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Lyngbyatoxin Biosynthesis: Sequence of Biosynthetic Gene Cluster and Identification of a Novel Aromatic Prenyltransferase

Daniel J. Edwards and William H. Gerwick*

College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

Received April 13, 2004; E-mail: bill.gerwick@oregonstate.edu

Lyngbyatoxins A−C (**1**−**3**) are potent skin irritants produced by *Lyngbya majuscula* and cause a condition known as "Swimmer's Itch" off Oahu, Hawaii.[1,2] They exert this activity through potent activation of protein kinase C (PKC) and are chemically and pharmacologically related to the teleocidin[3] and olivoretin[4] metabolites isolated from *Streptomyces* spp. Previous biosynthetic studies on teleocidin in *S. blastmyceticum* showed that the indolactam-V core **4** is assembled from L-Val, L-Trp, and methionine,[5] and that the monoterpene portion is derived from the nonmevalonate pathway.[6] Here we report the molecular cloning of the lyngbyatoxin (*ltx*) biosynthetic gene cluster from a Kahala Beach, Oahu, collection of *L. majuscula*, as well as the biochemical characterization of a novel aromatic prenyltransferase that transfers a geranyl group as the final step in the biosynthesis of lyngbyatoxin A (**1**).

To gain access to the enzymes responsible for some of the intriguing biosynthetic steps for lyngbyatoxin assembly, the cloning of the biosynthetic gene cluster was undertaken. A genomic fosmid library was constructed from a lyngbyatoxin-producing strain of *L. majuscula* in the copy control fosmid vector pCC1FOS (Epicentre, Madison, WI). We predicted that the (−)-indolactam V (**4**) core structure of **1**−**3** would be partially assembled by a nonribosomal peptide synthetase (NRPS) system that contained a C-terminal reductase (Red) domain (Figure 1). Thus, a highly specific probe was isolated by PCR from the genomic DNA of this *Lyngbya* strain using degenerate primers based on the A3 motif of the adenylation (A)-domains[7] and Red1 motif of NRPS-associated Red-domains[8] (Supporting Information). This 1.4 kilobase (kb) probe was used to isolate fosmid clones that were analyzed by restriction mapping and Southern blot experiments. Resultantly, a single fosmid clone, fos-DE3-86, was chosen for DNA sequencing. Fragments were subcloned into pBluescript II SK(+), and a 17.1 kb region harboring the *ltx* gene cluster was sequenced.

The *ltx* gene cluster spans a 11.3 kb region and consists of four open reading frames (ORFs) that are all transcribed in the same direction (Figure 1, see Supporting Information for table of deduced gene functions). The first ORF, *ltxA*, encodes for a two-module

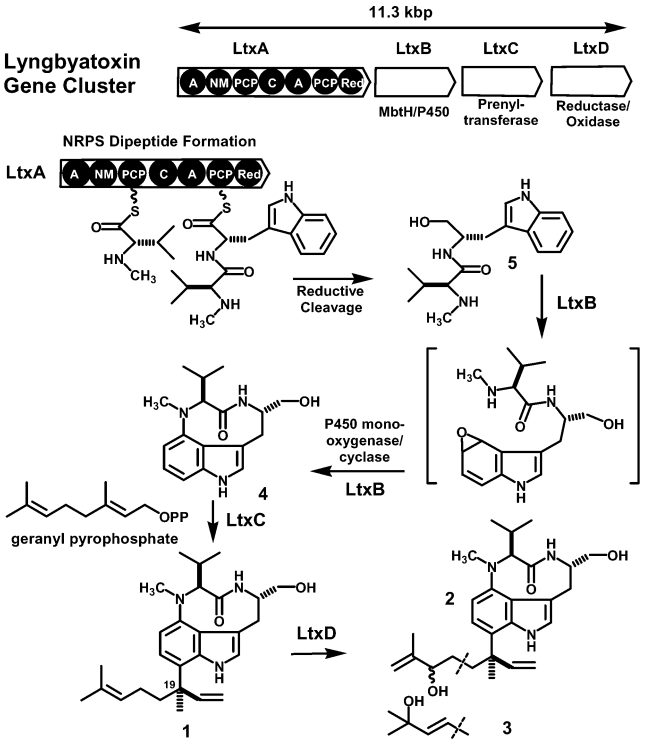

Figure 1. Lyngbyatoxin biosynthetic gene cluster and proposed biosynthesis of lyngbyatoxins A (**1**), B (**2**), and C (**3**).

NRPS protein. The first module contains an A-domain that is specific for L-Val, according to established A-domain binding pocket designations,[9] 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 Red-domain that is presumably responsible for the NADPH-dependent reductive release of *N*-Me-L-Val-L-Trp from the NRPS to generate **5**. The second ORF, *ltxB*, encodes for an unusual cytochrome P450 monooxygenase that contains a small N-terminal domain of about 80 amino acids that is highly similar to MbtH,[10] an unknown domain found in a number of diverse NRPS containing pathways. LtxB is most likely involved in the oxidation of the indole ring of **5** and may also be involved in the subsequent cyclization to form **4**. The third ORF, *ltxC*, encodes for a protein having very little similarity to other known enzymes. The fourth ORF, *ltxD*, encodes for a protein related to a diverse family of oxidase/reductase-type proteins. LtxD may be involved in the conversion of lyngbyatoxin A (**1**) into the minor metabolites lyngbyatoxin B (**2**) and C (**3**).

