Biosynthetic Studies of the DSP Toxin DTX-4 and an Okadaic Acid Diol Ester

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Stable isotope incorporation experiments with ^{13}C- and ^{18}O-labelled precursors show that all the carbon atoms in DTX-4 **2** and an okadaic acid diol ester **3** are derived from acetate and glycolate, and also identify the acetate and glycolate derived oxygen atoms.

Okadaic acid **1** (OA) is the parent of a group of related polyether metabolites called the diarrhetic shellfish poisoning (DSP) toxins that are produced by some species of dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*.[1] The water-soluble toxin dinophysistoxin-4 (DTX-4) **2**[2] is a composite of OA, the side chain present in the diol ester **3**,[3] and a C_{14} sulfated acyl chain. All the carbon atoms of the OA skeleton, except for C-37 and C-38, are derived from acetate,[4,5] and we have demonstrated recently that glycolate, and not glycine, provides the C-37/C-38 starter unit in **1** and dinophysistoxin-1 (DTX-1) **4**.[6] Although C-47 to C-52 of **3** were also shown to be derived from acetate, C-45 and C-46 of this compound were not enriched, leading us to speculate that an intact unit of glycolate could also provide the C45/C46 starter unit in the diol ester side chain. Similarly, the sulfated C_{14} acyl side chain in **2** could be derived from glycolate followed by the addition of six acetate units or entirely from seven intact units of acetate. Here we report the results of stable isotope experiments to determine the origin of the carbon and oxygen atoms in **2** and **3**.

Isotopically labelled acetate† and glycolate‡ precursors were administered in the usual way[6] to cultures of *Prorocentrum lima* (Mahone Bay strain Pa), and purification of the butanol and ether soluble fractions of the methanolic extract of the cells gave **2** and **3** respectively, as previously described.[2,3] The absolute percentage of ^{13}C at each site in **2** and **3** was measured quantitatively by ^{1}H and ^{13}C NMR, and the proportions of ^{13}C from the scrambled label and natural abundance material were determined by a refinement of methods previously reported.[7]

The predominately acetate origin of **2** and **3** was established by the incorporation of [1,2-$^{13}C_2$] acetate at uniform levels (**2**: 5.9%; **3**: 5.0% total ^{13}C, isolated from the same labelling experiment) at all the positions except C-37, C-38, C-45 and C-46. The pattern and extent of incorporation of intact acetate units (Fig. 1) was determined from the intensities of ^{13}C singlet, doublet and multiplet resonances, and from matching of

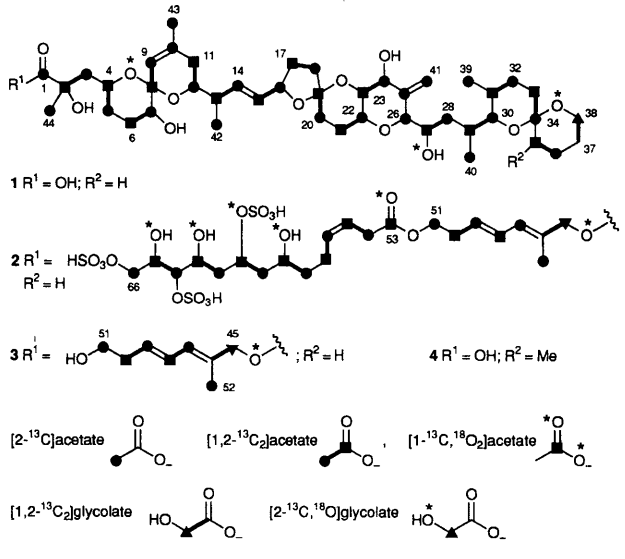

Fig. 1 Incorporation of stable isotopes into DTX-4 **2** and okadaic acid diol ester **3**

^{13}C–^{13}C coupling constants. In **2**, 14 enriched carbon atoms were derived from only a single carbon of acetate; thirteen of these were labelled in feeding experiments with [2-^{13}C acetate] while one carbon (C-53) was labelled from the C-1 of acetate. The origin of the methyl groups from C-2 of acetate, rather than methionine, is unusual but has been observed in a few microbial products.[8] A small proportion (*ca.* 0.2%) of the 5.9% ^{13}C originated from scrambled label and *ca.* 1.05% from acetate at natural ^{13}C abundance. The proportions of multiplet to doublet resonances of carbons which are part of a doubly-labelled unit, or of doublet to singlet resonances at other labelled carbons, showed that condensation of the acetate units occurred at a concentration of *ca.* 14%, subsequent dilution with natural abundance compound reducing the final level to 5.9%.

