5

The Secondary Metabolites and Biosynthetic Gene Clusters of Marine Cyanobacteria. Applications in Biotechnology

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Abstract

Marine cyanobacteria have proven to be one of the most versatile marine producers of secondary metabolites. Many of these metabolites demonstrate antiproliferative activity (*e.g.* curacin A, dolastatins), acute cytotoxic activity (*e.g.* apratoxin, hectochlorin) or have specific neurotoxic activity (*e.g.* kalkitoxin, antillatoxin), making them invaluable as potential therapeutic leads or pharmacological tools. The predominant biogenetic theme in cyanobacterial natural products chemistry is the integration of polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) along with a variety of unusual tailoring or modifying enzymes, and accounts for the tremendous structural diversity of their metabolites. Only recently has the genetic architecture of several cyanobacterial biosynthetic gene clusters been determined, and studies to understand and exploit this biosynthentic machinery present an exciting new frontier. This chapter will summarize the properties of several notable metabolites from marine cyanobacteria that have clinical or pharmacological applications followed by a detailed account of their biosyntheses at the molecular genetic level and their potential applications in biotechnology.

1. Introduction

Cyanobacteria, also known as blue-green algae, are ancient (ca. 2 x 10^9 years) photosynthetic prokaryotes which inhabit a wide diversity of habitats including

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open oceans, tropical reefs, shallow water environments, terrestrial substrates, aerial environments such as in trees and rock faces, and fresh water ponds, streams and puddles (Whitton and Potts, 2000) . Indeed, it is the luxuriant growth of cyanobacteria in these latter environments which has earned their undistinguished nickname of "pond scum". However, their abilities to survive adverse conditions by forming resistant spores, opportunistically colonizing microhabitats and surviving under conditions of high UV-flux through production of UV-absorbing pigments, has made them one of the most successful life forms on Earth! An additional feature, the rich elaboration of biologically-active natural products, has assisted some of these organisms to survive in predator-rich tropical reef ecosystems. As a result, tropical marine cyanobacteria, particularly the filamentous forms such as *Lyngbya* sp. or *Symploca* sp., have been exciting sources of novel natural products with therapeutic and biotechnological potential.

Cyanobacteria have a rich complement of photosynthetic pigments, including chlorophyll a and b, as well as several accessory pigments (phycoerythrin, phycocyanin, and allophycocyanin). Phycoerythrin has found application in biotechnology as a conjugate to antibodies that then allow visualization of cellular constituents and processes (Batard et al., 2002), and chlorophyll is being explored for its cancer chemoprevention activity (Egner et al., 2001). Freshwater varieties of cyanobacteria, notably *Aphanazomenon flos-aquae*, are reported to possess immune stimulating properties, although the specific chemical compounds responsible for this property have not yet been characterized (Pugh et al., 2001). Other cyanobacteria such as *Spirulina* are a rich source of digestible protein with a complete complement of essential amino acids, and have provided a considerable market for its cultivation and sale as a health food.

The chemical investigation of marine cyanobacteria for their unique natural products began with the pioneering work of Richard Moore at the University of Hawaii. In the early 1970's his laboratory published several surveys of marine cyanobacteria from the Pacific showing that they were rich in potential anticancer and antiviral substances (Moore, 1978; 1981), including several groundbreaking reports on the unique structures of potent toxins from these life forms (Cardellina et al., 1979; Kato and Scheuer, 1974). Early work along these lines showed that cyanobacteria, as versus most other marine life forms at the time, were rich in nitrogen-containing natural products. Indeed, most marine cyanobacterial natural products can be biosynthetically rationalized as combining structural units from amino acid metabolism with fatty acids or their component pieces (*e.g.* acetate). Subsequently, freshwater cyanobacteria were shown to have comparable biosynthetic capabilities and to produce an equally exciting assortment of bioactive lipopeptides. In the 1980's and 1990's, our laboratory initiated parallel investigations of marine cyanobacteria from the Caribbean, and found that they were also rich in lipopeptide constituents, some of which had powerful bioactivities relevant to potential cancer chemotherapy (*e.g.* curacin A (**8**))(Gerwick et al., 1994a). In the late 1990's, efforts in the Moore, Gerwick and several other laboratories continued to reveal the rich metabolic capabilities of marine cyanobacteria. They are now widely recognized to be one of the richest of all marine life in this regard, both as free living organisms as well as when living in association with marine invertebrates (*e.g.* sponges and tunicates) (Burja et al., 2001; Gerwick et al., 2001).

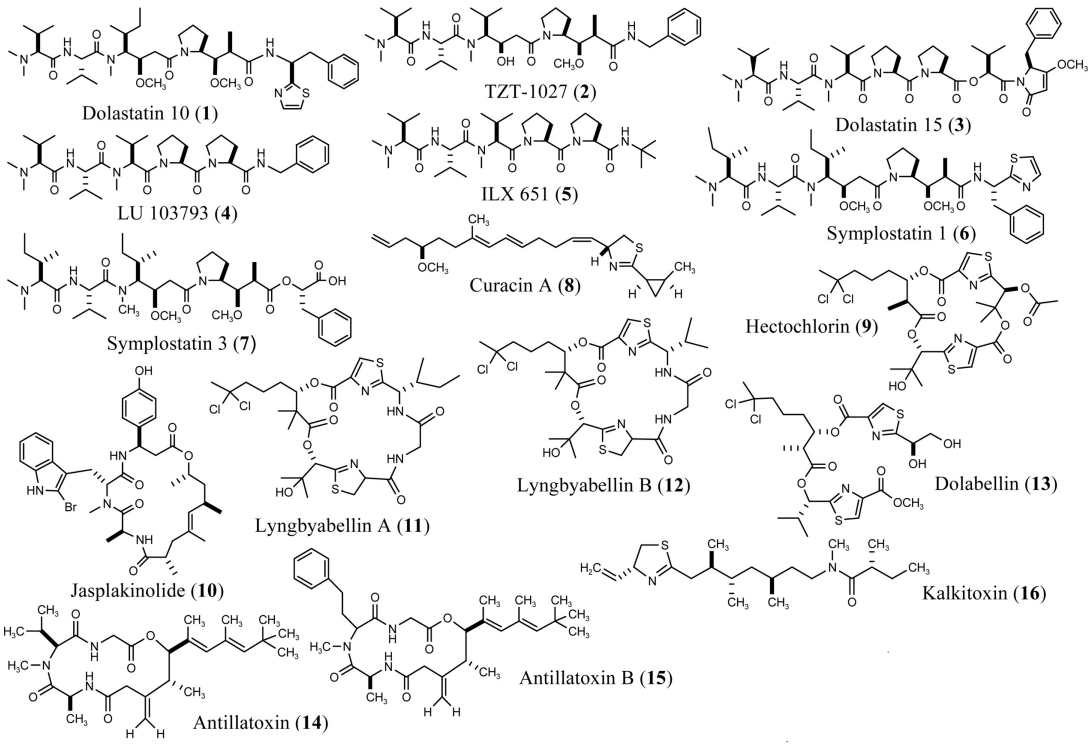
The ecological role that cyanobacterial natural products play has been hypothesized based on their fundamental biological properties. For example, a *Lyngbya majuscula* from Curaçao yielded a suite of metabolites with quite specific biological properties, including those with toxicity to arthropods, those toxic to fish, and those toxic to gastropods. In complement, it can be speculated that this cocktail of metabolites has the capacity to defend these luxuriant strands of cyanobacteria against a broad assortment of potential predators. Detailed experimental investigation of the chemical ecological role of marine cyanobacterial natural products has occurred in Guam and Florida through the efforts of Valerie Paul and her laboratory, and has generally shown that marine cyanobacteria are chemically defended against predation by generalist feeders (Nagle and Paul, 1999). Specialized predators, such as certain sea hares (*e.g*. *Dolabella* sp. and *Stylocheilus* sp.), preferentially feed on chemically-rich marine cyanobacteria, and their larvae utilize the secondary metabolite profile to identify suitable substrates for metamorphosis.

This chapter is organized along the following lines. The first section recounts a brief summary of several prominent marine cyanobacterial products which are in clinical evaluation or at advanced stages of preclinical testing. Interestingly, these are almost all in the area of cancer, and while this is partially explained by the focus of research funding and hence the bioassays which have been applied, it may also reflect on their essential properties and hence the underlying purposes to which marine cyanobacteria employ these molecules. The last five years has seen a dramatic increase in our knowledge of how marine cyanobacteria create their unique metabolites, particularly so at the molecular genetic level. The next two sections of the chapter will review these exciting developments as well as provide reference to the numerous genome sequencing projects that are underway with cyanobacteria. From our knowledge of the scope and character of marine cyanobacterial metabolism, we can hypothesize that a great many "marine invertebrate" (*e.g*. sponges, tunicates) metabolites actually derive from metabolic processes of associated or symbiotic cyanobacteria. This intriguing issue is updated in the ensuing section entitled "The Symbiosis Question". A final section of the chapter summarizes our ideas concerning some of the more interesting challenges and opportunities in future studies of marine cyanobacterial natural products.

2. Prominent lead compounds

2.1. Tubulin-binding compounds

Microtubules play many significant roles in cell biology. The formation of microtubules results from the polymerization of the subunit protein tubulin, first into αβ-heterodimer which subsequently binds end to end with other heterodimers forming a protofilament, which in turn interacts to form sheets and eventually microtubules. Specifically, microtubule assembly and motility are crucial for the formation of the spindle apparatus during cell replication and mitosis where microtubule fibers direct the separation of sister chromatids into the resulting daughter cells. In the case of rapidly dividing cancer cells, microtubule assembly has been an important target in the development of new chemotherapeutic agents. Various drugs have been developed to disrupt the process of mitosis and cause


Dolastatin 10 (1)
TZT-1027 (2)
Dolastatin 15 (3)
LU 103793 (4)
ILX 651 (5)
Symplostatin 1 (6)
Symplostatin 3 (7)
Curacin A (8)
Hectochlorin (9)
Jasplakinolide (10)
Lyngbyabellin A (11)
Lyngbyabellin B (12)
Dolabellin (13)
Antillatoxin (14)
Antillatoxin B (15)
Kalkitoxin (16)

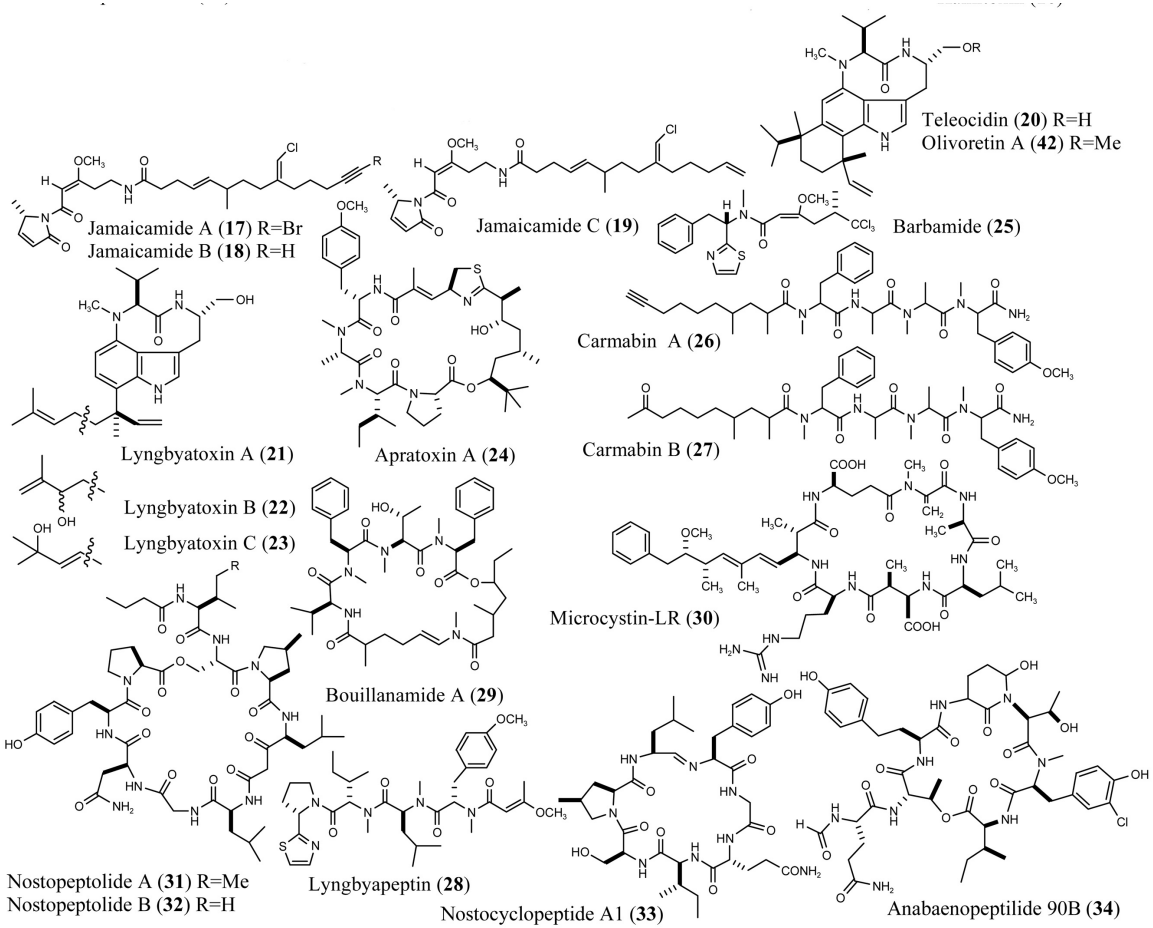


Figure 1. Chemical structures of natural products and derivatives **1-34** discussed in text.

catastrophic cell death by either stabilizing microtubule complexes (*e.g*. Taxol) or causing the depolymerization of the tubulin protein complex (*e.g*. Vinblastine).

In recent decades an increasing number of antimitotic marine natural products (and derivatives) have emerged which target intracellular tubulin. Here, we will discuss several antimitotic compounds isolated from marine cyanobacteria or their specialist predators, which are currently under evaluation as therapeutic lead candidates.

*2.1.1. Dolastatin 10 (**1**)*

The pharmacological properties of the marine mollusc (family Aplysiidae) commonly known as the 'sea hare' or 'nudibranch' has been known since pre-Christian times. It was reported that their toxic secretions were used by Agrippina (mother of Nero; A.D. 37-70) to poison potential competitors for the throne of Rome, thus ensuring the ascension of her son (Pettit et al., 1976). Since those earliest nefarious uses, our understanding of sea hare chemistry and the resulting mechanisms of action has become significantly more sophisticated.

