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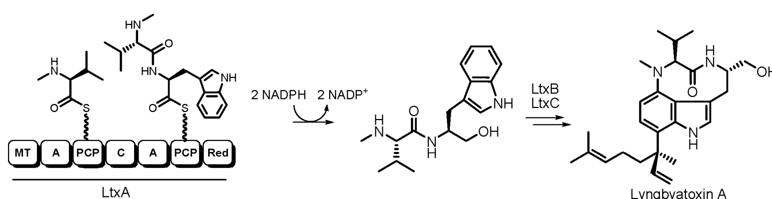

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The Lyngbyatoxin Biosynthetic Assembly Line: Chain Release by Four-Electron Reduction of a Dipeptidyl Thioester to the Corresponding Alcohol

Jay A. Read and Christopher T. Walsh*

Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, 240 Longwood Avenue Boston, Massachusetts 02115

Received September 24, 2007; E-mail: christopher_walsh@hms.harvard.edu

The lyngbyatoxins A−C are secondary metabolites isolated from the cyanobacteria *Lyngbya majuscula*.[1] Like the structurally related teleocidins (Figure 1a), these compounds are potent tumor promoters that operate by competitively binding to protein kinase C (PKC).[2] PKC is a family of serine/threonine kinases that mediate a range of signal transduction pathways in mammalian cells. As a result, small molecules able to specifically modulate the activity of a particular PKC isozyme are targets for cancer therapy.[3]

The gene cluster responsible for production of lyngbyatoxins A−C is composed of four proteins: LtxABCD.[4] LtxA is a nonribosomal peptide synthetase (NRPS) comprising seven domains that prepares **1**, a linear precursor to lyngbyatoxin A in a typical assembly-line fashion (Figure 1b).[5] The amino acid building blocks L-Val and L-Trp are activated as their aminoacyl-AMPs by two adenylation (A) domains. The activated aminoacyl-AMPs are then tethered to the subsequent peptidyl carrier protein (PCP) domain through a covalent thioester linkage with the phosphopantetheinyl arm of the PCP. L-Val is modified by a S-adenosyl methionine (SAM)-dependent methyl transferase (MT) domain. Formation of the peptide bond between *N*-Me-L-Val and L-Trp is catalyzed by the lone condensation (C) domain. Finally, the resulting dipeptide **1** is released from the LtxA scaffold in a reductive manner by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase (Red) domain. The Red domain is derived from the short chain dehydrogenase/reductase (SDR) superfamily of enzymes, as it possesses the signature YXXXK catalytic motif as well as a Rossman fold for NAD(P)H binding.[6] Two additional enzymes, the P-450 enzyme LtxB and the prenyltransferase LtxC, are required to convert the LtxA product **1** to lyngbyatoxin A.

The majority of NRPSs terminate peptide synthesis with thioesterase (TE) domains that either (i) hydrolyze the thioester linkage, releasing the free peptide acid, or (ii) catalyze intramolecular macrocyclization to produce cyclic macrolactam or macrolactone products.[5] LtxA is one of only a handful of NRPSs that do not use a TE domain, but rather release the mature peptide product reductively with the aid of a NAD(P)H cofactor.[7−14] The first such reaction was reported for α-aminoadipate reductase (Lys2), an enzyme required for lysine biosynthesis in yeast.[7] Lys2 catalyzes the two electron reduction of α-aminoadipoyl-*S*-pantetheine-PCP to α-aminoadipate semialdehyde. Recently, it was demonstrated that the macrocyclic imine moeity in nostocyclopeptide forms spontaneously following the two electron reduction of the PCP-bound peptide to release the free aldehyde.[8] Similar enzyme activities may be inferred from examining the gene clusters responsible for producing pseurotin,[9] fusarin C,[10] and equisetin.[11] Although the chemical structure of gramicidin A suggests a four electron reduction of the respective phosphopantetheinyl thioester, it was shown that a second NADPH-dependent dehydrogenase (LgrE) is required to reduce the aldehyde intermediate to the primary alcohol product.[12] As the gene cluster responsible for producing lyngbyatoxin A contains a second SDR enzyme LtxD, one might expect a similar two enzyme/four electron reduction system in operation. However, in contrast with this expectation, we report that the C-terminal domain of LtxA catalyzes a NADPH-dependent four electron reduction of a PCP-bound peptidyl thioester.

