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Phytochemistry 67 (2006) 314–322
PHYTOCHEMISTRY
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Biosynthetic intermediates and stereochemical aspects of aldehyde biosynthesis in the marine diatom *Thalassiosira rotula*

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Received 19 September 2005; received in revised form 9 November 2005

Abstract

Intermediates of the aldehyde biosynthesis in *Thalassiosira rotula* are investigated. Use of labeled precursors and cell preparations proves production of 2*E*,4*Z*-octadienal (**1a**) from 6*Z*,9*Z*,12*Z*-hexadecatrienoic acid (C16:3 ω − 4) through the lipoxygenase-dependent intermediate (9*S*)-9-hydroperoxyhexadeca-6,10,12-trienoic acid. On the contrary, synthesis of 2*E*,4*Z*,7*Z*-decatrienal (**2a**) involves mainly EPA (C20:5 ω − 3) by a 11*R*-lipoxygenase, as suggested by identification of chiral 11*R*-HEPE (12% e.e.) in the diatom extracts. Consistently with the necessity to have a rapid transport and metabolization of the intermediate hydroperoxides, we show that lipoxygenase and lyase activities are both found in the same subcellular fraction of the microalga.
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Keywords: *Thalassiosira rotula*; Aldehyde; Oxygenase; Hydroperoxide lyase; Hydroperoxide; Oxylipins

1. Introduction

Production and ecological role of phytoplankton oxylipins (oxygenase-derived derivatives of fatty acids) have recently attracted the attention of marine scientists in relation to the negative impact that diatom polyunsaturated aldehydes (PUAs) (**1**–**5**) seem to play on the reproduction of the main planktonic herbivores, the crustacean copepods (Miralto et al., 1999; Ianora et al., 2003, 2004). Production of **1**–**5** has been reported in approximately 30 different diatom species, showing metabolite level and composition apparently dependent on species and strain (Wichard et al., 2005). In analogy with plants, biosynthesis of diatom PUAs is suggested to involve lipoxygenase-mediated oxidation of polyunsaturated fatty acids (PUFAs) (Pohnert, 2005) and, in this view, it has been demonstrated that the diatoms *Thalassiosira rotula* and *Skeletonema costatum* transform in vitro labeled C_{16} and C_{20} PUFAs into labeled aldehydes (Pohnert, 2000; d'Ippolito et al., 2003, 2004). However, except for the studies of Shimizu (Shimizu, 1996) and Jüttner (Wendel and Jüttner, 1996; Jüttner and Durst, 1997) on freshwater species, currently no lipoxygenase intermediate has ever been described in marine diatoms, neither further proof of the involvement of lipoxygenase pathway is available in the literature.

Abbreviations: CET, carbethoxyethylidene; CET-TPP, carbethoxyethylidene-triphenylphosphorane; EPA, eicosapentaenoic acid; GC–MS, gas chromatography–mass spectrometry; HTrA, 6,9,12-hexadecatrienoic acid (C16:3 ω − 4); HTA, 6,9,12,15-hexadecatetraenoic acid (C16:4 ω − 1); 9*S*-HHTrE, (9*S*)-9-hydroxy-6*Z*,10*E*,12*Z*- hexadecatrienoic acid; 9-HPHTrE, 9-hydroperoxy-6*Z*,10*E*,12*Z*-hexadecatrienoic acid; 11-HEPE, 11-hydroxyeicosapentaenoic acid; HPL, hydroperoxide lyase; LOX, lipoxygenase; NMR, nuclear magnetic resonance; PUA, polyunsaturated short chain aldehydes.

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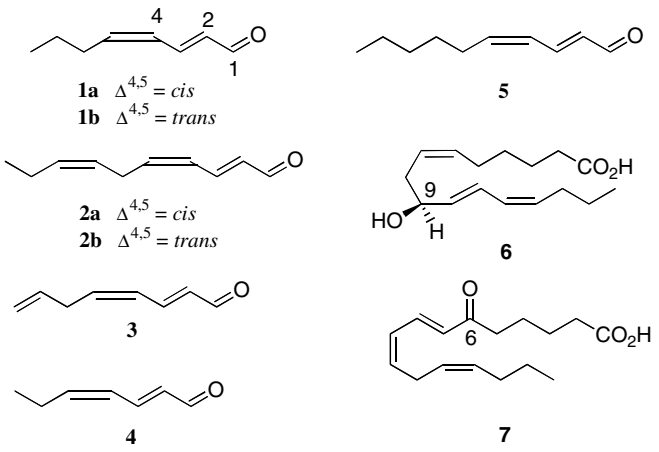
0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.11.012

In the course of our chemical studies on marine phytoplankton (d'Ippolito et al., 2002a; 2002b), we have recently characterized in *T. rotula* a series of new oxylipins, such as (9*S*)-9-hydroxy-6*Z*,10*E*,12*Z*- hexadecatrienoic acid (9*S*-HHTrE, **6**) and 6-keto-7*E*,9*Z*,12*Z*-hexadecatrienoic acid (6-KHTrE, **7**), all derived from C_{16} fatty acids (d'Ippolito et al., 2005). The presence of these compounds, which is very intriguing at physiological and ecological levels, brought our attention back to the enzymatic oxidation of fatty acids in this microalga and, in particular, to the substrate specificity and stereochemical aspects of the biosynthesis of PUAs. Here, we describe the prominent results of these investigations. The study, which provides the first characterization of the intermediates involved in the synthesis of marine diatom aldehydes, is based on enzymatic preparations and feeding experiments with labeled precursors. As part of our examination, we tested the localization of the biosynthetic pathway leading to aldehydes and the lipoxygenase activity in subcellular fractions of the diatom.



