# Biosynthesis of Anatoxin-a in *Anabaena flos-aquae* and Homoanatoxin-a in *Oscillatoria formosa*

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The carbon skeleton of anatoxin-a is derived from acetate and glutamate with retention of C-1 of the amino acid.

Strains of the cyanobacterium *Anabaena flos-aquae* produce two alkaloidal toxins, anatoxin-a[1] and anatoxin-a(s),[2] which, although structurally unrelated, exhibit similar pharmacological responses in animals shortly after the toxins are injected or ingested. Since the original isolation from strains of *Anabaena*, anatoxin-a has been detected in a variety of other species and genera.[3] As part of a program to elucidate the biosynthesis of toxins produced by blue–green algae, we have recently reported on our results concerning anatoxin-a(s) biosynthesis.[4] Here we present the first results of our investigation of the biosynthesis of anatoxin-a **1** in *A. flos-aquae* and its homologue homoanatoxin-a **2**[5] in *Oscillatoria formosa*.

Structurally, **1** and **2** appear to be related to the tropane class of alkaloids found in higher plants,[6] *e.g.* tropine and cocaine, having a substituted azabicyclo[4.2.1]nonene instead of an azabicyclo[3.2.1]octane ring system. This structural similarity might also be reflected in the biosynthetic pathways by which these compounds are assembled in the producing organisms. The tropanes are formed from ornithine/arginine *via* the diamine putrescine with addition of a $C_3$ fragment derived from acetate.[7] For the biosynthesis of **1** and **2**, we considered this as well as several other biogenetic schemes, most of which differed from one another by the number and presumed location of acetate-derived carbon atoms. Thus, acetate was chosen for the first feeding experiments.

Sodium [1.2-$^{13}C_2$]acetate, in an admixture with the natural abundance material to suppress interunit coupling, was administered to *Anabaena flos-aquae* strain 37 that produces **1** and to *Oscillatoria formosa* strain NIVA CYA-92 that produces **2**.

The $^{13}C$ NMR spectra of the samples that were isolated from the freeze-dried algal material by adaption of a known method[8] showed $^{13}C$ enrichment (specific incorporation above natural abundance: **1**, 4.5%; **2**, 27%) in several carbon atoms of each of the alkaloids. The homonuclear coupling pattern indicated that three pairs of carbon atoms, C-1,-8; C-3,-4, and C-2,-10, were derived intact from the bond-labelled precursor, satellites being observable in the signals due to these carbons in the 125 MHz $^{13}C$ NMR spectra of **1** and **2**. C-11 was also enriched in each compound, but no $^1J$ coupling was observable. No enrichment was seen in the signals due to C-5, C-6, and C-7 of **1** and C-5, C-6, C-7, and C-12 of **2**.

Thus, the molecules are only partially derived from polyketide precursors and, surprisingly, the terminal exocyclic carbon atoms do not represent an acetate or propionate derived starter unit, but appear to constitute the chain terminating group.

In both **1** and **2**, the acetate-derived $C_2$ unit, C-1,-8, had suffered lower dilution than the other two $C_2$ units, C-3,-4 and C-2,-10, and had therefore entered the toxins by a different route. This observation and the complete absence of enrichment in C-5, -6 and -7 appears to indicate that the fragment C-5,-6.-7,-8,-1 arises from a source other than acetate alone. The close proximity of the nitrogen atom of the toxin to this $C_5$ unit suggested a possible derivation of this fragment from an amino acid precursor, specifically a member of the glutamic acid family.

In a first experiment [1-$^{13}C$]-(*S*)-glutamate, together with sodium [2,$^{13}C$]acetate, was administered to *A. flos-aquae* strain 37. Addition of the acetate precursor was intended to serve as an internal standard to evaluate the level of incorporation, if any, of the carboxy-labelled amino acid substrate. Furthermore, it might prove possible to detect 'bond labelling' of the newly formed bond C-4,5, should labelled amino acid and labelled acetate be incorporated into the same molecule.

The $^{13}C$ NMR spectrum [Fig. 1(*a*)] of the sample of **1** isolated from this experiment showed a marked increase in signal intensity in the signal due to C-5, the carbon atom expected to be derived from the carboxyl group of glutamic acid, and in that due to C-4, derived from the methyl group of acetate (specific enrichment above natural abundance: C-4, 6%; C-5, 7%). In addition, satellites were observed in the signals due to C-5 and C-4, ($^1J$ 33 Hz), confirming the formation of a new bond between C-1 of glutamic acid and the methyl group of acetate.

