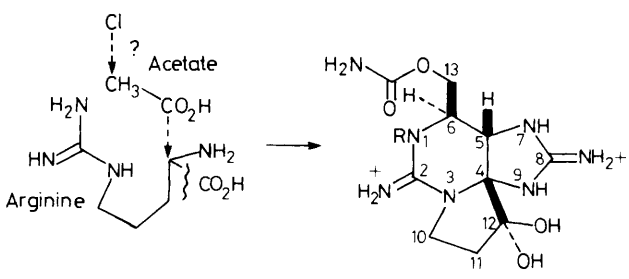
Biosynthesis of Saxitoxin Analogues: The Origin and Introduction Mechanism of the Side-chain Carbon

Sandeep Gupta, Manuel Norte, and Yuzuru Shimizu*

Department of Pharmacognosy and Environmental Health Sciences, College of Pharmacy, The University of Rhode Island, Kingston, Rhode Island 02881, U.S.A.

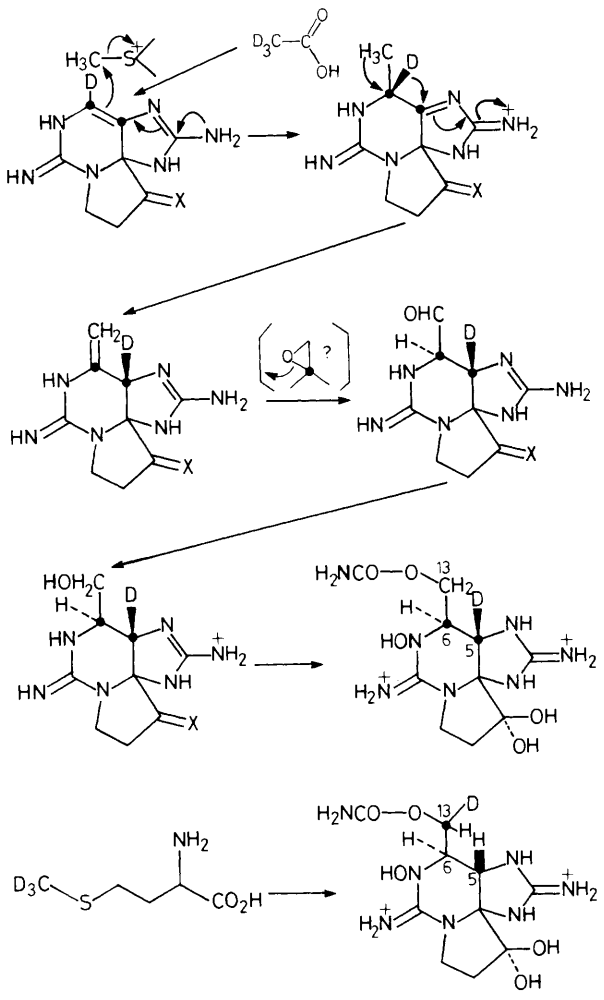
Feeding experiments on *Aphanizomenon flos-aquae* have revealed that the side-chain carbon, the C-13, of neosaxitoxin is derived from methionine methyl group by an electrophilic attack of *S*-adenosylmethionine on a dehydro-intermediate, followed by a hydride migration and a proton loss, further elaboration of the side chain probably involves intermediacy of an aldehyde.