2. Results and discussion

As shown in Fig. 1A, *T. rotula* lysates converted stereospecifically [6,7,9,10,12,13-$^2H_6$]-6*Z*,9*Z*,12*Z*-hexadecatrienoic acid (d_6-HTrA) into tetradeuterated 2*E*,4*Z*-octadienal (**1a**) (Scheme 1). Like in *S. costatum* (d'Ippolito et al., 2003), deuterium was found at C1, C2, C4 and C5 of the derived aldehyde **1a** (Supplementary Information) in harmony with a lyase-dependent conversion of the lipoxygenase product 9-hydroperoxy-6*Z*,10*E*,12*Z*-hexadecatrienoic acid (9-HPHTrE) (Scheme 1). Trace of this metabolite [*m*/*z* 319 ($M + Na^+$), 303 ($M - O + Na^+$); UV $\lambda_{max} = 236$ nm] could be detected only in fresh diatom extracts, but its role in the synthesis of octadienal was corroborated by analysis of the products derived from metabolism of d_6-HTrA. In fact, the enzymatic oxidation of this labeled acid by raw diatom preparations resulted in the formation of a deuterated product, [$^2H_6$]-9-hydroperoxy-6*Z*,10*E*,12*Z*-hexadecatrienoic acid (d_6-9-HPHTrE), that eluted similarly to the hydroperoxide present in the fresh diatom extracts and had an absorbance maximum at 236 nm and mass spectrum in agreement with the hexadeuterated derivative [*m*/*z* 325 ($M + Na^+$), 309 ($M - O + Na^+$)] of 9-HPHTrE. Mild reduction of the incubation mixture with trimethylphosphite (TMP) led to complete disappearance of the aforementioned compound and induced a clear increase of the peak at 19.5 min due to the deuterated derivative (*m*/*z* 309, UV $\lambda_{max} = 234$ nm) of [6,7,9,10,12,13-$^2H_6$]-9-hydroxy-6*Z*,10*E*,12*Z*-hexadecatrienoic acid (Fig. 1B and C). Chiral analysis of this product after purification on RP-column showed the formation of a single chiral product (95% e.e.) that was recognized as the methyl ester of the hexadeuterated derivative of 9*S*-HHTrE (**6**) by comparison with the authentic sample of the chiral hydroxy ester. Considering the selectivity of TMP for hydroperoxide reduction and the stereospecificity of the hydroperoxy/alcohol conversion, the data accurately reflected the 9*S* lipoxygenase activity of the cell lysates.

Synthesis of decatrienal (**2**) has never been investigated rigorously although a probable involvement of eicosapentaenoic acid has been put forward in consideration of the conversion of deuterated arachidonic acid into decadienal (**5**) (Pohnert, 2002). This latter aldehyde (**5**) is not produced by our strain of *T. rotula*, therefore the substrate/product relationship in the synthesis of C_{10} aldehydes was tested by feeding experiments with ^{3}H-labeled EPA (about 5.8×10^5 cpm). Incubation of this precursor with raw diatom extracts led to incorporation of radioactivity in 2*E*,4*Z*,7*Z*-decatrienal (**2a**, 1.2×10^5 cpm, 12%) and in the polar fraction containing hydroxy- and hydroperoxy derivatives of EPA. The specific labeling of **2a** was proved by radio-HPLC analysis of the corresponding CET-derivative before and after chemical modifications (Fig. 2), in agreement with the method recently used for *S. costatum* (d'Ippolito et al., 2004). On the contrary, accurate analysis of the polar products revealed the presence of labeling (0.6×10^5 cpm, 6%) in a single peak eluted at the same retention time of 11-HEPE methyl ester (31.0 min). To confirm this identification, we investigated the oxidation of EPA in fresh extracts of *T. rotula* before and after treatment with TMP. LC–MS/MS analyses of these fractions after methylation with diazomethane demonstrated the presence of a single C_{20} monohydroxy methyl ester (data not shown) eluted at 31.0 min. The LC retention time, as well as UV and ESI^+ MS data (λ_{max} at 236 nm and molecular ion at *m*/*z* 355 ($C_{21}H_{32}O_3^{\bullet}Na^+$)) of this peak were indistinguishable from authentic 11-HEPE. The stereochemistry of this hydroxyacid was established by chiral HPLC. Fig. 3A shows the elution of racemic methyl esters of commercially available 11-HEPE. The *R* and *S* enantiomers were resolved in two well-separated peaks at approximately 19 min. 11-HEPE methyl ester isolated from *Thalassiosira* was eluted as an asymmetric doublet (Fig. 3B) at the same retention time of the commercial *R*/*S* 11-HEPE, indicating the presence of a non-racemic mixture containing a slight predominance of the *R* isomer (12% e.e.) (Fig. 3C). This proved the presence in diatom extracts of 56% 11*R*-HEPE, a lipoxygenase metabolite previously