Of the other carbon atoms, shown by experiment to be derived from acetate, C-11 and C-2 were enriched relative to their neighbours C-3 and C-10 and are therefore derived from the methyl group of acetate. C-8, one of the acetate-derived carbon atoms of the unit C-5,-6,-7,-8,-1 was not enriched. Thus, the administration of glutamic acid effectively suppressed the incorporation of acetate into this fragment. This result suggests

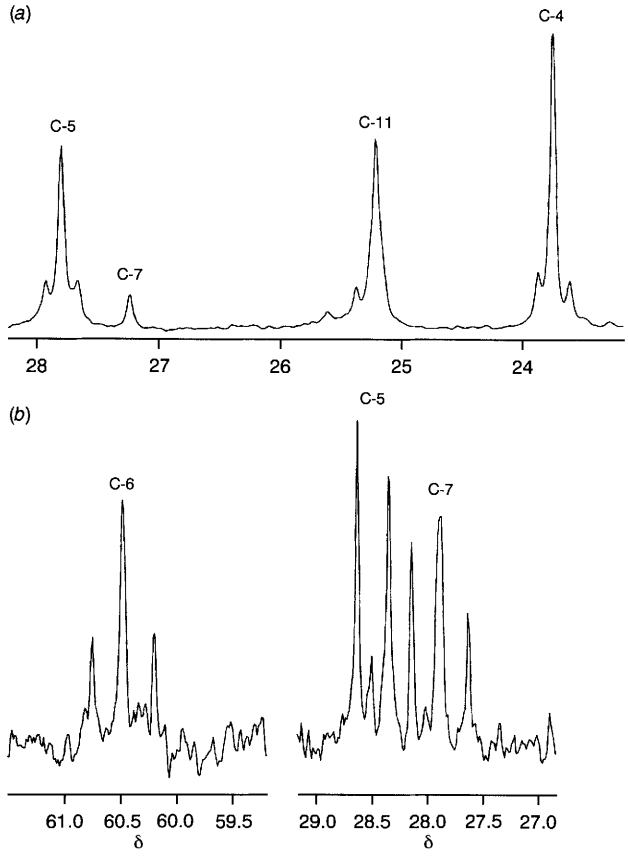


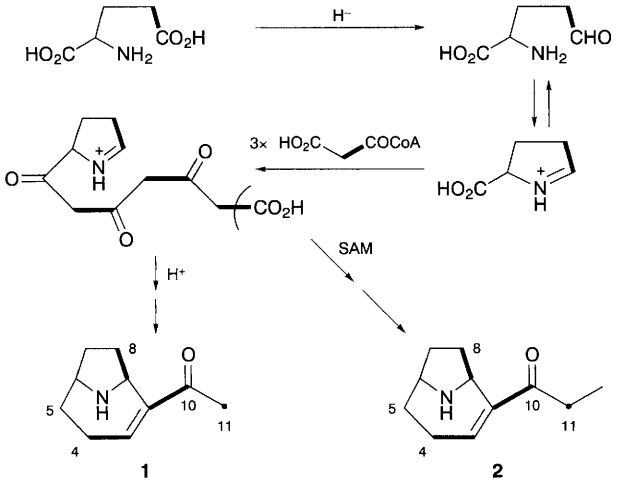

**Fig. 1** Sections of the 125 MHz $^{13}C$ NMR spectrum of anatoxin-a in $D_2O$–5% TFA. (*a*) Sample isolated from the [1-$^{13}C$]glutamate/[2-$^{13}C$]acetate experiment. (*b*) Sample isolated from the (*S*)-[$^{13}C_5$]glutamate experiment.

that this portion of the molecule may indeed be derived from glutamic acid or a compound closely related to it.

Conclusive evidence that carbon atoms C-5,-6,-7,-8,-1 of **1** are derived from glutamic acid came from a feeding experiment in which [$^{13}C_5$]-(*S*)-glutamate was administered to *A. flos-aquae* strain 37. Toxin isolated from this experiment showed strong enrichment (9% above natural abundance) in these carbon atoms. The homonuclear coupling pattern observed in the 125 MHz $^{13}C$ NMR spectrum of this sample [Fig. 1(*b*)] suggests that the complete carbon chain of the precursor amino acid is incorporated intact into the toxin.

A reasonable biosynthetic reaction sequence based on the results presented here is shown in Scheme 1. We postulate that glutamic semialdehyde is the central intermediate that serves as the starter unit for the polyketide fragment. It may be derived by reduction of a glutamate derivative or by transamination/oxidation of ornithine or by oxidation of proline.[9] The relative importance of these three routes cannot be assessed on the basis of the experiments described here.

The results described here provide unambiguous proof that C-1 of glutamic acid is retained during the formation of toxin **1** and is not lost by decarboxylation. This observation is incompatible with the view[10] that the carbon atoms of the pyrrolidine ring of **1** are derived in the same way as the corresponding fragment in the tropane alkaloids. Thus, the structural similarity between **1** and the tropane alkaloids is not reflected in the biochemical mechanism by which the carbon skeleton of **1** is assembled in the alga. Based on the qualitative agreement in the labelling pattern observed in **1** and **2** derived from sodium [$^{13}C_2$]acetate, we postulate that C-5,-6,-7,-8,-1 of **2** are derived in the same way as the corresponding carbon atoms in **1**.




**Scheme 1** A plausible biogenetic scheme for anatoxin-a and homoanatoxin-a

It is our hope that a precise understanding of the biosynthesis of toxins **1** and **2** will aid in the development of molecular probes for toxic strains of *Anabaena flos-aquae* that are of ever-increasing concern in environmental toxicology.[11]

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