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New C_{16} fatty-acid-based oxylipin pathway in the marine diatom *Thalassiosira rotula*

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An unprecedented series of C_{16} oxylipins (**1–8**) has been characterized from the marine diatom *Thalassiosira rotula*. Absolute stereochemistry of the major alcohols **2** and **3** was determined to be 9*S* by spectroscopic and chemical methods. All the described products are formally derived by unprecedented enzymatic oxidation of C_{16} fatty acids. Conversion of hexadeca-6,9,12-trienoic acid (C16:3 ω-4) into **3** unequivocally established the occurrence of (at least) a specific 9*S*-oxygenase activity. To the best of our knowledge, the present data reveal for the first time the existence of an organic network of oxygenase-mediated transformations that require C_{16} fatty acids as substrates in living cells.

Introduction

Oxylipins have been emerging as important signal transduction molecules widely distributed in animals and plants. To date, the family is known to embrace several different metabolites that share a common origin from the oxygenase-catalyzed oxygenation of C_{18} and C_{20} polyunsaturated fatty acids.[1] Following the recent report on the ecological role of polyunsaturated aldehydes in marine diatoms,[2] we first proved the original capability of the marine microalga *Skeletonema costatum* to transform glycolipid-derived hexadecatrienoic acid (HTrA,[3] C16:3 ω-4) to octadienal through an oxygenase pathway.[4] More recently, we have established that such an ability is common also to another diatom, namely *Thalassiosira rotula*, that produces the major antiproliferative aldehydes octadienal and decatrienal by enzymatic oxidation of chloroplastic HTrA and eicosapentenoic acid (EPA, C20:5 ω-3).[5] In the course of these studies, we noticed that the extract of the microalga also contained several other oxylipins apparently not correlated to the formation of aldehydes. The work described herein is the first account of this research, reporting the identification and biogenesis of the major metabolites, all formally derived by unprecedented enzymatic oxidation of C_{16} fatty acids.

Results and discussion

Axenic cultures of *T. rotula* were obtained and manipulated as described in the Experimental section. Keto- and hydroxy-fatty acid derivatives were purified as methyl esters **1–8** after methylation of the diatom extract with ethereal diazomethane. The structures of the two major derivatives **1** and **2** were easily determined as (7*E*)-9-ketohexadec-7-enoic acid methyl ester and (7*E*)-9-hydroxyhexadec-7-enoic acid methyl ester by ESI$^+$-MS spectrometry and NMR spectroscopy. In particular, in-source collision-induced dissociation[6] spectra (cone voltage = 90 V) of **1** exhibited a diagnostic ion at *m*/*z* 165, which derives from the cleavage of the conjugated double bond between C7 and C8; in addition, TOCSY and HMBC spectra clearly indicated the presence of two separate spin systems from C2 to C8 and from C10 to C16 (Table 1). The *trans* stereochemistry of the double bond was established on the basis of the H7–H8 coupling constant (*J* = 15.8 Hz). Similar results were obtained for **2**, and the structural relationship between the two oxylipins was proved by DIBAL reduction of **1**, to give racemic **2**. The absolute stereochemistry at C9 of this latter metabolite (**2**) was determined in agreement with the method recently proposed by Williamson and co-workers.[7] However, to achieve the determination on the microscale, the methodology was slightly modified by inverting the order of the reactions reported in the literature. Therefore, dichlorodimethylsilane was first added to a solution of the 9-hydroxy methyl ester **2** (1.4 µmol) in dry pyridine (200 µl), and then the resulting silyl derivative was reacted *in situ* with an excess of (*R*)- or (*S*)-α-(trifluoromethyl)benzyl alcohol to give the diastereomeric **2a** and **2b**. After purification on silica gel, both compounds were fully characterized by NMR spectroscopy. Differences in the ^{1}H chemical shifts ($\delta_R - \delta_S$) of the diastereomeric [α-(trifluoromethyl)benzyloxy]dimethylsilyloxy (*R*- and *S*-PhTFE) derivatives **2a** and **2b** (Fig. 1) indicated the *S* configuration of the secondary alcohol, thus characterizing compound **2** as the methyl ester of (9*S*)-9-hydroxyhexadec-7-enoic acid.

