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STEROLS AND PHYLOGENY OF THE ACIDOPHILIC HOT SPRINGS ALGAE *CYANIDIUM CALDARIUM* AND *GALDIERIA SULPHURARIA*

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Key Word Index—*Cyanidium caldarium*; *Galdieria sulphuraria*; Cyanidiophyceae; Rhodophyta; red algae; cyanobacteria; sterols; phylogeny; chemotaxonomy.

Abstract—The sterols of *Cyanidium caldarium* and *Galdieria sulphuraria* were analysed. These unicellular blue-green eukaryotic algae are acido-thermophilic and have a wide global distribution. The following sterols were found: ergosterol, ergost-7-enol, ergosta-7,22-dienol, chondrillasterol and 22-dihydrochondrillasterol. Ergost-7-enol was the predominant compound in cells of both algae grown in air or pure carbon dioxide. In addition 24-methylcholesta-5,7,22*E*,24(24') tetraen-3β-ol was identified for the first time in algal lipids. The phylogenetic aspects of the presence of these sterols are discussed.

INTRODUCTION

Sterols are distributed in almost all living organisms including some prokaryotic bacteria, and the algal genera exhibit a wide spectrum of sterols [1–3]. For example, ergosterol and sitosterol have been observed in many algal divisions as well as in higher plants. Cholesterol was detected in Rhodophyta (red algae) in addition to the presence of C_{27}, C_{28} and C_{29}-sterols [1–3]. The Chlorophyta (green algae) is characterized by the presence of sitosterol, ergosterol and other sterols. The cyanobacteria (blue-green algae) exhibit a large spectrum of sterols including cholesterol, ergosterol, chondrillasterol, sitosterol and campesterol [4]. The sterol content of organisms may serve in many cases as a systematic marker of the taxon. However, the correlation of sterol composition and the taxonomic position of an organism does not always apply and there are exceptions to the 'typical' sterol within a specific taxon. Klein and Cronquist [5] have pointed out that similar sterols observed in several algal divisions make the distribution of these compounds a poor phylogenetic criteria for taxa above the algal order.

Since sterols are ubiquitous constituents of cellular membranes, we were interested in examining the lipids of *Cyanidium* species which thrive in extreme conditions, such as acidic hot springs, and are able to grow under a pure atmosphere of carbon dioxide [6, 7]. Our initial survey of the sterols of *Cyanidium caldarium* was performed 20 years ago and has been thus far, the sole report of *C. caldarium* sterols [3, 8]. Since this initial study, Merola *et al.* [9] revised the *Cyanidium* population and established three genera (*Cyanidioschyzon*, *Cyanidium*, *Galdieria*) within the algal class Cyanidiophyceae (see the recent review [10] on the Cyanidiaceae and the discussion of Seckbach [11] on the confusion among these species). We have now reanalysed the sterols of a pure culture of *C. caldarium* and also identified the sterols of *Galdieria sulphuraria*.

RESULTS AND DISCUSSION

The sterol compositions of the hot spring algae *C. caldarium* and *G. sulphuraria* grown in air or under pure carbon dioxide are presented in Table 1. The comparison of the current and previous analyses [3, 8] of cyanidiophycean sterols with other algae are presented in Table 2. The following unsaturated C_{28}- and C_{29}-sterols were identified by GC-MS of the TMS derivatives: 24-methylcholesta-5,7,22,24(24') tetraen-3β-ol (1) (ergosta-5,7,22,24(24')-tetraenol); 24-methylcholesta-5,7,22-trien-3β-ol (2) (ergosterol); 24-methyl-5α-cholesta-7,22-dien-3β-ol (3) (ergosta-7,22-dienol, stellasterol); 24-methyl-5α-cholest-7-en-3β-ol (4) (ergost-7-enol); 24-ethyl-5α-cholesta-7,22-dien-3β-ol (5) (chondrillasterol); 24-ethyl-5α-cholest-7-en-3β-ol (6) (22-dihydrochondrillasterol). The C-24 configurations were not determined. For the purpose of this report they are assumed to have the 24β-configuration for the assignment of trivial names. Ergost-7-enol was the predominant compound in both *C. caldarium* and *G. sulphuraria* (Table 1). There was a small difference in the sterol profiles for both algal species when grown in air or carbon dioxide. Our previous analyses