The observation that C-45 and C-46 were enriched from scrambling of [1,2-$^{13}C_2$]acetate to the same small extent (**2**: 1.8% ^{13}C; **3**: 1.6% ^{13}C) as C-37 and C-38, and without incorporation of any intact doubly-labelled units, led us to postulate that the former carbons shared origins from glycolate already established for C-37 and C-38 in OA and DTX-1.[6] The ^{13}C NMR spectra of **2** and **3** (Fig. 2) isolated from the [1,2-$^{13}C_2$] glycolate experiment showed satellites due to ^{13}C–^{13}C coupling only for C-37 and C-38)**2**: $J_{C\text{-}37/C\text{-}38}$ = 35.4 Hz; **3**: $J_{C\text{-}37/C\text{-}38}$ = 35.1 Hz) and C-45 and C-46 (**2**: $J_{C\text{-}45/C\text{-}46}$ = 47.9 Hz; **3** $J_{C\text{-}45/C\text{-}46}$ = 48.2 Hz). The ^{13}C enrichment at these positions (**2**: 1.7%; **3**: 1.6%) was entirely due to the incorporation of intact glycolate units without scrambling. Smaller enrichment from glycolate is likely due to lower precursor concentration compared with acetate, and perhaps a less efficient penetration of membranes. There was no evidence of ^{13}C from glycolate at any other site in these molecules, either through direct incorporation or scrambling. As discussed previously,[6] [1,2-$^{13}C_2$] acetate can result in the production of [1-^{13}C]- and [2-^{13}C] glycolate via oxaloacetate and then conversion to pyruvate and hydroxypyruvate.[9]

Because it has been established that an intact glycolate unit acts as a starter unit for DSP toxin biosynthesis,[6] it was important to determine if the C-2 hydroxy of glycolate was also

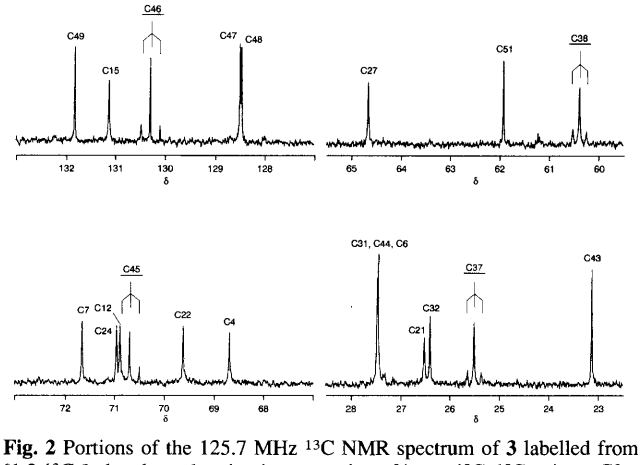

Fig. 2 Portions of the 125.7 MHz ^{13}C NMR spectrum of **3** labelled from [1,2-$^{13}C_2$] glycolate, showing incorporation of intact ^{13}C–^{13}C units at C37, C38 and C45, C46. These positions are enriched to 1.6% ^{13}C; other positions are at natural ^{13}C abundance.

involved in the formation of the first spiroketal ring system. The incorporation of intact ^{13}C–^{18}O bonds from labelled precursors is determined by characteristic upfield ^{13}C shifts of the ^{18}O-substituted carbon atoms.[10] The ^{13}C NMR spectra of **3** labelled from [2-^{13}C, ^{18}O]glycolate displayed ^{18}O isotopically shifted peaks for C-38 ($\Delta\delta$ = −0.023 ppm) and C-45 ($\Delta\delta$ = −0.031 ppm). This finding, coupled with the fact that >80% of the ^{13}C–^{18}O bonds in the glycolate precursor are retained in **3**, reveals the primary role of glycolate oxygen in spiroketal formation as well as formation of the ester linkage.