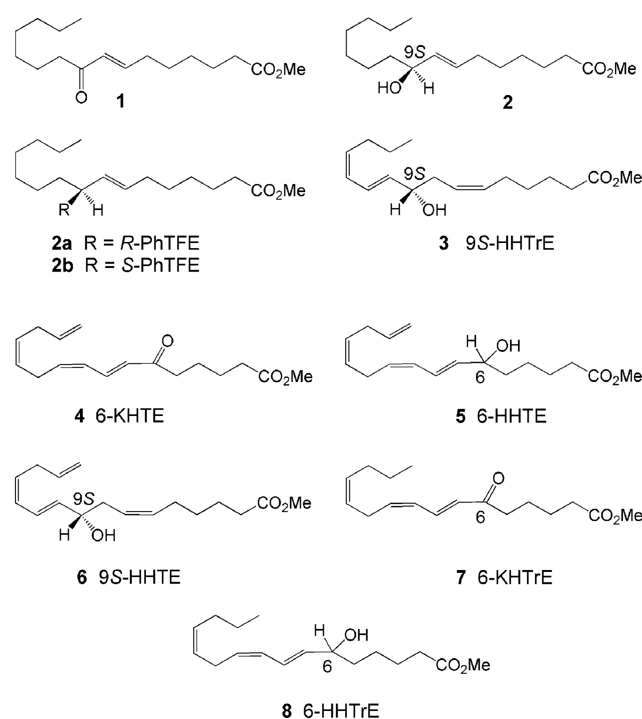

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Table 1 NMR data (C_6D_6, 600 MHz) for oxylipins **1–5** from *T. rotula*. Complete assignments were determined on the basis of 1D and 2D NMR experiments

	1		**2**		**3**		**4**		**5**
	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]	^{1}H[a]
C1	—	173.2	—	173.4	—	173.0	—	172.9	—
C2	2.08, t, 7.4	33.4	2.09, t, 7.4	33.7	2.06, t, 7.4	33.7	2.04, t, 6.9	33.8	2.03, t, 7.2
C3	1.48, q, 7.4	24.4	1.54, q, 7.4	24.9	1.51, q, 7.4	24.5	1.48, m	24.4	1.48, m
C4	1.08, m	28.5	1.16, m	28.8	1.20, q, 7.4	29.4	1.53, m	28.9	1.26, m
C5	1.15, m	27.9	1.25, m	29.5	1.90, q, 7.4	20.9	2.13, t, 7.2	40.6	1.36, m
C6	1.80, q, 7.0	31.8	1.91, q, 7.0	32.2	5.42, m	125.5	—	198.2	3.88, q,
C7	6.64, dt, 15.8 and 7.0	145.5	5.50, dt, 15.3 and 7.0	130.5	5.40, m	132.0	5.95, d, 15.3	129.9	5.52, dd, 14.9 and 6.0
C8	6.0, d, 15.8	130.5	5.43, dd, 15.3 and 6.6	134.2	2.19, m 2.25, m	35.8	7.58, dd, 15.3 and 11.5	135.4	6.53, dd, 14.9 and 10.8
C9		198.6	3.93, m	72.7	4.05, m	71.8	5.88, t, 11.5	127.1	6.0, t, 10.8
C10	2.27, t, 7.4	39.9	1.55, m 1.43, m	37.7	5.60, dd, 15.1 and 5.9	136.0	5.55, dt, 11.5 and 7.5	138.8	5.40, m
C11	1.64, m, 7.4	24.0	1.33, m	29.9	6.65, dd, 15.1 and 10.9	125.2	2.76, t, 7.5	26.0	2.90, t, 6.3
C12	1.22, m	28.8	1.24,[c] m	29.1	6.07, t, 10.9	128.5	5.27, m	127.4	5.40,[c] m
C13	1.20,[c] m	31.6	1.24,[c] m	32.2	5.38, m	132.0	5.38, m	127.9	5.40,[c] m
C14	1.20,[c] m	31.6	1.24,[c] m	32.2	2.09, q, 7.4	29.7	2.63, t, 6.2	31.2	2.73, t, 6.0
C15	1.23, m	22.5	1.26, m	22.5	1.32, m	22.7	5.70, m	136.2	5.72, m
C16	0.86, t, 7.4	13.8	0.88, t, 7.0	14.0	0.83, t, 7.4	13.4	4.97, dd, 10.1 and 1.5 5.02, dd, 17.1 and 1.5	115.0	4.98, dd, 10.1 and 1.5 5.05, dd, 17.1 and 1.5
OCH_3	3.36, s	50.8	3.35, s	50.8	3.34, s	50.8	3.31, s	50.5	3.33, s

[a] ^{1}H NMR data is given in the form: δ/ppm, multiplicity, J/Hz. [b] ^{13}C NMR data is given in δ/ppm. [c] Overlapping signals.

