Biosynthesis of Anatoxin-a(s). (2*S*,4*S*)-4-Hydroxyarginine as an Intermediate

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(2*S*,4*S*)-4-Hydroxyarginine is an intermediate in the biosynthesis of anatoxin-a(s) from L-arginine.

Anatoxin-a(s) **1** is one of several potent neurotoxins produced by strains of the cyanophyte *Anabaena flos-aquae*. It exhibits strong anticholinesterase activity *in vitro*[1] and *in vivo*[2] and has been implicated in animal deaths resulting from consumption of algal-contaminated drinking water.[3]

In a recent report[4] from this laboratory, it was shown that the carbons of the triaminopropane backbone and the guanidino function are derived from the amino acid L-arginine **2**. Two modifications of the precursor have to be achieved in the process. Firstly, one of the terminal nitrogens of the open chain guanidine moiety has to be joined to C-4 to form the heterocyclic ring. Secondly, the C-glycyl moiety (C-1, C-2, and N_α) has to be lost and replaced by a dimethylamino group.

To differentiate among several possible mechanisms for these transformations, we decided to probe the fate of the protons by feeding DL-[3,3,4,4,5,5-$^2H_6$]arginine† **2a** to the cyanophyte.

The precursor was fed in two 450 mg portions to four 8 l cultures of *A. flos-aquae* NRC 525-17 on days 10 and 14 after inoculation and the cells were then allowed to metabolize for another 7 days. The alga was harvested by membrane-filtration and the toxin was isolated as previously described.[4]

The 76 MHz ^2H NMR spectrum of the toxin **1a** obtained from this experiment (4 mg) displayed three broad resonances of essentially equal intensity (0.3% specific incorporation above natural abundance) and separated from the solvent peak. The signals at δ 4.75 and 4.0 could be assigned to single deuteriums on C-5 and C-4, respectively.[5] The remaining signal at δ 3.5 was due to either a second deuterium on C-4 or a single deuterium on C-6. Whereas the corresponding signals overlap in the ^1H NMR spectrum of **1**, these two resonances are clearly separated in the ^1H NMR spectrum of **3** [Fig. 1(*a*)].[5] The labelled toxin **1a** was therefore subjected to catalytic hydrogenation (5% Pd–C, MeOH, 1 atm, 4 h, room temp.) to hydrogenolyze the N–O bond. The resulting **3a** also displayed three signals in its ^2H NMR spectrum [Fig. 1(*b*)], two of which (at δ 4.45 and 3.95) corresponded to single deuteriums on C-5 and C-4, respectively. The third signal (δ 3.5) had to be due to a second deuterium on C-4, since a deuterium on C-6 would have shown a signal at either δ 3.05 or 3.2.[5] This meant that **1a** also had two deuteriums on C-4 and did not possess any deuterium on C-6 above natural abundance.

Two conclusions can be drawn from this experiment regarding the mechanism by which L-arginine is converted to the toxin. First of all, both hydrogens on C-3 or arginine are lost as a consequence of replacing the C-glycyl moiety with a dimethylamino group, implying that an intermediate having a keto functionality at C-3 is formed. Secondly, only one of the hydrogens on C-4 of arginine is lost, suggesting that the cyclization step does not proceed *via* a 4-oxoarginine intermediate. If the latter had been the case, both hydrogen atoms would have been lost from C-4. The observed distribution of label is compatible, however, with a 4-hydroxyarginine intermediate being involved in the cyclization. (2*S*,4*S*)-4-Hydroxyarginine (*erythro*-4-hydroxyarginine) **4** has been detected in the NRC 525-17 strain of *A. flos-aquae*[4] and has been proposed to be an intermediate on the pathway to the toxin.[6]

To establish the intermediacy of (2*S*,4*S*)-4-hydroxyarginine **4** experimentally, (2*S*,4*S*)-[3,3,4,5,5-$^2H_5$]-4-hydroxyarginine **4a** was synthesized using a published procedure[7]‡ and fed to the cyanophyte as follows: Two 8 l cultures of the algae were allowed to grow for 18 days, at which time they appeared healthy and dark green. A sterile aqueous solution of **4a** (65 mg) was added to each culture and the two batches were allowed to grow for another 6 days.§ The ^2H NMR spectrum of the anatoxin-a(s) **1a** (3 mg) isolated from this experiment [Fig. 1(*c*)] displayed the same pattern of signals (0.4% specific incorporation above natural abundance) observed previously in the spectrum of the toxin **1a** from the DL-[3,3,4,4,5,5-$^2H_6$]-arginine feeding experiment (only **2a** incorporated).

