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Photodegradation of unsaturated fatty acids in senescent cells of phytoplankton: photoproduct structural identification and mechanistic aspects

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Abstract

The light-induced degradation of unsaturated fatty acids in killed phytoplanktonic cells seems to involve mainly singlet oxygen and to a lesser extent radical oxidation. These processes afford isomeric allylic hydroperoxyacids, which are then either reduced to the corresponding hydroxyacids or cleaved to ω-oxocarboxylic acids and aldehydes (Hock cleavage). α,ω-Dicarboxylic acids are also produced by subsequent oxidation of the corresponding ω-oxocarboxylic acids. These different compounds constitute useful markers of photooxidative alterations of phytoplanktonic populations. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Photodegradation; Unsaturated fatty acids; Phytoplankton

1. Introduction

If in healthy cells of phytoplankton the primary route for energy from the photoexcited chlorophyll singlet state (^{1}Chl) is to the fast photochemical reactions of photosynthesis [1], during senescence this pathway in not functional. Thus, an accelerated production of the longer live triplet state (^{3}Chl) (by intersystem crossing), and toxic oxygen species ($^1O_2$, $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$) (by reaction of ^{3}Chl with ground state oxygen) [2] would be expected. The rate of formation of these potentially damaging species can then exceed the quenching capacity of the photoprotective system of the cells and photodegradation can occur [3].

Unless complexed with water-soluble proteins, chlorophylls would tend to remain associated with other hydrophobic cellular compounds, such as membrane lipids, in phytodetritus [4]. Due to the enhanced likelihood of interactions between photoexcited chlorophyll and lipids [4], and to the well known longer lifetime of singlet oxygen in hydrophobic environment [5], the photooxidative effect of chlorophyll sensitization is strongly amplified within detritus derived from phytoplankton.

It has previously been demonstrated that chlorophylls [4], chlorophyll phytyl chain [6], carotenoids [4], and sterols [7,8] could be quickly photodegraded in senescent phytoplanktonic cells. Since unsaturated fatty acids generally predominate in algal lipids (particularly in the photosynthetic membranes [9]), these compounds are particularly susceptible to photooxidation [10]. The photosensitized oxidation of unsaturated fatty acids has been studied mainly in solution [11,12], and there are relatively few works that deal with their photodegradation in chloroplast membranes [13].

This paper first describes the structural identification of unsaturated fatty acid photoproducts in senescent cells of phytoplankton. Furthermore, some mechanisms are proposed to explain the formation of the main photoproducts.

2. Experimental details

2.1. Biological material

The chlorophyte *Dunaliella* sp. was grown under nonaxenic conditions at constant illumination (150 μEin m^{-2} s^{-1}) in 500 ml f/2 medium [14]. The cultures were harvested by centrifugation (8000 × g) 10 days after subculturing. The concentrated cells were sonicated for 15 min at 0°C (Sonifier

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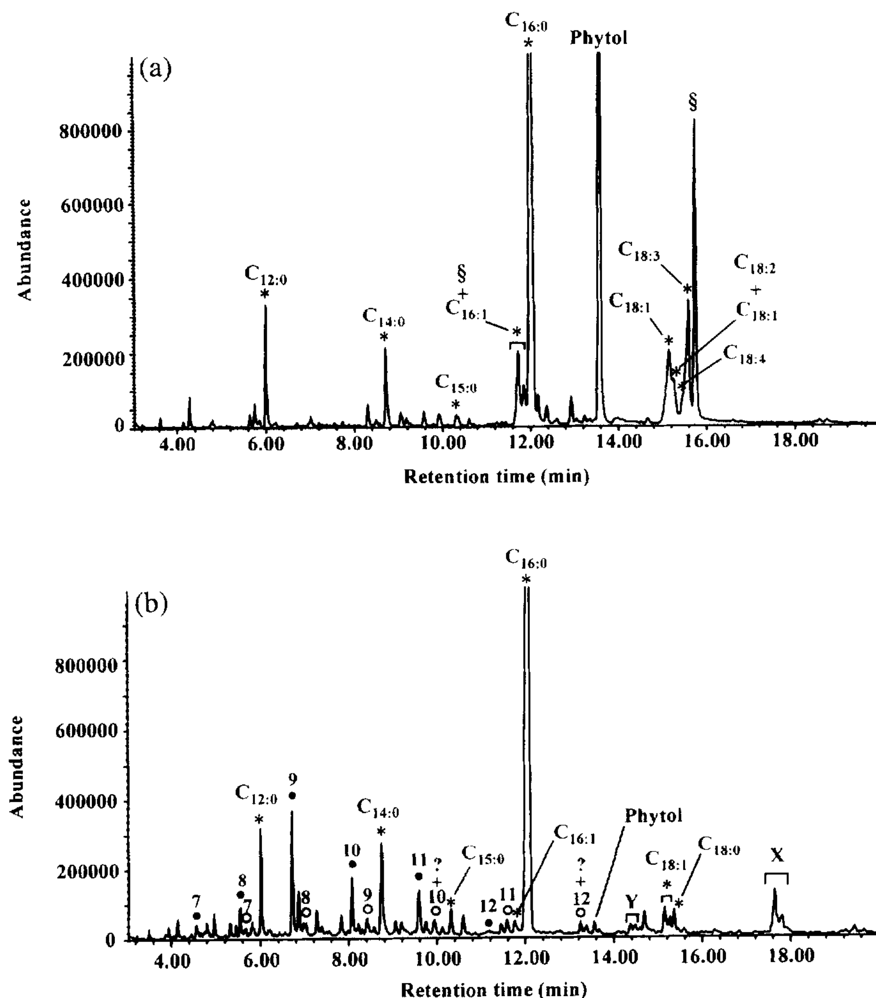