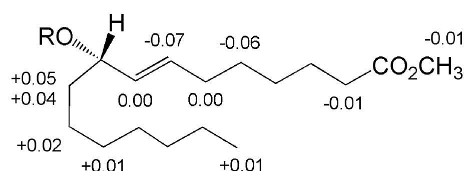


Fig. 1 Absolute stereochemistry of alcohol **2**. $\Delta\delta$ values ($= \delta_R - \delta_S$) are indicated. R = *R*- or *S*-PhTFE.

Compound **3** exhibited UV absorbance (λ_{max} 235 nm) and a ESI$^+$-MS pseudomolecular ion (*m/z* 303.5 for $[C_{17}H_{28}O_3 + Na]^+$) indicative of a hydroxy derivative of the HTrA methyl ester. The ^{1}H NMR spectrum of this material after purification confirmed this suggestion, showing the typical signals of a hydroxylated methine group (H-9, δ 4.05) coupled to a *trans*,*cis*-diene moiety (H-10/H-13, δ 5.60, 6.65, 6.07 and 5.38) and of diastereotopic allylic protons (H_2-8, δ 2.25 and 2.19). The other NMR resonances (Table 1) were in agreement with the structure depicted, proving that compound **3** is the 9-hydroxy derivative of (6*Z*,9*E*,12*Z*)-hexadeca-6,9,12-trienoic acid methyl ester (here named 9-HHTrE). To corroborate the enzymatic origin, we decided to determine the absolute stereochemistry of **3** by application of the strategy described in Scheme 1. Hydrogenation of the 9*S* alcohol **2** with 5% palladium on carbon gave the *S* enantiomer (**9a**), and $NaBH_4$ reduction of the ketone **1** gave the racemic mixture (**9a**/**9b**) of 9-hydroxyhexadecanoic acid methyl ester. Comparison of these products by chiral APCI$^+$ HPLC allowed us to discriminate the enantiomeric peaks (Scheme 1C), thus providing a tool suitable for the characterization of **3**. In fact, Pd-catalysed hydrogenation of **3** led to a single derivative that was recognized as the *R* isomer of 9-hydroxyhexadecanoic methyl ester (**9b**) by elution on the chiral column. This assigned the structure of 95% optically pure (6*Z*,9*S*,10*E*,12*Z*)-9-hydroxyhexadeca-6,10,12-trienoic acid methyl ester to **3**. The origin of **3** was investigated by incubating *Thalassiosira* homogenates with d_6-HTrA prepared as described previously.[4a] After extraction and methylation of the resulting oil, LC-MS analysis of the peak at 19.5 min indicated the presence of 9*S*-HHTrE methyl ester [*m/z* 303.5 (M + Na$^+$)] together with its hexadeuterated analogue [*m/z* 309.5 (M + Na$^+$)] (Fig. 2), thus proving the enzymatic conversion of HTrA to **3**. No product was obtained by incubating the deuterated precursor with boiled preparations of the diatom, thus further supporting the enzymatic origin of **3**.

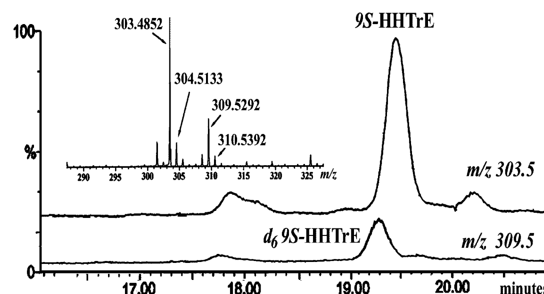
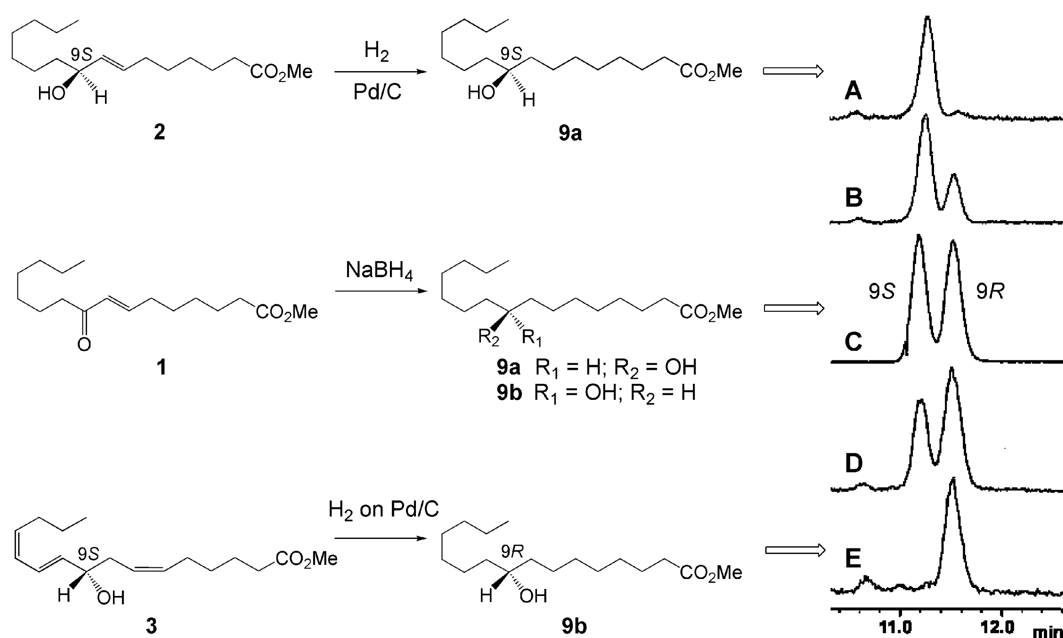


