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Production of Octadienal in the Marine Diatom *Skeletonema costatum*

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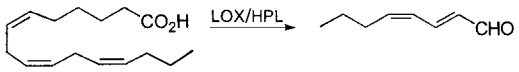
ABSTRACT



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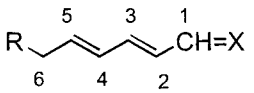
Marine diatoms produce α,β,γ,δ-unsaturated aldehydes that have detrimental effects on the reproduction of their natural predators. The production of these defensive metabolites is suggested to involve enzymatic oxidation of polyunsaturated fatty acids. In this paper, feeding experiments with labeled precursor provide clear evidence in support of the origin of octadienals 1 and 2 from 6,9,12-hexadecatrienoic acid (5), thus proving the involvement of novel lipoxygenase/lyase activity for the oxidation of C_{16} fatty acids.

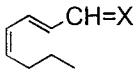
Damaged or wounded marine diatoms produce α,β,γ,δ-unsaturated aldehydes that have been suggested to induce detrimental effects on the reproduction of planktonic predators, the herbivore copepods.[1] In particular, we have recently reported that damaged cells of *Skeletonema costatum*, one of the main species forming algal blooms in world's oceans, produce four major compounds (**1**−**4**) responsible for the abortive and teratogenic effects in zooplankton herbivores.[2]

In contrast to the great deal of interest on the ecological role of these aldehydes,[1,3] very little is known about the molecular mechanisms leading to their production in the microalga. It is generally accepted that this family of oxylipins may derive from breakdown of C_{20} or C_{18} polyunsaturated fatty acids by lipoxygenase (LOX)/hydroperoxide lyase (HPL) activity. The origin of octadienals **1** and **2** and octatrienal **3** of *S. costatum*, is, however, not a simple issue, since the double-bond position of these compounds (belonging to ω-4 and ω-1 series) is not consistent with the composition of LOX-sensitive C_{20} or C_{18} fatty acids in the diatom.[2a,4]

In this paper, we describe feeding experiments with deuterated precursors that substantiate the formation of **1** and **2** from 6,9,12-hexadecatrienoic acid (**5**) by lipoxygenase pathway. To the best of our knowledge, formation of octadienals in *S. costatum* is the first evidence for LOX-mediated oxidation of C_{16} fatty acids under conditions very similar to those occurring during wound-activated cellular response.



1. R = -CH_2CH_3; X = O
1a. R = -CH_2CH_3; X = $C_5H_8O_2$
3. R = -CH=CH_2; X = O
3a. R = -CH=CH_2; X = $C_5H_8O_2$
4. R = -CH_3; X = O
4a. R = -CH_3; X = $C_5H_8O_2$



2. X = O
2a. X = $C_5H_8O_2$

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(1) (a) Miralto, A.; Barone, G.; Romano, G.; Poulet, S. A.; Ianora, A.; Russo, G. L.; Buttino, I.; Laabir, M.; Cabrini, M.; Giacobbe, M. G. *Nature* **1999**, *402*, 173. (b) Pohnert, G. *Angew. Chem., Int. Ed.* **2000**, *39*, 4352.
(2) (a) d'Ippolito, G.; Romano, G.; Iadicicco, O.; Miralto, A.; Ianora, A.; Cimino, G.; Fontana, A. *Tetrahedron Lett.* **2002**, *43*, 6133. (b) d'Ippolito, G.; Iadicicco, O.; Romano, G.; Fontana, A. *Tetrahedron Lett.* **2002**, *43*, 6137.
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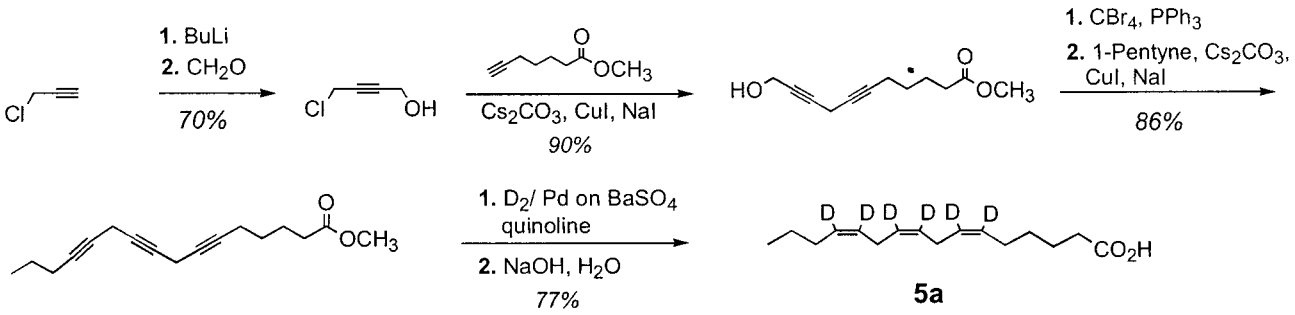
Scheme 1. Synthesis of hexadecatrienoic-d_6 Acid (**5A**)



