Labeling Pattern of Okadaic Acid from $^{18}O_2$ and [$^{18}O_2$]Acetate Elucidated by Collision-Induced Dissociation Tandem Mass Spectrometry

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Abstract: Okadaic acid (**1**) is a polyether compound produced by the marine dinoflagellate *Prorocentrum lima*. Its biosynthesis attracts considerable attention since the carbon skeleton has been shown to be synthesized via an unusual route. However, a very limited amount of information is available for the formation of its ether rings. We applied collision-induced dissociation tandem mass spectrometry (CID MS/MS) to the elucidation of the ^{18}O-incorporation pattern of okadaic acid. The extensive determination of $^{18}O/^{16}O$ ratios for each product ion bearing differing numbers of incorporated ^{18}O atoms resulted in the complete assignment of the labeled positions with accurate isotope ratios; the positions labeled from molecular oxygen ($^{18}O_2$) were O(1)/O(2), O(3), O(5), O(6), O(8), O(9), O(10), and O(12). Those labeled from [$^{18}O_2$]acetate were O(4), O(6), O(7), and O(11) (oxygen atoms are numbered beginning with those of carboxylic acid as O(1)/O(2) to O(13) in ring G). These incorporation patterns suggest that the cyclization of ether rings C, D, and E occurs via a β-epoxide intermediate at C22−C23, and the carboxylic acid is formed by Baeyer−Villiger oxidation.

Introduction

Okadaic acid (**1**), which was initially isolated from sponges[1] and later shown to be a metabolite of marine dinoflagellates,[2] is a well-known natural product because of its usefulness in biological research as a specific inhibitor of protein phosphatases. The biosynthetic route to **1** is markedly different from those of classic polyethers produced by actinomycetes. The ^{13}C-labeling patterns from [1,2-^{13}C]acetate have suggested that, besides the usual C_2 elongation seen in actinomycetous polyketide synthesis, 3-hydroxy-3-methylglutarate (HMG) and other carboxylates generated via the tricarboxylic acid (TCA) cycle are involved in the biosynthesis of **1** (Figure 1).[3,4] In addition to the carbon skeleton, the origin of oxygen atoms is of great interest because the structure of **1** is best characterized by the presence of 7 ether rings, for which the mechanism of formation is yet unknown; no experimental data have been reported as to the stereochemistry of a plausible precursor bearing epoxide/ketone groups. Needham et al. reported the origin of three oxygen atoms on the basis of ^{18}O-induced shifts in ^{13}C NMR; O(4) and O(11) were labeled from [1-^{13}C,$^{18}O_2$]acetate and O(13) from [2-^{13}C,^{18}O]glycolate (Figure 1).[5]

In comparison with conventional ^{13}C NMR methods, mass spectrometry requires far smaller amounts of samples. This huge reduction in sample size is a great advantage when precious isotope-labeled chemicals must be used in feeding experiments or when the organism used in the experiment produces only a very small amount of the metabolite. We have successfully applied collision-induced dissociation tandem mass spectrometry (CID MS/MS) to the structure determination of polyether compounds.[6,7] In the course of these studies, we became aware that most of the oxygen atoms present in polyether structures could be distinguished on the basis of the fragmentation occurring at particular sites of ether rings (see the inset structure in Figure 4). In this paper, we report the successful application of CID MS/MS to the elucidation of the oxygen incorporation pattern of okadaic acid (Figure 1), which demonstrates the usefulness of MS/MS methods for the comprehensive determination of isotope-labeled positions in natural products.[8]

Experimental Section

Cultivation of *Prorocentrum lima*. The dinoflagellate *Prorocentrum lima*, which is known to produce okadaic acid,[2] was collected in

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(8) Applications of mass spectrometry to the biosynthesis of natural products have been reported; e.g., (a) Chang, T. T.; Tsou, H. R.; Siegel, M. M. *Anal. Chem.* **1987**, *59*, 614−617. (b) Tsou, H. R.; Rajan, S.; Chang, T. T.; Fiala, R. R.; Stockton, G. W.; Bullock, M. W. *J. Antibiot.* **1987**, *40*, 94−99. (c) Serhan, C. N.; Nicolaou, K. C.; Webber, S. E.; Veale, C. A.; Dahlen, S.-E.; Puustinen, T. J.; Samuelsson, B. *J. Biol. Chem.* **1986**, *261*, 16340−16345.