Fig. 2 RP LC-MS analysis of *Thalassiosira* homogenates incubated with d_6-HTrA. ESI$^+$-MS spectrum of the peak eluted at 19.5 min (inset) and chromatographic profiles of methyl esters of 9*S*-HHTrE (**3**) and d_6-9*S*-HHTrE (d_6-**3**). obtained by ion extraction at *m/z* 303.5 ($C_{17}H_{28}O_3 + Na^+$) and 309.5 ($C_{17}H_{22}D_6O_3 + Na^+$), respectively.

Compound **4** showed an ESI$^+$-MS pseudomolecular ion at *m/z* 299 ([M + Na]$^+$) consistent with the formula $C_{17}H_{24}O_3$. The strong UV maximum at 282 nm indicated the presence of extended conjugation suggestive of an α,β,γ,δ-unsaturated ketone. Consistently, NMR experiments (Table 1) indicated the presence of one keto group at δ 198.2 (C6) separating two independent spin systems. One of these embraced most of the deshielded signals, including the diene residue H-7/H-10, one disubstituted double bond H-12/H-13 (δ 5.27 and 5.38) and the terminal methylene H-15/H_2-16 (δ 5.70, 4.97 and 5.02). The second fragment extended between two methylene groups at δ 2.04 (H_2-2) and 2.13 (H_2-5) through two shielded signals attributable to H_2-3 (δ 1.48) and H_2-4 (δ 1.53). The stereochemistry of the double bonds was identified as 7*E*,9*Z*,12*Z* on the basis of the



Scheme 1 Chiral analysis of **3** from *Thalassiosira rotula*. The 9*S* and 9*R* enantiomers of hydroxyhexadecanoic acid methyl esters (**9a** and **9b**, respectively) were prepared by hydrogenation of **2** and **3**, respectively. Racemic hydroxyhexadecanoic acid methyl ester (**9a/9b**) was obtained by $NaBH_4$ reduction of **1**. **(A) 9a**; **(B) 9a/9b + 9a**; **(C) 9a/9b**; **(D) 9a/9b + 9b**; **(E) 9b**.

coupling constants of the vinyl protons (15.3, 11.5 and 7.5 Hz, respectively), thus yielding the overall structure of compound **4** as the methyl ester of (7*E*,9*Z*,12*Z*)-6-ketohexadeca-7,9,12,15-tetraenoic acid (here named 6-KHTE).[8]

The ^{1}H NMR data of compound **5** (Table 1) show a strong resemblance with that of **4**, providing evidence for the presence of a *cis*,*trans* diene system, one isolated double bond and one terminal olefin. Clear differences in the spectra of the two compounds were only evident in the high-field region, which in compound **5** features a distinctive resonance due to an allylic alcohol (H-6, δ 3.88). Considering the molecular formula, $C_{17}H_{26}O_3$ (calculated from the ESI$^+$-MS molecular ion at *m/z* 301 [M + Na$^+$]) and the hypsochromic shift of the UV absorption (λ_{max} at 236 nm), these data established for **5** the structure of the methyl ester of (7*E*,9*Z*,12*Z*)-6-hydroxyhexadeca-7,9,12,15-tetraenoic acid (here named 6-HHTE). Purified from *T. rotula*, **5** eluted as a single enantiomer using chiral HPLC, but the absence of reference compounds and the small amount of the compound did not allow us to determine the absolute stereochemistry.

