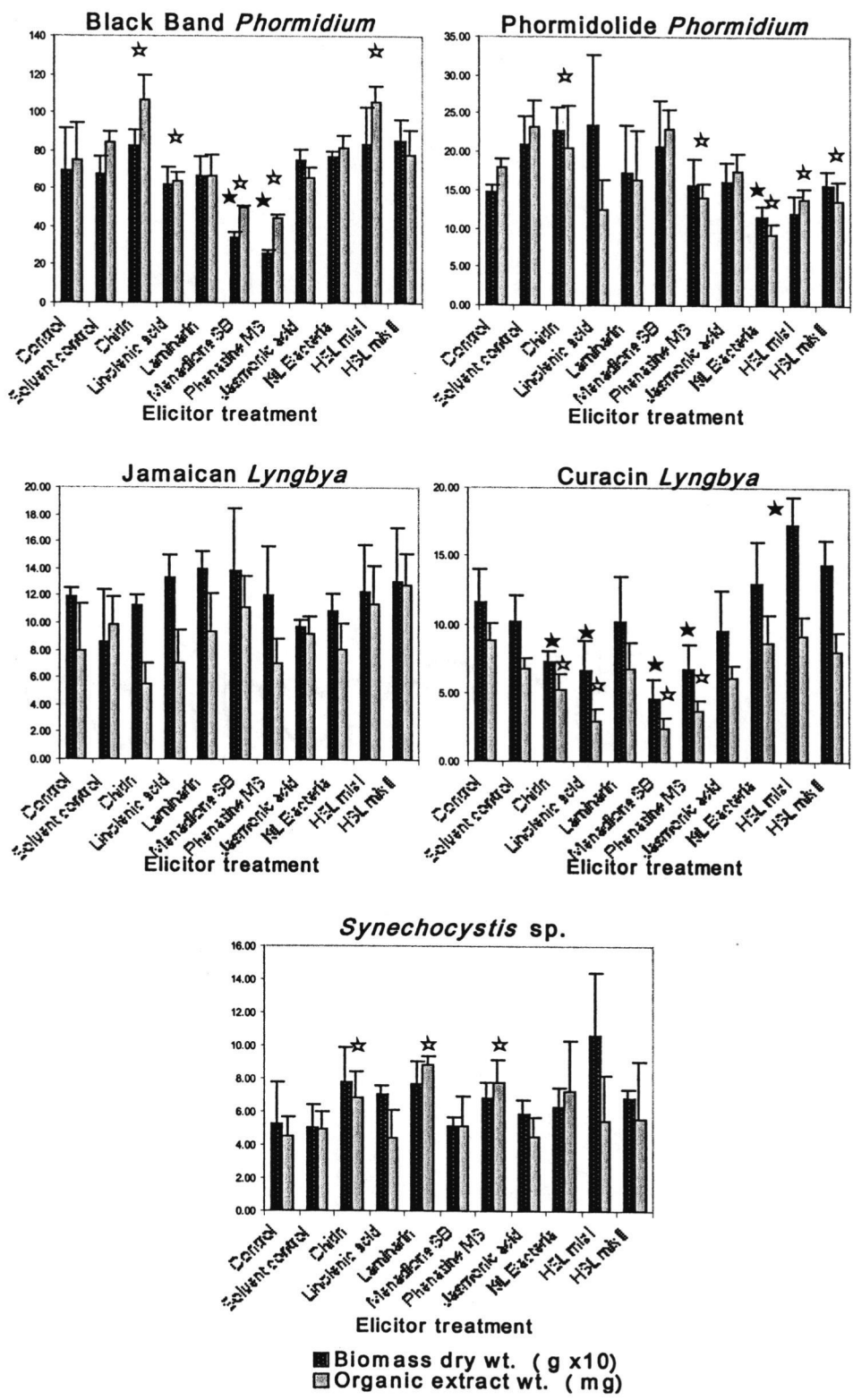


Figure II.3: Biomass and extract weights in the five cyanobacteria; asterisks indicate significant differences from controls (p < 0.05).

Effects on Chemical Composition

Following extraction, the effects of the different treatments on the chemical diversity of the organic extracts were assessed. Initial comparisons were done by 2 D TLC, with visualization of the compounds present under UV light and by acid spray charring. Further analysis was pursued by gradient PDA RP-HPLC in an attempt to quantify any changes in chemical composition compared with the controls, as well as confirm any results apparent from inspection of the 2 D TLC plates.

Variations were apparent in the pigment profiles of all 5 species, as well as in the lipids and minor UV-active compounds. Initially, one of the three replicates of each elicitor treatment was randomly picked for chemical diversity and biological activity analysis. The differences observed in the 2 D TLC profile of one particular treatment replicate were analyzed for significance by performing 2 D TLCs of the other two replicates, in parallel with several control replicates. This verification procedure allowed us to exclude most of the differences observed as due to inherent variation between samples, related possibly to biological variation and/or processing of samples up to the organic extract stage, rather than the effects of a particular elicitor treatment.

Typical 2 D TLCs and gradient PDA RP-HPLC profiles of the extracts from control cultures are shown in Figures II.4 and II.5, respectively. Sustainable results were obtained in only a few cases, and those were subjected to further analysis. In particular, in 'Black Band' *Phormidium* samples treated with menadioneSB and 19L Bacteria, an additional charring compound appeared on TLC and this occurrence was further investigated. Chemical characterization of this compound was attempted by isolation from preparative TLC followed by GC-MS analysis, but no significant peaks were obtained. Thus, the compound was either a) unstable to this isolation protocol, b) not soluble in hexanes or c) its molecular mass was higher

than the detection limit of the GC-MS (500 Da). The migration pattern on the TLC plate was more consistent with either the first or third hypothesis, since the R_f was 0.71 in a 50% hexanes/EtOAc solvent system. Another hurdle consisted in the very limited amount of sample obtained, since it represented only a very minor component of the organic extract.

In order to continue the study of the putative newly elicited compound from the 'Black Band' *Phormidium*, the menadioneSB and 19L bacteria treatments were scaled up, as well as a control, in four replicates (two the same size as previously - 0.5 L flasks, and two in 1.5 L flasks). The results proved that the same UV active charring spot appeared in both the treatments and the controls upon TLC analysis with high amounts of extract (300 μ g spotted), such that the difference was quantitative in nature (Figure II.6).

Furthermore, HPLC profiling of the extracts of 'Black Band' *Phormidium* exposed to different elicitors, as well as controls was performed. Upon inspection of the PDA-HPLC gradient profiles, three UV-active candidate peaks were identified. The extracts were injected repeatedly, and typical HPLC profiles are shown in Figure II.7.

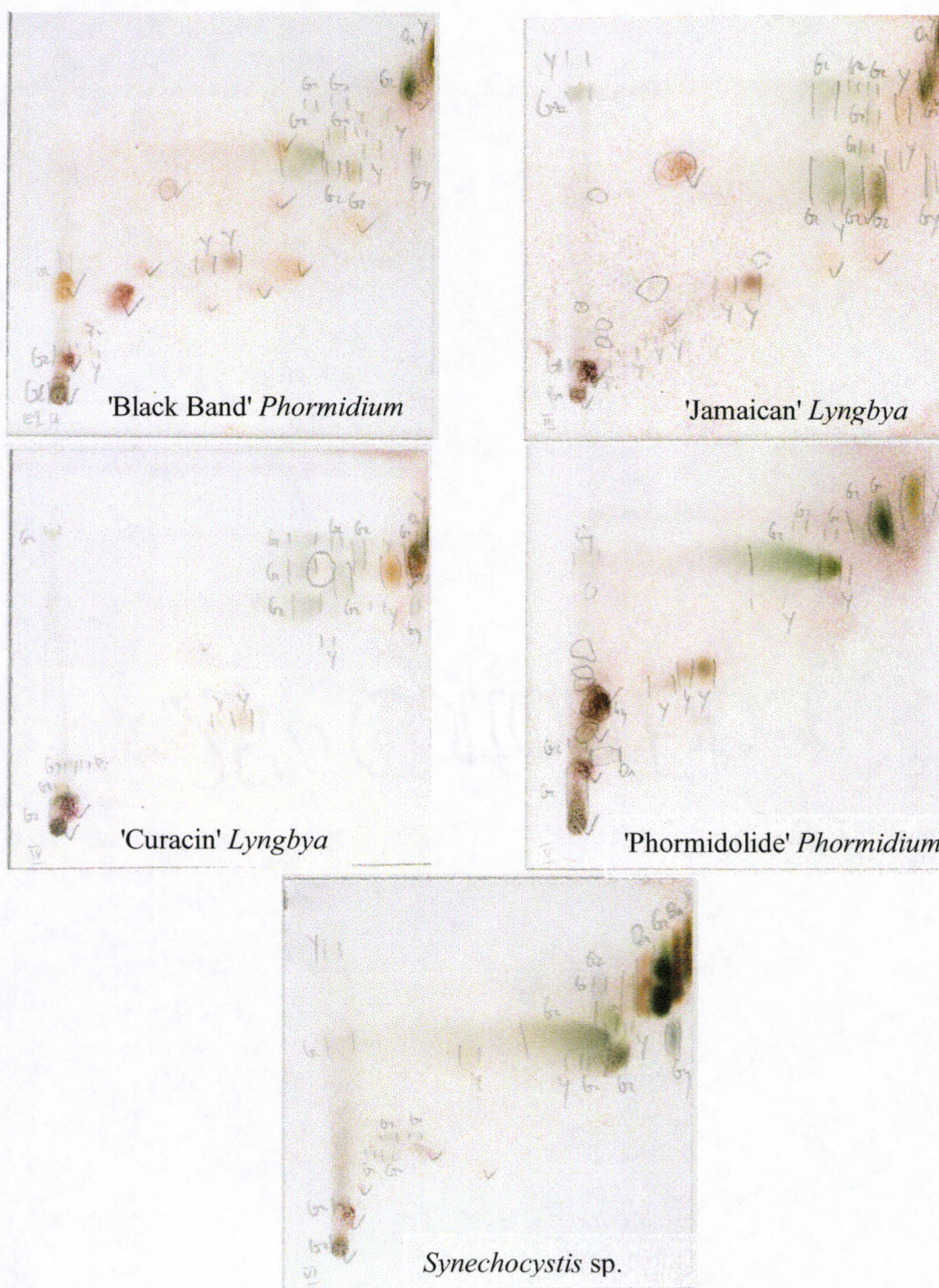


Figure II.4. 2 D TLC profiles of the five cyanobacterial control organic extracts. (Pigments are identified by vertical bars, colors by letter, UV-active spots are circled and charring compounds are represented by check marks).

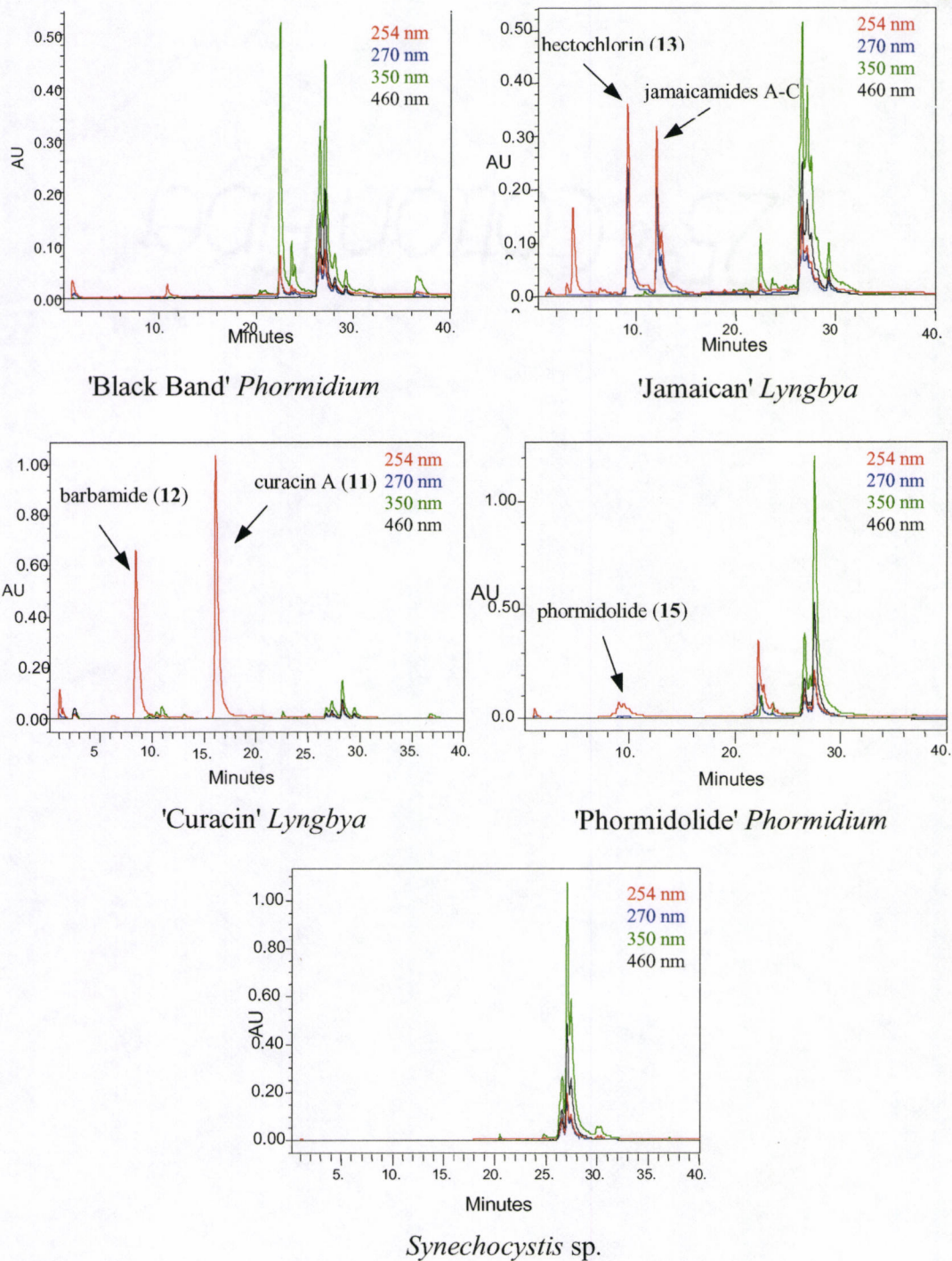


Figure II.5. PDA-HPLC profiles of organic extract controls of the five cyanobacteria.

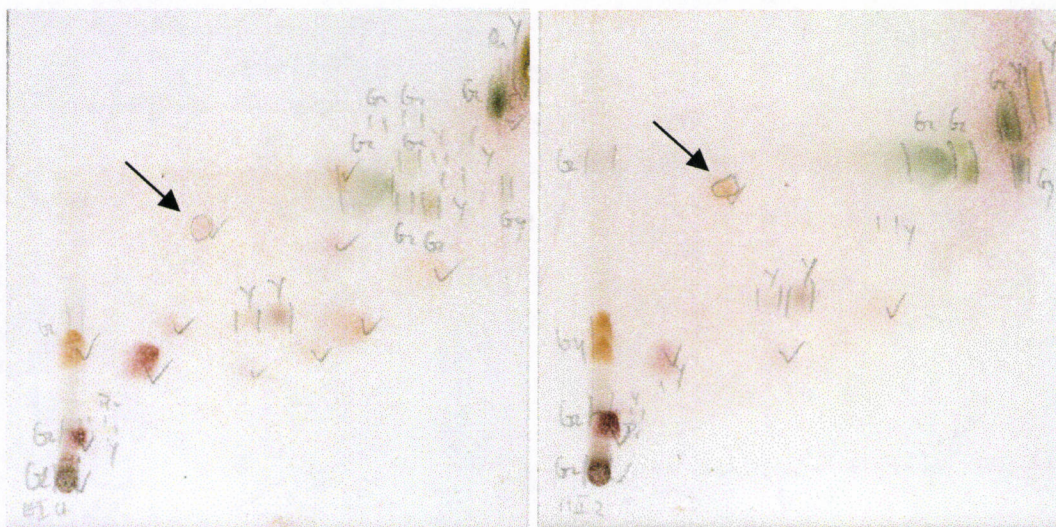


Figure II.6. 2 D TLCs of the Control and MenadioneSB-treated 'Black Band' *Phormidium* cultures (arrows identify compound upregulated in these experiments)

Of the three peaks, only the one with an elution time of 6.1 minutes (indicated by the arrow) exhibited the expected enhancement in the MSB treatment relative to control, as well as UV absorbance at 254 nm, which is necessary for UV visualization on the TLC plate.

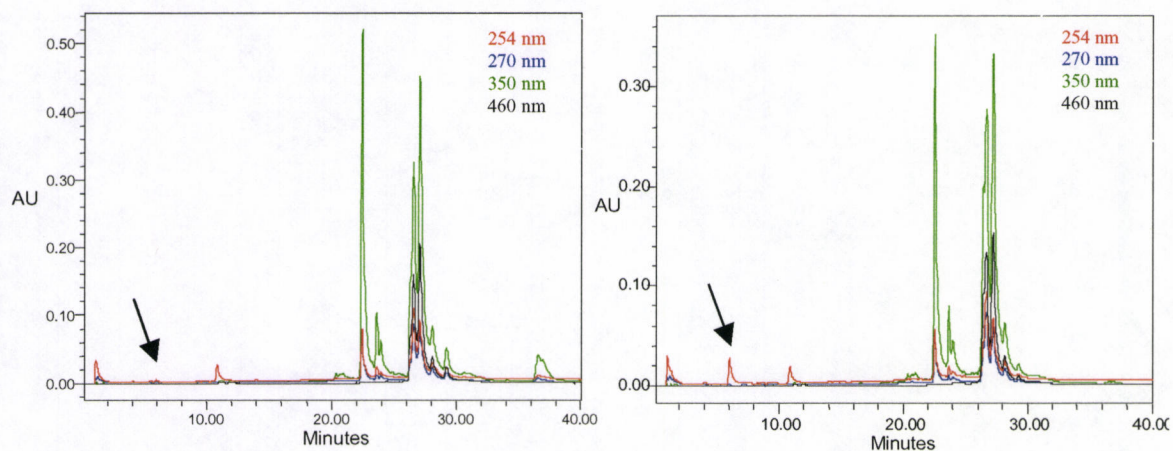


Figure II.7. Gradient PDA-HPLC profiles of Control (right) and MenadioneSB-treated (left) 'Black Band' *Phormidium* cultures. Arrow - peak at 6.1 minutes.

All three peaks were collected and subjected to 2 D TLC analysis, which confirmed that the peak eluting at 6.1 minutes was the UV-active charring compound observed initially on TLC. The other two peaks did not contain any components observable under the UV-lamp (wavelength 254 nm) or any acid charring compounds. The peak at 6.1 minutes yielded approx. 1 mg of material from 10 successive injections of the MSB-treated extract. Structural characterization was attempted by collecting ^1H NMR data, in which only traces of a relatively complex molecule were observed. The material was also submitted for FAB-MS analysis and yielded a major peak at m/z 530.4 Da. This molecular size would explain the initial failure to detect any significant components in the preparative TLC - eluted material by GC-MS (detection limit 500 Da). Further studies were not possible, since structural elucidation on the small amount potentially available, in absence of informative NMR signals, was deemed unpractical.

At the same time, phenazineMS - treated 'Curacin' *Lyngbya* showed an enhancement in a UV active spot in the preliminary TLC analysis, but the result was not reproduced in the other two replicates of the phenazineMS treatment. This occurrence was attributed to biological variation combined with experimental error due to the fact that phenazineMS acted as a potent growth inhibitor in this cyanobacterium.

Synechocystis sp. showed a potentially "new" charring yet non-UV-absorbing lipid in the 19L bacteria treatment that was pursued for confirmation, but the results were inconclusive because of the small size of the organic extracts obtained from this cyanobacterium, which resulted from its slower growth rate. At the same time, analysis by PDA RP-HPLC was impossible due to lack of an UV chromophore in the potentially elicited compound, such that comparative analytical HPLC between treatment and control could not be performed under these conditions.

Biological Activity Effects

None of the species analyzed presented activity in the antimicrobial assays performed by the disc diffusion method at a test concentration of 0.5 mg extract/disc, either in the treatments or the controls. Three of the five cyanobacterial extracts under study were active in the brine shrimp assay: 'Jamaican' *Lyngbya*, 'Curacin' *Lyngbya* and 'Phormidolide' *Phormidium*. Results of the brine shrimp assays are depicted in Figure II.8; no significant effects of the elicitor treatments were manifest in any of the three species.

Our finding that the 'Black Band' *Phormidium* (*Phormidium corallyticum*) extract had no antimicrobial or brine shrimp toxic activity is in agreement with a recently published study that suggested this cyanobacterium is not the one responsible for 'pathogenicity' in the 'black band disease' of corals (Cooney *et al.*, 2002). The cited study performed a characterization of the bacterial community involved in the coral disease by screening of 16S rDNA sequences and showed an unidentified cyanobacterium distinct from *Phormidium corallyticum* was present in all samples taken from diseased corals, while *P. corallyticum* was absent from some of the samples.

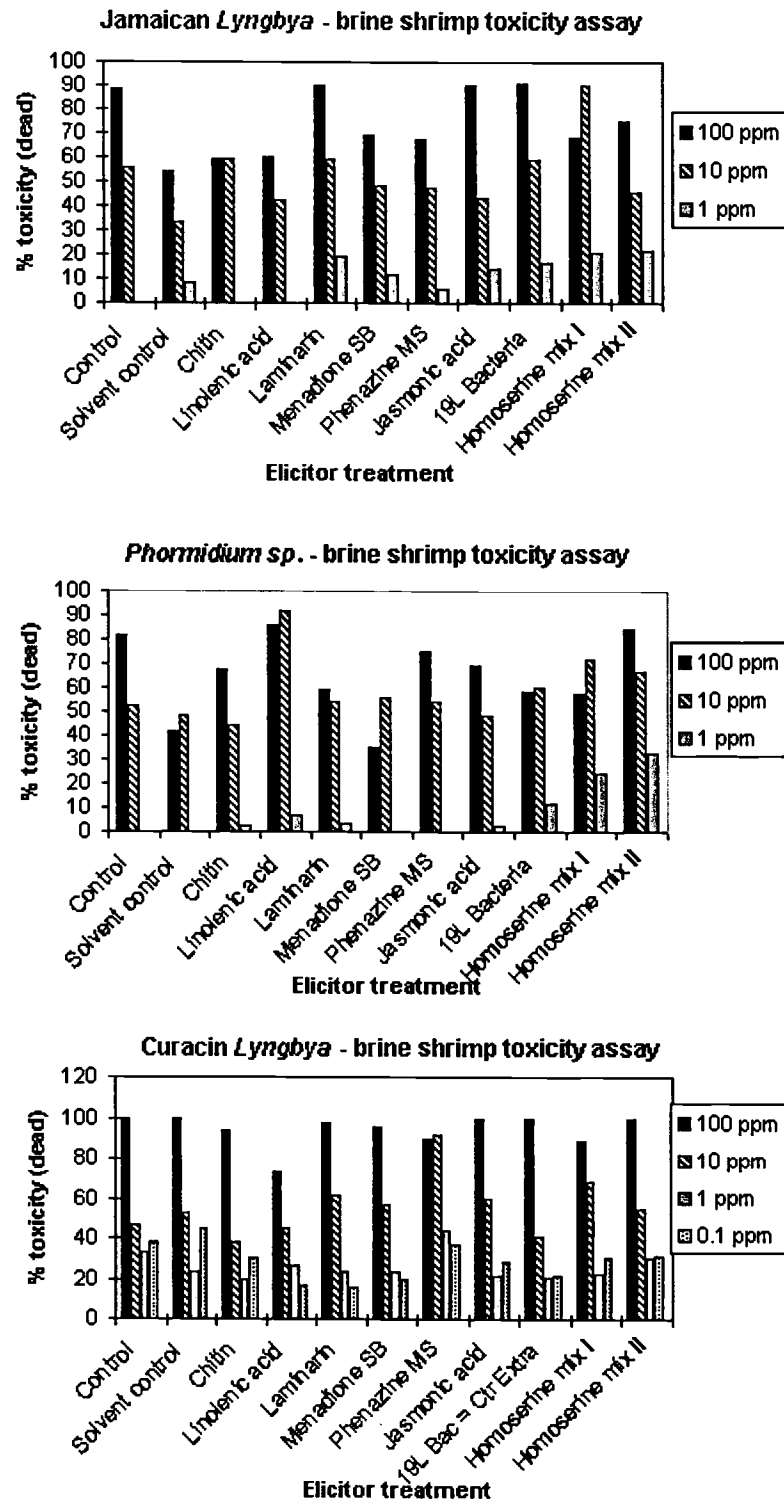


Figure II.8. Brine shrimp toxicity assay results.

Conclusions

Elicitation resulting in phytoalexin production has been previously defined as 1) the production of a novel compound or 2) an increase in the production of an already present secondary metabolite upon treatment of an organism with a small molecule elicitor (Radman *et al.*, 2003). In an investigation into the possible elicitor effects of a given compound, two types of results can be envisaged within the second aspect of the definition. The first possibility would be an absolute increase in production of one metabolite while the biomass, extract yield and other extract components are largely unaffected. A second scenario would comprise only a relative increase of the metabolite of interest due to a decrease in other extract components concomitant with a reduction of the total extract weight, which would result in a change in the ratio of the extract components. The second possibility would give the same apparent enhancement in the compound of interest as the first scenario, by TLC or even HPLC analysis, since some of the extract components decreased could consist of various lipids, which can be UV inactive and sometimes not char.