The extraordinary biological activity of the cyanobacteria - browsing sea hare *Dolabella auricularia* has been noted since 1972, and in 1987 through much effort and many expeditions to the Indian Ocean, the structure of the active constituent, dolastatin 10 (**1**) was finally elucidated (Pettit et al., 1987). Indeed dolastatin 10 displayed exceptional activity against the P388 lymphocytic leukemia cell line (NCI) with ED_{50}= 4.6 x 10^{-5} μg/mL, which at the time was the most potent antineoplastic agent known! Subsequently, dolastatin 10 was isolated from field collections of the marine cyanobacterium *Symploca* sp, clarifying that the true biosynthetic source is the cyanobacterium and not the sea-hare. This unique linear pentapeptide is composed of four novel amino acid residues (dolavaline, dolaisoleucine, dolaproline, and dolaphenine) and valine. Evidence for this linear pentapeptide structure was obtained by extensive high resolution mass spectroscopic and ^{1}H (COSY, 2D-J resolved) and ^{13}C NMR methodologies. The residue sequence was unambiguously assigned on the basis of several low resolution mass spectral fragmentation techniques.

A putative mechanism for its potent bioactivity was reported soon after the structure was solved, and involved the binding of dolastatin 10 (and possibly other antimitotic peptides and depsipeptides) near the exchangeable nucleotide and Vinca alkaloid sites on microtubules (Bai et al., 1990). Initial work by Hamel et al. showed that dolastatin 10 inhibits microtubule assembly *in vitro* and subsequently blocks cytokinesis. Additionally, dolastatin 10 was shown to noncompetitively inhibit the binding of radiolabeled vinblastine and vincristine to tubulin as well as the tubulin-dependent hydrolysis of GTP. It was proposed that dolastatin 10 binds in a 'peptide groove' within the β-subunit of tubulin. Molecular modeling suggests that the chiral centers of dolavaline, valine, and dolaisoleucine bind in a manner that requires the dolaphenine moiety to sterically block access to the Vinca alkaloid and exchangeable nucleotide binding sites (Bai et al., 1990). Structure activity relation (SAR) studies with synthetic analogs provided evidence that the γ-carbon of the dolaisoleucine must be in the *S*-configuration for maximal activity, while the α and β carbons of dolaproline are relatively unimportant. Further evidence of noncompetitive inhibition of Vinca alkaloid binding was realized by observation

that tubulin polymerization and nucleotide binding are substantially diminished at sub-stoichiometric concentrations of dolastatin 10.

Phase II clinical trials of dolastatin 10 (**1**) as an antitumor agent, evaluated the antitumor efficacy of dolastatin 10 in patients with measurable recurrences of platinum-sensitive ovarian carcinoma in relation to the nature and degree of toxicity. Unfortunately, it was concluded that dolastatin 10 had minimal activity against platinum-sensitive ovarian carcinoma under these test conditions (Hoffman et al., 2003). However, stimulated by these results several synthetic analogs (*e.g.* TZT-1027) have appeared and are currently in clinical trials.

*2.1.2. TZT-1027 (**2**)*

Also known as Soblidotin, TZT-1027 (**2**) is a structural analog of dolastatin 10 synthesized in the by Teikoku Hormone Mfg. Co. (Miyazaki et al., 1995). Designed to have enhanced antitumor activity and reduced inadvertent somatic toxicity, this compound differs from its parent structure by removal of the thiazole ring from the dolaphenine moiety, resulting in a terminal ethyl benzylamide functionality. TZT-1027 has shown therapeutic efficacy against lung and renal carcinoma (Natsume et al., 2001), VEGF-secreting lung cancer (Natsume et al., 2003) and has recently been applied in a directed drug delivery system by targeting the up-regulated adhesion molecule, E-selectin found in the epithelium of prostate carcinomas (Bhaskar et al., 2003; Newman and Cragg, 2004).

*2.1.3. Dolastatin 15 (**3**)*

In 1989 the cyanobacteria browsing sea hare, *D. auricularia* was shown to contain yet another exceptionally cytotoxic molecule, dolastatin 15 (**3**). Akin to dolastatin 10, this new depsipeptide displayed exceptional activity against the National Cancer Institute's (NCI) P388 lymphocytic leukemia cell line with an ED_{50}= 2.4 x 10^{-3}μg/mL. Dolastatin 15, was discovered via bioassay guided fractionation and purified as a minor fraction equaling 4 x 10^{-7} % (6.2 mg from 1600 kg wet sea hare), (Pettit et al., 1989) providing evidence that the compound is assimilated by the sea hare from its cyanobacterial diet. The structure of (**3**) is a linear heptadepsipeptide composed of dolavaline (N, N-dimethyl valine), valine, N-Me-valine, proline (x2), 2-hydroxyisovaleric acid, and the novel residue dolapyrrolidone (Dpy). The identity of these residues was determined by various high-field one and two dimensional NMR techniques. The new Dpy moiety was proposed to originate biosynthetically from N-acetyl-phenylalanine methyl ester via intramolecular condensation (Pettit et al., 1989). Definitive evidence for this structural feature was deduced by NOE and 2D-HMBC NMR experiments and supported by collision-activated MS/MS and high resolution EIMS. Furthermore, precedence for such a biosynthetic modification has been reported in the literature from multiple marine organisms (Gerwick et al., 2001) and provides additional support to the idea that the cyanobacteria are the ultimate source of many marine metabolites isolated from other sources (Sings and Rinehart, 1996).

Recently, it has been demonstrated that dolastatin 15 also binds to the αβ-heterodimer of tubulin (Cruz-Monserrate et al., 2003). Although dolastatin 15 was shown to bind in the Vinca alkaloid domain of the tubulin complex, a definitive binding site was not identified. In fact, (**3**) was shown to weakly bind tubulin

(K_d ~30 μM) with concurrent weak inhibition of cryptophycin 1 binding, the consequences of which are still unclear. Further work is ongoing to determine the molecular mechanism of activity for dolastatin 15, although it has been dropped from preclinical trials due to general toxicity.

Not surprisingly, this unique natural product with its unusual structural moieties has stimulated much interest in the organic synthesis of related compounds, and a number of analogs have been produced with varying degrees of efficacy (see below).

2.1.4. LU-103793 (**4**)

A structure-simplified synthetic derivative of dolastatin 15 (**3**), LU-103793 (**4**) (Cemadotin), is a stable, water soluble molecule that has displayed outstanding activity against a variety of tumor models. Structurally, the C-terminal 2-hydroxyisovaleryl-dolapyrrolidone unit of dolastatin 15 was replaced with a simple benzylamide in LU-103793 (Hu and Huang, 1999). Prior it is public release, this compound exhibited quite exciting antimitotic activity. Specifically, cell lines treated with LU-103793 arrested in the G2-M of the cell cycle due to the depolymerization of the microtubule spindle apparatus (de Arruda et al., 1995). Moreover, in turbidity assays with bovine brain microtubules, LU-103793 was shown to inhibit microtubule polymerization in a concentration dependent manner with an IC_{50}= 7 μM. Subsequently, LU-103793 has been evaluated in five phase I clinical trials and phase II trials are on-going in breast, lung, ovarian, prostate, and colon cancer patients (Amador et al., 2003). However, several undesirable side effects have been recently observed and have lead to the discontinuation of some of the phase II clinical trials (Newman and Cragg, 2004).

2.1.5. ILX-651 (**5**)

Another synthetic derivative of dolastatin 15 (**3**), ILX-651 (**5**) (Synthadotin), has been shown to have a novel molecular mechanism of action in that it inhibits microtubule nucleation, in contrast to the microtubule stabilization (*e.g.* taxanes and epothilones) or tubulin polymerase inhibitors (*e.g.* Vinca alkaloids and dolastatins). Structural modifications include replacement of the C-terminal 2-hydroxyisovaleryl-dolapyrrolidone unit of dolastatin 15 with a simple *tert*-butlyamine moiety. This alteration presumably improves its pharmacological properties and bioavailability, thus enhancing the 'therapeutic window' over previous analogs of dolastatin 15 (Ebbinghaus et al., 2004). Moreover, the substantial cardiovascular toxicity observed with the parent compound and other dolastatins is not observed for ILX-651. The phase II clinical trial concluded that Synthadotin (ILX-651) is a safe, well tolerated treatment in locally advanced and metastatic melanoma patients (Ebbinghaus et al., 2004). Currently, the license holder of this compound (ILEX Oncology) is conducting further phase II clinical trials for patients with locally advanced or metastatic non-small cell lung carcinoma (NSCLC) and hormone-refractory prostate cancer previously treated with Docetaxel (www.ilexonc.com).

2.1.6. Symplostatin 1(**6**)

In 1998, the first natural dolastatin 10 analog, symplostatin 1 (**6**) was discovered by direct isolation from the cyanobacterium *Symploca hydnoides*. Symplostatin 1 differs

from dolastatin 10 by the addition single CH_2 in the dolavaline moiety resulting in a terminal N,N-dimethylisoleucine versus the terminal N,N-dimethylvaline(Harrigan et al., 1998a). This compound exhibited potent cytotoxicity IC_{50}= 0.3 ng/mL against a human nasopharyngeal carcinoma cell line (KB), compared to < 0.1ng/mL for dolastatin 10. Symplostatin 1 was also shown to induce microtubule loss by 80% at 1 ng/mL when tested in A-10 cells. It has been concluded that the mechanism of action of symplostatin 1 must be similar if not identical to that determined for dolastatin 10 (Harrigan et al., 1998a). In further analysis, symplostatin 1 displayed efficacy against a number of cancer cell lines and caused the formation of abnormal mitotic spindles and accumulation of cells in metaphase. These effects were observed at concentrations that elicited only minor loss of interphase microtubules. Cell cycle analysis indicated that symplostatin 1 caused G_2/M arrest, a finding consistent with its effects on mitotic spindles. Moreover, it was shown to inhibit the assembly of purified tubulin, suggesting that tubulin is its intracellular target. Evaluation of this aspect revealed potent inhibition of both endothelial cell proliferation and invasion, with *in vivo* efficacy against murine colon 38 and murine mammary 16/C cell lines (Mooberry et al., 2003). In addition to the exciting biological activity, this discovery offered further evidence that these potent peptides and depsipeptides are of cyanobacterial origin.

*2.1.7. Symplostatin 3 (**7**)*

Another natural analogue of dolastatin 10 was discovered via bioassay guided fractionation of the organic extract of a Hawaiian variety of *Symploca* sp. VP452. Named symplostatin 3 (**7**), this compound differs from dolastatin 10 only in the C-terminal unit, where dolaphenine is substituted by a 3-phenyllactic acid moiety (Luesch et al., 2002). Symplostatin 3 displayed IC_{50} values of 3.9 nM and 10.3 nM against KB and LoVo cell lines, respectively. Thus these analogs are approximately 100- fold less potent than dolastatin 10 (**1**). Therefore, the dolaphenine subunit is important for the molecular mechanism of action of the drug; however, subsequent synthetic modifications to this unit have indicated that the thiazole ring is not crucial for bioactivity (Pettit et al., 1998). Similar to symplostatin 1, symplostatin 3 caused microtubule depolymerization in A-10 cells (0.1 μg/mL caused partial disruption and 1.0 μg/mL caused total depolymerization). As a result of microtubule disruption a breakdown of the cell nucleus into 'micronuclei' was observed. These effects on the microtubule network are identical to those observed for dolastatin 10 (**1**) and symplostatin 1 (**6**), although symplostatin 3 (**7**) was the least potent of the three (Luesch et al., 2002).

2.1.8. Curacin A (8)

Not all antimitotic marine natural products have an overt peptidic nature and the highly lipophilic natural product curacin A (**8**) is a good example. Isolated from a Caribbean collection of the cyanobacterium *L. majuscula*, the structure of curacin A consists of an interesting thiazoline ring containing a 14-carbon alkyl chain with conjugated diene and terminal olefin, and a methyl substituted cyclopropyl moiety (Gerwick et al., 1994a). Curacin A was originally shown to be active against a Vero cell line, and subsequent assessment in the NCI 60 cell line assay revealed potent antiproliferative and cytotoxicity with some selectivity

for colon, renal, and breast cancer cell lines. It was later shown that treatment of curacin A resulted in the depolymerization of purified tubulin induced by either glutamate or microtubule-associated protein-dependent microtubule assembly, with an IC_{50}= 4.0 μM and IC_{90}=6.0 μM. Further testing revealed that curacin A (**8**) binds tightly at the colchicine site of tubulin. Curacin A stimulates the uncoupled GTPase reaction typical of colchicine site agents, and indirect observations were consistent with curacin A binding rapidly and dissociating slowly from tubulin (Blokhin et al., 1995). Additionally, curacin A was shown to inhibit formation of the Cys239-Cys354 cross-link in β-tubulin (Luduena et al., 1997). Furthermore, under conditions ideal for tubulin polymerization, high concentrations of curacin A induced formation of aberrant tubulin polymers appearing similar to a twisted cable of fine spiral filaments (Hamel et al., 1995). Based on these results a series of semi-synthetic analogs were produced to explore the structure activity relations in this new drug class. The structural modifications included reduction and *E*-to-*Z* transitions of the olefinic bonds in the alkyl side chain; disruption of and configurational changes in the cyclopropyl ring, thiazoline moiety, and substituent modifications at the C10 methyl- and C13 methoxy groups. This work revealed that the most crucial portions of curacin A for tubulin interaction seems to be the thiazoline ring and the side chain at least through C4, and the portions of the side chain including the C9-C10 olefinic bond, and the C10 methyl group (Verdier-Pinard et al., 1998). Recently, some new directions have emerged with construction of synthetic combinatorial libraries using solution phase and fluorous scavenging techniques, resulting in new analogs with improved bioavailability and efficacy for a complete summary see (Wipf et al., 2004).

2.2. Actin-binding compounds

The actin cytoskeleton is a dynamic network of filaments, which in association with several proteins, plays an important role in cell shape, motility and signal transduction that in turn impact a number of processes such as embryonic development, repair of tissue damage, immune response and tumor formation (Spector et al., 1999; Fenteany and Zhu, 2003). The actin cytoskeleton and actin associated proteins undergo considerable modification in transformed tumor cells and play an important role in the abnormal growth of cancer cells and their ability to adhere and metastasize (Jordan and Wilson, 1998). Actin and actin-associated proteins therefore make exciting targets for developing new chemotherapeutic agents. Molecules which target actin directly either disrupt actin by destabilizing the filaments or induce hyperpolymerization and stabilize filaments (Giganti and Friederich, 2003). There are a number of natural products which target actin filaments and offer exciting opportunities for the development of new anticancer agents or valuable tools to study the actin cytoskeleton and dynamics in cells. The following sections describe the characteristics of actin-targeting compounds derived from marine cyanobacteria.