In addition to compounds **1–5**, lysis of *T. rotula* cells produced a complex group of minor oxylipins (Fig. 3). Although these compounds occurred at levels too low to allow their isolation, a combination of LC-MS/MS and NMR techniques proved to be suitable to recognize at least three major classes of metabolites derived by oxidation of HTrA, hexadecatetraenoic acid (HTA,[3] C16:4 ω-1) and octadecatetraenoic acid. In particular, chemical investigation of the raw methylated extracts of the microalga were consistent with the presence of the methyl esters of (6*Z*,10*E*,12*Z*,15)-9-hydroxyhexadeca-6,10,12,15-tetraenoic acid (**6**, named 9-HHTE), (7*E*,9*Z*,12*Z*)-6-ketohexadeca-7,9,12-trienoic acid (**7**, named 6-KHTrE) and (7*E*,9*Z*,12*Z*)-6-hydroxyhexadeca-7,9,12-trienoic acid (**8**, named 6-HHTrE). Notably, synthesis of these compounds was drastically inhibited when diatom cells were boiled prior to the lysis.

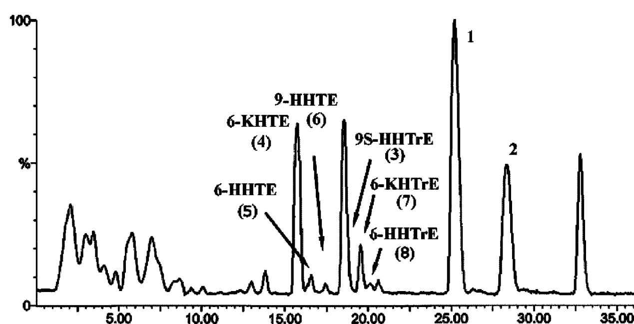


Fig. 3 LC-ESI$^+$-MS profile of oxylipins from methylated extracts of *T. rotula*.

HTrA metabolism was assayed to document oxylipin synthesis in *T. rotula*. Microsomal origin was indicated by production of deuterated **3**, **7** and **8** after incubation of crude homogenates and sub-cellular fractions of the diatom with d_6-HTrA. The levels of **7** and **8** were significantly increased in comparison to the natural samples, thus allowing us to be more confident with the structure assignment. In particular, MS/MS fragmentation of the pentadeuterated derivative of **7** (ESI$^+$ *m/z* 306 [M + Na]$^+$, $C_{17}H_{19}D_5O_3$) indicated a fragmentation α to the keto function, consistent with the LOX-derived structure of **7**. Synthesis of deuterated derivatives of HTrA was not observed in 102 000*g* supernatant, thus confirming that membrane-bound proteins are responsible for the synthesis of the aforementioned compounds. Consistent with the hypothesis that the oxylipin profile reflects LOX arsenal, microsomal fractions of *T. rotula* were incubated with HTrA, and lipoxygenase activity was directly measured by a specific spectrophotometric method described in the literature.[8] Using HTrA as substrate, we found that the diatom microsomes gave a clear coloration dependent on the addition of the fatty acid.

The presence in diatom extracts of other enzymatic activities involved in the lipoxygenase pathway (*e.g.*, hydroperoxide lyases) and organic compounds, make the isolation and characterization of *Thalassiosira* lipoxygenase very difficult. Preliminary data were obtained only by native isoelectric focusing (IEF) after precipitation of the diatom proteins by ammonium sulfate. The lipoxygenase activities were directly localized on the polyacrylamide gels by a specific staining technique[9] using *o*-dianisidine as developer and HTrA (lane B), linoleic acid (lane C) and EPA (lane D) as substrates (Fig. 4). One major band was observable with HTrA, while the gel revealed more activities when stained with EPA. The positive bands presented average pH values of 5.63 suggesting a pI (protein isoelectric point) near this value. A more detailed pI determination, carried out using the same ampholyte pH gradient with a double gel length

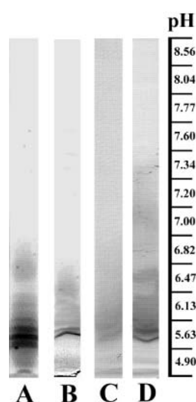
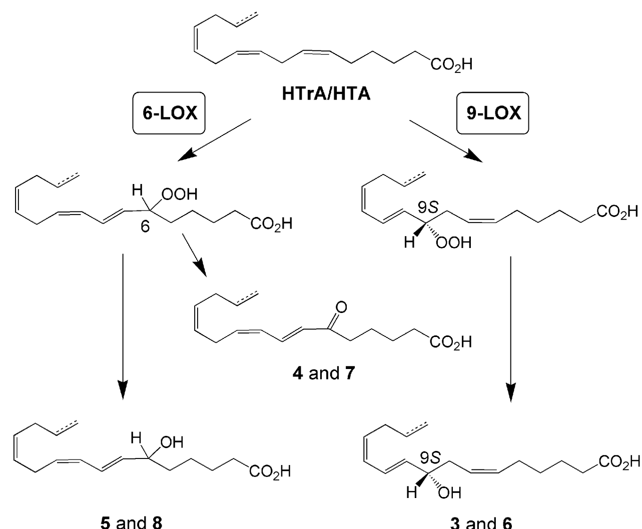