Fig. 1. Total ion current chromatograms of the silylated extracts E_2 of *Dunaliella* sp. broken cells (A) untreated and (B) exposed to 66 Ein m^{-2}. Fatty acids (*), ω-oxocarboxylic acids (•), α,ω-dicarboxylic acids (○), contaminants (§). The numbers indicate the carbon chain lengths.

250, Bransonic) to provide disruption of cellular structure [15].

2.2. Photodegradation experiments

Broken phytoplanktonic cells were distributed in pyrex flasks containing 100 ml of supernatant to which had been added 1 ml of a 0.1 M solution of mercuric chloride. The flask contents were irradiated for various durations (with magnetic stirring) using two 30 W fluorescent lamps (Osram, fluora) at 20°C. Irradiance (as Photosynthetically Available Radiations (PAR)) was measured using a Licor LI 1000 data logger equipped with an LI 1935A spherical quantum sensor. Dark controls were carried out in parallel.

2.3. Treatment of phytoplanktonic cells

After filtration on GF/F (Whatman) paper, the filter was extracted in dichloromethane/acetone (1:1, 3×30 ml) with sonication. The solvents were removed by means of rotary evaporation and the residue was divided in two. One half was reduced (15 min) in methanol (25 ml) by excess $NaBH_4$ [16]. After reduction, 25 ml of water and 2.8 g of KOH were added and the mixture was saponified by refluxing for 2 h. After saponification, the content of the flask was acidified with hydrochloric acid (pH 1) and extracted three times with toluene. The combined toluene extracts were dried on Na_2SO_4, filtered and concentrated to give an extract E_1. The other half of the residue was taken up in 300 μl of a mixture of pyridine and acetic anhydride (2/1) and allowed to react at 50°C for 2 h. After evaporation to dryness under nitrogen, the residue was saponified and treated as above described for the first half to give an extract E_2.

2.4. Silylation

After evaporation of the solvent, the different extracts were taken up in 400 μl of a mixture of pyridine and BSTFA (Supelco) (3:1, v/v) and allowed to silylate at 50°C for 1 h. Following evaporation to dryness under nitrogen, the residue was taken up in ethyl acetate and analysed by gas chromatography/electron impact mass spectrometry (GC/EIMS).