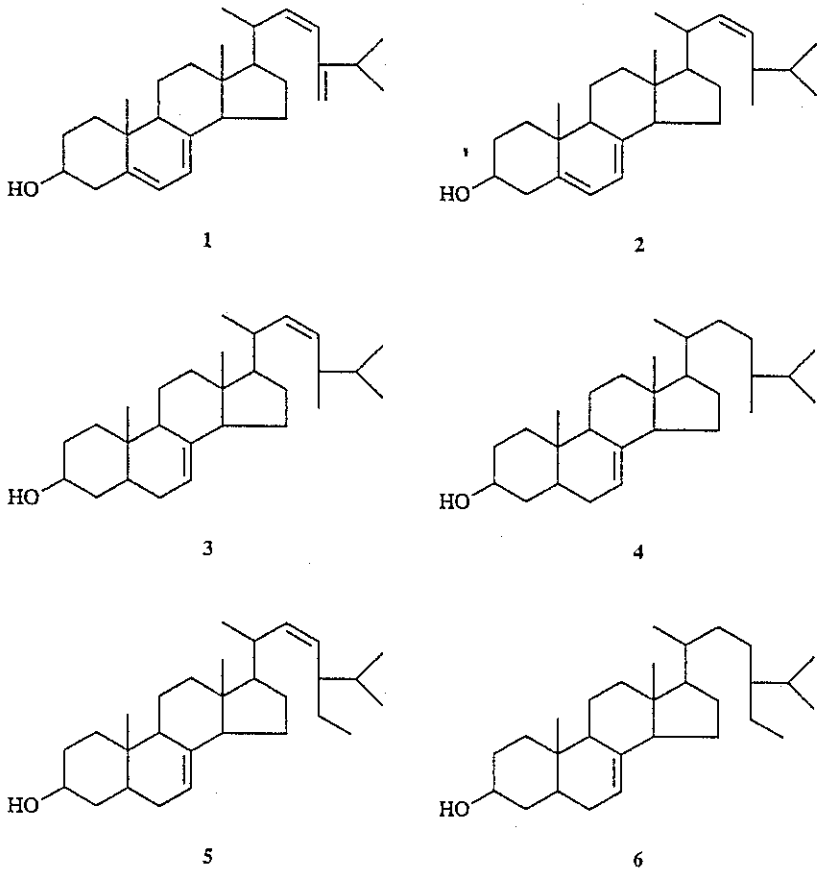

Table 1. Sterol content of thermo-acidophilic algae grown on air or pure carbon dioxide

	Air grown species		CO_2 grown species			
Algal sterol*	*Cc*	*Gs*	*Cc*	*Gs*	RR_t†	$[M]^+$ *m/z*
Ergosta-5,7,22,24(24')-tetraen-3β-ol (1)	9.43	6.16	5.09	6.91	1.43	466
Ergosterol (2)	0.23	3.77	2.59	9.33	1.47	468
Ergosta-7,22-dienol (3)	11.18	14.95	20.58	13.65	1.49	470
Ergost-7-enol (4)	20.68	61.82	40.78	36.65	1.58	472
Chondrillasterol (5)	27.44	6.77	13.27	15.27	1.62	482
22-Dihydro-chondrillasterol (6)	18.62	5.62	15.36	14.84	1.69	486
Unknown	11.81	0.91	2.33	3.35	1.76	—

*The sterol composition of *Cc* = *Cyanidium caldarium* and *Gs* = *Galdieria sulphuraria* is expressed in mol%. The sterols were analysed as the TMS ether derivatives.

†Relative retention times (RR_t) are relative to cholestane (1.00).

