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New Tricks from Ancient Algae: Natural Products Biosynthesis in Marine Cyanobacteria

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Abstract

Summary—Cyanobacteria, among Earth's oldest organisms, have evolved sophisticated biosynthetic pathways to produce a rich arsenal of bioactive natural products. In consequence, cyanobacterial secondary metabolites have been an incredibly fruitful source of lead compounds in drug discovery efforts. Investigations into the biochemistry responsible for the creation of these compounds, complemented by genome sequencing efforts, are revealing unique enzymatic mechanisms not described or rarely described elsewhere in the natural world. Herein, we discuss recent advances in understanding the biosynthesis of three cyanobacterial classes of natural product: mixed polyketide synthase/non ribosomal peptide synthetase (PKS/NRPS) metabolites, aromatic amino acid-derived alkaloids, and ribosomally encoded cyclic peptides. The unique biosynthetic mechanisms employed by cyanobacteria are inspiring new developments in heterologous gene expression and biotechnology.

B. Introduction

Unarguably, natural products have played an enormous role in the development of modern medicines, especially in the areas of cancer and infection [1]. The last 30 years has seen a focused and purposeful exploration of the marine environment for drug leads from natural products, and recently it has emerged that marine microbes are an especially rich source of structurally novel and bioactive compounds [2••,3]. One particularly noteworthy group, the cyanobacteria, produces a surprisingly diverse array of metabolites that derive largely from the integration of non-ribosomal peptide synthetases with polyketide synthases [4-6]. These nitrogen-rich frameworks are often decorated with unusual modifications such as halogenations, methylations and oxidations. Many have potent cellular toxicity due to inhibition of tubulin or actin mediated processes, although a growing number have other more novel sites of action [7,8]. Hence, there is great interest to better understand how these unusual structures are created, with special focus on mechanistic biochemistry, gene evolution, transcriptional regulation and biosynthetic logic [9,10]. It is hoped that these studies will

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Dedicated to Ralph A. Lewin, longtime inspirational professor of phycology at Scripps Institution of Oceanography and discoverer of *Prochloron* – 1921-2008.

improve access to the full richness of cyanobacterial natural products as well as an ability to employ these gene clusters and biosynthetic gene motifs in heterologous expression and combinatorial biosynthesis.

C. Curacin A biosynthesis

Curacin A, a metabolite isolated from a Curaçao strain of the marine cyanobacterium *Lyngbya majuscula* [11], exhibits potent anti-proliferative and cytotoxic activity against colon, renal, and breast-cancer derived cell lines [12]. Its biosynthetic pathway was identified and partially characterized by genetic and precursor labeling studies [13] as a mixed polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) (Figure 1). Based on this initial analysis, it was predicted that a number of genes encoding unusual catalytic domains and enzymes were present in this pathway, including a GCN5-related *N*-acetyltransferase (GNAT)-like domain in the chain initiation module, a HMG enzyme cassette, an α-KG dependent non-heme halogenase, and a sulfotransferase (ST) domain in the chain termination module (Figure 1). These intriguing catalytic elements embedded within the Cur biosynthetic system illustrate the combinatory flexibility of modular polyketide assembly lines as well as provide valuable objectives for mechanistic enzymology and protein evolution studies.

The GNAT-like domain ($GNAT_L$) in the CurA chain initiation module is an ideal example of functional diversification and gain-of-function in an enzyme scaffold. Previously, the GNAT superfamily of enzymes was only reported to catalyze *N*-acetyl transfer from acetyl-CoA or ACP to primary amino groups on diverse acceptor substrates [14]. In curacin A as well as a series of other biosynthetic pathways, GNAT-like domains embedded in chain initiation modules were predicted to transfer acetyl groups to the terminal thiol of their adjacent ACP phosphopantetheine arm (ACP_Ls) [13,15••,16••,17-18]. Unexpectedly, malonyl-CoA was found to be loaded onto CurA ACP_L, but only an acetyl product was detected on the ACP phosphopantetheine (PPant) arm [19••]. Thus $GNAT_L$ was demonstrated to catalyze decarboxylation of malonyl-CoA to form acetyl-CoA followed by *S*-acetyltransfer to the adjacent ACP_L (Figure 1). Both of these steps represent new functional activities for the GNAT superfamily.

