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Origins of Oxygen Atoms in a Marine Ladder-Frame Polyether: Evidence of Monooxygenation by ^{18}O-Labeling and Using Tandem Mass Spectrometry

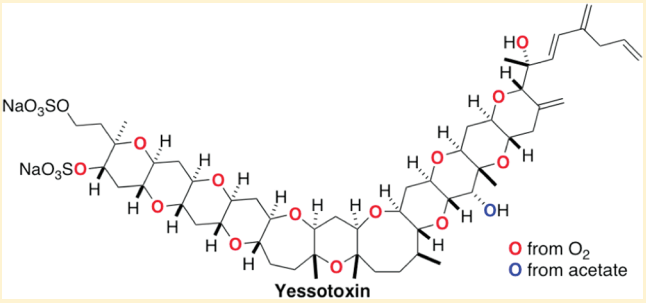
Masatoshi Yamazaki,[†] Miho Izumikawa,[†] Kazuo Tachibana,[†] Masayuki Satake,[†,*] Yoshiyuki Itoh,[‡] and Masahiro Hashimoto[‡]

[†]Department of Chemistry, School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

[‡]MS Business Unit, JEOL Ltd., 3-12 Musashino, Akishima, Tokyo 196-8558, Japan

Supporting Information

ABSTRACT: Yessotoxin is a ladder-frame polyether produced by the dinoflagellate *Protoceratium reticulatum*. Previous labeling experiments using ^{13}C-acetate established the unique assembly of the carbon chain from intact and cleaved acetate units. The origins of ether and hydroxy oxygens in the molecule, which would yield further information regarding the assembly of the ladder-frame structure, have yet to be established. In this study, we describe the incorporation of ^{18}O in one experiment where the dinoflagellate was cultured under $^{18}O_2$ atmosphere and in a second where the culture media was supplemented with [$^{18}O_2$]acetate. Labeled yessotoxin obtained from these experiments was subjected to collision-induced dissociation tandem mass spectrometry to determine the positions of ^{18}O-incorporation pattern in the molecule. Detailed analyses of product ions from the fragmentation processes led to the identification of ^{18}O-labeled positions and the incorporation ratios. The data revealed that the ether oxygens were labeled from $^{18}O_2$ and the hydroxy oxygen on C32 was derived from [$^{18}O_2$]acetate. These results support a proposed biosynthetic mechanism of marine ladder-frame polyethers that a polyene precursor was oxidized by a monooxygenase after acetate condensation.



INTRODUCTION

The ladder-frame polyethers which have a *trans*-fused ether ring scaffold in the molecule are representative secondary metabolites produced by dinoflagellates.[1] Their complicated structures and potent biological activities have fascinated synthetic chemists, and a considerable body of synthetic work has been reported.[2] In contrast, the extremely limited production by dinoflagellate metabolites and lack of genetic information has hampered studies of their biosynthesis. The early ^{13}C-labeling experiments of brevetoxins another groups of polyether toxins were reported independently by Nakanishi and Shimizu.[3,4] The ^{13}C-labeling pattern of the brevetoxins was obviously different from that of polyethers from actinomycetes because the methyl groups were derived from methionine and the methyl of acetate (m−c, m: an acetate methyl; c: an acetate carbonyl). In addition, the carbon chain contained intact and cleaved acetate units. Thus, the most striking feature of ^{13}C-labeling patterns of the brevetoxins is a methyl−methyl (m−m) coupling from acetates. After those studies, few biosynthetic studies of the ladder-frame polyethers were reported, though detailed studies of the okadaic acid compounds produced by another dinoflagellate *Prorocentrum lima* established that these unusual biosynthetic features were not confined to fused ring polyethers and, in fact, are a common occurrence among all dinoflagellate metabolites.[5−9] Yessotoxin (YTX), a ladder-frame polyether with two sulfate esters, was first isolated from Japanese scallops,[10] but later the dinoflagellate *Protoceratium reticulatum* was identified as the biogenetic origin.[11] With the producing organism in culture, YTX was considered a good example to clarify ^{13}C-labeling patterns of the ladder-frame polyethers since it contains two simple six-membered ether ring tetrad sequences (rings A−D and H−K) which are often found in ladder-frame polyethers. ^{13}C-labeling experiments of YTX were conducted as shown in Figure 1.[12] These labeling results showed that the six-membered ether ring tetrad sequences were constructed by repetition of a C3-unit (m−m−c), which displayed m−m coupling between an isolated methyl and an intact acetate unit. A biosynthetic starter of YTX was glycolate similar to okadaic acid.[13] In addition, all ether carbons except C19 were labeled from acetate methyl. This suggests that the acetate methyls are oxidized after polyketide chain construction. It has been proposed that a ladder-frame scaffold is formed by a cascade of epoxide-opening events of the transformation of a polyepoxide precursor which is derived from an *E* polyene.[14,15] In order to elucidate ether ring formation, ^{18}O-labeling