The molecular basis of the origin of the important neurotoxins, saxitoxin (**1**) and its analogues,[1,2] has been of particular interest especially with regard to the toxigenesis of the dinoflagellates and blue–green alga which cause extremely deleterious blooms. Earlier attempts to study the biosynthesis of these toxins in the marine dinoflagellate *Gonyaulax tamarensis* were hampered owing to the difficulties associated with the culturing of the organism and its nonheterotrophic nature which results in the nonacceptance of the exogenous organic material.[2] However, subsequent isolation of the toxin producing fresh water blue–green alga *Aphanizomenon flos-aquae*[3] greatly facilitated the feeding studies to understand the biosynthetic origin of these toxins. In a previous Communication[4] we reported that the tricyclic skeleton of the toxin molecule is formed by the Claisen-type condensation of an acetate unit, or its derivative, onto the amino bearing α-carbon of arginine followed by decarboxylation, introduction of a guanidine, moiety, and cyclization (Scheme 1). An important unsettled question was the origin of the side-chain carbon, the C-13 in the molecule. A high level of specific enrichment at positions 5 and 6 in neosaxitoxin by the feeding of [1,2-$^{13}C_2$]acetate suggested the origin of C-13 by a C_1 donor. However, when ^{13}C labelled formate, a general C_1



(**1**) R = H
(**2**) R = OH

Scheme 1



Scheme 2

precursor, was fed to the culture of a toxin producing strain of *Aphanizomenon flos-aquae*[3] and the isolated neosaxitoxin analysed by ^{13}C n.m.r. spectroscopy no specific enrichment could be observed.[4] This observation made us speculate that the carbon might be introduced by CO_2 prior to the Claisen condensation. Acetyl coenzyme A can react with CO_2 to give rise to malonyl coenzyme A which after reduction and hydrolysis can form 3-hydroxypropionate. Either malonate or 3-hydroxypropionate could serve as a substrate in the toxin biosynthesis. However, feeding of [1,2-$^{13}C_2$]-3-hydroxypropionate to *A. flos-aquae* did not show any incorporation in the toxin.[5] Pulsed feeding of the ^{13}C labelled bicarbonate to *A. flos-aquae* also did not result in any specific enrichment. However, the free β-alanine isolated from the algal extract in the same experiment did show selective incorporation at the β-carbon thus confirming that the pulsed feeding of labelled bicarbonate was indeed operative in the alga.[6]

Meanwhile, in another experiment it was discovered that the feeding of [1,2-$^{13}C_2$]glycine to *A. flos-aquae* resulted in specific enrichment at the C-13 of the toxin molecule. Glycine can act as a one-carbon donor by a carbon transfer to tetrahydrofolate which can then transfer the carbon to homocysteine to form methionione, which as *S*-adenosylmethionine is a versatile carbon donor. Also, glycine and serine *in vivo* usually exist in a reversible equilibrium and any one of these can act as a substrate for tetrahydrofolate for carbon transfer.[7] To test this possibility, [3-^{13}C]serine was fed to *A. flos-aquae* and the resultant toxin indeed showed selective labelling at C-13.† Subsequently, the ^{13}C-labelled methyl group of methionine was found to be incorporated into the C-13 of neosaxitoxin with high efficiency in *A. fiso-aquae*.‡ Since methyltetrahydrofolate is a methyl donor in the formation of methionine, the reverse is not the normal case,[8] we believe that the side-chain carbon is introduced by the methylation of the ring system with *S*-adenosylmethionine (SAM). To investigate this further, [methyl-D_3, $^{13}C_1$] methionine was fed to *A. flos-aquae*.§ The ^{13}C n.m.r. spectrum of the isolated neosaxitoxin (**2**) showed, besides the natural abundance singlet signal of C-13, a triplet (δ 60.4, J_{C-D} 23.6 Hz) (Figure 1A).[9] The clear spin–spin coupling pattern, the isotope effect to the chemical shift (δ 0.24),[10] and the absence of other signals indicated that the enriched methylene carbon carries only one deuterium atom. This is interesting because usually in the process of transfer of the methyl carbon from SAM to an electron rich species, three protons of the methyl carbon would be expected to be retained in the product, and thus two in the 13-methylene.