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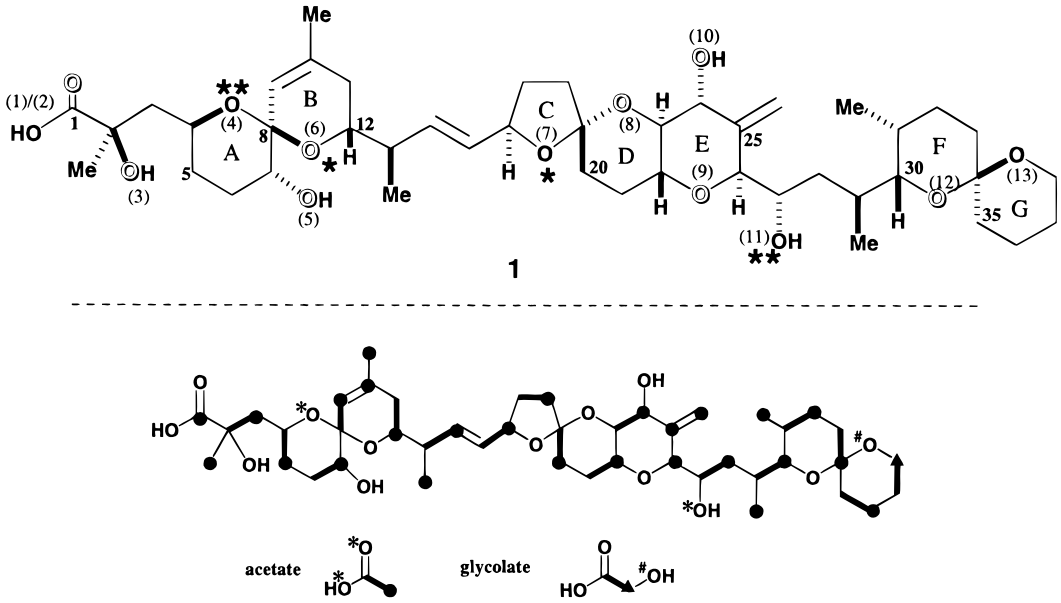


Figure 1. ^{18}O-labeling pattern of okadaic acid (**1**) elucidated in this study (top) and ^{13}C-acetate incorporation pattern from the literature (bottom).[3−5] O denotes an oxygen atom that was significantly labeled from $^{18}O_2$. O* and O** (below and above 30%) were labeled from [$^{18}O_2$] acetate. For a precursor ion $(M - H)^-$ at *m*/*z* 805 that possesses only one ^{18}O atom, ^{18}O-incorporation ratios in each oxygen atom were determined as follows: from $^{18}O_2$, one of O(1)/O(2) 11%, O(3) 7%, O(5) 15%, O(6) 14%, O(8) 15%, O(9) 19%, O(10) 8%, and O(12) 9%, the ratios for the other sites were less than 2%; from [$^{18}O_2$]acetate, O(4) 32%, O(6) 13%, O(7) 5%, and O(11) 31%, the ratios for the other sites were less than 3%.

Motobu, Okinawa, Japan. The organism was isolated as a clonal but not axenic culture. Feeding experiments for the incorporation of molecular oxygen were carried out in a 100-mL sealed flask containing the medium with the head space filled with N_2-$^{18}O_2$ (4:1), and those for labeling from acetate under conventional conditions[2] were conducted in the presence of 1 mM [$^{18}O_2$]AcONa. The medium consisting of seawater supplemented with f/2 enrichment[9] was inoculated with a seed culture (∼3000 cells/mL), and maintained at 22−27 °C for 30−50 days with an 18 h/6 h light/dark cycle. When the cell density reached about 14000 cells/mL, the cells were harvested by filtration and subjected to extraction.