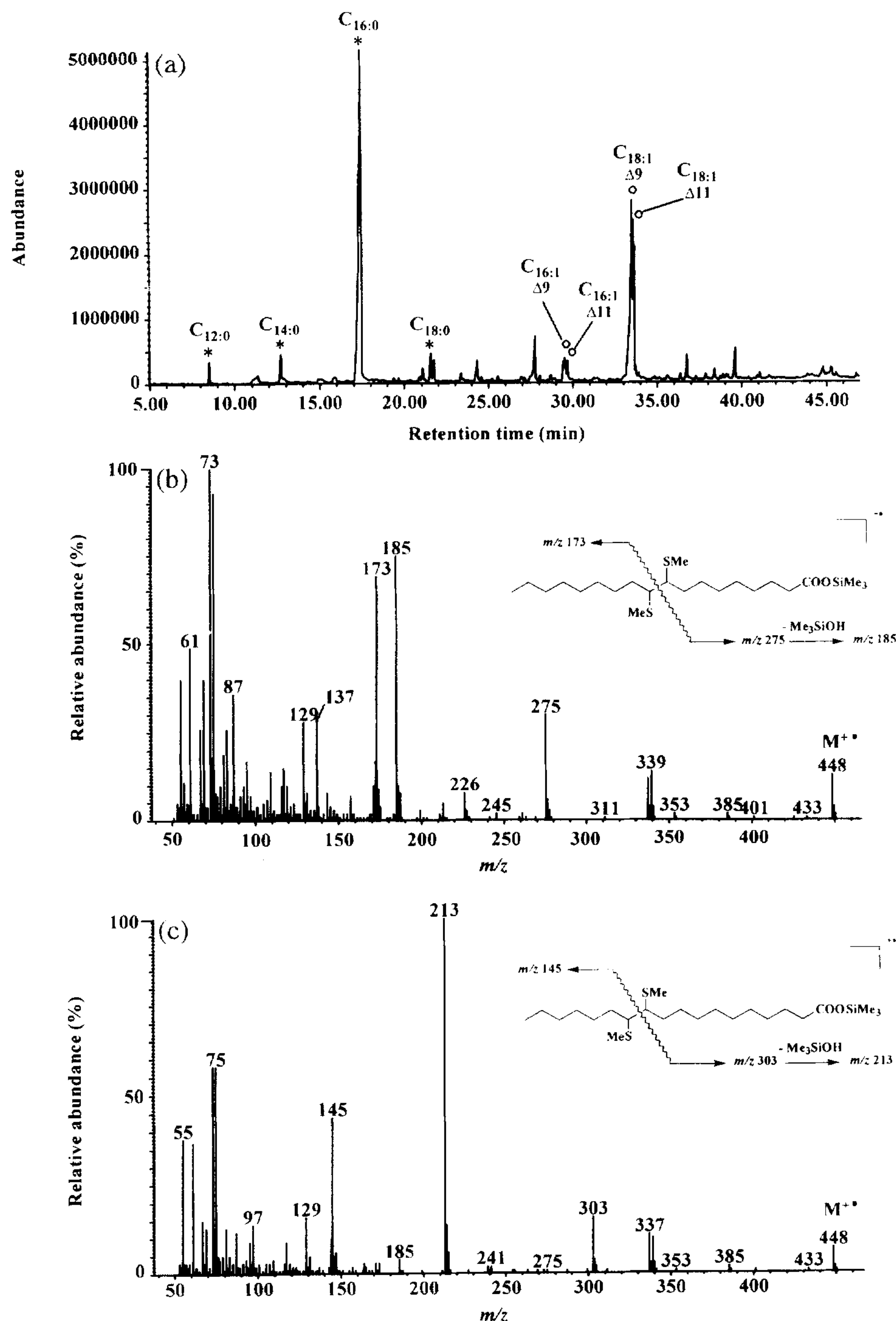


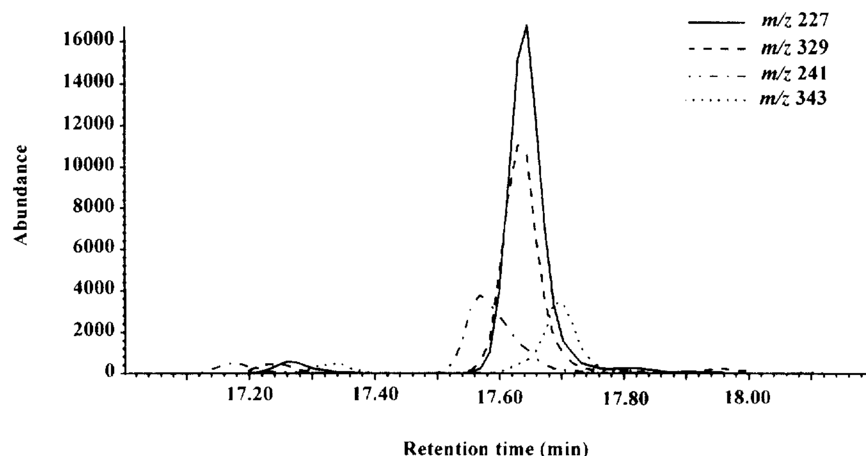
Fig. 2. (A) Total ion current chromatogram of the silylated extract E_2 of nonirradiated *Dunaliella* sp. broken cells after DMDS treatment. (B) Electron impact mass spectrum of (silylated) 9,10-bis(methylthio)-octadecanoic acid. (C) Electron impact mass spectrum of (silylated) 11,12-bis(methylthio)-octadecanoic acid.

2.5. DMDS treatment

Following the procedure initially described by Vincenti et al. [17], the extract to derivatize was dissolved in 250 μl of hexane, 250 μl of dimethyl disulfide (DMDS) and 125 μl of an iodine solution (60 mg of iodine in 1 ml of diethyl ether). The reaction mixture was held at 50°C for 24 h and then diluted with hexane. The reaction was quenched with 2 ml of 5% $Na_2S_2O_3$ and the hexane layer was pipetted off. The solution was extracted twice with hexane, the hexane extracts were combined, dried on Na_2SO_4, filtered and the solvent was evaporated. The residue was then silylated as described above and analyzed by GC/EIMS.