In the course of the studies presented herein two instances of possible elicitation were encountered. One comprised an increase in biomass and/or organic extract production following treatment with chitin, laminarin, and HSL mix I in several of the cyanobacteria under study. The second one involved the apparent increase of an unknown lipid component in cultures of *Phormidium corralyticum* treated with the oxidative stress agent phenazineMS.

The first instance manifested itself differently between the various cyanobacteria species under study. Chitin promoted an increase in organic extract weight in both *Phormidium* species, as well as in *Synechocystis*, while being an inhibitor of growth in the 'Curacin' *Lyngbya*. Laminarin appeared to increase organic extract weight in the *Synechocystis* sp. and didn't affect any other species.

HSL mix I increased organic extract weight in one *Phormidium* and 'Curacin' *Lyngbya*, while decreasing it in the 'Phormidolide' *Phormidium*.

The second elicitation situation required a distinction between an absolute increase in the amount of the lipid compound, which would constitute true elicitation, and an apparent increase, due to a decrease in quantity of other components of the extract concomitant with decreasing total extract weight. Alas, the enhancement in production was stronger in the PMS treatment, which simultaneously acted as a potent growth inhibitor and was clearly toxic to the organism, inducing a lower production of pigments and other lipid charring compounds in the extract (Figure II.5). However, a modest enhancement was also observed in the 19L Bacteria treatment of 'Black Band' *Phormidium*, a treatment which did not influence biomass or organic extract weights. Thus, we conclude that we did indeed find indications of elicitation phenomena taking place in some of the cyanobacteria, in response to plant oxidative stress mediators and Gram-negative bacteria signaling molecules. Further studies are needed to draw more definitive conclusions and investigate further this phenomenon in cyanobacteria.

EXPERIMENTAL

Cyanobacterial culture conditions: Cultures were obtained from specimens collected by scuba diving from different tropical locations: Curaçao in the case of 'Black Band' *Phormidium* and 'Curacin' *Lyngbya*, Indonesia for 'Phormidolide' *Phormidium* and *Synechocystis*, and Jamaica for the 'Jamaican' *Lyngbya*.

Cyanobacteria were grown in triplicate 1 L flasks containing 0.5 L of SWBG11 medium, at 28°C with a 16 hrs light/8 hrs dark regime (5.4 to 7.0 μ Einsteins). Three days after inoculation, elicitors were added to the flasks, dissolved in 0.5 ml of either DMSO or water. After 24 or 30 days, cells were harvested by filtration, except for *Synechocystis sp.*, where harvesting was done by centrifugation, and the biomass was frozen at - 80 °C until extraction.

Elicitor treatments: The putative elicitors were introduced as follows: jasmonic acid, 5 μ M in DMSO; chitin, 200 mg/L; laminarin, 66 mg/L in water; linolenic acid, 240 μ M in DMSO; 19L Bacteria, 1 ml overnight culture/L; phenazineMS, 1 μ M for 'Black Band' *Phormidium*, 0.5 μ M for all others in water; menadioneSB, 2 μ M for black band, 1 μ M for all others in water; HSL mixes I and II, 10 ng/L of each HSL for a total of 50 ng/L mix in DMSO. All values are final concentrations in the flask.

Extraction protocol: The biomass was ground with mortar and pestle in 2:1 CH₂Cl₂/MeOH, infused at room temperature for 40 min in the same solvent, then boiled 2 x 15 min in fresh solvent. The resulting extracts were combined and dissolved in ether, yielding the "organic" extract. In the case of *Synechocystis sp.*, the biomass was freeze-dried prior to extraction.

2 D TLC's: 300 μ g of extract was spotted on Silica Gel 60F₂₅₄ aluminum plates and developed with 10% MeOH in CHCl₃ in the first dimension, followed by 50% EtOAc/hexanes in the second dimension. UV-active spots were observed under a UV lamp, and pigments, UV-active spots and compounds charring upon heating

after 50% H₂SO₄ spray were marked in pencil on the plate. Pigment colors were identified by letters: G - green, Gy - grey, O - orange and Y - yellow.

Gradient PDA-HPLC profiles: 200 µg of extract were dissolved in 10 µl MeOH and subjected to RP-HPLC on a Waters SymmetryShield RP-18 column (5 µm particle size, 3.9 x 150 mm) in a gradient from 70% MeOH/water to 100 % MeOH, 1 ml/min flow, over 20 minutes followed by a MeOH wash at 2 ml/min for an additional 25 minutes. Detection was achieved by an in-line Waters 996 PDA detector.

Antimicrobial Assay: The antimicrobial assays were performed according to the disc diffusion method as described (Gerwick *et al.*, 1989), with 0.5 mg extract/disc, using a panel of 5 bacteria: *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11775), *Staphylococcus aureus* (ATCC 12600), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 14028) and one yeast *Candida albicans* (ATCC 14053).

Brine shrimp toxicity assay: The brine shrimp toxicity assay was performed as previously described, using *Artemia salina* (Meyer *et al.*, 1982). Extracts were used at three concentrations (100, 10 and 1 ppm) in duplicate experiments. Assays were also run at 0.1 ppm for very toxic extracts (Curacin *Lyngbya*, Jamaican *Lyngbya*, Phormidolide *Phormidium*). Results (dead and live shrimp) were tallied 24 hr after the introduction of the extracts.

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CHAPTER THREE

BIOSYNTHETIC STUDIES ON PHORMIDOLIDE, A BRINE SHRIMP TOXIC
MARINE CYANOBACTERIAL MACROLIDE

ABSTRACT

Phormidolide (**1**) is a brine shrimp toxic ($LC_{50}=1.5 \mu\text{M}$) macrolide produced by a cultured *Phormidium* sp. originally collected from Indonesia. Its complex structure is derived from a polyketide synthase pathway, but possesses unique and intriguing features which prompted interest for investigation of its biosynthetic origin. Preliminary studies on the timetable of production of this secondary metabolite in the 'life cycle' of the culture were performed in order to facilitate subsequent biosynthetic feeding experiments. Phormidolide synthesis in stationary culture is parallel to growth and biomass production until at least 10 weeks after inoculation. Stable isotope incorporation experiments confirmed the polyketide nature of this compound and established a new example of pendant methyl functionality originating from C-2 of acetate. In this metabolite, both S-adenosyl methionine (SAM) and acetate contribute to the formation of the pendant methyl groups. SAM contributes pendant methyl groups placed on backbone carbon atoms derived from C-2 of acetate, while C-2 acetate-derived methyl groups are placed on backbone atoms derived from C-1 of acetate. Efforts for establishing the starter unit biogenesis were not fully successful, despite numerous feedings with putative ^{13}C -labeled precursors; it is proposed that the starter unit is a three carbon unit such as phosphoenol pyruvate or glycerate, but cyanobacterial metabolism in light does not allow intact incorporation from $[\text{U-}^{13}\text{C}]$ glucose.

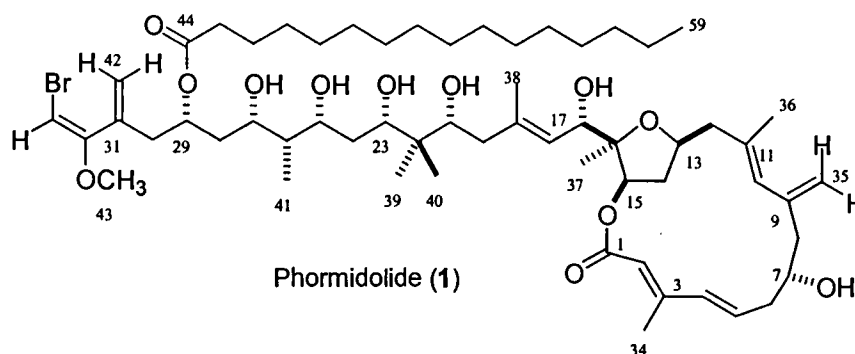
INTRODUCTION

In the course of our laboratory's natural products mechanism-based screening program for new potential anticancer lead compounds, the extracts of three cultures of the marine cyanobacterium *Phormidium* sp. showed potent and specific inhibitory activity to Ras-Raf protein-protein interaction (Finney and Herrera, 1995). This interaction has been identified as a critical component in the mitogen-activated signal transduction cascade that is upregulated in a number of cancer types, and is therefore an attractive site at which to find new inhibitors (Pumiglia *et al.*, 1995). Analysis of these *Phormidium* extracts by TLC and NMR showed a single major secondary metabolite to be present in all three isolates. However, Ras-Raf assay guided fractionation led to the isolation of a novel and very minor chlorophyll-type pigment as the active component; its isolation, structure elucidation and biological properties will be presented elsewhere (Singh and Gerwick, unpublished). Nevertheless, the major component accounted for up to 20% of the extractable lipids in some cultures and was found to possess potent brine shrimp toxicity ($LC_{50} = 1.5 \mu\text{M}$).

These studies led to the isolation of phormidolide (1), a novel bromine-containing macrolide (Williamson *et al.*, 2002). Its complex structure with eleven stereocenters has posed a number of challenging questions in the course of structure elucidation, prompting extensive use of novel NMR methods (Williamson *et al.*, 2001), as well as the recently developed J-based configuration analysis (Matsumori *et al.*, 1999; Murata *et al.*, 1999).

Phormidolide (1) constitutes an interesting target for biosynthetic studies due to its unique structure comprising a high level of modification of the skeleton with hydroxyl and pendant methyl groups, a high level of unsaturation and a unique vinyl bromide functionality at one terminus of the molecule. Preliminary

inspection of the structure revealed that (1) is a polyketide synthase-derived natural product.

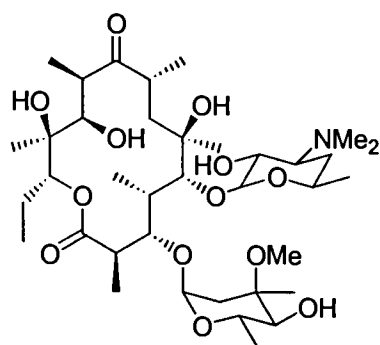


Polyketide synthases (PKSs) are large multifunctional enzyme systems that catalyze the formation of metabolites with carbon chains formed by extension of a "starter unit", often acetyl CoA, propionyl CoA or more complex material, e.g. C₇ units derived from the shikimate pathway. The extender units are most often malonyl CoA or methylmalonyl CoA derived from acetate and propionate, respectively. The main chain-building step is a decarboxylative condensation analogous to the chain elongation step of fatty acid biosynthesis. PKSs and fatty acid synthases (FASs) show strong genetic, structural and mechanistic similarities. The key difference is that in PKSs the reactions of ketoreduction, dehydration and enoyl reduction that follow the condensation step in FASs are not obligate, such that a remarkable structural diversity ensues in the products (Carreras *et al.*, 1997; Bentley and Bennett, 1999).

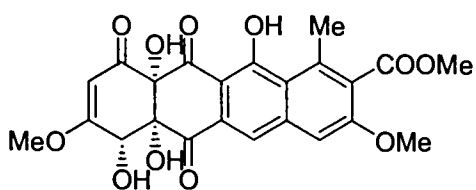
Three major types of PKSs have been described (Hopwood, 1997): type I (modular), analogous to vertebrate FASs, type II (aromatic), related to bacterial FASs, and type III belonging to the chalcone synthase superfamily, found in plants and more recently in bacteria (Moore and Hopke, 2001). Type I PKSs are large multifunctional enzymes organized in modules where each module is used only

once and catalyzes the addition and further modification of one extender unit to the growing polyketide chain. Thus, processing of the growing polyketide chain is done sequentially, immediately following condensation of each acetate unit and the completed product is released by a thioesterase at the end. Generally, the gene sequence specifies directly the structure of the product through the presence/activity of ketoreductase, dehydratase and enoyl reductase domains, such that predictions can be made from the DNA sequence (Hopwood, 1997; Katz, 1997; Staunton and Wilkinson, 1998). Type II PKSs are multienzymatic complexes which include a heterodimeric ketosynthase-chain length factor complex, an acyl carrier protein (ACP) and a malonylCoA:ACP malonyltransferase where each component is used iteratively to catalyze sequential condensations. The completed product undergoes further modification and cyclization to yield the final structure (Hutchinson and Fujii, 1995). Finally, type III PKSs are structurally and mechanistically different from both type I and type II enzymes and use free CoA thioesters as substrates without the involvement of ACPs (Moore *et al.*, 2002).

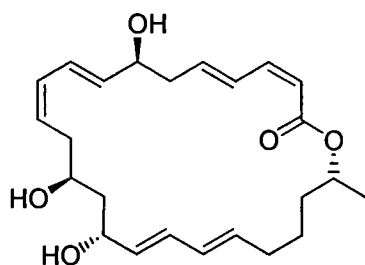
Numerous bioactive metabolites with important pharmaceutical applications pertain to the polyketide family of natural products. Members of this class show a multitude of biological activities, such as antimicrobial - erythromycin (2), tetracenomycin (3); antiviral - the macrolactins (4); immunosuppressant - rapamycin (5), anticancer: - bryostatin (6) and the epothilones (7); antifungal activity - swinholide (8) (Fenical, 1993; Carte, 1996; Shu, 1998; Newman *et al.*, 2000), depicted in Figure III.1.



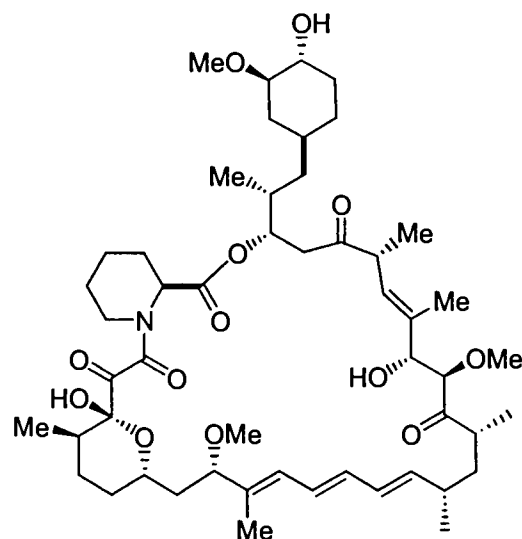
Erythromycin A (2)



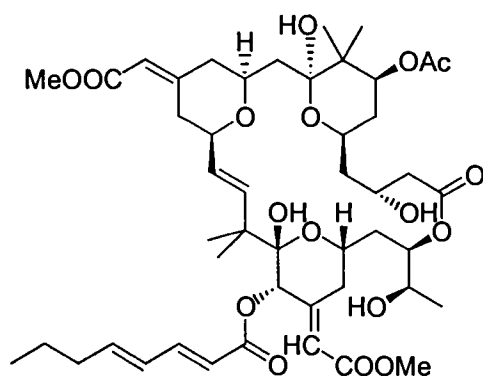
Tetracenomycin C (3)



Macrolactin A (4)



Rapamycin (5)



Bryostatin 1 (6)

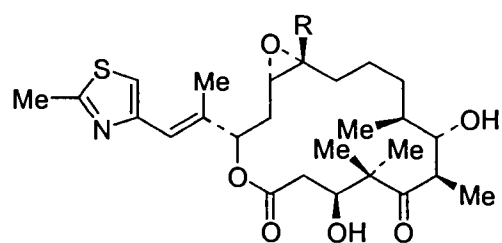
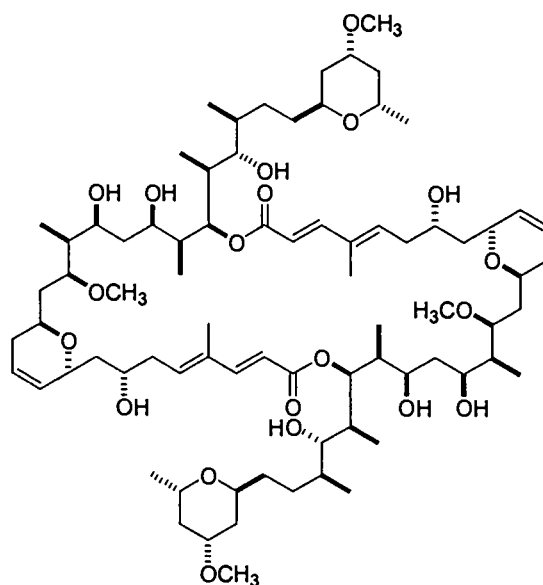
Epothilones A R = H
(7) B R = Me

Figure III.1. Examples of bioactive polyketides.



Swinholide A (8)

The potential development of these natural products as therapeutic agents requires a basic understanding of their molecular and genetic assembly, since often times organic synthetic approaches are unpractical, due to the large number of steps and/or instability of the compound. However, frequently the producing organisms are unculturable and thus cannot be used as sources of compound in sufficient quantities. Another direction that shows great promise is combinatorial biosynthesis, where entire pathways coding for potentially useful compounds are cloned, expressed and manipulated in heterologous hosts that are more amenable to genetic manipulation and subsequently used to produce 'unnatural' natural products (Khosla, 2000).

One of the first steps toward this approach comprises biosynthetic feeding studies in order to determine the number and type of enzymes in the biosynthetic pathway. In the last 50 years, the field of polyketide biosynthesis has made

impressive advances, from the first tentative feeding studies (Birch *et al.*, 1955) to deciphering the molecular architecture and mechanisms of action of these fascinating and diverse enzymes (Staunton and Weissman, 2001). Our laboratory has been proficient in cultivating and maintaining several strains of marine cyanobacteria with bioactive metabolites, including the phormidolide-producing *Phormidium* sp. collected from Indonesia. This availability of cultured organisms has greatly facilitated biosynthetic and genetic studies of the pathways responsible for active natural product formation in marine cyanobacteria.

Secondary metabolite production in bacterial cultures generally attains a peak soon after reaching the growth peak, as was observed at the onset of the studies on bacterial metabolism (Leudeking, 1967). It follows that the production should be maximal in the early stationary phase, followed by a decline toward the late stationary phase. This 'general pattern' of secondary metabolite production was shown to be also true in cyanobacteria by studies on curacin A production in *Lyngbya majuscula* cultures undertaken in our laboratory (Rossi *et al.*, 1997). Similar results were obtained in the study of scytonemin accumulation in cultures of the soil-dwelling cyanobacterium *Scytonema ocellatum* (Patterson and Bolis, 1993). A deviation from this model was found in the production of microcystins by two *Microcystis* species, when maximum toxin production was observed during the exponential phase, with subsequent decrease in the stationary phase. In this case the production peak closely preceded the growth peak (Watanabe *et al.*, 1989). Thus, experiments investigating the timing of phormidolide production in *Phormidium* culture were necessary.

Phormidolide is a suitable model compound for the study of the characteristics of polyketide biosynthesis in cyanobacteria because of its structural features and the relatively high yield of production by the *Phormidium* strain cultured in our laboratory. The present study was designed to yield further

understanding of the orchestration of polyketide biosynthesis in cyanobacteria, as well as tools that could later serve for production of cyanobacterial natural products by recombinant expression and possible generation of a library of derivatives by culture or genetic manipulations.

RESULTS AND DISCUSSION

Studies on the timing of phormidolide biosynthesis in the Phormidium sp. culture

Knowledge of the timing of production of the metabolite of interest is necessary for optimal design of labeled precursor feeding studies. In order to achieve a good level of incorporation of the metabolic precursors, they should be added to the culture at a time when de novo production of the metabolite is very active. At the same time, the experiments need to be scheduled such that an appropriate amount of labeled metabolite of interest can be isolated for analysis with minimal dilution from unlabeled metabolite preexistent in the culture.

Anecdotal observations gathered during the isolation of phormidolide for its structural elucidation suggested that the metabolite accumulates in increasing amounts in aging cultures and therefore production is not slowing down after the early stationary phase. These observations conflicted with literature reports where a decrease in production of the secondary metabolites is observed after entrance into the stationary phase (Watanabe *et al.*, 1989; Patterson and Bolis, 1993; Rossi *et al.*, 1997).

The experiment was set up over a period of 10 weeks, in 1 L flasks containing 500 ml SW-BG11 media, with 5 replicates per time point and samples randomly selected for analysis once per week. Growth was assessed by wet and dry biomass weights (before and after extraction, respectively), while phormidolide production was measured quantitatively using a standard curve in a gradient RP HPLC-based method.

The results showed that phormidolide production tends to increase toward the later stages of growth; however, they also appeared to indicate that the culture did not attain the stationary growth phase during the time course of the experiment (Figure III.2). Practically, the growth curve was almost linear and

phormidolide production paralleled growth with a slight tendency to acceleration after an initial lag.

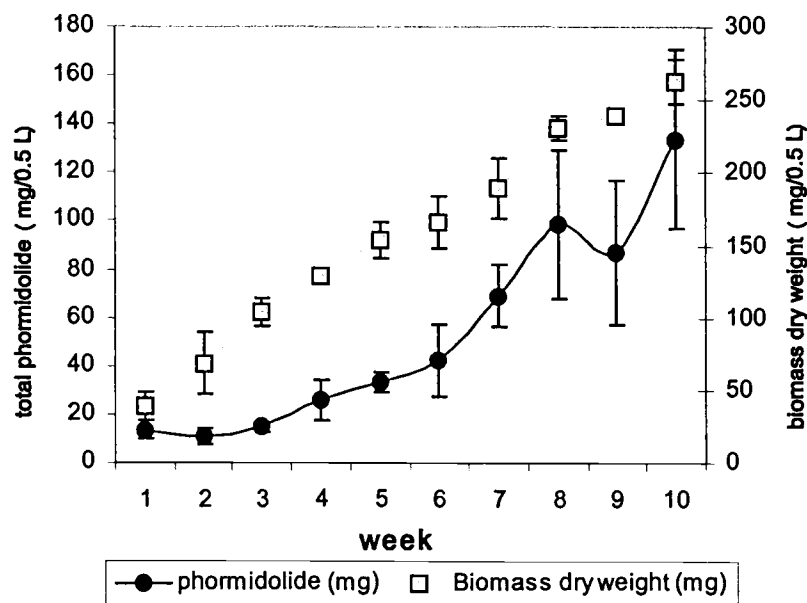


Figure III.2. Time-course experiment of production of phormidolide in culture.

In the case of the labeled precursor feeding experiments presented in this study, a period of growth of 6-8 weeks after inoculation was considered optimal, with addition of the labeled precursors in the last week. This experimental design afforded sufficient phormidolide for spectroscopic experiments with minimal dilution and non-specific catabolism of the metabolic precursors within a reasonable time frame of experiments.

Biosynthetic hypothesis of phormidolide (1)

Initial inspection of the macrolide phormidolide suggested it is a product of a type I (modular) PKS with a carbon skeleton derived from a three carbon starter unit and fifteen intact malonyl CoA-derived acetate units (Figure III.3). The presence of the methoxy group on C-32 in the proposed starter unit suggested methylation by SAM of a hydroxyl group and singled out pyruvate as the most likely precursor of the three-carbon unit, since it could be directly extended by a PKS (Figure III.3A). Alternatively, the starter unit could be derived through SAM methylation of an acetate unit (Figure III.3B), as was shown in the case of borophycin, a boron-containing polyketide from the cyanobacterium *Nostoc linckia* (Hemscheidt *et al.*, 1994).

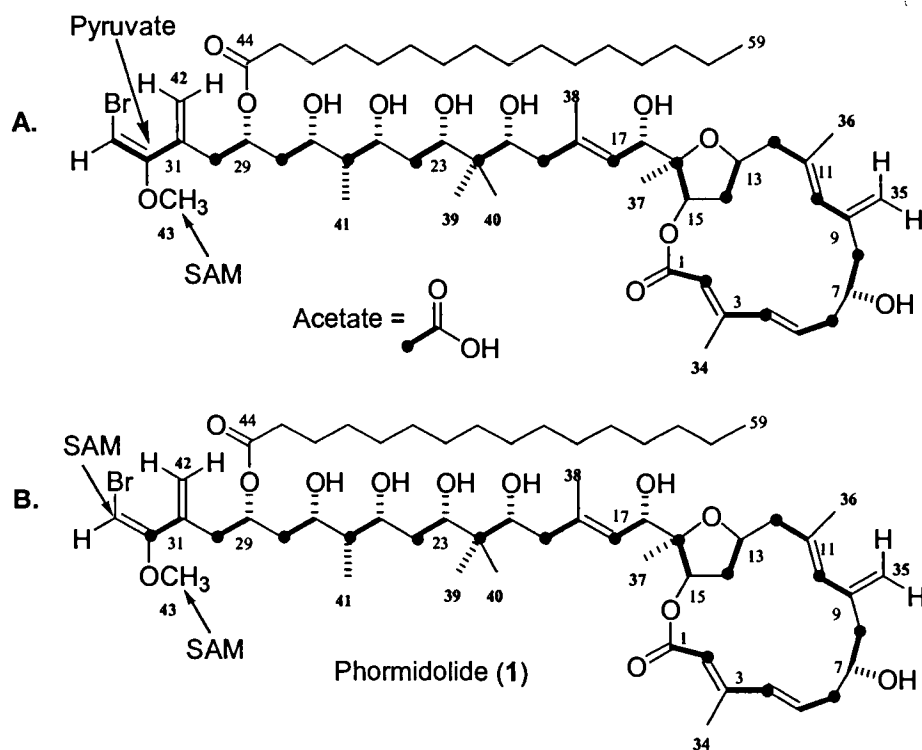


Figure III.3. Proposed biosynthetic origin of phormidolide (1), with two alternatives for the origin of the starter unit depicted in A and B.

The presence of the vinyl bromide functionality in phormidolide makes hypothesis A much more attractive, since the enolization of pyruvate would create the hydroxyl group needed for methylation at the same time with a terminal alkene which could be brominated to obtain the complete starter unit of phormidolide (Figure III.4).

