J|A|C|S
COMMUNICATIONS

Published on Web 05/13/2006

# Formation of Halogenated Medium Chain Hydrocarbons by a Lipoxygenase/Hydroperoxide Halolyase-Mediated Transformation in Planktonic Microalgae

Thomas Wichard and Georg Pohnert*

*Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland, and Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany*

Received November 22, 2005; E-mail: georg.pohnert@epfl.ch

Marine organisms are a rich source for halogenated metabolites. These range from simple halomethanes produced in macroalgae to the most complex secondary metabolites found in sponges, ascidia, and other invertebrates.[1,2] Nevertheless, only few pathways for enzymatic halogenation are known.[2] Among the halogenating enzymes, haloperoxidases are the most widely distributed, although $FADH_2$-dependent halogenases and *S*-adenosylmethionine transforming fluorinating enzymes have been identified, as well.[3] Recently, a novel class of non-heme $Fe^{2+}$, α-ketoglutarate, and $O_2$-dependent halogenases has been added to this known enzymatic repertoire.[4] Several halogenated fatty acids and fatty acid derived metabolites have been previously reported. In most cases, their biosynthesis is dependent on the above-mentioned pathways, but few products, for which the biosynthesis might be explained by nucleophilic attack of a halogenide, have been identified, as well.[5] Here we describe a new pathway to halogenated natural products in the marine diatom *Stephanopyxis turris*.

Diatoms are very abundant unicellular algae at the bottom of the marine food chain. Their primary production is responsible for more than 20% of the global carbon fixation. In recent years, certain diatom species came into the focus of chemical ecologists due to the production of α,*β*,*γ*,*δ*-unsaturated aldehydes, such as 2,4-octadienal and 2,4,7-decatrienal. This aldehyde release is suggested to be involved in an indirect chemical defense targeted against herbivores.[6] The volatile unsaturated aldehydes, as well as *ω*-oxo acids, such as (5*Z*,8*Z*,10*E*)-12-oxo-5,8,10-dodecatrienoic acid (12-ODTE **9**), are produced via lipoxygenase-mediated pathways which are initiated upon cell disruption.[6,7] During a screening of diatoms for their ability to generate unsaturated aldehydes, we revealed that more than 30% of the investigated species produce these potential defensive metabolites.[8] Included is *S. turris*, which releases 12-ODTE **9**, a metabolite earlier described from the freshwater diatom *Asterionella formosa*.[7] In this alga, eicosapentaenoic acid **1** is transformed to 12-ODTE **9** and 1,3*E*,5*Z*-octatriene.[7] Contrary to our expectations, *S. turris* did not produce elevated amounts of the octatriene, indicating that another pathway is involved in the formation of **9**. Since the wounded algae exhibited an intense flowery smell, we concluded that other volatiles are most likely formed during fatty acid transformation. For characterization of this bouquet, we performed a headspace solid-phase microextraction (SPME)[7] of stationary cultures (10−40 mL, $1 \times 10^4$ cells $mL^{-1}$), which had been concentrated by filtration prior to wounding by sonication.[8] One dominant volatile metabolite and several minor components were identified (Figure 1). The characteristic isotope patterns of the molecular ions pointed to chlorinated volatiles. EI spectra showed a dominant loss of M-35, and the accurate mass was in agreement with chlorinated unsaturated C8 hydrocarbons (TOF-MS $^{12}C_8{}^1H_{13}{}^{35}Cl$ calculated, 144.0706; measured, 144.0718 (**3**), 144.0706 (**4**)). Characteristic fragments (e.g., base peak *m*/*z* =

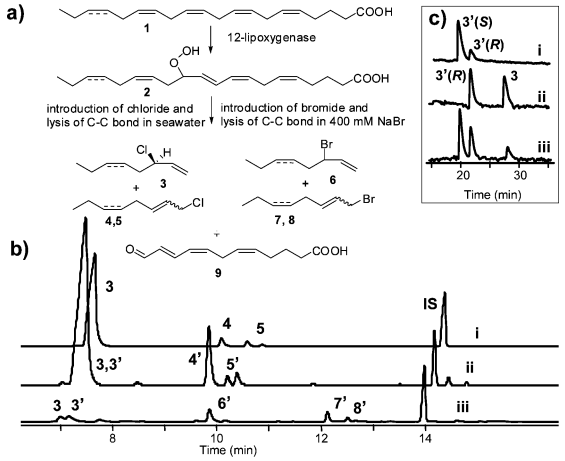


***Figure 1.*** (a) Lipoxygenase/hydroperoxide halolyase-mediated transformation of eicosanoids. The numbers refer to metabolites derived from eicosapentaenoic acid **1** (dashed double bond present); numbers with a ′ refer to *ω*6-arachidonic acid derived metabolites (dashed double bond not present). (b) (i) SPME of *S. turris* wounded in seawater; (ii) as (i) in the presence of *ω*6-[$^2H_8$]-arachidonic acid; and (iii) in the presence of *ω*6-arachidonic acid in 400 mM buffered NaBr, IS = 2-decanone. (c) The inset shows the chiral GC separation of **3** and **3′**: (i) synthetic mixture of predominantly (3*S*)-**3′**; (ii) *S. turris* extract; (iii) co-injection.

