Pure Appl. Chem., Vol. 79, No. 4, pp. 481–490, 2007.
doi:10.1351/pac200779040481

Chemistry of oxylipin pathways in marine diatoms*

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Abstract: Oxylipins are important signal transduction molecules widely distributed in animals and plants where they regulate a variety of events associated with physiological and pathological processes. The family embraces several different metabolites that share a common origin from the oxygenase-catalyzed oxidation of polyunsaturated fatty acids. The biological role of these compounds has been especially studied in mammalians and higher plants, although a varied and very high concentration of these products has also been reported from marine macroalgae. This article gives a summary of our results concerning the oxylipin chemistry of marine diatoms, a major class of planktonic microalgae that discourage predation from their natural grazers, zooplanktonic copepods, using chemical warfare. These apparently harmless microscopic cells produce a plethora of oxylipins, including short-chain unsaturated aldehydes, hydroxyl-, keto-, and epoxyhydroxy fatty acid derivatives, that induce reproductive failure in copepods through abortions, congenital malformations, and reduced larval growth. The biochemical process involved in the production of these compounds shows a simple regulation based on decompartmentation and mixing of preexisting enzymes and requires hydrolysis of chloroplast-derived glycolipids to feed the downstream activities of C_{16} and C_{20} lipoxygenases.

Keywords: chemical ecology; lipid biochemistry; biosynthesis; structure elucidation; isolation.

INTRODUCTION

Eighteen years ago, at the 16th Symposium on the Chemistry of Natural Products in Kyoto [1], our group described the fascinating complexity of the structure of sarain-A, a sponge-derived product that Overman's group has only recently been able to obtain by chemical synthesis [2]. On the contrary, the present contribution is completely devoted to very simple molecules, the diatom oxylipins, which play a relevant ecological role not only for the producing organisms but, more in general, for the stability of the marine ecosystem. By definition, oxylipins are oxygenase-mediated oxygenated derivatives of fatty acids [3] that are encountered in many organisms, including plants [4] and algae [5], where they play a crucial role in eco-physiological processes [6,7].

Pure Appl. Chem.* **79, 467–823 (2007). An issue of reviews and research papers based on lectures presented at the 25th International Symposium on Chemistry of Natural Products (ISCNP-25) and 5th International Conference on Biodiversity (ICOB-5), held jointly in Kyoto, Japan, 23–28 July 2006, on the theme of natural products.
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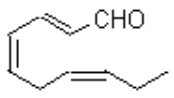
Thirty-five years ago, Isaacs described in "The Nature of Ocean Life" [8] the marine food chain, where the primary producers, the microscopic plants of the phytoplankton, are harvested by the zooplankton, the animal component of the plankton, which is in turn fed upon by top predators including important commercial fish species. However, the predator–prey interactions that occur within this food chain are much more complex than originally realized. Unable to move from their predators, diatoms, a major microalgal class comprising the phytoplankton, protect themselves from their principal predators, the copepods, by producing an arsenal of chemical compounds that compromise future generations of these small crustaceans. This unique form of chemical defense, which does not deter grazing but causes predator populations to crash, was investigated by Ianora et al. when they questioned, for the first time, "…whether diatoms are a good or poor food for copepod reproduction" [9]. In 1999, Miralto et al. published a milestone paper reporting the isolation of three bioactive aldehydes (**1–3**) from the diatom *Thalassiosira rotula* [10]. These molecules, commonly referred to as PUAs (polyunsaturated aldehydes), negatively impacted copepod populations by either drastically reducing fecundity and/or hatching success and larval recruitment. Furthermore, PUAs, despite their very simple chemical structure, were demonstrated to inhibit cleavage of sea urchin, polychaete, and ascidian embryos and replication of human tumor cell lines [11–13]. The paper by Miralto and coworkers opened a very stimulating international debate [14,15] that prompted investigation on several different aspects of diatom–copepod interactions, including the isolation of other metabolites and the elucidation of the biochemical pathways involved in the biosynthesis of PUAs.