The insertion of an HMG enzyme cassette in the curacin pathway reveals an interesting convergence of two distinct biosynthetic systems, type I polyketide synthases and enzymes involved in isoprenoid assembly. In the curacin A pathway, this cassette catalyzes β-branching modification during the biosynthetic assembly line process at β-carbonyls of the polyketide chain elongation intermediate (dehydration and decarboxylation) [20•]. These modifications are combined with halogenation and cyclopropane ring formation to generate the unusual β-branched cyclopropyl group in the final product [21]. A most interesting aspect of this cassette is how it is structured and incorporated into PKS modules. It comprises tandem triple ACPs (ACP_I, ACP_{II}, ACP_{III}), a discrete ACP (ACP_{IV}), ketosynthase-like decarboxylase (KS), HMG-CoA synthase (HCS), and a dehydratase (ECH_1)/decarboxylase (ECH_2) pair related to the crotonase superfamily of enzymes (Figure 1) [13,20•]. The polyketide intermediate tethered to the tandem triple ACPs undergoes multiple modifications to convert a β-carbonyl group to β-branched methyl group. The HCS catalyzes a key Claisen condensation using C-2 of acetate as a nucleophile, the PhyH halogenase chlorinates at the γ position [21], and then ECH_1 and ECH_2 catalyze the consecutive dehydration and decarboxylation of the chlorinated HMG-like intermediate to form the β-branched product [22•]. Related β-branching modifications have been observed in several biosynthetic pathways, and provide chemical diversity by variation in the number and type of HMG cassette enzymes [23,24] or by using alternative starter units [25] to create additional structural variation in natural products. The recently solved curacin ECH_2 structure suggests that specific amino acid residues in a hypervariable region play a role

in regiochemical control of ECH_2 decarboxylation, which might provide a facile strategy for metabolic diversification [26].

D. Jamaicamide

Jamaicamide A is a neurotoxic metabolite of a Jamaican collection of *Lyngyba majuscula* that possesses several highly intriguing structural features, including an interdigitated polyketide and NRPS overall construction with acetylenic bromide, pendant vinyl chloride, and terminal pyrrolidone ring functionalities [27••]. The original isolation work also mapped out the biosynthetic components and characterized the gene cluster coding for jamaicamide assembly and tailoring reactions. One key insight from the gene cluster analysis was realization that a six-carbon carboxylic acid serves as the starter unit for the pathway. An innovative mass spectrometric method was subsequently used to gain insight into the relative timing of bromination to form the bromoalkyne functionality [28•]. Recombinant proteins JamA [hexanoyl acyl carrier protein (ACP) synthetase] and JamC (ACP) were provided with ATP and a choice of substrates, hexenoic and bromo-hexynoic acids (Figure 2). The results of incubation were queried by Fourier transform ion cyclotron resonance mass spectrometry (FT ICR MS) and only a hexenoyl chain was shown to be tethered to JamC via a thioester bond. The enzyme system was completely unreactive to bromohexynoic acid under any circumstances, showing conclusively that bromination occurs subsequent to hexanoic, hexenoic or hexynoic acid activation and covalent tethering.

E. Lyngbyatoxin

Lyngbyatoxin, a prenylated cyclic dipeptide, was originally isolated from a Hawaiian strain of the marine cyanobacterium *Lyngbya majuscula* and shown to be responsible for a condition known as “Swimmer's Itch” due to its potent activation of protein kinase C [29]. Subsequently, the lyngbyatoxin gene cluster was cloned from this strain, and found to be composed of only four genes that code for several novel biochemical features [30]. The first gene encodes LtxA, a didomain NRPS required for assembly of the dipeptide that terminates with a reductase domain for release of a presumed primary alcohol. LtxB is a cytochrome P450 believed to oxidize the indole ring and possibly act in the cyclization of the molecule. LtxC is a reverse prenyltransferase, and LtxD functions as a short chain acyl dehydrogenase involved in formation of the saturated geranyl chain in lyngbyatoxins B and C. Recently, mechanistic aspects of the reductive off-loading of the dipeptide were studied using alternative substrates and the PCP/reductase components of the NRPS (Figure 2) [31]. Using the N-acetylcysteamine thioester (S-NAC) analog of the proposed natural dipeptide thioester substrate, the four electron reduction of the thioester to a primary alcohol was conclusively demonstrated. Furthermore, performing the reaction in $H_2{}^{18}O$ allowed deduction of an aldehyde intermediate in this reaction sequence, and use of chiral forms of ^2H-labeled NADPH showed only the pro-*S* hydrogen was transferred in both reductive steps.

