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Dinoflagellate and other microalgal toxins: chemistry and biochemistry

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Abstract - The origin of the side-chain portion of paralytic shellfish toxins has been determined along with some details of the biosynthetic steps. Saxitoxin of an unknown origin was discovered in the common mackerels, *Scomber scombrus* Linn. A small pennate diatom, *Amphora coffaeiformis* Cl. was identified as a source of a new type of shellfish toxin, domoic acid found in Canadian mussels.

INTRODUCTION

Many seafood-borne toxins, which cause serious health problems, are actually derived from microalgae. For example, paralytic shellfish poisons (PSP), such as saxitoxin, neosaxitoxin, and gonyautoxins, are actually produced by *Gonyaulax* spp. and some other dinoflagellates (ref. 1). Neurotoxic shellfish poisons (NSP) in Gulf of Mexico are produced by a naked dinoflagellate, *Gymnodinium breve*. The toxic principles, represented by brevetoxin A (ref. 2) and brevetoxin B (ref. 3), are also potent ichthyotoxins. Their unique structural features of linear polycyclic ether system with comformationally flexible large-member rings are of particular interest vis-a-vis their interactions with sodium channels. Another example of such toxins is the diarrhetic shellfish toxins derived from *Dinophysis* spp. (ref. 4). It is generally felt that there are more unrecognized seafood toxins derived from microalgae. The most recent event with Canadian mussels described in this paper supports such conception.

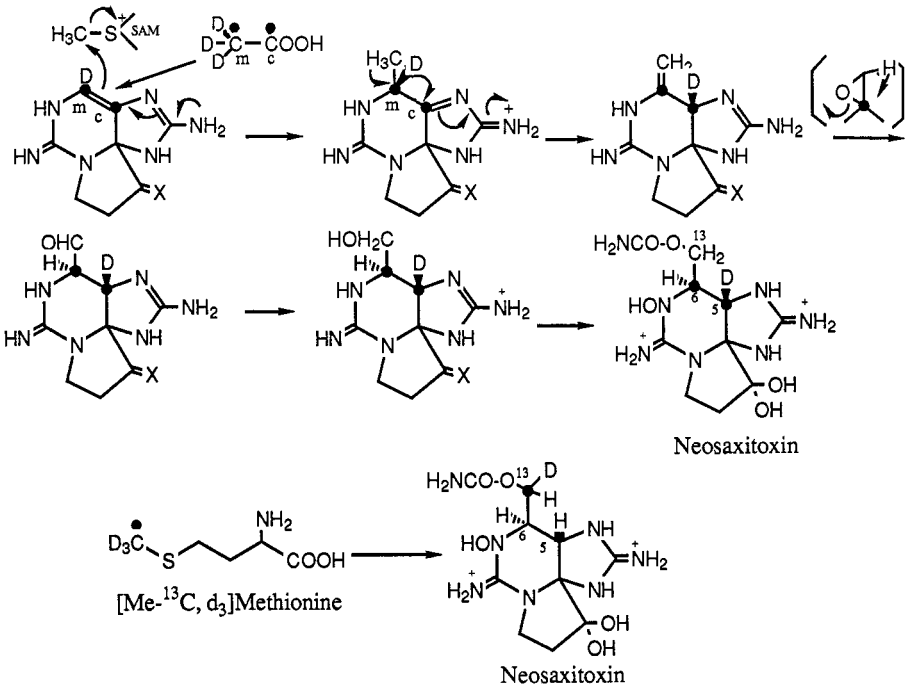
The toxigenesis in these deleterious organisms is, however, not well known. Clearly it is essential to understand the basic mechanism of toxigenesis for the genetic manupulation of the organisms or the production of specific secondary metablites. The determination of the molecular origins of the toxins and involved biochemical steps are deemed to be the first step to approach the complicated problem.