*2.2.1. Hectochlorin (**9**)*

Hectochlorin (**9**), was isolated from the marine cyanobacterium *L. majuscula* collected from Hector Bay, Jamaica, and Boca del Drago Beach, Bocas del Toro, Panama (Marquez et al., 2002). The planar structure was deduced by one- and

two-dimensional NMR spectroscopy, and X-ray crystallography was used to determine the absolute stereochemistry (Marquez et al., 2002). Hectochlorin was initially shown to be a potent antifungal agent in preliminary antimicrobial bioassays. Further testing showed that Ptk2 cells (derived from the rat kangaroo, *Potorous tridactylus*) treated with hectochlorin showed an increase in the number of binucleated cells as result of arrest of cytokinesis. This result is consistent with an interference with the actin cytoskeleton (Marquez et al., 2002). Hectochlorin is similar in its activity to jasplakinolide (or jaspamide) (**10**) in its ability to promote hyperpolymerization of actin (Crews et al., 1986; Zabriskie et al., 1986). The main biochemical difference between jasplakinolide and hectochlorin is that while the former can displace fluorescently labeled phalloidin from actin polymers, the latter is unable to do so, suggesting that the two have distinct interactions with actin. Hectochlorin was also evaluated against the *in vitro* panel of 60 different cancer cell lines (National Cancer Institute) and showed strong potency towards cell lines in the colon, melanoma, ovarian and renal sub-panels. It had a flat dose-response curve against most cell-lines which is characteristic of compounds that are antiproliferative but not directly cytotoxic (Marquez et al., 2002).

*2.2.2. Lyngbyabellins A (**11**) and B (**12**)*

The lyngbyabellins (**11,12**) were isolated from collections of *L. majuscula* from the South Pacific and Caribbean, and bear structural resemblance to hectochlorin and dolabellin (**13**) (Luesch et al., 2000a; Luesch et al., 2000b; Milligan et al., 2000; Sone et al., 1995). The structure of lyngbyabellin A was determined using 2D NMR techniques and its absolute stereochemistry was determined by chiral HPLC analysis. Lyngbyabellin A (**11**) exhibited IC_{50} values of 0.03 μg/mL and 0.50 μg/mL against KB cells (human nasopharyngeal carcinoma cell line) and LoVo cells (human colon adenocarcinoma cell line), respectively. It was also shown to disrupt the microfilament network in fibroblastic A-10 cells at concentrations between 0.01-5.0 μg/mL. However, when treated with a higher concentration of lyngbyabellin A many cells became binucleate, an observation which is consistent for compounds inhibiting cytokinesis (Luesch et al., 2000b). The structure of lyngbyabellin B **(12)** was determined using a combination of 1D and 2D NMR spectroscopy and its stereochemistry was proposed using a combination of NMR and chiral GC/MS analysis. Lyngyabellin B was found to be less toxic than lyngbyabellin A with IC_{50} values of 0.10 μg/mL and 0.83 μg/mL against KB and LoVo cell lines, respectively. Lyngbyabellin B had the same effect as hectochlorin on PtK2 cells, in that an increased number of binucleate cells were observed when the cells were treated with10 μM of the agent (Marquez et al., 2002). These findings suggest that actin is the likely cellular target of the lyngbyabellins.

2.3. Neurotoxic compounds

*2.3.1. Antillatoxin (**14**)*

The crude extract of a Curaçao collection of *L. majuscula* was found to be highly ichthyotoxic and molluscicidal. Fractionation and subsequent purification led to the discovery of the potent lipopeptide ichthyotoxin, antillatoxin (Orjala et al., 1995b). The structure of antillatoxin was elucidated using several spectroscopic

methods including HR FABMS, IR and NMR. Antillatoxin (**14**) is one of the most ichthyotoxic metabolites isolated to date from a marine cyanobacterium (LD_{50} = 0.05 μg/mL), and is exceeded in potency only by the brevetoxins (Baden et al., 1984). Initial pharmacological studies showed that antillatoxin was acutely neurotoxic and rapidly induced morphological changes in rat cerebellar granule neurons (CGC's), including blebbing of neurite membranes. However, the toxicity was remarkably reduced when cells were treated with NMDA receptor antagonists like dextrophan and MK-801, indicating that the toxicity of antillatoxin was mediated through an NMDA receptor- dependent mechanism (Berman et al., 1999). Antillatoxin was later shown to be a powerful activator of voltage-gated sodium channels and resembled brevetoxin in this respect. But unlike brevetoxin which is known to bind site 5 on the α- subunit of voltage-gated sodium channels, the binding site of antillatoxin is unknown and remains to be identified (Li et al., 2001). The unique biological activity of antillatoxin combined with its unusual structure spurred several synthetic efforts, and the total synthesis of antillatoxin was reported by two groups (White et al., 1999; Yokokawa and Shioiri, 1998). However, spectroscopic analyses of the synthetic versions showed that they were different from "natural antillatoxin". Eventually, the synthetic 4R,5R stereoisomer (**14**) which was identical to natural antillatoxin was reported by the Shioiri group (Yokokawa et al., 1999). In addition to resolving some initial stereochemical issues, the syntheses of several stereoisomers of antillatoxin facilitated SAR studies and a recent report showed that naturally occurring antillatoxin was about 25-fold more potent than any of its stereoisomers (Li et al., 2004).

*2.3.2. Antillatoxin B (**15**)*

Antillatoxin B (**15**) was isolated from *L. majuscula* collected from Puerto Rico and the Dry Tortugas, and is a lipopeptide closely related to antillatoxin (**14**) although with a larger *N*-methyl homophenylalanine instead of an *N*- methyl valine residue (Nogle et al., 2001). The structure of antillatoxin B was deduced by a combination of spectroscopic methods and by comparison with antillatoxin. Antillatoxin B was also found to be ichthyotoxic (LC_{50} =1.0 μM) and a potent activator of voltage-gated sodium channels in mouse neuro-2a neuroblastoma cells (EC_{50} =1.77 μM). However, in both cases, the potency is about 10-fold less than that of antillatoxin (LC_{50} = 0.1 μM and EC_{50}= 0.18 μM); thus the substitution of the larger N-methyl homophenylalanine residue for the smaller N-methyl valine markedly impacts the potency in this drug class.

*2.3.3.Kalkitoxin (**16**)*

Kalkitoxin (**16**) was first isolated from a Caribbean collection of *L. majuscula* and exhibited potent brine shrimp and fish toxicity (Wu, 1996). Subsequently, the compound was re-isolated in very small quantities from various Caribbean collections of *L. majuscula* (Wu et al., 2000). Structural elucidation was accomplished by various 2D NMR methods and stereochemistry was resolved using Marfey's analysis. Kalkitoxin was toxic to rat CGC's and had an LC_{50} =3.86 nM and this effect could be inhibited by the addition of NMDA receptor antagonists (Berman et al., 1999). There is also evidence indicating that kalkitoxin is a blocker of voltage-sensitive Na^+ channel in mouse neuro-2a cells (EC_{50} = 1 nM) (Wu et

al., 2000). In an inflammatory disease model, kalkitoxin was found to inhibit the release of IL-β-induced $sPLA_2$ (secreted phospholipase A_2), a key enzyme in the inflammatory cascade (IC_{50} = 27nM) (Wu et al., 2000; Tan et al., 2000).

*2.3.4. Jamaicamides A-C (***17-19***)*

A strain of *L. majuscula* collected from Hector's Bay, Jamaica yielded a series of novel and functionalized lipopeptides, jamaicamides A-C (**17-19**) (Edwards et al., 2004). The structure of the jamaicamides was deduced using a combination of NMR spectroscopy including an ACCORD-1, 1-ADEQUATE experiment. Jamaicamaide A (**17**) was particularly unique in its presence of an acetylene bromide functionality. The jamaicamides exhibited cytotoxicity against H-460 human lung cell line and mouse neuro-2A neuroblastoma cell lines with an $LC_{50} \approx 15$ μM for both cell lines and showed sodium channel blocking activity at a concentration of 5 μM. From a combined precursor feeding study and a molecular genetics approach a fascinating mixed NRPS and PKS pathway was deduced.

2.4. Protein kinase C activators

Protein kinase C (PKC) plays an important role in cellular communication. Diacylglycerol (DG), obtained from the breakdown of membrane phosphoinositol, functions as a second messenger and activates PKC which in turn triggers cellular response by phosphorylating key downstream proteins (Ohno and Nishizuka, 2002). Tumor promoting phorbol esters bear three-dimensional structural similarities to membrane diacylglycerols and hence are capable of activating PKC *in vitro* (Castagna et al., 1982). Indole alkaloids such as the teleocidins (**20**) and lyngbyatoxins (**21-23**) can also activate PKC, but the activation is persistent and causes downregulation of the enzyme which ultimately leads to tumor promotion.

*2.4.1. Lyngbyatoxins A-C (***21-23***)*

Lyngbyatoxin A (**21**) was isolated from a Hawaiian shallow-water variety of *L. majuscula* and is believed to be responsible for a condition known as 'swimmer's itch'. The structure of lyngbyatoxin A is closely related to the teleocidins, metabolites produced by several *Streptomyces* sp. (Cardellina et al., 1979). The structure of lyngbyatoxin A was elucidated using high resolution mass spectrometry and NMR analyses and is the first indole alkaloid to be reported from a marine cyanobacterium. This highly inflammatory compound had an LD_{100} =0.3 mg/kg in mice which was comparable to the toxicity of the teleocidins. It was also found to be ichthyotoxic and exposure to 0.15 μg/mL resulted in death of fish in 30 minutes (Cardellina et al., 1979). Ensuing pharmacological studies showed that lyngbyatoxin A (**21**) and teleocidin were potent tumor promoters in a manner similar to that of the phorbol esters when tested in mice (Fujiki et al., 1981). To confirm whether lyngbyatoxin was produced by the cyanobacterium or merely associated with it, collections of *L. majuscula* from Kahala Beach, Hawaii were re-examined and this led to the isolation of two related metabolites, lyngbyatoxin B (**22**) and lyngbyatoxin C (**23**) (Aimi et al., 1990). In an effort to understand the interaction of lyngbyatoxin A with PKC, several analogs were synthesized and it was found that the lactam ring is essential for PKC activation and that the hydrophobic group attached to C-7 actually downregulates PKC production (Basu et al., 1992; Kozikowski et al., 1991).

2.5. Other bioactive cyanobacterial metabolites

*2.5.1. Apratoxin A (**24**)*

L. bouillonii isolated from Apra Bay, Guam initially mis-identified as *L. majuscula* was found to produce a very potent cytotoxin, apratoxin A (**24**) (Luesch et al., 2001b). The structure of this mixed polyketide-peptide was deduced using a variety of 2D-NMR techniques, and the amino acid configurations were determined using chiral HPLC analysis. Apratoxin exhibits an $IC_{50} = 0.52$ nM and 0.36 nM against KB and LoVo cells, respectively but its exact mode of action at present is uncharacterized (Luesch et al., 2001b). The overall unusual structure of apratoxin A and its potent activity, prompted several synthetic efforts and the total synthesis of apratoxin was reported by Chen and Forsyth (2004). Further pharmacological studies along with the synthesis of several structural analogs may enable the determination of the biological target of this potent molecule.

*2.5.2. Barbamide (**25**)*

Barbamide (**25**) was obtained from the lipid extract of a Curaçao collection of *L. majuscula* and possesses several unique structural elements including a trichloromethyl group (Orjala and Gerwick, 1996). Standard spectroscopic techniques were used for the structural elucidation of barbamide and stereochemistry was deduced by a combination of degradation and biosynthetic methods. The presence of several structurally intriguing elements in barbamide led to a detailed examination of its biosynthesis including the cloning and characterization of the barbamide biosynthetic gene cluster (Sitachitta et al., 1998; 2000; Chang et al., 2002). Preliminary bioassays indicated that barbamide possesses anti-molluscicidal activity ($LC_{50} = 10.0$ μg/mL). However, the compound was found to be inactive in other assays and the full extent of its biological properties remains unknown.

3. Advances and applications of molecular genetics

With an ever increasing inventory of natural product biosynthetic genes available in the public databases, it has become possible to utilize this genetic information to develop highly specific cloning strategies to isolate new biosynthetic gene clusters. This has been especially true of the modular biosynthetic systems encoded by nonribosomal synthetase (NRPS) and polyketide synthase (PKS) gene clusters. Inherent difficulties of culturing marine organisms, such as cyanobacteria, have hampered biosynthetic studies in these organisms. In addition the lack of effective techniques to genetically manipulate cyanobacteria has slowed research efforts in this area compared to some of the terrestrial counterparts, such as the actinomycetes. In recent years the ability to culture certain marine cyanobacteria, in particular *Lyngbya majuscula,* and improved methods to isolate DNA and generate genomic libraries from environmental samples, has made it possible to intiate molecular genetic studies with these organisms.

A productive collaboration between Dr. David Sherman's Laboratory at the University of Michigan and Dr. William Gerwick's Laboratory at Oregon State University has led to the isolation and characterization of several complete cyanobacterial biosynthetic gene clusters, including those encoding the production of barbamide (**25**), curacin A (**8**), jamaicamide A (**17**), and lyngbyatoxin A (**21**).

Sequencing of putative pathways encoding the biosynthesis of the carmabins (**26-27**), hectochlorin (**9**), apratoxin (**24**), lyngbyapeptin (**28**), and bouillonamide (**29**) are currently underway. Isolation of these pathways provides the foundation for the development of heterologous expression systems that can be used to produce biologically active compounds and their derivatives in quantities useful for biomedical testing and human utilization. In addition, the activity of unique tailoring enzymes can be harnessed and used to derivatize therapeutic lead compounds *in vitro* or as engineered components in genetic recombination experiments.

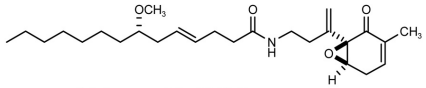
3.1. Gene clusters from freshwater cyanobacteria

The elucidation of several freshwater cyanobacterial pathways, including those encoding the biosynthesis of microcystin-LR (**30**), nostopeptolides (**31-32**), nostocyclopeptide (**33**) and anabaenopeptilide (**34**) has provided the foundation for further genetic studies in marine counterparts and will be briefly reviewed here.