F. Scytonemin

Scytonemin is a dimeric indolic-phenolic alkaloid that acts as a passive sunscreen in the protection of cyanobacteria against ultraviolet light in marine and freshwater environments [32,33]. Studies of its unique structure, gene cluster, and enzymatic mechanisms have given new insights on the biosynthesis of this compound class in cyanobacteria [33-35]. In 2007, a scytonemin deficient *Nostoc punctiforme* ATCC 29133 mutant was created using random transposon mutagenesis [34]. Analysis of this mutant revealed the transposon was embedded within NpR1273, an open reading frame (ORF) that encodes a putative protein with a signal peptide. NpR1273 was one of 18 contiguous ORFs, likely all co-transcribed [34], and shown to be upregulated by UV light based on semi-quantitative reverse transcriptase-PCR analysis (CM Sorrels *et al.*, unpublished). These ORFs appear to be involved in tryptophan and tyrosine

biosynthesis and have sequence similarity to corresponding operons in five other cyanobacterial species (CM Sorrels *et al.*, unpublished). Bioinformatics analyses showed that NpR1271-NpR1274 had little sequence similarity to other characterized proteins and led to the prediction that they were involved in scytonemin biosynthesis [34]. Further analysis of the cluster prompted a recent investigation into the mechanistic biochemistry required for scytonemin assembly through study of NpR1275 and NpR1276 (Figure 3) [35]. NpR1275, which resembles a leucine dehydrogenase, was shown to catalyze the oxidative deamination of tryptophan to form indole-3-pyruvic acid (IPA). IPA and *p*-hydroxyphenylpyruvic acid then act as the substrates for an acyloin reaction catalyzed by NpR1276, a homolog to a thiamine diphosphate (ThDP) dependent acetolactate synthase. The acyloin product arises from a single β-ketoacid regioisomer, indicative of a highly selective reaction by the ThDP-dependent NpR1276. The mechanism of this early reaction in scytonemin biosynthesis has rarely been described in any other natural system [35].

G. Prochloron biosynthesis

In 1976, a new subclass of algae, the Prochlorophyta, was proposed to describe a unique symbiont living in association with didemnid ascidians from tropical Pacific locations [36]. This symbiont, given the generic name "*Prochloron*" [37], is now recognized as a cyanobacterium despite its plant-like use of chlorophylls a and b and lack of phycobilins. Although *Prochloron* has evaded culture attempts, this cyanobacterium is now known to be the biosynthetic source of the patellamides, cyclic peptides first isolated from one of the host ascidians, *Lissoclinum patella* [38••]. Unlike other peptides from cyanobacteria, the patellamides are remarkable because they are generated from a ribosomally encoded gene cluster rather than from a NRPS mediated process. The *pat* gene cluster is composed of seven ORFs (*patA*-*patG*) in which the precursor peptide *patE* directly encodes the amino acids found in patellamides A and C. While none of the other genes in the *pat* cluster appear to function as epimerases, nonenzymatic epimerization of amino acids to D-isomers appears possible (e.g., see the lissoclinamides below) [39]. The involvement of this gene cluster in patellamide biosynthesis was confirmed through successful heterologous expression of patellamide A in *E. coli* [3,38,40].

In a PCR based screening for the *patE* gene from *Prochloron spp.* obtained from 46 tropical Pacific ascidian samples [41], over 20 *patE* variants were detected. The majority of these gene clusters were virtually identical, except for the patellamide amino acid encoding region. The variable regions in the clusters were predicted to correspond to three cyclic peptide classes: the patellamides, ulithiacyclamides, and lissoclinamides. Engineered mutation of the region encoding the ulithiacyclamides led to heterologous expression of a novel compound, eptidemnamide. Using quantitative PCR, it was found that each *Prochloron* strain likely contains a single pathway, and thus, ascidians can store an entire chemical library by harboring multiple strains of the cyanobacterium.

