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# Biosynthesis of Polyunsaturated Short Chain Aldehydes in the Diatom *Thalassiosira rotula*

**Alexandra Barofsky and Georg Pohnert***

*Ecole Polytechnique Fédérale de Lausanne (EPFL), Institut des sciences et ingénierie chimiques, LECH, Batochimie, CH-1015 Lausanne, Switzerland*

*georg.pohnert@epfl.ch*

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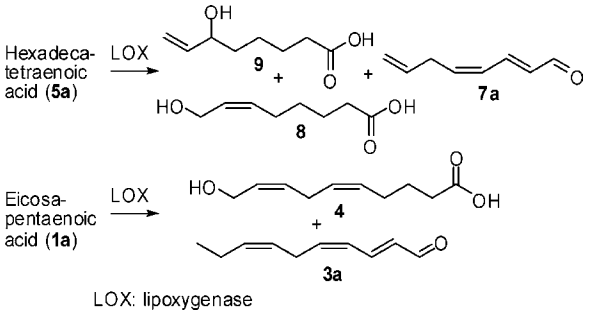
## ABSTRACT




**The diatom *Thalassiosira rotula* releases polyunsaturated short chain aldehydes (PUA) such as 2*E*,4*Z*,7-octatrienal (7a) and 2*E*,4*Z*,7*Z*-decatrienal (3a) upon wounding. Using labeling experiments and synthetic standards, we demonstrate that the mechanism of fatty acid transformation does not follow established lipoxygenase/hydroperoxide lyase pathways known from higher plants or mammals but rather relies on a unique transformation of polyunsaturated hydroperoxy fatty acids. These intermediates are transformed to PUA and short chain hydroxylated fatty acids, which are novel oxylipins.**

Diatoms are unicellular algae at the base of the marine food web. These algae were regarded as a generally good food source for herbivorous copepods. In recent years this view was challenged because feeding on several diatoms, such as *Thalassiosira rotula* (CCMP 1647), results in reproductive failure of the herbivores due to suppression of their egg hatching success.[1] Moreover, malformations of the offspring of these zooplankters have been observed when females were fed on certain diatom-rich diets.[2] Polyunsaturated aldehydes (PUA) such as 2*E*,4*Z*,7-octatrienal (**7a**) and 2*E*,4*Z*,7*Z*-decatrienal (**3a**) were determined to be responsible for causing those embryonic development failures and are thus considered as teratogens.[1−5] A broader survey revealed that ca. 30% of diatom species are capable of producing α,*β*,*γ*,*δ*-unsaturated aldehydes.[3−5]

The biosynthesis of these oxylipins is initiated by lipases releasing polyunsaturated fatty acids from galacto- and phospholipids.[6,7] The precursor of decatrienal (**3a**) is eicosapentaenoic acid (**1a**),[3] whereas hexadeca-6,9,12,15-tetraenoic and hexadeca-6,9,12-trienoic acid (**5a/b**) are the source for octatrienal (**7a**) and octadienal (**7b**), respectively.[8,9] The

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transformations of these free fatty acids are mediated by lipoxygenases that produce the hydroperoxy fatty acids **2a/b** and **6a/b** as last known intermediates toward the PUA.[4,10] Despite progress in the elucidation of the biosynthetic pathways involved, nothing is known about the mechanism of further enzymatic transformations.

The biosynthesis of volatile aldehydes from hydroperoxy fatty acids is widespread in plants, animals, and algae and generally occurs simultaneously with the release of a second fatty acid fragment.[11,12] In the case of PUA from *T. rotula*, the formation of an additional acidic C8-fagment in the biosynthesis of octadienal (**7b**) and octatrienal (**7a**) from C16 fatty acids and of a C10 fragment in the biosynthesis of decatrienal (**3a**) from eicosapentaenoic acid is thus likely. Using an approach based on ultra performance liquid chromatography−mass spectrometry (UPLC−MS), we show that the transformation of the intermediate hydroperoxy fatty acids **2a/b** and **6a/b** results in novel hydroxylated C8 and C10 oxylipins.