2.6. Identification and quantification of photoproducts by GC/EIMS

These compounds were identified by comparison of their retention times and mass spectra with those of standards.



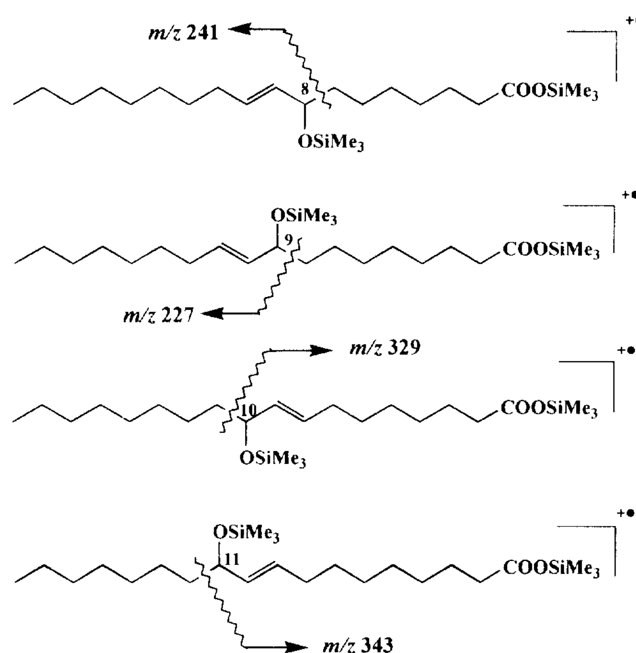


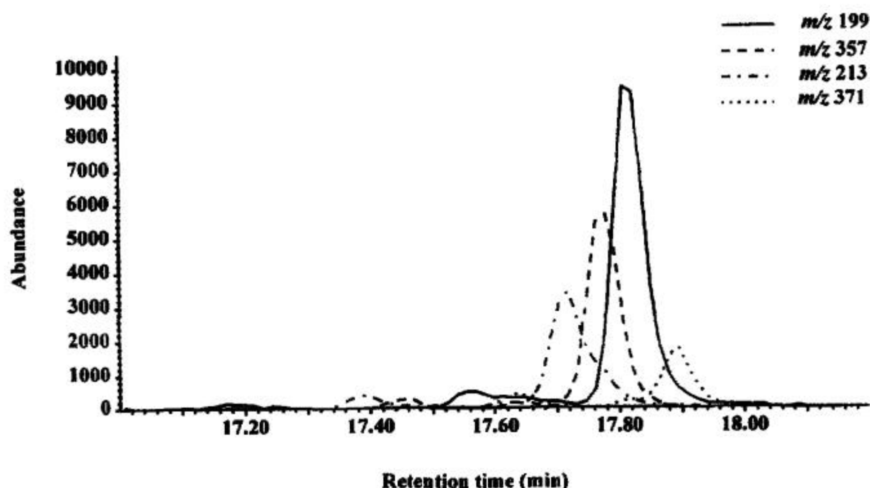
Fig. 3. Mass chromatograms of m/z 227, 241, 329 and 343 revealing the presence of four Δ9 octadecenoic acid deriving isomeric hydroxyacids in the group of unresolved peaks named X in Fig. 1B.

GC/EIMS analyses were performed with a HP 5890 series II plus gas chromatograph equipped with two injectors (spitless and on column) and connected to a HP 5972 mass spectrometer. Different chromatographic conditions were used: (A): 15 m×22 mm (i.d.) fused capillary column coated with BPX35 (SGE): oven temperature programmed from 80°C to 150°C at 30°C min^{-1} and then from 150°C to 310°C at 4°C min^{-1}; carrier gas pressure (He), 0.48 bar; spitless injector temperature, 300°C. (B) (for DMDS derivatives): 30 m×0.25 mm (i.d.) fused capillary column coated with HP5 (Hewlett Packard): oven temperature programmed from 60°C to 130°C at 30°C min^{-1} and then from 130°C to 300°C at 4°C min^{-1}; carrier gas pressure (He), 1.04 bar; on column injector temperature, 50°C. The following mass spectrometric conditions were employed: electron energy, 70 eV; source temperature, 170°C; cycle time, 1.5 s.

2.7. Standard compounds

α,ω-Dicarboxylic acids were purchased from Sigma. ω-Hydroxycarboxylic acids were produced by reduction of the corresponding α,ω-dicarboxylic acid in dry diethyl ether with half of the stoichiometric quantity of $AlLiH_4$. Oxidation of the foregoing ω-hydroxycarboxylic acids with CrO_3/pyridine in dry dichloromethane [18] gave the corresponding ω-oxocarboxylic acids.