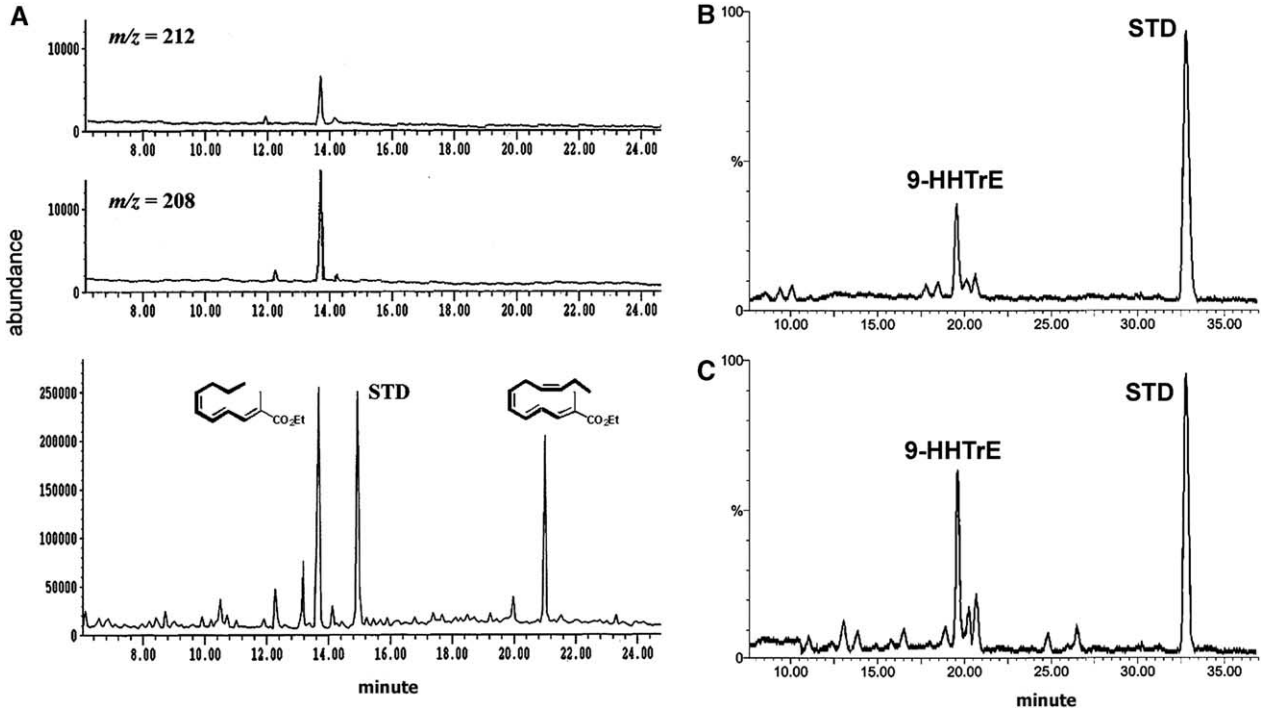


Fig. 1. Biosynthesis of octadienal (**1**) in cell homogenates of *T. rotula*. GC–MS analysis (A) of CET-PUAs from cells incubated with d_6-HTrA (STD = 4-*trans*-decenal as internal standard): Total GC profile (bottom), GC profile after ion extraction at *m/z* 212 for d_4-octadienal (top) and 208 for octadienal (middle); LC–MS/MS detection of deuterated 9*S*-HHTrE (**6**) before (B) and after (C) reduction of extract incubated with d_6-HTrA (STD = 16-hydroxyhexadecanoic acid methyl ester). The depicted profiles were obtained by ion extraction at *m/z* 309 corresponding to the pseudomolecular formulas of methylated d_6-HHtrE ($C_{17}H_{22}D_6O_3 + Na^+$) and 16-hydroxyhexadecanoic acid ($C_{17}H_{34}O_3 + Na^+$).

isolated from mussel ovarians (Coffa and Hill, 2000). Interestingly, the structures of decatrienal (**2a**) and 11*R*-HEPE are both consistent with a biogenetic origin from lipoxygenase-mediated oxidation of EPA at C11 (Scheme 1) and, therefore, their presence strongly supports the intermediate formation of 11*R*-HPEPE as primary intermediate in the diatom lysates (Scheme 1). Like reported above for 9*S*-HPHTrE, the C_{20} hydroperoxide was not directly characterizable in our preparations, although the presence of a TMP-labile product showing MS/MS data consistent with the structure of 11-HPEPE, namely *m/z* 371 $[M + Na]^+$, 355 $[M + Na]^+$–O, 337 $[M + Na]^+$–O, $-H_2O$, could be observed by ESI^+ LC–MS/MS in the diatom extract. In agreement with the enzymatic origin, synthesis of decatrienal and 11*R*-HEPE was completely abolished in homogenates of boiled cells incubated with radioactive EPA.

The apparently low stereocontrol of EPA oxidation was a quite surprising result that arises the question about the substrate specificity of the oxidative pathway(s) of *T. rotula*. To address this point, several free fatty acids, including HTrA (12 μmol), EPA (10 μmol), arachidonic acid (10 μmol), linoleic acid (11 μmol), linolenic acid (11 μmol) and octadecatetraenoic acid (C18:4 ω − 3), were incubated with fatty acid-depleted preparations of the microalga (1.2×10^9). These fractions were prepared as described in Section 4 by ultrafiltration of diatom's supernatant lysates over membranes with 10,000 cutoff (Fig. 4). As expected, HTrA and EPA were converted at largest extent to 2*E*,4*Z*-octadienal (**1a**, 4.7 ± 0.8 μmol) and 2*E*,4*Z*,7Z-decatrienal (**2a**, 3.9 ± 1.1 μmol), respectively. Like in experiments with labeled precursors and consistently with the supposed lipoxygenase catalysis, the 2*E*,4*E*-isomers (**1b** and **2b**) were not detected, whereas we observed clear formation of 9-HHTrE and 11-HEPE (data not shown). Octadecatetraenoic acid (C18:4 ω − 3), that is a minor component of the diatom glycolipids, was also converted to 2*E*,4*Z*,7Z-decatrienal (**2a**, 1.6 ± 0.7 μmol), while 2*E*,4*Z*-decadienal (3.6 ± 0.7 μmol) was obtained from arachidonic acid. However, considering the significant differences in the conversion rate observed with EPA, AA and octadecatetraenoic, eicosanoids seem to be the preferred substrate for the synthesis of C_{10}-aldehydes. In contrast, α-linolenic, linoleic, palmitoleic and oleic acid were apparently not involved in the formation of PUAs, being their conversion very low (0.3 ± 0.2 μmol for α-linolenic) or inexistent (data not shown). Heat denaturation of the retentates abolished the generation of aldehydes.

