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Evidence that Biosynthesis of the Neurotoxic Alkaloids Anatoxin-a and Homoanatoxin-a in the Cyanobacterium *Oscillatoria* PCC 6506 Occurs on a Modular Polyketide Synthase Initiated by L-Proline

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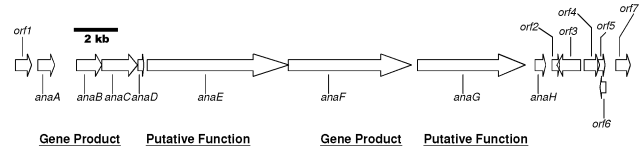
Anatoxin-a and homoanatoxin-a are neurotoxins produced by certain species of cyanobacteria. These alkaloids are potent agonists of the nicotinic acetylcholine receptor,[1] and they provoke the rapid death of animals by acute asphyxia. Several such cases, due to ingestion of water contaminated by cyanobacteria, have been reported from different places around the world,[2] and it is now recognized that the presence of cyanobacterial toxins in water bodies and water supplies has major implications for public health and the environment.[3]

Our knowledge about cyanobacteria that produce anatoxin-a and homoanatoxin-a is limited, and our goal is to contribute to the characterization of this interesting class of microorganisms using biochemical as well as genomic approaches. An important issue is finding a specific genetic marker for these neurotoxic cyanobacteria and in particular to find the genes responsible for the biosynthesis of the toxin. Such markers would be useful for the detection of cyanobacteria that produce anatoxin-a. On the basis of feeding experiments using labeled precursors, Hemscheidt et al.[4] proposed that anatoxin-a results from the condensation of three acetate units on (*S*)-1-pyrroline-5-carboxylate, which is derived from Glu, followed by a decarboxylation. Homoanatoxin-a would result from methylation of anatoxin-a. However, the individual chemical steps of this pathway have not been elucidated.

We recently identified a DNA sequence, which we called ks2, that is specific for *Oscillatoria* cyanobacteria producing anatoxin-a or homoanatoxin-a.[5] This ks2 sequence was shown to code for a polyketide synthase (PKS), a fact that was consistent with the proposed biosynthetic scheme. To gain further insight into cyanobacteria that produce anatoxin-a and homoanatoxin-a, we have initiated the sequencing of the genome of *Oscillatoria* Pasteur Culture Collection (PCC) 6506, which produces these neurotoxins. Among the contigs reconstructed to date, we have identified a 29 kb DNA fragment containing the ks2 sequence. The sequence of this genomic fragment was analyzed, and we found several genes (Figure 1) that we annotated (Table S1 in the Supporting Information) using Psi-Blast similarity searches and specialized web servers[6] for PKS domain prediction.

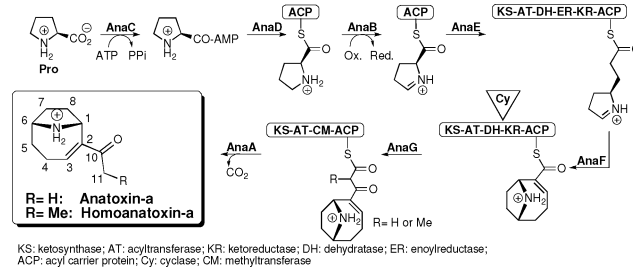
The deduced function of the proteins in this candidate cluster immediately suggested a concise and plausible biosynthetic pathway to anatoxin-a and homoanatoxin-a, which is outlined in Scheme 1.



Gene Product	Putative Function	Gene Product	Putative Function
Orf1	Cyclase	AnaE	Modular Type I Polyketide Synthase
AnaA	Type II Thioesterase	AnaF	Modular Type I Polyketide Synthase
AnaB	Proline dehydrogenase	AnaG	Modular Type I Polyketide Synthase
AnaC	Proline Adenylation Protein	AnaH	Transposase
AnaD	Acyl Carrier Protein		

Figure 1. Organization of the putative gene cluster responsible for anatoxin-a and homoanatoxin-a biosynthesis in *Oscillatoria* PCC 6506. The sequence of this region has been deposited in GenBank (accession number FJ477836). The full annotation of each open reading frame is given in Table S1.

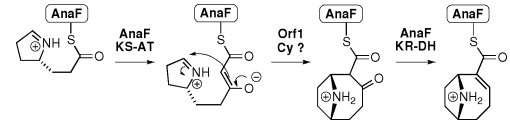
Scheme 1. Proposed Biosynthetic Route to Anatoxin-a and Homoanatoxin-a Starting from Proline



KS: ketosynthase; AT: acyltransferase; KR: ketoreductase; DH: dehydratase; ER: enoylreductase; ACP: acyl carrier protein; Cy: cyclase; CM: methyltransferase

The first three steps are likely similar to those described for the biosynthesis of pyrrole-containing secondary metabolites,[7] with adenylation of proline, loading on an ACP, and oxidation by a flavoenzyme. Although the position of the double bond can only be guessed at the moment, the oxidation product is very likely the (*S*)-1-pyrroline-5-carboxylate thioester derivative because the imine is the perfect electrophile in the subsequent proposed Claisen-type cyclization step (Scheme 2).