S. costatum was grown in a 10 L tank for 1 week and then harvested by centrifugation at 1436*g*. In agreement with Gouygou,[4] the diatom contained a large fraction of polyunsaturated C_{16} fatty acids (65% of whole fatty acid content), including 6,9,12-hexadecatrienoic acid (C16:3 ω-4, 26% of C_{16} fatty acid content) and 6,9,12,15-hexadecatetraenoic acid (C16:4 ω-1, 12% of C_{16} fatty acid content).[2a] Docosaesenoic (C22:6 ω-3), eicosapentaenoic (C20:5 ω-3), and myristic (C14:0) acids were the only other species significantly present in the organism, whereas only a trace of C_{18} fatty acids was detectable. For the biosynthetic experiments, frozen samples of the diatom (1.5×10^6cell/g of wet weight) were allowed to warm slowly to room temperature. Then, the cells were suspended in 1 mL/g of distilled water, and [6,7,9,10,12,13-$^2H_6$]-6,9,12-hexadecatrienoic acid (**5a**) was added.[5] The resulting suspensions were sonicated for 60 s and extracted with acetone/H_2O (1:1, three times). After partition in a separatory funnel with CH_2Cl_2, the organic extracts were concentrated at reduced pressure and derivatized with carbetoxyethylidenetriphenylphosphorane (CET-triphenylphosphorane) as previously descibed.[2] Purification on silica gel column led to the mixture of CET-aldehydes **1a**−**4a** that was both analyzed by GC−MS and resolved by HPLC. The hexadeuterated acid **5a** was prepared as reported in Scheme 1, following a general procedure based on coupling of propargyl halide with terminal alkyne in the presence of cesium carbonate.[6] Reduction of the resulting polyalkyne by D_2/Pd on $BaSO_4$ as catalyst gave good yield (77%) of the deuterated C_{16} ester, from which pure [6,7,9,10,12,13-$^2H_6$]-6,9,12-hexadecatrienoic acid (**5a**, D_6 91%) was quantitatively obtained by basic hydrolysis.[7]

GC−MS analysis of the mixture of CET derivatives obtained from cells treated with **5a** showed clear labeling of **1a** and **2a**.[2] In fact, the MS spectra of these compounds (Figure 1) showed significant M + 4 peaks (natural $C_{13}H_{20}O_2^+$, *m/z* 208*;* labeled $C_{13}H_{16}D_4O_2^+$, *m/z* 212) that well agreed with the retention of four deuterium atoms. The presence of these isotopomers was also confirmed by the isotopic clusters associated to fragments generated by diagnostic loss of ethyl (natural $C_{11}H_{15}O_2^+$, *m/z* 179*;* labeled

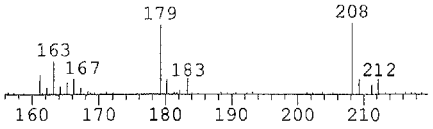


Figure 1. EI-MS spectrum of **2a** from cells of *S. costatum* fed with **5a**.

$C_{11}H_{11}D_4O_2^+$, *m/z* 183) and ethoxyl (natural $C_{11}H_{15}O^+$, *m/z* 163*;* labeled $C_{11}H_{11}D_4O^+$, *m/z* 167) radicals in the mass spectra of **1a** and **2a** (Figure 1).[2] As expected, no trace of labeling was detectable in the other compounds (**3a** and **4a**), supporting the prediction of a substrate-specific process.

The localization of the deuterium atoms, on the other hand, was clearly established by NMR spectroscopy. In fact, the ^2H NMR spectrum of **1a** isolated from treated cells showed three signals attributable to ^2H-2 (δ 6.42), ^2H-4 (δ 6.20), and ^2H-5 (δ 5.91) (Figure 2a). The resonance of the fourth deuterium atom (^2H-1) was only apparently missing, since

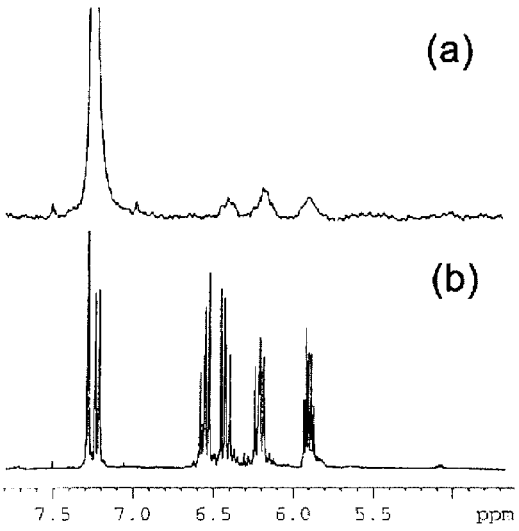


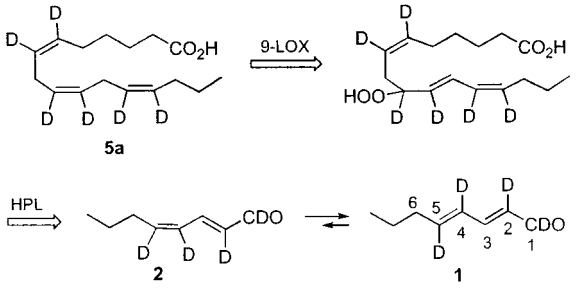
Figure 2. Downfield regions of ^2H NMR (top) and ^1H NMR (bottom) of the CET derivatives of labeled (a) and commercially available (b) *trans,trans*-2,4-octadienal (**1a**).