This result indicates that (2*S*,4*S*)-4-hydroxyarginine **4** is an intermediate on the pathway from L-arginine to the toxin. Furthermore, the (*S*)-configuration at C-4 for **4** and the (*R*)-stereochemistry at C-5 for the toxin suggest that the ring closure proceeds by an S_N2-type process, since overall inversion is observed.

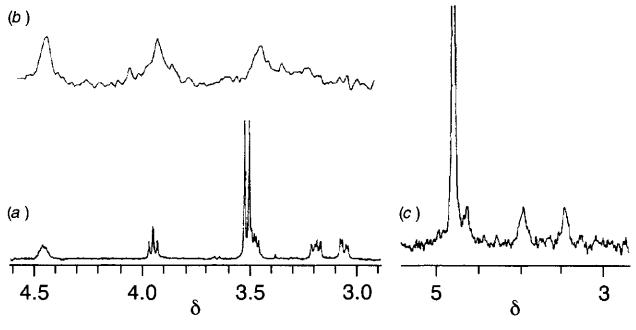
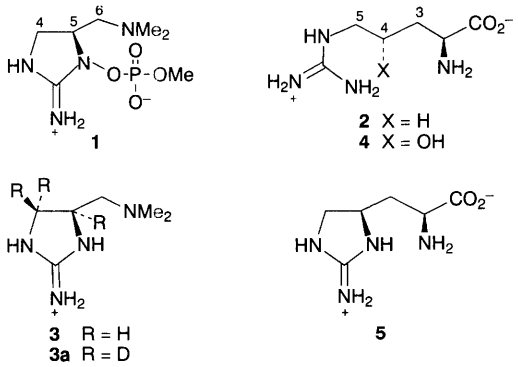
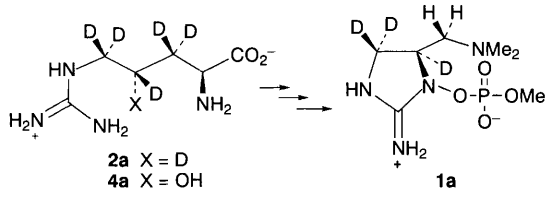


Fig. 1 Comparison of the δ 3.0–4.5 regions of the (*a*) 500 MHz ^1H and (*b*) 76 MHz ^2H NMR spectra of a mixture of [4,4,5-$^2H_3$]-**3a** (0.4% ^2H above natural abundance) and methyl phosphate produced from hydrogenolysis of [4,4,5-$^2H_3$]-anatoxin-a(s) **1a** biosynthesized from L-[3,3,4,4,5,5-$^2H_6$]-arginine **2a**. Spectra determined in ^2H-depleted H_2O (solvent presaturation used to obtain ^1H NMR spectrum); however, (*c*) contains 0.5% TFA. The doublet at δ 3.51 in (*a*) is assigned to methyl phosphate. In (*c*) is shown the 76 MHz ^2H NMR spectrum of **1a** biosynthesized from (2*S*,4*S*)-[3,3,4,5,5-$^2H_5$]-4- hydroxyarginine **4a**.





Scheme 1 Fate of the deuteriums in the biosynthesis of [4,4,5-$^2H_3$]-anatoxin-a(s) **1a** from L[3,3,4,4,5,5-$^2H_6$]-arginine **2a** and (2*S*,4*S*)-[3,3,4,5,5-$^2H_5$]-4-hydroxyarginine **4a**

At present, it is unknown whether the cyclization precedes or follows the loss of C-1, C-2 and N_α. If the former process is operative, then the known cyclic amino acid enduracididine[8] **5** should be an intermediate on the pathway.

Experiments to establish the intermediacy of **5** and the mechanisms of ring closure and chain shortening are in progress.

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Footnotes

† DL-[3,3,4,4,5,5-$^2H_6$]-Arginine was synthesized from [1,1,2,2,3,3-$^2H_6$]-propane-1,3-diol[9] by routine synthetic methodology.

‡ The deuterium label was introduced into the *N,N'*-dibenzamido-4-oxo-L-ornithine methyl ester intermediate by acid-catalyzed H/D exchange followed by reduction with $NaBD_4$. (2*S*,4*S*)-[3,3,4,5,5-$^2H_5$]-4-Hydroxyornithine was isolated as described[7] and displayed only one signal in its 300 MHz ^1H NMR spectrum at δ 3.63 (in D_2O) for H-2, indicating greater than 95% deuteration at C-3, C-4 and C-5.

§ The timing of addition of the tracer to the culture is crucial for the success of the experiment. (2*S*,4*S*)-4-Hydroxyarginine appears to have mild bacteriostatic activity since cultures to which the tracer had been added 6 days after inoculation did not grow to the expected cell density and did not produce labelled toxin.

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