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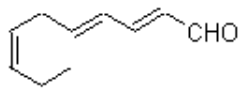

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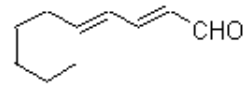

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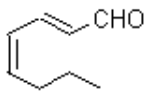

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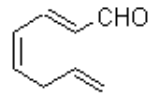

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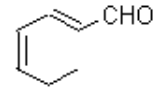

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DISCUSSION

Our research began with the study of the diatom *Skeletonema costatum*, a species responsible for major late winter diatom blooms lasting several weeks to months in the North Adriatic Sea. When ingested, this and other diatom species induce strong abortive effects on egg development in the copepod *Calanus helgolandicus*, leading to 100 % reduction in egg viability after a few days of feeding on this diet, even though fecundity remains unchanged for several weeks (Fig. 1). The embryos that do manage to hatch are morphologically abnormal with body tissues that are marked positively for apoptosis (i.e., cell death), and die soon after hatching.

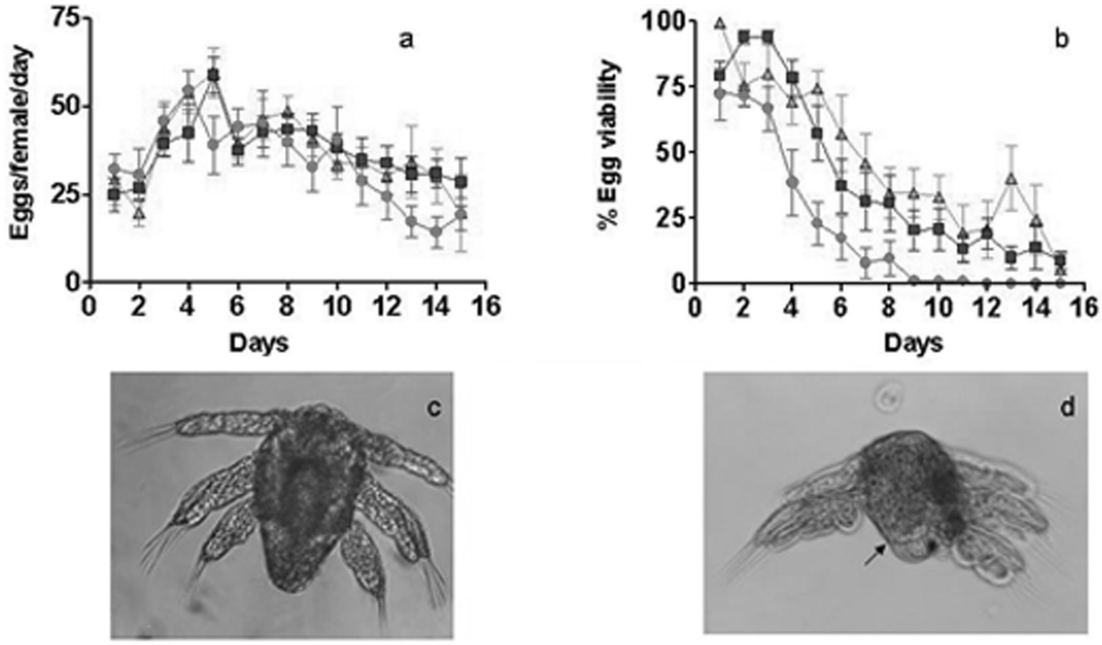

Fig. 1 Effect of diatom diets on the copepod *Calanus helgolandicus*. (a) Egg production and (b) hatching success of the copepod fed 16 days on three diatom diets. Mean values (n = 15) and SD: ■ *Thalassiosira rotula*; ● *Skeletonema costatum*; ▲ *Lauderia borealis*. Microscopy images of (c) normal nauplius (control) and (d) abnormal hatched nauplius after incubation in aldehydes. Arrow indicates missing posterior appendix.

Chemical studies on *S. costatum* led to the discovery of three new aldehydes, namely octa-2,4-dienal (**4**), octa-2,4,7-trienal (**5**), and hepta-2,4-dienal (**6**) [16] and, showed, for the first time, that *S. costatum* cultures and North Adriatic phytoplankton resident bloom populations had almost identical gas chromatography/mass spectrometry (GC/MS) profiles as those of the volatile components (Fig. 2) [17]. Compounds **4–6** were successively described also in the diatom *Thalassiosira rotula*, where they form a toxic pool that includes also the previously reported deca-2,4,7-trienal (**1**) [18].