[3, 8] showed that ergosterol, ergosta-7,22-dienol, sitosterol and campesterol were the major sterol components. Cholesterol and 7-dehydrositosterol were reported [3, 8] at low concentration in lyophilized *C. caldarium* cells (which was then the only alga known in this class) but the cultures were harvested after growth for a few weeks (at their stationary phase) in a rich carbon dioxide atmosphere. For the present study the algal material was taken from very active (log phase) young cultures (see Experimental) and in contrast to our initial study, almost no cholesterol, sitosterol or campesterol were detected. However, trace amounts of unidentified compounds, which may be sterols, were noted. Klein and Cronquist [5] have commented that the relative concentrations of

Table 2. The distribution of algal sterols in the Cyanidiophyceae and their comparison with related algal divisions*

Sterol/algae†	1	2	3	4	5	6	Sito	Campe	Choi
C. caldarium and *G. sulphuraria*	+	+	+	+	+	+	−(nd)	−(nd)	−(nd)
C. caldarium 1972 analyses‡	—	+	+	—	—	—	+	+	+
Cyanobacteria	—	+	—	+	+	+	+	+	+
Rhodophyta	—	+	—	—	—	—	+	+	+
Chlorophyta	—	+	+	+	+	+	+	—	+

* + = presence; — = absence; nd = not determined; Choi = cholesterol; Sito = sitosterol; Campe = campesterol.
† See text and Table 1 for the numbers representing the various algal sterols.
‡ Includes 5,6-dihydroergosterol (ergosta-7,22-dienol) and 7-dehydrositosterol.

different sterols in algae may vary with the age and the method of cultivation and this may explain the differences observed between our two investigations.

Our combined sterol data suggest they may be phylogenetic markers and lend tentative support to the taxonomic position of the Cyanidiophyceae among the Rhodophyta or pre-rhodophytes [3, 8, 10–12]. The rhodophytes possess cholesterol, ergosterol, sitosterol and campesterol as their main constituents [1, 2, 4, 8, 13]. The sterols observed in *C. caldarium* and *G. sulphuraria* have also been recognized in cyanobacteria (Table 2) and this may somewhat support the transitional phylogenetic position of the acidothermophilic eukaryotes.

It is interesting that a similar sterol profile to the cyanidiophytes can also be observed in the Chlorophyta [4, 5, 13–15]. It is generally recognized that both algal groups (Cyanidiophyceae and Chlorophyceae) are not related and do not share a common phylogenetic origin. Pollio *et al.* [16] analysed the sterols of *Dunaliella acidophila*, a unicellular, biflagellate, wall-less green alga which is an acidophilic organism (as the Cyanidiaceae). However, *Dunaliella*, *Cyanidium* and *Galdieria* showed no common features in terms of sterols. The difference may be related to the fact that the two Cyanidiophyceae are thermophilic algae both possessing a heavy cell wall, while *Dunaliella* is a mesophilic chlorophyte which does not possess a cell wall.

The cyanidiophytes have been considered as a 'transitional algal group' bridging between the cyanobacteria and the lower red algae [7, 10–12, 17, 18]. Based on ribosomal RNA sequence studies, the Cyanidiophyceae can be regarded as an early evolved algal eukaryotic class. Because the Cyanidiophytes show primitive features among the Rhodophyta [10] and since the rhodophytes are the first [19], or among the earlier evolved photosynthetic nucleated organisms [20] as supported by rRNA sequencing, it is reasonable to conclude that the *Cyanidium* algal class is among the transitional group bridging prokaryotes and lower eukaryotic algae.

Although the sterol content revealed in the present study does not fully support the 'bridge' proposition, there are some indications in favour of the connection between the Cyanidiaceae from the one side, and the blue-green and red algae on the other side. For example, ergosterol was reported in three species of cyanobacteria [4] and in two *Porphyridium* (Rhodophyta) species [1, 4]. *Cyanidium* and *Galdieria* are closely related to *Porphyridium*-like rhodophytes and were placed in the order Porphyridiales or into the algal family Porphyridiaceae [17, 18]. Furthermore, chondrillasterol and 22-dihydrochondrillasterol (in addition to cholesterol, sitosterol and campesterol [1, 3, 8]) have been detected (Tables 1 and 2) in the cyanidiophytes as well as in cyanobacteria [4].