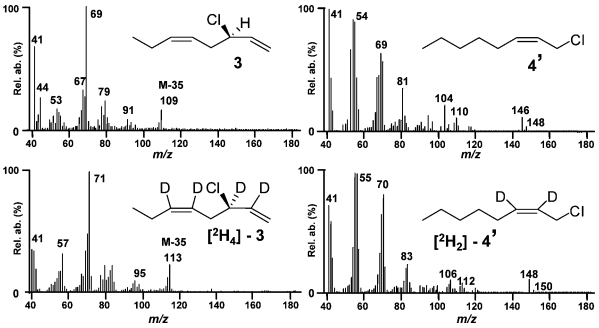


***Figure 2.*** Top: MS (EI) spectra of *S. turris* volatiles. Below: Transformation products of *ω*3-[$^2H_8$]-arachidonic acid (left) and *ω*6-[$^2H_8$]-arachidonic acid (right).

69) suggested that an unsaturated fatty acid derived C5 terminus is still present (Figure 2). Following biosynthetic considerations, we proposed the structures of (5*Z*)-3-chloro-1,5-octadiene **3**, (2*Z*,5*Z*)-1-chloro-2,5-octadiene **4**, and its (2*E*,5*Z*)-isomer **5**. Retention time and mass spectra of synthetic standards matched those of the algal metabolites confirming their identity.

The simultaneous production of C12 and C8 metabolites suggested that *S. turris* transforms C20 fatty acids by a new halogenating lyase activity. For further elucidation of the mechanism, *ω*6-[$^2H_8$]-labeled arachidonic acid **1′** was applied to *S. turris*. The mass shift of Δ6 observed in LC/MS experiments indicated that the

 **10.1021/ja057942u CCC: $33.50** **© 2006 American Chemical Society**

labeled precursor was incorporated (rate ca. 27%) into 12-ODTE **9**. In parallel, we could identify labeled 1- and 3-chlorooctenes **3′**−**5′** (Figures 1 and 2). If $\omega$3-[$^2H_8$]-labeled arachidonic acid was administered, the chlorooctadienes **3**−**5** were formed, but (8*Z*,10*E*)-12-oxo-8,10-dodecadienoic acid was found as the second fragment (Supporting Information). These experiments show that C20 fatty acids with varying degree of unsaturation and varying double bond position are transformed into C12-oxo acids and C8 halocarbons by *S. turris*.

At this stage, it was not clear if the halocarbons are formed directly by an enzyme-controlled reaction or if an abiotic transformation in the NaCl-rich seawater is responsible for the halogenation. Since such a secondary reaction would result in racemization, the stereochemistry of enzymatically produced 3-chlorooct-1-ene **3′** was investigated. Chiral GC/FID analysis of the enzyme product was carried out in comparison with a synthetic standard (Supporting Information). 3-Chlorooct-1-ene **3′** is of >98% ee (3*R*) (Figure 1) and thus directly generated by enzymatic halogenation. Since no racemization occurred and since the product ratio of **3** to **4** and **5** remained constant even after prolonged exposure to seawater, we conclude that the involved enzyme(s) release a mixture of these chlorinated C8 metabolites.

Incubations of *S. turris* in different media revealed that the introduced chlorine originates from seawater. If a cell pellet was resuspended in deionized water before sonication, no formation of halogenated metabolites and 12-ODTE **9** was observed (data not shown). However, if the algae were incubated in 100 mM Tris/HCl pH 7.8 containing 400 mM NaBr, the preferred formation of 1- and 3-bromooctadienols (**6**−**8**) was observed (Figure 1, Supporting Information). The supply of halogen ions from the surrounding water evidently determines product formation. The pseudohalogene $SCN^-$ was not accepted, and NaF inhibited product formation completely (see below).

For the further characterization of the transformation resulting in the cleavage of a hydrocarbon chain and the introduction of a halogen, prototypic reactions might not be adopted. However, it has been observed in the moss *Physcomitrella patens* that arachidonic acid can be activated to 12-hydroperoxyeicosatetraenoic acid (12-HpETE), which is further transformed to 12-ODTE **9** and a mixture of 1- and 3*R*-octenol.[9] Both, the introduction of oxygen and the subsequent cleavage of the intermediate are catalyzed by a multifunctional lipoxygenase.[9] Similarity of the 12-ODTE **9** precursor and the kinetics of oxo acid formation (Supporting Information) prompted the investigation for related fatty acid activation in *S. turris*. On addition of the hydroperoxide reducing agent ebselen[10] (100 $\mu$M final concentration) and 100 $\mu$g of $\omega$6-[$^2H_8$]-arachidonic acid before cell disruption, the intermediate [$^2H_8$]-12-HpETE could be trapped as the corresponding hydroxy fatty acid and characterized in LC/MS experiments (Supporting Information). This experiment confirms a pronounced lipoxygenase activity in the wounded cells. Incubation of *S. turris* with 2 $\mu$g of synthetic 12-HpETE proved that this hydroperoxide is indeed a substrate for the halogenating enzyme. In the absence of this substrate, *S. turris* only releases the chlorooctadienes **3**−**5** from eicosapentaenoic acid since it lacks endogenous arachidonic acid for the production of the chlorooctenes **3′**−**5′** (verified by analysis of the total fatty acids). After addition of 12-HpETE, however, de novo production of **3′**−**5′** from this intermediate was observed. As a control, the cell pellet was heated for 15 min to 120 °C before adding 12-HpETE. This treatment completely inactivated the involved enzymes and prevented the reaction. Hydroperoxy fatty acids are thus direct intermediates en route to the chlorinated products. Addition of 200 mM NaF afforded decoupling of the lipoxygenase and the halogenating activity. NaF exclusively inhibits fatty acid hydroperoxide formation due to the formation of a fluoride−$Fe_3^+$ complex,[11] leaving the transformation of externally supplied 12-HpETE to 12-ODTE and **3′**−**5′** active (Supporting Information). On the basis of our findings, we propose the name hydroperoxide halolyase for the newly characterized enzyme. However, the detailed enzyme mechanism has yet to be fully established.