When [2-$^2H_3$-1,2-$^{13}C_2$]acetate was fed to *A. flos-aquae* under identical conditions, we first observed a total loss of the deuterium atoms in the biosynthetic neosaxitoxin which, in retrospect, was due to a rather facile exchange. However, after a brief incubation (2 days),¶ the ^{13}C n.m.r. spectrum of the isolated neosaxitoxin showed a partial retention (*ca.* 40%) of deuterium on the methine carbon, the C-5, whose signal appeared as a composite of a natural abundance singlet (δ 56.36), a doublet of nondeuteriated enriched ^{13}C (δ 56.29, J_{C-C} 38.04 Hz), and an apparently overlapped triplet–triplet (δ 56.16, J_{C-C} 38.04 Hz, J_{C-D} 19.02 Hz), which is shifted upfield due to a deuterium isotope effect (Figure 1B).†† Since it is unequivocally established that C-5 is derived from the carboxyl carbon of the acetate,[4] the deuterium atom on C-5 must have come from C-6 (by a deuteride shift) which, in turn, is derived from the acetate methyl group. The results of the above experiments strongly suggest that the side chain carbon of the toxin is introduced in the fashion seen in the alkylation

† ^{13}C n.m.r. spectra of neosaxitoxin derived from the feeding of the [1,2-$^{13}C_2$]glycine and [3-$^{13}C_1$]serine are available as supplementary material.

‡ A 20 fold enhancement of the C-13 signal of neosaxitoxin was observed. The spectrum is available as supplementary material.

§ D,L-[Methyl-$^{13}C_1$,D_3]methionine (99% ^{13}C, 98% d) was purchased from MSD isotopes, Canada. To a ten-day old culture of *A. flos-aquae* (3 × 10 l), D,L-[methyl-$^{13}C_1$,D_3]methionine was fed (0.033 mmol/l culture). The culture was allowed to grow for five days and then harvested. Neosaxitoxin (2.8 mg) and a small amount of saxitoxin were isolated by a combination of Bio Gel P2 gel filtration and Bio Rex-70 ion exchange chromatography as reported earlier (Y. Oshima, L. J. Buckley, M. Alam, and Y. Shimizu, *Comp. Biochem. Physiol.*, 1977, **57C**, 31). For ^{13}C n.m.r. measurements the toxin sample was passed through a small column (50 mg) of Chelex-100 resin in deionised H_2O. The spectrum was recorded in D_2O–H_2O mixture (1:3), pH 2.2, HCl) on a Bruker instrument at 125.8 MHz. Sweep width, 29412 Hz, acquisition time, 0.56, a number of scans, 19000.

¶ Twenty-day old culture of *A. flos-aquae* (4 × 10 l) was incubated with [2-$^2H_3$; 1, 2-$^{13}C_2$]acetate-sodium salt (0.079 mmol/l culture) for two days. The culture was harvested and neosaxitoxin (2.48 mg) isolated.

†† ^{13}C n.m.r. measurement was done in D_2O : H_2O mixture (1 : 3, pH 2.4, HCl) on Bruker instrument at 125.8 MHz. Sweep width, 29412 Hz, acquisition time, 0.56 s, number of scans, 25 000. Only a small β-effect (<0.03 p.p.m.) was observed with C-6, whose signal appeared as broad ended peaks compared to those observed for (**2**) obtained previously from a [1,2-$^{13}C_2$]acetate feeding experiment.