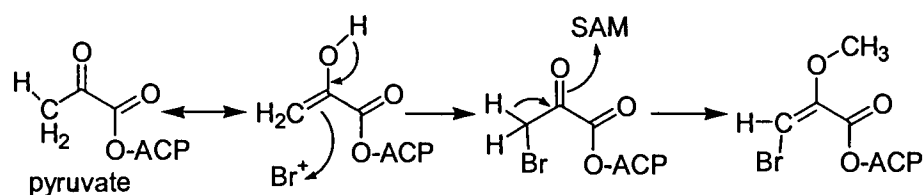


Figure III.4. Proposed mechanism for the formation of the phormidolide starter unit from pyruvate.

Thus, the methoxy group C-43 was postulated to derive from SAM, but the origin of the other 8 pendant methyl groups (C-34 through C-42) was uncertain, with three possibilities being considered: SAM, propionate derived from methylmalonyl CoA incorporation, or C-2 of acetate. The latter biogenetic mechanism has been previously demonstrated in biosynthetic studies of oncorhyncolide (Needham *et al.*, 1992), virginiamycin (Kingston and Kolpak, 1980) and amphidinolide (Sato *et al.*, 2000). Recent studies in our laboratory showed that the pendant vinyl chloride group in jamaicamide A (9) is derived from C-2 of acetate (Nogle, Ph.D. thesis, 2002, Figure III.5) through an HMG CoA synthase-like mechanism.

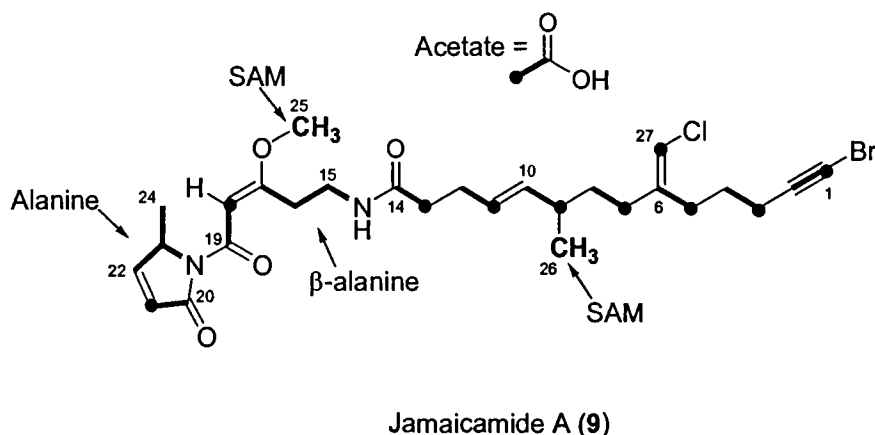


Figure III.5. Biogenetic units of jamaicamide A.

This process is thought to involve addition of an acetate unit to a ketone group in the growing polyketide chain, immediately after the addition of the next acetate unit, followed by decarboxylation, formation of a double bond and facultative reduction (Figure III.6).

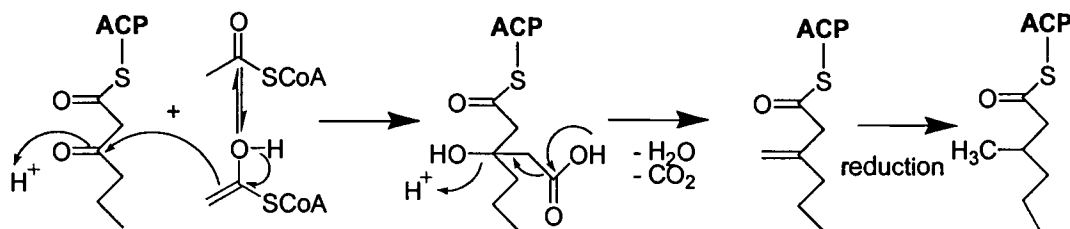


Figure III.6. Proposed pathway for incorporation of pendant methyl groups from C2 of acetate through HMG CoA synthase-like mechanism.

In order to confirm this biogenetic hypothesis and determine the origin of the 8 pendant methyl groups, various stable isotope labeled precursors were supplied to cultures of *Phormidium*. The results were obtained by harvest of the biomass followed by extraction, isolation of phormidolide and analysis of the ^{13}C NMR spectra of phormidolide samples from the different feedings.

[¹³C]-Acetate feedings of Phormidolide

The first isotope feeding experiments conducted were [1-¹³C] acetate, [2-¹³C] acetate and [1,2-¹³C₂] acetate, performed as described in the experimental section. The ¹³C NMR spectrum of phormidolide isolated from the [1-¹³C] acetate feeding experiment showed enhancement in carbons 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29 when compared to natural abundance phormidolide, indicating they all derive from C1 of acetate, as expected (Figures III.3, III.7 and Table III.1). However, no enrichment was observed at position 31 of phormidolide, which showed that the starter unit is not derived from methylated acetate and thus supported alternative A in Figure III.3. Reciprocally, analysis of the ¹³C NMR spectrum of phormidolide isolated from the [2-¹³C] acetate feeding experiment showed that carbons 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 are derived from C2 of acetate, as depicted in figures III.3, III.7 and Table III.1.

An extremely interesting result from the feeding with [2-¹³C] acetate was the enrichment observed in the pendant methyl groups 34, 35, 36, 38 and 42, indicating these are also derived from C2 of acetate through the previously discussed HMG CoA synthase-like mechanism (Figure III.6 and Table III.1). Next, a feeding with [1,2-¹³C₂] acetate was performed which confirmed the incorporation of intact acetate units in the polyketide backbone. The overlapping of ¹³C signals in this large molecule and the presence of multiple carbon atoms residing in very similar environments, which results in the coupling constants being the same or nearly the same, precluded deriving the entire coupling pattern from the 1D ¹³C NMR (Figure III.8 and Table III.1). A new ACCORD-ADEQUATE pulse sequence was devised which allowed inverse detection of the coupled ¹³C-¹³C pairs with great sensitivity (Williamson *et al.*, 2001). The labeled acetate feedings also revealed no ¹³C incorporation at positions 31, 32, 33 of phormidolide (1) and thus established unequivocally that the starter unit is not acetate-derived. This result

also excludes any intermediates from the Krebs cycle from being involved in the starter unit, since they are all derived from, and hence labeled by, acetate.

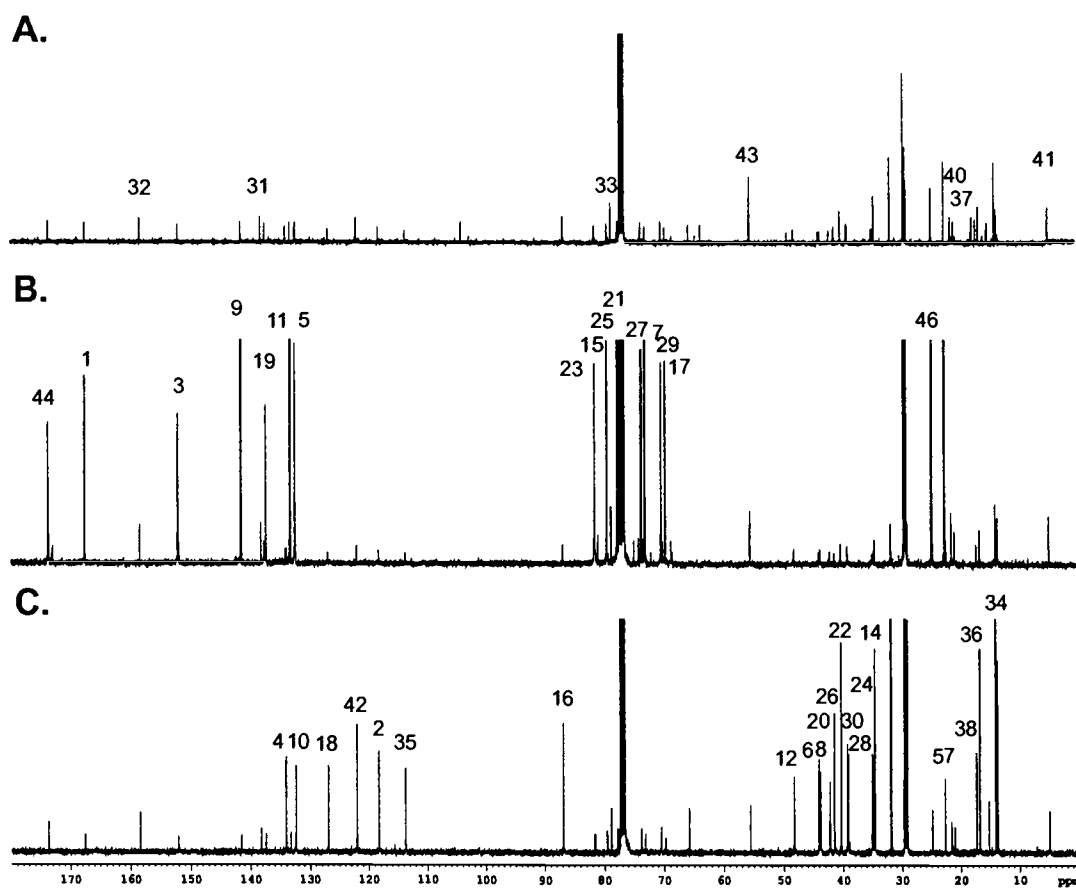


Figure III.7. ^{13}C NMR spectra of phormidolide at natural abundance (A), from cultures fed $[1\text{-}^{13}\text{C}]$ acetate (B) and from cultures fed $[2\text{-}^{13}\text{C}]$ acetate (C).

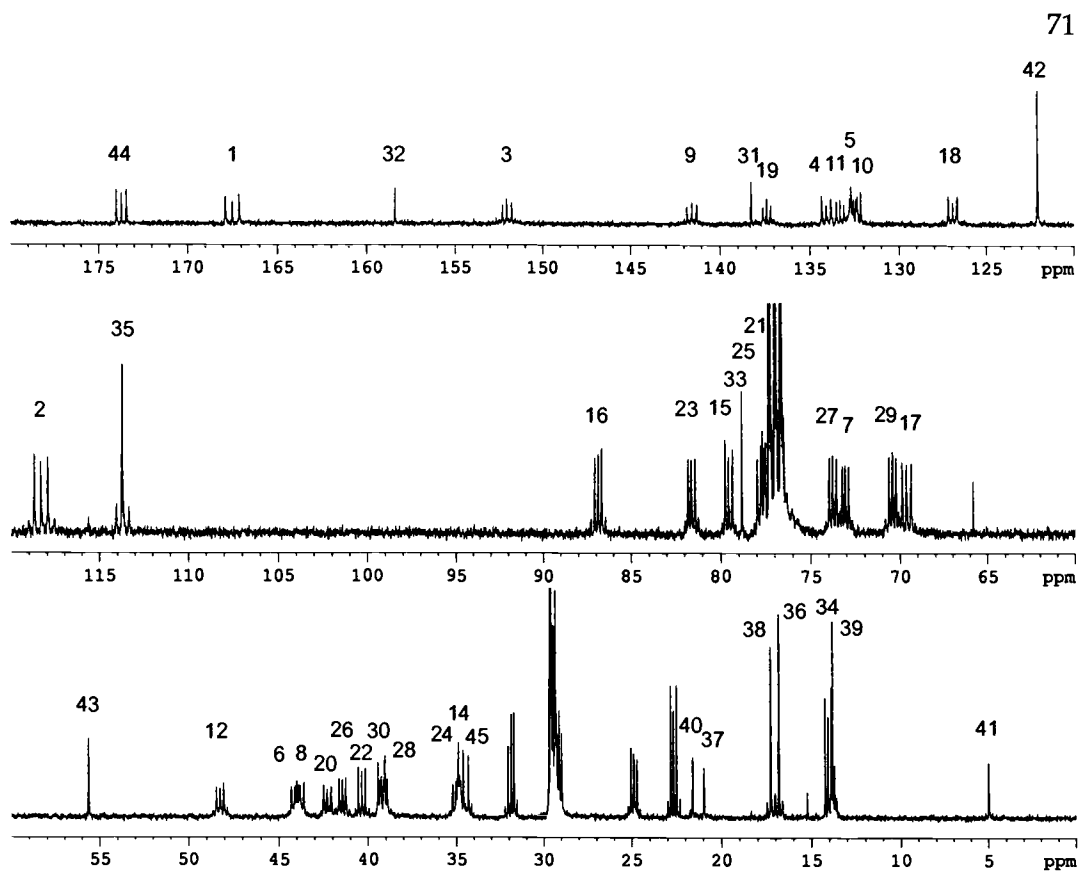


Figure III.8. ^{13}C NMR spectrum of phormidolide isolated from cultures fed $[1,2-^{13}\text{C}_2]$ acetate.

[1-¹³C, ¹⁸O₂]-Acetate feeding

An additional experiment - feeding of [1-¹³C, ¹⁸O₂] acetate was also performed, and the analysis of the ¹³C NMR spectrum (Figure III.9) revealed expected upfield isotopic shifts of *ca.* 0.3 ppm (Vedegas, 1987) due to the presence of ¹⁸O at carbons C-1, C-15, C-29 and C-44 in phormidolide (**1**). Smaller isotopic shifts (0.01-0.02 ppm) were present at carbons C-17, C-21, C-23, C-25 and C-27, which were initially difficult to observe. These results confirmed that all the expected acetate-derived oxygen atoms are retained in phormidolide biosynthesis.

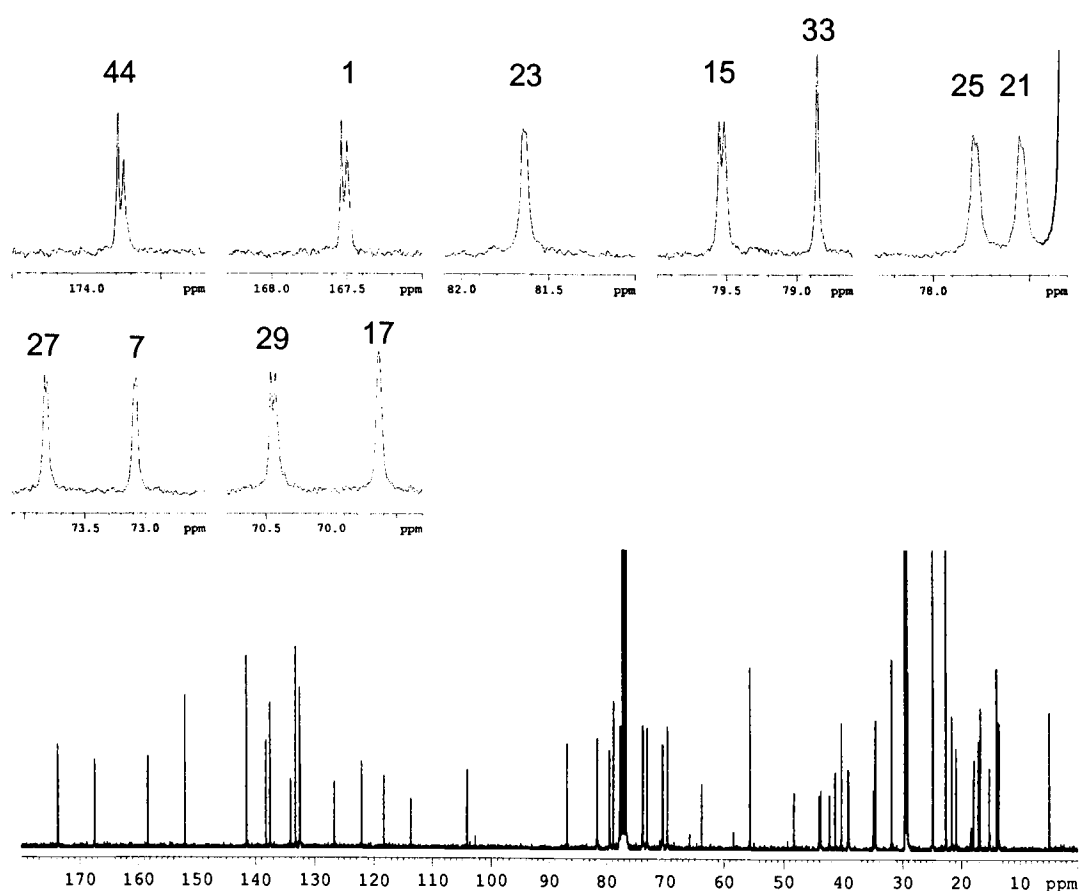


Figure III.9. ¹³C NMR spectrum of phormidolide (**1**) from a culture supplemented with [1-¹³C, ¹⁸O₂] acetate with enlargements of the carbon resonances manifesting isotopic shifts.

[²H₃, 1-¹³C]-Acetate feeding

One flask of *Phormidium* was grown in presence of [²H₃, 1-¹³C] acetate for an investigation of the fate of the hydrogen atoms in acetate in the course of biosynthesis. A maximum of 2 deuterium atoms can be retained on every C-2 of acetate-derived carbon in the backbone, when only condensation and possibly ketoreduction occur. Only one deuterium atom can be retained from labeled acetate in units containing a double bond. The retention of deuterium is observable in the ¹³C NMR spectrum through β-isotopic shifts, and the number of peaks present at a given carbon atom resonance is indicative of the number of deuterium atoms retained, since each isotopic species (CH₂, CHD and CD₂) gives rise to a separate peak.

Results (Figures III.10 and III.11) showed the expected number of deuterium atoms retained in the majority of cases: 1 atom at C-2, C-4, C-10 and C-18, and 2 atoms at C-6, C-20, C-24 and C-28. However, a few exceptions were present - C-7 and C-25 displayed no isotope shifts, and thus C-8 and C-26 didn't retain any deuteriums instead of the 2 and 1 expected, respectively, while C-11 and C-29 showed only one isotope shift, and thus C-12 and C-30 retained only one deuterium instead of the expected two. The anomaly at C-26 could be justified by label loss through the subsequent SAM methylation.

The observation that the other 3 sites (C-8, C-12 and C-30) with anomalous behaviors are vicinal to sites of methylation on C-1 of acetate through the HMG-CoA synthase-like mechanism suggests that this process determines the additional loss of deuterium compared to normal polyketide synthesis. However, this observation contradicts the mechanism presented in Figure III.6, since the protons on C-2 of acetate on either side are not involved in the proposed mechanism. These considerations support a different mechanism, presented in Figure III.12, which corroborates with observations in our laboratory relative to the presence of

additional sequences homologous to enoyl dehydratase in modules containing the HMG-CoA synthase-like activity in the gene cluster for jamaicamides A-C (Dan Edwards, personal communication).

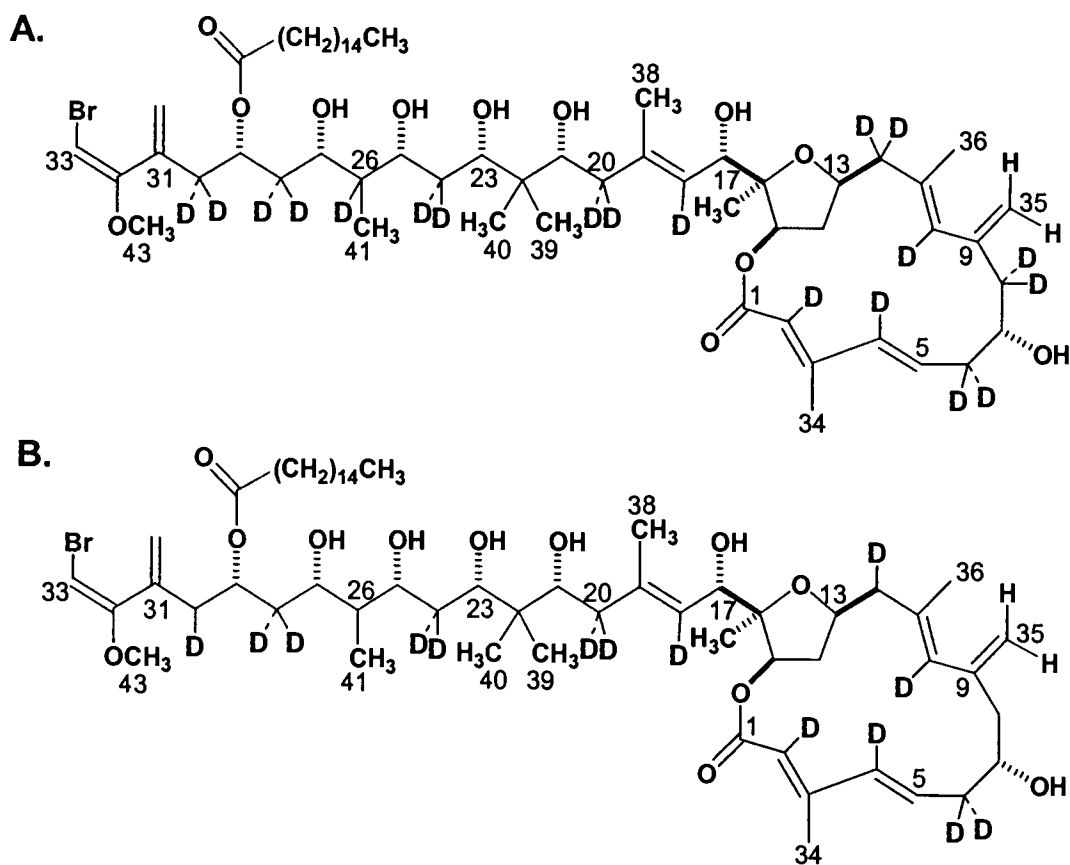


Figure III.10. Results of [$^2\text{H}_3$, $1\text{-}^{13}\text{C}$] acetate feeding experiment for phormidolide (1) (A - expected and B - observed incorporation).

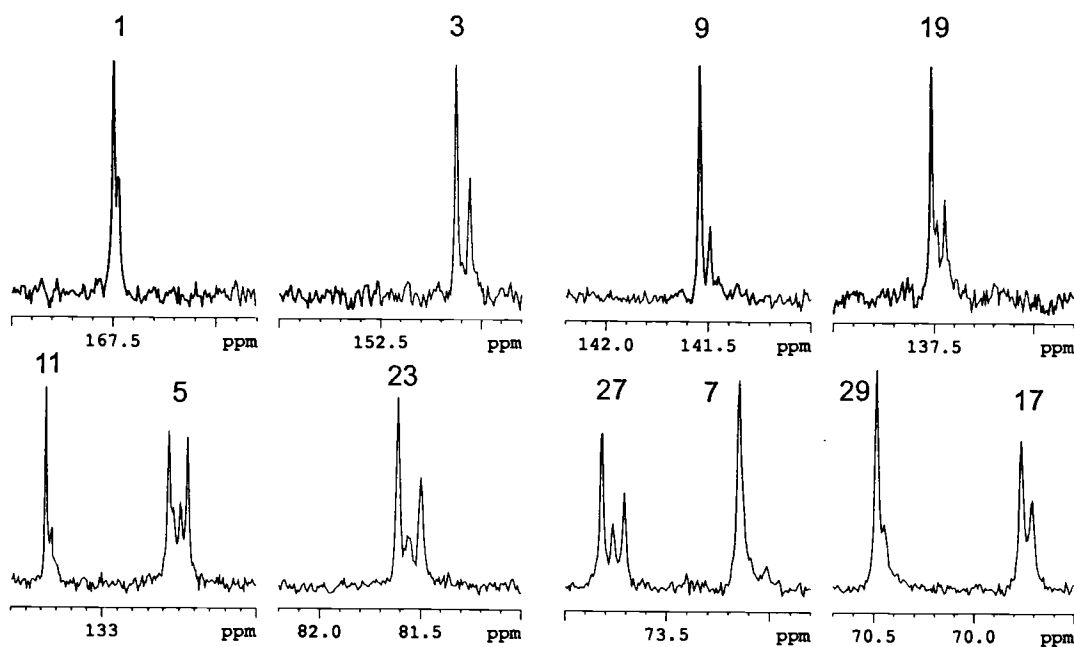


Figure III.11. Selected enlargements from the ^{13}C NMR spectrum of phormidolide (1) from a culture supplemented with $[\text{2H}_3, 1\text{-}^{13}\text{C}]$ acetate.

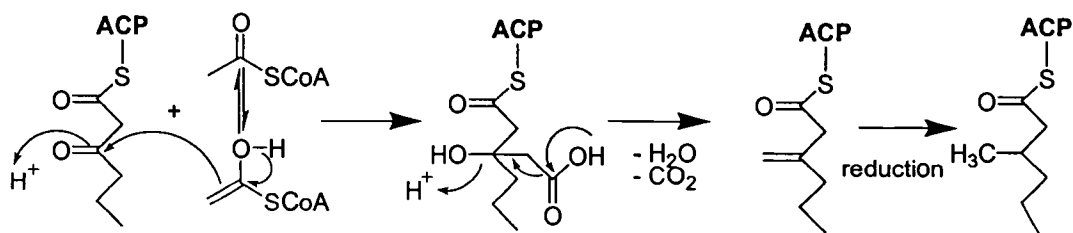


Figure III.12. Revised proposal for incorporation of pendant methyl groups from C2 of acetate through HMG CoA synthase-like mechanism.

S-[methyl-¹³C] methionine feeding experiment

In order to establish the origin of the other four pendant methyl groups and the carbon in the methoxy group, an *S*-[methyl-¹³C] methionine feeding was undertaken. The ¹³C NMR spectrum of phormidolide isolated from this treatment showed very intense enrichment of carbons in positions 37, 39, 40, 41, 43 and proved these carbon atoms are introduced through SAM - mediated methylation on the backbone atoms derived from C2 of acetate or a hydroxyl group, respectively. Results are depicted in Figure III.13 and Table III.1. Up until this point, the origin of the entire backbone of phormidolide and of the pendant methyl groups except the three-carbon starter unit had been determined.