To further validate the involvement of lipoxygenase activity in the diatom biosynthesis of PUAs, we compared the subcellular localization of the aldehyde route and LOX activity in cells of *T. rotula*. The synthesis of aldehydes was monitored by conversion of d_6-HTrA (0.5 mg) into d_4-octadienal and, in the absence of genetic and biochemical data, LOX activity was measured in agreement with Anthon

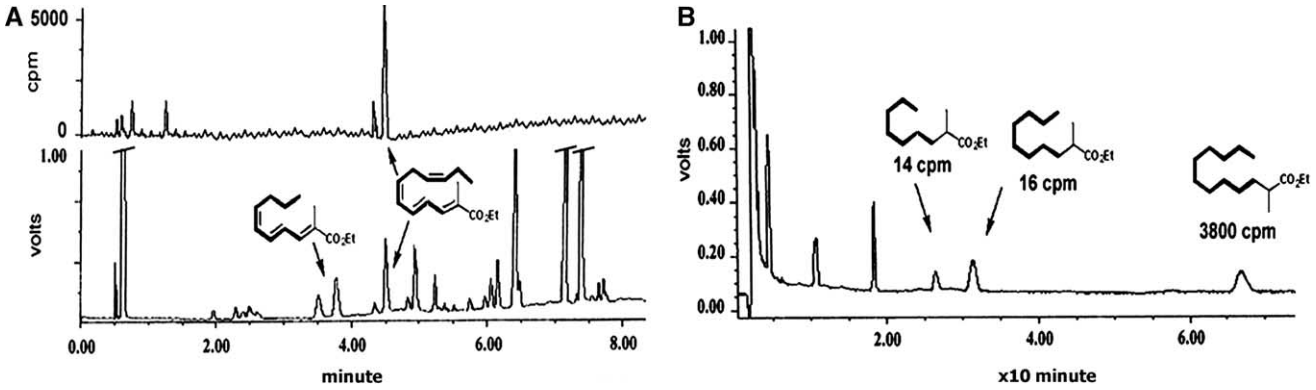


Fig. 2. Biosynthesis of decatrienal in cell homogenates of *T. rotula* (A) HPLC analysis of CET-aldehydes from cells incubated with ^{3}H-EPA: elution followed by UV 210 nm (below) and radio (top) detectors; (B) HPLC analysis (210 nm) of hydrogenated CET-aldehydes from cells incubated with ^{3}H-EPA. Derivative of CET-heptadienal ($T_R = 26.8$ min), CET-octadienal ($T_R = 32.0$ min) and CET-decatrienal ($T_R = 67.2$ min).

and Barrett (2001) using EPA as exogenous substrate. As shown in Fig. 5, major capability of producing both lipid hydroperoxides (expressed as absorbance at 598 nm/μg of total protein) and deuterated aldehyde (192.3 ± 7.2 fg/cell) occurred in 102,000*g* pellet, proving an identical localization of aldehyde synthesis and lipoxygenase activity in *T. rotula*. In terms of acid/aldehyde conversion, subcellular fractions were apparently more efficient than raw homogenates, reflecting probably a rapid depletion of the aldehydes in consequence of the interaction with nucleophiles (for example, amine groups of protein) largely present in raw extracts.

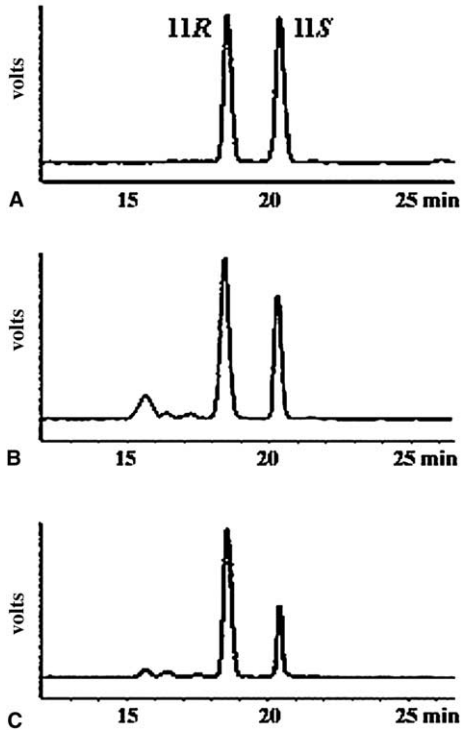


Fig. 3. Chiral analysis of 11-HEPE from *Thalassiosira* homogenates. (A) Resolution of enantiomers of racemic 11-HEPE methyl ester; (B) elution of 11-HEPE methyl ester from extract of *T. rotula*; (C) co-elution of 11-HEPE methyl ester from extract of *T. rotula* plus authentic 11R-HEPE methyl ester.