Purification of Okadaic Acid for MS Experiments. The harvested cells were extracted with MeOH. The extract was treated with methanolic 0.2 M NaOH to recover **1** from its conjugated form. Since okadaic acid seemd to be present predominantly as a free acid in the algal cells, most of the oxygen atoms in the C1-carboxyl group remained unchanged after the hydrolysis; a small portion of ^{18}O might be replaced with ^{16}O, but this does not significantly influence the results of this study. After neutralization with aqueous AcOH, the solution was extracted with EtOAc. The extract was dissolved in MeCN−H_2O (6:4) and passed through a small column of ODS (∼0.5 mL) with the same solvent. The final HPLC purification was carried out on a ODS column (YMC-pack, ODS AM120 AS-5, 10 × 250 mm) with MeCN−H_2O−AcOH (60:40:0.2) at the flow rate of 2 mL/min. Okadaic acid was eluted at around 23 min when monitored at 210 nm.

CID MS/MS Spectra. CID MS/MS spectra were measured with a JMS-HX110/HX110 tandem mass spectrometer (JEOL) equipped with a variable dispersion array detector (JEOL, MS-ADS11). Okadaic acid (∼1 μg) dissolved in MeOH was mixed with 2,2′-dithiodiethanol and subjected to CID MS/MS measurements in the negative-ion FAB mode. Helium was introduced to cause the dissociation at a pressure that reduced the intensity of precursor ions to 30%.

Calculations of Gross Incorporation Ratios. The incorporation ratios from $^{18}O_2$ and [$^{18}O_2$]acetate for the whole molecule were calculated by using the molecular related ions in the conventional FAB spectra (Figure 2) as follows. The theoretical distribution of the isotopomers due to naturally occurring ^{13}C for the molecular ion $(M - H)^-$ could be calculated on the basis of the peak intensity of a ^{13}C-free ion appearing at *m*/*z* 803.[10] To estimate the ^{18}O-derived portion in the ion peak at *m*/*z* 805, the contribution of ions bearing two ^{13}C atoms was subtracted. From the ion peak at *m*/*z* 807, the contributions of both ions bearing four ^{13}C atoms and those bearing one ^{18}O and two ^{13}C atoms were deducted. The portions due to naturally occurring ^{13}C were subtracted from isotope peaks at *m*/*z* 805, 807, and 809 in the FAB spectra (Figure 2), which gave the portion of ^{18}O-derived ions. The sum of these portions due to ^{18}O relative to the total ions was regarded as the gross incorporation of ^{18}O in **1**.

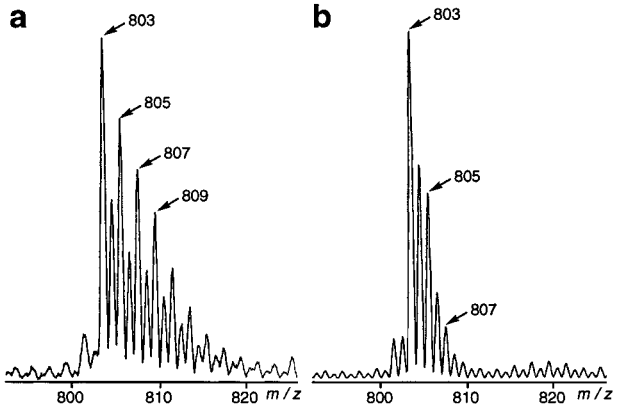


Figure 2. Molecular-related ions of okadaic acid (**1**) labeled from $^{18}O_2$ (**a**) and [$^{18}O_2$]acetate (**b**) on conventional negative ion FAB mass spectra.