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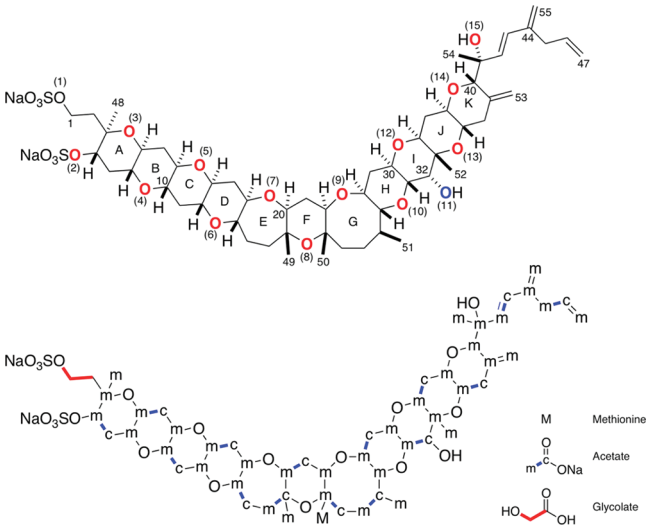

Figure 1. ^{18}O-incorporation pattern of yessotoxin (YTX) in this work and ^{13}C-labeling pattern.[12] O (red) was derived from $^{18}O_2$ and O (blue) was from [$^{18}O_2$]acetate. ^{18}O-incorporation ratios in each oxygen atom were determined as follows: from $^{18}O_2$, O(2) 10%, O(3) 2%, O(4) 9%, O(5) 4%, O(6) 16%, O(8) 4%, O(9) 5%, O(10) 10%, O(12) 17%, O(13) 2%, O(14) 10%; from [$^{18}O_2$]acetate, O(11) 29%; for a precursor ion $[M - SO_3Na + H - Na]^-$ at m/z 1063 which possesses only one ^{18}O atom. O(1) originated from C2-OH of glycolate, supported by ^{13}C-labeling pattern. The ratios of O(7) and O(15) could not be calculated because of small peaks and this method.

experiments are essential to verify monooxygenation of polyene and epoxide-opening events. Previously, the multiple ^{18}O-labeling patterns of okadaic acid were elucidated by comparison of peak heights of isotope-labeled signals using a collision-induced dissociation tandem mass spectrometry (CID MS/MS) method.[5e,f] Thus CID MS/MS is a promising method for determination of the ^{18}O-incorporation patterns of polyether compounds. The applications of CID MS/MS for YTX were well studied because a negative charge localized at the terminal sulfate ester caused charge remote fragmentation and simplified its product ion spectrum.[16] In this paper, we report the labeling pattern of all oxygen atoms in YTX using MALDI-SpiralTOF-TOF tandem mass spectrometry experiments.[17]