3. Conclusion

The data presented above demonstrate that the diatom *T. rotula* possesses an enzymatic arsenal capable of transforming different fatty acids to 2*E*,4*Z*-octadienal (**1a**) or 2*E*,4*Z*,7*Z*-decatrienal (**2a**) (Scheme 1). In particular, analysis of the biochemical intermediates supports the presence of a 9*S* lipoxygenase activity that converts HTrA to 2*E*,4*Z*-octadienal (**1a**) via the stereospecific formation of (9*S*)-HPHTrE. This is consistent with the biosynthesis previously described in *S. costatum* and supports the hypothesis that C_{16} PUFAs serve as specific substrates for uncharacterized lipoxygenase(s) in these marine microalgae (d'Ippolito et al., 2003, 2004). On the contrary, synthesis of decatrienal (**2a**) appears to depend mainly on EPA (C20:5 ω − 3). The identification of 56% 11*R*-HEPE (12% e.e.) suggests the involvement of an "impure" 11*R*-lipoxygenase activity through the formation of 56% 11*R*-HPEPE as primary intermediate (Scheme 1). This activity might be due to a definite *R*-lipoxygenase or to a non-specific activity of the enzyme that catalyses the synthesis of 9*S*-HPHTrE from the C_{16}-fatty acid HTrA. Without a complete purification of the enzyme(s) it is however impossible to make any conclusion to this regard, although the non-specific capability of the raw enzymatic preparations to convert EPA (C20:5 ω − 3) and octadecatetraenoic acid (C18:4 ω − 3) to decatrienal pushes us to incline for the second option.

In contrast to other authors (Miralto et al., 1999; Pohnert and Boland, 2002), we did not detect decadienal (**5**) in the extracts[1] of *T. rotula*. However, the significant

[1] The report of trace of decadienal in a previous paper of ours (d'Ippolito et al., 2002a) was due to an misassignment of a very minor isomer of CET-2,4,7-decatrienal.

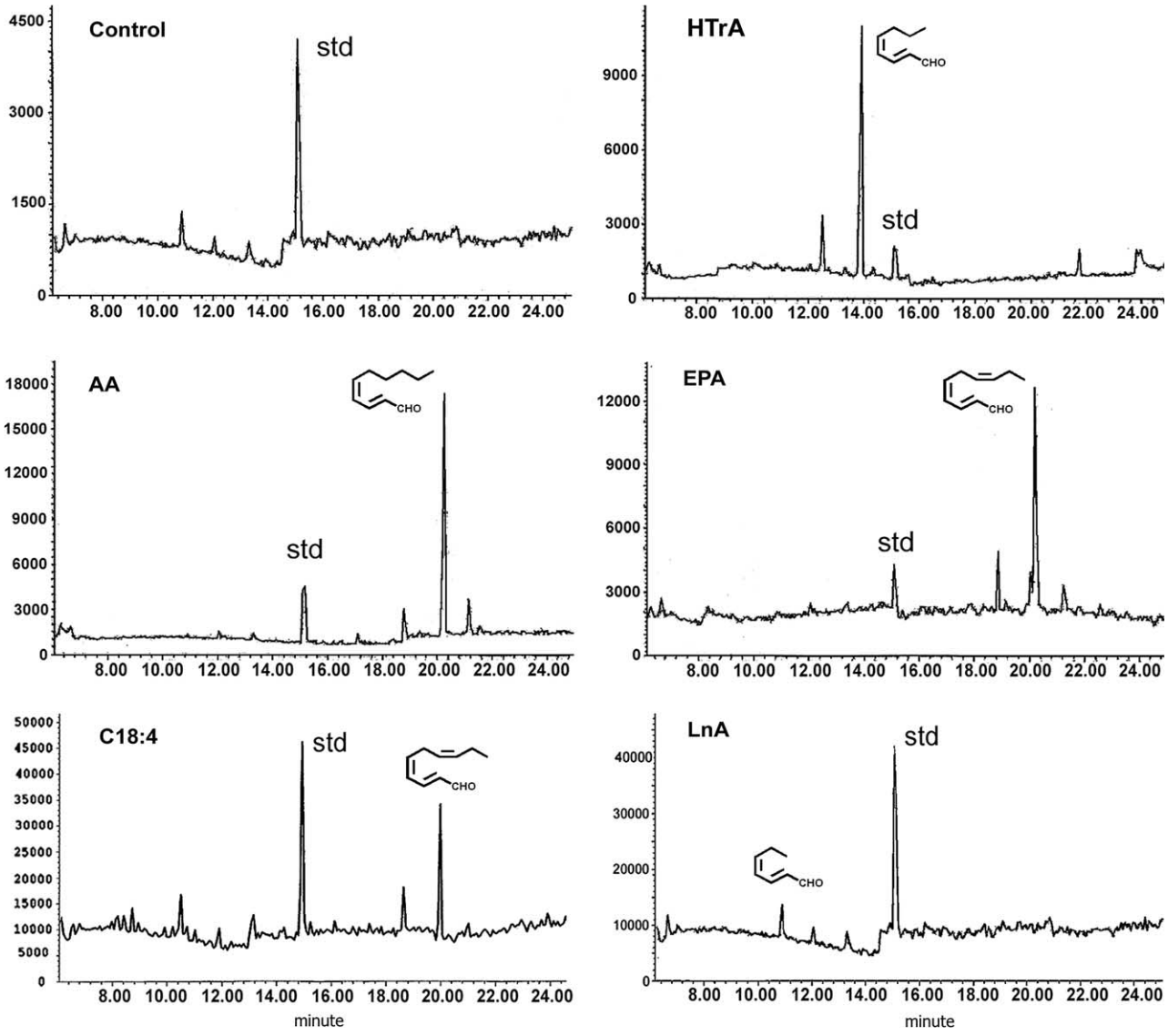


Fig. 4. Conversion of free PUFAs by YM10 Amicon retentates of *T. rotula*. GC profiles of derivatized aldehydes after incubation of: HTrA = 6,9,12-hexadecatrienoic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid; C18:4 = 6,9,12,15-octadecatetraenoic acid; LnA = α-linolenic acid. 4-*trans*-Decenal (30 μg) was used as internal standard. For simplicity, the CET moiety is not showed in the structures.

differences in the conversion of EPA, AA and ω − 3 polyunsaturated C_{18} fatty acids (octadecatetraenoic acid and linolenic acid) (Fig. 4) by diatom preparations confirm that eicosanoids are the preferred substrates for the synthesis of C_{10} aldehydes (**2a** and **5**). In this view, the absence of **5** in the extracts is as a result of the deficiency of arachidonic acid in the diatom (data not shown).