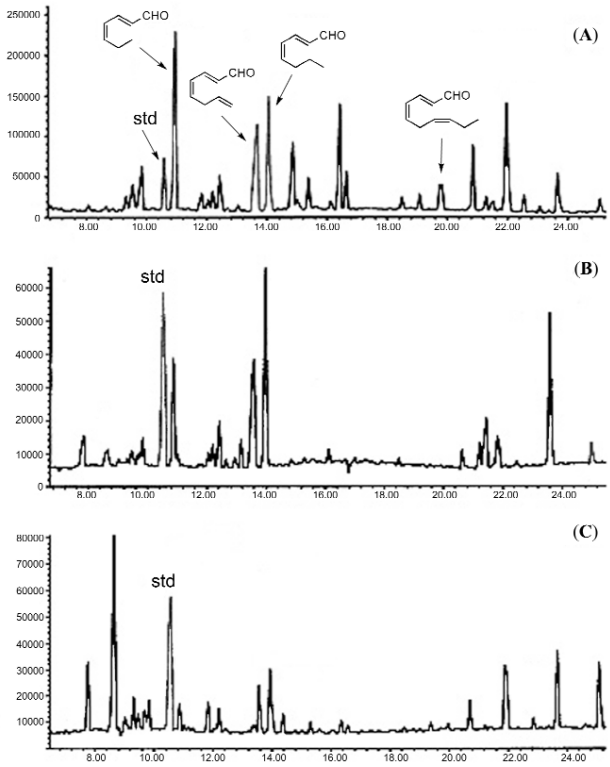

Fig. 2 GC/MS profiles of PUAs from marine diatoms. (**A**) *T. rotula*, (**B**) *S. costatum*, and (**C**) phytoplankton from the North Adriatic Sea. Analyses were carried out after derivatization with CET-TPP (see text). Decenal was used as internal standard. Data presented at the 1st Workshop on Diatom-Copepod Interactions, Ischia (Italy) 2002.

Analysis of diatom PUAs was greatly facilitated by using a new method of derivatization based on carbethoxyethylidene-triphenylphosphorane (CET-TPP) which leads to derivatives that are easily detectable by routine GC/MS analysis and also allows for the isolation of the products for NMR characterization [18]. By these means, no aldehydes were found in extracts of intact diatoms according to Pohnert's arguments that cell damage triggers production of decadienal (**3**) in *T. rotula* [19]. The same author also proposed the origin of **3** from arachidonic acid (AA) and of deca-2,4,7-trienal (**1**) from eicosapentaenoic acid (EPA) [20]. While the role of AA appeared to be questionable because this fatty acid is absent in *T. rotula*, later experiments with radiolabelled probes identified rigorously EPA as the precursor of heptadienal (**6**) in *S. costatum* and decatrienal in *T. rotula* (Fig. 3) [21]. In these experiments, no radioactivity was detected in C_8-aldehydes **4** and **5** recovered from the cultures of both microalgae. Bearing in mind that polyunsaturated C_{16}-fatty acids (C_{16} PUFAs) constitute almost 30 % of the total fatty acids in diatoms, we began investigating whether this pool of lipids played a role in the biosynthesis of aldehydes. In particular, our attention was attracted by 6,9,12-hexadecatrienoic (HTrA) and 6,9,12,15-hexadecatetraenoic (HTA) acids, two major fatty acid components of diatom glycolipids, which showed chemical structures fitting the unusual ω-4 and ω-1 arrangement present in octadienal (**4**) and octatrienal (**5**). A deuterated analog of HTrA with deuteration of the olefinic positions (d_6-C16:3 ω4) was synthesized and added to cell-free preparations of either *S. costatum* [22] and *T. rotula* [23]. After purification, electron impact/mass spectrometry (EI/MS) and ^{2}H NMR analysis not only proved the labelling of octadienal but also allowed location of deuterium atoms at positions 1, 2, 4, 5 of **4**, thus providing the first direct evidence of the involvement of lipoxygenase (LOX) pathways in