(4) Berge, J. P.; Gouygou, J. P.; Dubacq, J. P.; Durand, P. *Phytochemistry* **1995**, 39, 1017.

(5) Feeding experiments with deuterated 6,9,12-hexadecatrienoic acid were repeated several times using concentration of the precursor ranging from 8.3 to 15.6 micromol/g of wet cells.

it fell under the large peak of $CDCl_3$ (δ 7.26).[8] The assignment was rigorously proved by comparison with the ^1H NMR spectrum of a commercially available sample of derivatized *t,t*-octadienal (**1a**) (Figure 2b). The labeling pattern is fully consistent with the mechanism summarized in Scheme 2 that

Scheme 2. Biosynthesis of Deuterated Octadienals **1** and **2** in Damaged Cells of *S. costatum*



involves the oxidation of 6,9,12-hexadecatrienoic acid (**5**) by LOX/HPL activity. As previously reported,[1a,2a,3e] no detectable level of octadienal was found in intact cells of *S. costatum*. Analogously, octadienal production was totally inhibited when the acid **5a** was added to a boiled sample of the diatom, confirming that the aldehydes are not formed by a spontaneous, auto-oxidative process.

In conclusion, this study proves that damaged cells of *S. costatum* are able to produce octadienals **1** and **2** from 6,9,-12-hexadecatrienoic acid by lipoxygenase pathway. As outlined in the biogenetic proposal (Scheme 2), *trans,cis*-octadienal (**2**) is the former product of the oxidation of hexadecatrienoic acid (**5**), whereas *trans,trans*-octadienal (**1**) is probably derived by a nonenzymatic process involving the isomerization of the C-4/C-5 double bond of **2**.

In previous papers, it has been shown that another marine diatom, *Thalassiosira rotula*, is able to produce C_{10} aldehydes by oxidation of polyunsaturated eicosanoic fatty acids.[3c] Our experiments and the structural differences of **1**−**4** seem to indicate that *S. costatum*[2a] possesses, at least, two lipid-oxidizing systems differing for the substrate affinity. In fact, in analogy with the formation of aldehydes in *T. rotula*,[3c] heptadienal (**3**, C7:2 ω-3) is probably derived by LOX-dependent peroxidation of eicosapentaenoic acid (C20:5 ω-3), and whereas the production of octadienals (**1** and **2**, C8:2 ω-4) and octatrienal (**3**, C8:3 ω-1) is fulfilled by oxidation of 6,9,12-hexadecatrienoic acid (C16:3 ω-4) or 6,9,12,15-hexadecatetraenoic acid (C16:4 ω-1), respectively.

In agreement with a wound-activated response, it has been reported that the LOX-dependent formation of aldehydes in *T. rotula* is retained in seawater over several minutes.[3c] Analogously, oxidation of **5** and production of C_8 aldehydes continued for a few minutes under the conditions used during our experiments. On these grounds, since only trace levels of free fatty acids are found in intact cells,[9] the mobilization of endogenous **5** from lipid reserves may be the limiting step in the formation of toxic aldehydes at sea. In this regard, it is significant that the aldehyde content nearly resembles the composition of polyunsaturated fatty acids. However, the factors that can elicit the process might be various and further studies are required to understand the molecular mechanism leading to the production of these toxic compounds.

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Supporting Information Available: NMR and MS spectra of the biosynthetic precursor and target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) 6,9,12-Hexadecatriynoic acid methyl ester (0.82 mmol) was added to 10 mL of MeOH containing a catalytic amount of 5% Pd on $BaSO_4$ and quinoline. The reaction was vigorously stirred under D_2 atmosphere for 16 h. After filtration on paper, the clear solution was evaporated. The reaction mixture was purified on silica gel and then hydrolyzed by 10% NaOH in EtOH to give [6,7,9,10,12,13-$^2H_6$]-6,9,12-hexadecatrienoic acid (**5a**, 77%).

(8) The intrinsic limits of the ^2H NMR spectroscopy (e.g., fast relaxation delay) do not allow reduction of the line broadening of the signals. The observed $CDCl_3$ resonances are due to the natural abundance of deuterium in $CHCl_3$.

(9) The level of the free fatty acids in nonstressed cells was established after gentle centrifugation of the diatom culture and subsequent extraction of the fresh pellet with hot methanol.