ELSEVIER

Journal of Photochemistry and Photobiology A: Chemistry 111 (1997) 139–144

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A: CHEMISTRY

Rates and mechanism of light-dependent degradation of sterols in senescent cells of phytoplankton

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Received 14 May 1997; accepted 15 July 1997

Abstract

Irradiation of killed cells of *Skeletonema costatum* resulted in a quick photodegradation of the sterol components of this algae. Light-dependent degradation rates of these compounds showed a good fit to apparent first-order kinetics. Identification of the main photoproducts using gas chromatography/electron impact mass spectrometry analyses demonstrated that sterol photodegradation in killed cells of phytoplankton involved pure type II (i.e. involving singlet oxygen) photoprocesses. 5α- and 6α/6β-hydroperoxides, which constitute the bulk of the sterol photoproducts, appeared to be relatively stable in phytodetritus and are proposed as potential markers of photodegradation processes in the marine environment.

Keywords: Sterol photodegradation; Senescent phytoplanktonic cells; Type II photoprocesses; 5α- and 6β-hydroperoxides

1. Introduction

The structural diversity of sterols allows them to be used as indicators of biological activity in the oceans [1]. Indeed, these compounds possess structural features, such as positions of double bonds and patterns of side-chain alkylation, which are restricted to a few groups of organisms [2].

Though sterols have a long geological record and constitute excellent biomarkers for tracing diagenetic transformations in sediments [3], in oxic environments only a small part of the sterols produced in the euphotic zone reaches the sediments. As an example, Gagosian and Nigrelli [4] have calculated that at most 0.3% of sterols produced by phytoplankton in the Sargasso Sea is deposited on the ocean floor.

During an *in vitro* study of the photodegradation of n-C_{37} alkenones in senescent cells of *Emiliania huxleyi* [5], we recently observed that 24α-methylcholesta-5,22(E)-dien-3β-ol (which is the major sterol of this algae) was quickly photodegraded. This very interesting result suggests that the photochemical processes associated with phytodetritus must play an important role in the degradation of sterols in the euphotic layer of the oceans.

In dead phytoplanktonic cells, as the fast photochemical reactions of photosynthesis are not functional, an accelerated rate of ^{3}Chl (chlorophyll at the triplet state) formation would thus be expected [6]. The rates of formation of ^{3}Chl and toxic oxygen species ($^1O_2$, $O_2^{\bullet -}$, HO$^\bullet$, HOO$^\bullet$) (formed by reaction of ^{3}Chl with ground state oxygen ($^3O_2$)) [7] can then exceed the quenching capacity of the photoprotective system of the cell and photodegradation can occur [8]. If type I [9] and type II [10,11] photo-oxidation of these compounds have been extensively studied in solution, there are no texts of studies that deal with the photodegradation of sterols in senescent phytoplanktonic cells.

The present study examines the rates, pattern and mechanism of light-dependent degradation of sterols in senescent cells of *Skeletonema costatum*, which is a widespread diatom present in coastal waters [12].

2. Experimental details

2.1. Biological material

Non-axenic *Skeletonema costatum* was obtained from IFREMER (Nantes) collection and grown in 500 ml F/2 medium [13] at 20°C using a 12 h light/12 h dark regime. The cultures were harvested by centrifugation (8000*g*) during the stationary phase for maximum yield. Prior to experiments the concentrated cells were sonicated for 15 min at 0°C

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1010-6030/97/$17.00
PII S1010-6030(97)00221-9

(Bransonic Sonifier 250) to provide some disruption of cellular structures [14].

2.2. Photodegradation experiments

Broken phytoplanktonic cells were distributed in pyrex flasks containing 50 ml of supernatant to which has 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 193SA spherical quantum sensor. Dark controls were carried out in parallel.

2.3. Treatment

After filtration on GF/F (Whatman) paper, total lipids were extracted in dichloromethane/acetone (1:1, 3 × 30 ml) with sonication. Solvents were removed by means of rotary evaporation and hydroperoxides were reduced (15 min) in methanol (25 ml) by excess $NaBH_4$ [15]. After reduction, 25 ml of water and 2.8 g of potassium hydroxide were added and the mixture was saponified by refluxing for 2 h. After saponification, the content of the flask was extracted three times with hexane and the combined hexane extracts were dried on Na_2SO_4, filtered and concentrated. The residue thus obtained was taken up in 250 μl of a mixture of pyridine and BSTFA (Supelco) (3:1, v/v) and allowed to silylate at 50°C for 2 h. Following evaporation to dryness under nitrogen, the residue was taken up in ethyl acetate and analyzed immediately by gas chromatography/mass spectrometry.