RESULTS AND DISCUSSION

Cultures of $^{18}O_2$ and [$^{18}O_2$]Acetate Feeding Experiments. For $^{18}O_2$ feeding experiments, the dinoflagellate *Protoceratium reticulatum* was cultured under the atmosphere of N_2−$^{18}O_2$ (4:1) in a flask sealed by a silicon cap to avoid the exchange of $^{18}O_2$ with $^{16}O_2$ and supplemented with natural abundance 2.5 mM sodium bicarbonate as carbon source. Original nonlabeled YTX from inoculated cells complicated calculation of the incorporation ratio. Initial cell density of the $^{18}O_2$ feeding experiment was 1/10 of that of nonlabeled culture conditions.

For [$^{18}O_2$]acetate feeding experiments, 2.5 mM sodium [$^{18}O_2$]acetate was added as a carbon source into the culture media of *P. reticulatum* under an atmosphere of N_2−O_2 (4:1) for 22 days.

Positions and Ratios of $^{18}O_2$ Feeding Experiments. Chromatographic purification led to the isolation of ^{18}O-labeled YTX (13 μg) from cultured cells and ^{18}O-labeled YTX (67 μg) from 150 mL of the culture filtrate, respectively. The negative MALDI-SpiralTOF mass spectrum of the ^{18}O-labeled YTX yielded the isotope distribution. The desulfate ion $[M - SO_3Na + H - Na]^-$ at m/z 1061 was a dominant ion of YTX ($C_{55}H_{80}O_{21}S_2Na_2$) in the MALDI negative ion mass spectrum and the peak intensity of its mono-^{18}O-labeled peak at m/z 1063 indicated that ^{18}O enrichment from $^{18}O_2$ was estimated to be 10% (Figure 2).

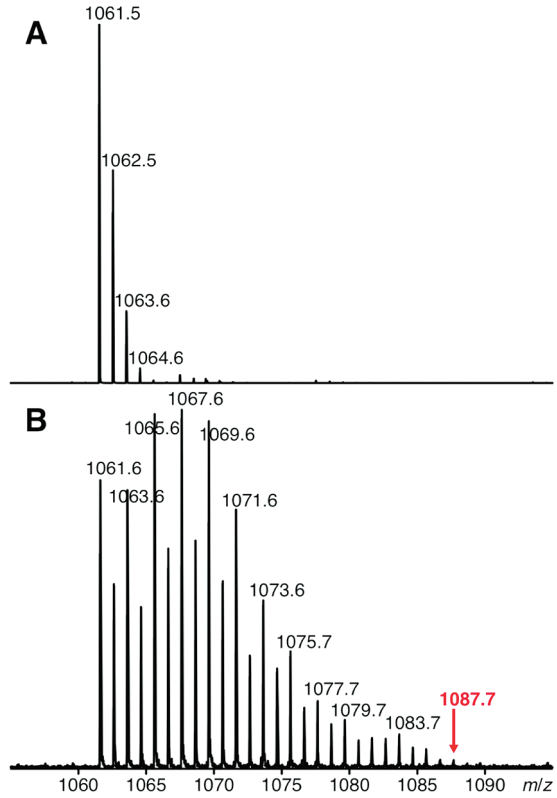

Figure 2. MALDI-SpiralTOF mass spectra of (A) intact and (B) $^{18}O_2$-labeled YTX.