Calculations of ^{18}O-Incorporation Ratios for Each Oxygen Site. In the ion peak selected as a precursor, peaks of two isotopomers are superpositioned. One is for ^{18}O-labeled ions and the other is for ions derived from naturally occurring ^{13}C (e.g., 15% of the ion peak at *m*/*z* 805 in Figure 2a is due to those bearing two ^{13}C atoms but no ^{18}O). Therefore, to obtain an accurate ^{18}O/^{16}O ratio, the portion due to ^{13}C-derived ions must be subtracted. The contribution of ^{13}C to the ion peak at *m*/*z* 805 could be estimated from the peak intensity of the ^{13}C-free ions at *m*/*z* 803. Since ^{13}C atoms are distributed evenly throughout the structure of **1**, the portion of ^{18}O-derived and ^{13}C-derived ions in the precursor peak at *m*/*z* 805 could be calculated to be 85% and 15%, respectively, according to a method reported by Biemann.[10]

(9) Guillard, R. R. L.; Ryther, J. H. *Can. J. Microbiol*. **1962**, *8*, 229−239.

(10) Biemann, K. *Mass Spectrometry, Organic Chemical Application*; McGraw-Hill Book: New York, 1962; pp 204−250.

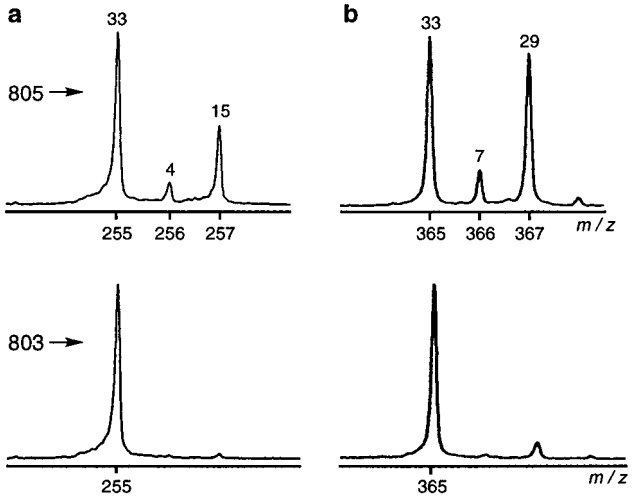


Figure 3. Partial product ion spectra of $^{18}O_2$-labeled (*m*/*z* 805) and unlabeled (*m*/*z* 803) okadaic acid (**1**) for *m*/*z* 257/255 (**a**) and 367/365 (**b**). The numbers at the peaks are peak heights in mm.

To explain how the ^{18}O-incorporation ratio was calculated for each oxygen site from product ion spectra, we take ion peaks at *m*/*z* 257/255 originating from the precursor of *m*/*z* 805 as an example (see Figure 3a). The ^{18}O-contributing portion to the ion peak at *m*/*z* 257 ($Ic_{(257)}$) can be obtained by subtracting the ^{13}C-contributing portion ($Io_{(257)}$) from the total ions ($I_{(257)}$). In the ion peak at *m*/*z* 255, in addition to the product ions generated by a loss of an ^{18}O atom, there are the ions that originated from $^{13}C_2$-derived precursor ions (*m*/*z* 805) and shifted to *m*/*z* 255 due to the loss of two ^{13}C atoms during fragmentation. The contribution of the ions that lost ^{18}O during fragmentation can be determined by subtracting the contribution of these ^{13}C-related ions from the ion peak at *m*/*z* 255. The following calculations give the $^{18}O/^{16}O$ ratio for the *m*/*z* 257/255 pair (their compositions are $C_{13}H_{19}{}^{16}O_4{}^{18}O$ and $C_{13}H_{19}{}^{16}O_5$, respectively).