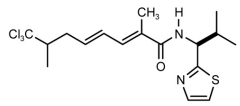
*3.1.1. Microcystin-LR (***30***) biosynthetic pathway*

The microcystins are a group of cyanotoxins that have been the subject of intensive research due to their acute hepatotoxic properties. This toxicity results from the ability of the microcystins to inhibit the eukaryotic protein phosphatases 1 and 2A. This family of toxins consists of about 65 cyclic heptapeptides and is produced by many species of bloom- forming cyanobacteria belonging to the genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nodularia* and *Oscillatoria* (Briand et al., 2003). The isoforms share a common cyclo [Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z] where X and Z are the two variable amino acid units, Adda is 3-amino-9-methoxy-2,6,8 trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid and Mdha is N-methyl dehydroalanine. Microcystin-LR is the most commonly occuring isoform, and has L-leucine (X) and L-arginine (Z) as the variable amino acids (Tillett et al., 2000). Biosynthetic feeding studies carried out with labeled precursors (Moore et al., 1991) and biochemical studies (Arment and Carmichael, 1996) indicated that these compounds were synthesized by polyketide synthases and nonribosomal peptide synthetases.

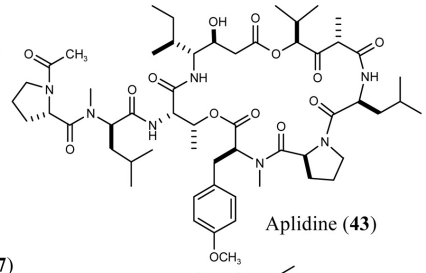
The entire gene cluster involved in the biosynthesis of microcystin-LR has been cloned and sequenced from *Microcystis aeruginosa* PCC7806 (Tillett et al., 2000) and *Microcystis aeruginosa* K-139 (Nishizawa et al., 2000) making it the first cyanobacterial hybrid PKS/NRPS cluster to be characterized. Recently, the gene cluster for microcystin biosynthesis has also been cloned and sequenced from a *Planktothrix* spp. (Christiansen et al., 2003) and *Anabaena* strain 90 (Rouhiainen et al., 2004). The 55 kilobase (kb) gene cluster consists of ten open reading frames (ORF's), *mcyA-J*. McyABCDE and G are nonribosomal peptide synthetases and polyketide synthases that are involved in the incorporation of a β-amino-polyketide unit Adda, glutamate, serine, alanine, leucine, aspartate and arginine. McyJ, McyF, McyI and McyH are tailoring enzymes which are involved in O-methylation, epimerization, dehydration and localization, respectively (Tillett et al., 2000). A notable feature of this compound is the presence of an unusual polyketide-extended amino acid Adda which is synthesized by McyG, D and E. The first step involves the activation of the precursor phenylacetate by the adenylation domain of McyG in an ATP-dependent manner. The activated phenylacetate undergoes four ketide



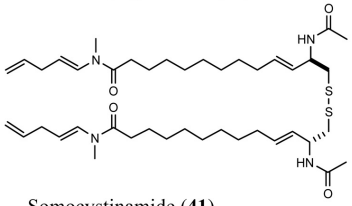
Malyngamide H (**35**)



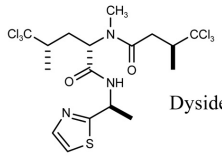
Herbamide B (**36**)



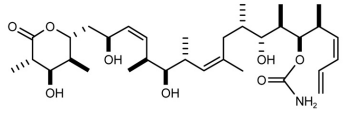
Aplidine (**43**)



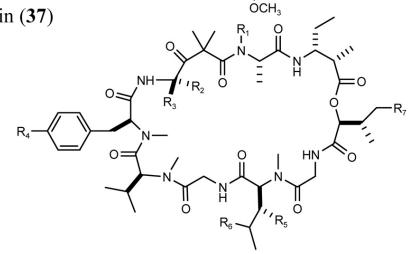
Somocystinamide (**41**)

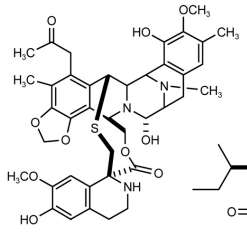


Dysidenin (**37**)

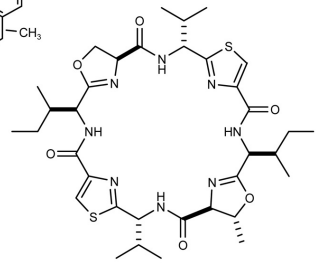


Discodermolide (**45**)





Ecteinascidin (**44**)



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Majusculamide C (**46**)	H	CH_3	H	OCH_3	CH_3	H	CH_3
Dolatstatin 11 (**47**)	H	CH_3	H	OCH_3	H	CH_3	CH_3
Dolastatin 12 (**48**)	CH_3	CH_3	H	H	H	CH_3	CH_3
Epidolastatin 12 (**49**)	CH_3	H	CH_3	H	H	CH_3	CH_3
Lyngbyastatin 1 (**50**)	CH_3	CH_3	H	OCH_3	H	CH_3	CH_3
Epilyngbyastatin 1 (**51**)	CH_3	H	CH_3	OCH_3	H	CH_3	CH_3

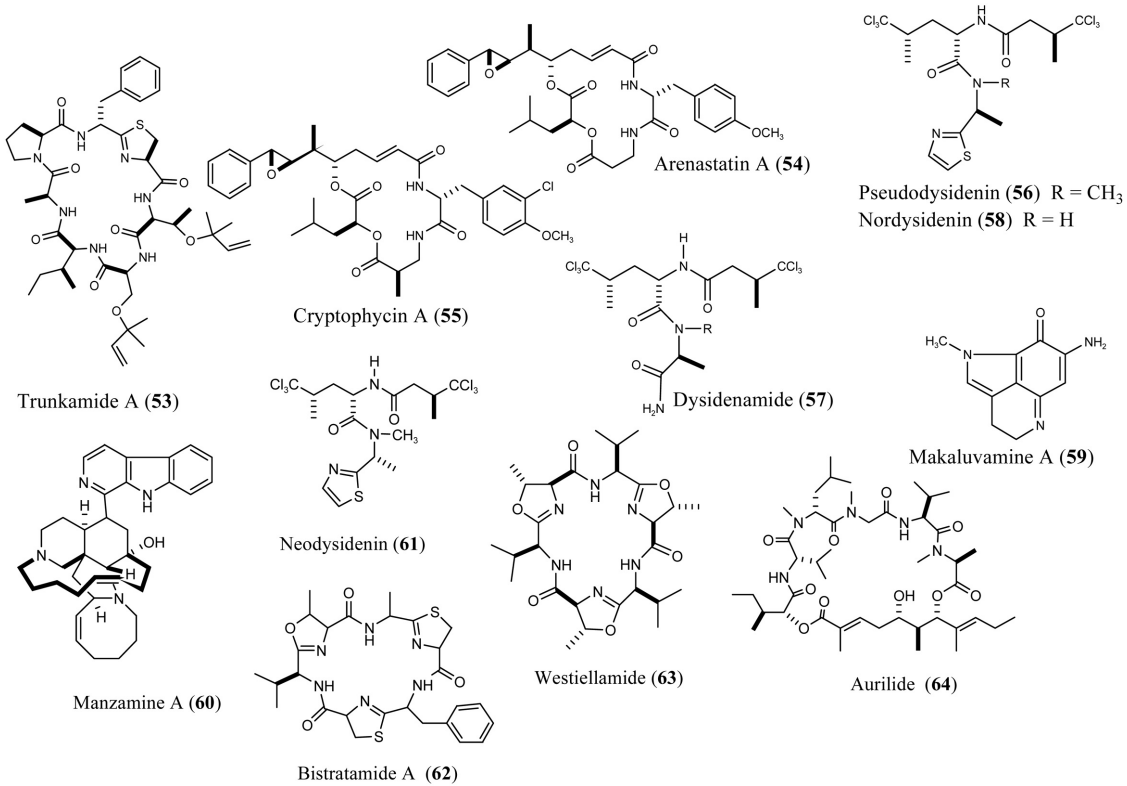


Figure 2: Chemical structures of natural products and derivatives **35-64** discussed in text.

extensions catalyzed by the polyketide modules in McyD and E before the addition of an amino group by the aminotransferase domain in McyE. McyE is also involved in the activation and γ-condensation of D-glutamate. McyAB and C are peptide synthetases that catalyze the incorporation of the remaining five amino acids. The bimodular McyA protein is presumably involved in the addition of the N-methyl-dehydroalanine moiety and D-alanine. McyB also has two NRPS modules and catalyzes the incorporation of L-serine and D-β-methyl aspartate. The addition of the last amino acid, L-arginine is catalyzed by MycC, which also contains the thioesterase domain involved in cyclization and release of microcystin (Tillett et al., 2000).

Transcriptional analyses of the microcystin gene cluster revealed that it is composed of two polycistronic operons *mcyABC* and *mcyDEFGHIJ*, which share a common central promoter located between *mcyA* and *mcyD*. Additionally, two light dependent transcription initiation sites have been identified for each *mcyA* and *mcyD*. Another interesting aspect revealed in this study is the presence of multiple internal promoters which are believed to be functional under certain conditions (Kaebernick et al., 2002). The microcystin biosynthetic gene cluster exhibits unique features of PKS and NRPS with the organization of domains in a manner that enables the biosynthesis of over 65 isoforms. Nevertheless, MALDI-TOF mass spectrometry has allowed detection of single isoforms from a particular genetic variant (Erhard et al., 1997). Genetic characterization of *mcyABC* genes from closely related strains of *Microcystis* showed that genetic variations in *mcyB1* and *mcyC* is highly prevalent as a result of recombination events and these variations along with the relaxed substrate specificity of the adenylation domain in McyB1 may enable the biosynthesis of the various isoforms of microcystins (Mikalsen et al., 2003). It is also interesting to note that all of the microcystin producing genera also have closely related non-producing strains. Horizontal gene transfer has always been speculated as one of the main reasons for the infrequent distribution of microcystins among these genera. However, recent phylogenetic studies have shown that the microcystin genes are very ancient, and were originally present in a common ancestor and that the non-producing strains of cyanobacteria probably resulted from loss of these genes (Rantala et al., 2004).

*3.1.2. Nostopeptolide A1(**31**), A2 (**32**) and Nostocyclopeptide (**33**)*

3.1.2.1. Biosynthetic pathways

Terrestrial cyanobacetria are also well known sources of cytotoxic peptides and depsipeptides that are derived from nonribosomal peptide synthetases. The gene clusters for nostopeptolide A1 and nostocyclopeptide, a group of related peptides from *Nostoc* GSV224 (Hoffmann et al., 2003) and *Nostoc* ATCC53789 (Becker et al., 2004) respectively, have been recently cloned and sequenced, adding to the growing repertoire of cyanobacterial hybrid PKS-NRPS gene clusters. The nostopeptolide gene cluster (*nos*) spans a region of about 40 kb and consists of eight ORF's. The entire cluster is believed to be organized into two operons, *nosABC* and orf-5 and *nosEFG*. *NosABC* encode for PKS/NRPS domains that appear to be co-linear with regards to the biosynthesis of the polypeptide. The adenylation domain

of first module in NosA shows relaxed substrate specificity for the hydrophobic amino acids valine, leucine and isoleucine. This is in accordance to the structures of nostopeptolidesA1 (**31**) and A2 (**32**) in which the first residue is either isoleucine or valine respectively. NosEFG encode for a zinc dependent dehydrogenase, a reductase and an ABC transporter, respectively. NosE and F are believed to be involved in the biosynthesis of the methyl-Proline (MePro) subunit. The product of *orf-5* is also presumably involved in the formation of MePro (Hoffmann et al., 2003). Subsequently, a detailed study on the biosynthesis of Me-Pro by NosE and NosF was reported by Luesch et al. (Luesch et al., 2003).

The nostocyclopeptide (**33**) gene cluster (*ncp*) consists of seven ORF's *ncpA-F* spanning a region of 33 kb (Becker et al., 2004). NcpA and B are peptide synthetases involved in the activation and incorporation of tyrosine, glycine, glutamine, isoleucine, serine, MePro and phenylalanine/leucine residues. NcpDEC are homologous to NosEF and orf-5, respectively, and are believed to be involved in the biosythesis of the 4-methylproline residue from L-leucine. NcpF is similar to an ABC transporter and maybe involved in exporting the compound out of the cell. NcpG is homologous to a family of enzymes that hydrolyze compounds containing D-amino acids. This enzyme is believed to be involved in hydrolyzing the D-Gln residue resulting in degradation of nostocyclopeptide within the cell. A unique feature of the pathway is the presence of a reductase domain with an NAD(P)H binding site at the C-terminal end of NcpB. This reductase is believed to catalyze a NAD(P)H mediated cleavage of the PCP-tethered heptapeptide as a linear aldehyde which ultimately reacts with the amino group of the N-terminal tyrosine residue to form a stable imine.

*3.1.3. Anabaenopeptilide (**34**) biosynthetic pathway*

The gene cluster involved in the biosynthesis of the anabaenopeptilides was isolated from *Anabaena* strain 90 (Rouhiainen et al., 2000). The gene cluster spanning 29kb consists of four genes, *apdA-D*. ApdAB and D encode typical peptide synthetases which are involved in the incorporation of the L-amino acids, glutamine, threonine, tyrosine and amino 6-hydroxy 2-piperidone. A unique feature of this gene cluster is the presence of a formyl-transferase domain in ApdA which is believed to formylate the glutamine starter unit. ApdC is believed to be a putative halogenase which is involved in chlorination of the tyrosine moiety.