The desulfated ion and its isotopic ions were used as a precursor ion for high-energy CID MS/MS experiments. The isotopic ion at m/z 1087 that was 26 Da larger than that of the intact YTX suggested 13 of 15 oxygens attached on the skeletal carbons were labeled from $^{18}O_2$. YTX has 11 ethers, two hydroxys, and two sulfates bearing oxygens. The locations and ratio of labeled oxygens were determined from peak intensity of product ions measured by a MALDI-SpiralTOF-TOF. Intense product ions were generated by a series of fragmentations containing an ether oxygen as shown in Figure 3, and each oxygen could be assigned to a particular product ion. The incorporation ratio of each oxygen labeled from $^{18}O_2$ ranged from 2 to 17%. Low incorporation ratios at such O(3) and O(13) were supposed to be caused by errors of peak height. A small peak at m/z 405 hampered the calculation of incorporation ratio at O(7). Although the incorporation ratio at O(1) was calculated as 4%, the ^{13}C-labeling pattern by glycolate and the incorporation ratio (10%) at O(2) from $^{18}O_2$ indicated that O(1) was derived from C2-OH of glycolate.[5c] Therefore, all ether oxygens were labeled from $^{18}O_2$. The ratio of O(15) was not determined because product ions higher than at m/z 924 formed by loss of the C41−C55 side chain including O(15) were not observed. However, Figure 4 demonstrated evidence of ^{18}O incorporation into O(15). Product ions from the fully labeled precursor ion at m/z

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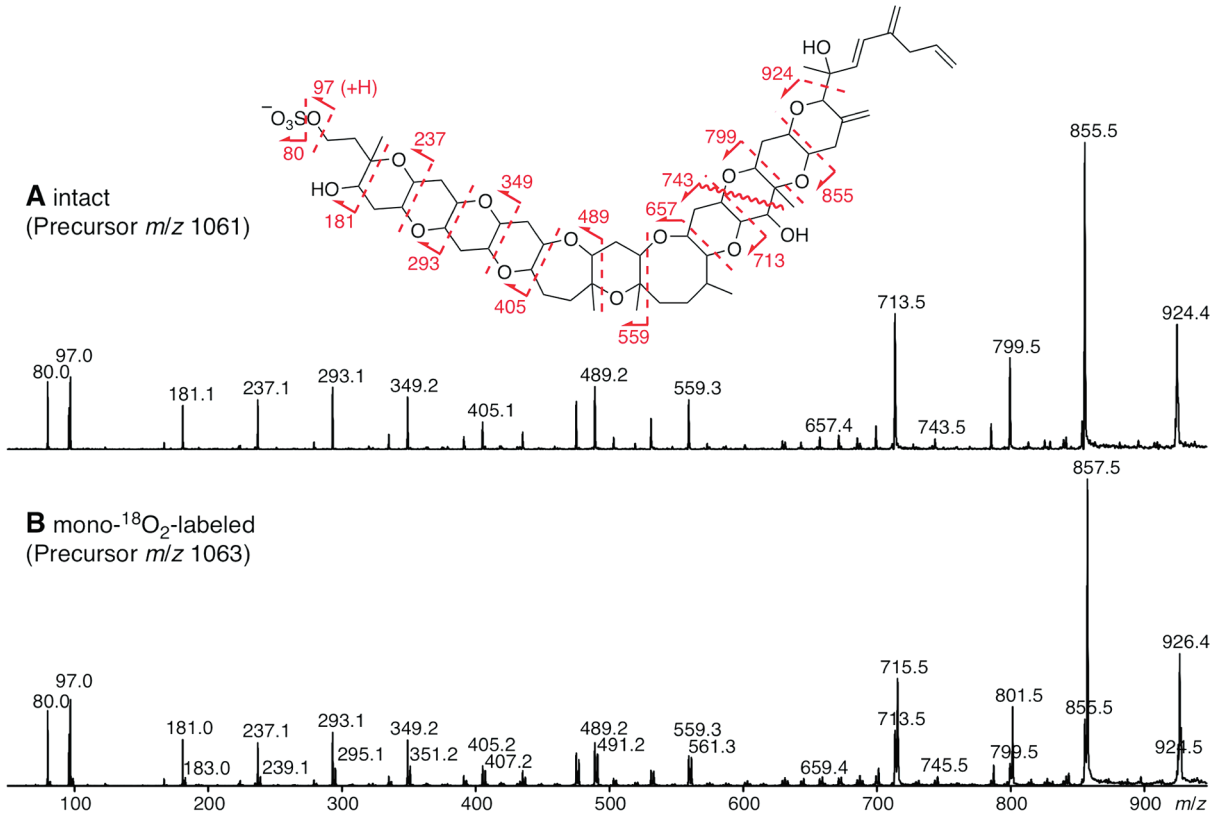

Figure 3. Cleavage site of YTX and product ion spectra of (A) intact (precursor at *m*/*z* 1061) and (B) mono-$^{18}O_2$-labeled YTX (precursor at *m*/*z* 1063).