Theoretical percentages of ^{13}C-derived ions appearing at *m*/*z* 257, 256, and 255 are $\boldsymbol{C(31,2)/C(44,2)} = 8.3\%$, $(\boldsymbol{C(13,1)} \times \boldsymbol{C(31,1)})/\boldsymbol{C(44,2)} = 42.6\%$, and $\boldsymbol{C(13,2)/C(44,2)} = 49.1\%$, respectively, where $C(n,m)$ is the number of combinations of n things taken m at a time: $C(n,m) = n!/m!(n-m)!$, $\boldsymbol{C(31,2)/C(44,2)}$ and $(\boldsymbol{C(13,1)} \times \boldsymbol{C(31,1)})/\boldsymbol{C(44,2)}$ correspond to the respective cases in which two and one ^{13}C atom is present in the lost fragments (thus, no and one ^{13}C atom resides in the product ions), and $\boldsymbol{C(13,2)/C(44,2)}$ corresponds to the case where two ^{13}C atoms remain in the product ions. The total height for the ion peaks at *m*/*z* 257, 256, and 255 is 52 mm. The sum of the portion attributable to ^{13}C-derived ions should be 7.8 mm (=52 mm × 15%; the portion of ^{13}C-derived ions in the precursor is 15%), and therefore those for *m*/*z* 257 and 255 are 0.6 (=7.8 × 8.3%) and 3.8 mm (=7.8 × 49.1%), respectively.

Since the relative peak intensities for ^{18}O-bearing product ions are approximately the same as those for ^{16}O-bearing product ions throughout the MS/MS spectra, the probability of breaking a C−^{18}O bond seems to be equal to that for a C−^{16}O bond. Thus, the percentage of ^{18}O incorporation for the product ion can be obtained as follows.

$$^{18}\text{O: } Io_{(257)} = I_{(257)} - Ic_{(257)} = 15 - 0.6 = 14.4 \text{ mm}$$

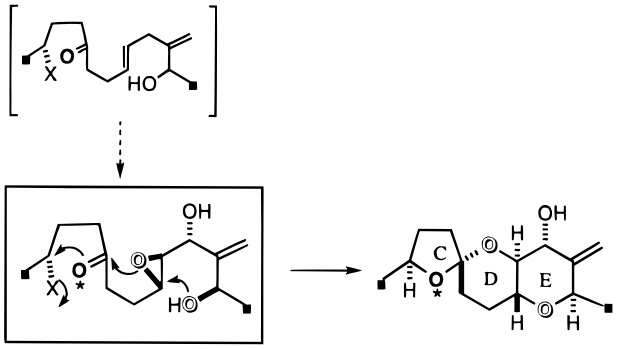
$$^{16}\text{O: } Io_{(255)} = I_{(255)} - Ic_{(255)} = 33 - 3.8 = 29.2 \text{ mm}$$

Therefore, ^{18}O incorporation in the ion peak at *m*/*z* 257 is 14.4/(29.2 + 14.4) = 33%.

By similar calculations, the ^{18}O-incorporation ratio for the ion peaks at *m*/*z* 367/365 was determined to be 47%. Since there is one oxygen atom between the cleavage sites for *m*/*z* 367/365 and *m*/*z* 257/255 (Figure 4), the difference between these two ratios should correspond to ^{18}O incorporation (14%) at O(6).

When the difference between two vicinal ion peaks was two mass units, as seem for *m*/*z* 745/747 and 421/423, their relevant incorporation ratios were not directly calculated since the peak of ^{18}O-bearing ions with the lighter mass overlaps that of the unlabeled ions with the heavier mass. In these cases, we selected other product ions for the calculation of the incorporation ratios such as those corresponding to *m*/*z* 663 for 745, *m*/*z* 477 for 423, and *m*/*z* 464 for 421. The MS/MS spectra used for the determination of the incorporation ratios are presented in the Supporting Information.