In summary, we have shown that *S. turris* transforms C20 fatty acids by a lipoxygenase-mediated reaction to intermediate hydroperoxides. These are then employed for the production of new halogenated metabolites by an unprecedented pathway (Figure 1). Our work thus adds a fundamentally new biogenetic transformation to the limited set of known halogenating enzymatic reactions. It is especially interesting in the context of biogeochemical cycling to identify a planktonic alga as a new source for halocarbons. Halogenated metabolites play a significant role for the functioning of atmospheric processes.[12] Until now, mainly methyl halides from microalgae have been considered from plankton.[13] Adding a new and potentially widely distributed marine source of halogenated metabolites might influence the general view of plankton impact on atmospheric processes. Further studies will have to show if the newly identified metabolites contribute to infochemical-mediated processes of microalgae.

**Acknowledgment.** This study was funded by the Deutsche Forschungsgemeinschaft PO 628/3. The Max-Planck-Gesellschaft is acknowledged for financial support.

**Supporting Information Available:** Additional figures showing the decoupling of lipoxygenase and the tentative hydroperoxide halolyase activity, trapping of 12-HpETE and the time course of 12-ODTE formation, as well as experimental details. This material is available free charged via the Internet at http://pubs.acs.org.

## References

(1) Gribble, G. W. *J. Chem. Educ.* **2004**, *81*, 1441−1449.
(2) Murphy, C. D. *J. Appl. Microbiol.* **2003**, *95*, 203−203.
(3) (a) Butler, A.; Carter-Franklin, J. N. *Nat. Prod. Rep.* **2004**, *21*, 180−188. (b) Dong, C. J.; Flecks, S.; Unversucht, S.; Haupt, C.; van Pee, K. H.; Naismith, J. H. *Science* **2005**, *309*, 2216−2219. (c) Dong, C. J.; Huang, F. L.; Deng, H.; Schaffrath, C.; Spencer, J. B.; O'Hagan, D.; Naismith, J. H. *Nature* **2004**, *427*, 561−565.
(4) Blasiak, L. C.; Vaillancourt, F. H.; Walsh, C. T.; Drennan, C. L. *Nature* **2006**, *440*, 368−371.
(5) (a) Dembitsky, V. M.; Srebnik, M. *Prog. Lipid Res.* **2002**, *41*, 315−367. (b) Todd, J. S.; Proteau, P. J.; Gerwick, W. H. *Tetrahedron Lett.* **1993**, *34*, 7689−7692.
(6) Pohnert, G. *ChemBioChem* **2005**, *6*, 946−959.
(7) Pohnert, G. *Angew. Chem., Int. Ed.* **2000**, *39*, 4352−4354.
(8) Wichard, T.; Poulet, S. A.; Halsband-Lenk, C.; Albaina, A.; Harris, R.; Liu, D. Y.; Pohnert, G. *J. Chem. Ecol.* **2005**, *31*, 949−958.
(9) (a) Wichard, T.; Gobel, C.; Feussner, I.; Pohnert, G. *Angew. Chem., Int. Ed.* **2005**, *44*, 158−161. (b) Senger, T.; Wichard, T.; Kunze, S.; Gobel, C.; Lerchl, J.; Pohnert, G.; Feussner, I. *J. Biol. Chem.* **2005**, *280*, 7588−7596.
(10) Noguchi, N.; Yoshida, Y.; Kaneda, H.; Yamamoto, Y.; Niki, E. *Biochem. Pharmacol.* **1992**, *44*, 39−44.
(11) Meier, B.; Scherk, C.; Schmidt, M.; Parak, F. *Biochem. J.* **1998**, *331*, 403−407.
(12) (a) Laturnus, F.; Haselmann, K. F.; Borch, T.; Gron, C. *Biogeochemistry* **2002**, *60*, 121−139. (b) Quack, B.; Wallace, D. W. R. *Global Biogeochem. Cycles* **2003**, *17*, Art. No. 1023.
(13) Scarratt, M. G.; Moore, R. M. *Mar. Chem.* **1998**, *59*, 311−320.

JA057942U