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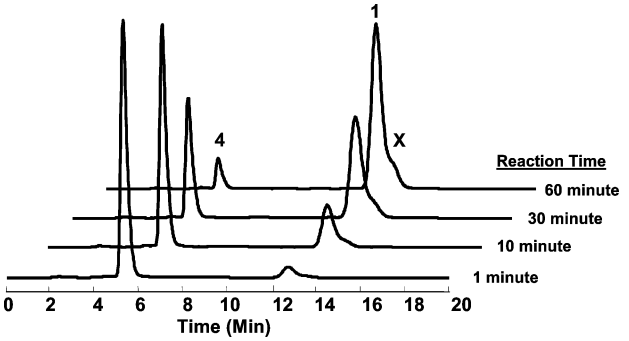

Figure 2. HPLC analysis of in vitro synthesis of lyngbyatoxin (**1**) from (−)-indolactam-V (**4**) and GPP by LtxC.

Because LtxC was unlike any known protein, this enzyme was selected as the most likely candidate to catalyze the prenyltransferase conversion of **4** to **1**. To test this hypothesis, the *ltxC* gene was amplified from fos-DE3-86 by PCR, and the resultant product was cloned into the *Nde*I-*Xho*I sites of pET20a to yield the expression construct pDE101. Introduction of pDE101 into *E. coli* BL-21(DE3) resulted in the overproduction of LtxC as a His_6-tagged protein. LtxC was purified by standard affinity chromatography using Ni−NTA resin (Qiagen, Valencia, CA).

Although the isolation of **4** has not been reported from *L. majuscula*, we nevertheless predicted that the biosynthesis of lyngbyatoxin A (**1**) proceeds through **4** since several teleocidin-producing strains of *Streptomyces* have been found to produce **4**.[11] Therefore, purified LtxC (1 μM) was incubated with **4** (0.1 mM, EMD Biosciences, Madison, WI) and geranyl pyrophosphate (GPP) (0.1 mM) in the presence of 2 mM $MgCl_2$ for varying lengths of time (200 μL scale). Each reaction was extracted twice with EtOAc (200 μL), dried under vacuum, and resuspended in 20 μL of 80% MeOH in H_2O for HPLC analysis on a Microsorb-MV 100-5 phenyl column (80:20 MeOH/H_2O isocratic, detection at 280 nm, Figure 2). Detailed ^{1}H NMR and HRMS analyses confirmed the chemical identity of this product as lyngbyatoxin A. Geranyl addition to the indolactam results in the formation of a new and stereogenic quaternary carbon center (C-19). CD was used to verify that the configuration at C-19 of the enzymatic product was *R*, identical to the naturally occurring **1**.[2] The shoulder observed at 13 min (compound x) was also observed in authentic **1** isolated from field-collected *L. majuscula.* The identity of x was previously investigated but is still not known (J. Cardellina, personal communication).

Indole alkaloids that contain prenylated tryptophan moieties at their core represent an important class of natural product for which considerable synthetic and biosynthetic interest exists.[12] Unfortunately, very little is known about the enzymes involved in the prenylation of the indole core, with dimethlyallyltryptophan synthase (DMAT), which is involved in ergot alkaloid biosynthesis in the fungus *Claviceps*, representing the only known example of this enzyme type.[13] On the other hand, several aromatic prenyltransferases (enzymes catalyzing the formation of a carbon−carbon bond between a prenyl group and an aromatic nucleus) have been identified.[14] These include, in addition to DMAT, the soluble CloQ and NovQ.[15] Like the soluble DMAT and CloQ, LtxC lacks an obvious prenyl diphosphate binding site, and it exhibits catalytic activity in the absence of divalent cations. Despite these similarities, LtxC shows very little sequence similarity to these other aromatic prenyltransferases. 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 **1** as previously proposed.[16] Access to the soluble LtxC now allows for a detailed mechanistic study of this reverse prenylation reaction.

In summary, the *ltx* gene cluster is one of only a few biosynthetic gene clusters identified to date from marine cyanobacteria[17] and is the first pathway sequenced for the family of (−)-indolactam-V-containing PKC modulators, including the teleocidins and olivoretins. In this work we present preliminary biochemical characterization of a new type of prenyltransferase that catalyzes the last step in the biosynthesis of lyngbyatoxin A (**1**). Further biochemical investigations of LtxC and other components of the Ltx pathway will provide for intriguing mechanistic studies and lay a foundation for utilization of the Ltx pathway to generate new PKC modulators.

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Supporting Information Available: Table of deduced functions for genes of lyngbyatoxin cluster (GenBank accession AY588942), PCR primer sequences, and CD spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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