Several years ago, we started to study the biosynthesis of saxitoxin anlogues in order to get basic information about the production of the unique molecules in the red tide organisms. Fortunately, we have been able to identify all the building blocks of the tricyclic perhydropurine system, and also some of the intricate biosynthetic mechanisms.

BIOSYNTHESIS OF SAXITOXIN ANALOGUES

In previous works (ref. 5,6), we showed that two units of actate are incorporated at C-5 - C-6 and C-10 - C-11 in neosaxitoxin. It was also shown by double labeling studies that the connectivity between N-2 and C-2 of arginine are incorporated intact into the toxin molecule. The results point to the pathway which involves Claisen-type condensation of acetate or its derivative with arginene. The remaining question was how the side-chain carbon, C-13 is introduced. Our initial attempt to feed labeled formate as a general precursor of C1 units did not result in any enrichment (ref. 5). The assumption was that if any of precursors in the C1 pool was involved, formate would be incorporated. Thus the failure of formate feeding made us disregard C1 precusors and focus on CO_2 as an alternate source. It was speculated that CO_2 could be incorporated into malonate, and condense with arginine. The reduction of carboxyl group to carbinol could take place before or after condensation. However, repeated attempts to pulse-feed $^{13}CO_2$ to a toxin-producing strain of *Aphanizomenon flos-aquae* could not effect the selective enrichment of C-13.

Meanwhile, it was accidentally discovered that [2,3-^{13}C]glycine could enrich C-13 in neosaxitoxin exclusively (ref. 7). Furthermore [3-^{13}C]serine, and [Me-^{13}C]methionine were also found to enrich the carbon effectively. Thus the side-chain carbon is actually derived from a C1 unit. The direct precusor was determined to be S-adenosylmethionine (SAM), since methionine was far more efficiently utilized than the other C1 donors. The unsuccessful feeding of formate was probably due to the rejection of the compound by the organism.



In order to investigate the mechanism of methyl introduction from SAM and further conversion to carbinol, double-labeled tracer studies have been carried out. First, [Me-13C-Me-d3]methionine was fed to *A. flos-aquae*. Isolated neosaxitoxin carried only one deuterium on the methylene carbon, since the NMR signal of the exogenous 13C appeared as a clear triplet split by spin-spin coupling with a single deuterium nucleus (I=1). In another experiment, [1,2-13C]acetate-d3 was fed to the same organism. Initally we observed a loss of acetate deuterium by rather fast exchange in the culture. However, by a concentrated brief contact, we were able to isolate neosaxitoxin with retaining some deuterium (ca. 40 %). The location of the deuterium was determined to be C-5, because its 13C-NMR signal appeared as a up-field shifted sextet as results of couplings with neighboring C-6 and deuterium. The signal for C-6 appeared as a doublet split by C-5. Since C-5 is known to be derived from the carboxyl group of acetate (ref. 5), the deuterium must have come from C-6 by migration. Methylation of double bond and consecutive hydride ion shift are commonly seen in biochemical alkylations. The following sequence: the epoxidation of terminal methylene, the opening of the epoxide to aldehyde and reduction to carbinol may well explain both of the above described deuterium loss and hydride ion shift.

IDENTIFICATION OF SAXITOXIN IN MACKERELS

Saxitoxin analogues are now known to be produced by a variety of organisms. They include dinoflagellates: *Gonyaulax* spp., *Pyrodinium bahamense* var. *compressa*, *Gymnodinium catenatum*; a blue-green alga: *Aphanizomenon flos-aquae*; and red algae: *Jania* spp. The occurrence of the unique molecules in taxonomically varied species and productivity difference in the same species may suggest the presence of common vectors as bacteria.

While the bacterial procuction of saxitoxin is hotly discussed, we have confirmed the presence of saxitoxin in the common Atlantic mackerel, *Scomber scombrus* Linn. The finding may be highly significant with regard to the source of the toxin.