Fig. 4 IEF PAGE of *Thalassiosira* lipoxygenases. Lane A = total protein; lane B = HTrA-dependent LOX activity; lane C = linoleate-dependent LOX activity; lane D = EPA-dependent LOX activity. Gel was stained with Coomassie for total protein and with *o*-dianisidine and fatty acid for LOX activity.

(about 13 cm), gave values between 5.61 and 5.76 (data not shown), in agreement with other known lipoxygenases from rabbit reticulocyte (pI = 5.5),[10] from *Pleurotus ostreatus* (pI = 5.1)[11] and lipoxygenase isoenzymes from germinating barley (pI = 5.2–5.3).[12]

In conclusion, cells of *T. rotula* have an oxidizing activity capable of converting polyunsaturated fatty acids to a variety of unprecedented oxylipins. Although some minor components could be generated non-enzymatically *in vitro*, the high stereospecificity of **2** and **3** (compound **5** also occurs as a single enantiomer but we were not able to determine its absolute stereochemistry) implies that enzymes participate in their synthesis. Preliminary data on the molecular characterization of the lipoxygenase activities suggest the presence of more than one activity (Fig. 4), in agreement with the oxylipin profile showing metabolism of at least C_{16} and C_{20} fatty acids. In fact, besides the synthesis of decatrienal from EPA,[13] the data substantiate unambigously the presence of a 9*S*-specific lipoxygenase that triggers the synthesis of **3** from HTrA. Yet, structural and biochemical considerations also suggest an enzymatic origin of **4–8** through C9 or C6 oxidation of HTrA and HTA (Scheme 2). Unfortunately, the IEF bands (pI = 5.61–5.76) were too close to allow us to discriminate between the different activities. The low protein quantities and the lipophilic nature of *Thalassiosira* LOXs have so far prevented further purification, as well as the determination of the molecular weight in SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).



Scheme 2 Biosynthetic proposal of the origin of oxygenated C_{16} derivatives of *T. rotula*.

We are currently studying the origin and role of oxylipins in *T. rotula*. In particular, in analogy with plants, we are considering if these and other uncharacterized compounds *in vivo* may act as chemical messengers in diatom/diatom communication, or may be parts of a signal transduction system that serves to protect diatom cells from various stresses (oxidant injury, predation, pathogen interaction). In this regard, it interesting to note that **3** is derived from the same hydroperoxide that is involved in the formation of the ecologically relevant aldehydes octadienal and octatrienal.[13]

Experimental

General

Solvents were purchased from Carlo Erba (Milano) and distilled prior to use. Silica-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 an R401 differential refractometer and 490E UV multiwavelength detector. GC-MS data were obtained by a Hewlett Packard 5989B mass spectrometer equipped with a 5890 Series II Plus gas chromatograph. LC-MS analysis was performed on a Qtof-*micro* mass spectrometer (Waters) equipped with an ESI or APCI source and coupled with an HPLC Alliance 2695 system. NMR spectra were registered on a Bruker AMX 400 or a Bruker DRX 600 equipped with inverse TCI Cryoprobe®. [6,7,9,10,12,13-$^2H_6$]hexadeca-6,9,12-trienoic acid (d_6-HTrA) was prepared as previously described.[4a]

Cell culturing

Axenic coltures of *T. rotula* were prepared as described in Miralto *et al.*[2a] Briefly, diatoms were grown in Guillard's (F/2) Marine Enrichment Basal Salt Mixture Powder (Sigma–Aldrich) medium, on a 12 h light/12 h dark cycle, and a light intensity of 20.9 J mol^{-1} µm^{-2} s^{-1}. Cells were kept in 10 L tanks for 1 week and then harvested by centrifugation at 1200*g* in a swing-out rotor. The procedure for preparing and maintaining axenic cultures is identical to that reported in ref. 3.