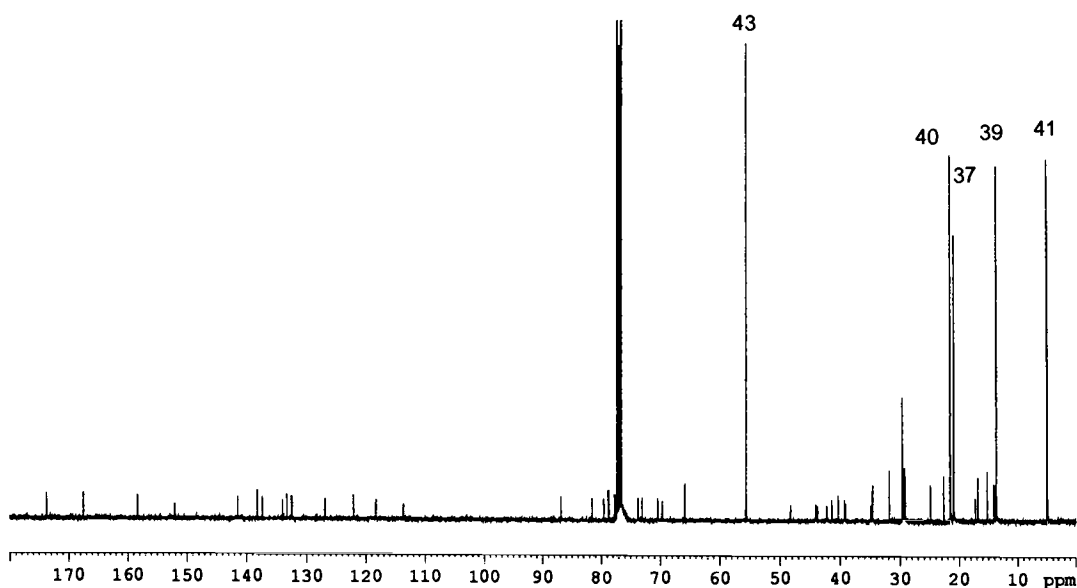


Figure III.13. ¹³C NMR spectrum of phormidolide isolated from a culture treated with *S*-[methyl-¹³C] methionine.

Table III.1. Relative enhancements of carbon resonances from phormidolide biosynthetic feeding experiments.

position	Chemical shift (ppm) ^a	[1- ¹³ C] acetate	[2- ¹³ C] acetate	¹ J _{CC} values from [1,2- ¹³ C ₂] acetate (Hz)	[CH ₃ - ¹³ C] methionine
1	167.5	20.6	0.6	77.4	0.0
2	118.3	0.2	9	77.4	-0.1
3	152.1	32.8	1.3	52.1	0.5
4	134.1	0.3	9.2	52.1	-0.2
5	132.6	12.8	0.4	41.2	-0.3
6	44.1	0.6	11.8	41.4	0.1
7	73.1	16.9	0.6	38.3	-0.2
8	43.8	0.3	10.0	38.3	-0.1
9	141.5	15.4	0.2	54.4	-0.2
10	132.4	1.2	5.5	54.4	-0.4
11	133.4	17.5	0.7	41.4	-0.1
12	48.3	0.2	10.2	41.4	0.0
13	76.7				
14	34.8	0.1	8.6	31.4	-0.2
15	79.6	19.7	0.6	39.1	-0.4
16	86.9	0.4	10.3	39.1	-0.1
17	69.7	15.7	0.4	49.8	-0.3
18	127	-0.3	8.6	49.8	-0.1
19	137.4	11.6	0.3	41.4	-0.4
20	42.3	0.2	11	41.4	0.0
21	77.5	9.1	0.4	37.4	-0.4
22	40.4	-0.4	5.2	38.3	-0.6
23	81.6	19.1	1.4	39.1	0.2
24	35.1	-0.3	7.4	39.4	-0.2
25	77.8	12.2	0.3	38.3	-0.4
26	41.5	0.0	9.7	38.3	-0.2
27	73.8	11.1	0.4	39.1	-0.2
28	39.2	-0.3	6.3	39.1	-0.3
29	70.5	8.6	0.0	39.1	-0.5
30	39.3	-0.4	4.3	38.3	-0.5
31	138.3	0.4	0.0		-0.5
32	158.4	-0.2	-0.1		-0.7
33	78.8	0.2	0.1		-0.5
34	13.9	0.5	9.0	39	-0.3
35	113.8	1.2	16.7	71.3	0.6
36	16.8	0.0	5.8	42.2	-0.4
37	21	1.7	0.8		10.9
38	17.3	0.2	9.9	43.8	0.1
39	13.7	0.8	0.8		8.8
40	21.6	0.6	0.2		6.5
41	5	0.2	0.0		5.3
42	122.1	-0.4	3.5		-0.5
43	55.6	0.0	0.0		4.7
44	173.7	8.6	0.2	57.5	-0.5

^a Referenced to CDCl₃ centerline, 77.0 ppm. Bold numbers indicate ¹³C enrichment

[1-¹³C] Pyruvate and [3-¹³C] alanine feeding experiments

In an attempt to establish the origin of the starter unit, a culture flask with *Phormidium* was provided with [1-¹³C] pyruvate and phormidolide was isolated and subjected to ¹³C NMR spectroscopic evaluation. The results showed no specific incorporation of label into any of the positions in the starter unit (carbons 31, 32, 33). However, an overall enrichment of approximately 4% was observed due to the catabolism of pyruvate to acetate and ¹³CO₂, subsequent reuptake of the labeled carbon source and random incorporation into phormidolide (data not shown). An alternate experiment using [3-¹³C] alanine was also undertaken, and no specific incorporation in the starter unit was observed in this case, either.

[1-¹³C] Propionate feeding experiment

Labeled propionate was also introduced in the culture to test its incorporation into the starter unit, although the possibility was considered quite unlikely due to the extensive modifications needed to obtain the functionalities in phormidolide. No specific incorporation was observed, in accordance to expectations. Other labeled precursors were then supplied to cultures in the search for the elusive precursor to the starter unit.

[1-¹³C] Glycine feeding experiment

[1-¹³C] labeled glycine was fed under the assumption that it will be transformed to serine in the course of normal metabolism by the cyanobacterium, and serine could provide the three carbon starter unit upon deamination. No specific incorporation was observed from this experiment.

[U-¹³C] Glucose and [U-¹³C] glycerol feeding experiments

Several reports in the literature described that in cases when some precursors were not able to give positive incorporation results, a different metabolite that feeds into the same pathway was able to enter the cells and demonstrated the origin of the substructure under study. Glucose is one of the most easily assimilable precursors, and thus labeling studies with glucose were performed next. The ¹³C NMR spectra of phormidolide isolated from these experiments showed incorporation in the starter unit; a representative spectrum from the 16 day feeding is presented in Figures III.14 and III.15. However, the coupling pattern observed was consistent with a symmetric doubly labeled intermediate being incorporated in both orientations, due to couplings observed between C-31 and C-32 (63.6 Hz) and separately between C-32 and C-33 (93.5 Hz) and not with the intact incorporation of a three carbon fragment. A subsequent feeding with [U-¹³C] glycerol produced identical results.

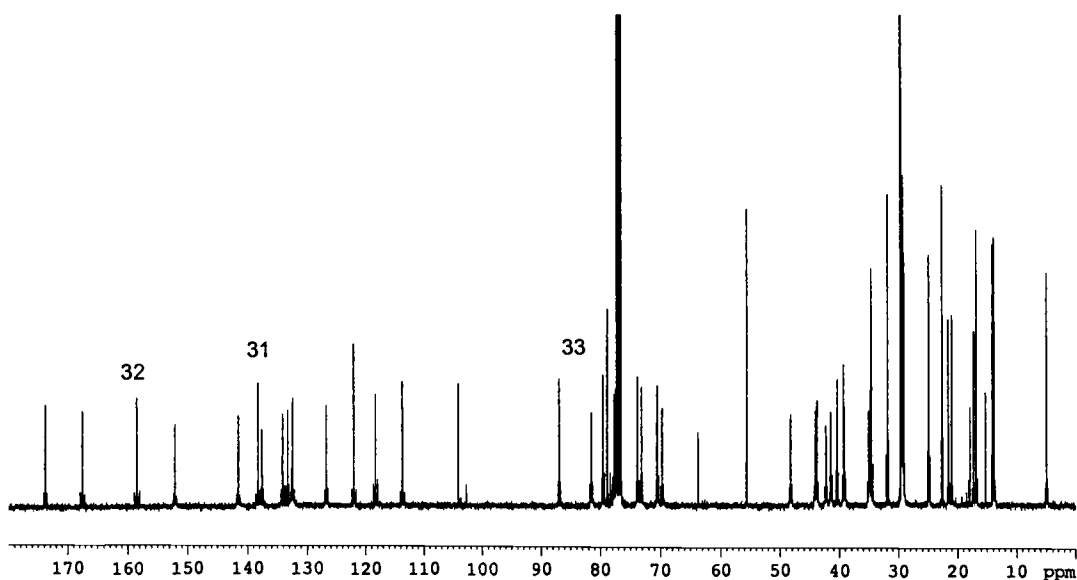


Figure III.14. ¹³C NMR spectrum of phormidolide from a culture grown in presence of [U-¹³C] Glucose.

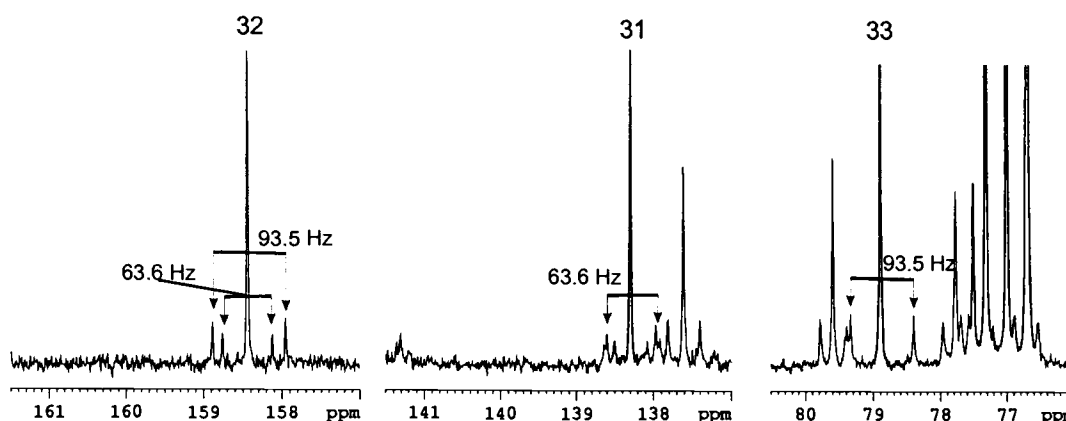


Figure III.15. Selected enlargements of the ^{13}C NMR spectrum of phormidolide from a culture treated with $[\text{U-}^{13}\text{C}]$ Glucose.

Intact incorporation of a three-carbon unit is expected to give rise to a complex coupling pattern at C-32 instead of two separate and easily identifiable couplings.

One possible explanation for this incorporation pattern is provided by the high activity reported for enzymes in the Calvin cycle in lighted growth conditions, which results in CO_2 fixation from air. In this process, an unlabeled carbon atom from the atmosphere is introduced next to labeled coupled carbons from glucose. Due to the particular labeling pattern observed, a symmetrical intermediate must be involved; another difficulty resides in explaining the total lack of incorporation of intact units - if production of the three carbon unit precursor occurred simply by glycolysis, both three carbon coupled and two carbon coupled starter units should be observed.

A recent article provides metabolic flux analysis data in *Synechocystis* cultured in both heterotrophic (in the dark) and mixotrophic (in light) growth conditions based on amino acid labeling patterns following feeding with $[\text{U-}^{13}\text{C}]$ glucose (Yang *et al.*, 2003). The results obtained for cyanobacterial metabolism in mixotrophic growth (simultaneous feeding with glucose and exposure to light)

showcased unique characteristics that add an important contribution to the understanding of the labeling pattern obtained in the present study. In the dark, the major pathway for glucose utilization is the pentose phosphate pathway, with glycolysis playing a secondary role. With exposure to light, the Calvin cycle becomes the major pathway of glucose utilization to such an extent that the estimated fluxes through phosphofructokinase (for glycolysis) and transketolase (as a start point for glucose utilization in the Calvin cycle) were 4.4 and 76.4, respectively. It follows that the extent to which intact coupled three carbon units are produced from utilization of labeled glucose is much smaller than we had envisaged, possibly rendering them undetectable. A schematic presentation of these considerations is rendered in Figure III.16, with one pass through the Calvin cycle starting from universally labeled glucose. It is evident that several passes will serve to increase the population of two coupled carbons or uncoupled three carbon units, diminishing the intact three carbon coupled population. It is conceivable then that the starter unit for phormidolide is a three carbon unit derived from glucose such as phosphoenol pyruvate, or glycerate, but intact incorporation cannot be observed due to intrinsic characteristics of cyanobacterial metabolism.

Further investigations were attempted by culturing the cyanobacterium in the dark, under heterotrophic conditions of feeding with [U-¹³C] glucose diluted 1:1 with unlabeled glucose, in order to hinder atmospheric CO₂ fixation. However, the cyanobacterium did not adapt well to the dark culture conditions and as a result, after harvest and extraction no phormidolide could be isolated for analysis. Thus, the exact identity of the starter unit of phormidolide remains a mystery that might not be elucidated unless the biosynthetic gene cluster is cloned and sequenced. In that event, the sequence homologies and possibly substrate specificities of the enzymes present for the first steps of the pathway might shed further light on the nature of the starter unit.

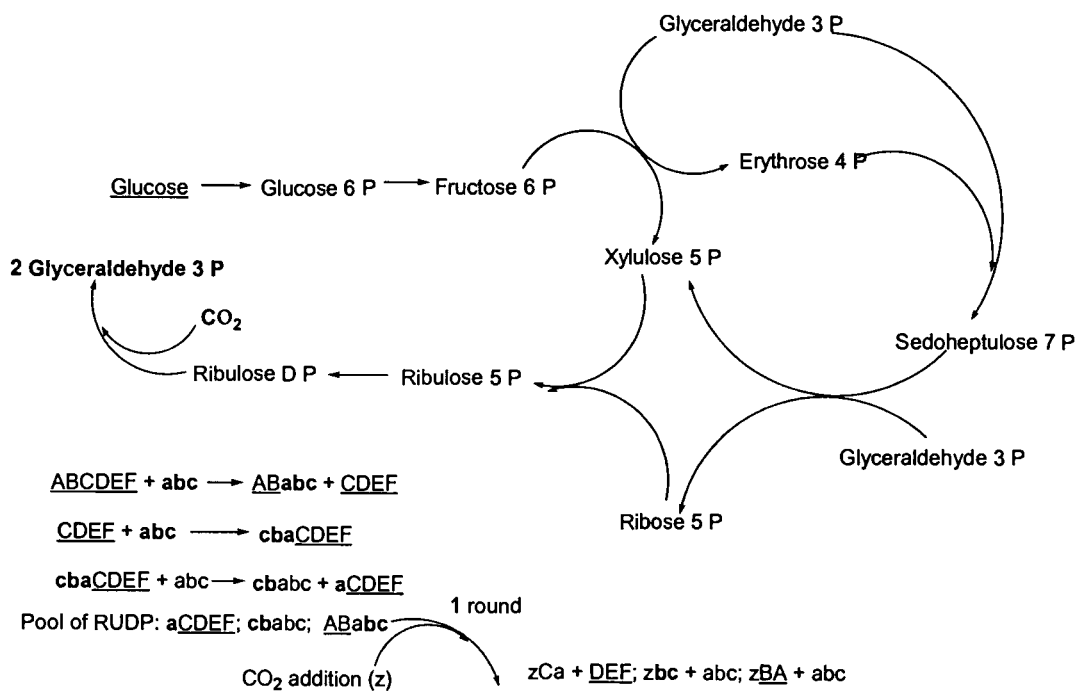


Figure III.16. Schematic representation of the fate of [U-¹³C] Glucose fed to cyanobacterial cultures under mixotrophic conditions.

EXPERIMENTAL

Culture conditions: *Phormidium* sp. was grown in flasks containing SWBG11 medium, at 28°C with a 16 hrs light/8 hrs dark regime (5.4 to 7.0 μ Einsteins). For the timeline of phormidolide production experiments, volume of cultures was 500 ml, while for feeding experiments either one or two Fernbach flasks with 1.5 L of media were used. After 64 or 70 days, cells were harvested by filtration and the biomass was frozen at - 80 °C until extraction.

Extraction protocol: The biomass was ground with mortar and pestle in 2:1 CH₂Cl₂/MeOH, infused at room temperature 40 min. in the same solvent, then boiled 2 x 15 min. in fresh solvent. The resulting extracts were combined and retaken in ether, yielding the total organic extract.

Isolation of phormidolide: In a typical experiment the crude extract was then subjected to VLC (TLC grade silica gel) with a range of solvents (0 to 100% EtOAc/hexanes followed by MeOH wash). The fraction eluted with 100% EtOAc was further fractionated in NP- HPLC (dual Phenomenex Luna 10 μ m silica, 2 x 250 mm x 4.6 mm) with a 60% Hexanes/ 35% EtOAc/ 5% IPA solvent system, 4.5 ml/minute flow rate and yielded pure phormidolide.

Gradient PDA-HPLC quantitation: 200 μ g of extract were dissolved in 10 μ l MeOH and subjected to RP-HPLC on a Waters SymmetryShield RP-18 column (5nm diameter, 3.9 x 150 mm column) in a gradient from 85% MeOH/water to 100 % MeOH, 1 ml/min flow, over 20 minutes followed by MeOH wash 2 ml/min for an additional 25 minutes. Detection was afforded by an in-line Waters model 480LC PDA detector. Known quantities of phormidolide were injected and the area under the peaks was used to define a standard curve.

[1-¹³C] Acetic acid feeding: [1-¹³C] acetic acid (45 mg total/flask) diluted 1:1 with unlabeled sodium acetate was introduced in 2 culture flasks containing 1.5 L

SWBG-11 on days 49, 51, 53 and the biomass was harvested on day 56 (8.07 g wet weight, 1.57 g dry weight). 19 mg of phormidolide were isolated after extraction, VLC and HPLC from 103 mg organic extract.

[2-¹³C] Acetic acid feeding: [2-¹³C] acetic acid (45 mg total/flask) diluted 1:1 with unlabeled sodium acetate was introduced in 2 culture flasks with 1.5 L SWBG-11 on days 49, 51, 53 and the biomass was harvested on day 56 (10.25 g wet weight, 1.69 g dry weight). 22 mg of phormidolide were isolated after extraction, VLC and HPLC from 120 mg organic extract.

Sodium [1, 2-¹³C₂] acetate feeding: [1, 2-¹³C₂] acetate (30 mg total/flask) diluted 1:2 with unlabeled sodium acetate was introduced in 2 culture flasks containing 1.5 L SWBG-11 on days 54, 56, 58 and the biomass was harvested on day 61 (7.04 g wet weight, 1.47 g dry weight). 17 mg of phormidolide were isolated after extraction, VLC and HPLC from 163 mg organic extract. Two other label incubation periods, 16 days and 24 days, were also performed in addition to this 7 days experiment, with analogous results.

Sodium [1-¹³C, ¹⁸O₂] acetate feeding: [1-¹³C, ¹⁸O₂] acetate (50 mg total) diluted 1:1 with unlabeled sodium acetate was provided to one culture in 1.5 L SWBG-11 on days 70, 72, 75 and the biomass was harvested on day 79 (7.3 g wet weight, 1.2 g dry weight). 30 mg of phormidolide were isolated after extraction, VLC and HPLC from 144 mg organic extract.

Sodium [²H₃, 1-¹³C] acetate feeding: [²H₃, 1-¹³C] acetate (30 mg total/flask) diluted 1:2 with unlabeled sodium acetate was introduced in 2 culture flasks containing 1.5 L SWBG-11 on days 50, 52, 54 and the biomass was harvested on day 57 (7.19 g wet weight, 1.1 g dry weight). 31 mg of phormidolide were isolated after extraction, VLC and HPLC from 172 mg organic extract.

S-[Methyl-¹³C] methionine feeding: S-[Methyl-¹³C] methionine (70 mg total/flask) was fed to 2 culture flasks containing 1.5 L SWBG-11 on days 49, 51, 53 and the

biomass was harvested on day 56 (14.8 g wet weight, 1.67 g dry weight). 28 mg of phormidolide were isolated after extraction, VLC and HPLC from 158 mg organic extract.

Sodium [1-¹³C] pyruvate feeding: [1-¹³C] pyruvate (30 mg total) diluted 1:3 with unlabeled sodium acetate was provided to one culture in 1.5 L SWBG-11 on days 74, 78, 81 and the biomass was harvested on day 83 (5.2 g wet weight, 0.9 g dry weight). 13 mg of phormidolide were isolated after extraction, VLC and HPLC from 109 mg organic extract.

[3-¹³C] Alanine feeding: [3-¹³C] alanine (54 mg total) was provided to one culture in 1.5 L SWBG-11 on days 70, 72, 75 and the biomass was harvested on day 79 (7.7 g wet weight, 1.2 g dry weight). 29 mg of phormidolide were isolated after extraction, VLC and HPLC from 133 mg organic extract.

Sodium [1-¹³C] propionate feeding: [1-¹³C] propionate (50 mg total) was provided to one culture in 1.5 L SWBG-11 on days 50, 52, 54 and the biomass was harvested on day 58 (6 g wet weight, 0.95 g dry weight). 24 mg of phormidolide were isolated after extraction, VLC and HPLC from 118 mg organic extract.

[1-¹³C] Glycine feeding: [1-¹³C] glycine (100 mg total) was provided to one culture in 1.5 L SWBG-11 on days 70, 74, 77 and the biomass was harvested on day 88 (8.8 g wet weight, 1.21 g dry weight). 3 mg of phormidolide were isolated after extraction, VLC and HPLC from 159 mg organic extract.

[U-¹³C] Glucose feeding: [U-¹³C] glucose (200 mg total) diluted 1:2 with unlabeled glucose was introduced in a 1.5 L *Phormidium* culture in SWBG-11 on days 51 and 59 and the biomass was harvested on day 66 (5.73 g wet weight, 1.1 g dry weight). 28 mg of phormidolide were isolated after extraction, VLC and HPLC from 197 mg organic extract. In a different experiment, the same amount of glucose was added on days 51 and 59, but the harvest was on day 75 for a total of 24 days label

incubation instead of 16 days, and 26.8 mg phormidolide were obtained from 219 mg extract (6.87 g wet, 1.15 g dry weight).

[U-¹³C] Glycerol feeding: [U-¹³C] glycerol (130 mg total/flask) was fed to 2 culture flasks containing 1.5 L SWBG-11 on days 74 and 77 and the biomass was harvested on day 84 (13.2 g wet weight, 2 g dry weight). 19.5 mg of phormidolide were isolated after extraction, VLC and HPLC from 283 mg organic extract.

Calculation of ¹³C Enrichments from Precursor Feeding Experiments. The relative ¹³C enrichments from exogenously supplied isotopically labeled precursors were calculated as follows. All ¹³C NMR spectra were recorded in CDCl₃ on a Bruker AM400 NMR spectrometer operating at a ¹³C resonance frequency of 100.61 MHz, and processed with 1.0 Hz line broadening (zgpg Bruker pulse program). For each experiment, carbon resonance intensities for the natural abundance and enriched sample were tabulated and the enriched sample was normalized to natural abundance C43 for the labeled acetate feeding experiments and to C1 for the S-[Methyl-¹³C] methionine experiment. Enrichment was calculated as Normalized integral enriched minus Integral natural abundance divided by Integral natural abundance for each carbon resonance (enriched minus natural/natural, in other words).

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CHAPTER FOUR

INVESTIGATIONS OF THE HALOGENATION PROCESS INVOLVED IN
THE BIOSYNTHESIS OF PHORMIDOLIDE

ABSTRACT

Phormidolide (**10**) is a brine shrimp toxic ($LC_{50}=1.5 \mu\text{M}$) macrolide possessing an unique terminal vinyl bromide functionality. The producing *Phormidium* sp., originally collected from Indonesia, has been successfully established in culture in our laboratory. The characteristics of the halogenation reaction involved in the biosynthesis of phormidolide were studied by varying the halogen ion content in the culture media. *Phormidium* sp. grown in bromide-enriched, bromide-depleted and simultaneously bromine-depleted and iodine-enriched media was extracted and any phormidolide-type compounds were isolated and analyzed by NMR and mass spectrometry. In all of these instances, 'phormidolide-like' compounds were produced by the cyanobacterium. In the bromine-depleted growth conditions, phormidolide was not detected, but two novel analogs of phormidolide were produced: debromophormidolide (**11**) and iodophormidolide (**12**), in a ratio of 2:1. The yield of phormidolide was significantly increased (up to three fold) upon bromine supplementation of the culture medium, as well as the yield of iodophormidolide (ten fold) in the iodine supplemented and bromine depleted growth conditions. Debromophormidolide was not produced in this latter experiment.

INTRODUCTION

The high concentration of halide ions in the marine environment determines that a major theme in the secondary metabolism of marine organisms is the presence of halogenated compounds, in greater proportion than in metabolites from terrestrial and fresh water plants or cyanobacteria (Faulkner, 2000; Gerwick *et al.*, 2001). Many of these halogenated metabolites manifest biological activities, such as: antitumor - halomon (1), antibiotic - pentabromopseudilin (2), antiviral - eudistomin C (3) or cytotoxic - methyl chlorosarcophytoate (4) (Burkholder *et al.*, 1966; Carte, 1996).