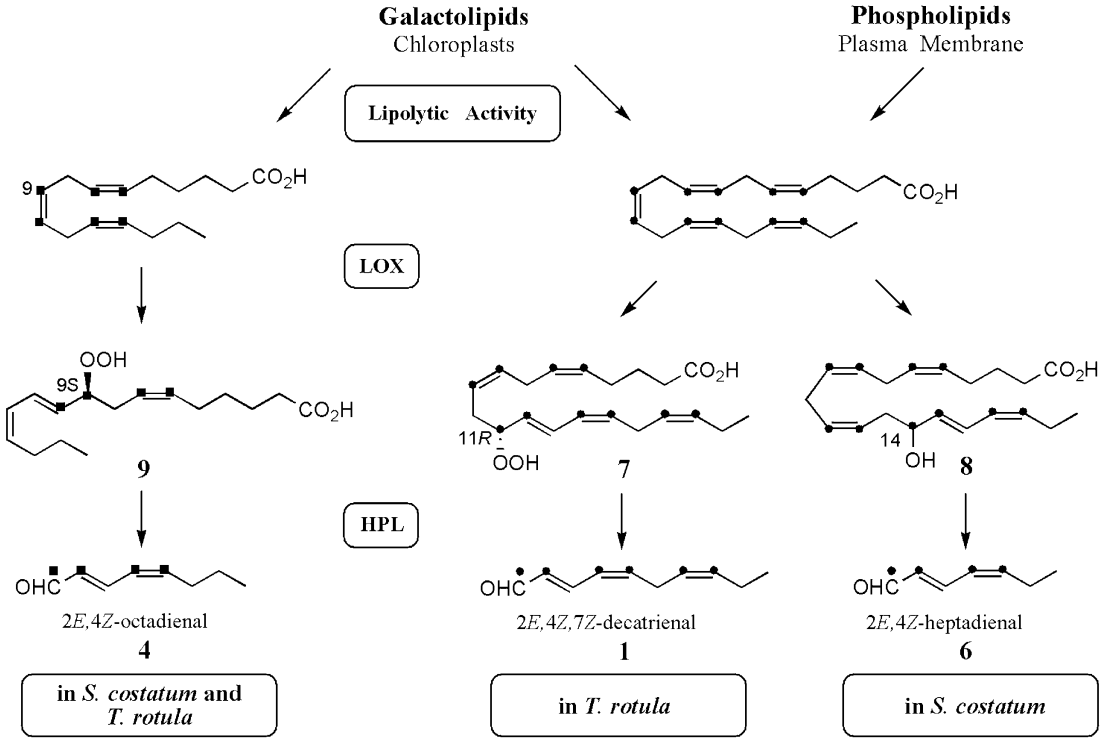

Fig. 3 Biosynthesis of PUAs in *T. rotula* and *S. costatum.* ■ = deuterium; ● = tritium.

PUA synthesis (Fig. 3). Later, an independent study proved that deuterated HTA (C16:4 ω1) was the precursor of octatrienal [24].

As shown in Fig. 3, synthesis of decatrienal (**1**) suggests LOX attack at C-11 of EPA (**7**) in *T. rotula*, whereas heptadienal (**6**) of *S. costatum* should derive by a similar attack at C-14 (**8**). Analogously, synthesis of octadienal (**4**) and octatrienal (**5**) requires LOX peroxidation at C-9 of HTrA and HTA (**9**). The elusive chemistry of the intermediate hydroperoxides does not permit routine analysis of these compounds, although their presence can be proved by indirect evidence. In fact, a transient peak was detected by LC/MS after the addition of d_6-HTrA (C16:3 ω4) to cell-free cultures of *T. rotula* [23]. The short-lived product had a molecular weight ($M+Na^+$ *m/z* 325) in agreement with a hydroperoxide derivative of d_6-HTrA and, after reduction with trimethylphosphite (TMP), was converted to d_6 9-hydroxy-6,10,12-hexadecatrienoic acid. This last product was the deuterated analog of the hydroxyacid **10**, named 9-HHTrE, isolated and characterized as the major component of *Thalassiosira* extract. The *S* absolute stereochemistry (98 % e.e.) at C-9 of 9-HHTrE (**10**) was assigned by chemical correlation with the co-occurring (9*S*)-9-hydroxy-7-hexadecenoic acid [25], the configuration of which was inferred by the procedure recently suggested for allylic alcohols by Williamson et al. [26]. Analysis of lysed cells of *T. rotula* also provided evidence for the presence of hydroperoxide **7**. Chiral HPLC of the methyl ester of the corresponding 11-HEPE (**11**) revealed a slight prevalence of the 11*R* enantiomer (12 % ee).