Extraction, purification and analysis of oxylipins

About 25 g of frozen cells were extracted as previously described.[14] Briefly, the cell pellets were sonicated in distilled water (1 mL g^{-1} cell pellet) for 1 min, and left for 30 min. Acetone (25 mL) was then added to the suspension and the resulting solution was centrifuged at 2500*g* for 5 min. The cell pellet was extracted with acetone–H_2O 1 : 1 (25 mL × 2). The supernatants were pooled together and extracted with the same volume of CH_2Cl_2 (100 mL × 3). The organic layers were combined, dried over Na_2SO_4 and then evaporated under reduced pressure. The crude extract was methylated with ethereal diazomethane and successively fractionated on silica gel column by a polarity gradient system (Et_2O in petroleum ether). Further purification steps were performed by reverse phase HPLC (Phenomenex C-18, 100A, 5 µm, 250 × 4.6 mm) eluting with MeOH–H_2O 80 : 20 (1 mL min^{-1}) and monitoring the elution at 210 nm. Every purified fraction was analyzed by LC-ESI$^+$-MS under the same conditions. About one-tenth of the column flow was sent to the ESI source. Alternatively, the crude extracts were dissolved in MeOH at a final concentration of 1 mg mL^{-1} and directly analyzed by LC-ESI$^+$-MS/MS as described above.

Stereochemistry of (7*E*)-9-hydroxyhexadec-7-enoic acid (2)

Dichlorodimethylsilane (54 µmol) was added by a syringe to a solution of the alcohol **2** (0.4 mg, 1.4 µmol) in dry pyridine (200 µl) under argon. The reaction was kept at 75 °C for 45 min and then split in two equal parts. These aliquots were then reacted with (*R*)- and (*S*)-α-(trifluoromethyl)benzyl alcohol (108 µmol) dissolved in 200 µl of dry pyridine. After 30 min. at

75 °C, the reactions were quenched by the addition of 400 μl of MeOH. The resulting solutions were dried under reduced pressure and purified on silica gel by eluting with petroleum ether–diethyl ether 95 : 5 to give **2a** and **2b**.

Stereochemistry of (6*Z*,9*E*,12*Z*)-9-hydroxyhexadeca-6,9,12-trienoic acid methyl ester (3)

Racemic 9-hydroxyhexadecanoate methyl ester (**9a/9b**) and (9*S*)-9-hydroxyhexadecanoate methyl ester (**9a**) were prepared by $NaBH_4$ reduction of **1** and hydrogenation of **2**, respectively. 9-Ketohexadec-7-enoic acid methyl ester (**1**, 0.8 mg) in MeOH was stirred overnight with excess $NaBH_4$. The reaction mixture was acidified with 5% H_2SO_4 and extracted with diethyl ether three times. The ethereal fractions were combined and evaporated at reduced pressure to give 0.4 mg of racemic 9-hydroxyhexadecanoic acid methyl ester (**9a/9b**). (7*E*,9*S*)-9-Hydroxyhexadec-7-enoic acid methyl ester (**2**, 0.3 mg) and **3** (0.2 mg) were purified from diatom extracts as described above and hydrogenated on 5% palladium on (activated) carbon in MeOH (0.5 mL). The reactions were stirred under H_2 for 6 h and then paper-filtered to give the saturated derivatives **9a** and **9b**, respectively. For the chiral analysis, the above products were first purified on RP HPLC (Kromasil C-18, MeOH–H_2O 80 : 20, flow 1 mL min^{-1}) and then injected onto a Chiralcel OD–H column (Baker) using *n*-hexane–isopropanol (98 : 2) (flow 1 mL min^{-1}) monitoring the elution by APCI-MS.

Chiral analysis of (7*E*,9*Z*,12*Z*)-6-hydroxyhexadeca-7,9,12,15-tetraenoic acid methyl ester (5)

Compound **5** was purified from raw extracts of *T. rotula* by RP HPLC, eluting with MeOH–H_2O 80 : 20 (1 mL min^{-1}), λ = 236 nm. The peak was collected and re-injected onto a chiral-HPLC column (Chiralcel OD-H), using hexane–isopropanol (98 : 2) at a flow rate of 1 mL min^{-1} (detection $\lambda = 236$ nm).

Conversion of d_6-HTrA into d_6-HHTrE (d_6-3)

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 [6,7,9,10,12,13-$^2H_6$]hexadeca-6,9,12-trienoic acid (d_6-HTrA) (15.6 μmol g^{-1} of wet cells). The suspension was sonicated for 1 min, diluted with MeOH and centrifuged at 2500*g* for 5 min. The supernatant was recovered, diluted with water and extracted with CH_2Cl_2 three times. The organic extracts were evaporated at reduced pressure and methylated with ethereal diazomethane. After removing the organic solvent, the oily residue (5.3 mg) was dissolved in MeOH (1 mg mL^{-1}) and analyzed by RP LC-MS as described above. For experiments with inactivated enzymatic preparations, diatom cultures were centrifuged as described above and fresh pellets were kept in a boiling water bath for 10 min.