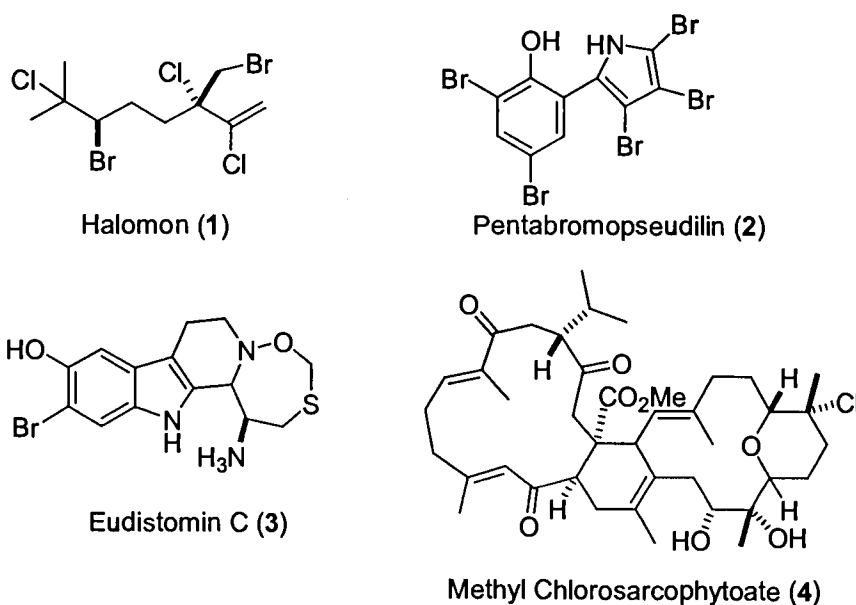
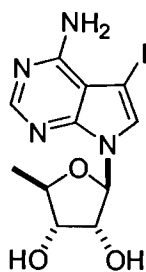


Figure IV.1. Bioactive halogenated metabolites of marine origin.

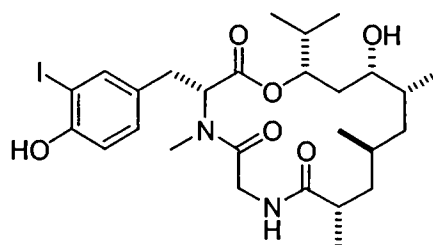
The study of these halogenated compounds is particularly interesting because the identification of the mechanisms of halogenation and the isolation of novel halogenating enzymes can have important industrial applications in

addition to providing a reliable source of the bioactive halogenated metabolites for clinical studies. One direction in which the knowledge derived from studies of the biosynthesis of halogen-containing metabolites can be applied is the 'ecologically friendly' production of halogenated compounds. Another direction in which this type of research can lead is the finding of novel solutions for the 'natural' biodegradation of contaminating halogenated wastes.

Despite the extreme difference in concentration between the different halide ions in seawater (chlorine 19,000 mg/L, bromine 65 mg/L and iodine 1 mg/L), marine organisms are able to select and preferentially incorporate bromine and even iodine into natural product structures, as in the case of deoxyiodotubercidin (5, Davies *et al.*, 1984), a potent adenosine kinase inhibitor from the red alga *Hypnea valetiae* or dolicolide (6, Shiwata *et al.*, 1994), a novel antitumor agent.



5'-Deoxy-5-Iodotubercidin (5)



Dolicolide (6)

As a result of this capability, bromine-containing secondary metabolites abound in marine species, while terrestrial organisms produce mainly chlorinated compounds. In some cases, the same skeleton has been isolated from both marine and terrestrial species, the only difference being the type of halogen present (van Pee, 1996). For example, pentabromopseudilin (2) was isolated from several marine species: *Pseudomonas bromoutilis*, *Chromobacteria* and *Alteromonas*

luteoviolaceus, while the chlorinated analog, pentachloropseudilin was found in a terrestrial *Actinoplanes* species (Cavalleri et al., 1978).

Marine cyanobacteria have been shown to possess a number of unique halogen-containing functional groups like the aromatic iodo group of dolicolide (6), the trichloromethyl group of barbamide (7), the aromatic bromo groups of the rivularins (e.g. 8), the vinyl chloro group of many of the malyngamides (e.g. malyngamide A, 9, shown in Figure IV.2.), and the vinyl bromide of phormidolide (10). It is thus clear that marine cyanobacteria contain unique and distinctive metabolic capabilities to incorporate covalent halogen atoms into their natural product structures.

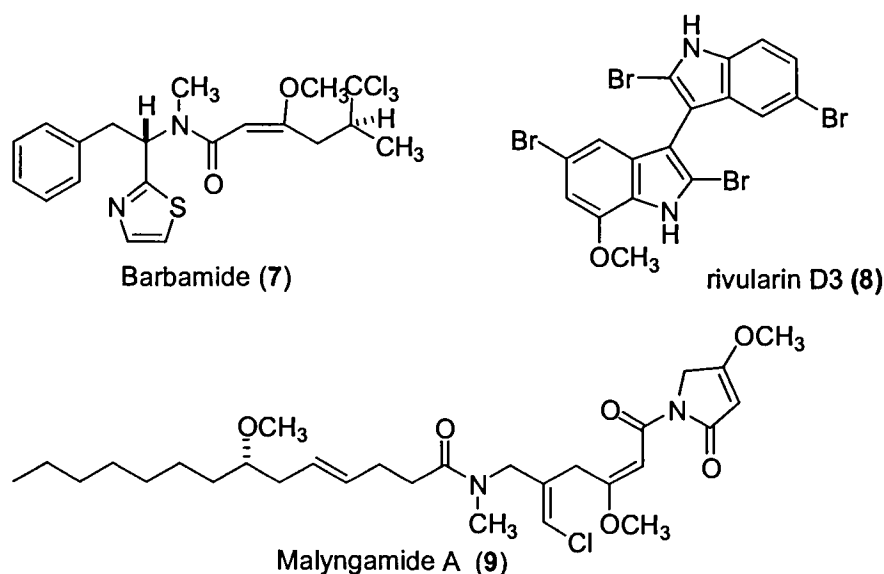
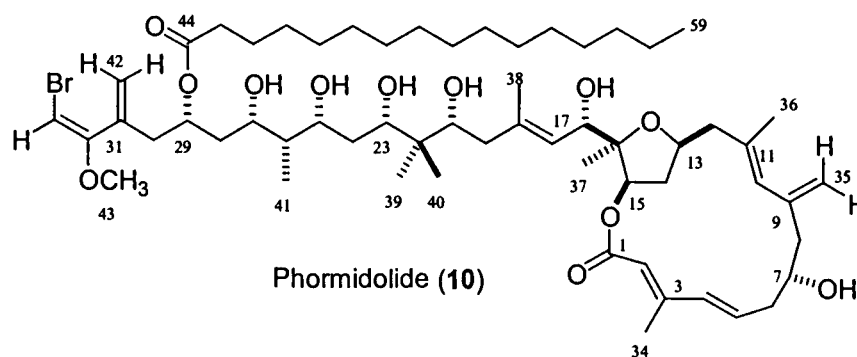


Figure IV.2. Bioactive halogenated metabolites of marine cyanobacterial origin.

Two major classes of halogenases have been described to date. The first class, which comprises most of the known enzymes, possess halogenation activity coupled with peroxidase activity and are hence called haloperoxidases. The second class was recently discovered on the basis of gene sequence analysis and

includes NADH-dependent halogenases, such as the one involved in the biosynthesis of pyrrolnitrin (Keller *et al.*, 2000; van Pee *et al.*, 2000). Several types of haloperoxidases have been characterized on the basis of their cofactor requirements: heme-containing haloperoxidases resembling cytochrome P450s, vanadium-containing haloperoxidases, and metal-free haloperoxidases (Butler and Walker, 1993; Hemrika *et al.*, 1997). Haloperoxidases have broad substrate specificities and high K_M values and are generally capable of introducing several different halogen atoms in the substrate molecule, for example chloroperoxidases can use chlorine, bromine, and iodine, depending on the ion present in the reaction mixture, while bromoperoxidases introduce bromine and iodine, but not chlorine. (Wiesner *et al.*, 1990)



As discussed in the previous chapter, phormidolide (10) is an intriguing brine shrimp toxic polyketide metabolite with a unique terminal vinyl bromide functionality and it is produced by a cultured *Phormidium* sp. The studies presented in this chapter were undertaken in order to explore the characteristics and specificity of the halogenating enzyme involved in phormidolide biosynthesis. The effects of varying the halogen content of the culture medium on phormidolide production and yield were assessed.

RESULTS AND DISCUSSION

Phormidolide production in bromine-enriched medium

Previous reports have indicated that in some cases, the concentration of bromine ions in the growth medium can be a limiting factor in the production of brominated metabolites, as for the production of pentabromopseudilin (2) by *Alteromonas luteoviolaceus*. In this case, the yield was increased 10 fold through addition of bromide to the usual culture medium (Hanefeld *et al.*, 1994).

The phormidolide producing *Phormidium* sp. was cultured in media supplemented with 0.5 g/L and 1g/L NaBr, respectively, the biomass was harvested and extracted in 2:1 CH₂Cl₂/MeOH and then the organic extract was subjected to fractionation by VLC and NP HPLC. Phormidolide yield was increased approximately 2.5 fold in both cases (50 and 54 mg, respectively, versus 19 mg) compared to the normal SW-BG11 culture conditions, which showed that bromine ion concentration in the culture medium is indeed a limiting factor in the biosynthesis of phormidolide.

Studies in bromine-depleted culture medium

For these experiments, *Phormidium* sp. was grown in a defined artificial seawater medium, MBL, instead of the normal SW-BG11 medium used in our laboratory, in order to facilitate replacement of bromine with chlorine while keeping the same ionic concentration for all other ions. Upon harvest, extraction and isolation of the product following the protocol routinely used for phormidolide isolation, a phormidolide-like peak was obtained and was subjected to characterization by NMR. The ¹H and ¹³C NMR spectra were extremely similar to those of phormidolide, except for the region between C-29 and C-33, which led to the conclusion that a phormidolide analog had been obtained. Mass spectral

analysis showed a LRFABMS peak at m/z 1021, corresponding to $[M_{(10)}+Na-Br]^+$, since the authentic phormidolide LRFABMS was at m/z 1099. The ^{13}C NMR spectrum showed two 'new' carbon signals, at 115.6 and 83.5 ppm. Inspection of the 2 D multiplicity-edited HSQC spectrum showed a correlation between two proton signals at 5.00 and 5.53 ppm and a carbon at 115.6 ppm, while in the HMBC spectrum, the 115.6 ppm carbon signal was correlated to the methyl protons at 3.59 ppm assigned to the methoxy group at C-43. The signal at 83.5 showed correlations to protons at 4.15 and 4.44 ppm, respectively in the HSQC spectrum, and a weak correlation to the same methyl protons. These correlations in conjunction with the mass spectrometry data indicated that the analog was a debromo derivative, with a terminal olefin replacing the former vinyl bromide functionality. However, closer analysis of the 2 D data revealed an additional carbon signal at 44 ppm correlated to a proton at 5.1 ppm in the HSQC spectrum, which remained unassigned until we determined that the compound under study was in fact a mixture of two very close structural analogs of phormidolide. Further HPLC separation was attempted using different columns and solvent systems, and the two analogs were successfully separated by RP HPLC on a phenyl-hexyl Luna column eluted with 100% MeOH (Figure IV.3).

The two analogs were present in 2:1 ratio, and analysis of the 1 D and 2 D NMR spectra confirmed debromophormidolide (**11**) as the major component, while the minor component was identified as the iodinated analog of phormidolide, iodophormidolide (**12**).

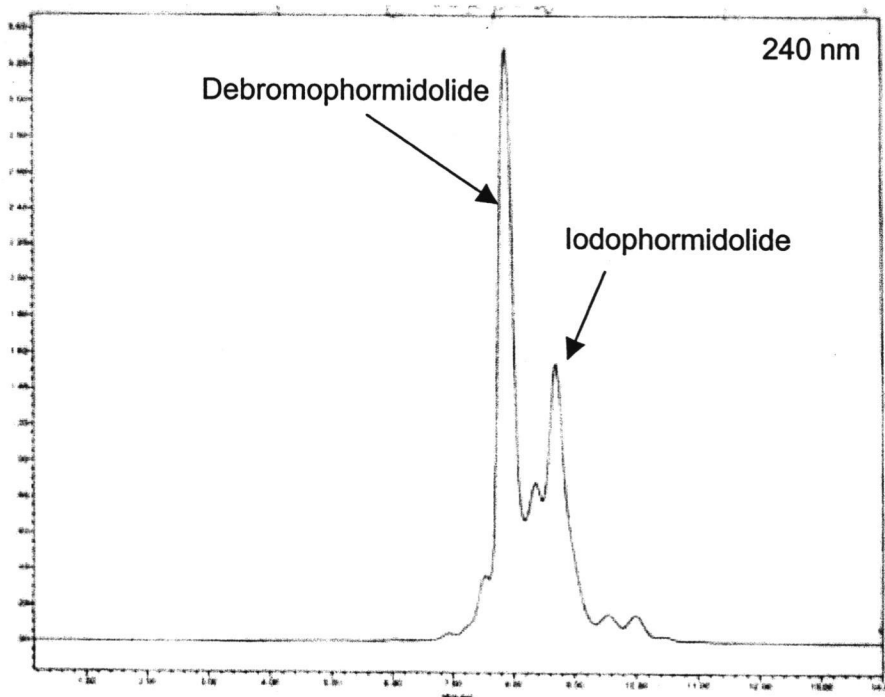
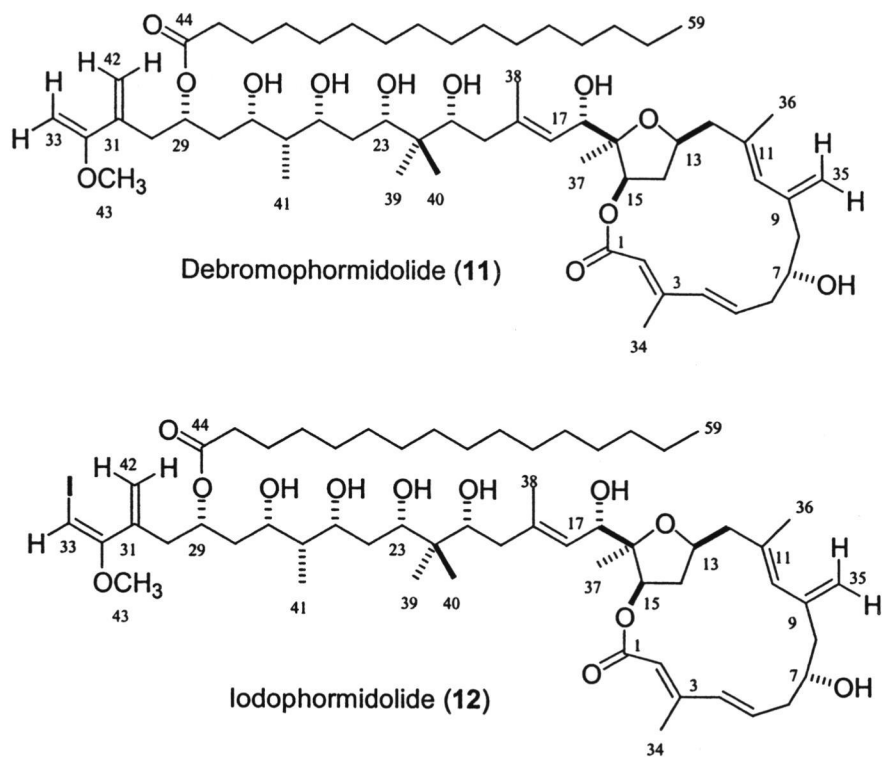


Figure IV.3. HPLC separation of debromophormidolide (11) and iodophormidolide (12).

The structures of the two analogs were determined on the basis of ^1H , ^{13}C , multiplicity edited HSQC and HMBC NMR spectra by analogy with the assigned spectra for phormidolide (Figures IV.4- IV.10) and table II.1. Key HMBC correlations are represented in Figure IV.10.

Indeed, upon submission of (12) for mass spectral analysis with a wider window, a LRFABMS peak at m/z 1147 was observed, corresponding to $[\text{M}+\text{Na}]^+$, or $[\text{M}_{(11)}+\text{I}+\text{Na}]^+$. In the NMR spectra, a diagnostic shift for the presence of iodine for C-33 at 44 ppm with an attached proton at 5.1 ppm in the HSQC spectrum combined with correlations between the proton at 5.1 ppm and C-31 (δ 139.9) and between the methyl protons at 3.59 and C-33 (δ 44) in the HMBC spectrum confirmed the assignment of the structure of iodophormidolide (12). The stereochemistry of the iodine-containing terminal double bond was determined by ROE correlations between the methoxy group H_3 -43 and the vinylic proton H-33 to be E, the same as in phormidolide (10).

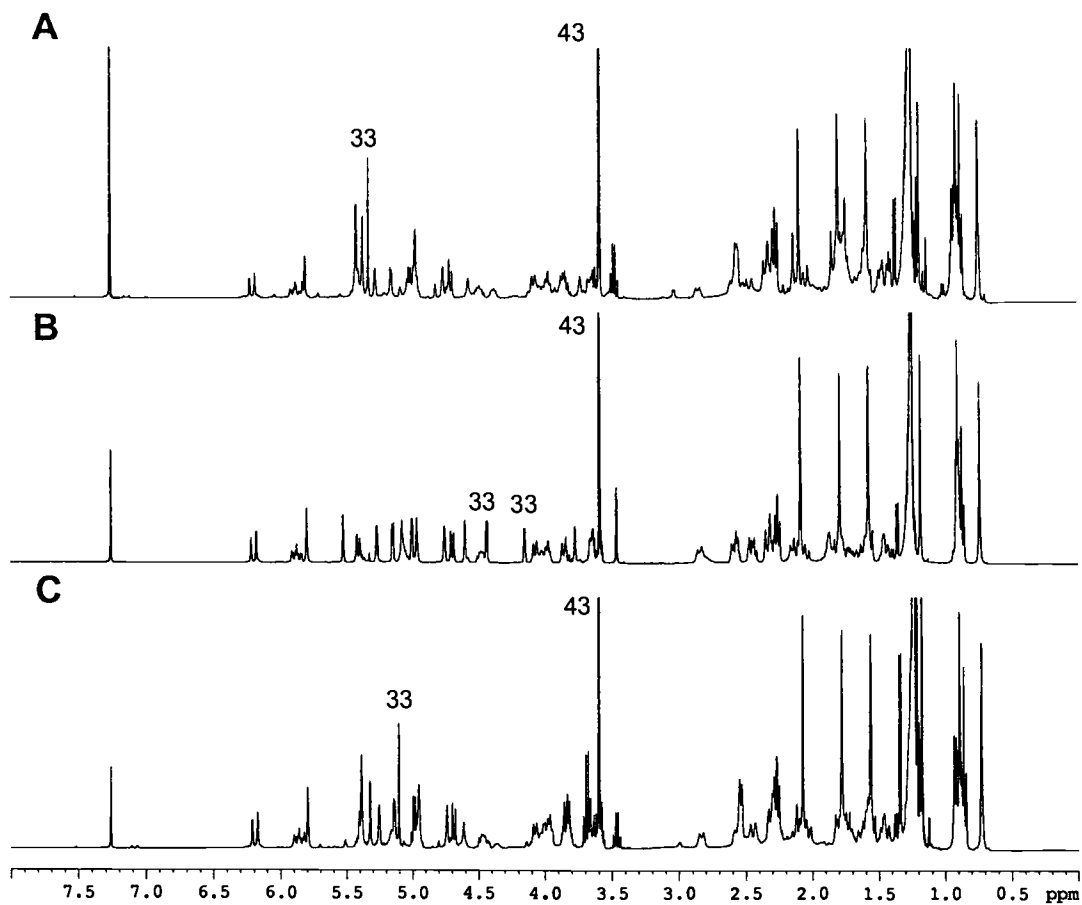


Figure IV.4. Comparative ^1H NMR spectra of phormidolide (A), debromophormidolide (B) and iodophormidolide (C).

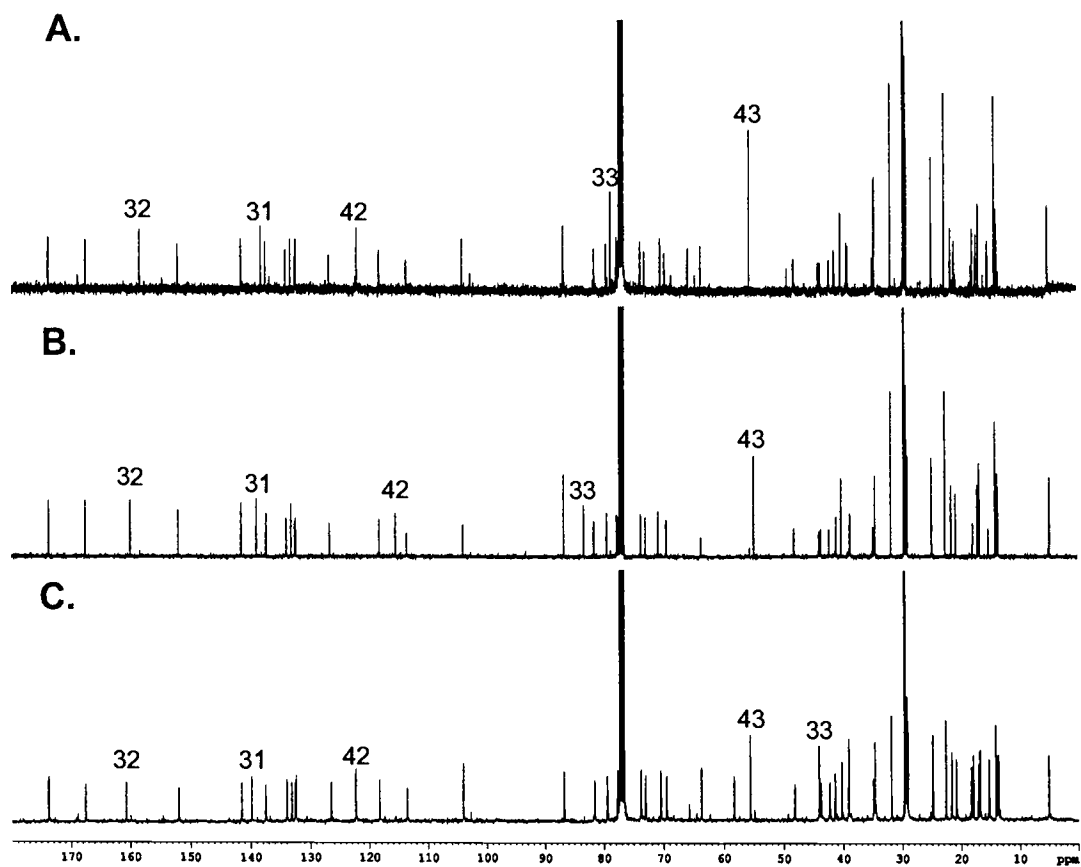


Figure IV.5. Comparative ^{13}C NMR spectra of phormidolide (A), debromophormidolide (B) and iodophormidolide (C).

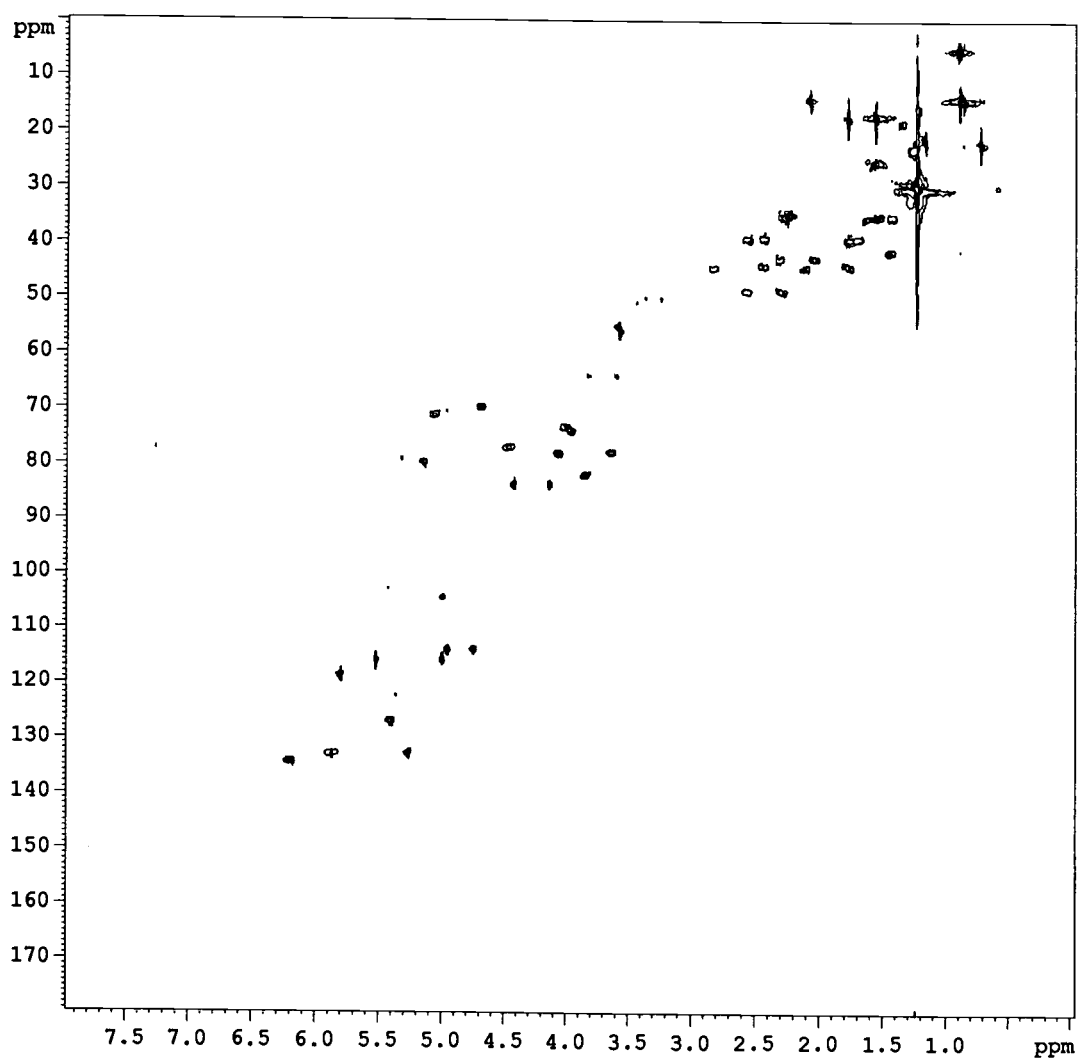


Figure IV.6. Multiplicity edited HSQC spectrum of debromophormidolide (11).