Isomeric C_{18} allylic hydroperoxyacids were produced from Δ9 and Δ11 octadecenoic acids (Sigma): either by photosensitized oxidation in pyridine in the presence of haematoporphyrin as sensitizer [19], or by thermal autoxidation [20]. Reduction of these compounds in methanol with $NaBH_4$ [16] afforded the corresponding hydroxyacids.



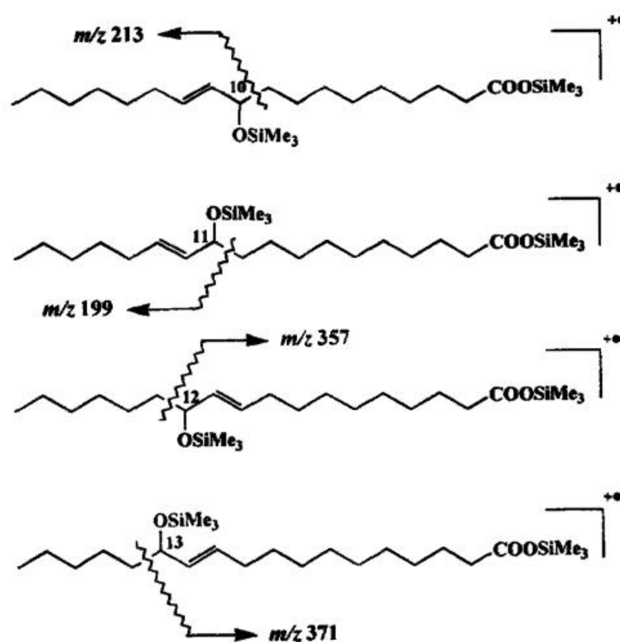


Fig. 4. Mass chromatograms of m/z 199, 213, 357 and 371 revealing the presence of four Δ11 octadecenoic acid deriving isomeric hydroxyacids in the group of unresolved peaks named X in Fig. 1B.

3. Results and discussion

The GC/EIMS analysis of extract E_2 obtained from healthy cells of *Dunaliella* sp. is given in Fig. 1A. Peak identification is based on comparison of retention times and mass spectra with those of standards. The most abundant unsaturated fatty acids in this organism are $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ and $C_{18:4}$. $C_{16:1}$ acids are also present but in smaller proportions (these compounds are strongly overvalued in Fig. 1A due to their coelution with contaminants).

To establish the position of the double bond in the monounsaturated fatty acids, this extract was treated with dimethyl disulfide (DMDS) [17]. The double bond positions were determined from the mass spectra of the DMDS derivatives (Fig. 2B and C) on the basis of the fragments formed by cleavage between the carbons bearing the thioether groups. It appears that the mono-unsaturated fatty acids of *Dunaliella* sp. are in fact Δ9 and Δ11 isomers (Fig. 2A).

In killed phytoplanktonic cells, the photodegradation rate of unsaturated fatty acids logically and quickly increases with their degree of unsaturation [8]. So after exposure to 66 Ein m^{-2} (Fig. 1B), we can note in extract E_2 the disappearance of poly-unsaturated fatty acids, whereas there remain some weak amounts of mono-unsaturated fatty acids.

Parallel to the photodegradation of unsaturated fatty acids, we observed the production of ω-oxocarboxylic and α,ω-dicarboxylic acids (Fig. 1B) (the ω-oxocarboxylic acids being reduced to their corresponding ω-hydroxycarboxylic acids in extracts E_1). These compounds, which are lacking in dark controls, range from C_7 to C_{12} with a maximum at C_9.

Table 1
Isomeric distribution of oleic acid ($C_{18:1\omega9}$) hydroperoxides

Experiment	8-Hydroperoxide (%)	9-Hydroperoxide (%)	10-Hydroperoxide (%)	11-Hydroperoxide (%)
In killed cells of *Dunaliella* sp. after exposure to 13 Ein m^{-2}	3.8	45.6	47.4	3.2
In killed cells of *Dunaliella* sp. after exposure to 46 Ein m^{-2}	13.7	39.7	35.1	11.5
In killed cells of *Dunaliella* sp. after exposure to 66 Ein m^{-2}	12.2	45.7	31.4	10.7
In killed cells of *Dunaliella* sp. after exposure to 105 Ein m^{-2}	11.7	44.5	33.8	10.0
After type II photosensitized oxidation in solvents[a]	–	50	50	–
After radical oxidation in solvents[a]	27	23	23	27

[a][11].