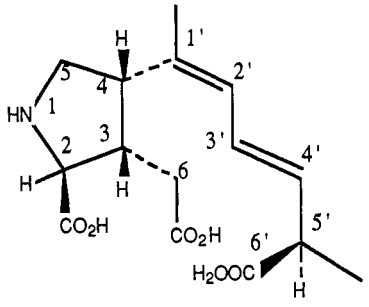
In December, 1987, health officials in New England states were alerted for possible toxicity of mackerels. A number of hunchback whales were found dead at Massachusetts beaches, and their stomach contents showed large quantities of mackerels (ref. 8). Examination showed that extracts of the mackerel livers kill mice with a typical PSP syndromes. Livers from 20 kg of mackerels, which were caught off Rhode Island coast, were extracted with aqueous methanol. The extract, which had the toxicity of about 800 mouse

units, was treated with charcoal. The charcoal extract was further purified by Bio-Gel P-2 and Amberlite IRC-50 resin (ref. 9). About 150 mouse units (*ca.* 30 ug) of the toxin was brought to a pure form and compared with an authentic sample of saxitoxin in two TLC systems (ref. 1), two paper electrophoresis systems (ref. 10) and the sodium channel specific test using the mouse neuroblastoma cell line Neuro 2A (ref. 11). In all cases, the mackerel toxin and the authentic sample were identical.

This surprising occurrence of saxitoxin in mackerels seems to be rather ubiquitous. All samples of mackerels caught other parts of New England showed more or less the same level of toxicity (about 2 mouse units per gram of liver). It does not seem to be seasonal or regional, because a preserved specimen of mackerels caught off the coast of New Jersey in April, 1987 also showed the same level of toxicity. On the other hand, Japanese specimens of the Pacific mackerel, *Scomber japonicus* Houttyne caught at two locations were completely devoid of toxicity. It is not certain if this absence of saxitoxin is due to the difference of species or their diets. At any case, it is difficult to explain this rather uniform toxin presence in the Atlantic mackerels by the food-chain originating from dinoflagellate blooms, which are normally regional and sporadic.

CANADIAN MUSSEL POISON

In November, 1987, mussels cultured at Cardigan Bay, Prince Edward Island, Canada caused poisonings among a large population. Neurological symptoms including amnesia were observed with the patients. Two deaths were reported.
Ip injections of the mussel extracts caused scratching motions in mice, convulsion and deaths. The toxic principle was identified as domoic acid, a potent glutamate agonist (ref. 12). Some minor components, probably double bond isomers were also found.
Domoic acid was first isolated from the red alga, *Chondria armata* by Daigo (ref. 13). The alga had been used in the southern part of Hapan as a folklore anthelmintic. However, the source of domoic acid in the filter-feedig mussels, which were cultured by the hanging method must be planktonic.



Domoic Acid

d value (ppm) B72-52-2 YSN 440, 300 MHz

C-2H 3.98 d, C-3H 3.09 dddd, C-4H 3.88 ddd, C-5H 3.48 dd, 3.74 dd, C-6 2.45 dd, 2.71, C-1'-Me 1.80, C-2'H 6.11 d, C-3'H 6.29 dd, C-4'H 5.76 dd, C-5'H 3.21 dd, C-5'-Me 1.21 d.

We have isolated 30 species of organisms: bacteria, cyanobacteria and diatoms from the toxic mussels. The HPLC analysis of their cultures revealed that one small pennate diatom, prodcuces domoic acid. Isolation work was performed with a large culture, and domoic acid was confirmed by HPLC, TLC and NMR. The organism was identified as *Amphora coffaeiformis* Cl., a rather common but diverse benthic diatom by Dr. P. E. Hargraves of University of Rhode Island.

Diatoms constitute an important basis of ocean production and are generally considered to be benign. This study, however demonstrates that they can be also sources of deleterious compounds. It is also an intriguing question if there is any connection between domoic acid producing algae and diatoms, since symbiotic relationship between two organisms may be possible (ref. 14).

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