Scheme 1



Results and Discussion

The dinoflagellate *Prorocentrum lima* was cultured for 30−50 days[2] in a flask with the head space filled with $N_2-{}^{18}O_2$ (4:1) or with a medium containing 1 mM[$^{18}O_2$]AcONa. From the harvested cells that were cultured in 70 mL of the medium, approximately 5 μg of okadaic acid (**1**, $C_{44}H_{68}O_{13}$, MW 804) was obtained, which was sufficient for several runs of the MS/MS experiments. On the basis of the isotope distribution in the conventional FAB spectra (Figure 2), we estimated the gross incorporation ratio of ^{18}O in **1** from molecular oxygen ($^{18}O_2$) to be 14% and that from [$^{18}O_2$]acetate to be 6%. The product ion spectra of ^{18}O-labeled **1** (Figure 5) were measured for a series of precursor ions corresponding to *m*/*z* 803, 805, 807, and 809. As seen in Figure 4, all the oxygen sites could be distinguished by CID MS/MS experiments[7] and ^{18}O-incorporation ratios from $^{18}O_2$ and [$^{18}O_2$]acetate were determined for virtually all oxygen sites (Figure 1).

The ^{18}O-labeling pattern disclosed several intriguing features in the biosynthesis of **1**. Two oxygen sites, one each of O(1)/O(2) and O(13), were labeled from neither $^{18}O_2$ nor [$^{18}O_2$]-acetate. The high incorporation ratios at O(4) and O(11) from [$^{18}O_2$]acetate are also consistent with the results reported by Needham et al.[5] However, for the sites with a low incorporation ratio, such as O(6) and O(7), the MS/MS method seems to be better than the conventional NMR methods since the weak relative intensity of ^{18}O-bearing ^{13}C signals, in addition to a very minute shift induced by the ^{18}O isotope, often hampers the separation of these signals from ^{16}O-bearing ones.

Regarding the mechanism of ether-ring formation, the MS/MS experiments provided valuable information. The incorporation pattern for O(7) and O(8) indicates that the cyclization of rings C, D, and E occurs via a β-epoxide intermediate (Scheme 1), which partly mimics the cyclization mechanism reported for actinomycetous polyethers.[11] However, this is the first example, among dinoflagellate polyethers, of assigning the stereochemistry of the epoxide precursor and the direction of nucleophilic additions upon cyclization. At the terminal carboxylic acid, only one of two oxygen atoms O(1)/O(2) was labeled from O_2 but not from [$^{18}O_2$]acetate (Figure 6). This observation and the fact that C1 originates from a methyl carbon of acetate[5] suggest that

(11) Cane, D. E.; Celmer, W. D.; Westley, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594−3600.