In order to localize the LOX activities responsible for aldehyde synthesis, *T. rotula* homogenates were fractionated by centrifugation. LOX and HPL activities, tested by recovering labelled d_4-octadienal from d_6-HTrA, were mainly present in the microsomial pellet [23]. Anthon-Barret's bioassay using EPA as a substrate also confirmed the presence of LOX-derived hydroperoxides in this fraction. Further confirmation of the origin of the aldehydic pool from EPA and C_{16}-fatty acids was obtained from experiments with diatom preparations obtained by ultrafiltration of lysed cells on YM10 Amicon.

Suspensions of the resulting retentates in F2 gave rise to octadienal (**4**) and decatrienal (**1**) from HTrA and EPA, respectively [23]. Diatom retentates were also used to address the problem as to the origin of the fatty acids feeding the downstream LOX activities. In fact, an earlier study with *T. rotula* had proposed phospholipids as the primary pool of lipids involved in the biosynthesis of PUAs [19]. However, analysis of the fatty acids present in this diatom did not appear in agreement with the proposal, since HtrA and HTA, the precursors of C_8 aldehydes, were practically present only in chloroplastic glycolipids. To address this apparent contradiction, the different classes of complex lipids were isolated from *T. rotula* and incubated with F-2 suspensions of the microalgal retentates. No detectable products were obtained from the triglycerides, whereas phospholipids yielded decatrienal (**1**) and glycolipids decatrienal (**1**) and octadienal (**4**), in accordance with our hypothesis [27]. In *S. costatum*, incubation of glycolipids and phospholipids labelled with tritiated EPA gave results similar to those recorded with *T. rotula*, even though EPA-derived heptadienal (**6**) seemed to be preferentially synthesized in homogenates incubated with glycolipids [21]. Diatom monogalactosyldiacylglycerol has a prokaryotic structure, with the exclusive presence of C_{16} PUFAs at the position *sn-2* of glycerol and EPA and other polyunsaturated acids localized at *sn*-1 [21,27]. Since fatty acids are hydrolyzed from both glycerol sites, it is very likely that the putative lipolytic enzyme does not possess positional specificity. On the contrary, the mechanism of phospholipid hydrolysis remains unclear. Phospholipase A_2 has been suggested as a key enzyme in the pathway leading to aldehyde production in *T. rotula*, but no molecular evidence has documented the involvement of this class of enzymes to date. On the contrary, the results of incubation experiments discussed above show a substrate specificity that is comparable to the hydrolyzing activity of lipolytic acyl hydrolases (LAHs) described also in leaves and tubers of land plants [28,29]. Although we could not exclude that more than one enzyme might contribute to the hydrolytic process in diatoms, the involvement of LAHs in the synthesis of toxic aldehydes is consistent with recent results on the molecular basis of chemical defense in terrestrial plants [30,31].

A wound-activated mechanism for the production of aldehydes has been proposed to justify the local increase of aldehyde concentrations required to induce reproductive failure in grazing copepods [19]. In fact, in a continuous moving fluid such as marine water, a mechanism based on leakage of aldehydes would be inappropriate since most of the molecules would be washed away before an effective concentration level could be achieved. On the contrary, production of these molecules only after cell lysis would enhance the local concentration, since the toxic factors would be released directly into the body of grazers. In the laboratory, this process can be mimicked by the mechanical rupture of cells by sonication. As reported in Fig. 4, formation of aldehydes begins soon after sonication and steadily increases for several minutes. Interestingly, when aldehydes are removed by keeping the cell suspension for 15 min under vacuum, the process of production is re-initiated, giving rise to higher levels. These results demonstrate that diatom enzymes are active for a substantial period in seawater. The production curve suggests that the process reaches a steady state between consumption of the substrates (fatty acids) and synthesis of the end products (aldehydes). When the level of the end products (aldehydes) is artificially changed, the equilibrium is disturbed until a new balance is reached after removal of the perturbation.