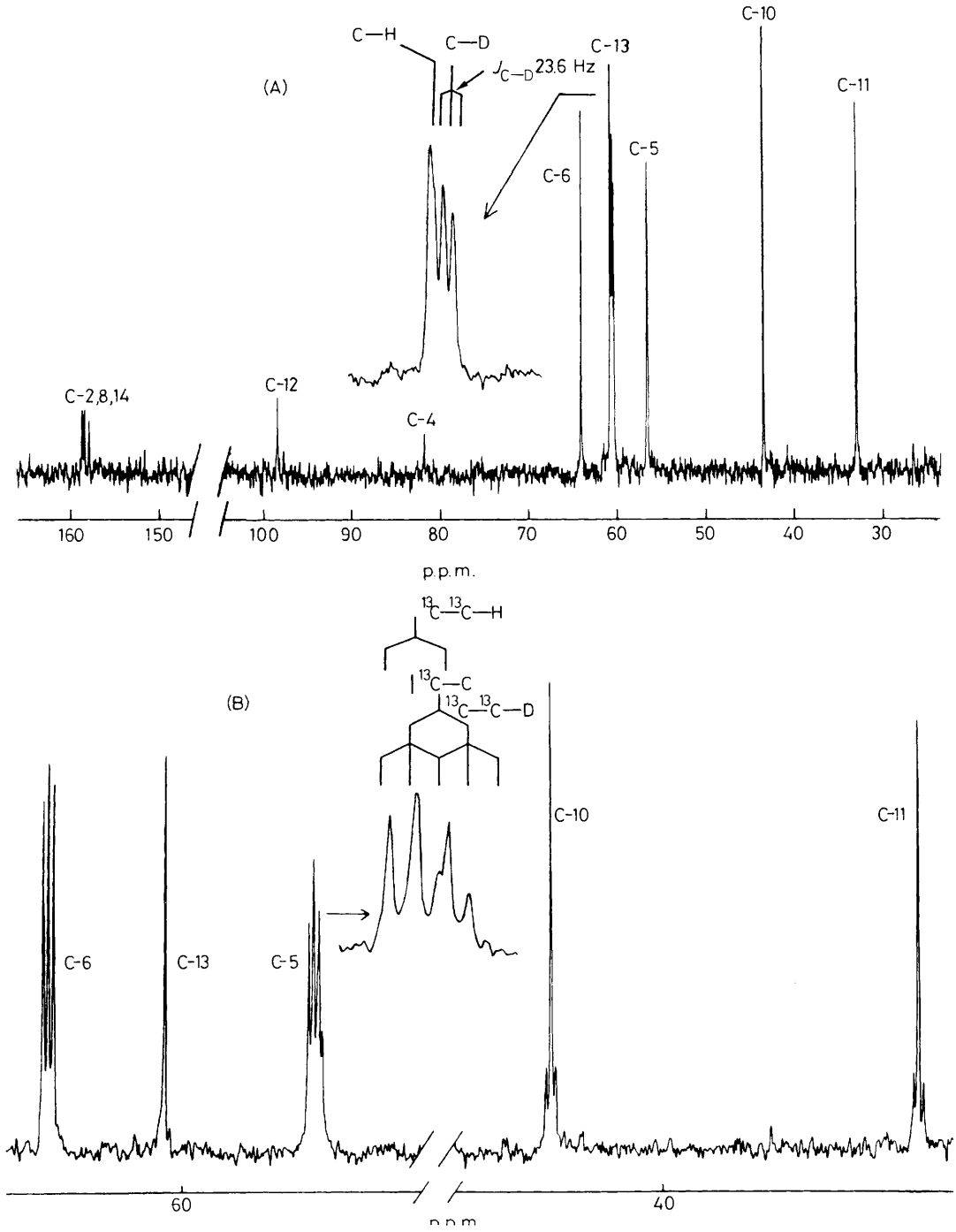


Figure 1. ^{13}C n.m.r. spectra of neosaxitoxin (**2**) isolated from *Aphanizomenon flosaquae* fed with [methyl-$^{13}C_1$,D_3]methionine (A) and $^{13}CD_3{}^{13}CO_2Na$ (B).

of many natural products, *i.e.* electrophilic attack on a double bond followed by the migration of a hydride ion and elimination of a proton (Scheme 2).[11] The conversion of the resulting terminal methylene to the carbinol may proceed through epoxide formation followed by opening to an aldehyde and reduction. Such a process that involves the intermediacy of an oxidised substrate (an aldehyde in this case) can explain the retention of only one deuterium atom in the product from the trideuteriated methyl group of the methionine.

These results along with the earlier work establish the molecular origin of all the carbons of saxitoxin and its analogues.

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