Scheme 2. Proposed Mechanism for the Cyclization Step



After transfer to the PKS AnaE, the thioester would be elongated by two carbons with complete reduction. The growing chain would then be transferred to AnaF for elongation and cyclization. The cyclization may be catalyzed by Orf1, since this predicted protein

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shows significant similarity to the C-terminal domain (Cy) of StiJ in *Stigmatella aurantiaca*, which has been speculated to play an analogous role in the biosynthesis of the polyketide stigmatellin.[8] The bicyclic thioester would then be transferred to AnaG for condensation and methylation. The proposed biosynthetic scheme readily accounts for the synthesis of both anatoxin-a and homoanatoxin-a by the same PKS. PCC 6506 produces predominantly homoanatoxin-a (99%) and minor amounts (1%) of anatoxin-a,[9] suggesting that *S*-AdoMet-dependent methylation is faster than subsequent release of the chain from the multienzyme. A thioesterase is presumably responsible for chain release, which would lead to 11-carboxyanatoxin-a or 11-carboxyhomoanatoxin-a, respectively, as plausible enzyme-free intermediates. We did not detect such compounds in cellular extracts of PCC 6506, although the presence of 11-carboxyanatoxin-a has been previously reported in extracts of *Aphanizomenon issatschenkoi*.[10] Decarboxylation to give anatoxin-a and homoanatoxin-a, respectively, may be spontaneous or catalyzed by an as-yet unidentified enzyme. It remains to be established whether or not the putative type-II thioesterase AnaA plays a role in chain release. Similar enzymes are common features of many modular PKS- and nonribosomal peptide synthetase-mediated biosynthetic pathways, where they are proposed to help reactivate misacylated multienzymes.[11]

We carried out feeding experiments using ^{2}H-, ^{13}C-, or ^{15}N-labeled precursors and PCC 6506 as the toxin producer. The homoanatoxin-a thus produced was extracted and analyzed by GC−MS and NMR (Figures S1−S11 in the Supporting Information). The incorporation of labeled acetate and L-[U-^{13}C−^{15}N]Glu was in agreement with Hemscheidt's proposal.[4] However, we observed that the nitrogen of Glu was lost, probably during a rapid transamination between α-ketoglutarate and Glu. Thus, Glu may not be the actual substrate of the adenylation protein, AnaC. Using L-[U-^{13}C−^{15}N]Arg, we observed the formation of homoanatoxin-a with six extra mass units in the bicyclic moiety of the toxin. This is consistent with the incorporation of Pro that derives from Arg via ornithine in the primary metabolism. Using L-[U-^{13}C−^{15}N]Pro or L-[U-^{13}C]Pro, we observed a high incorporation (63%) of all C's and the N of this compound in the bicyclic moiety of homoanatoxin-a. Using L-[*methyl*-$^{2}H_3$]Met, we confirmed that the C_{12}-methyl of homoanatoxin-a derives from Met via a *S*-AdoMet-dependent methylation reaction.[12] Taken together, these results are consistent with, though they do not rigorously prove, our proposal that Pro, which can be readily produced from either Glu or Arg in primary metabolism, provides the starter unit for anatoxin-a and homoanatoxin-a biosynthesis.

Efforts to obtain genetic confirmation of the identity of the cluster by specific gene disruption or deletion in PCC 6506 have to date been unsuccessful. However, it is encouraging that we have found a consistent correlation, for 13 axenic strains of cyanobacteria in the PCC, between the presence of the putative *ana* gene cluster (probed using specific PCR primers for each of *anaE*, *anaF*, *anaG*, and *anaC*) and the production of anatoxin-a and homoanatoxin-a in cell extracts[2b] (Table S2).

To gain more direct insight into the role of the putative adenylation enzyme AnaC, we undertook the expression of that enzyme and of the ACP AnaD as recombinant His-tagged proteins in *Escherichia coli* (Figure S12). The affinity-purified AnaC was assayed using the ATP−PPi exchange reaction and found to be specific for Pro (specific activity = 36 nmol mg^{-1} min^{-1}, Figure S13). Apo-AnaD was readily transformed into holo-AnaD using the Sfp phosphopantetheinyl transferase and further transformed into the Pro thioester using AnaC, as probed by mass spectrometry (Table S5). Neither Glu nor ornithine was an AnaC substrate.

Although much remains to be done to deconvolute the detailed enzymology of the pathway, these data (together with the close fit of the deduced functions in the candidate *ana* cluster to those required for anatoxin-a and homoanatoxin-a biosynthesis in PCC 6506) provide strong evidence that we have successfully identified the gene cluster for production of these remarkable toxins.

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Supporting Information Available: Materials and methods, GC−MS and NMR analyses, and protein biochemistry. This material is available free of charge via the Internet at http://pubs.acs.org.

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