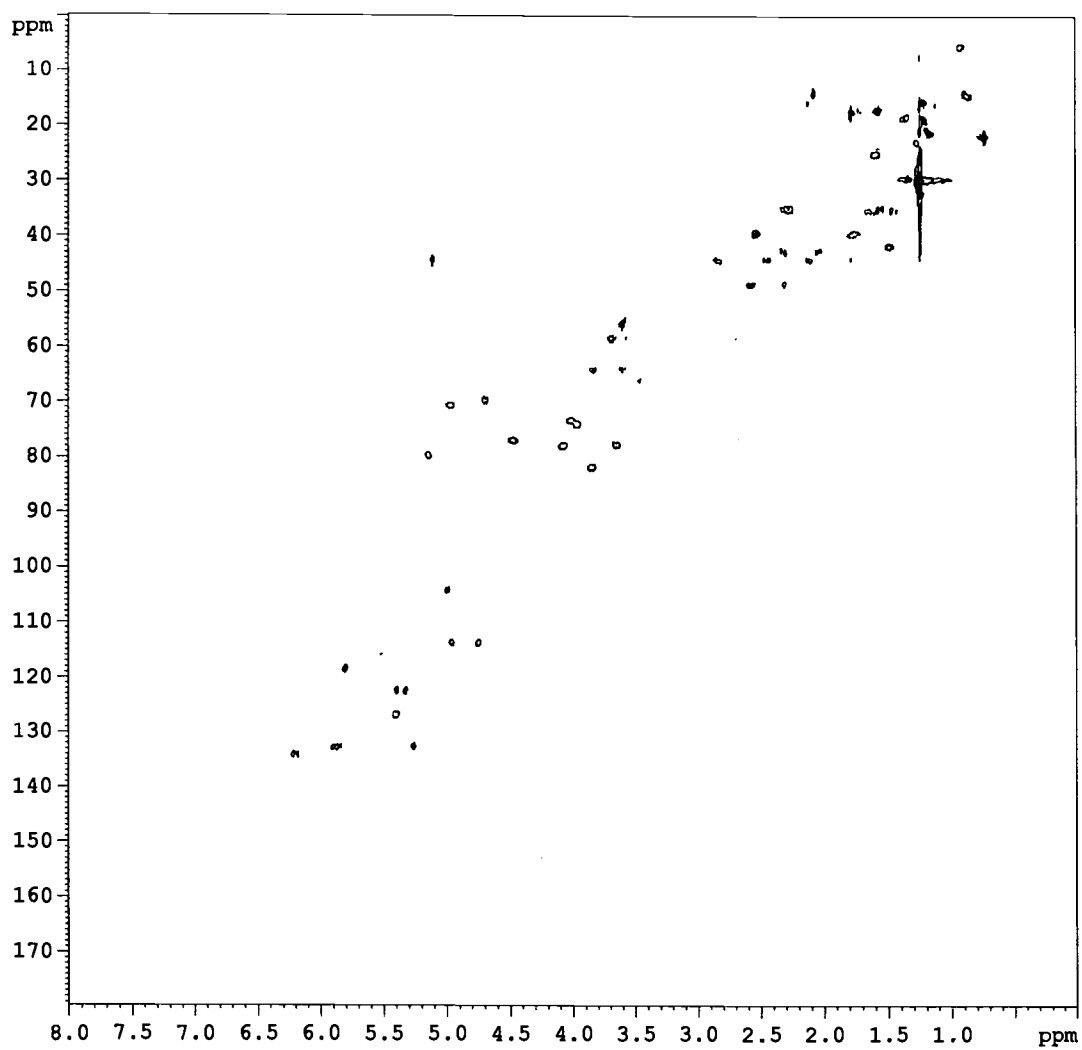


Figure IV.7. Multiplicity edited HSQC spectrum of iodophormidolide (12).

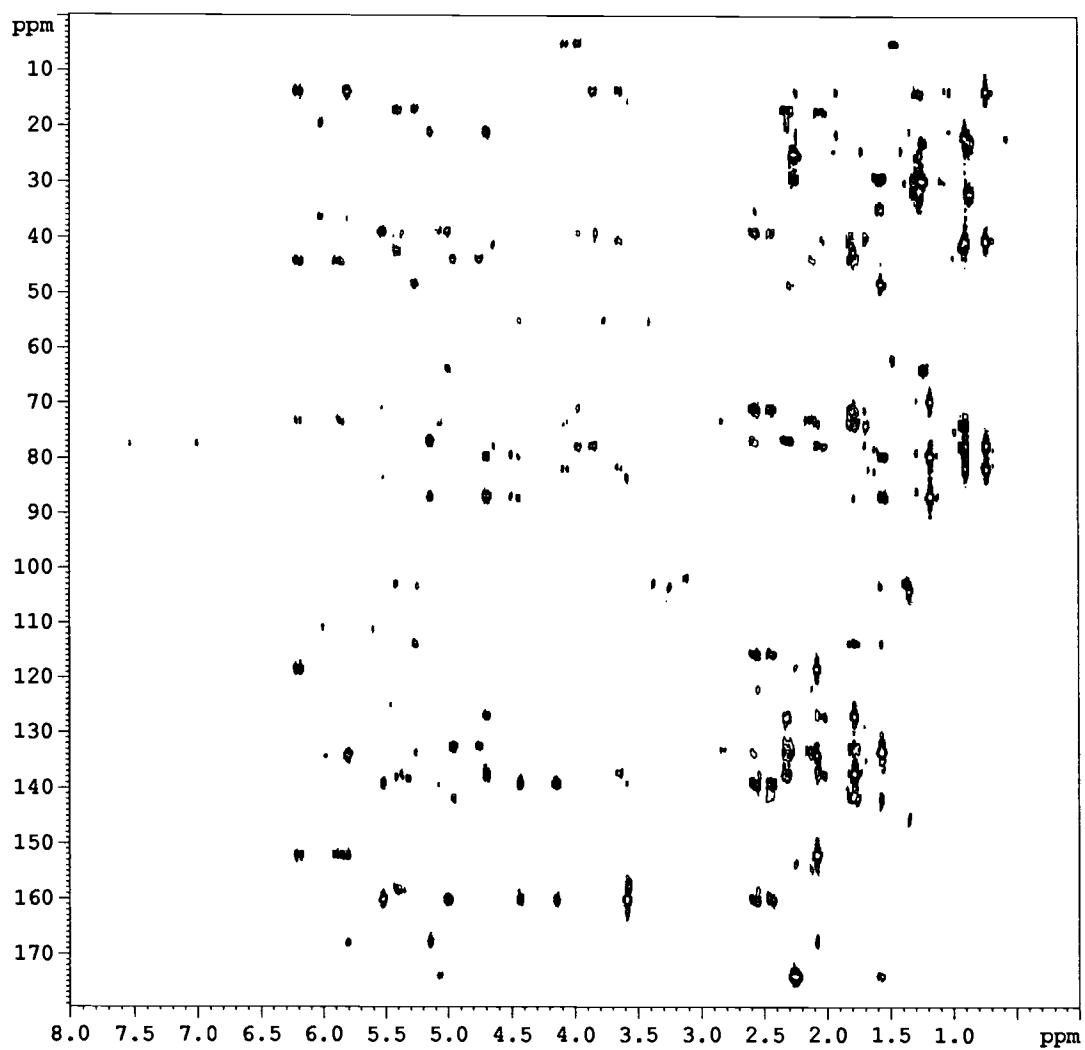


Figure IV.8. HMBC spectrum of debromophormidolide (11).

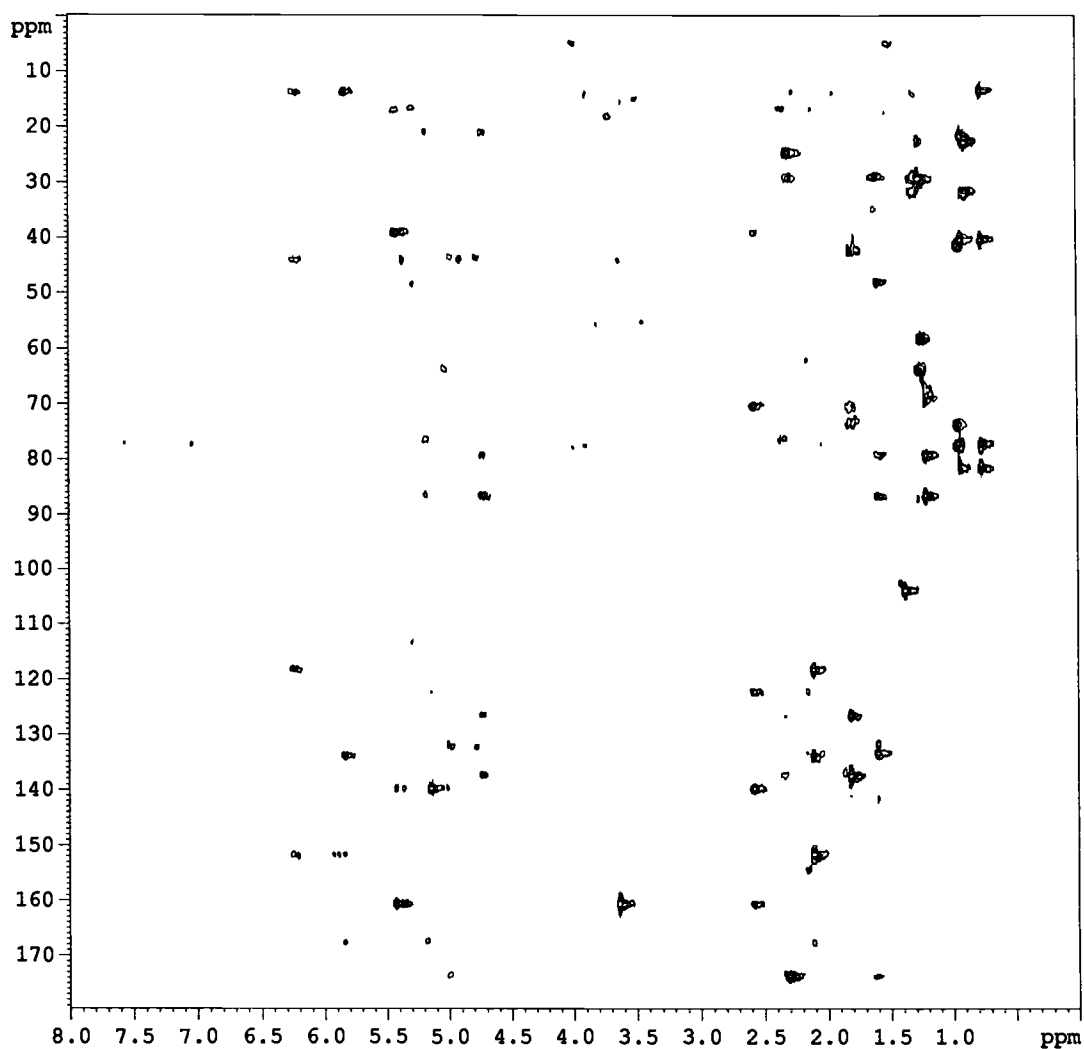


Figure IV.9. HMBC spectrum of iodophormidolide (12).

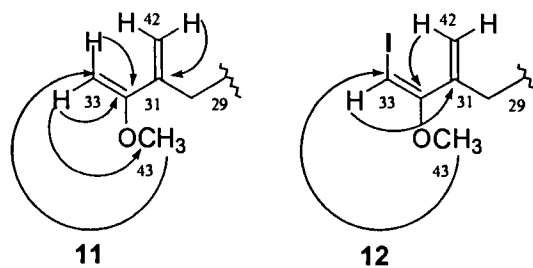


Figure IV.10. Key HMBC correlations in debromophormidolide (11) and iodophormidolide (12).

Table IV.1. Chemical shifts of phormidolide (10), debromophormidolide (11), and iodophormidolide (12).

Phormidolide			Debromophormidolide			Iodophormidolide		
1	167.5. s		1	167.5. s		1	167.5. s	
2	118.3. d	5.80	2	118.3. d	5.80	2	118.3. d	5.80
3	152.1. s		3	152.1. s		3	152.1. s	
4	134.1. d	6.20	4	134.1. d	6.20	4	134.1. d	6.20
5	132.6. d	5.88	5	132.6. d	5.88	5	132.6. d	5.88
6	44.1. t	2.85	6	44.1. t	2.85	6	44.1. t	2.85
		2.13			2.13			2.13
7	73.1. d	4.05	7	73.1. d	4.05	7	73.1. d	4.03
8	43.8. t	2.46	8	43.8. t	2.46	8	43.8. t	2.46
		1.81			1.81			1.81
9	141.6. s		9	141.6. s		9	141.6. s	
10	132.5. d	5.28	10	132.5. d	5.28	10	132.5. d	5.26
11	133.4. s		11	133.2. s		11	133.4. s	
12	48.3. t	2.33	12	48.3. t	2.33	12	48.3. t	2.33
		2.58			2.58			2.58
13	76.7. d	4.48	13	76.7. d	4.48	13	76.7. d	4.48
14	34.8. t	1.57	14	34.8. t	1.57	14	34.8. t	1.57
		2.33			2.33			2.33
15	79.6. d	5.15	15	79.6. d	5.15	15	79.6. d	5.14
16	86.9. s		16	86.9. s		16	86.9. s	
17	69.7. d	4.70	17	69.6. d	4.70	17	69.7. d	4.70
18	127.0. d	5.40	18	126.8. d	5.40	18	126.6. d	5.40
19	137.5. s		19	137.5. s		19	137.5. s	
20	42.3. t	2.06	20	42.4. t	2.06	20	42.3. t	2.06
		2.34			2.34			2.34
21	77.6. d	3.65	21	77.6. d	3.65	21	77.6. d	3.65
22	40.4. s		22	40.4. s		22	40.4. s	
23	81.7. d	3.86	23	81.7. d	3.86	23	81.7. d	3.86
24	35.1. t	1.65	24	35.1. t	1.65	24	35.1. t	1.65
		1.47			1.47			1.47
25	77.8. d	4.08	25	77.8. d	4.08	25	77.8. d	4.08
26	41.5. d	1.49	26	41.2. d	1.49	26	41.2. d	1.49
27	73.8. d	3.97	27	73.8. d	3.97	27	73.8. d	3.97
28	39.2. d	1.76	28	38.8. d	1.76	28	38.8. d	1.76
		1.79			1.79			1.79
29	70.4. d	5.0	29	70.9. d	5.0	29	70.6. d	4.97
30	39.3. t	2.57	30	38.9. t	2.57	30	38.9. t	2.57
		2.57			2.57			2.57
31	138.3. s		31	139.1. s		31	139.9. s	
32	158.4. s		32	160. s		32	160.7. s	
33	78.8. d	5.33	33	83.5 t	4.15(Z): 4.44 (E)	33	44 d	5.1
34	13.9. a	2.07	34	13.9. a	2.07	34	13.9. a	2.07
35	113.8. t	4.76	35	113.8. t	4.76	35	113.8. t	4.75
		4.98			4.98			4.96
36	16.8. a	1.58	36	16.8. a	1.58	36	16.8. a	1.58
37	21.0. a	1.19	37	21.0. a	1.19	37	21.0. a	1.19
38	17.3. a	1.80	38	17.1. a	1.80	38	17.1. a	1.80
39	13.7. a	0.91	39	13.7. a	0.91	39	13.7. a	0.91
40	21.6. a	0.74	40	21.6. a	0.74	40	21.6. a	0.73
41	5.0. a	0.92	41	4.8. a	0.92	41	4.8. a	0.92
42	122.1. t	5.37	42	115.6. t	5.53	42	122.4. t	5.39
		5.42			5.00			5.32
43	55.6. a	3.59	43	54.9. a	3.59	43	55.6. a	3.59
44	173.7. s		44	173.8. s		44	173.7. s	
45	34.6. t	2.27 2.27	45	34.6. t	2.27 2.27	45	34.6. t	2.27 2.27
46	24.9. t	1.61 1.61	46	24.9. t	1.61 1.61	46	24.9. t	1.61 1.61
47-	29.7. t	1.25 1.25	47-	29.7. t	1.25 1.25	47-	29.7. t	1.25 1.25
57	22.7. t	1.30 1.30	57	22.7. t	1.30 1.30	57	22.7. t	1.30 1.30
58	31.9. t	1.25 1.25	58	31.9. t	1.25 1.25	58	31.9. t	1.25 1.25
59	14.1. a	0.87	59	14.1. a	0.87	59	14.1. a	0.87

Spectra were recorded on a Bruker AM 400 spectrometer and referenced to CDCl₃.

Studies in bromine-depleted and iodine-enriched medium

A 1.5 L culture of *Phormidium* was grown in the bromine-depleted MBL supplemented with NaI 0.5 g/L, and in this case only iodophormidolide (**12**) was detected upon extraction and fractionation. The yield of iodophormidolide was greatly enhanced, approximately ten fold (35 mg versus 3 mg) compared with the bromine-depleted MBL treatment.

Biological activity of the phormidolide analogs

The brine shrimp toxicity assay was performed on the two phormidolide analogs (**11**) and (**12**) and the results showed that iodophormidolide was as active as phormidolide (LD₅₀ 1.5 µg/ml), while debromophormidolide was more active, with an LD₅₀ of approximately 0.5 µg/ml.

CONCLUSIONS

The studies presented in this chapter showed that bromine concentration in seawater is a limiting factor in phormidolide production by this cyanobacterium, due to the 2.5 fold increase in phormidolide yield seen upon bromine supplementation. Furthermore, the phormidolide biosynthetic pathway is constitutively expressed and is likely to be biologically relevant to the organism, since *Phormidium* grown in Br-depleted media produces 2 phormidolide analogs, a debromo derivative and an iodo derivative, introducing iodine in place of bromine from the trace amounts present in the media. Studies in conditions of iodine supplementation showed a 10 fold increase in iodophormidolide production in the absence of bromine. Due to the introduction of iodine, but not chlorine under conditions of bromine depletion, a profile previously observed in other bromoperoxidases (Ahern *et al.*, 1980; Manthey and Hager, 1981), we presume that the enzyme involved in phormidolide biosynthesis is a bromoperoxidase. This hypothesis could be investigated using a standard bromoperoxidase assay (bromination of monochlorodimedone) in the crude extract of *Phormidium* filaments.

EXPERIMENTAL

Culture conditions: *Phormidium* sp. was grown in flasks containing 1.5 L SWBG11, MBL, bromine-depleted MBL (MBL-Br) or iodine-supplemented bromine-depleted MBL medium, at 28°C with a 16 hrs light/8 hrs dark regime (5.4 to 7.0 μ Einsteins). After 120 or 130 days, cells were harvested by filtration and the biomass was frozen at - 80 °C until extraction.

Media composition for halogen ion influence studies: Since the salt formula of Instant Ocean® is proprietary, MBL's trace mineral solution recipe (Lyman and Fleming, 1940) was used to allow modification of the bromide and iodide ion concentrations of the artificial seawater in the SWBG11 medium. For Br supplementation studies, 0.5 g/L and 1 g/L NaBr were added to one flask with 1.5 L SWBG11 immediately before inoculation. For the bromine ion depleted medium, Instant Ocean® solution was replaced by MBL trace mineral solution prepared without sodium bromide, with use of replacement sodium chloride. A control experiment where 'normal', bromine-containing MBL was used in the preparation of the SWBG11 culture medium was also undertaken in order to verify culture viability. For the iodine-enriched medium, the previously prepared MBL-Br was supplemented with 1g/L sodium iodide.

Extraction protocol: The biomass was ground with mortar and pestle in 2:1 CH₂Cl₂/MeOH, infused at room temperature 40 min. in the same solvent, then boiled 2 x 15 min. in fresh solvent. The resulting extracts were combined and dissolved in ether, yielding the total organic extract.

Isolation of phormidolide, debromophormidolide and iodophormidolide: In a typical experiment the crude extract was then subjected to VLC (TLC grade silica gel) with a range of solvents (0 to 100% EtOAc/hexanes followed by MeOH wash). The fraction eluted with 100% EtOAc was further fractionated in NP- HPLC (dual

Phenomenex Luna 10 μm silica, 2 x 250 mm x 4.6 mm) with a 60% Hexanes/ 35% EtOAc/ 5% IPA solvent system, 4.5 ml/minute flow rate to yield phormidolide or the phormidolide-type product mixture. The mixture of debromophormidolide and iodophormidolide was then subjected to RP-HPLC on a phenylhexyl Phenomenex column (0.7 ml/min 100 % MeOH, Luna 5 μ) and yielded the pure compounds **11** (6 mg) and **12** (2.8 mg).

Debromophormidolide (11): pale yellow oil; $[\alpha]^{23}_{\text{D}} + 31$ (*c* 0.2, CHCl_3); UV (MeOH) λ_{max} 236 nm (log ϵ 4.43), 270 nm (log ϵ 4.21); IR (neat) 3395, 2917, 2849, 1717, 1442, 1374, 1311, 1229, 1083 cm^{-1} ; ^1H and ^{13}C NMR data, see Table IV.1; LRFABMS (positive ion, 3-nitrobenzylalcohol) observed $[\text{M}+\text{Na}]^+$ at m/z 1021.

Iodophormidolide (12): pale yellow oil; $[\alpha]^{23}_{\text{D}} + 33$ (*c* 0.21, CHCl_3); UV (MeOH) λ_{max} 236 nm (log ϵ 4.49), 270 nm (log ϵ 4.2); IR (neat) 3342, 2921, 2849, 1722, 1591, 1442, 1321, 1137, 1088, 1035 cm^{-1} ; ^1H and ^{13}C NMR data, see Table IV.1; LRFABMS (positive ion, 3-nitrobenzylalcohol) observed $[\text{M}+\text{Na}]^+$ at m/z 1147.

Brine shrimp toxicity assay: The brine shrimp toxicity assay was performed as previously described, using *Artemia salina* (Meyer *et al.*, 1982). Extracts were used at five concentrations (100, 10, 1 and 0.1 ppm) in duplicate experiments. Results (dead and live shrimp) were tallied 24 hr after the introduction of the extracts.

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CHAPTER FIVE

PHORMIDAMIDE, A NOVEL CYTOTOXIC BROMINATED PEPTIDE FROM
A CULTURED MARINE *PHORMIDIUM* SP.

ABSTRACT

A novel halogenated cytotoxic peptide, phormidamide (9) has been isolated in the course of biosynthetic studies on the toxic macrolide phormidolide (8) in a cultured *Phormidium* sp. originally collected from Indonesia. Extensive NMR spectroscopy experiments on phormidamide at natural abundance and isolated from feedings of labeled precursors have led to the proposed structure (9), but final verification could not be obtained. The structural elucidation has been challenging due to the high number of quaternary carbons present in the phormidamide skeleton, generated from multiple halogen, ketide and hydroxyl group substituents. X-ray crystallographic data acquisition is being awaited for confirmation of the planar structure and elucidation of the stereochemistry. Phormidamide is potently cytotoxic to mouse neuroblastoma cells with an LD₅₀ of 1 µg/ml.

INTRODUCTION

Marine cyanobacteria have long been established as rich sources of chemically diverse bioactive metabolites. A major theme in the secondary metabolites isolated from marine cyanobacteria is constituted by the production of structurally unique toxic peptides derived from mixed PKS - NRPS metabolic pathways (Gerwick *et al.*, 2001). Another characteristic of marine cyanobacterial metabolism is the high incidence of halogenated, especially brominated, metabolites (see chapter 4).

Frequently, one cyanobacterial collection is found to produce several bioactive metabolites pertaining to different chemical classes. For example, a single strain of *Lyngbya majuscula* collected from Hector bay, Jamaica and cultured in our laboratory yielded two types of bioactive metabolites - hectochlorin (1, Marquez *et al.*, 2001) and the jamaicamides A-C (2-4, Nogle, PhD Thesis, 2002). Hectochlorin (1) is an antifungal and actin polymerization inhibitor and jamaicamides A and C display sodium channel blocking activity against a mammalian neuroblastoma cell line (Edwards *et al.*, in preparation). Structurally, hectochlorin (1) is a chlorinated lipopeptide similar to dolabellin (5) isolated from *Dolabella auricularia* (Sone *et al.*, 1995) and the lyngbyabellins (6,7) from *L. majuscula* (Luesch *et al.*, 2000; Milligan *et al.*, 2000) while the jamaicamides are a chemically distinct group of molecules bearing a slight structural resemblance to the malyngamide family of *L. majuscula* metabolites (Cardellina *et al.*, 1978; Ainslie *et al.*, 1985; Gerwick *et al.*, 1987; Orjala *et al.*, 1995; Wu *et al.*, 1997; Milligan *et al.*, 2000), as shown in Figure V.1.