It has been reported that synthesis of aldehydes begins immediately after cell lysis (Pohnert, 2000), thus implying that the proteins responsible for the oxidative metabolism of C_{16} and C_{20} PUFAs are expressed constitutively in the diatom. Following this line of reasoning, the presence of both lipoxygenase and lyase activities in the same subcellular fraction is in agreement with the necessity to have a rapid transport and metabolization of the intermediate hydroperoxides. Furthermore, the intimate association of these activities explains the very low levels of detectable hydroperoxides that, in this model, are quickly and continuously removed by transformation into aldehydes.

4. Experimental

4.1. General

Solvents were purchased from Carlo Erba (Milano) and distilled prior to use. 6*Z*,9*Z*,12*Z*-Hexadecatrienoic acid (HTrA) and [6,7,9,10,12,13-$^2H_6$]-6*Z*,9*Z*,12*Z*-hexadecatrienoic acid (d_6-HTrA) were prepared in agreement with Fontana and co-workers (d'Ippolito et al., 2003). [5,6,8,9,

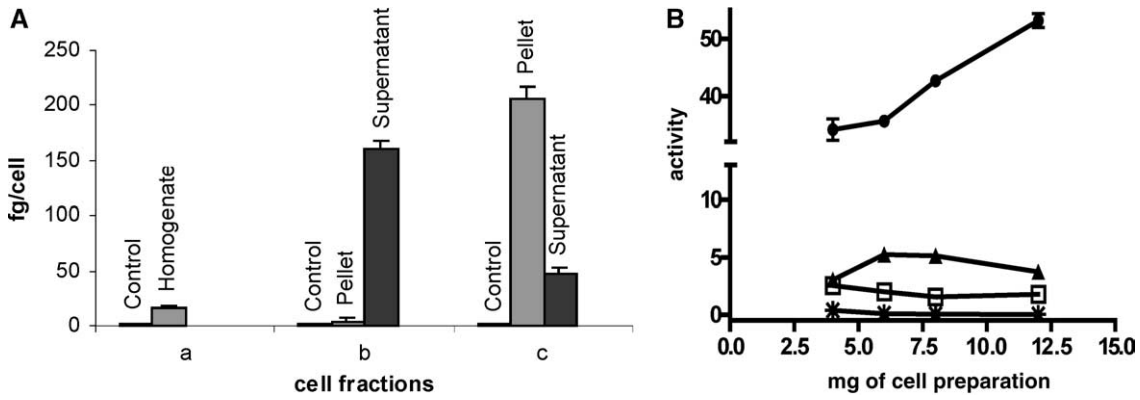
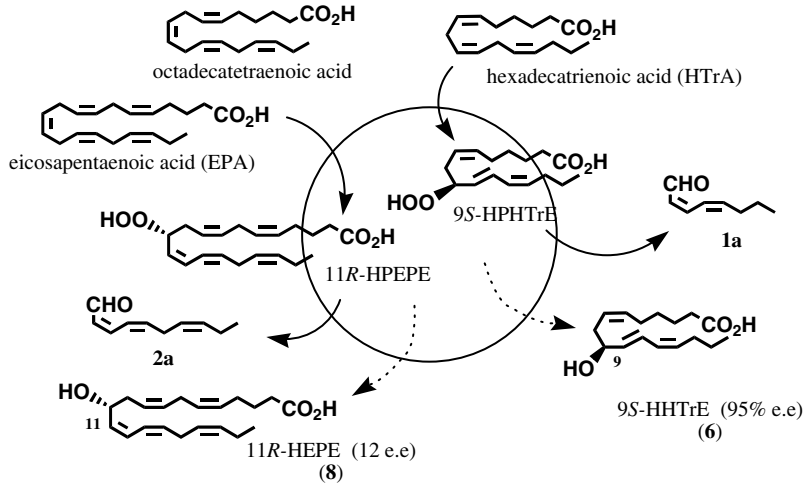


Fig. 5. (A) Production of d_4-octadienal by cell preparations of *T. rotula* derived by (a) crude homogenate + 0.5 mg of d_6-HTrA, (b) 9600*g* centrifugation + 0.5 mg of d_6-HTrA, (d) 102,000*g* centrifugation + 0.5 mg of d_6-HTrA. Control experiments were carried out by adding d_6-HTrA to boiled preparations. (B) Lipoxygenase activity vs number of diatom cells. Assays were performed in triplicate in agreement with the spectrophotometric method of Anthon and Barret. Lipoxygenase activity = Δ_{598}/μg of protein. ● = 102,000*g* pellet; (▲) = 9600*g* supernatant; (□) = 102,000*g* supernatant; (∗) = 9600*g* pellet.



Scheme 1. Proposed lipoxygenase/lyase pathway leading to octadienal (**1**) and decatrienal (**2**) in *T. rotula*. The dashed arrows indicate the chiral alcohols, 9*S*-HHTrE (**6**) and 11*R*-HEPE (**7**), obtained by diversion of the unstable hydroperoxides. The oxygenated derivatives of octadecatetraenoic acid were not characterized.

