Biosynthesis of the Neurotoxin Domoic Acid by the Marine Diatom *Nitzschia pungens* forma *multiseries*, determined with [^{13}C]-Labelled Precursors and Nuclear Magnetic Resonance

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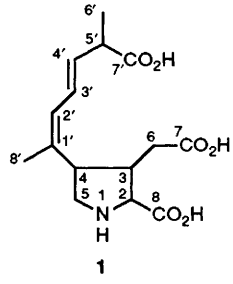
Labelling experiments with [1-^{13}C]acetate and [1,2-^{13}C]acetate provide evidence that the neurotoxin domoic acid, produced by the diatom *Nitzschia pungens*, is derived by the novel condensation of a geranyl unit with an activated citric acid cycle derivative and subsequent cyclisation to form a proline ring system.

Domoic acid **1** is a neuroexcitatory amino acid identified as the toxin responsible for an outbreak of shellfish poisoning in eastern Canada in 1987,[1] and recently implicated in the deaths of aquatic birds on the West Coast of the USA.[2] Domoic acid is produced by the marine diatom *Nitzschia pungens* forma *multiseries*,[3] the first reported case of a marine toxin originating from a diatom.

Nothing is known about the biogenesis of domoic acid or other kainoids.[4] Cyclisation of glutamic acid *via* the γ-semialdehyde, as in the established route to proline biosynthesis, does not appear to be a likely biosynthetic pathway for domoic acid. This would require, after initial assembly of the proline moiety, two separate steps involving carbon–carbon bond formation at inactivated positions of the ring. Comparison of domoic acid with kainic acid **2**, which is produced by several marine macroalgae,[5] suggests a common biosynthetic pathway in which the proline ring system is generated by condensation of an isoprenoid unit (such as isopentenyl pyrophosphate for kainic acid, or geranyl pyrophosphate for domoic acid), with a common activated C_5 unit, possibly from the citric acid cycle, as illustrated in Scheme 1.

Although Scheme 1 proposes that the proline ring system is



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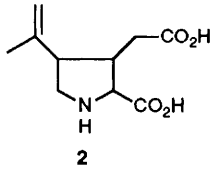

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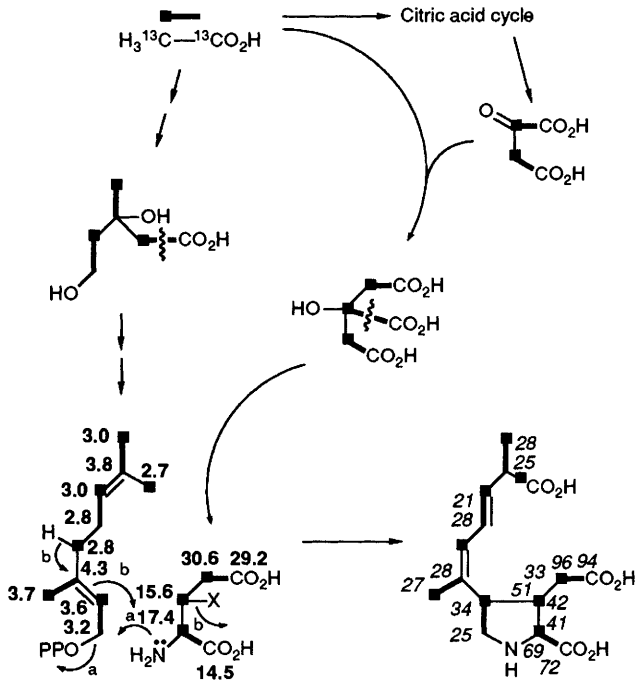
generated through condensation of two different biogenetic units, it is interesting to note that both putative biogenetic units are entirely derived from acetate. Consequently, if this biosynthetic pathway is utilised by *N. pungens*, uptake of doubly-labelled acetate would result in labelling of every carbon in the domoic acid molecule.

In two separate labelling experiments, a 12 l bacteria-free unialgal culture of *N. pungens*[3,6] was pulsed three times (0.67 mmol dm^{-3} each pulse, 2 mmol dm^{-3} total fed to culture) with labelled [1-^{13}C]acetate 99.7% ^{13}C, or [1,2-^{13}C]acetate 99.7% ^{13}C (MSD Isotopes, Canada), at first immediately before the cessation of exponential growth, and thereafter at 24 and 48 h. Three days after the last pulse, the cells were filtered, centrifugated, then extracted with 60% aqueous methanol and domoic acid was isolated.[1] The compound (*ca.* 100 μg) was dissolved in D_2O at pH 1.6 for NMR spectroscopy at 125 MHz (^{13}C) or 500 MHz (^{1}H). Both ^{1}H and ^{13}C spectra of domoic acid have been assigned previously at this pH.[7]

The ^{13}C NMR spectrum of domoic acid labelled from [1-^{13}C]acetate showed substantial enrichment (>9% ^{13}C) at the carboxyl carbon C-7 and significant but lesser enrichment (>3% ^{13}C) at carboxy carbon C-8. This supports the proposal that one part of the proline ring is derived from a citric acid cycle intermediate. However, the small amount of labelled product available for this experiment (<100 μg), and the relatively low enrichment, precluded quantitation of ^{13}C enrichment at other labelled positions.