Continuing exploration of other cyanobacteria, both free living as well as obligate symbionts, for biosynthetic gene clusters homologous to the patellamide pathway [42] has revealed that this ribosomally encoded process is widespread (more than 100 compounds have been identified or implied from gene analysis), and given rise to a new term to describe these cyclic peptides as the 'cyanobactins' (Figure 4) [43•]. Recent advances with the cyanobactins include structural diversification through the biosynthetic addition of side chains such as prenyl groups (patellins, trunkamide), and improved recombinant expression of symbiont natural products [44•].

H. Visualizing biosynthesis

Most organisms exist in Nature as complex assemblages. For example, marine cyanobacteria are commonly found living in association with various invertebrates, such as sponges and tunicates (e.g. see above), and are richly populated themselves by diverse heterotrophic bacteria. Thus, it can be quite difficult to rigorously determine the actual biosynthetic source of a given metabolite. Nevertheless, a variety of gene-based [44•,45••] as well as mass spectrometric imaging techniques are emerging to provide powerful insights to these long standing questions. A recent illustration involved MALDI imaging to demonstrate that mixtures of cyanobacterial filaments could be easily distinguished by their metabolite profile, and that chlorinated peptides could be localized to regions of tissue from the sponge *Dysidea herbacea* populated by the cyanobacterium *Oscillatoria spongeliae* [2••,46]. Other soft ionization techniques such as Desorption Electrospray Ionization (DESI) and Direct Analysis in Real Time (DART) will certainly find innovative application in answering questions concerning the biosynthesis of natural products from complex assemblages, including those containing cyanobacteria [47,48].

I. Conclusions

We are at a particularly interesting juncture in our understanding of how the remarkable natural product structures of marine cyanobacteria are created, mainly as a result of modular NRPS and PKS pathways with a variety of novel tailoring steps. Functional groups of unprecedented structure, such as a variety of pendant one carbon units at C-1 positions of polyketides (terpene-like β branches) and halogen atoms located at traditionally unreactive sites (radical halogenases) [49,50], are now comprehensible in terms of their biosynthetic origin. While the functions of these enzyme systems are now better appreciated, the mechanistic features are only slowly being revealed and much exciting biochemical investigation remains. Moreover, new pathways of assembly with only partially defined biosynthetic processes are being described in some cyanobacteria, such as the *Prochloron* symbionts of ascidians. While these studies are making rapid progress, realization of the ultimate goal of pathway engineering and heterologous expression will require significant advances in underlying gene manipulation technologies as well as understanding of the regulation, storage, and excretion of these metabolites. Indeed, these ancient prokaryotic algae have much new biochemistry to teach us!