3.2 Gene clusters from marine cyanobacteria

*3.2.1. Barbamide (**25**) biosynthetic pathway*

A single collection of *L. majuscula* from Curaçao, produces several bioactive secondary metabolites including barbamide (**25**), curacin A (**8**), carmabin A (**26**) and B (**27**), antillatoxin B (**15**), and malyngamide H (**35**) (Gerwick et al., 1994a; Hooper et al., 1998; Nogle et al., 2001; Orjala and Gerwick, 1996; Orjala et al., 1995a; Yoo and Gerwick, 1995). Thus, this one strain variant provides a treasure trove of biosynthetic machinery to produce secondary metabolites with many unique structural features. The genetic architecture and catalytic domain organization of the barbamide gene cluster (*bar*) cluster is generally co-linear and contains twelve open reading frames (ORFs) (Chang et al., 2002) (**Figure** 3). It is synthesized

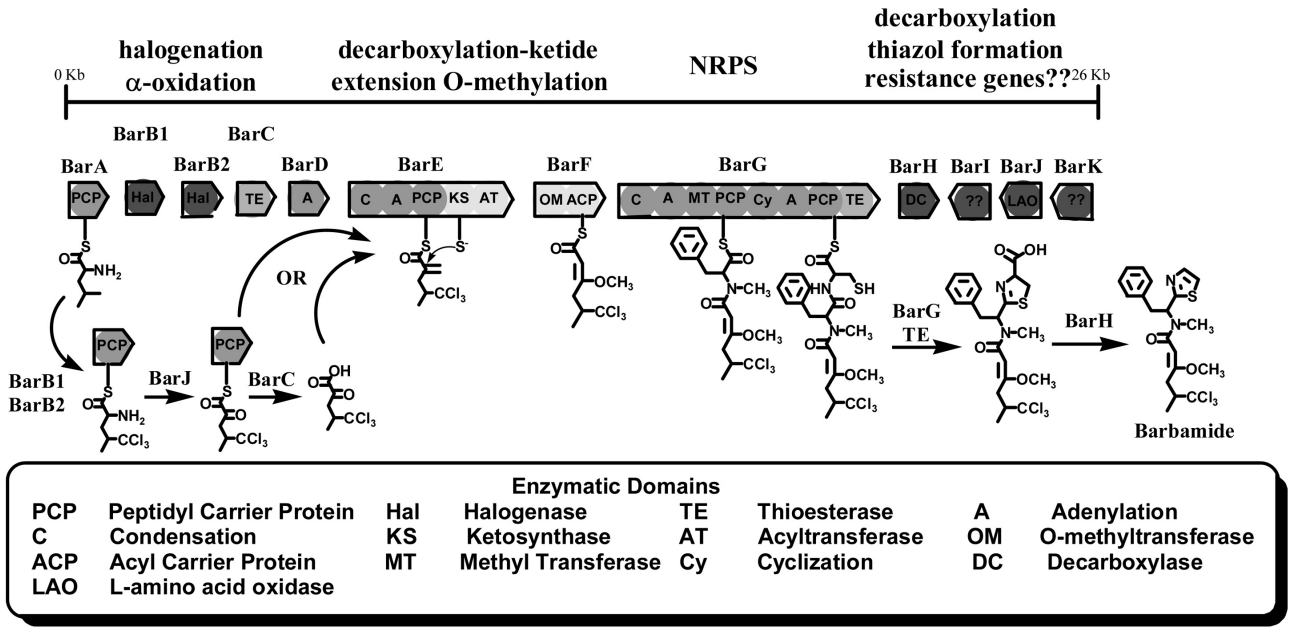


Figure 3: Genetic architecture of the biosynthetic gene cluster and proposed biosynthesis of barbamide (**25**).

by a mixed PKS/NRPS system with unusual features that include a stand-alone peptidyl carrier (PCP) and unusual adenylation (A) domains, as well as a PKS module that is encoded on two separate ORFs. In addition, the biosynthetic system encodes several tailoring enzymes involved in (1) the chlorination, α-oxidation, and decarboxylation of leucine to form a trichloroisovaleric acid moiety, and (2) the oxidative decarboxylation of the cysteine residue at the end of the assembly line to form the terminal thiazole ring (Figure 3). The genetic architecture of the bar cluster is supported by several stable isotope feeding experiments that demonstrate that barbamide is derived from acetate, two S-adenosylmethionine (SAM) derived methyl groups and three amino acids; L-leucine, L-phenylalanine, and L-cysteine (Sitachitta et al., 1998; 2000; Williamson et al., 1999).

Perhaps the most intriguing facet of barbamide biosynthesis is the formation of the unique trichlormethyl functionality. The incorporation of either [2-^{13}C]-leucine or [2-^{13}C]-5,5,5-trichloroleucine during barbamide biosynthesis suggests that L-leucine or an α-keto derivative of L-leucine is the actual substrate for the unique chlorination reactions (Sitachitta et al., 2000). In addition, the chlorination reactions have been shown to be stereospecific, occurring only at the pro-S methyl group of leucine with no detectable activation of the site by adjacent functionalities (Sitachitta et al., 2000). These data suggest that the trichlorination of barbamide occurs through a novel mechanism requiring an enzyme-catalyzed reaction and possibly the formation of an intermediate radical species. BLAST anaylsis combined with preliminary biochemical data suggests that the BarA-BarE and BarJ proteins are involved in the conversion of leucine to the trichloroisovaleric acid. BarD houses a leucine/trichloroleucine-specific A domain, BarA contains a stand-alone PCP domain, BarC shares high sequence homology with known thioesterases (TE) and BarB1 and BarB2 are believed to be the putative halogenases (Figure 3). BarE contains an A domain that shows high activity for both the non-chlorinated and trichlorinated α-keto derivatives of L-leucine, as well as modest activation of trichloroleucine (P.M. Flatt, unpublished). Thus, it is predicted that the conversion of L-leucine to the chlorinated α -keto derivative occurs by the action of BarB1 and BarB2 while the substrate is bound as a thioester intermediate to BarA. The chlorinated α-keto intermediate could then be released and incorporated as the loading module in BarE to initiate barbamide biosynthesis (Figure 3). Thioester intermediates have previously been shown to be the substrate for tailoring reactions, such as hydroxylation, giving precedence for this type of reaction (Chen et al., 2001). Furthermore, a thioester formation assay has shown that BarD activates leucine and is required for covalent bond formation between leucine and BarA (P.M. Flatt, unpublished). BarC can mediate the release of leucine from BarA, *in vitro*, suggesting that BarC may be involved in the release and transfer of the intermediate product from BarA to BarE (P.M.Flatt, unpublished). BarB1 and BarB2 are 89% identical to each other, and 43% identical to the SyrB2 protein in the syringomycin biosynthetic gene cluster (a protein believed to be involved in the chlorination of threonine) (Zhang et al., 1995). BarB1 and BarB2 also show sequence homology with phytanoyl-CoA hydroxylases, suggesting that the enzyme mechanism may require 2-oxoglutarate and iron (II) as cofactors (Kershaw et al., 2001; Mukherji et al., 2001). Preliminary data, supporting this cofactor requirement, has shown that recombinantly expressed BarB1 and BarB2 can convert 2-oxoglutarate into

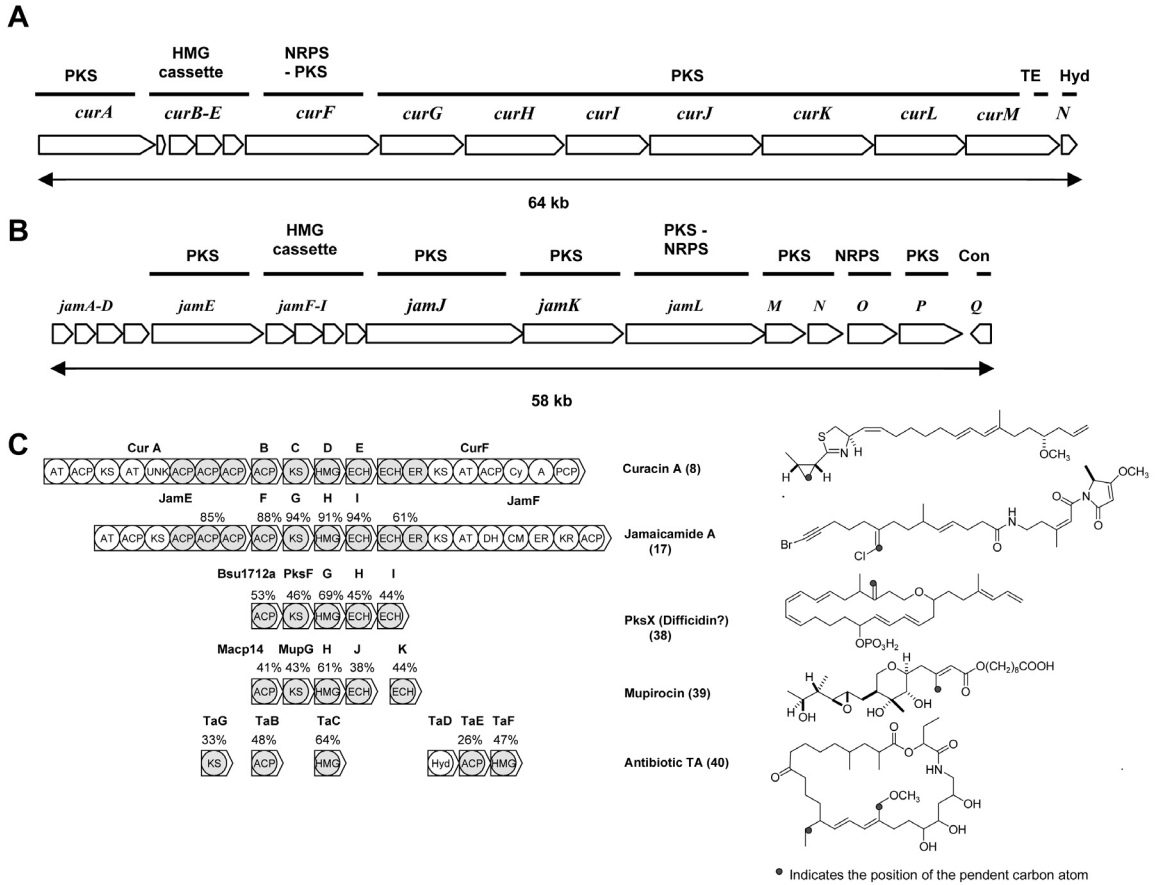


Figure 4. (A) The genetic architecture of the curacin A (**8**) biosynthetic pathway; (B) the genetic architecture of jamaicamide A (**17**) biosynthetic pathway; and (C) the putative gene cassette required for the addition of the pendent carbon atom from acetate to a nascent polyketide is conserved in several biosynthetic pathways. Alignment of this gene cassette from the curacin (**8**), jamaicamide A (**17**), pksX or difficidin (**38**), mupirocin (**39**) and antibiotic TA (**40**) biosynthetic gene clusters (figure adapted from Zhang et al, 2004).

succinate in the absence of substrate (P.M. Flatt, unpublished). In addition, BarJ, which shares homology with L-amino-acid oxidases, is proposed to function as the α-oxidase that converts trichloroleucine to the α-keto-derivative. Similar to phytanic acid metabolism, we propose that the α-keto-derivative is decarboxylated by the KS domain of BarE and ketide extended to begin barbamide biosynthesis (Figure 3). The cleaved formyl group would easily be hydrolyzed from the PCP domain of BarE to complete enzyme turnover (Figure 3). Precedence for the production of a formyl-CoA byproduct has been demonstrated during the metabolism of 3-methyl-branched fatty acids (Croes et al., 1997). BarG is predicted to catalyze the addition of N-methyl phenylalanine and cysteine followed by cleavage from the enzyme complex by the internal TE domain residing in BarG. The released product must then undergo oxidative decarboxylation, possibly mediated by BarH, to form the final product, barbamide (**25**) (Figure 3).

Overall, the mechanisms predicted for barbamide chlorination is catalyzed by a new class of halogenase enzyme, and could be used as a model system for studying mechanisms of halogenation in other microorganisms. BarB1 and BarB2 homologs have been found in other cyanobacterial biosynthetic gene clusters which require halogenation. Homologs with greater than 80% sequence identity have been isolated from strain variants of *L. majuscula* and the cyanobacterial sponge-symbiont, *Oscillatoria spongeliae* that produce other trichlorinated metabolites, including herbamide B (**36**) and dysidenin (**37**) (P.M.Flatt et al, manuscript submitted). Homologs sharing < 35% sequence identity to BarB1 and BarB2 have also been identified in the jamaicamide A (**17**) pathway (JamE) (Edwards et al., 2004) and the putative hectochlorin (**9**) biosynthetic gene cluster (A.V. Ramaswamy, unpublished) and are thought to mediate either the production of a vinyl chloride functionality in the case of jamaicamide A and a gem-dichloromethyl functionality in the case of hectochlorin.

*3.2.2. Curacin A (**8**) biosynthetic pathway*

In addition to the barbamide biosynthesic gene cluster, the curacin A (**8**) biosynthetic gene cluster was also isolated from the 19L strain of *L. majuscula* (Chang et al., 2004). Several stable isotope feeding experiments were instrumental in determining that the biosynthesis of curacin A is derived from one cysteine, ten acetate units, and two S-adenosyl methionine-derived methyl groups, as well as providing several unique mechanistic insights (Chang et al., 2004).

A particularly notable feature of the pathway involves a unique biochemical mechanism for cyclopropyl ring formation. Feeding experiments using [2-^{13}C] acetate revealed that an acetate unit is fragmented via decarboxylation of the C-1 carboxylate during the biosynthetic process, leading to an isolated carbon atom deriving from C-2 of acetate at the C-20 position. The corresponding curacin A biosynthetic gene cluster has revealed a cassette of genes that are predicted to be involved in cyclopropyl ring formation via a novel enzyme that bears homology with 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) synthases (**Figure** 4A). The gene cassette includes the tandem ACP domains found at the end of CurA, the four small ORFs encoding CurB, CurC, CurD and CurE and the first two domains of CurF. Homologs for the *curB-F* cassette can be found in other cyanobacterial pathways, demonstrating that this is a common tailoring mechanism in cyanobacterial

secondary metabolism. These include the putative carmabin biosynthetic gene cluster also isolated from the L19 strain (Sherman laboratory, unpublished) and the jamaicamide gene cluster (*jam*) isolated from a strain variant of *L. majuscula* collected in Hector Bay, Jamaica (Edwards et al., 2004). Additional homologous genes can be found in the "*pksX*" pathway from *Bacillus subtilis*, possibly involved in the biosynthesis of difficidin (**38**), the mupirocin (**39**) pathway (*mup*) from *Pseudomonas fluorescens*, and the antibiotic TA (**40**) gene cluster from *Myxococcus xanthus* (Albertini et al., 1995; El-Sayed et al., 2003; Paitan et al., 1999). The unique feature of all of these pathways is that they require tailoring enzymes that introduce and modify a pendant carbon atom to the nascent polyketide chain during assembly (Figure 3C). The remarkable similarity of the curacin enzymes (CurA-CurE) to the jamaicamide enzymes (JamE-JamI) suggests that these two pathways may share a common late-stage intermediate, such as a β-vinyl-acyl group, before cyclization to yield the cyclopropyl ring structure in curacin biosynthesis or the vinyl chloride in jamaicamide biosynthesis (Figure 4C).

The predicted mechanism of cyclopropyl ring formation in curacin A (**8**) involves the condensation of an acetate unit with an acetoacetyl-ACP to form (S)-3-hydroxy-3-methylgutaryl-ACP (HMG-ACP). This process is thought to be mediated by the *curD*-encoded HMG-CoA synthase. Processing of this intermediate via decarboxylation and dehydration yields an isopentenyl-ACP intermediate that is subsequently isomerized to form the cyclopropyl ring structure. The CurC protein shares homology with KS^Q domains found in numerous modular PKS systems (Bisang et al., 1999) although it contains a Ser residue in place of the invariant Cys residue in the active site. Thus, instead of mediating a condensation reaction, CurC is predicted to act solely as a decarboxylase and thus, is a candidate for mediating the decarboxylation of the HMG-ACP intermediate. Dehydration and isomerization of the resulting intermediate could then be processed by the ECH domains encoded by *curE* and *curF* to yield the cyclopropyl ring. Alternatively, a novel unknown domain in the CurA protein that shares homology with phytanoyl-CoA hydroxylase might contribute to formation of the cyclopropyl ring.