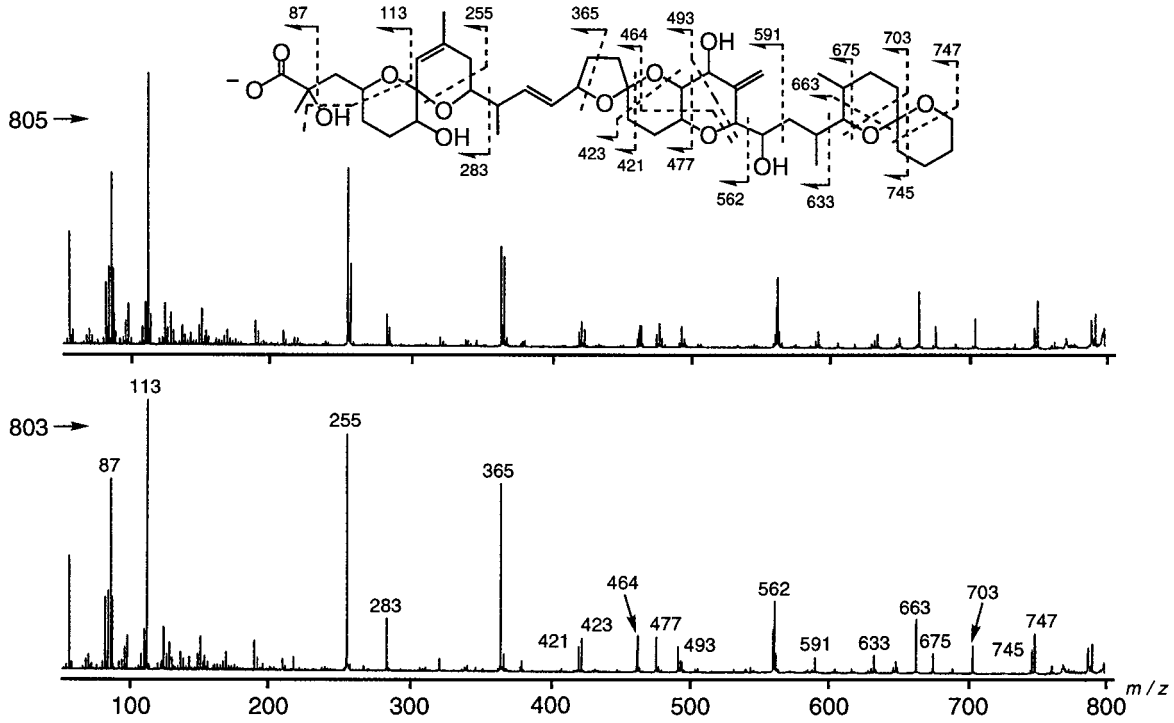


Figure 4. CID MS/MS product ion spectra of $^{18}O_2$-labeled (*m*/*z* 805) and unlabeled (*m*/*z* 803) okadaic acid (**1**) with its fragmentation pattern.

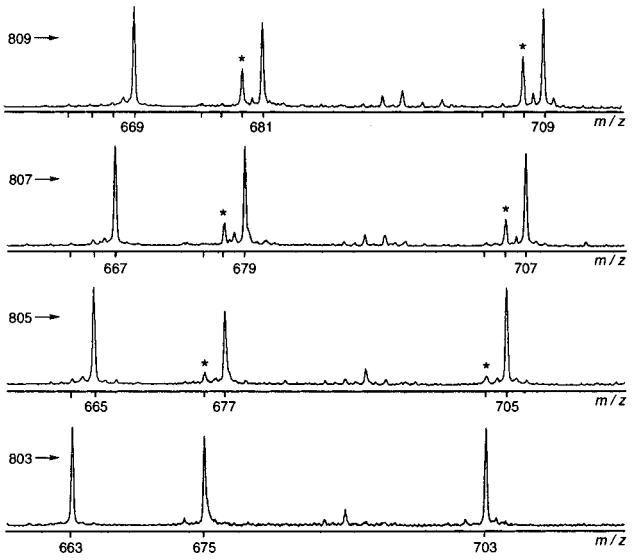


Figure 5. Partial product ion spectra of okadaic acid (**1**) labeled from $^{18}O_2$ for precursor ions corresponding to *m*/*z* 809, 807, 805, and 803. The product ions appearing as singlet peaks at *m*/*z* 665 for an 805 precursor, *m*/*z* 667 for an 807 precursor, and *m*/*z* 669 for an 809 precursor are generated by double bond cleavage at C30−C31 and C34−O(12) (Figure 4), and possess oxygen atoms from O(1) to O(12) but not O(13). These observations show that a loss of O(13) does not result in the deprivation of the ^{18}O isotope, which indicates that O(13) is not labeled from $^{18}O_2$. In contrast, the product ions due to 675 and 703 fragmentations, both of which lose O(12) and O(13), are accompanied by two mass-unit lighter ions (*) with their intensity increasing as the mass of the precursor ion increases from *m*/*z* 805 to 809. These observations reveal that the lighter ions (*) are generated by a loss of ^{18}O and thus O(12) is labeled from $^{18}O_2$.