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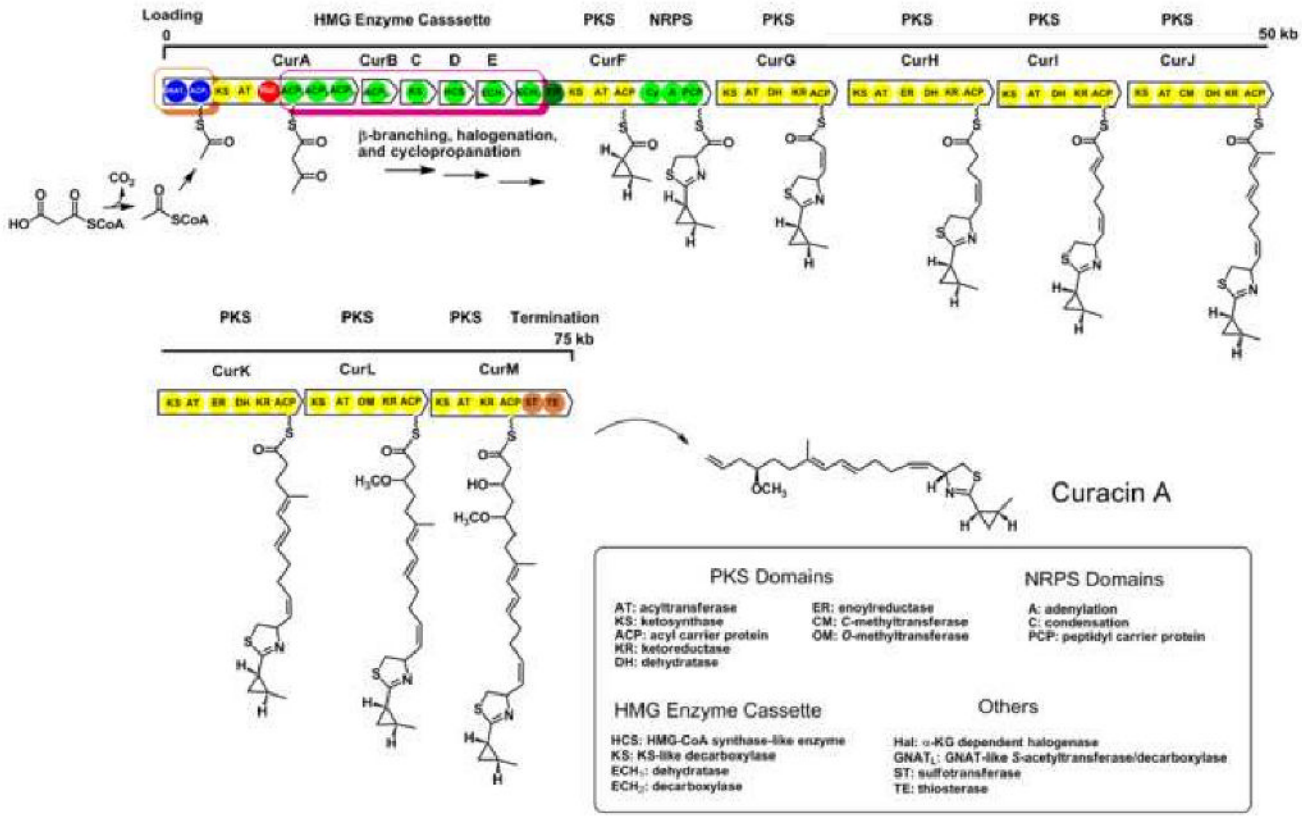

Figure 1.
The curacin A biosynthetic pathway. The chain initiation module and HMG enzyme cassette are highlighted in orange and magenta, respectively.