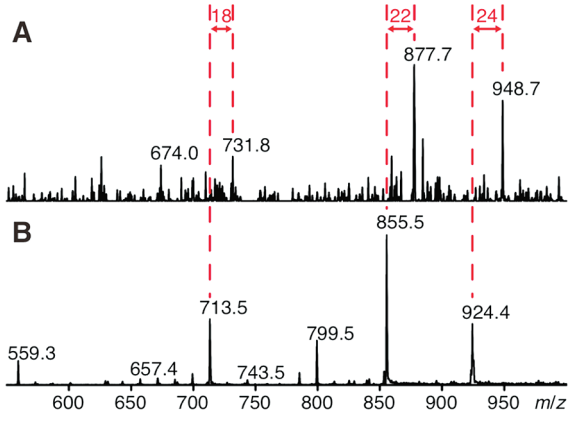

Figure 4. Partial product ion spectra of (A) fully $^{18}O_2$-labeled (precursor at *m*/*z* 1087) and (B) intact YTX (precursor at *m*/*z* 1061).

1087 which contained 13 labeled oxygens were observed at *m*/*z* 948, 877, and 731. Those product ions corresponded to product ions at *m*/*z* 924, 855, and 713 from intact YTX, respectively. The differences between corresponding peaks were 24, 22, and 18, and decreased 2 Da by every product ion, indicating that each fragment has an ^{18}O-labeled ether oxygen. The product ion at *m*/*z* 948, corresponding to the ion at *m*/*z* 924, is 24 Da larger than the ion at *m*/*z* 924, which contains 12 labeled oxygen atoms. Thus, one ^{18}O-labeled oxygen atom was lost from the C41−C55 side chain by bond cleavage between C40−C41. Therefore, O(15) was deduced to be labeled from $^{18}O_2$. In addition to O(15), O(14) was also derived from $^{18}O_2$; however, these oxygen atoms would be introduced not during an epoxidation step but a during m−m coupling formation step by a Favorskii-type rearrangement.[5c,d]

Positions and Ratios of [$^{18}O_2$]Acetate Feeding Experiments. Following the same purification procedures as before, YTX (31 μg) was obtained from the cells supplemented with [$^{18}O_2$]acetate, while an additional 66 μg was obtained from 150 mL of the medium. The negative MALDI-SpiralTOF mass spectrum of the [$^{18}O_2$]acetate-labeled YTX gave an isotope-incorporated pattern (Figure 5). An intense desulfated isotopic ion was observed at *m*/*z* 1063. The whole ^{18}O-incorporation ratio was estimated to be 15%. The product ion spectra as shown in Figure 6 showed the peak intensity of an isotopic ion at *m*/*z* 715 from [$^{18}O_2$]acetate-labeled YTX apparently reduced after loss of 32-OH. The incorporation ratio at O(11) was calculated as 29% and was remarkably larger than those of other

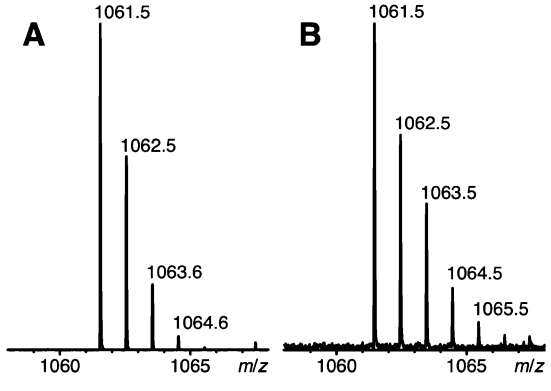

Figure 5. MALDI-SpiralTOF mass spectra of (A) intact and (B) [$^{18}O_2$]acetate-labeled YTX.