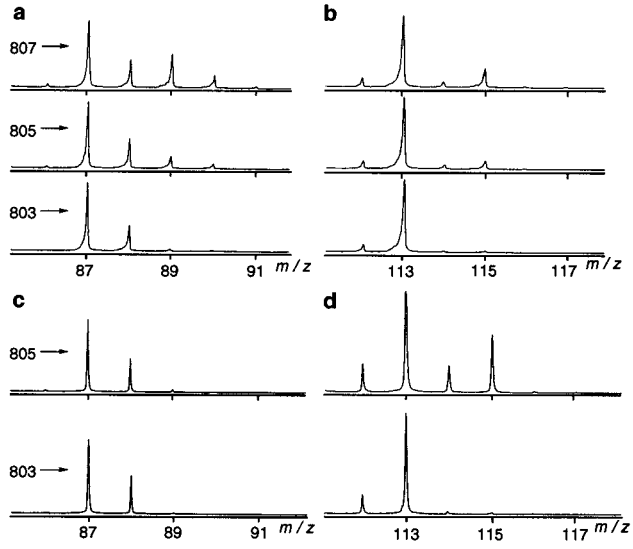


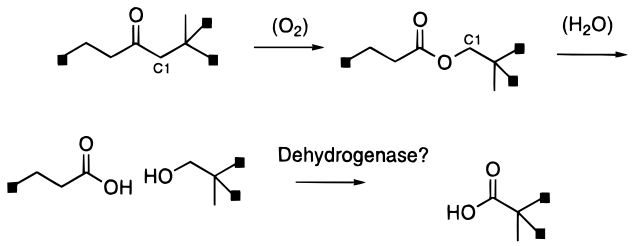
Figure 6. Partial product ion spectra of okadaic acid (**1**) labeled from $^{18}O_2$ and [$^{18}O_2$]acetate for *m*/*z* 89/87 and 115/113: **a** and **b**, product ion spectra of precursor ions of *m*/*z* 807, 805, and 803 labeled from $^{18}O_2$; **c** and **d**, product ion spectra of precursor ions of *m*/*z* 805 and 803 labeled from [$^{18}O_2$]acetate. The incorporation of ^{18}O from $^{18}O_2$ in O(1)/O(2) and/or O(3) was supported by the weak but significant peak at *m*/*z* 89 in **a** (805) while the absence of a peak at *m*/*z* 89 in **c** revealed the lack of incorporation in O(1)/O(2) or O(3) from [$^{18}O_2$]acetate. Note the absence of an ion peak at *m*/*z* 117 in **b** (807); this suggests that only one of O(1)/O(2) was labeled from $^{18}O_2$. The prominent ion peak at *m*/*z* 115 in **d** (805) and the absence of an ion peak at *m*/*z* 89 in **c** (805) clearly indicate that [$^{18}O_2$]acetate is incorporated in O(4) but not in O(1/2) or O(3).

the carboxylic acid of **1** is formed by Baeyer−Villiger oxidation, as has been proposed for an ester group of DTX4.[12] The incorporation of an oxygen atom from H_2O could be caused by dehydrogenase-catalyzed oxidation (one of the possible routes is illustrated in Scheme 2).

The mechanism by which O(6) was significantly labeled from both $^{18}O_2$ and [$^{18}O_2$]acetate remains unclear. This unusual labeling behavior might be explained by competitive incorporation from acetate and indirectly from molecular oxygen via oxidative formation of a building block. Another possible

(12) Wright, J. L. C.; Hu, T.; McLachlan, J. L.; Needham, J.; Walter, J. A. *J. Am. Chem. Soc*. **1986**, *118*, 8757−8758.

Scheme 2



explanation is that ring B is formed via a diketone intermediate at C8 and C12 followed by reduction of the ketone at C12, in which the ketone oxygen atoms would be able to interchange during the keto−ketal conversion.

Applications of MS to biosynthetic studies, especially to the origin of oxygen atoms, have been reported by Serhan et al.[8c] The present study also indicates the usefulness of MS for labeling experiments with heteroatoms. Furthermore, CID MS/MS is proven to be a powerful tool for natural products with a limited sample size, and particularly useful for simultaneously assigning multiple sites for isotope incorporations. Further labeling experiments with other oxygen sources using CID MS/MS are currently underway.

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Supporting Information Available: Product ion spectra of CID MS/MS experiments with $^{18}O_2$- and [$^{18}O_2$]acetate-labeled okadaic acid (6 pages). See any current masthead page for ordering and Internet access instructions.

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