As expected, the ^{13}C NMR spectra of [1-^{13}C, $^{18}O_2$]acetate labelled **2** and **3** showed substantial ^{13}C enrichments (**2**: 5.4; **3**: 5.9%) for all the carbon atoms derived from C-1 of acetate (Fig. 1). The OA moiety of **2** and **3** displayed ^{18}O isotopically shifted peaks only for C-4 (**2**: $\Delta\delta$ = −0.029 ppm; **3**: $\Delta\delta$ = −0.030 ppm) and C-27 (**2**: $\Delta\delta$ = −0.026 ppm; **3**: $\Delta\delta$ = −0.024 ppm). No detectable isotope shifts were observed for C-2, C-8, C-19 or C-23, the other oxygen-bearing carbon atoms in the OA skeleton derived from C-1 of acetate. In the C_{14} sulfated side chain of **2**, ^{18}O isotopically shifted peaks were observed for all five of the oxygen-bearing carbon atoms derived from C-1 of acetate (C-53, $\Delta\delta$ = −0.037 ppm; C-59, $\Delta\delta$ = −0.025 ppm; C-61, $\Delta\delta$ = −0.041 ppm; C-63, $\Delta\delta$ = −0.022 ppm; C-65, $\Delta\delta$ = −0.016 ppm).

These labelling experiments have established an interesting relationship between the biosynthesis of the OA and the diol portions (C-45 to C-52) of **2** and **3**. Both moieties require an intact unit of glycolate, including the C-2 hydroxyl oxygen, as a starter unit. In the case of the diol, the glycolate carboxyl carbon (C-46) is subsequently alkylated with a methyl group derived from acetate. Furthermore, in both cases the glycolate starter unit is extended with acetate units and terminates with decarboxylation of an acetate group. In view of the structure of **2** and the fact that glycolate is used as a starter unit in other portions of the molecule, it is perhaps surprising that glycolate is not also used as a starter unit for the sulfated chain. Instead, the labelling data indicate that C-54 through C-65 are derived from six intact units of acetate, C-66 from acetate methyl and C-53 from acetate carboxyl carbon. In addition, the oxygen retention at C-53 is the same as that observed for the other acetate-derived oxygen atoms in the sulfated side chain. This finding puts considerable restraints on the formation of the latter chain. A reasonable explanation is that the sulfated acyl unit is derived from a C_{16} polyketide in which both the starter and terminal acetate groups are cleaved. Alternatively, the labelling pattern can be explained by the incorporation of a malonate starter unit formed by carboxylation of an intact acetate unit with $^{13}CO_2$ derived from the C-1 of acetate. Although rare, there have been reports of malonate as a starter unit in other polyketide-derived metabolites.[11–14]

As many as six oxygen atoms in the OA portion of **2** and **3** could, in principle, be derived from ^{18}O-labelled acetate, but the labelling data revealed that only two positions, C-4 and C-27, retain labelled oxygen (Fig. 1). Until now no ^{18}O labelling studies have been reported for other marine polyethers, although by comparison with the bacterial polyether monensin A,[15,16] it is likely that oxidation reactions involving molecular oxygen may be important in the formation of the spiroketal ring systems in **2** and **3**. In contrast, all the oxygenated carbons of the sulfated side chain labelled from C-1 of acetate (*i.e.* C-53, C-59, C-61, C-63 and C-65) retain ^{18}O. Unlike the elaboration of acetate units to form the OA/DTX-1 skeleton, the retention of ^{18}O in the sulfated side chain reflects a more typical bacterial or fungal polyketide pathway. Regardless of the possible biosynthetic mechanisms employed, the uniform enrichment of all the acetate-derived carbon atoms in **2** strongly suggests that the moieties used to form **2** are assembled at the same time from the same acetate and glycolate pools. Thus as **1** is formed, it is quickly converted to **2**, likely the ultimate product of DSP toxin biosynthesis.

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Footnotes

† Cultures were administered [1,2-$^{13}C_2$: 0.91 mmol dm^{-3}]sodium acetate (99%), [2-^{13}C: 0.91 mmol dm^{-3}]sodium acetate (99%), [1-$^{13}C_2{}^{18}O_2$: 0.99 mmol dm^{-3}]sodium acetate (99% ^{13}C, 86% ^{18}O). The ^{18}O-labelled precursor was prepared from iodomethane, potassium [^{13}C]cyanide (99%) and [^{18}O]water (95%).[17]

‡ Cultures were administered [1,2-$^{13}C_2$: 0.33 mmol dm^{-3}]calcium glycolate (99%) synthesized from [1,2-$^{13}C_2$]bromoacetic acid (99%),[18] and [2-$^{13}C^{18}O$: 0.33 mmol dm^{-3}]calcium glycolate (99% ^{13}C, 20% ^{18}O) and synthesized from [2-^{13}C]bromoacetic acid (99%) and [^{18}O]water (50%).[18]

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