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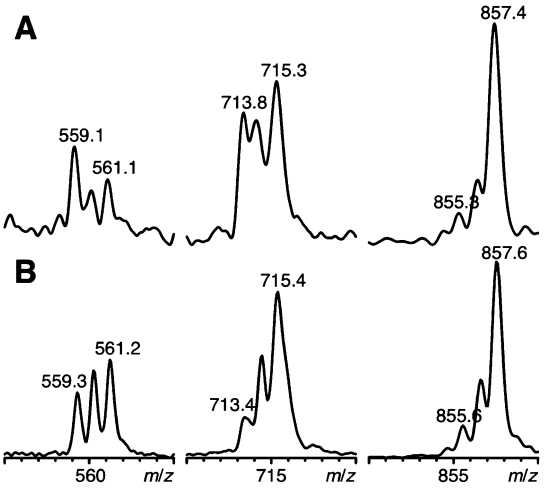

Figure 6. Partial product ion spectra of (A) [$^{18}O_2$]acetate-labeled and (B) intact YTX. Each precursor ion was selected at m/z 1063.

positions. The ^{13}C-labeling experiments support that the O(11) at C32 was derived from the acetate carbonyl which was retained during the biosynthesis of YTX.

CONCLUSION

This is the first report of the origin of the oxygen atoms in a marine ladder-frame polyether. All of the ether oxygens in YTX were labeled from $^{18}O_2$. The previous ^{13}C feeding experiments revealed that the ether carbons except C19 in YTX were derived from acetate methyls. These results support the proposed fused ether ring formation mechanism that an *E*-polyene precursor is stereoselectively epoxidized by a monooxygenase to produce a polyepoxide precursor and then *endo-tet* cyclization of the resultant polyepoxide precursor proceeded successively to biosynthesize a ladder-frame polyether.[14,15,18] Moreover, the incorporation of O(2) from $^{18}O_2$ also indicates O(2) originated from an epoxide oxygen by the epoxide-opening process. Thus, an epoxide-opening starts at ring K and the *endo-tet* closure by the epoxide-opening event proceeds from tail (ring K) to head (ring A) direction (Figure 7).[19] The terminal ring in the dinoflagellate cyclic ether have an oxygenated functional group (ester, alcohol, and sulfate) from the epoxide-opening event. The carbon at C4 in YTX has an *S* configuration analogous with the carbons, C5 in brevetoxin-B, C18 and C31 in brevisin, C11 in brevisamide isolated from *Karenia brevis*,[20] and C50 and C54 in gymnocins A and B,[21] respectively, from *Karenia mikimotoi*. Thus a dinoflagellate monooxygenase seems to form predominantly an all (*S*,*S*)-epoxide intermediate via an *E*-polyene intermediate.

EXPERIMENTAL SECTION

General Information. Labeled reagents, $^{18}O_2$ gas (96.5 atom %), sodium [$^{18}O_2$]acetate (95 atom %), and other chemicals were used without further purification. MALDI mass spectra and high-energy CID product ion mass spectra were acquired with a MALDI-SpiralTOF-TOF tandem mass spectrometer.

Cultivation of $^{18}O_2$ Feeding Experiments. The dinoflagellate *Protoceratium reticulatum* was inoculated at the final cell density of 460 cells/mL and cultured in 100 mL Erlenmeyer flasks each containing 75 mL of medium at 17 °C for 23 days. The flasks were filled with a seawater medium enriched with GSe nutrients containing antibiotics (penicillin G, 50 units/mL; polymyxin B, 50 units/mL; streptomycin, 50 units/mL; chloramphenicol, 0.5 μg/mL; neomycin, 2.5 μg/mL; chlortetracycline, 10 μg/mL) and then sealed by silicon caps. Nitrogen (56 mL) and ^{18}O-oxygen (14 mL) gases were replaced with the same volume of the medium by a syringe. Sodium bicarbonate was added at the final concentration of 2.5 mM.