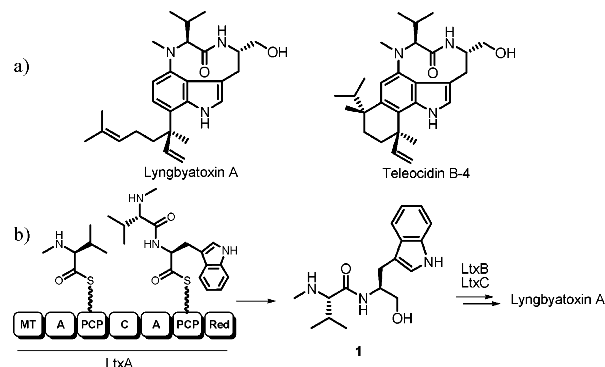

Figure 1. (a) Structures of Lyngbyatoxin A and Teleocidin B-4; (b) biosynthesis of Lyngbyatoxin A.

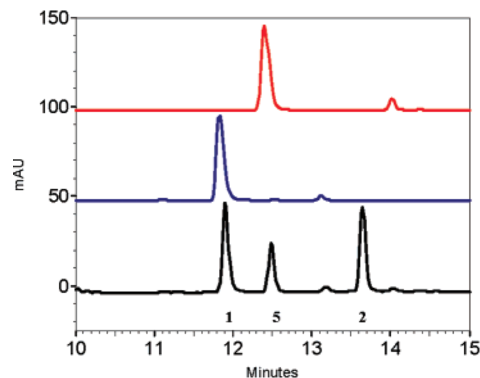

Figure 2. HPLC traces of LtxA-PCP-Red reduction of **2** (black), synthetic **1** (blue), and synthetic **5** (red).

A previous study has shown a Red domain to be inactive in the absence of its upstream PCP domain.[8] Accordingly, the PCP-Red didomain of LtxA was expressed as a N-terminal His_6 fusion protein. LtxA-PCP-Red was purified by elution from a Ni^{2+}-NTA resin with a gradient of imidazole, followed by size exclusion chromatography, which indicated that the 60 kDa protein exists in soluble form as a monomer (Supporting Information).

A common approach used in the study of NRPS systems is to prepare synthetic mimics of the peptide phosphopantetheinyl thioesters and feed them to the terminal TE domains. These mimics are recognized by the TE domains and processed in an identical fashion to the PCP-bound substrates. To that end, we prepared

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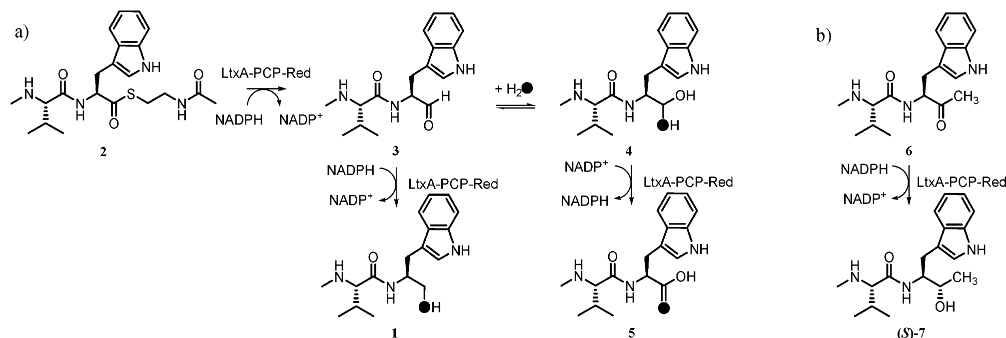
Scheme 1. A Comparison of the LtxA-PCP-Red Catalyzed Reduction of (a) *N*-Me-L-Val-L-Trp-SNAc **2** and (b) the Methyl Ketone Analogue **6**