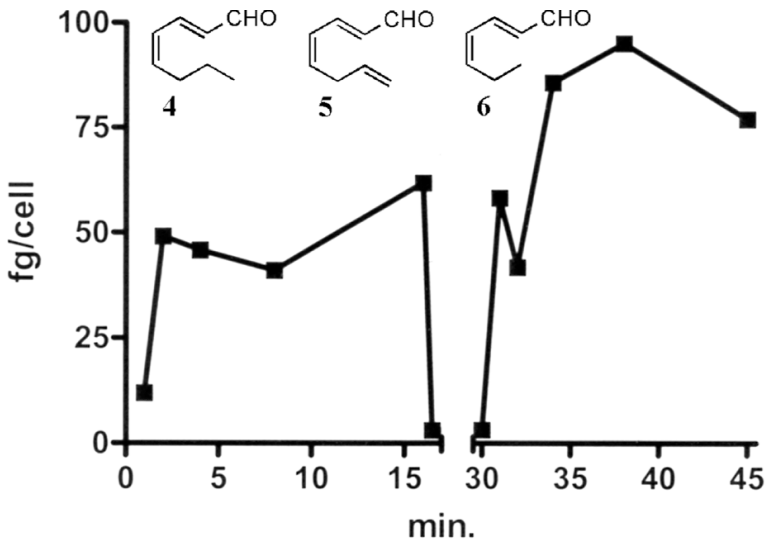

Fig. 4 Kinetics of PUA synthesis by diatom cells. Formation of aldehydes in lysed cells of *T. rotula* was tested by GC/MS after derivatization with CET-TPP. The biosynthesis was followed for 45 min in marine water at room temperature. After 16 min, the homogenates were treated with a vacuum pump for 14 min and then the quantity of aldehydes newly measured at regular intervals of time. *2E*-decenal was used as internal standard. These results are means ± SEM of three different experiments.

The above studies have clarified many details of the chemical processes leading to the production of bioactive aldehydes in diatoms. However, as in the case of hexenal and nonenal in terrestrial plants, synthesis of PUAs in marine diatoms is only a minor part of a broader biochemical scenario that has been recently emerging. In fact, during our work on aldehydes, we noticed that diatoms produce a plethora of other fatty acid derivatives. As shown in Figs. 5 and 6, for the majority of these compounds, structural and biochemical considerations supported a lipoxygenase origin for their production through oxidation of EPA (C20:5 ω-3), HTrA (C16:3 ω-4), and HTA (C16:4 ω-1). In *T. rotula*, together with the previously described 11*R*-HEPE (**11**) and 9*S*-HHTrE (**10**), we found several other oxylipins (**12–16**) derived from oxidation at C-9 and C-6 of C_{16} PUFAs [25]. The absolute stereochemistry of **13** and **15** remains undetermined even though their methyl esters appeared as single peaks on elution from a chiral column. In *S. costatum*, the C_{16}-oxylipin pathway exhibits analogies with that of *T. rotula* leading to compounds **10** and **12** through 9*S*-LOX [32]. Nevertheless, the two diatoms differed for the EPA metabolism that is apparently absent in *T. rotula*, whereas they lead to synthesis of 5*R*-HEPE (**17**, 87 % ee) and 15*S*-HEPE (**18**) in *S. costatum* [32]. Enzymatic control of the process is inferred by the high enantiopurity of the isolated alcohols and directly confirmed by isolation of 15*S*-HpEPE (**26**), the primary product of 15-LOX oxidation of EPA. The methyl ester of this product showed a molecular peak at *m/z* 371 ($M + Na^+$) in ESI^+ MS and the diagnostic ^1H NMR signal at δ 4.42 for H-15, that shifted to higher fields by reduction with TMP. Presence of hydroxy derivatives of EPA was also revealed in two species of *Chaetoceros*, namely, *C. socialis* and *C. affinis*, even though both microalgae were completely lacking in aldehydes [32]. Interestingly, the two diatoms displayed very different effects on copepods. Hatching success in the copepod *C. helogalandicus* reared on *C. socialis* was only moderately reduced, whereas with *C. affinis* hatching declined to zero after seven days [32]. Furthermore, in analogy with the diatoms *S. costatum* and *T. rotula*, copepods fed on *C. affinis* bred nauplii with evident malformations of the swimming appendages, and this is associated with apoptotic progression. No such effects were observed with *C. socialis*. The occurrence of EPA derivatives, such as 14-HEPE (**20**), and the concomitant absence of aldehydes in the two species of toxic *Chaetoceros* implies the involvement