A group of nonresolved peaks (named X) also appeared during irradiation (Fig. 1B). These peaks correspond to isomeric allylic mono-hydroxyoctadecenoic acids, which have been characterized by GC/EIMS on the basis of the cleavage at the carbon bearing $-OSiMe_3$ group (Figs. 3 and 4). There are in fact two groups of hydroxyacids arising respectively from the oxidation of Δ9 (Fig. 3) and Δ11 (Fig. 4) octadecenoic acids. These compounds are present in similar quantities in extracts E_1 and E_2, and we failed to detect significant amounts of isomeric mid-chain ketoacids (formed by acetylation of hydroperoxides [21]) in extracts E_2. Consequently, it can be concluded that allylic hydroxyoctadecenoic acids are formed by reduction of the corresponding hydroperoxides in killed cells of *Dunaliella* sp. and not thermally during gas chromatographic analyses. Small quantities of isomeric hydroxyhexadecanoic acids were also detected (group of unresolved peaks named Y in Fig. 1B), but we failed to identify hydroxyacids resulting from the photodegradation of the poly-unsaturated fatty acids.

During radical oxidation of mono-unsaturated fatty acids, hydrogen atom abstraction from the allylic methylenes produces two allylic radicals in which electrons are delocalized

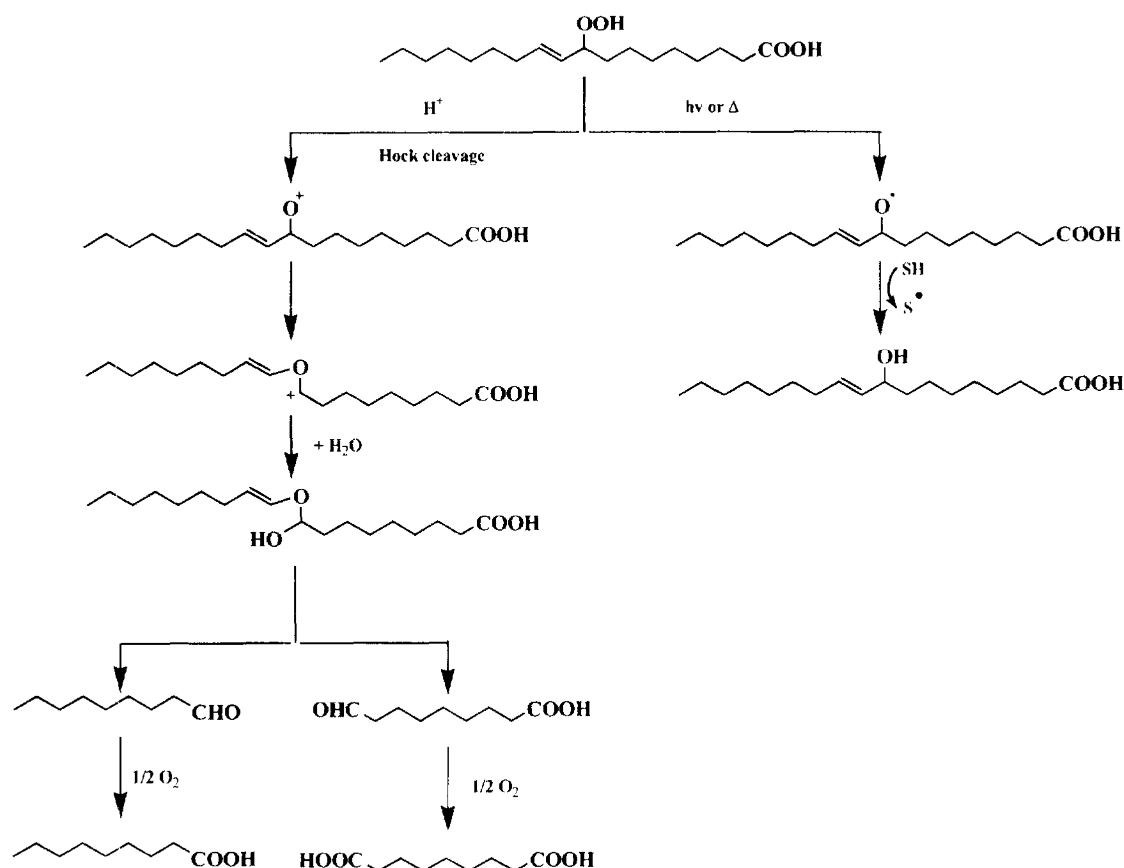


Fig. 5. Proposed pathways for the degradation of 9-hydroperoxyoctadec-10-enoic acid in senescent cells of *Dunaliella* sp..