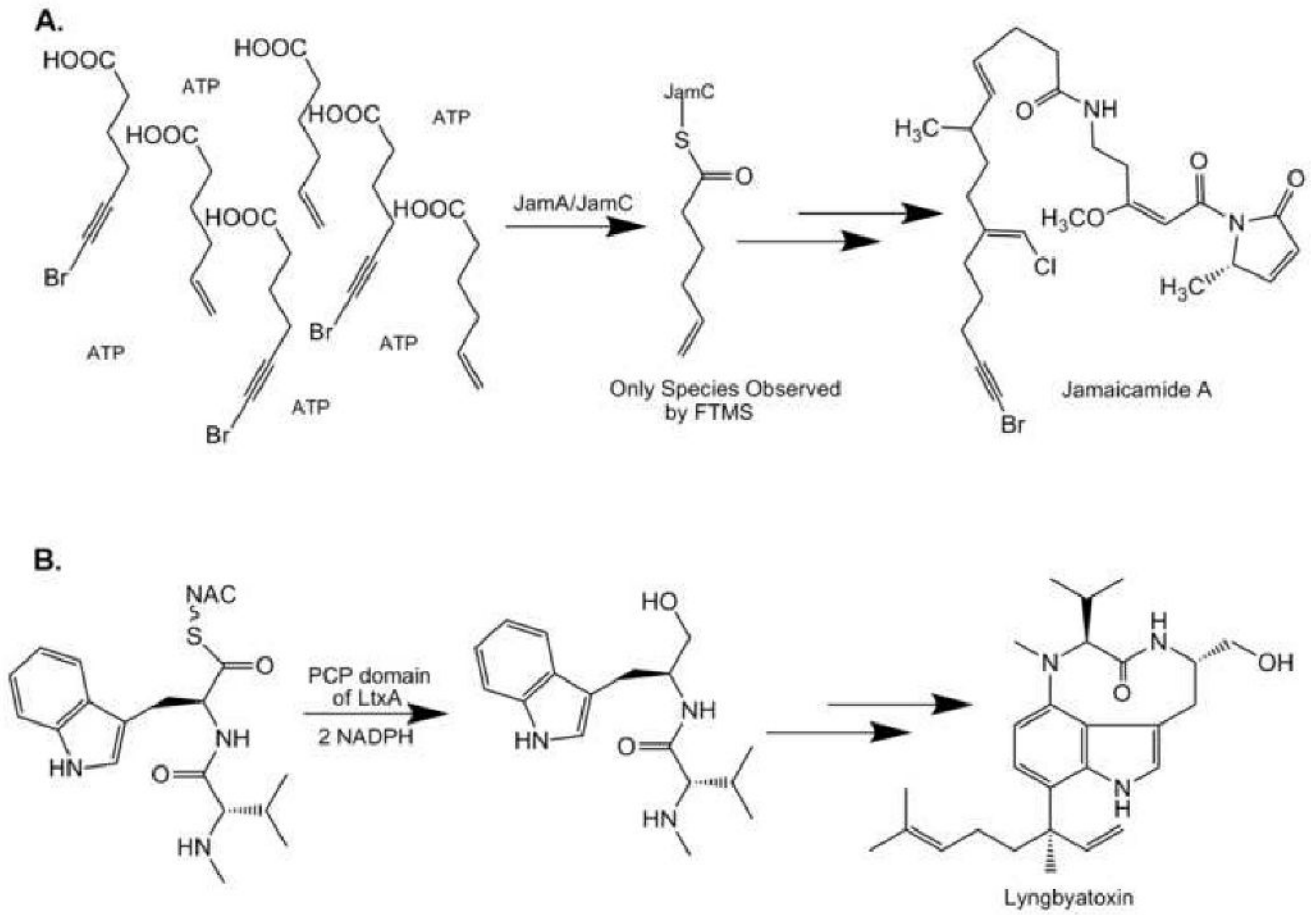

Figure 2.

A) Mass Spectrometric method for determining the identity of preferred substrate initiating the jamaicamide A biosynthetic pathway [28•]. B) Reductive off-loading of the NRPS tethered dipeptide intermediate in lyngbyatoxin biosynthesis shown to be a $4e^-$ reduction involving two equivalents of NADPH [31].