N-Me-L-Val-L-Trp-SNAc **2** and incubated it with purified LtxA-PCP-Red in the presence of NADPH and EDTA. Upon initiation of the reaction, a decrease in the absorbance at 340 nm was observed and HPLC−MS analysis indicated two new products were formed (Figure 2). The new products were confirmed to be the expected reduction product **1** and *N*-Me-L-Val-L-Trp **5** by coelution with authentic standards. This result demonstrates that LtxA releases its linear peptide product using a four electron reduction, as originally proposed by Gerwick.[4]

The obvious intermediate in the reduction of a thioester to a primary alcohol is the aldehyde. Our attempts to prepare the putative aldehyde intermediate synthetically proved unsuccessful, but we were able to establish the intermediacy of aldehyde **3** indirectly, through the enzyme-dependent incorporation of an ^{18}O label into the primary alcohol **1**. When LtxA-PCP-Red was incubated with **2**, NADPH and EDTA in buffer containing 50% $H_2^{18}O$, incorporation of ^{18}O label into both the alcohol and acid products was observed by HPLC−MS analysis (Supporting Information), suggesting the reaction pathway illustrated in Scheme 1a. Transfer of a hydride from NADPH to **2** would yield the aldehyde **3**. This intermediate could then undergo a dismutation reaction and either (i) accept a second equivalent of hydride from a second molecule of NADPH to give the alcohol **1** or (ii) equilibrate to the hydrate **4** by reversible addition of a molecule of water, followed by transfer of a hydride back to the enzyme-bound $NADP^+$ to yield *N*-Me-L-Val-L-Trp **5**. The oxidation product **5** is only formed in the presence of both enzyme and cofactor, arguing that its formation is truly a redox process and not simply hydrolysis. Incorporation of ^{18}O label into the primary alcohol product argues that the aldehyde, in equilibrium with the hydrate, be an intermediate in the reduction of the peptidyl thioester; it is difficult to imagine an alternative scenario whereby a solvent-derived oxygen atom would be transferred to the alcohol product during reduction.

The stereochemistry of the reduction reaction was probed using an alternative substrate, the methyl ketone **6** (Scheme 1b). LtxA-PCP-Red reduces **6**, yielding the secondary alcohol (*S*)-**7** as the sole reduction product (Supporting Information). The reduction of **6** is analogous to the second reduction step in Scheme 1a and demonstrates that the cofactor delivers a hydride to the *re* face of the carbonyl group. Furthermore, it was observed that only the pro-*S* hydride of NADPH is transferred in the enzymatic reduction. Reduction of *N*-Me-L-Val-L-Trp-SNAc **2** with LtxA-PCP-Red using enzymatically prepared[15] *S*-(4-^{2}H)NADPH as a cofactor resulted in incorporation of two equivalents of ^{2}H into the product **1**. Conversely, no deuterium incorporation was observed when *R*-(4-^{2}H)NADPH was used as the cofactor.

Two enzymes that catalyze mechanistically related four-electron reduction (or oxidation) reactions are 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase[16] and UDP-glucose dehydrogenase.[17] Like LtxA-PCP-Red, these enzymes perform a four-electron reduction (or oxidation) reaction, proceeding through an aldehyde intermediate. HMG-CoA reductase and UDP-glucose dehydrogenase are distinct from LtxA-PCP-Red in that the substrate for the latter is covalently tethered to a protein scaffold, whereas the substrates for the two former enzymes are small molecules. To our knowledge, this is the first example in which an enzyme is demonstrated to catalyze the four-electron reduction of a PCP−phosphopantetheinyl−thioester. As such, this reactivity is an alternative to the typical thioesterase chain termination in the context of NRPSs.

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Supporting Information Available: Synthetic procedures, spectrometric data, and enzyme assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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