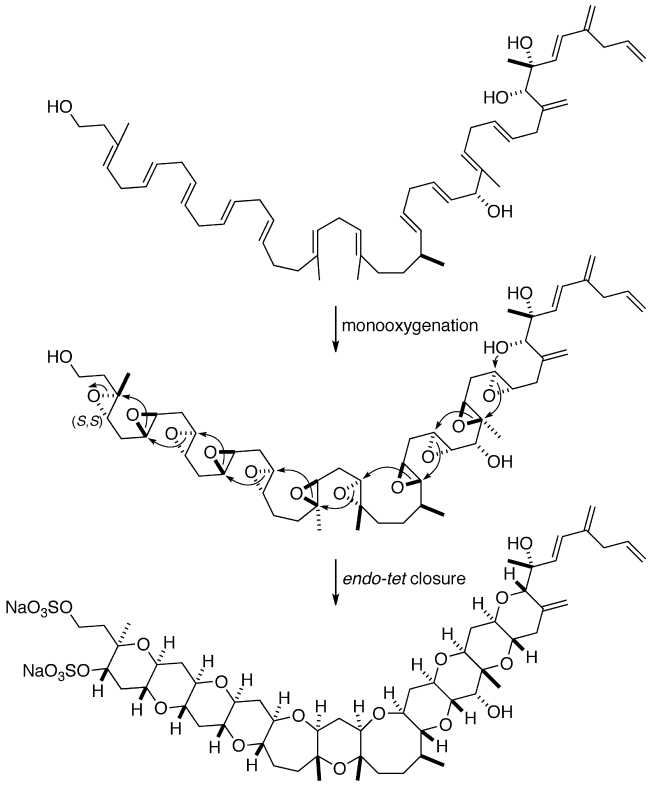

Figure 7. Presumed biosynthesis of yessotoxin via a polyepoxy intermediate.

Cultivation of [$^{18}O_2$]Acetate Feeding Experiments. *Protoceratium reticulatum* at an initial concentration of 460 cells/mL was grown in 100 mL Erlenmeyer flasks each containing 75 mL of medium at 17 °C for 22 days. The flasks were filled with a seawater medium enriched with GSe nutrients containing antibiotics. Nitrogen (56 mL) and oxygen (14 mL) gases were replaced with the same volume of the medium by a syringe. Sodium [$^{18}O_2$]acetate was added at the final concentration of 2.5 mM.

Purification of Yessotoxin. Harvested cells by filtration were extracted with MeOH. After evaporation of the solvent, the extract was partitioned between H_2O and ethyl acetate. The H_2O layer was extracted with 1-butanol. The extract was dissolved in $CHCl_3$–MeOH (1:1) and loaded on an alumina column. The column was washed with $CHCl_3$–MeOH (1:1) and MeOH and then eluted with 1% NH_4OH–MeOH (1:1). The final fraction was passed through an ODS cartridge column, and final purification was performed on an ODS-HPLC column with MeOH–MeCN–H_2O (2:1:2). Filtered medium was passed through a resin column. The column was washed with H_2O and eluted with MeOH. YTX in the MeOH fraction was purified by an ODS cartridge and an ODS-HPLC.

MALDI-SpiralTOF MS and SpiralTOF-TOF Measurements. The ^{18}O-labeled YTX was dissolved in MeOH, mixed with liquid matrix (3-aminoquinoline/α-cyano-4-hydroxycinnnamic acid), and subjected to MALDI-SpiralTOF MS and high-energy CID MS/MS measurements in negative-ion mode. The mass spectra and product ion mass spectra were recorded with a laser irradiation at 349 nm, a laser frequency at 250 Hz, −20 kV of acceleration voltage in the first TOF stage. In case of the product ion mass spectrum measurements, all the product ions were reaccelerated for 9 kV in the second TOF stage.