In the course of the biosynthetic investigations of the brominated macrolide phormidolide (8), described in chapter 3, a comparatively minor UV-active component was observed by TLC and UV-detected NP-HPLC. Further fractionation by HPLC led to the isolation of phormidamide, a novel highly

modified halogenated peptide. Phormidamide showed potent cytotoxicity ($LC_{50} = 1 \mu\text{g/ml}$) in the mouse neuroblastoma cell assay run routinely in our laboratory.

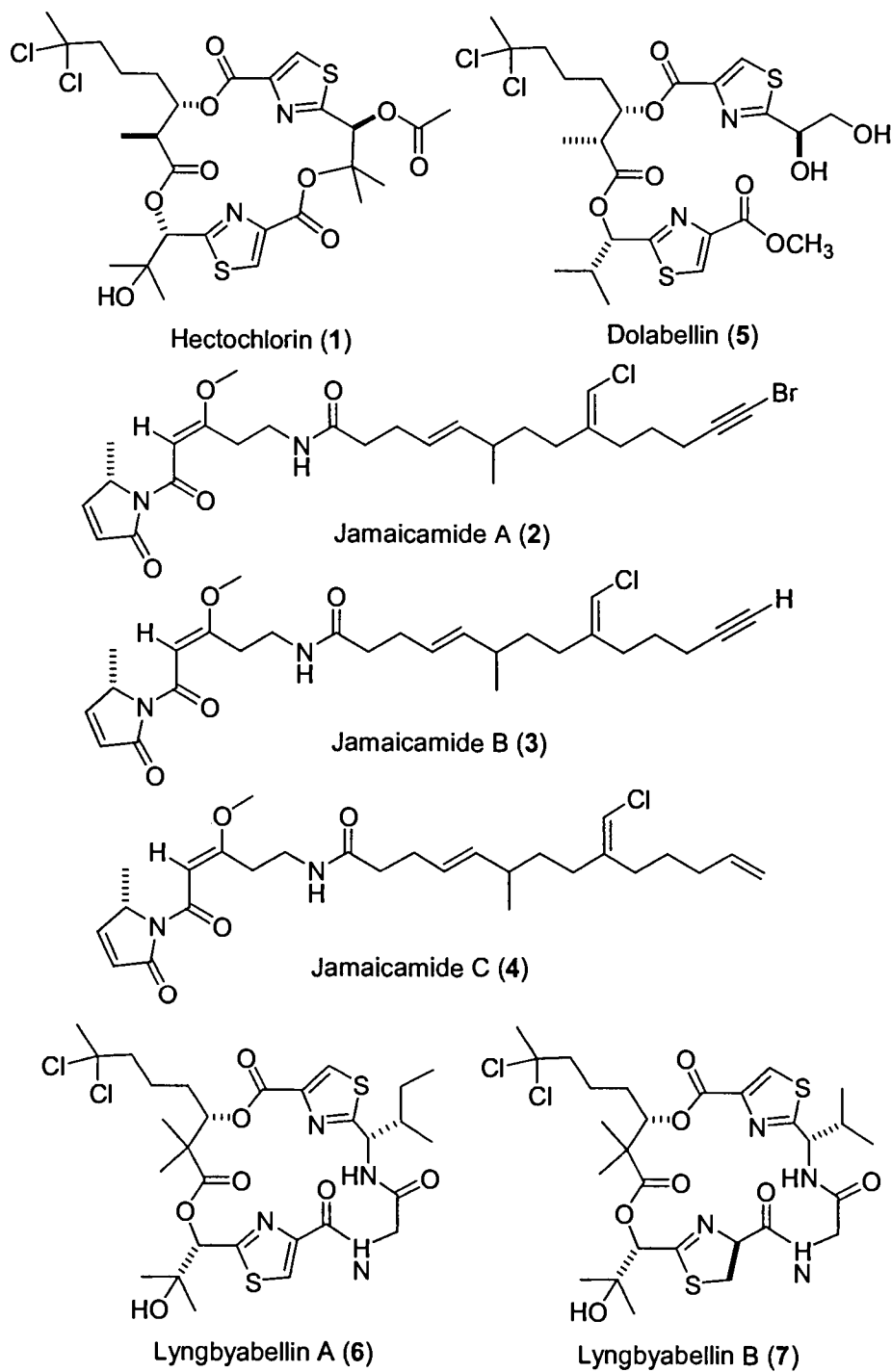
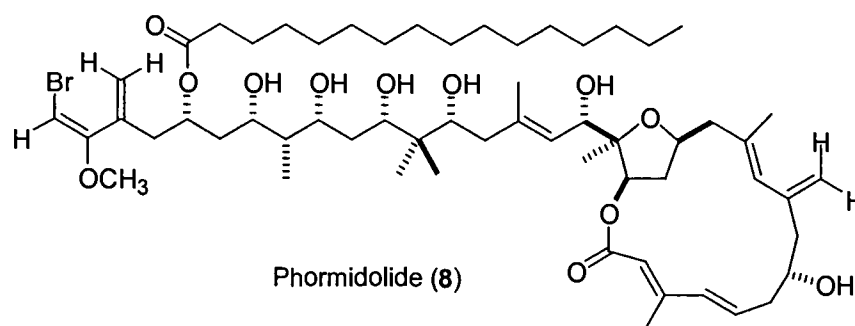


Figure V.1. Bioactive marine halogenated peptides.



To our knowledge, phormidolide (8) was the first bioactive secondary metabolite reported in the literature from the genus *Phormidium*, despite the fact that this genus is relatively widespread in the aqueous environment and belongs to the same family, Oscillatoriaceae, as *Lyngbya*, from which several hundred structurally interesting and bioactive compounds have been reported. This work shows that the genus *Phormidium* also possesses biosynthetic capabilities for the production of unique secondary metabolites, and thus further chemical, biosynthetic and genetic investigations are warranted.

This chapter presents the isolation, structural investigation and proposed structure of phormidamide, a novel cytotoxic brominated and chlorinated cyclic peptide obtained from the same *Phormidium* sp. strain that produces phormidolide.

RESULTS AND DISCUSSION

The marine cyanobacterium *Phormidium* sp. was collected in 1995 from Indonesia and a live sample was adapted to laboratory culture conditions and yielded the novel brine shrimp toxic polyketide-derived macrolide, phormidolide (8, Williamson *et al.*, 2002). The same cultured strain was used in studies on the biosynthesis of phormidolide described in chapters 3 and 4. In the course of these investigations, an additional more polar UV-active material was observed on the TLC plate in the phormidolide-containing silica gel vacuum liquid chromatography (VLC) fraction. This late eluting material was subsequently collected separately in the NP HPLC separation routinely used for phormidolide isolation and subjected to a final purification over NP HPLC in 94% EtOAc/isopropyl alcohol to yield phormidamide (9).

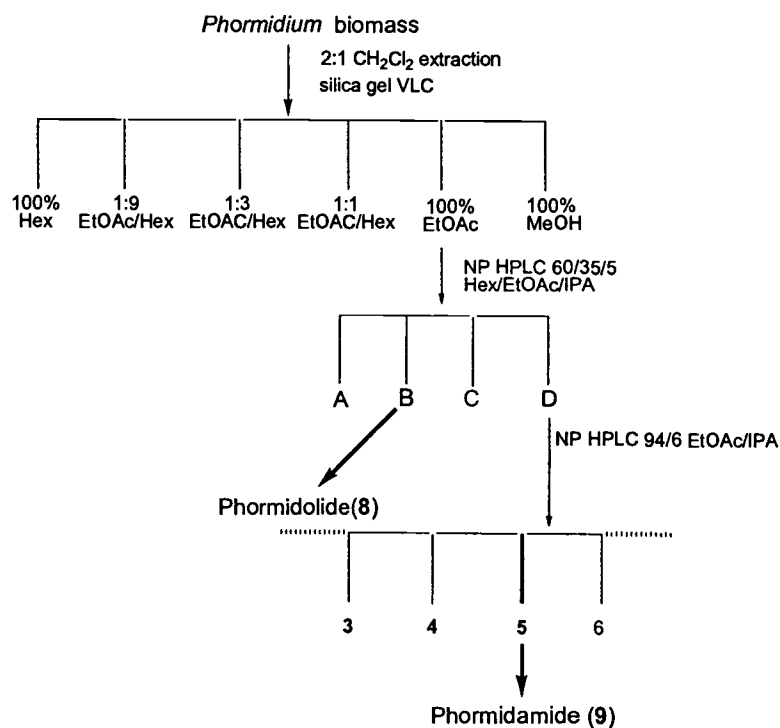


Figure V.2. Typical isolation and purification of phormidamide (9).

Phormidamide was isolated as a clear oil (evaporated to dryness from methanol) or a white powder (from ethyl acetate) and showed a LRFABMS peak at m/z 919.1 in 3-nitrobenzoic acid that was initially presumed to represent $[M+H]^+$ but proved subsequently to correspond to $[M+Na]^+$ upon further analysis by FABMS in a different matrix (oxalic acid/thioglycerol/glycerol = OTG), when a base peak with m/z 897.1 (a difference of 22) was obtained for $[M+H]^+$. MALDI-TOF mass spectrometry confirmed this interpretation, since both molecular species are present in the mass spectrum (Figure V.3.). The molecular ion species showed a complex pattern indicative of multiple halogen atoms in the molecule, which was consistent with the presence of either Br_2Cl or $BrCl_3$ in (9).

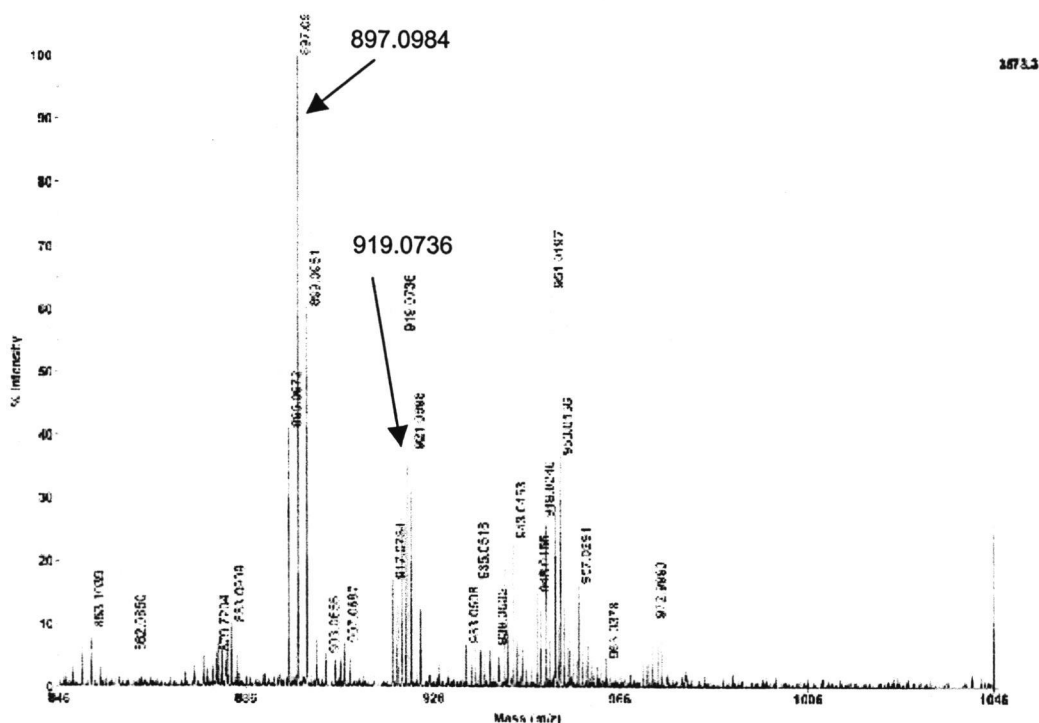


Figure V.3. MALDI-TOF spectrum of phormidamide (9).

Inspection of the ^1H and ^{13}C NMR spectra (Table V.1 and Figures V.4, V.5) allowed for the identification of 35 carbon resonances in phormidamide, including 7 quaternary carbons with chemical shifts between δ 165-186 ppm, indicative of carbonyl carbons involved in ester or amide bonds.

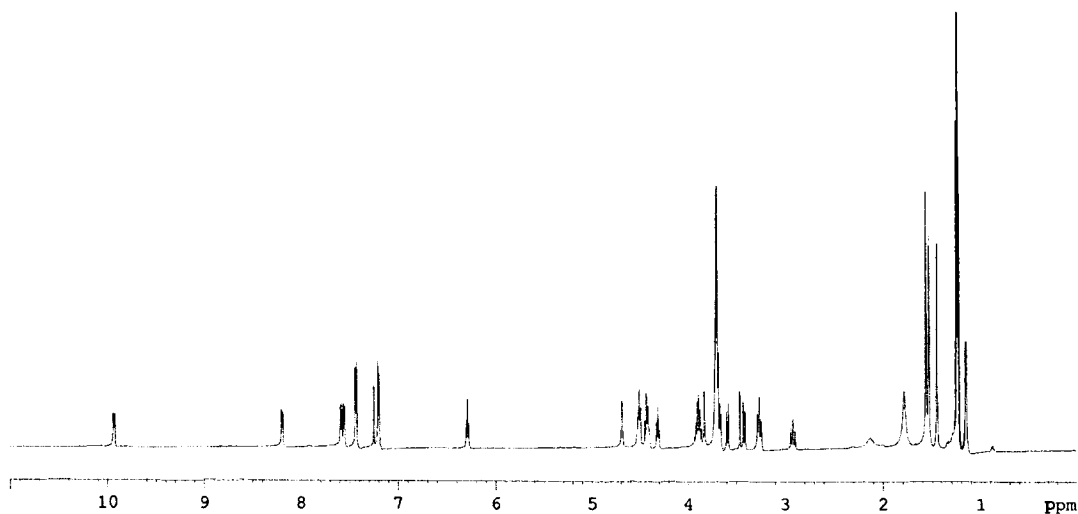


Figure V.4. ^1H NMR spectrum of phormidamide (9).

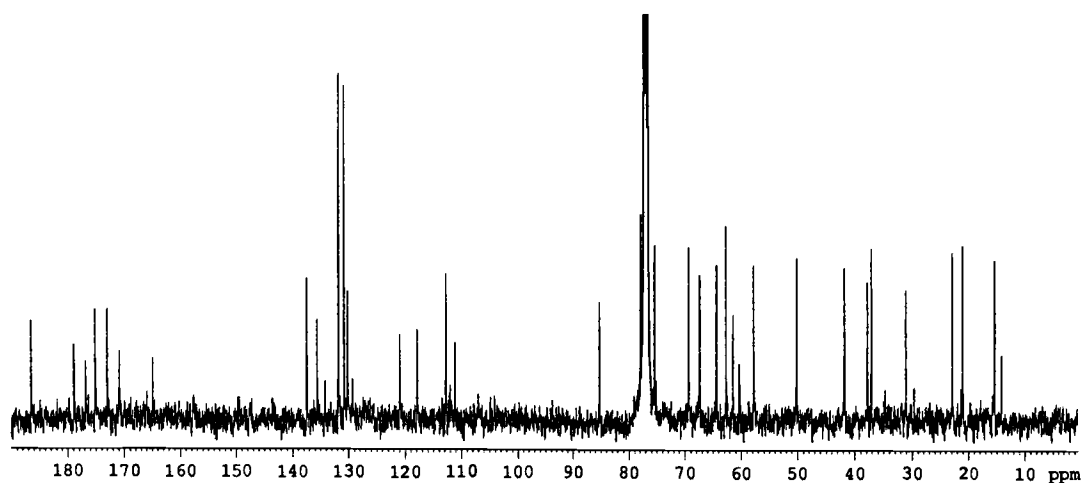


Figure V.5. ^{13}C NMR spectrum of phormidamide (9).

The spectra also exhibited aromatic resonances in the region 120-135 ppm in the carbon spectrum characteristic for a substituted phenylalanine moiety, as well as two ^1H NMR shifts of δ 8.07 and δ 10.1 ppm, respectively, consistent with protons attached to nitrogen. The quaternary oxymethines in conjunction with the phenylalanine-type resonances and the presence of amide protons suggested that phormidamide is derived from a NRPS metabolic pathway. The compound was thus submitted for amino acid analysis, but the results were negative; no 'conventional' amino acids were identified.

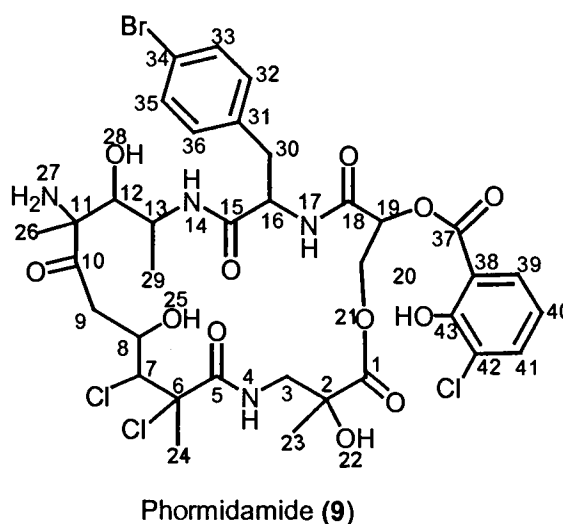


Figure V.6. Proposed structure of phormidamide (9).

The challenge in the structural elucidation of phormidamide is derived from the exceptionally high number of quaternary carbons combined with the abundance of heteroatoms (14 quaternary carbons out of a total number of 35 and 20 heteroatoms proposed on the basis of the molecular ion pattern in the mass spectrum, number of valences of quaternary carbons to fulfill and the downfield chemical shifts of the carbonyl carbons that are indicative of ester or amide

functions). Based on the mass spectrometry, NMR and chemical modification results the proposed structure of phormidamide is shown in Figure V.6, corresponding to the molecular formula $C_{35}H_{42}BrCl_3N_4O_{12}$, with exact mass m/z of $[M+H]^+$ 895.1126.

Initial interpretation of the 1D 1H and ^{13}C NMR data as well as the 2D multiplicity edited HSQC, HMBC and HSQC-TOCSY spectra permitted assembly of several partial structures: the bromophenylalanine moiety in the region N14 to C18, fragments C18 - C19 - C20 - C37 and N14-C13-C29-C12. Furthermore, a few additional groups of atoms were identified from the HMBC data within which the direct connectivities could not be assigned: C1-C2-C3-C23, C5-C6-C7-C8-C9-C24, C10-C11-C12-C26 and the aromatic ring with a conjugated carbonyl carbon C37-C43 (Table V.1 and Figures V.7 - V.9).

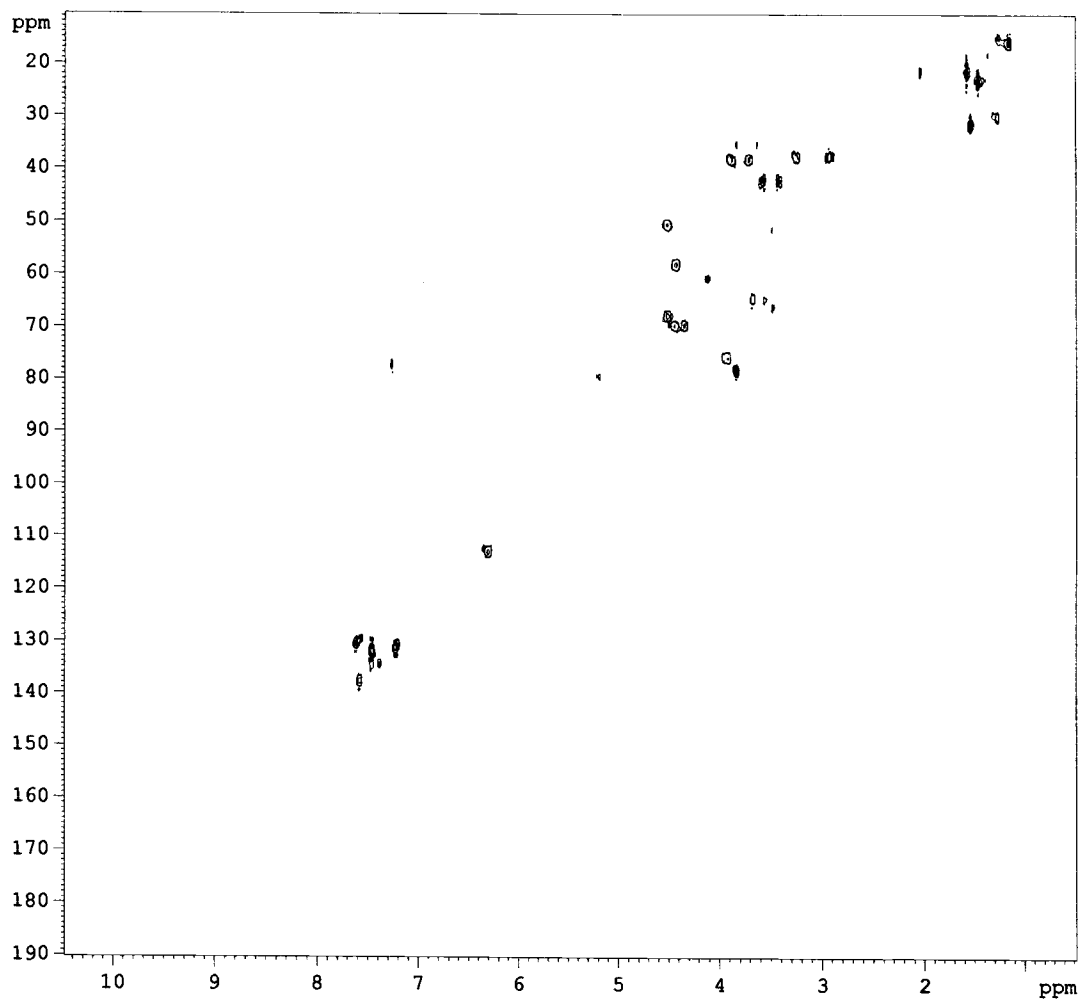


Figure V.7. Multiplicity edited HSQC spectrum of phormidamide (9).

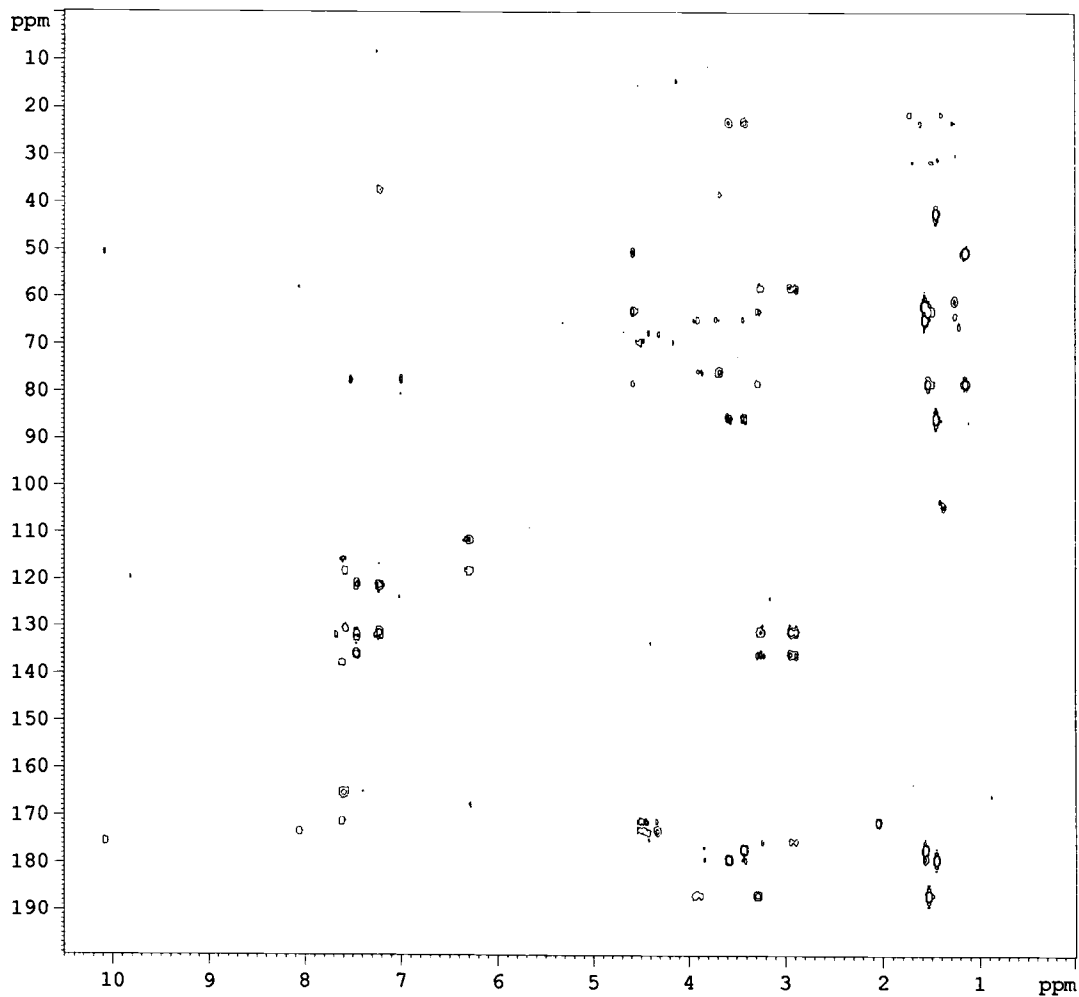


Figure V.8. HMBC spectrum of phormidamide (9).