11,12,14,15,17,18-$^3H_{10}$]-Eicosapentaenoic acid ($^3H_{10}$-EPA) was obtained from ICN Pharmaceuticals. Prior to use, $^3H_{10}$-EPA (10 μCi) was mixed with natural EPA (3 mg) and purified by flash chromatography on silica gel (elution diethyl ether/*n*-hexane 50/50 v/v) to give 1.8 mg of pure EPA (36,100,000 cpm). 9*S*-HHTrE (**6**) was purified from fresh extracts of *T. rotula* in agreement with Fontana and co-workers (d'Ippolito et al., 2005). (±)11-HEPE and 11(*R*)-HEPE were purchased from Cayman Chemicals. Except where noted, all other chemicals were obtained from Sigma–Aldrich. Si-gel chromatography was performed using precoated Merck F254 plates and Merck Kieselgel 60 powder. HPLC purifications were carried out on a Waters chromatograph equipped with Packard Radiomatic Flo-One radiodetector and 490E UV multiwavelength detector. A Packard 1600TR liquid scintillation analyser monitored ^{3}H-incorporation. GC–MS data were obtained by a Hewlett & Packard 5989B mass spectrometer equipped with a 5890 Series II Plus gas chromatograph. Routine NMR analysis of products was carried out on Bruker AMX 500 and Bruker Avance DPX 300 spectrometers. Amicon YM10 were purchased by Millipore S.p.A.

4.2. Cell culturing

Axenic cultures of *T. rotula* were prepared as described in Miralto et al. (1999). Briefly, diatom was grown in Guillard's (F/2) Marine Enrichment Basal Salt Mixture Powder (Sigma–Aldrich) medium, on a 12L:12D cycle, and a light intensity of 175 mmol m^{-2} s^{-1}. Cells were kept in 10 l tanks for 1 week and then harvested by centrifugation at 1200*g* in

swing-out rotor. The procedures for preparing and maintaining axenic cultures are reported elsewhere (Ianora et al., 1996).

4.3. Aldehyde analysis

The diatom lipids were extracted and analyzed by GC–MS as previously described (d'Ippolito et al., 2002a). Initial experiments were carried out by purification of the aldehydes on silica gel and subsequent derivatization with carbetoxyethylidene-triphenylphosphorane (CET-triphenylphosphorane) (d'Ippolito et al., 2002a). Alternatively, the derivatization was carried out on the crude extract and the resulting oil in MeOH (1 μg/μl) was directly analyzed on GC–MS (HP5 column) by a temperature gradient from 130 to 220 °C at 3 °C/min (injector temperature 240 °C, detector temperature 260 °C, nitrogen flow 1 ml/min).

4.4. Incubation experiments with deuterated HTrA

The microalgae harvested in stationary phase were centrifuged at 1200*g* for 10 min at 16 °C and the resulting cell pellet (1.2×10^8 cells) was suspended at 4 °C in 2 ml of F/2 medium prior to the addition of d_6-HTrA (15.6 μmol/g of wet cells). The suspension was sonicated for 1 min and the CH_2Cl_2 extract obtained was divided in three aliquots as previously described. The first aliquot was used for GC–MS analysis after derivatization with CET-TPP. The second aliquot was used as control, and the third one was treated with 20 wt% of TMP in CH_2Cl_2 at 4 °C for 30 min. This reaction mixture was evaporated to dryness, diluted with water and extracted with CH_2Cl_2 for three times. The organic extracts were evaporated and methylated with diazomethane prior to the analysis on RP LC-MS (Kromasil C-18) with MeOH/H_2O 80:20, flow 1.0 ml/min (detection UV at 236 nm). 16-Hydroxyhexadecanoic acid was used as internal standard.

4.5. Incubation experiment with radioactive EPA

In triplicate, 0.6 mg of radioactive EPA (1.2×10^7 cpm/mg) was added to a F/2 suspension of a fresh pellet (3 g wet weight) of diatoms harvested in stationary phase. As described above, the pellet was sonicated for 1 min and let to stand for 30 min prior to the extraction with acetone (1 ml/g of cells). The suspension was centrifuged at 2000*g* for 5 min. The supernatant was transferred and the pellet was washed twice with fresh acetone/H_2O (2 ml/g of cells). The supernatants were combined and a little aliquot (1/100 of the whole suspension) was diluted with 10 ml scintillation cocktail and counted for radioactivity (3.9×10^6 cpm). Analogously, the pellet was suspended in F/2 (12 ml) and counted (1.7×10^6 cpm). The supernatant was partitioned against CH_2Cl_2 to give 15 mg of organic extract (6.5×10^4 cpm/mg) that was directly derivatized by CET-TPP as described above. Silica gel fractionation of the resulting material led to six major fractions containing: #1 apolar compounds (0.3 mg, below blank), #2 pigment (0.5 mg, below blank), #3 CET-aldehydes (1.2 mg, 1.3×10^5 cpm/mg, 14.0% of yield), #4 sterols and fatty acids (1.5 mg, 8253 cpm/mg), #5 fatty acids (7.2 mg, 8.1×10^5 cpm/mg, 58.6% of yield), #6 polar components (1.7 mg, 6.9×10^4 cpm/mg, 12.0% of yield). Each fraction was characterized by ^{1}H NMR in $CDCl_3$. Fraction #3 was dried at reduced pressure and part of this material (5.0×10^4 cpm) was further fractionated on RP-HPLC (gradient CH_3CN/H_2O from 65:35 to 85:15 v/v, flow 1.0 ml/min) equipped with both a Flo-One (Perkin Elmer) radiodetector and UV detector. The remaining part of fraction #3 (about 6.7×10^4 cpm) was hydrogenated with 5% Pd/C as described above. After 6 h, the reaction was stopped and filtered on paper. The resulting solution was dried at reduced pressure, dissolved in 0.5 ml of CH_2Cl_2/MeOH 1:1 and analyzed on RP-HPLC (Kromasil C-18) eluting with CH_3CN/H_2O 78:22, flow 1.0 ml/min, and monitored by UV detector at 210 nm and radiodetector. Fraction #6 was methylated with ethereal diazomethane and further analyzed before and after TMP reduction (see above) on RP-HPLC (isocratic MeOH/H_2O 80:20 v/v, flow 1.0 ml/min) equipped with both Flo-One (Perkin Elmer) radiodetector and UV (210 nm) detector. 11-HEPE methyl ester was used as reference.