2.4. Gas chromatography/electron impact mass spectrometry

Gas chromatography/electron impact mass spectrometry (GC/EIMS) analyses were carried out on a Hewlett–Packard 5890 serie II plus gas chromatograph connected to a HP 5972 mass spectrometer. The following operating conditions were employed: 15 m × 0.22 mm (i.d.) fused capillary column coated with BPX35 (SGE); oven temperature programmed from 80 to 150°C at 30°C min^{-1} and then from 150 to 310°C at 4°C min^{-1}; carrier gas pressure (He), 0.48 bar; on column injector temperature, 50°C; source temperature, 170°C; electron energy, 70 eV.

Sterols and their photoproducts were identified by comparison of their retention times and mass spectra with those of commercial or synthesized standards. Quantitative determinations were based on integrator data, which were calibrated with external standards.

2.5. Standard compounds

Cholest-5-en-3β-ol, 24-methylcholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol were obtained from Aldrich chimie. Photosensitized oxidation of these sterols in pyridine in the presence of haematoporphyrin [10] and subsequent purification by preparative thin layer chromatography (TLC) [10,16] gave the corresponding 5α- and 6α/6β-hydroperoxides. Allylic rearrangement of 5α-hydroperoxides to 7α-hydroperoxides and epimerization of the latter to 7β-hydroperoxides were carried out at room temperature in chloroform [17]. Subsequent reduction of these different hydroperoxides in methanol with excess $NaBH_4$ afforded the corresponding diols. Hydrogenation of these different diols was carried out at atmospheric pressure and temperature with Raney nickel as the catalyst in methanol [18].

3. Results and discussion

In accordance with previous investigations [2], GC/EIMS analyses of living cells of *Skeletonema costatum* revealed the presence of cholest-5-en-3β-ol, 24-methylcholest-5-en-β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol and 24-ethylcholest-5-en-3β-ol as major sterols. 24-Methylcholesta-5,24(28)-dien-3β-ol being not commercially available, it was identified by comparison of its mass spectrum with mass spectral data described in the literature [19].

In order to determine the photodegradation rates of these sterols, we irradiated broken cells of *Skeletonema costatum* for different times. The light-dependent degradation rates thus obtained show a good fit to apparent first-order kinetics (Table 1). Sterols are photodegraded in broken cells at a similar rate to that of the chlorophyll phytyl chain ($D_{1/2} \approx 7.4$ Ein m^{-2} at 20°C) [20]. The apparent first-order rate constants of these four sterols are very closed (Table 1) and the presence of an additional double bond in the molecule of 24-methylcholesta-5,24(28)-dien-3β-ol does not increase significantly its photodegradation rate. This surprising result can be easily explained if we consider that the photodegradation of sterols in broken cells of phytoplankton is a type II photoprocess. Indeed, singlet oxygen ($^1O_2$) reacts practically two hundred times more slowly upon a disubstituted double

Table 1
Apparent first-order rate constants (k_1)[a] for light-dependent degradation of sterols in senescent cells of *Skeletonema costatum* ($n = 5$)

Sterol	k_1 (Ein m^{-2})$^{-1}$	Regression r^2	$D_{1/2}$ (Ein m^{-2})
Cholest-5-en-3β-ol	0.085	0.96	8.15
24-Methylcholest-5-en-3β-ol	0.090	0.94	7.70
24-Methylcholesta-5,24(28)-dien-3β-ol	0.100	0.98	6.93
24-Ethylcholest-5-en-3β-ol	0.084	0.94	8.25

[a] The rate constants represent the opposite of slopes of regression determined as $\ln(C/C_0) = -k_1 D$, where C is the concentration at the time of sampling, C_0 the initial concentration, and D the cumulative light dose (Ein m^{-2}, PAR). $D_{1/2}$ is the light exposure under which the compounds are reduced to half of their initial concentrations.

bond such as the additional $\Delta^{24(28)}$ double bond of 24-methylcholesta-5,24(28)-dien-3β-ol than upon trisubstituted double bonds such as Δ^5 double bonds [21].