To avoid the effort of a large scale culturing and the purification of the metabolites from a reactive matrix, we selected an in situ approach based on the comparison of chromatographic and spectroscopic properties of crude diatom extracts with synthetic reference compounds. Initially, candidate molecules were synthesized on the basis of biosynthetic considerations that were inspired by the formation of other known diatom oxylipins.[4] The biosynthesis of $\omega$-oxo acids, such as 12-oxo-5*Z*,8*Z*,10*E*-dodecatrienoic acid, that harbor the same $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde element as the PUA in question is well understood. This $\omega$-oxo-acid is biosynthesized by hydroperoxide lyases or halolyases from 12-hydroperoxyeicosapentaenoic acid with the concomitant release of unsaturated or chlorinated hydrocarbons as second cleavage fragments.[3,13] In direct analogy to these known mechanisms we reasoned that, for example, 5*Z*,7*E*,9-decatrienoic acid might result of a hydroperoxide lyase mechanism during the formation of decatrienal (**3a**). Alternatively, halolyase mechanisms might result in 8-chloro-6-octenoic acid and 6-chloro-7-octenoic acid as additional fragments during the formation of **7a/b**. These hypothetic products were synthesized and then compared with the metabolites found in diatom extracts. Investigations were performed using concentrated cell suspensions of *T. rotula* that were wounded by sonication. After 10 min (sufficient time for the wound-activated production of PUA[3]), the suspensions were centrifuged and methanol was added. After a second centrifugation, the solution was directly submitted to UPLC−MS analysis (see Supporting Information for experimental details). Comparison of the chromatographic and spectroscopic properties of the synthetic standards with chromatograms from *T. rotula* extracts revealed no natural products with identity to the hypothetic metabolites (data not shown). Hence, the mechanism of fatty acid transformation into octadienal (**7b**), octatrienal (**7a**), and decatrienal (**3a**) does not follow one of the established pathways in diatoms.

Evaluation of the chromatographic and spectroscopic data from UPLC−MS investigations of the *T. rotula* extracts revealed tentative signals of oxylipins (M − H = 157, M − H − $H_2O$ = 139 and M − H = 183, M − H − $H_2O$ = 165), which were in accordance with hydroxylated unsaturated C8 and C10 fatty acids (Figure 1). For structural elucidation we

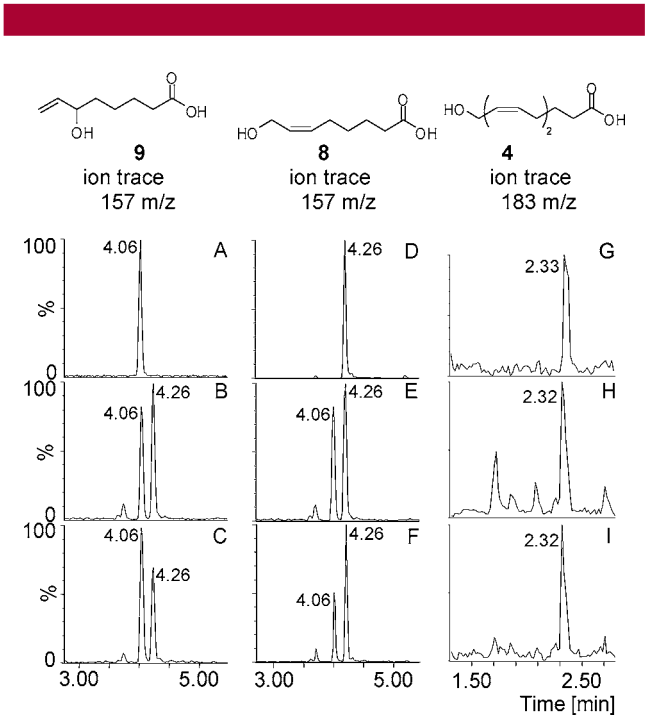


**Figure 1.** Monitoring of the pseudomolecular ion (M − $H^-$) in UPLC separations[15]: (A) synthetic 6-hydroxy-7-octenoic acid (**9**); (D) synthetic 8-hydroxy-6*Z*-octenoic acid (**8**); (G) synthetic 10-hydroxy-5*Z*,8*Z*-decadienoic acid (**4**); (B, E, H) *T. rotula* extract; (C, F, I) co-injection of the *T. rotula* extract of B, E, and H and the respective synthetic standards.

synthesized the candidate oxylipins 6-hydroxy-7-octenoic acid (**9**), 8-hydroxy-6*Z*-octenoic acid (**8**), 8-hydroxy-6*E*-octenoic acid, and 10-hydroxy-5*Z*,8*Z*-decadienoic acid (**4**) according to known procedures.[14]

The UPLC−MS comparison of these standards with the metabolites in *T. rotula* extracts shows identical retention times and high-resolution mass spectra[16] of **9**, **8**, and **4**. 8-Hydroxy-6*E*-octenoic acid was not detected.