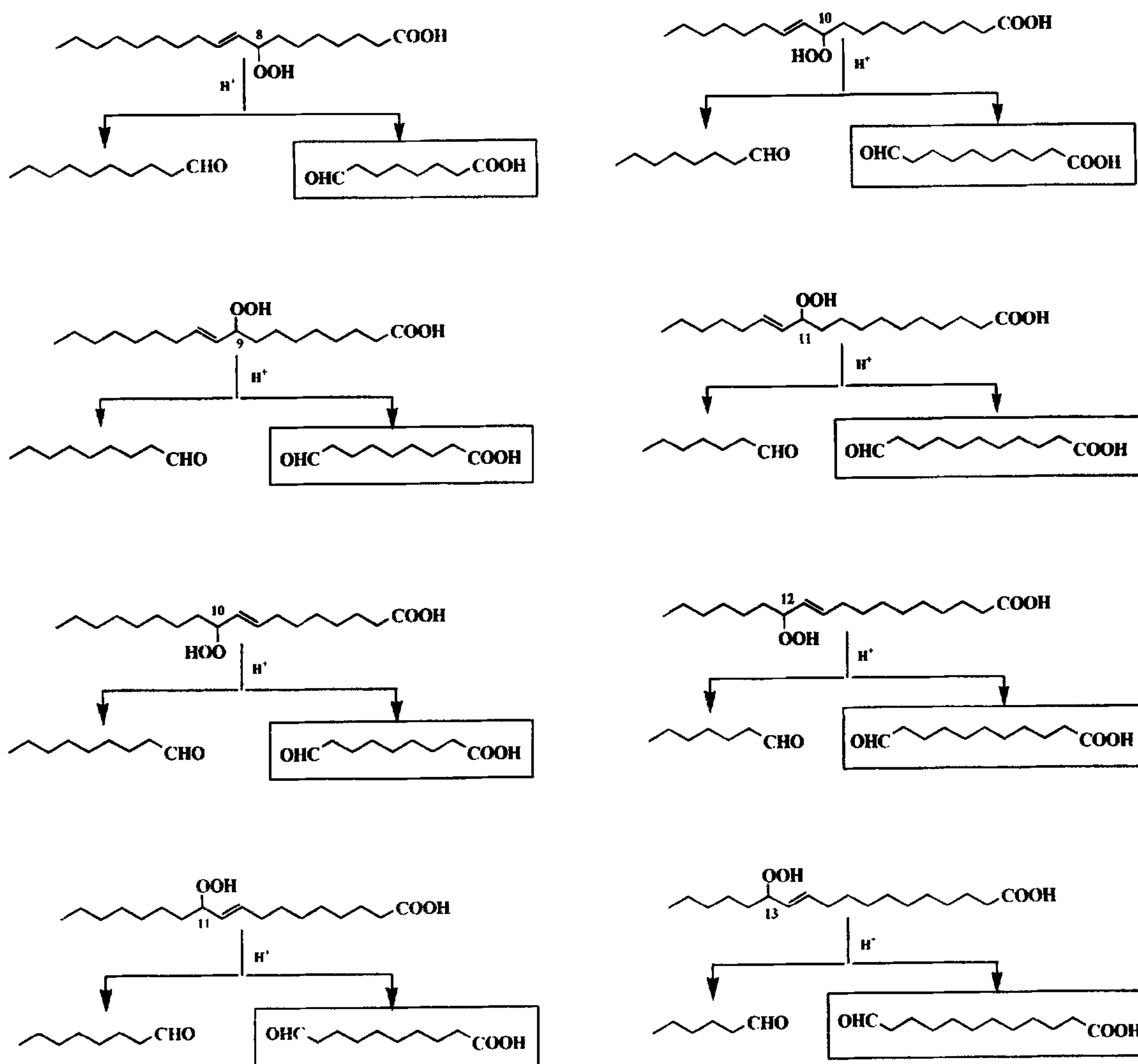


Fig. 6. Hock cleavage of isomeric hydroperoxides resulting from the photooxidation of octadecenoic acids.

through three-carbon systems. Further reaction of these radicals with ground state oxygen produces a mixture of four hydroperoxides in practically equal proportions [12] (Table 1). In contrast, singlet oxygen ($^1O_2$) reacts with the carbon–carbon double bond of these compounds by a classical concerted 'ene' mechanism [22] and yields only two hydroperoxides [12] (Table 1). If we compare the isomeric distribution of Δ9 octadecenoic acid (oleic acid) hydroperoxides (quantified after reduction to their corresponding secondary alcohols) in killed cells of *Dunaliella* sp. and after oxidation with radicals or $^1O_2$ (Table 1), it appears that the photodegradation of mono-unsaturated fatty acids in phytodetritus involves mainly $^1O_2$. Due to the initiation of fatty acid radical autoxidation by the singlet oxygen oxidation products (i.e., 9- and 10-hydroperoxides) initially formed [12,23], the proportion of 8- and 11-hydroperoxides increases with incubation time (Table 1). During the irradiation, we also observed an inexplicable decrease in the 10-hydroperoxide proportion (Table 1).