If sterols are practically unchanged in dark controls (Fig. 1(a)), after irradiation GC/EIMS analyses showed the presence of epimeric 3β,7- and 3β,6-diols corresponding to each sterol component (Fig. 1(b)). Mass spectra of (disilylated) 3β,7-diols exhibit strong $[M-HOSiMe_3]^+$ peaks (Fig. 2), whereas these of (disilylated) 3β,6-diols show characteristic $[M-143]^+$ ion signals (Fig. 3) [22]. Trimethylsilyl derivatives of epimeric 3β,7-diols present similar EI mass spectra and can only be differentiated on the basis of their retention times. On the other hand, mass spectra of trimethylsilyl derivatives of epimeric 3β,6-diols present some differences which

1- Squalene
2- Cholest-5-en-3β,7α-diol
3- Cholest-4-en-3β,6β-diol
4- Cholest-5-en-3β-ol
5- 24-Methylcholest-5-en-3β,7α-diol
6- 24-Methylcholesta-5,24(28)-dien-3β,7α-diol
7- Cholest-4-en-3β,6α-diol
8- Cholest-5-en-3β,7β-diol
9- 24-Methylcholest-4-en-3β,6β-diol
10- 24-Methylcholesta-4,24(28)-dien-3β,6β-diol
11- 24-Ethylcholest-5-en-3β,7α-diol
12- 24-Methylcholest-5-en-3β-ol
13- 24-Methylcholesta-5,24(28)-dien-3β-ol
14- 24-Methylcholest-4-en-3β,6α-diol
15- 24-Ethylcholest-4-en-3β,6β-diol
16- 24-Methylcholest-5-en-3β,7β-diol
17- 24-Methylcholesta-4,24(28)-dien-3β,6α-diol
18- 24-Methylcholesta-5,24(28)-dien-3β,7β-diol
19- 24-Ethylcholest-5-en-3β-ol
20- 24-Ethylcholest-4-en-3β,6α-diol
21- 24-Ethylcholest-5-en-3β,7β-diol
* n-Alkanols

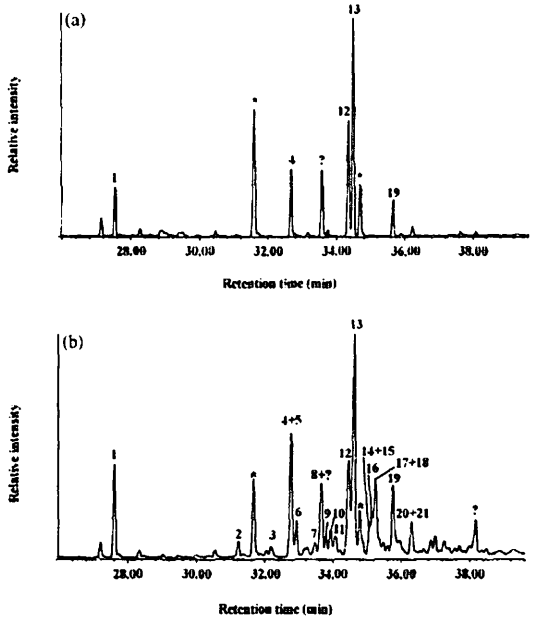


Fig. 1. Partial total ion chromatogram showing the sterol region after injection of reduced ($NaBH_4$) neutral lipids of *Skeletonema costatum* broken cells: (a) incubated for 5 days in darkness and (b) exposed to 35 Ein m^{-2} (5 days).

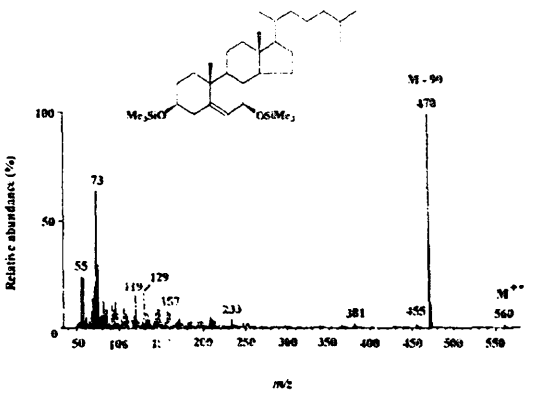


Fig. 2. Electron impact mass spectrum of (disilylated) 24-methylcholest-5-en-3β,7β-diol.