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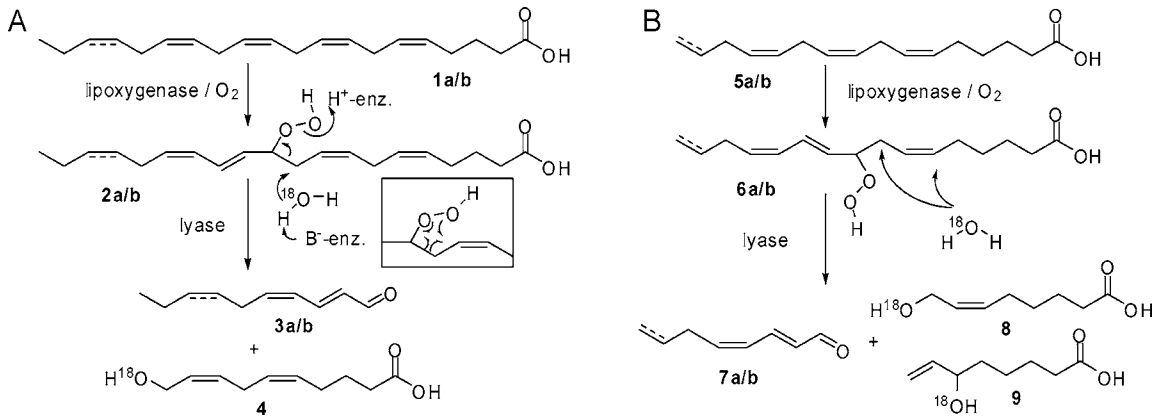
(15) **Separation conditions.** A solvent system of $a$ = 0.1% acetic acid in water and $b$ = acetonitrile was used. A−F: 100 mm Acquity UPLC BEH $C_{18}$ column (2.1 mm, 1.7 $\mu$m), 4 min 0−20% B, 5 min 25% B, 6 min 100% B, 6.5 min 0% B with a flow rate of 0.45 mL/min. G−I: 50 mm Acquity UPLC BEH $C_{18}$ column (2.1 mm, 1.7 $\mu$m), 4 min 0−50% B, 5 min 100% B, 5.5 min 0% B with a flow rate of 0.6 mL/min.

**Scheme 1.** Suggested Biosynthesis of (A) Decatrienal (**3a**), Decadienal (**3b**), and 10-Hydroxy-5*Z*,8*Z*-decadienoic Acid (**4**) and (B) Octatrienal (**7a**), Octadienal (**7b**), 6-Hydroxy-7-octenoic Acid (**9**), and 8-Hydroxy-6*Z*-octenoic Acid (**8**)[a]




[a] The insert in A shows an alternative schematic homolytic mechanism that was ruled out. Molecules with a higher degree of unsaturation are labeled with **a**.

Co-injection of the synthetic standards with the *T. rotula* extract proved unambiguously the identity of the natural products (Figure 1). Interestingly, a mixture of the 6- and 8-hydroxy octenoic acids **9** and **8** was detected, but the 10-hydroxy fatty acid **4** was found as the dominant C10 fragment in the extract (Figure 2).

In *T. rotula* decatrienal (**3a**) is produced from eicosapentaenoic acid (**1a**), and externally added arachidonic acid (**1b**) is metabolized to 2*E*,4*Z*-decadienal (**3b**).[7] This property was used to explore if indeed the production of the C10 aldehydes and 10-hydroxydeca-5,8-dienoic acid (**4**) is biosynthetically linked as depicted in Scheme 1.

Addition of 5,6,8,9,11,12,14,15-[$^2H_8$]-arachidonic acid and subsequent sonication of the cells leads to the formation of [$^2H_4$]-decadienal, which was identified by GC−MS after solid-phase microextraction.[7] Investigation of the same sample using LC−MS showed that labeled [$^2H_4$]-10-hydroxy-5,8-decadienoic acid (**4**) was formed. In addition, a second signal of a labeled hydroxylated C10 fatty acid with high-resolution mass and UV identical to those of [$^2H_4$]-**4** was observed (Figure 2). Comparable incorporation rates for the sum of these C10 hydroxy acids (14%) and for the sum of the C10 aldehydes **3a/b** (15%) were observed. The biosynthesis of the hydroxy acids thus occurs concomitantly with the formation of the PUA (Scheme 1). In full accordance were experiments with 9,10-[$^2H_2$]-hexadeca-6,9,12,15-tetraenoic acid (**5a**) that allowed comparative GC−MS and LC−MS investigations to prove the biogenetic relation of 6-hydroxy-7-octenoic acid (**9**) and 8-hydroxy-6*Z*-octenoic acid (**8**) to the unsaturated C8-aldehydes **7a/b** (Scheme 1, Supporting Information).