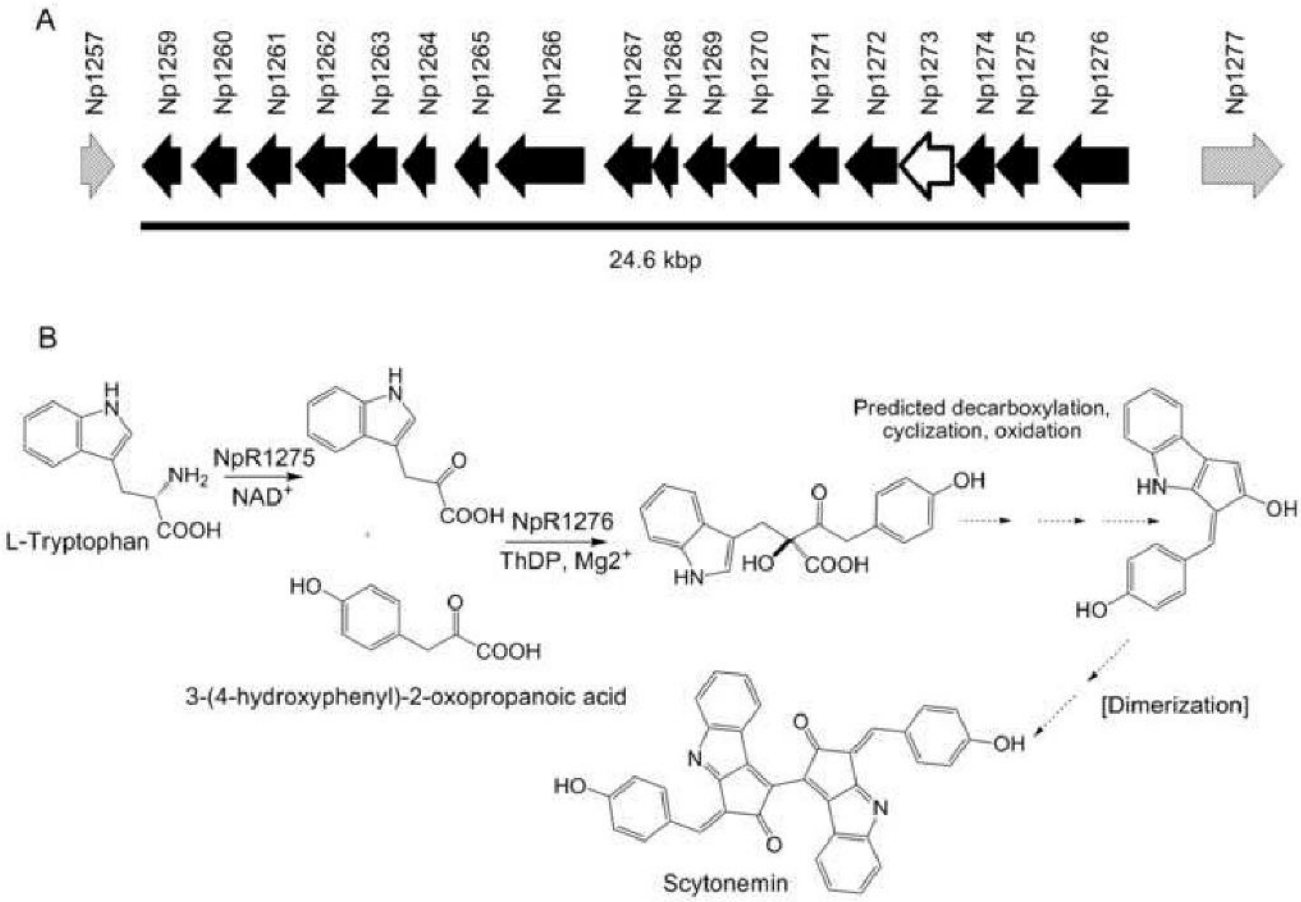

Figure 3.

A) Proposed scytonemin gene cluster shown by solid black arrows with transposon insertion site indicated with a white arrow outlined in black. Patterned arrows represent boundary genes of the proposed cluster [34]. B) Summary of results from initial studies in the biochemistry of scytonemin biosynthesis including functions of NpR1275 and NpR1276 [35]. Predicted gene functions of the scytonemin gene cluster are: **NpF1257** - Short-chain dehydrogenase; **NpR1259** - Hypothetical protein; **NpR1260** - 3-deoxy-D-arabino-heptolosonate-7-phosphate (DAHP) synthase; **NpR1261** - Anthranilate phosphoribosyltransferase; **NpR1262** - Tryptophan synthase (β subunit); **NpR1263** - Putative tyrosinase; **NpR1264** - Tryptophan synthase (α subunit); **NpR1265** - Indole-3-glycerol phosphate synthase; **NpR1266** - Anthranilate synthase; **NpR1267** - 3-Dehydroquinate synthase; **NpR1268** - Dithiol-disulfide isomerase; **NpR1269** - Prephenate dehydrogenase; **NpR1270** - Putative glycosyltransferase; **NpR1271** -Hypothetical protein; **NpR1272** - Hypothetical protein; **NpR1273** - Hypothetical protein; **NpR1274** - Hypothetical protein; **NpR1275** - Leucine dehydrogenase; **NpR1276** - Thiamine diphosphate requiring enzyme; **NpR1277** - PAS/PAC sensor signal transduction histidine kinase.