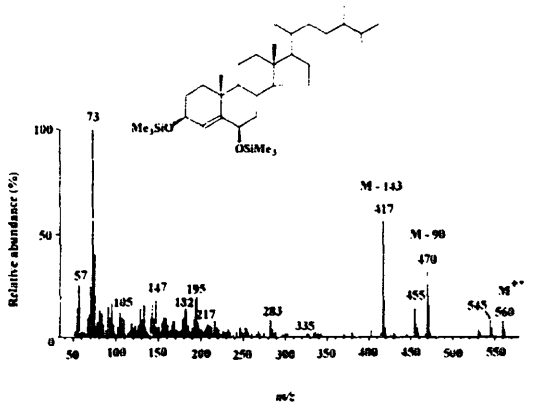
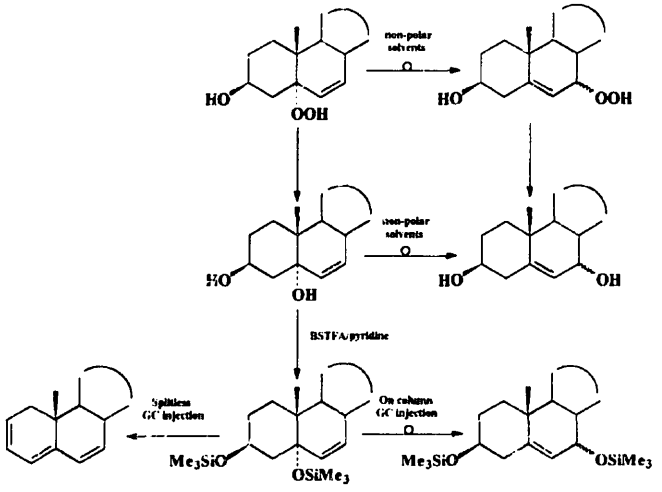


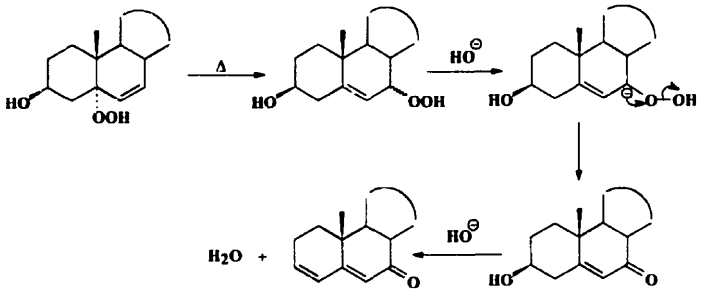
Fig. 3. Electron impact mass spectrum of (disilylated) 24-methylcholest-4-en-3β,6β-diol.

can be related to their axial or equatorial configuration. Indeed, the presence of axial Me_3SiO– groups gave rise to molecular ions of low abundance and prominent ions produced by loss of Me_3SiOH [22]. Due to the lack of standards, diols deriving from 24-methylcholesta-5,24(28)-dien-3β-ol have been tentatively identified on the basis of their mass spectral data. The lack of photoproducts arising from the photo-oxidation of the $\Delta^{24(28)}$ double bond of this sterol confirms the poor photoreactivity of this double bond.

Type II ($^1O_2$) photo-oxidation of sterols gives mainly 5α-hydroperoxides and to a lesser extent 6α/6β-hydroperoxides [10,11], whereas type I (free radical) photoreactions give 7α/7β-hydroperoxides [9]. 5α-Hydroperoxides are relatively unstable and may undergo reduction or allylic rearrangement to 7α-hydroperoxides, which in turn epimerize to 7β-hydroperoxides [23,24] (Scheme 1). Such conversions can occur during the photoreaction itself or during product isolation and analysis [25]. On-column GC injection of silylated standards of 3β,5α-diols revealed that these compounds also undergo intense allylic rearrangements into the chromatograph, whereas splitless injection resulted in a complete desilylation (Scheme 1).



Scheme 1.



Scheme 2.

It is important to note that the quantity of unsaturated diols strongly decreases if the reduction with $NaBH_4$ is omitted before the alkaline hydrolysis. This strong decrease and the concomitant formation of cholesta-3,5-dien-7-one, 24-methylcholesta-3,5-dien-7-one and 24-ethylcholesta-3,5-dien-7-one (known to be produced during alkaline hydrolysis of sterol hydroperoxides (Scheme 2) [26,27]) demonstrate that the reduction of hydroperoxides to the corresponding diols weakly operates in broken cells of *Skeletonema costatum*.

At this time we cannot be certain whether pure type II photoprocesses are occurring, followed by light-independent rearrangements, or whether a mixed type I–type II mechanism applies. In order to eliminate double bonds and avoid allylic rearrangements, the residue obtained after saponification was subsequently hydrogenated in methanol with Raney nickel as catalyst. Comparison of mass spectra and retention times with those of standards allowed to identify cholesta-3β,5α-diol, cholesta-3β,6α/6β-diols, 24-methylcholesta-3β,5α-diol, 24-methylcholesta-3β,6α/6β-diols, 24-ethylcholesta-3β,5α-diol and 24-ethylcholesta-3β,6α/6β-diols in this hydrogenated extract. EI mass spectra of (monosilylated) 24-methylcholesta-3β,5α-diol and (disilylated) 24-methylcholesta-3β,6β-diol are given as examples in the Figs. 4 and 5. On the other hand, we failed to detect significant quantities of 3β,7α- and 3β,7β-saturated diols.