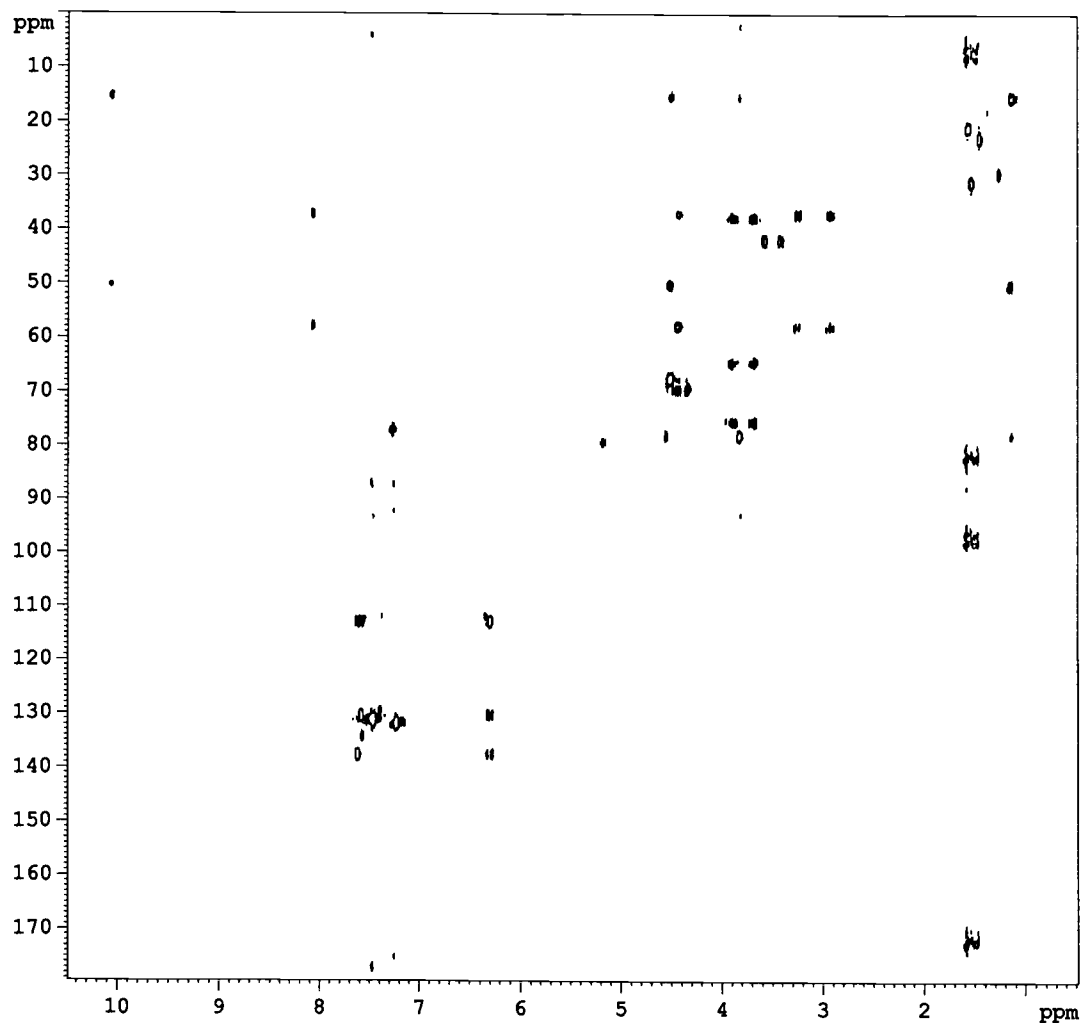


Figure V.9. HSQC - TOCSY spectrum of phormidamide (9).

In order to overcome this early impasse, phormidamide was isolated from a culture supplemented with $[U-^{13}C]$ glucose and an INADEQUATE spectrum was recorded (Figure V.10). Interpretation of the INADEQUATE data combined with the HMBCs allowed unambiguous connectivities to form 6 partial structures, A through F, which comprised the entire carbon skeleton of phormidamide, depicted in Figure V.11.

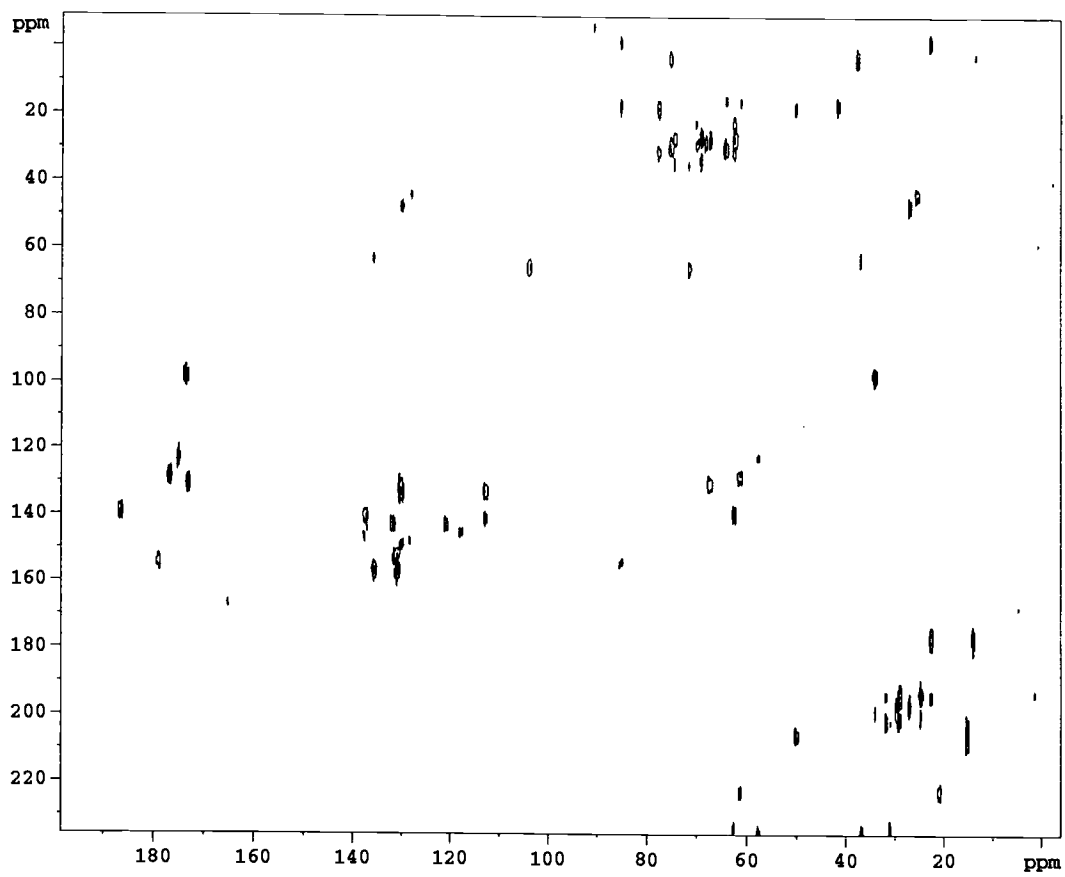


Figure V.10. ^{13}C NMR INADEQUATE spectrum of phormidamide (9).

Table V.1. NMR spectral data for phormidamide (9) in CDCl₃.

position	¹³ C	¹ H (J in Hz)	HSQC-TOCSY	HMBC ^a	INADEQUATE
1	178.9, s				2
2	85.4, s				1, 3, 23
3	41.8, t	3.42 d (11.7) 3.59 d (11.7)		1, 2, 5, 23	2
4					
5	176.8, s				6
6	61.5, s				5, 7, 24
7	64.4, d	3.67 d (8.1)	8, 9	6, 8, 9, 24	6, 8
8	75.4, d	3.92	7, 9	7, 9, 10	7, 9
9	37.8, t	3.72 dd (1.7, 10) 3.88 dd (9.5, 10)	7, 8, 20	7, 8, 10, 11	8
10	186.5, s				11
11	62.7, s				10, 12, 26
12	77.9, d	3.84	OH-28, 13, 29	26	11, 13
13	50.2, d	4.51 m	12, 29	11, 12, 29	12, 29
NH-14		10.1 d (7.7)	12, 13, 29	13, 15, 29	
15	175.2, s				16
16	57.8, d	4.42 m	NH-14, 16, 30	15, 18, 30	15, 30
NH-17		8.07 d (7)		16, 18, 30	
18	173, s				19
19	67.4, d	4.509 m		18, 20, 37	18, 20
20	69.4, t	4.34 m 4.44 m		18, 19, 37	19
21					
22					
23	22.9, q	1.45		1, 2, 3	2
24	21.1, q	1.56		1, 5, 6, 7	6
25					
26	31.1, q	1.53		10, 11, 12	11
NH-27		3.29		10, 11, 12	
OH-28		4.59 d (1.5)		10, 11, 12, 13, 26	
29	15.4, q	1.14 d	12, 13, 30	12, 13	13
30	37.1, t	2.92 dd (14.2, 11.6) 3.26 m	16	15, 16, 31, 32, 36	16, 31
31	135.7, s				30, 32, 36
32	130.9, d	7.22 d (8.2)		30, 32, 34	31
33	131.9, d	7.46 d (8.2)		32, 34, 35, 36	34
34	121, s				33, 35
35	131.9, d	7.46 d (8.2)			34
36	130.9, d	7.22 d (8.2)			31
37	171, s				
38	117.9, s				39
39	137.5, d	7.58 dd (7.8, 1.9)		37, 38, 41, 43	38, 40
40	112.8, d	6.3 t (7.8)	39, 41	38, 39, 41, 42	39, 41
41	130.2, d	7.62 dd (7.8, 1.9)	39, 40	37, 38, 39, 43	40
42	111.2, s				43
43	165, s				42

^a Proton showing HMBC correlation to indicated carbon.

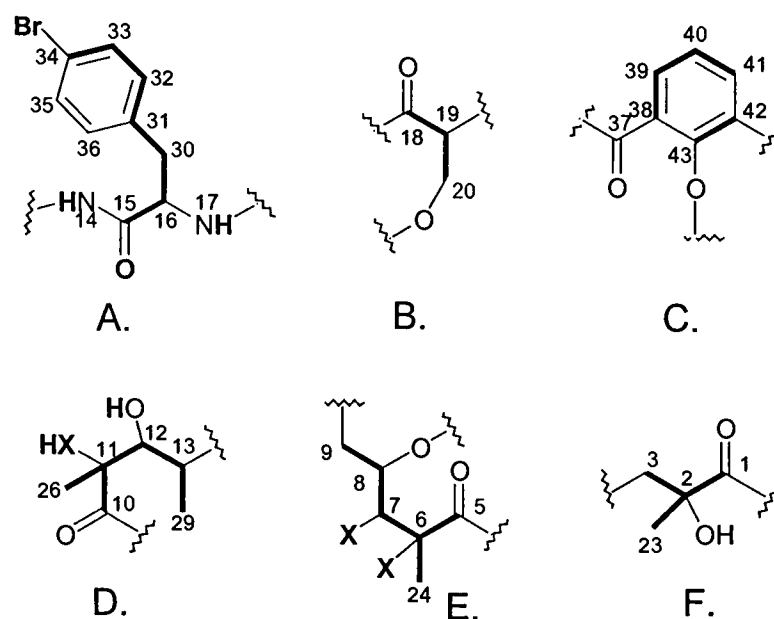


Figure V.11. Partial structures of phormidamide (9) from the INADEQUATE data. Connected carbons are represented with bold lines, and observed protons on heteroatoms in the HMBC spectrum are also bold.

The protons placed on heteroatoms, presumably O-28 at C-12 and N-27 at C-21, were observed in the HMBC spectrum and also partly confirmed by a qualitative-scale acetylation of phormidamide, which yielded a derivative with one acetyl group (δ 2.15). It is conceivable that steric hindrance protects the phenolic hydroxyl group at C-43 from acetylation, while the hydroxyl at C-2 is geminal to a methyl group which would hinder acetylation. Methylation with diazomethane was also attempted, but no methylated product resulted, which suggested free carboxylic acid groups are absent in phormidamide.

HMBC correlations permitted connecting partial structures A and D through correlations between the amide proton at N14 and C-13, C-29, C-12 and A and B through HMBC correlations between the amide proton at N-17 and the carbonyl carbon C-18. Thus, three of the previous 6 partial structures could be connected, leaving only 4 fragments. Furthermore, HMBC correlations could be

observed between the proton attached to C-8 and the carbonyl C-10, as well as between the protons at C-9 and C-10 and the proton at δ 3.29 ppm attached to N-27 and C-10. Another key HMBC was from the protons on C-3 to the carbonyl C-5 and from the protons on C-19 and C-20 to the carbonyl C-37, as depicted in Figure V.12.

The next challenge was assigning the heteroatoms (nitrogen, chlorine and oxygen) on the quaternary carbons and methine groups that were in close vicinity by HMBC but could not be directly connected due to chemical shift considerations. Unfortunately, despite numerous types of HMBC experiments, the carbonyl C-1, the proposed phenolic C-43 and the methylene C-20 could not be connected to any resonances in the spectrum except for those already shown and confirmed by the INADEQUATE experiment, and thus, the proposed structure of phormidamide could not be unequivocally established.

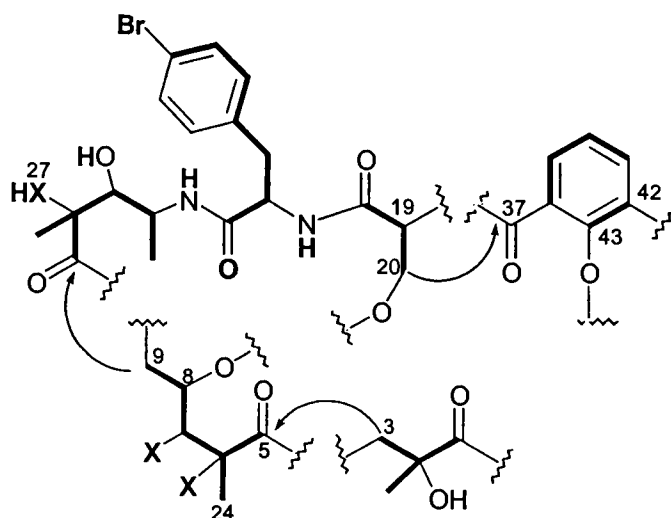


Figure V.12. Partial structures of phormidamide with key HMBC correlations represented by arrows.

In arriving at the final proposed structure of phormidamide (9) depicted in Figure V.6, nitrogen N-4 was inserted between C-3 and the carbonyl C-5 due to the upfield shift of C-5 at δ 179 ppm, incompatible with a ketone, and C-3 (δ 41.8), while the heteroatoms at C-6 and C-7 were assigned as chlorine, which is compatible with the observed downfield shifted resonances (δ 61 and 64 ppm, respectively). Another possible functional group consistent with these chemical shifts would be an epoxide bridge between C-6 and C-7, but this alternative would leave out the two chlorine atoms and determine a significant (54 units) difference in mass. The closure of the lactone ring C20-O21-C1 was proposed due to a) the chemical shift of H₂-20 at 4.34 and 4.44 ppm, consistent with an ester bond rather than a primary alcohol functionality and b) the probable absence of a free carboxylic acid group as determined from the compound's resistance to methylation. The free amine functionality at N-27 was proposed on the basis of mass and confirmed through an ¹⁵N HSQC experiment, with the other proton present at 1.53 ppm, buried under the methyl peak of C-26. An alternative could have been a sulfur atom in position 27 instead of nitrogen, but the expected characteristic stretch for an SH was not observed in the IR spectrum. Furthermore, isolation of secondary metabolites with an intact reduced sulfhydryl group is highly unlikely after the lengthy process of isolation and purification used for obtaining phormidamide, as well as unprecedented in the literature. This alternative would have also necessitated the closure of another lactone ring in order to keep within the observed mass.

The remaining chlorine atom was placed on C-42, since it is conceivable that in this highly unusual system, with the contribution of conjugation to the exocyclic carbonyl C-37, the carbon resonance of C-42 could be shifted upfield as far as 111 ppm. We had initially envisaged the presence of a bromine atom at C-42 in order to explain this upfield shift, but the molecular ion pattern of the

compound is not as compatible with Br_2Cl , the molecular mass does not accommodate another bromine atom, and Br_2Cl instead of BrCl_3 would leave one valence unoccupied on one of the quaternary carbons C-6 or C-7. If the possibility of an epoxide bridge at C6 - C7 and a bromine atom at C-42 is considered, there is no chlorine left in the molecule and the molecular ion pattern as well as a mass difference of 11 units exclude this possibility.

Due to the crystalline nature of phormidamide, and the exhaustion of all conceivable NMR experiments that could assist in its final structure elucidation, a sample was submitted for X-ray crystallographic analysis. The first attempt at collecting data was not successful because the crystals obtained were too small to afford quality data collection. A second attempt on a higher resolution instrument could completely solve the structure, as well as establish the stereochemistry, which would be extremely difficult to tackle through spectroscopic methods.

As an interesting note, in the course of repeated isolation efforts for obtaining more phormidamide for NMR spectroscopy and crystallization, (9) was isolated from the bromine-supplemented cultures discussed in Chapter 4 and the yield was increased four fold (15 and 16 mg versus 4 mg in 'normal' SW-BG11). Isolation was also attempted from the culture grown in bromine-depleted MBL, but phormidamide could not be detected after NP-HPLC in this instance.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 243 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. All NMR spectra were recorded on Bruker DRX600 and AM400 spectrometers with the solvent CDCl_3 used as an internal standard (δ_{C} 77.0; δ_{H} 7.26). Chemical shifts are reported in ppm and coupling constants (J) are reported in Hz. Mass spectra were recorded on a Kratos MS50TC mass spectrometer. HPLC isolations were performed using a Waters Millipore Lambda-Max model 480 LC spectrophotometer with Waters 515 HPLC pumps.

Cyanobacterial collection and culture conditions: The marine cyanobacterium *Phormidium* sp. was initially collected by scuba diving from Sulawesi, Indonesia in 1994. It was grown in flasks containing 1.5 L SWBG11 medium, at 28°C with a 16 hrs light/8 hrs dark regime (5.4 to 7.0 $\mu\text{Einstein}$ s).

Extraction and isolation protocol: In a typical experiment, approximately 2 g biomass (dry weight) was ground with mortar and pestle in 2:1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$, infused at room temperature 40 min. in the same solvent, then boiled 2 x 15 min. in fresh solvent. The resulting extracts were combined and dissolved in ether, yielding 165 mg total organic extract. The crude extract was subjected to VLC with a range of solvents (0 to 100% EtOAc/hexanes followed by MeOH wash). The fraction eluted with 100% EtOAc (ca. 90 mg) was further fractionated in NP- HPLC (dual Phenomenex Luna 10 μm silica, 2 x 250 mm x 4.6 mm) with a 60% Hexanes/ 35% EtOAc/ 5% IPA solvent system, 4.5 ml/minute flow rate and yielded 12 mg of a late - eluting fraction containing phormidamide. This fraction was further purified by NP-HPLC (same column) in 94%EtOAc/IPA 2ml/min flow rate to yield 4.1 mg of phormidamide (9).

Biological assays: Activation of the voltage sensitive sodium channel and cytotoxicity in mouse neuro-2a neuroblastoma cells was determined as previously described (Manger *et al.*, 1995).

Phormidamide (9): clear colorless oil from MeOH; $[\alpha]_D^{25}$ 250 (*c* 0.24, CHCl₃); UV (MeOH) λ_{max} 224 nm (log ϵ 4.21), 258 nm (log ϵ 3.79), 348 nm (log ϵ 3.45); IR (neat) 3333, 3241, 3004, 2921, 2849, 1678, 1611, 1529, 1466, 1379, 1243, 1151, 1074, 1011 cm⁻¹; ¹H and ¹³C NMR data, see Table V.1; LRFABMS (positive ion, 3-nitrobenzylalcohol) *m/z* 919.1.

Production of ¹³C-enriched phormidamide (9): For the INADEQUATE NMR experiment, 2 x 1.5 L cultures of Phormidium sp. were provided with [U-¹³C] glucose (10 mg/L) on days 54, 56 and 58 post-inoculation. The cultures were harvested on days 60 and 78, respectively and (9) was purified as described above, then the two samples were combined for NMR spectra acquisition.

Culture conditions and isolation of phormidamide (9) from bromine-depleted or bromine supplemented media: Media were prepared as detailed in Chapter 4. Phormidamide isolation and purification was performed as described above.

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CHAPTER SIX

CONCLUSION

In the past 50 years, studies in the field of marine natural products have yielded an astounding abundance of secondary metabolites with novel chemical structures and potential pharmaceutical, biochemical and agricultural applications. Among the most productive sources of such structurally unique and bioactive agents are marine cyanobacteria, and in particular *Lyngbya majuscula*.

This research has investigated the biosynthetic and biochemical aspects of secondary metabolite production in five species of marine cyanobacteria cultured in the laboratory, with particular focus on a less-studied organism, *Phormidium*. Despite its abundance in locations all over the world, little is known about the chemistry and secondary metabolism of this cyanobacterium pertaining to the family Oscillatoriaceae, of which *Lyngbya majuscula* is also a member.

The first investigations undertaken have assessed the effects of introducing 19 compounds as putative 'elicitors' on the chemistry and bioactivity of five marine cyanobacterial species. The starting paradigm was that many cyanobacteria might possess latent biosynthetic capabilities that are 'switched on' only in conditions of stress, when they would be needed for the survival of the organism, otherwise constituting an unnecessary burden on the cellular economy. The results showed that growth, biomass production and the ratio of the extract components were influenced by some of the 'elicitors' in four of the five species tested, but production of a novel secondary metabolite or a significant change in the bioactivity of the extracts could not be observed. Similar studies had been

previously performed in plants, where upregulation or novel production of bioactive metabolites was obtained by elicitation. However, current knowledge on plant metabolism is much more extensive due to over a century of research in the fields of terrestrial plant natural products chemistry and ecology. The present study has furthered the understanding of the growth and metabolic characteristics of marine cyanobacteria, as well as led to more precise chemical characterization of the species in our laboratory's culture collection.

Next, the biosynthesis of a structurally unique brominated brine shrimp toxin, phormidolide, was investigated through stable isotope feeding experiments and altered halogen composition of the culture medium. Phormidolide is derived from a modular polyketide synthase pathway that is constitutively expressed at high levels in the *Phormidium* strain under study.

The biosynthetic feeding experiments established the origin of the pendant methyl groups on the polyketide backbone from both S-adenosyl methionine and C-2 of acetate, for methyl groups placed on backbone atoms derived from C-2 or C-1 of acetate, respectively. This result confirms an emerging trend in cyanobacterial metabolism, as opposed to bacteria and yeast, where pendant methyl groups are mainly derived from incorporation of methyl-malonyl CoA. The labeling pattern obtained from incorporation of deuterated acetate allowed new insight into the events involved in the recently described HMG CoA synthase-like mechanism for the biogenesis of pendant methyl groups from C-2 of acetate. These biosynthetic feeding experiments corroborate genetic evidence obtained in our laboratory and elsewhere that suggests there are additional enoyl hydratase/reductase type enzymes/activities that act in conjunction with the condensation unit in order to form the different functionalities found in products (methyl group, exocyclic double bond, etc). The starter unit of phormidolide is a three carbon unit, possibly phosphoenol pyruvate or glycerate, whose identity

could not be fully established because of the lack of intact incorporation from any of the putative precursor feedings. The labeling patterns obtained from [U-¹³C] glucose feeding are consistent, however, with other studies on cyanobacterial metabolism, showing high fluxes through the enzymes of the Calvin cycle and pyruvate decarboxylase under mixotrophic growth conditions in light, which reduces markedly the likelihood of finding intact three carbon unit incorporation.

The experiments on phormidolide production in bromine-depleted culture medium yielded two new phormidolide analogs, debromo and iodophormidolide, which retained brine shrimp toxicity (in fact, debromophormidolide has enhanced bioactivity relative to phormidolide). This result is suggestive of a defense chemical role for phormidolide, as well as the involvement of a bromoperoxidase in the halogenation of the starter unit. Addition of supplementary bromine and iodine to the media enhanced the production of phormidolide and iodophormidolide, respectively, proving that the concentration of halogen ions in the medium is a limiting factor for phormidolide biosynthesis.

Lastly, in the course of the biosynthetic investigations on phormidolide, a novel cytotoxic halogenated peptide, phormidamide, was isolated and its structural characterization was undertaken through the use of spectroscopic methods, particularly mass spectrometry and high field NMR. Phormidamide is potently cytotoxic to mouse neuro-2a neuroblastoma cells ($LD_{50} = 1 \mu\text{g}/\text{ml}$ or ca. $1.2 \mu\text{M}$) and presents an ongoing puzzle due to its unique structure and high degree of halogenation and derivatization of the envisaged peptide backbone. These give rise to an unusually high number of quaternary carbon atoms which has hindered the complete structural elucidation, leaving confirmation of the proposed structure of phormidamide to further X-ray crystallographic analysis.

The results presented herein further substantiate the tenet that marine cyanobacteria like *Phormidium* are capable of biosynthesizing structurally unique

compounds with important potential for the treatment of human disease or for use as agrichemicals or research chemicals. Moreover, since all of the investigations were performed on cultured species, it is evident that improved culture methodologies together with genetic engineering would greatly enhance our capability of exploring the immense reservoir of bioactive and structurally diverse metabolites contained in the Earth's most ancient organisms - cyanobacteria, as well as help meet supply demands while preserving the oceans as a viable and continuous resource.

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