In addition to the cyclopropyl ring, feeding experiments using [2-^{13}C] acetate revealed that a second acetate unit is also fragmented leading to an isolated carbon atom deriving from C-2 of acetate at the C-16 position. This decarboxylative dehydration is predicted to yield the characteristic C-15/C-16 olefin that terminates curacin biosynthesis. Several other cyanobacterial metabolites also share this molecular signature, including kalkitoxin (**16**) and somocystinamide (**41**), suggesting that this is a common termination in the biosynthetic modification of cyanobacterial metabolites. These unique aspects of the *cur* gene cluster will provide new opportunities for metabolic engineering of secondary metabolites as well as provide useful tools for the identification and analysis of additional pathways that share some of these same distinctive features.

*3.2.3. Jamaicamide A (**17**) biosynthetic pathway*

Biosynthetic feeding experiments to jamaicamide A (**16**) producing cultures of *L. majuscula* showed that it is derived from acetate, β-Ala, L-Ala, and methionine strongly supporting a mixed PKS/NRPS assembly of this lipopeptide. These feeding results were instrumental in developing an effective cloning strategy to

isolate the jamaicamide A biosynthetic gene cluster (Edwards et al., 2004). The cloning strategy was based on the initial isolation of PKS containing gene clusters by the use of a general PKS gene probe followed by the utilization of a HMG-CoA-like domain probe to specifically target the jamaicamide pathway. A HMG-CoA synthase-like enzyme was predicted to be necessary for incorporation of the pendent vinyl chloride of jamaicamide A, based on the labeled precursor feeding results and the previously derived sequence data from the *cur* gene cluster.

The 58 kb jamaicamide A (**17**) biosynthetic gene cluster (*jam*) is a remarkable example of a co-linear pathway for the assembly of a complex lipopeptide (Figure 4B). The assembly of jamaicamide A involves approximately thirty separate biochemical steps, and is rich in biochemical transformations novel to PKS or NRPS biosynthetic systems. The biosynthetic pathway is initiated by the generation and incorporation of a novel C-6 fatty acid starter unit with either a terminal double, triple, or brominated triple bond. Although the exact timing of the desaturation and bromination steps awaits further characterization, several predications can be made. It is likely that the novel 5-hexenoate and/or 5-hexynoate starter units are generated from hexanoic acid by the action of a fatty acid desaturase, JamB. Furthermore, based on an ATP-dependent activation experiment with the cloned and over-expressed JamA protein, it appears that these fatty acids are activated in an ATP dependent manner by the acyl-ACP synthetase protein JamA homologous to the recently classified acyl-AMP ligases (Trivedi et al., 2004). This is followed by the loading of this acyl group to JamC. The unusual nature of JamD along with its location in the biosynthetic pathway suggests that this enzyme may be involved in the terminal bromination reaction to generate the terminal brominated alkyne.

After a single PKS chain extension step catalyzed by JamE, a C-8 β-keto-thioester intermediate is generated that presumably undergoes a complex series of chemical modifications to generate the vinyl chloride functionality. Based on homologs in other PKS pathways, such as the curacin A (**8**) pathway described above, JamH, an HMG-CoA synthase homolog, likely plays a key role in the acetate condensation with the β-keto intermediate to generate an HMG-CoA-like intermediate. This intermediate provides a functionalized scaffold for further modifications. In jamaicamide A biosynthesis, further modifications are likely catalyzed by JamF, G, and I (decarboxylation, dehydration and halogenation) to form a one carbon vinyl chloride appendage (Figure 4C). As described for curacin biosynthesis, it appears that this cassette of genes is conserved in a number of different microbial pathways that require the introduction of pendent carbon atoms to a nascent lipopeptide. Next, three more acetate units are incorporated by a series of PKS modules in JamJ, JamK, and the first part of JamL. The reactions catalyzed by JamJ also include the incorporation of a methyl group by the action of an *S*-adenosylmethionine (SAM) dependent methyltransferase.

The final stages of jamaicamide A (**17**) biosynthesis involve a series of interdigitated NRPS and PKS catalyzed chain elongation steps to incorporate a β-alanine (Ala), an acetate (with O-methylation), an L-alanine, and another acetate unit in a linear fashion. Several interesting features are present in this region of the biosynthetic gene cluster. Although feeding experiments showed the incorporation of β-Ala into the jamaicamides (Edwards et al., 2004), an unexpected decarboxylase domain was found to be integrated into the NRPS module. It is

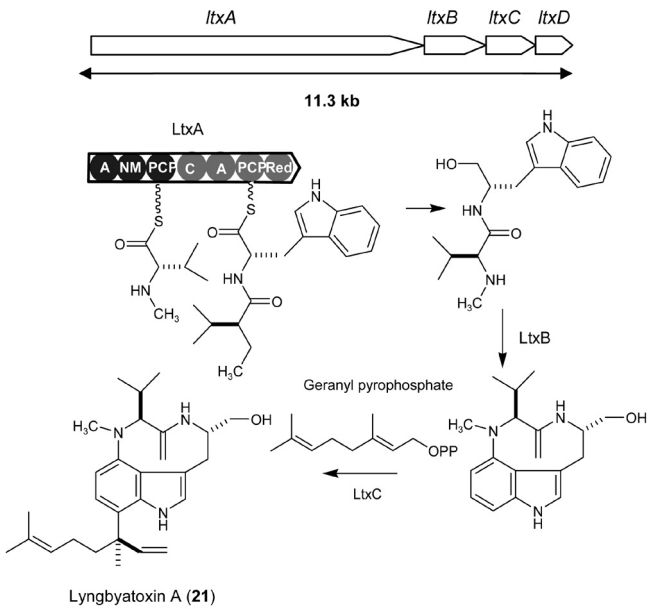


Figure 5. The genetic arichitecture of the lyngbyatoxin biosynthetic gene cluster and proposed biosynthesis of lyngbyatoxin A (**21**). LtxA (NRPS) A: adenylation domain; NM: N-methyl transferase domain; PCP: peptidyl carrier protein; C: condensation domain; Red: reductase domain. LtxB: P450, LtxC: prenyl transferase, LtxD: reductase/oxidase.

possible that the JamL adenlyation domain may have evolved to generate β-Ala *in situ* by the decarboxylation of the α-carboxylate of aspartic acid within the modular biosynthetic environment. Following β-Ala incorporation, a β-methoxy enone is generated by a two protein PKS module (JamM containing KS and AT domains and JamN containing O-methyltransferse and acyl carrier proteins) that incorporates an acetate unit followed by SAM-dependent O-methylation of the resulting enol. Similar PKS O-methylation domains have been found in the barbamide (**25**) (Chang et al., 2002) and myxothiazol (Silakowski et al., 1999) pathways. JamO incorporates an L-Ala unit into jamaicamide A and JamP incorporates the final acetate unit. Although uncertain, the steps that lead to pyrrolinone ring formation maybe catalyzed JamQ, an enzyme with weak similarity to NRPS condensation domains.

The jamaicamide A biosynthetic pathway represents a highly integrated mixed PKS/NRPS pathway identified to date, with two switch points between PKS and NRPS segments as well as two reverse switch points between NRPS and PKS segments. The jamaicamide pathway provides a rich bounty of novel genetic elements that encode for new enzymes catalyzing novel biochemical transformations. Some of these features include a novel alkynyl PKS starter unit, an HMG-CoA synthase containing gene cassette for pendent vinyl or vinyl chloride formation, incorporation of a decarboxylase domain into an NRPS module, and chain termination resulting in pyrrolinone ring formation.

3.2.4. Lyngbyatoxin A (**21**) biosynthetic pathway

Lyngbyatoxins A-C (**21-23**) (Aimi et al., 1990; Cardellina et al., 1979) are potent skin irritants produced by *L. majuscula* and cause a condition known as 'Swimmer's Itch' off Oahu, Hawaii and in Australia. They exert this activity through potent activation of protein kinase C (PKC) (Fujiki et al., 1981), and are chemically and pharmacologically related to teleocidin (**19**) (Fujiki et al., 1981) and olivoretin (**42**) (Hitotsuyanagi et al., 1984) metabolites isolated from terrestrial *Streptomyces* spp. The (-)-indolactam-valine (V) core structure of the lyngbyatoxins most likely derives from L-Val, L-Trp, and methionine, based on experimental chemical feeding results from teleocidin in *S. blastmyceticum* (Hagiwara et al., 1987; Irie et al., 1990).

The cloning of the *ltx* biosynthetic gene cluster was accomplished utilizing a highly specific PCR generated probe (Edwards and Gerwick, 2004). A 1.4 kilobase (kb) probe was isolated by PCR with degenerate primers and spanned an L-tryptophan (Trp) specific adenylation domain/peptidyl carrier protein/reductase domain. Use of this probe led to the isolation of the 11.3 kb *ltx* gene cluster comprised of four open reading frames (ORFs) that are all transcribed in the same direction (**Figure** 5). Lyngbyatoxin biosynthesis is initiated by a two module NRPS protein, LtxA. The first module contains an A-domain that is specific for L-valine (Val), according to established A-domain binding pocket designations, (Challis et al., 2000) an *N*-methylation (NM) domain, and a peptidyl carrier protein (PCP). The second module contains a condensation (C)-domain, an A-domain specific for L-Trp, a PCP, and a reductase domain (Red) that is responsible for the NADPH-dependent reductive release of N-Me-L-Val-L-Trp from the NRPS to N-Me-L-Val-L-tryptophanol. N-Me-L-Val-L-tryptophanol is most likely further modified by LtxB, an unusual cytochrome P450 monooxygenase. It has been proposed that LtxB catalyzes the oxidation of the indole ring of N-Me-L-Val-L-tryptophanol leading to the subsequent cyclization to form (-)-indolactam-V. LtxB also contains a small N-terminal domain of about 80 amino acids that is highly similar to MbtH (Quadri et al., 1998), an unknown domain found in a number of diverse NRPS containing pathways. Lyngbyatoxin is a relatively rare terpene containing bacterial natural product. The monoterpene portion of the teleocidins was shown to derive from the non-mevalonate pathway, (Irie et al., 1990; Irie et al., 1996) suggesting a similar source in the cyanobacterial derived lyngybatoxins. However, it is not known how the geranyl group becomes attached to the (-)-indolactam-V core. Over-expression, purification, and subsequent biochemical characterization of LtxC has shown that the geranyl group is transferred from geranyl pyrophosphate to (-)-indolactam-V to yield lyngbyatoxin A. A key difference between the reaction catalyzed by LtxC and all other known prenyltransferases is that prenylation occurs in a reverse fashion with attachment to the C-3 carbon of GPP to form a quaternary center. This reverse prenylation may proceed first via prenylation of the indole nitrogen followed by a Claisen-type rearrangement to yield lyngbyatoxin, as previously proposed for teleocidin biosynthesis (Irie et al., 1990; Irie et al., 1996). Access to the soluble LtxC now allows for a detailed mechanistic study of this reverse prenylation reaction. The fourth protein encoded by the lyngbyatoxin pathway is LtxD. It shows similarity to a diverse family of oxidase/reductase-type proteins. LtxD may be involved in the conversion of lyngbyatoxin A (**21**) into the minor metabolites lyngbyatoxin B (**22**) and C (**23**).

Table 1. List of completed and in-progress cyanobacterial genomes.

	Organism	Size(mb)	Status	Institution	Publication
1	*Gloeobacter violaceus*	4.66	Completed	KDRI	DNA Res (2003) 10,137-145
2	*Prochlorococcus marinus* subsp *pastoris* MED4	1.67	Completed	JGI / DOE	Nature(2003) 424,1042-1047
3	*Prochlorococcus marinus* MIT 9313	2.4	Completed	JGI / DOE	Nature(2003) 424,1042-1047
4	*Synechococcus* sp. WH 8102	2.43	Completed	JGI / DOE	Nature(2003) 424,1037-1042
5	*Prochlorococcus marinus* SS120	1.8	Completed	European Union/Genoscope	PNAS(2003) 100, 10020-10025
6	*Thermosynechococcus elongatus* BP-1	2.59	Completed	KDRI	DNA Res (2002) 9, 123-130
7	*Anabaena* PCC 7120	6.41	Completed	KDRI	DNA Res (2001) 8, 205-213
8	*Synechocystis* PCC 6803	3.57	Completed	KDRI	DNA Res (1996) 3, 109-136
9	*Acaryochloris marina*	n/a	In progress	Arizona State University/NSF	
10	*Anabaena variabilis* ATCC 29413	7.1	In prefinishing	Missourri State University/JGI/DOE	
11	*Crocosphaera watsonii* WH 8501	6.2	In progress	WHOI / JGI / DOE	
12	*Microcystis aeruginosa* PCC 7806	n/a	In progress	Institut Pasteur	
13	*Nostoc punctiforme* ATCC 29133	9.2	In finishing	JGI / DOE	Photosyn Res(2001) 70, 85-106
14	*Prochlorococcus marinus* MIT 9312	2.4	In progress	JGI / MIT/ DOE	
15	*Prochlorococcus marinus* NATL2A	2	In progress	JGI / MIT/ DOE	
16	*Prochloron didemni*	n/a	In progress	TIGR/Univ of Utah/UCSD/NSF	
17	*Spirulina platensis*	n/a	In progress	Human Genome Center, China	
18	*Synechococcus elonagtus* PCC 7942	2.7	In prefinishing	JGI, Texas A&M University, DOE	
19	*Synechococcus* sp PCC 9311	n/a	In progress	UCSD / TIGR/NSF	
20	*Synechococcus* sp PCC 9605	2.5	In progress	JGI / DOE	
21	*Synechococcus* sp PCC 9902	2.5	In progress	JGI / DOE	
22	*Synechococcus* sp PCC 6301	2.6	In progress	Nagoya University	
23	*Synechococcus* sp PCC 7002	3.2	In progress	Beijing University / Penn State University	
24	*Trichodesmium eythraeum* IMS 101	7.2	In prefinishing	WHOI / JGI / DOE	

Table adapted from Genome Online Database (GOLD); http:// www.genomesonline.org (Kyrpides et al, 1999). Abbreviations: KDRI: Kazusa DNA Research Institute; DOE: Dept. o Energy (USA); JGI: Joint Genome Research Institute; NSF: National Science Foundation; WHOI: Woods Hole Oceanographic Institute; MIT: Massachusetts Institute of Technology TIGR: The Genome Research Institute; UCSD: University of California, San Diego.

Isolation of the *ltx* gene cluster has helped explain several features of the biosynthesis of the (-)-indolactam-V containing family of PKC modulators, including the teleocidins and olivoretins, and has laid a foundation for the utilization of the *ltx* pathway to generate new PKC modulators. Future biochemical investigations of the lyngbyatoxin pathway will undoubtedly reveal interesting mechanistic insights into indole modification reactions catalyzed by LtxB and LtxC.