The observed product spectrum (Scheme 1) is unusual for the enzymatic break down of hydroperoxy fatty acids,[4,11,17,18] and we thus endeavored to obtain further insight into the cleavage mechanism. As we reasoned that both homolytic and heterolytic bond cleavage could be involved in the transformation of the hydroperoxides, we conducted experiments with $^{18}O$-labeled water to exploit the underlying mechanism. A possible heterolytic route could involve incorporation of water into the hydroxy acid, whereas oxygen from air would be incorporated in the case of a homolytic cleavage (Scheme 1).[18] When the medium was replaced with a 1:1 mixture of [$^{18}O$]- and [$^{16}O$]-water before wounding, approximately 50% incorporation of [$^{18}O$] into the hydroxy acid **4** was observed (Figure 2), whereas no labeled decatrienal (**3a**) could be detected. In direct analogy, both hydroxylated C8 acids **9** and **8** were labeled as well, indicating that the attack of water on C6 and C8 of the intermediate **6a/b** could result in the cleavage products **9** and **8** (Figure 3, Scheme 1).

Judging from the available data it is not possible to conclude if the product mixture of **9** and **8** results from a sloppy enzyme activity or rather from two different enzymes. In this context it is interesting to note that the terminus of the fatty acid can influence the cleavage mechanism. As can be seen from Figure 2D and E, transformation of arachidonic acid results to a higher degree in the formation of a second labeled hydroxylated C10 fatty acid while eicosapentaenoic acid is preferably transformed to **4**. Similar behavior was observed for a hydroperoxide lyase of the diatom *Gomphonema parvulum*, where formation of different hydrocarbons depends on the fatty acid substrate.[19]

The lyase activity of *T. rotula* enzymes utilizes water from the medium, presumably to assist the carbon carbon bond cleavage. It does not follow the same mechanisms as established hydroperoxide lyases, which operate on the basis of homolytic bond cleavage using a P450 system. This would result in hemiacetal formation and subsequent cleavage of the intermediate to two aldehydic down stream products.[11,18]

(16) Compound **9**: *m*/*z* calcd for M − H 157.0865, detected 157.0884. Compound **8**: *m*/*z* calcd for M − H 157.0865, detected 157.0884. Compound **4**: *m*/*z* calcd for M − H 183.1021, detected 183.1031.

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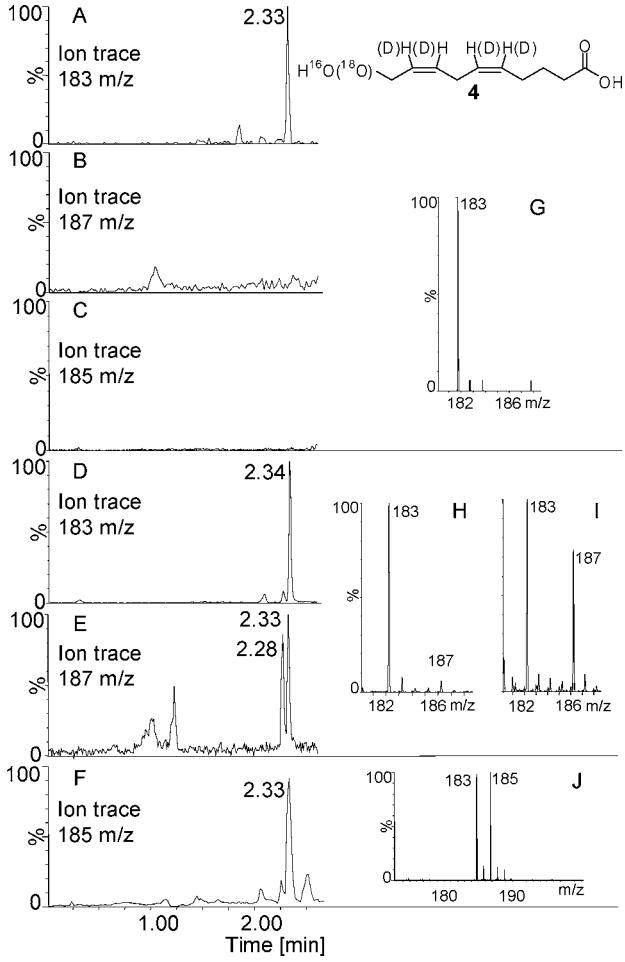