Allylic hydroperoxides can undergo two main degradative processes: (i) homolysis of the O–O bond leading to carbonyl (dehydration), alcoholic (reduction), fragmentation (β-scission) or oxirane (radical cyclization) products [22]; and (ii) heterolysis of the O–O bond leading to the formation of two carbonyl fragments (Hock cleavage), this proton-catalysed cleavage being initiated by migration of groups to positive oxygen [22].

In killed cells of *Dunaliella* sp. the homolysis of the O–O bond of unsaturated fatty acids deriving hydroperoxides involves mainly reduction to the corresponding secondary alcohols, since we failed to detect significant amounts of ketoacids (dehydration products), epoxyacids (radical cyclization products), alkenals and ω-oxoalkenoic acids (which must be their main fragmentation products).

Proton-catalysed cleavage of allylic hydroperoxides involves mainly the migration of the vinyl group [22] (Fig. 5) (the alkyl group can also migrate but at a much lesser extent). According to this pathway, allylic hydroperoxides resulting from the light-induced oxidation of unsaturated fatty acids can afford ω-oxocarboxylic acids and aldehydes (Fig. 5). In the presence of peroxides or molecular oxygen such compounds can then be easily oxidized respectively to car-

boxylic and α,ω-dicarboxylic acids [24,25]. Hock cleavage of hydroperoxides corresponding to the different isomeric hydroxyoctadecenoic acids identified provide an explanation for the formation of most of the ω-oxocarboxylic acids detected (Fig. 6). We failed to detect the corresponding aldehydes (ranging from C_6 to C_{10}) probably owing to their too high volatility.

Homologous series of ω-oxocarboxylic and α,ω-dicarboxylic acids (C_4–C_{14}; C_9 being maximum) have been detected initially by Kawamura and Gagosian [26] in marine aerosols. These authors attributed the formation of such compounds to the photooxidation (involving O_3, H_2O_2 or $HO^{\bullet}$) of unsaturated fatty acids in the marine atmosphere and surface seawater. The further detection of α,ω-dicarboxylic acids (C_8 and C_9) in marine sediments [27,28] and sediment traps [27] was interpreted as indicative of atmospheric transport of these photooxidation products rather than of an autochthonous formation [29], and these compounds have been proposed as useful markers for evaluating the importance of atmospheric input to the sea [28]. This role is called into question by the results obtained in the present work, since the photosensitized oxidation (involving mainly singlet oxygen) of unsaturated fatty acids in senescent phytoplanktonic cells, which can act in all the euphotic layer of the oceans, must constitute a nonnegligible source of ω-oxocarboxylic and α,ω-dicarboxylic acids in the marine environment.

4. Conclusions

Unsaturated fatty acids are quickly photodegraded in senescent cells of *Dunaliella* sp. As in the case of chlorophyll phytyl chain [6], sterols [7], and carotenoids [8], the photosensitized degradation of these compounds seems to involve mainly singlet oxygen. The key role played by singlet oxygen in the photodegradation of algal lipids in phytodetritus can be attributed to its well known longer lifetime in hydrophobic environments [5]. Radical oxidation of unsaturated fatty acids intervenes to a lesser extent and is probably initiated by the breakdown of singlet oxygen reaction products initially formed [23].

These different processes result in the formation of isomeric allylic hydroperoxyacids, which are then either reduced to the corresponding secondary alcohols or cleaved to ω-oxocarboxylic acids and aldehydes (Hock cleavage).

We failed to detect poly-hydroxyacids arising from the photosensitized oxidation of polyunsaturated fatty acids (and subsequent reduction). This result can be attributed to: (i) the instability of the corresponding hydroperoxides, or (ii) the involvement of cross-linking reactions (after homolytic cleavage) leading to the formation of macromolecular structures [30] nonamenable by gas chromatography.

Allylic hydroxyacids (with the same carbon number as the starting fatty acids) and C_7–C_{12} ω-oxocarboxylic and α,ω-dicarboxylic acids (with C_9 as the most abundant species) could constitute useful markers of photodegradative alterations and contribute to a better estimation of the degradation state of phytoplanktonic populations.

The photooxidative production of homologous ω-oxocarboxylic and α,ω-dicarboxylic acids during the senescence of phytoplankton calls into question the use of these compounds as tracers of atmospheric input to the sea.

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