Carbon spectra of domoic acid labelled from [1,2-^{13}C]-acetate were recorded,[8] and the concentration of domoic acid determined from ^{1}H and ^{13}C NMR spectra of the sample and ^{1}H spectra of a separate sucrose standard solution obtained under identical conditions. Percentages of ^{13}C incorporated at each position were calculated, and are shown on Scheme 1 in bold type against the corresponding positions on the second-last stage of the suggested biosynthetic pathway. Also shown, in italics, are the probabilities P_{AB} (%) that, when an indicated carbon A is ^{13}C, the adjacent carbon B along the bond shown is also ^{13}C. These quantities were calculated from the intensity distributions of multiplets due to ^{13}C–^{13}C coupling, by a refinement of methods previously described.[8]

The labelling pattern observed following incorporation of [1,2-^{13}C]acetate confirms the involvement of a citric acid cycle intermediate. The considerable variation in the dilution of isotope at the labelled positions (see Scheme 1) also supports the proposal that the toxin is biosynthesized from two different precursor units. The absolute enrichment at C-6 and C-7 (*ca.* 30% ^{13}C) is approximately twice as high as at C-2, C-3 and C-8 (*ca.* 16% ^{13}C), consistent with the direct incorporation of acetate onto oxaloacetate formed after multiple rounds of the citric acid cycle, redistribution of the label having occurred at the symmetric succinate intermediate.[9] The above enrichments for C-2, C-3 and C-6 were confirmed from the integrated intensities of ^{13}C satellites in the ^{1}H resonances of the corresponding protons. The high probabilities P_{67} and P_{76} (each *ca.* 95%), and P_{28} and P_{82} (each *ca.* 71%), show that intact doubly-labelled units were incorporated at the C-6, C-7 and C-2, C-8 positions, the lower proportion in the latter case being consistent with the formation of *ca.* 1/3 single label in multiple rounds of the citric acid cycle. The P_{23} and P_{32} values are identical for the C-2, C-3 bond and indicate, on average, a



Scheme 1 Suggested biosynthetic pathway for domoic acid, showing the positions of incorporation of intact doubly-labelled units, and single labels, originating from [1,2-^{13}C]acetate. Bold numerals are % ^{13}C at each position, determined from NMR experiments (error *ca.* ± 0.7% for C-4, C-5, C-1′ to C-8′; others *ca.* ± 2.5%). Numbers in italics are the probabilities P_{AB} (%) that, when an indicated carbon A is ^{13}C, the adjacent carbon B along the bond shown is also ^{13}C (error *ca.* ± 5% except P_{32} and P_{36} *ca.* ± 7%).

42% level of enrichment of the citric acid cycle intermediates at the time of formation of this bond. The P_{36} and P_{63} values show that the average ^{13}C enrichment of the C-3, C-2, C-8 unit was subsequently diluted to *ca.* 33% in the oxaloacetate pool at the time of condensation with a fresh acetate unit at > *ca.* 51%. After formation of the C-3, C-6 bond, further dilution with an approximately equal amount of natural abundance material reduced the absolute ^{13}C enrichments to the levels observed.

The absolute ^{13}C enrichment levels for the remainder of the molecule are substantially lower (3.3 ± 0.6% ^{13}C), suggesting the utilisation of another precursor in the overall biosynthetic scheme. The labelling pattern is compatible with an isoprenoid origin for this portion, which comprises C-4 and C-5 of the proline ring and C-1′ to C-8′ of the unsaturated sidechain. The ^{13}C–^{13}C satellites for these carbons and matching of carbon–carbon coupling constants showed definite incorporation of doubly-labelled units at C-4, C-5; C-1′, C-8′; C-3′, C-4′ and C-5′, C-6′. Evidence for a C-5′, C-7′ unit, which would indicate the possibility of free rotation about the C-4′, C-5′ bond prior to oxidation of the methyl group to generate the terminal carboxy group, was equivocal. There was some evidence of satellites (J_{CC} 55 ± 4 Hz) about the poorly relaxed C-7′ resonance, but the corresponding satellites at the C-5′ peak were not present, even though satellites for the coupling to C-6′ were clearly visible. As expected, no satellites were visible at the C-2′ resonance as this carbon is derived from a cleaved doubly-labelled unit during isoprenoid biosynthesis. Nevertheless, the ^{13}C enrichment at this position was the same as at other positions in the sidechain. The intensities of the satellites relative to the central peaks, when compared to the absolute ^{13}C probabilities, indicated that *ca.* 1% of the ^{13}C (mole fraction of domoic acid 0.008 ± 0.005) originated from intact doubly-labelled units, *ca.* 1% (mole fraction 0.978) from natural-abundance material, and *ca.* 1% (mole fraction

0.014) from enriched precursor that had been scrambled to single labels. Satellites owing to coupling between adjacent doubly-labelled units were not detectable, but low intensity might have precluded their observation.

The lower incorporation of acetate in the putative geranyl unit and high proportion of scrambled single label indicates that the acetate utilised for mevalonate synthesis is drawn from a different pool than that which supplies the citric acid cycle. Either the incorporation of exogeneous acetate is not favoured in the biosynthesis of the isoprenoid precursor, or the major proportion of the geranyl unit is biosynthesised early in the growth cycle before the label is added, and this unit is utilised for domoic acid biosynthesis only when the activated derivative from the citric acid cycle (perhaps 3-hydroxyglutamic acid) becomes available.

The data reveal a new route to the assembly of a proline ring structure by condensation of an activated glutamate derivative with an isoprenoid chain. Indeed, it is likely that this route will be a general pathway to all kainoids.

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