4. Overview of genome sequencing projects and their utility in studying secondary metabolism

Advances in biotechnology have resulted in high throughput sequencing of DNA, which in turn has revolutionized the potential for examining the genetic complexity of organisms and has led to the creation of an enormous database of both prokaryotic and eukaryotic DNA sequences. The convergence of advancements in technology and biology has triggered an explosion in the field of bioinformatics commonly referred to as the 'genomic revolution'. Post-genomic research areas such as transcriptomics and proteomics have further allowed researchers to decipher the complex nature of gene expression and regulation.

The 'genomics revolution' has revitalized the search for novel drug molecules from microbial sources. Sequence analyses of several bacterial genomes have revealed that there is often discrepancy between the number of peptide/polyketide secondary metabolite gene clusters harbored in the genome of an organism and the number of natural products that can be isolated, suggesting that genes encoding natural product biosynthesis are tightly regulated rather than constituitively expressed. Analysis of the genomes of industrially important organisms like *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* have revealed the presence of several such gene clusters (Bentley et al., 2002; Omura et al., 2001). These 'silent' or cryptic gene clusters can be cloned and expressed in a heterologous host to produce new natural products. A genomics guided approach using select genome sequence tags (GST's) has also been used successfully to identify and express several cryptic biosynthetic pathways involved in biosynthesis of enediyne antibiotics from *Streptomyces* sp. (Zazopoulos et al., 2003). Sequence information is invaluable in identifying regulators of secondary metabolite gene clusters through the use of well-designed microarrays (Askenazi et al., 2003). Once a positive regulatory element is identified, it can be used to evaluate conditions necessary to activate a previously dormant pathway or increase expression levels of actively transcribed gene clusters. The surge in whole genome sequencing has also made it necessary for the development of new bioinformatic tools for data analyses. Of particular interest to the field of polyketide/peptide natural product biosynthesis is the development of a web-based software program such as NRPS-PKS (http://www.nii.res.in/nrps-pks.html) (Ansari et al., 2004) which enables the analyses of the megasynthases involved in these biosynthetic pathways.

There are currently 24 cyanobacterial genomes at various stages of sequencing (**Table** 1) (Kyrpides, 1999). These sequences provide an opportunity to evaluate the molecular potential of different cyanobacterial strains to produce novel secondary metabolites. Generally, it appears that the unicellular bacteria belonging to the genera *Synechocystis* and *Synechococcus* lack the ability to produce NRPS/PKS-derived secondary metabolites. This has been validated by '*in silico*'

analysis and by a PCR approach using degenerate primers to amplify NRPS genes (Christiansen et al., 2001). Similarly, the recently sequenced genomes of various strains of *Prochlorococcus* also show no evidence for the presence of NRPS or PKS genes (Rocap et al., 2003; Dufresne et al., 2003). However, analyses of several isolates of the related *Prochloron* spp., which live exclusively in symbiosis with didemnid ascidians have revealed the presence of NRPS genes of unknown function (Schmidt et al., 2004). These findings further support the hypothesis that many natural products isolated from marine invertebrates are actually produced by the cyanobacterial symbiont present in these invertebrates. Insights gleaned from genome sequencing of *Prochloron didemni,* which is currently in progress will provide valuable information regarding the biosynthetic capabilities of these organisms.

Filamentous cyanobacteria, on the other hand, tend to harbor a number of gene clusters involved in secondary metabolite bioynthesis. Genome sequences of the filamentous cyanobacterium *Anabaena* PCC 7120 and *Anabaena variabilis* ATCC 29413 show the presence of several biosynthetic gene clusters involved in the production of natural products of PKS/NRPS origin (http://www.kazusa.or.jp/cyano/Anabaena/index.html). Similarly, analysis of the genome of the terrestrial cyanobacterium, *Nostoc punctiforme* has revealed the presence of at least 16 secondary metabolite gene clusters (Salomon et al., 2004).

The natural product chemistry of marine cyanobacteria is amazingly diverse and is a direct reflection of the biosynthetic capabilities of these organisms. *L. majuscula* is clearly the most prominent producer of novel secondary metabolites among marine cyanobacteria and accounts for the production of about 30% of all natural products reported from marine cyanobacteria (Burja et al., 2001). At present, four secondary metabolite biosynthetic gene clusters have been described from strains of *L. majuscula* (*bar*, *cur*, *jam*, *ltx*) (Edwards et al., 2004; Chang et al., 2002; 2004; Edwards and Gerwick, 2004). Additionally, unpublished research from our laboratory has revealed the presence of 'silent' or cyptic gene clusters in some strains of *L. majuscula*. An organism of such diverse metabolic expertise makes an exciting candidate for whole genome sequencing! Sequence information from this organism will provide a better understanding of the regulation of these gene clusters, their architecture in the genome and their probable role in the marine environment, thereby providing an opportunity to further exploit the biosynthetic potential of this unique organism. Functional genomic studies will be indispensable in the search for new bioactive compounds. It provides a well-defined blueprint that facilitates the design of rational approaches to search for and harness the gene clusters involved in the biosynthesis of valuable natural products.

5. The Symbiosis Question

Marine invertebrates have proven to be a rich source of biologically active, structurally unique secondary metabolites. Many of these metabolites have significant potential for development as therapeutic leads (Haygood et al., 1999; Belarbi et al., 2003; Haefner, 2003). For example, aplidine (**43**), a cyclic peptide from the ascidian *Aplidium albicans*, is now approved in Europe for the treatment of acute lymphocytic leukemia (Jimeno et al., 2004). Several others are also under clinical investigation for use as anticancer agents include ecteinascidin (**44**), from

the tunicate *Ecteinascidia turbinata* (Phase III) (Jimeno et al., 2004), dolastatin 10 (**1**), from the sea hare *Dolabella auricularia* (Phase II) (Haefner, 2003) and discodermolide (**45**), from the deep-sea sponge *Discodermia* spp. (Phase I) (Schwartsmann et al., 2003). Despite the discovery of numerous biologically active compounds from marine invertebrates, the development of these pharmaceutical leads has been slow, largely due to the inability to obtain enough of the biologically active compound from natural sources for clinical trials and development (Proksch et al., 2002). The desire to develop alternate methodologies for producing active metabolites has driven fundamental research into understanding, at a genetic level, the processes that control invertebrate secondary metabolism.

Marine invertebrates are largely sessile, filter-feeding organisms that contain complex assemblages of symbiotic microorganisms. Thus, when biologically active compounds are isolated from marine invertebrate sources, it is always uncertain if the invertebrate host or an associated microorganism is the actual producer. Observation that some metabolites occur in unrelated genera of marine invertebrates, as well as the isolation of related compounds from microbial sources, provides evidence that some natural products are actually produced by symbionts rather than their invertebrate hosts (Haygood et al., 1999; Osinga et al., 2001). For example, the sea hare, *D. auricularia* has yielded several cytotoxic agents that share similarity with cyanobacterial counterparts, including the anticancer agent dolastatin 10 (**1**) (Bai et al., 1990) and the cytotoxic agent, dolabellin (**13**) (Sone et al., 1995) The isolation of a dolastatin-10 (Luesch et al., 2001a) and related compounds, including symplostatin 1 (**6**) (Harrigan et al., 1998a), from the marine cyanobacteria *Symploca* spp., and the dolabellin-like compounds, lyngbyabellin A and B (**11**, **12**) from *L. majuscula* (Luesch et al., 2000b; 2000a; Milligan et al., 2000), suggests that sea hares obtain and accumulate bioactive compounds as a result of their cyanobacterial diet.

Similarities between structures of cyanobacterial origin have also been found in filter-feeding invertebrates as well. For example, the antifungal compound, majusculamide C (**46**) was originally isolated from a deep water collection of *L. majuscula* and subsequently re-isolated from the sponge, *Ptilocaulis trachys* (Carter et al., 1984; Williams et al., 1993). In parallel, metabolites closely related to majusculamide C were also isolated from the sea hare *Dolabella auricularia,* including dolastatin 11 (**47**) and dolastatin 12 (**48**) (In et al., 1994; McDonald and Ireland, 1992; Rashid et al., 1995). Furthermore, a study of several Guam collections of *L. majuscula* and *L. majuscula*/*Stylocheilus calcicola* yielded a re-isolation of dolastatin 12, its C-15 epimer (**49**), and two closely related compounds, lyngbyastatin 1 (**50**) and its C-15 epimer (**51**) providing more direct evidence of their cyanobacterial origin (Harrigan et al., 1998b).

Many didemnid family ascidians have also been shown to produce biologically active cyclic peptides, including the patellamides (*e.g.* patellamide A (**52**)and trunkamide (**53**) (Degnan et al., 1989a; Fu et al., 1998). Because many of these cyclic peptides resemble cyanobacterial metabolites and it is known that ascidians house cyanobacterial symbionts of the genus *Prochloron,* there is a high likelihood that *Prochloron* is the producer of these cyclic peptides. However, chemical localization studies have demonstrated that the patellamides accumulate in the tunic tissue of the ascidian and are not associated with the cyanobacterial

symbiont (Salomon and Faulkner, 2002). In light of these contradictory results, it cannot be ruled out that the compounds are in fact produced and secreted by the cyanobacterial symbiont where they would then accumulate in the ascidian tissues. Advancements in our understandings of the biosynthetic pathways required to produce cyclic pepetides and other secondary metabolites will undoubtedly help to resolve these conflicting issues. Experiments are quickly being developed to assess the biosynthetic capacities housed within hosts and their symbiotic partners. In the case of *Lissoclinum pattelum*, Schmidt et al, recently identified an NRPS gene sequence from a mixture of the ascidian and its symbiotic *Prochloron* spp. (Schmidt et al., 2004). Based on BLAST sequence homology and overall G/C base content, the NRPS domain appears to be of cyanobacterial origin, providing genetic evidence that the *Prochloron* symbiont possesses the biosynthetic capacity to make complex metabolites. Evidence for NRPS-like genes in the tunicates has yet to be found.

Perhaps the best studied sponge-symbiont relationship involves the sponge of the genus *Dysidea*. *Dysidea* sp. have abundant symbiotic cyanobacterium, predominantly *Oscillatoria spongelliae* (Thacker and Starnes, 2003). In fact, the resulting cyanobacterial biomass can account for up to 40% of the total sponge biomass! Furthermore, within the *Dysidea* genus, it appears that different species have an obligate requirement for specific populations of microbial symbionts. Thacker and Starnes (2003) found that three species of *Dysidea* from Guam (*D. herbacea*, *D. chlorea*, and *D. granulosa*) each contain a genetically distinct clade of *O. spongeliae* symbionts. Concurrently, biochemical analysis has shown that *Dysidea* spp. are renowned for their production of bioactive metabolites that share high structural similarity with cyanobacterial natural products. The Okinawan sponge *D. arenaria* yielded the cytotoxic compound arenastatin A (**54**) that shows high structural similarity with cryptophycin A (**55**), a cyclic peptide isolated from the terrestrial cyanobacterium *Nostoc* sp. (Trimurtulu et al., 1994; Smith et al., 1994; Kobayashi and Kitagawa, 1999). A related sponge, *Dysidea (Lamellodysidea) herbacea* and its *O. spongellieae* symbiont produces several chlorinated metabolites, including dysidenin (**36**) (Dumdei et al., 1997; Harrigan et al., 2001; MacMillan et al., 2000). The trichloromethyl group of barbamide (**24**) and the related cyanobacterial metabolites, pseudodysidenin (**56**), dysidenamide (**57**), nordysidenin (**58**), and herbamide B (**35**), strongly resembles that found in these various *Dysidea*-derived metabolites (Orjala and Gerwick, 1996; Jiménez and Scheuer, 2001). Cell separation studies using flow cytometry analysis has shown that the chlorinated metabolites are associated with the cyanobacterial symbiont. Most recently, molecular analysis of the sponge-symbiont biomass have resulted in the isolation of biosynthetic genes containing high similarity with the barbamide biosynthetic gene cluster (*bar*). Fluorescence in situ hybridization (FISH) techniques were used to localize the *bar*-like halogenase biosynthetic genes to the *O. spongellieae* symboint, demonstrating that the biosynthetic potential for compound production is housed within the cyanobacterial symbiont (P.M.Flatt et al, manuscript submitted).

Recognition that microbial symbionts are often important contributors to the biosynthesis of biologically active compounds opens up additional opportunities to develop sustainable methods for compound production. One could envision

the isolation and culturing of the associated microorganism as a viable option. However, isolating and culturing a producing microorganism from an invertebrate host has proven to be a difficult challenge. The microbial communities residing within marine invertebrates are often diverse and many appear to have an obligate requirement for the host. For example, one report characterized a total of 228 different bacterial species, 25 fungi, 3 actinomycetes, and 9 cyanobacterial strains from the sponge *Candidaspongia flabellate* (Burja and Hill, 2001). In addition, a limited screening discovered 64 bacterial symbionts from the sponge *Aplysina aerophoba* and 51 from *Theonella swinhoei,* representing seven different bacterial divisions (Hentschel et al., 2002). In some cases, bacteria account for up to 40% of the total sponge biomass (Vacelet and Donadey, 1977). Since the early 1990's, several research groups have explored the stable production of bioactive compounds through the isolation and culturing of marine invertebrate-derived symbiotic microorganisms, including unicellular bacteria, filamentous bacteria, and fungi. However, there are only a few limited examples where the same natural product substances have been isolated from both an invertebrate macroorganism and their associated prokaryotic microorganisms, and include makaluvamine A (**59**) from both *Zyzzya* sponges (Barrows et al., 1993) and *Didymium bahiense* myxomycetes (Ishibashi et al., 2001), manzamine A (**60**) from several sponges and a sponge-derived actinomycete (Hill et al., 2003), the polychlorinated metabolites, neodysidenin (**61**) (MacMillan et al., 2000) from *D. herbacea* and its epimer, pseudodysidenin (**56**) (Jiménez and Scheuer, 2001) from *L. majuscula,* and bistratamide A (**62**) from *Lissoclinum bistratum* (Degnan et al., 1989b) and westiellamide (**63**) from the terrestrial cyanobacterium *Westeillopsis prolifica* (Prinsep et al., 1992). In addition, the trichlorinated compound, herbamide B (**35**) has also been isolated from both the sponge *D. herbacea* (P. Crews, unpublished) and the cyanobacterium *L. majuscula* (Jiménez and Scheuer, 2001).

Alternatively, the isolation and characterization of biosynthetic genes from host-symbiont populations is a promising new approach to address the compound supply problem. The development of genetic techniques to screen sponge-symbiont metagenomes for genes related to secondary metabolite biosynthesis and then the use of these for in-situ hybridization experiments is a critical first step in determining the identity of the producing organism.