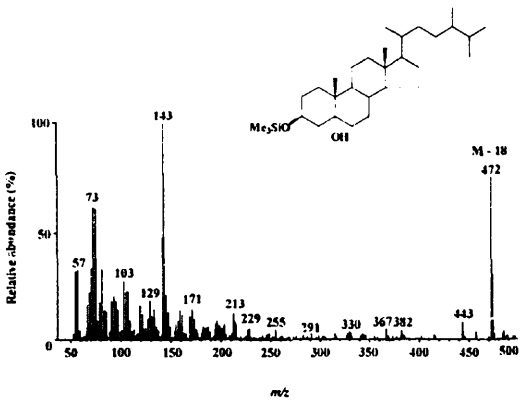


Fig. 4. Electron impact mass spectrum of (monosilylated) 24-methylcholesta-3β,5α-diol.

These results clearly establish that the photo-oxidation of sterols in senescent cells of phytoplankton involves pure type II photoprocesses. Singlet oxygen-mediated photo-oxidation

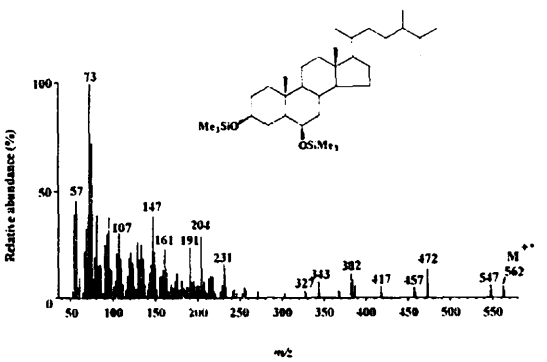


Fig. 5. Electron impact mass spectrum of (disilylated) 24-methylcholesta-3β,6β-diol.

of sterols in phytodetritus produces 6-hydroperoxides in considerably higher yields (ratio 6α/6β-hydroperoxides/5α-hydroperoxides = 0.34) than in homogeneous solutions [11]. This confirms previous observations made by Korytowski et al. [25] in cell membranes.

Allylic rearrangement does not occur significantly in broken cells of phytoplankton nor during the extraction, reduction and saponification processes employed. It occurs only in the chromatograph during the GC analysis of 3β,5α-diols. Korytowski et al. [25] have observed that 5α-hydroperoxides are more stable in membranes containing unsaturated phospholipids than in those containing saturated phospholipids. This stability was attributed either to hydrogen bonding between the unsaturated fatty acyl chain of phospholipids and 5α-hydroperoxides which could hinder the allylic rearrangement [28], or to differences of polarity in the carbon 7–10 zone of the fatty acyl chain (where sterols tend to localize in phospholipid/sterol bilayers [29]). On the basis of these hypotheses, the lack of allylic rearrangement in phytodetritus can be correlated with the well-known high Δ^9 unsaturated fatty acid content of phytoplankton [30,31].

4. Conclusions

The apparent first-order rates constants determined in the present study allow a prediction to be made that, in phytodetritus, sterols will be photodegraded to half their initial concentrations after exposure to 7–8 Ein m^{-2}. Assuming a surface irradiance of 60 Ein m^{-2} day^{-1} (a value representative of mid-latitude waters under a clear summer day [6]), it can be concluded that sterols contained in killed phytoplanktonic cells will be quickly photodegraded in the upper portion of the euphotic layer of the oceans.

Sterol photodegradation involves pure type II ($^1O_2$) photoprocesses and leads mainly to the formation of 5α- and 6α/6β-hydroperoxides. The surprising stability of these hydroperoxides in killed phytoplanktonic cells strongly suggests that in the marine environment non-negligible quantities of these photoproducts must be deposited on the ocean floor. Consequently, 5α- and 6α/6β-hydroperoxides may constitute potential tracers of photodegradation processes operating in the euphotic layer. This hypothesis requires rapid confirmation, since there is a real need of organic compounds stable and specific enough to act as efficient tracers of photodegradation processes. At this time, only 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (which is a photoproduct of the chlorophyll phytyl chain [32,33]) and some α,ω-diacids arising from the photodegradation of unsaturated fatty acids [30,31] have been proposed for such a use.

Acknowledgements

Thanks are due to Dr. A. Pastoureaud (IFREMER, La Rochelle) for her generous gift of the strain *Skeletonema costatum* used in this work. We also thank Dr. B. Beker (Centre d'Océanologie de Marseille), who kindly made cultures available for us. Mrs. S. Cuny is gratefully acknowledged for reviewing the text.

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