We detected 24-methylcholesta-5,7,22,24(24')-tetraen-3β-ol for the first time in an algal division. This compound has been found in fungi and particularly in yeast [21] and in the protozoan *Tetrahymena pyriformis* [22], and has been isolated from the insect *Tribolium confusum* [23]. We believe that this sterol was a constituent of the Cyanidiophyceae and does not reflect any source of contamination because (a) our algal inocula were always taken from actively growing axenic cultures; (b) we checked them often by microscopy and electron microscopy and (c) the algae were often washed with 0.5 M sulphuric acid and they were grown in very acidic media (pH 2) and at elevated temperature level (45°), where the chances for contamination are extremely remote. It is believed that sterol molecules have evolved in an oxygenated atmosphere and that their biosynthesis requires aerobic conditions [24, 25]. Such aerobic conditions were provided in the air grown cyanidiophycean cells. Since the sterol content was not lowered by growth in carbon dioxide atmosphere and in some cases the content was even higher than in air cultures (Table 1) we assume that during the continued illumination period the photosynthetically evolved oxygen was sufficient for sterol synthesis. Seckbach *et al.* [6] demonstrated that released photosynthetic oxygen is greater in carbon dioxide grown *Cyanidium* than in air cultured *Cyanidium*. On the other hand, when the peroxisomal activities were examined in *Cyanidioschyzon* (the most primitive member of the Cyanidiophyceae) cultured under pure carbon dioxide a sharp reduction in these activities was observed [7]. A similar drastic reduction has been observed in ethylene

production in the Cyanidiophyceae when cultured with pure carbon dioxide (Seckbach, J. and Starrett, D., unpublished results).

EXPERIMENTAL

Algal material and growth conditions. The algal growth and initial lipid analyses were performed at the Department of Biology and Institute of Geophysics and Planetary Physics of the University of California at Los Angeles. The lipid fractions of the algal extracts were re-analysed at the University of Tennessee/Oak Ridge National Laboratory, TN.

Active growing cultures (inoculated with algal suspensions which was removed successively ×3 from exponential phase) of the unicellular acido-thermophilic algae *C. caldarium* and *G. sulphuraria* were re-suspended in double strength mineral media [26] (supplemented with 5 ppm Fe as FeEDTA and acidified with H_2SO_4 to pH 2–3). Cells were autotrophically cultured in 2–20 l glass flasks and the suspensions were agitated with magnetically driven stirring bars and maintained at 45–48° inside conditional controlled incubators. Continuous illumination was provided from fluorescent tubes supplying an intensity of 15–30 $\mu E\ m^{-2}\ sec^{-1}$. Cultures were aerated with either a stream of air or with pure carbon dioxide (gases refiltered and humidified). After 7–14 days of intensive growth, the warm algal vessels (covered with aluminium foil or black cloth) were placed in the cold room overnight or up to 2 days. The upper layer of the growing medium was then decanted and the pptd cells were harvested by centrifugation at low speed (2000 *g* for 10 min). The pellets were washed with a fresh nutrient medium followed by H_2O, then re-centrifuged and stored in the deep freezer until analyses.

Lipid analysis. A known weight of wet algal material (1–2 g dry wt equiv.) was refluxed with 1 M KOH (in MeOH–H_2O, 19:1) for 4 hr and filtered though GF/C millipore. The nonsaponifiable fraction containing the sterols (and neutral compounds such as hydrocarbons and alcohols) was extracted with hexane and the aq. layer extracted with CH_2Cl_2 and added to the hexane extract. After acidification of the aq. soln (saponifiable fraction) the fatty acids were extracted with Et_2O. Both fractions were purified by TLC on silica gel. The non-saponifiable fraction served for sterol analyses.

Sterol analyses. The sterols in the non-saponifiable fraction were converted to trimethylsilyl ether derivatives by BSTFA [*N,O*-bis(trimethylsilyl) trifluoroacetamide +1% trimethylchlorosilane] in pyridine at 80° for 15 min. The TMS ethers of the sterols were examined by using a VG Trio-3 (3000 mass range) GC/MS with EI. A Resteck Rtx-5, 30 m length, 0.25 mm i.d. column was used. Temperature programming (GC) was from 200 to 310°, at average rate of 10° min^{-1} to 280° then 2° min^{-1} to 310°. The injector temp. and detector temp. were both set at 290°. Mass spectral parameters were electron current at 200 μA and electron energy at 70 eV.

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