However, the abundance of different microbial symbionts within an invertebrate host makes the isolation and characterization of individual biosynthetic gene clusters extremely difficult. From a conservative estimate of only 50 symbiotic species per marine sponge, an estimated 125,000 cosmid-containing colonies (estimated insert size ~40kb) would need to be screened from a total genomic library to fully cover the genomic sequences of each organism. However, through the careful selection of metabolites that are likely of microbial origin and a sponge-symbiont system that is limited to a few abundant symbiotic associates, as in the case of the *D. herbaceae – O. spongelliae* system, the screening and isolation of biosynthetic gene clusters can be feasible.

6. The Future
6.1. Challenges

6.1.1. Heterologous expression

The ability to functionally express cyanobacterially-derived natural product biosynthetic gene clusters in a heterologous host is an important goal of current research. Not only will such technologies enable increased production of structurally unique, biologically active compounds, but also will aid in the development of genetic recombination experiments. It is clear that advances in the expression of biosynthetic genes in metabolically engineered strains of *Escherichia coli* make it an attractive artificial host system. Results by Pfeifer et al., demonstrating the production of complex polyketides in *E. coli* have fostered the further development of this expression host in the construction of larger and more complex molecules (Pfeifer et al., 2002; 2001). For example, the partial expression of the epothilone pathway in *E. coli* has led to the precursor-directed biosynthesis of epothilone (Boddy et al., 2004). In this study, the last three ORFs from the epothilone biosynthetic gene cluster were cloned and expressed in *E. coli*. An intermediate substrate was then chemically synthesized and activated as the N-acetylcysteamine thioester to mimic the native S-ACP bound substrate. This was used in feeding experiments to produce epothilone. This combination of synthetic and biosynthetic approaches to natural product production is very attractive because it provides a flexible mechanism to introduce derivative structures into the nascent metabolite. With regard to the expression of cyanobacterial metabolic genes, our laboratory has expressed several functional recombinant proteins in *E. coli*. These include BarA, BarC, BarD, and BarE from the *bar* gene cluster, JamA and JamO from the *jam* gene cluster, and LtxB and LtxC from the *ltx* gene cluster. Thus, *E. coli* is proving to be an important host organism for the production of catalytically active cyanobacterial biosynthetic enzymes. However, most successful examples of heterologous expression have been made using *Streptomyces* sp. as an artificial host, including expression of the actinorhodin, epothilone, and rebeccamycin biosynthetic gene clusters (Malpartida and Hopwood, 1984; Tang et al., 2000; Sanchez et al., 2002). *Streptomyces* sp. have been very useful as heterologous host systems because they are easily grown, plasmids and cosmids for the expression and/or integration of biosynthetic gene clusters are well characterized, and they house many of the modifying and auxiliary enzymes required to produce functional pathways. However, early attempts by our group and others to express cyanobacterial pathways in *Streptomyces lividans* have been unsuccessful. Cyanobacterial gene clusters have shown high levels of molecular instability and are often rearranged within *Streptomyces*. In addition, low levels of protein expression could be attributed to inefficient binding and activation of cyanobacterial transcriptional elements, or possibly alternate codon usage may prevent efficient translation of protein production. Another important factor which hinders the heterologous expression of cyanobacterial gene clusters in *Streptomyces* is the difference in the %GC content in the genomes of the two groups. While most cyanobacteria typically have GC content of 30-42% (http://www.kazusa.or.jp/cyano/cyano.html), this value is much higher in *Streptomyces* (>70%) (Bentley et al., 2002; Omura et al., 2001).

Several studies have shown that the use of heterologous hosts that are closely related to the parental species is more effective and produces higher quantities of the desired natural product. This has been clearly demonstrated in the case of epothilone biosynthesis, where heterologous expression in *Myxococcus xanthus* produces higher yields of epothilone than in a *Streptomyces* counterpart (Julien and Shah, 2002; Tang et al., 2000). Two ideal candidates for the heterologous expression of cyanobacterial genes are *Nostoc punctiforme* ATCC 29133 and *Anabaena* PCC 7120. *Nostoc punctiforme* is a freshwater, filamentous cyanobacterium from the family Oscillatoriaceae, the same family as the genus *Lyngbya*. The genome of this strain has been sequenced and several molecular tools have been developed that allow for the design and development of genetic manipulation experiments (Meeks et al., 2001; Wong and Meeks, 2001). These include plasmid vectors used for the integration of DNA into the host genome as well as non-integrating, independently replicating vectors suitable for the expression of target genes (Wong and Meeks, 2001). In addition, technologies for gene transfer, such as triparental conjugation and electroporation have been well studied and used in targeted gene disruption-rescue experiments in this species (Cohen et al., 1994; Hagen and Meeks, 1999).

Additionally, several strains of *Nostoc sp*. have been shown to produce structurally complex mixed NRPS-PKS natural products similar in structure to those isolated and characterized from marine cyanobacteria, including cryptophycin A (**55**), the nostopeptolides (**31** and **32**), and the nostocyclopeptolides (**33**) (Trimurtulu et al., 1994; Hoffmann et al., 2003; Golakoti et al., 2001). BLAST analysis of *Nostoc punctiforme* ATCC 29133 genomic sequences has revealed the presence of several NRPS clusters, including one with high homology to the nostopeptolide pathway reported from *Nostoc* sp. GSV224 (Hoffmann et al., 2003; Salomon et al., 2004). These data suggest that *Nostoc punctiforme* ATCC 29133 will be well adapted to the functional expression of cyanobacterial biosynthetic genes and contains the required auxiliary proteins to facilitate an active biosynthetic enzyme complex.

6.1.2. Culturing

At present, only a small percentage of cyanobacteria have been successfully cultured in a laboratory and this is largely due to the challenges involved in isolation and purification of these organisms or because cyanobacteria from certain habitats are recalcitrant to laboratory culture techniques (Castenholz, 1988). Traditional microbiological methods are often unsuccessful because of the larger size and complex morphology of some of these cyanobacteria (Rippka, 1988). Isolation and purification methods are often not standardized and there are several factors which need to be optimized for the cultivation of specific cyanobacteria. Sampling techniques, transportation of the sample to the laboratory, media composition and physical parameters such as temperature, light and type of culture vessel are among the many factors which influence cultivation of cyanobacteria.

From a marine natural products perspective, it is important to possess cultures of field collected samples of cyanobacteria for a number of reasons. Since most bioactive metabolites are produced in minute quantities, access to cultures ensures a reliable supply of the metabolite for further chemical and biosynthetic characterization, and for biological evaluations as potential pharmaceuticals.

While some marine cyanobacteria occur as macroscopic tufts or mats that allow for easy collection and direct chemical characterization, a majority of the marine microalgae occur as planktonic or thinly encrusting forms (Gerwick et al., 1994b). In order to study the chemical diversity of these forms, it is important to develop effective culture methodologies.

Members belonging to Oscillatoriales, such as *Lyngbya*, *Phormidium* and *Oscillatoria* have been rich sources of bioactive molecules (Gerwick et al., 2001). These cyanobacteria are characterized by multicellular filaments that are encased in a mucilaginous sheath of variable thickness (Castenholz et al., 2001). Often, these filamentous forms occur in tight aggregates making it difficult to separate single filaments for isolation purposes. Moreover, some cyanobacteria simply do not grow well on solid media (Rippka et al., 1981). Field collected samples of cyanobacteria are also heavily contaminated with diatoms and heterotrophic bacteria that live in close association with the sheath material (Rossi et al., 1997; Cho et al., 2002). The filamentous morphology of these cyanobacteria presents a major obstacle, making it virtually impossible to remove all contaminating bacteria along the length and extensions of the filaments. Standard methods involving repeated washing and filtration usually do not remove these contaminants (Castenholz, 1988). While it is possible to treat cultures with select antibiotics at concentrations that will eliminate bacterial contaminants without affecting the cyanobacteria (Rippka, 1988; Ferris and Hirsch, 1991; Connell and Cattolico, 1996), it has been observed that antibiotic treatment can be ineffective because the mucilaginous sheath surrounding the cyanobacterial filaments protects the heterotrophic contaminants from the effects of the antibiotics (Cho et al., 2002; Vasquez-Martinez et al., 2004).

Another problem associated with the culture of cyanobacteria is their slow growth rate. Single cells or short filaments require several weeks or even months before visible growth can be observed. Doubling times often range from a day to several days or even weeks in some cases. For instance, the cyanobacterium *L. majuscula* exhibits a lag phase during the first six days after inoculation which is followed by a logarithmic phase for the next six days during which cell mass doubles (Rossi et al., 1997). In some cases, field collected samples have taken as long as six months to produce visible growth under culture conditions (Gerwick et al., 1994b).

Some cyanobacteria are inherently difficult to culture and this is well exemplified by *Prochloron* spp which live exclusively in symbiosis with didemnid ascidians (Lewin, 2002). The unique nature and pigment composition of this organism has initiated research in several areas. From the standpoint of natural products chemistry, this organism is believed to be the actual source of several bioactive molecules isolated from ascidians (Sings and Rinehart, 1996) and there is some genetic evidence to support this theory (Schmidt et al., 2004). Despite intensive efforts, all attempts to cultivate this organism outside its ascidian host have proven unsuccessful (Lewin, 1984) making it difficult to determine the exact biosynthetic origin of these compounds. The microenvironmental conditions that regulate the symbiosis of *Prochloron* spp. with didemnid ascidians is currently being investigated with the aid of microsensors and such studies will be critical in identifying conditions for attempting the isolation of *Prochloron* spp (Kuhl and Larkum, 2002). Despite these daunting challenges, a number of reports have

described the successful isolation and purification of certain cyanobacteria in order to exploit their rich chemistry from our group and from others (Burja et al., 2002; Gerwick et al., 1994b; Marquez et al., 2002; Rossi et al., 1997; Williamson et al., 2002).

6.2. Opportunities

6.2.1. Diverse chemistry

The study of marine cyanobacterial natural product chemistry has revealed a number of metabolic themes as well as novel and re-occurring structural motifs. An overriding theme is the rich integration of polyketide/peptide units derived from mixed PKS/NRPS biosynthetic systems. Continuing biochemical study of these pathways will hopefully answer key questions regarding the interactions in a modular PKS/NRPS environment. Other common themes include the incorporation of novel starter units (*e.g.* tertiary-butyl, terminal alkene, alkynes, and even a bromo-alkyne in jamaicamide A), a number of diverse halogenations, heterocylces, the incorporation of functionalized pendent carbons from the action of an HMGCS containing gene cassette, and the incorporation of diverse amino acid units (*e.g.* β-amino acids, N-methylated and O-methylated amino acids). The genetic features that encode for these functional units in marine cyanobacteria will provide a rich toolbox for the creation of structural diversity through pathway engineering and chemoenzymatic synthesis. However, to successfully utilize these features, key underpinning biochemical and genetic knowledge must be developed further. Biochemically, critical studies need to be performed to address the substrate tolerance of these enzymes and how these large modular proteins interact.

6.2.2. Co-linear arrangement of genes

The highly co-linear arrangement of genes-enzymes-final natural product structure is a well established paradigm that holds true for a large proportion of PKS and NRPS systems. The marine cyanobacterial systems studied to date, even with the rich integration of new domains and features, follow this paradigm closely. Further isolation and characterization of new biosynthetic pathways from marine cyanobacteria will provide valuable insights into their evolution and a better understanding of how nature has "combinatorialized" these biosynthetic genes in order to generate structural diversity. Clearly, a deeper level understanding of the natural evolution of these pathways is necessary to develop strategies by which to engineer these systems to generate new compounds.

6.2.3. Novel motifs for use in biotechnology

Overcoming problems in the heterologous and functional expression (the "expression wall") of marine cyanobacterial secondary metabolite pathways will allow the ready production of these fascinating compounds; however, we should not overlook opportunities to utilize individual enzyme(s) to carry out key transformations in a chemoenzymatic-type approach. Recent work in the labs of Marahiel and Walsh have demonstrated the utility of standalone TE domains in the robust cyclization of linear peptide and mixed peptide/polyketide metabolites to their final macrocyclic forms (Trauger et al., 2001; Kohli et al., 2002b; 2002a;

Watanabe et al., 2003; Boddy et al., 2003; Sieber et al., 2003). In these examples, the TE domain mediated cyclization proceeds with high regioselectively and near quantitative yield, in contrast to many standard chemical macrocyclization reactions. In fact, this technology has even been adapted to solid-phase synthesis of cyclic peptide libraries by the TE mediated release of linear peptide precursors from a solid support (Kohli et al., 2002b). Although the TE domains studied to date appear to demonstrate some substrate specificity towards these linear substrates, a degree of flexibility towards certain substitutions has been observed. Marine cyanobacteria are an especially rich source of bioactive cyclic peptide and peptide/polyketide structures, including the potent agents as apratoxin (**23**) (Luesch et al., 2001b), hectochlorin (**9**) (Marquez et al., 2002), and the aurilides (**64**) (Suenaga et al., 1996). Cloning of the TE domains from these responsible biosynthetic pathways would allow the development of these enzymes as standalone macrocyclization reagents to complement standard organic synthetic methods.

Another strategy by which biosynthetic pathways can be utilized to generate natural products or natural product-like compounds is by partial pathway expression and precursor directed biosynthesis. For example, in the case of the epothilone biosynthesis from the myxobacterium *Sorangium cellulosum*, an *E. coli* strain was engineered to express the last three modules of the epothilone biosynthetic pathway (epoD-M6, epoE, and epoF) and the substrate required to complement the biosynthetic enzymes was obtained by chemical synthesis. Under high-density cell culture conditions, this engineered *E. coli* strain processed exogenously fed synthetic substrate into epothilone C (Boddy et al., 2004).

7. Conclusions

Marine cyanobacteria are clearly an extraordinary source of complex and bioactive secondary metabolites, and there is a growing recognition that many of the more interesting natural products previously attributed to marine invertebrates are indeed of cyanobacterial origin. At present, only a few of the most obvious and easily grown marine cyanobacteria have been cultured in the laboratory; we can anticipate tremendous advances in this arena that will spur additional natural products investigations, increase our understanding of the complex metabolic relationships in symbiotic species, and in general, enhance their fruitful application in biotechnology through a detailed comprehension of their metabolic processes. The continued discovery of new compounds from these metabolically talented organisms will provide a range of novel and bioactive natural products which may be developed as potential therapeutics. Additionally, efforts focused at dissecting the biosynthesis of these metabolites at the molecular genetic level, detailed biochemical characterization of some of the unique tailoring enzymes, and whole-genome sequencing of several cyanobacteria will enable us to fully exploit and harness the enormous chemical diversity of these organisms.

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