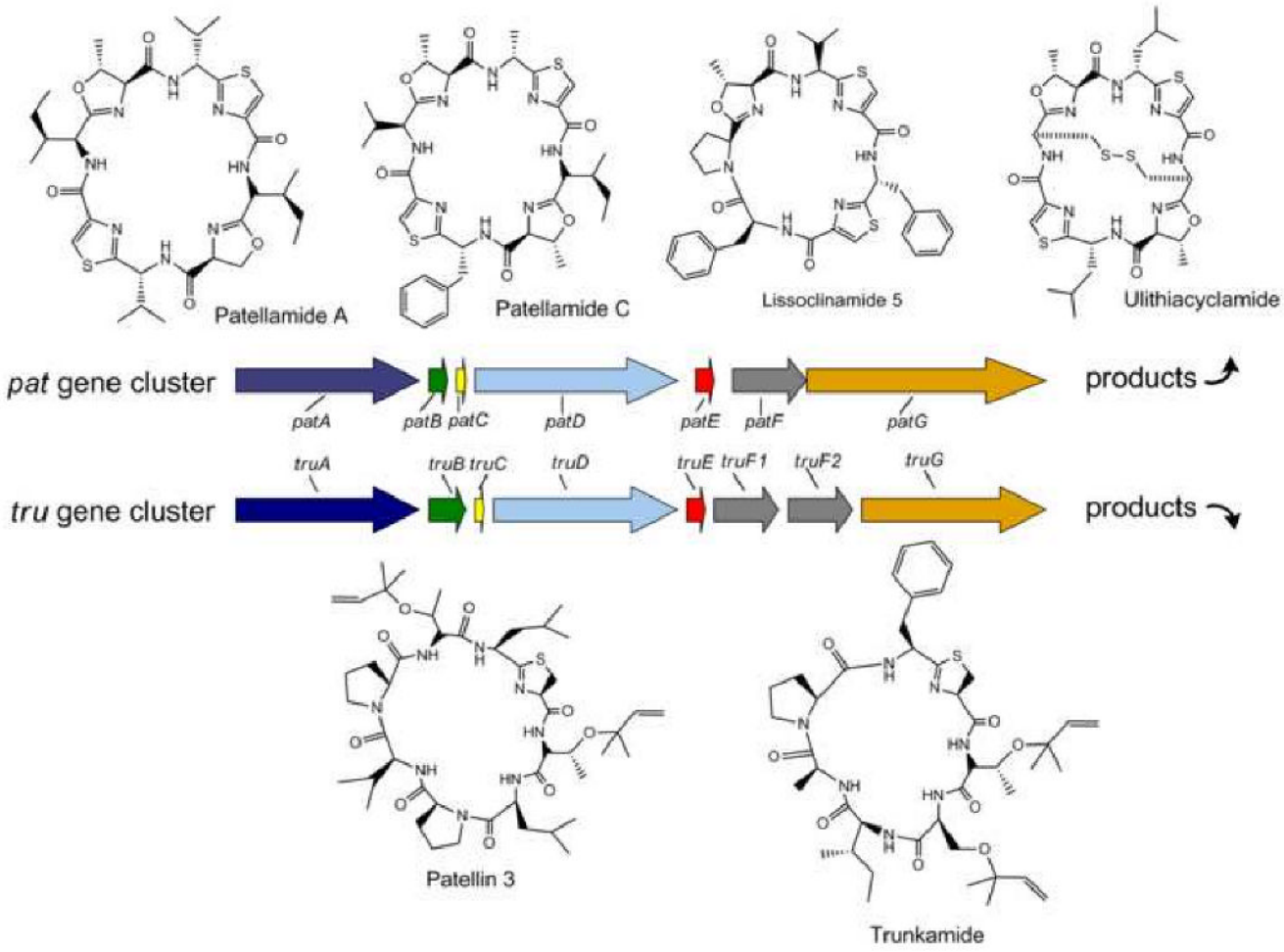

Figure 4.

Two examples of cyanobactin gene clusters from *Prochloron* spp. isolated from the ascidian *Lissoclinum patella* (represented by arrows) [43•]. Conserved genes between the patellamide (*pat*, top) and trunkamide (*tru*, bottom) clusters are shown in matching colors. Subsamples of compounds encoded by variations in *patE* and *truE* are provided above and below the gene clusters, respectively. The absolute stereoconfiguration of lissoclinamide 5 is based on recent synthetic efforts [39].