Oxylipin synthesis and LOX activity in subcellular fractions

To test the subcellular localization of the enzymatic activities related to oxylipin synthesis, cytosolic and microsomal fractions of *T. rotula* cells (1×10^9 cells) were prepared after sonication and successive centrifugation at 9600 and 102 000*g* in an F/2 medium. Pellets and supernatants were incubated with d_6-HTrA (0.5 mg) at room temperature for 30 min. After extraction and methylation, production of compounds **3**, **7** and **8** was monitored by LC-MS/MS on a RP column (using MeOH–H_2O 70 : 30 → 80 : 20 over 15 min, followed by MeOH–H_2O 80 : 20 for 40 min). An internal standard (16-hydroxyhexadecanoic acid methyl ester) was used to correct for losses during extraction. The process was totally inhibited by boiling the preparations for 10 min.

Using HTrA as an exogenous substrate, LOX analysis in diatom microsomes was carried out in triplicate with increasing concentrations of algal cells (1.42×10^6, 2.13×10^6, 2.84×10^6 and 4.26×10^6) in accordance with ref. 8. Activity was expressed as absorbance at 598 nm per μg of total protein. Protein content was determined using the Lowry protein assay, following the instructions of the manufacturer (Bio-Rad), with bovine serum albumin as standard.

Enzymatic sample preparation

About 5 g of frozen *T. rotula* cells were defrosted at room temperature and re-suspended in 20 mM sodium phosphate buffer pH 7.0. The mixture was sonicated (6 cycles of 20 s) in an ice bath, and the extract centrifuged in a Sorvall centrifuge at 10 600*g* for 45 min. The supernatant was recovered and proteins precipitated by 30% ammonium sulfate. The solution was stored at 4 °C for 2 h and centrifuged in a Sorvall centrifuge at 10 600*g* for 1 h. The supernatant was recovered, dialyzed against 4 L of 20 mM sodium phosphate pH 7.0 buffer, and concentrated on Centriplus 10 (Amicon) at 3000*g* in a Sorval centrifuge. The concentrated sample was used for IEF electrophoresis.

Identification of lipoxygenase activities on IEF

Native isoelectric focusing (IEF) was performed on a vertical electrophoresis cell (model Mini-PROTEAN and PROTEAN II, Bio-Rad) following the general guidelines provided by manufacturer. Polyacrylamide gels containing 7% of 30% (w/v) acrylamide–*N*,*N*′-methylene bis(acrylamide) (37.5 : 1) solution (Bio-Rad), 50% (v/v) glycerol, 5% (v/v) ampholyte (pH range 5–8) (Bio-Lyte 5/8 Ampholyte – Bio-Rad), 0.1% (v/v) TEMED and 0.04% (w/v) ammonium persulfate. Polyacrylamide solution was degassed before the TEMED and ammonium persulfate addition. Prior to IEF each sample was mixed with one half volume of an aqueous solution of 60% (v/v) glycerol and 4% (v/v) of ampholytes. The cathode solution was 25 mM NaOH and the anode solution 20 mM acetic acid. These solutions were cooled to 4 °C prior to electrophoresis. Electrophoresis was performed at room temperature for 1.5 h at 200 V constant voltage, then increased to 400 V constant voltage for an additional 1.5 h. When the electrophoresis was complete, the gel was cut and single lanes stained by protein and enzyme activity. Protein bands were detected by rapid protein staining as described by Reisner,[15] 0.04% (w/v) Coomassie Brilliant Blue G-250 and 3.5% (v/v) $HClO_4$, gels were destained with 5% (v/v) acetic acid. Staining for enzyme activity was carried out using the slightly modified procedure reported by Verhue and Francke.[9] HTrA (2 mg) and *o*-dianisidine (4 mg) were combined in 1.5 mL absolute ethanol and 0.5 mL of dimethylformamide. The mixture was diluted to 10 mL with 0.1 M sodium phosphate buffer pH 7.0. A similar mixture was prepared with EPA (1 mg) and linoleic acid (2 mg) as substrates. Gels were incubated in the presence of reaction mixtures for several hours (depending from enzyme activity) at 25 °C. After staining was completed, the gels were rinsed several times with distilled H_2O and digitally acquired by a HP ScanJet 4C scanner. In-gel determination of pH gradient was carried out cutting a gel lane every 5 mm, re-suspending these fragments in 1 mL of H_2O milliQ, and measuring the pH value of resulting solutions by a standard pH meter.

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