**Figure 2.** UPLC−MS of the *T. rotula* extract monitoring the pseudomolecular ions (M − H$^-$) for (labeled) 10-hydroxy-5*Z*,8*Z*-decadienoic acid (**4**): (A) ion trace 183 *m*/*z*; (B) ion trace 187 *m*/*z*; (C) ion trace 185 *m*/*z*; (D, E) same as A and B after addition of 5,6,8,9,11,12,14,15-[$^2H_8$]-arachidonic acid; (F) same as C after addition [$^{18}$O]-water (ion trace 185); (G) MS of **4**; (H) MS of **4** and [$^2H_4$]-**4** obtained after addition of labeled arachidonic acid **1b**; (I) same as H for the putative isomer of **4** (retention time 2.28 min); (J) MS of **4** and [$^{18}$O]-**4**.

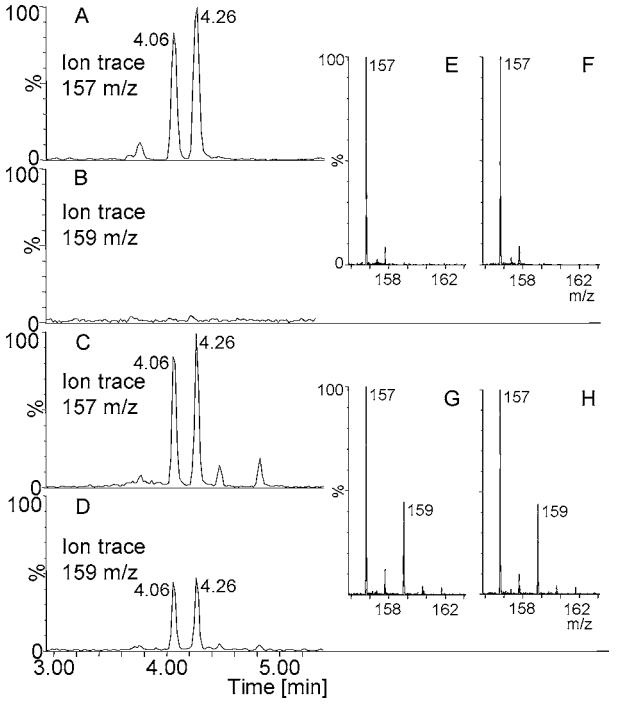


**Figure 3.** UPLC−MS of the *T. rotula* extract monitoring the signals for the (labeled) hydroxylated C8-acids **9** and **8**: (A) ion trace 157 *m*/*z* showing 8-hydroxy-6*Z*-octenoic acid (**8**) and 6-hydroxy-7-octenoic acid (**9**); (B) ion trace 159 *m*/*z*; (C, D) same as A and B generated by *T. rotula* in the presence of a 1:1 mixture of [$^{16}$O]- and [$^{18}$O]-water to *T. rotula*; (E) MS of 6-hydroxy-7-octenoic acid (**9**); (F) MS of 8-hydroxy-6*Z*-octenoic acid (**8**); (G) MS of **9** and [$^{18}$O]-**9**; (H) MS of **8** and [$^{18}$O]-**8**.

To the best of our our knowledge, the only similar transformation mechanism of fatty acid hydroperoxides has been reported from the moss *Physcomitrella patens*.[20] In this organism a bifunctional lipoxygenase catalyzes both the oxygenation of arachidonic acid and the lyase reaction leading to the formation of 1-octen-3-ol and 12-oxo-dodeca-5*Z*,8*Z*,10*E*-trienoic acid.[21] It has yet to be determined if the observed activity in the diatom *T. rotula* is also caused by one single multifunctional lipoxygenase.

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**Supporting Information Available:** Experimental details on diatom culturing, extraction and incubation experiments, and analytical protocols for UPLC−MS, GC−MS, and incorporation of labeled **5a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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