Calculation of ^{18}O-Incorporation Ratios. The incorporation ratio for each oxygen site was determined from the peak intensity of

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the product ion spectrum for the precursor at m/z 1063 by following calculations.[5e]

The desulfate ion peak at m/z 1063 is overlapped by three ion isotopomers, $^{12}C_{53}{}^{13}C_2H_{81}O_{18}S$ (^{13}C-bearing), $C_{55}H_{81}{}^{16}O_{17}{}^{18}OS$ (^{18}O-bearing), and $C_{55}H_{81}O_{18}{}^{34}S$ (^{34}S-bearing). The two ^{13}C- and ^{34}S-bearing ions are derived from naturally occurring ^{13}C and ^{34}S (theoretically 27% of the ion peak at m/z 1063 is due to the two ^{13}C and ^{34}S atoms but no ^{18}O). To estimate ^{18}O-incorporation ratio, contribution of the two ^{13}C- and ^{34}S-derived ions into the ion peak at m/z 1063 must be subtracted. Therefore, ^{18}O-derived ion in the precursor peak at m/z 1063 was calculated to be 73%.

Since ^{13}C or ^{34}S atoms are thought to be distributed equally everywhere in the structure of YTX, the percentage of two-^{13}C and ^{34}S ion peaks are considered to be retained in whole product ions. For instance, the incorporation ratio of the oxygen O(8) at ring F was determined as following. The oxygen at O(8) is located between the product ions at m/z 489 and 559. The product ion at m/z 489 was observed together with m/z 490 (m + 1) and 491 (m + 2), the total ions of which was regarded as corresponding to contribution of ^{13}C- or ^{34}S-bearing ions to the precursor at m/z 1063. Then, their contribution was figured out based on calculation of combinations for carbon number on the product ion.

Theoretical percentages of ^{13}C- and ^{34}S-bearing ions in the fragment ions at m/z 491, 490, and 489, which consist of 22 carbons, were obtained $(C(22,2) + 1)/(C(55,2) + 1) = 15.6\%$, $C(22,1) \times C(33,1)/(C(55,2) + 1) = 48.9\%$, and $C(33,2)/(C(55,2) + 1) = 35.5\%$, respectively, where $C(n,k)$ means combinations of n things taken k at a time: $C(n,k) = n!/k!(n - k)!$. The peak intensity at m/z 491, 490, and 489 were 41556.2, 8542.4, and 30580.4 (total 80679), and the sum of the portion related to ^{13}C- and ^{34}S-derived ions should be 21783.3 (= 80679 × 27%). On the basis of the subtraction of their contribution at m/z 491 and 489, the contribution of ^{18}O-bearing product ions could be calculated:

$$m/z\ 491: 30580.4 - 21783.3 \times 15.6\% = 33823.1$$

$$m/z\ 489: 34968.8 - 21783.3 \times 35.5\% = 27182.2$$

Therefore, the ^{18}O-incorporation in the ion peak at m/z 491 was 33823.1/(27182.2 + 33823.1) = 45%.

The similar calculation for the product ion at m/z 559 gave the ^{18}O-incorporation ratio to be 49%. The ^{18}O-incorporation at O(8) should correspond to the difference between two ratios at m/z 491/489 and 561/559 to be 4%. By the same calculation, the ^{18}O-incorporation ratio for each oxygen site was determined.

■ ASSOCIATED CONTENT

Supporting Information

Signal intensity of each product ion on CID MS/MS of mono-$^{18}O_2$- and [$^{18}O_2$]acetate-labeled YTX and product ion spectra of fully $^{18}O_2$- and [$^{18}O_2$]acetate-labeled YTX. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: msatake@chem.s.u-tokyo.ac.jp.

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