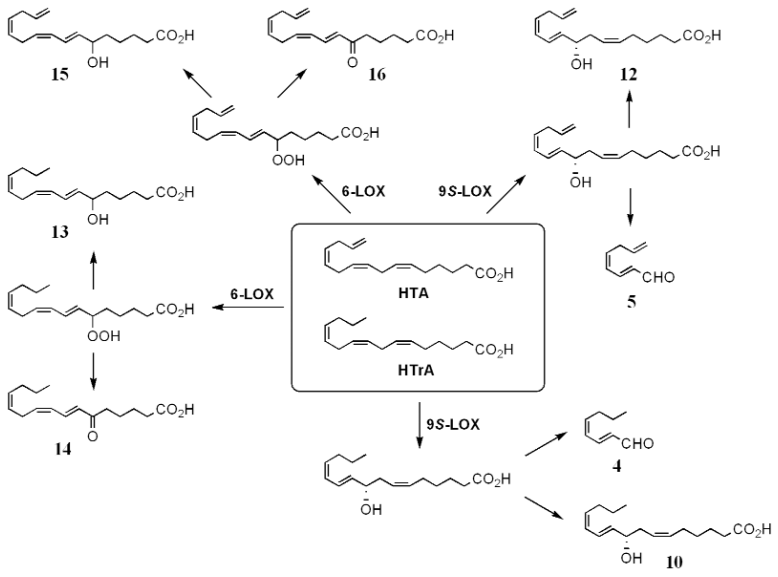

Fig. 5 LOX pathways leading to diatom oxylipins from HTrA and HTA.

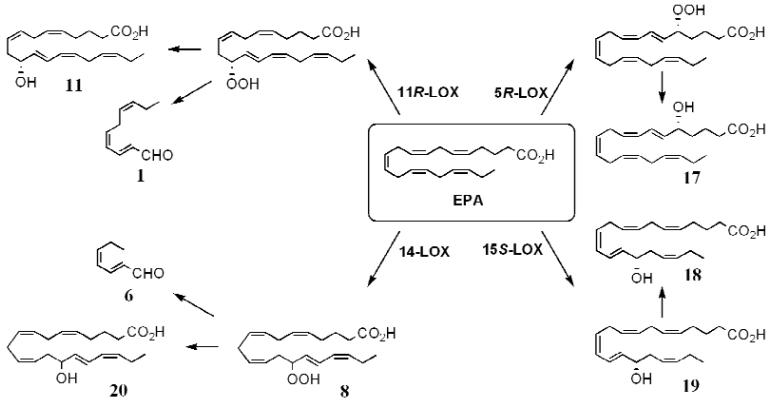

Fig. 6 LOX pathways leading to diatom oxylipins from EPA.

of mechanisms and/or products other than aldehydes in the induction of reproductive failure in copepods. It is significant that the different impact of the two species of *Chaetoceros* on copepod reproduction agrees perfectly with the difference in the LOX activities between the two microalgae in that higher LOX activities were associated with lower hatching success and vice versa.

CONCLUSIONS

Lysis of diatom cells triggers the production of a complex bouquet of compounds, including PUAs, hydroxyacids, and ketoacids, and is critically dependent on species-specific LOX activities. Oxylipin production starts within seconds after cell rupture, a fact that makes regulation through transcription and de novo protein biosynthesis of the required LOXs very unlikely. Following this line of reasoning, it is possible to assume a regulation of the process based on a simple segregation of constitutively expressed enzymes and substrates. After damage or in response to stress conditions, the cellular compartmenta-

tion is lost and enzymes are allowed to mix with substrates, thus triggering the activation of the fatty acid oxidation. The process, which leads to an increase in the local concentration of the relevant compounds, may not necessarily depend on a wound-activated response that, as traditionally defined, elicits gene activation and expression of specific proteins. The release of toxic metabolites by elaboration of constitutive lipids by pre-existing enzymes may be more appropriate to the defense mechanism of unicellular organisms than the wound-activated response characteristic of higher plants. Since chloroplastic galactolipids are the major source for the fatty acids involved in these processes, it is plausible that a galactolipase activity plays a crucial role in the control of biosynthesis. Finally, considering the well-established biological functions of oxylipins in terrestrial and marine organisms, the presence of the complex network of LOX products that our work has started to reveal opens intriguing questions about the role of fatty acid derivatives in the regulation of phytoplankton communities, suggesting an ecological scenario much more complex than hitherto suspected.

ACKNOWLEDGMENTS

The authors thanks the support of the EU Network of Excellence "Marine Biodiversity and Ecosystem Functioning (MARBEF)". This publication is contribution number MPS-07021 of MarBEF.

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