4.6. RP-HPLC analysis of hydroperoxy- and hydroxy-derivatives of HTrA and EPA

Lysed cells or preparations of lysed cells incubated with d_6-HTrA (0.5 mg) were obtained as described above. Analysis of this material was carried out by LC–MS/MS after methylation. Briefly, lipid extracts were dried, dissolved in HPLC mobile phase, purified on a Phenomenex C_{18} column, and eluted isocratically with MeOH/H_2O (from 70:30 to 80:20, v/v in 15 min, followed by 40 min of MeOH/H_2O 80:20) at a flow rate of 1.0 ml/min. Internal standard (16-hydroxy-hexadecanoic acid methyl ester) was used to correct for losses during extraction. Between injections the column was washed with methanol to remove any residual nonpolar lipids. Alternatively, extracts were methylated with diazomethane, reduced with TMP and analyzed by RP LC–MS/MS as reported above.

4.7. Chiral analysis

For determining the stereochemistry 9*S*-HHTrE, labeled or natural product was first purified on RP HPLC (Kromasil C-18, MeOH/H_2O 80/20, flow 1 ml/min) and then compared in APCI$^+$ LC–MS with an authentic standard by using Chiralcel OD-H column using *n*-hexane-isopropanol (98:2) (flow: 1 ml/min; UV detection $\lambda = 236$ nm). 11-HEPE was purified from raw extracts of *T. rotula* by RP-HPLC eluting with MeOH/H_2O 80:20 (1 ml/min), $\lambda = 236$ nm. The peak corresponding to the retention time of authentic 11-HEPE was collected and

re-injected on chiral-HPLC together with authentic standard under the conditions described above.

4.8. Localization of enzymatic activity

All the procedures were carried out at 4 °C unless otherwise indicated. Frozen diatom pellets (6×10^8 cells) were sonicated in 30 ml of F/2 medium for 1 min. The homogenate (about 27 ml) was centrifuged for 30 min at 9600*g*. The pellet was suspended in F/2 and centrifuged again. The resulting supernatants were pooled together (about 40 ml of suspension) and centrifuged for 2 h at 102,000*g*. The pellets of 9600*g* and 102,000*g* were suspended in 8 ml of F/2. These suspensions together with crude homogenate and supernatants at 9600*g* and 102,000*g* were used for the LOX assay and protein quantification. The same preparations were incubated for 30 min at 22 °C with 0.5 mg of d_6-HTrA. After addition of the internal reference (30 μg of 4-decenal), the reaction mixtures were extracted with dichloromethane and directly derivatized with CET-TPP. The analysis of the product was carried by GC–MS as described previously. Inactivated enzymatic preparations were obtained by boiling the preparations for 3 min before adding HTrA. Their processing was identical to that described for the active fractions. Protein content was determined using Lowry protein assay, following the protocol of the manufacturer's instructions (Bio-Rad), with bovine serum albumin as standard. LOX activity was determined in agreement with the recent method proposed by Anthon and Barrett (2001). The colorimetric response was optimized by two-step assay using hemoglobin, 32.5 μl of 25 mM EPA with algal suspensions containing different number of cells (1.42×10^6, 2.13×10^6, 2.84×10^6 and 4.26×10^6) in 200 μl F/2. LOX activity was expressed as absorbance at 598 nm (Δ_{598})/μg of protein and normalized with respect to control without EPA. All the experiments were performed in triplicate and data are presented as mean ± SD.

4.9. Substrate specificity

The diatom cells (about 1.2×10^9 cells) were lysed in F/2 medium as described above. Debris was removed by centrifugation at 9600*g* for 30 min at 4 °C, and clarified lysate was ultrafiltrated on Amicon YM10 membranes under N_2 flow. The retentate was extensively washed with F/2 medium, diluted to 10 ml in the same medium and divided in different aliquots. This material was incubated (21 °C for 30 min) with the following fatty acids: HTrA (3.0 mg), EPA (3.0 mg), arachidonic acid (3.0 mg), linoleic acid (3.0 mg) and α-linolenic acid (3.0 mg), [6,9,12,15]-octadecatetraenoic acid (3.0 mg), oleic acid (3.0 mg) and palmitoleic acid (3.0 mg). After extraction with organic solvent (CH_2Cl_2), the analysis of the incubation mixtures were analyzed for the presence of aldehydes and other oxygenated fatty acids by either GC–MS or LC–MS as previously described. d_6-HTrA was used as internal control. 4-Decenal (30 μg) and 16-hydroxyhexadecanoic acid were used as internal standard for aldehyde and hydroxyacids, respectively.

Acknowledgements

We thank Mr. A. Maiello, Mr. M. Di Pinto and Dr. F. Esposito for technical support in diatom culturing and chemical work-up. The project was partially supported by "Programma Nazionale di Ricerche in Antartide" and "Legge Regionale Campania n. 5/2002".

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.11.012.

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