Taxonomic Implication of Sterol Composition in the Genus *Chlorella*[1]

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ABSTRACT

Thirty-five isolates of the genus *Chlorella* were grown under standardized conditions and analyzed for sterol composition. Six different sterol synthetic patterns were found. Two patterns were characterized by nuclear unsaturation at C-5 and one type by nuclear unsaturation at C-7. The remaining isolates all synthesize sterols with a diunsaturated nucleus (C-5 + C-7) with various modifications. Subtype 1 synthesizes only C_{28} sterols, subtype 2 produces C_{28} and C_{29} sterols, and subtype 3 (1 isolate) produces C_{28} sterols with nuclear unsaturation at C-5 + C-8 in addition to the $\Delta^{5,7}$ C_{28} sterols seen in the first 2 subtypes. Comparison of the sterol composition of strains shared in common with taxonomic researchers showed good correlation with the taxonomic scheme of Fott and Novakova which has been confirmed by Kessler and coworkers using other biochemical markers.
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INTRODUCTION

Species of the genus *Chlorella* are nonmotile, unicellular algae belonging to the Chlorophyta or green algae. The genus is characterized by only a few distinguishing structural features including cell morphology (spherical or ellipsoidal), chloroplast size and shape, and presence or absence of pyrenoid. Sexual reproduction is unknown and vegetative reproduction is by autospore production; 2-32 autospores are derived from successive divisions of the nucleus and single chloroplast. With the completion of wall formation, these minatures of adult cells are simultaneously released through rupture of the parent cell wall.

Chlorella is ubiquitous in nature and has been isolated in diverse aquatic and aerial habitats and as symbionts in certain animals. Many members of this genus are highly adaptable to life under marginal conditions. Explosive growth rates under good conditions have earned it a reputation as an algal weed. *Chlorella* was first isolated and the genus described by a microbiologist, Beyerinck. Bold and Wynne suggest that *Chlorella* was probably the first alga to be grown extensively in axenic culture (1). Partly due to the ease of growth and manipulation, *Chlorella* has found extensive use in research in plant physiology. However, due to the minuteness of size where significant subcellular structures are at the limit of resolution of light microscopy and the few available distinguishing features, the identification of an individual isolate is difficult. The result is multiple species designations for the same isolate in different culture collections. The problem of proper identification is particularly acute due to the diversity of biochemical properties and physiological reactions found in different isolates. The latter has been used extensively as a basis for taxonomic schemes.

The algae, as a group, are known for a greater diversity in sterol production than any other group of organisms. It has been suggested that characterization of sterol composition can be used as a tool in biochemical taxonomy and in the establishment of phylogenetic relationships (2). Genera in some algal divisions are united by sterol synthetic patterns, but others, particularly the green algae, are characterized by varied sterol synthesis (3). *Chlorella* is unique in the variety of biosynthetic patterns encountered in members of one genus. Previous work with *Chlorella* species had shown that individual isolates synthesize a Δ^5, Δ^7, or $\Delta^{5,7}$ series of sterols (4-6). It was of interest to investigate both the sterol composition of a large number of isolates and the value of these data in the taxonomy of the genus.

METHODS

Chlorella isolates were grown axenically and heterotrophically in 15-ℓ carboys with glucose as a carbon source. Carboys were bubbled with filtered, compressed air and checked daily for contamination. Cells were harvested at the end of log phase growth with a Sharples Super Centrifuge and freeze-dried. Algal material was stored at -20 C until analysis. Ten g of freeze-dried cells were extracted with chloroform/methanol (2:1, v/v) using a Soxhlet. The crude lipid extract was saponified with alcoholic KOH (20% in 60% EtOH) and the unsaponifiable matter extracted into ether using a liquid-liquid extraction apparatus. Sterols were purified

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using alumina column chromatography. Separation of sterol components was effected by the use of $AgNO_3$/silicic acid columns (12% w/w) with a graded series of ether in hexane (4, 5, 6, 7, 8, 9, 16, 20 and 90%). Steryl acetates, prepared using acetic anhydride/pyridine overnight in the dark, were chromatographed on the $AgNO_3$/silicic acid column and separated according to the number and position of the double bonds.

Sterols were identified and quantitated by gas liquid chromatography (GLC) using a Varian Model 3700 gas chromatograph equipped with a Varian CDS 111 data system. The 6-ft glass column was packed with SE-30 (3%) on 100/120 mesh Gas Chrom Q. Sterols were chromatographed using helium as a carrier gas at 245 C and 20 ml/min. Retention times relative to cholesterol were calculated for unknowns and compared to appropriate authentic standards run in conjunction with the unknown. Mass spectral analyses were conducted on representative samples using an LKB 9000 GC/MS equipped with a Varian SS-100 mass spectra data system. The 0.75% SE-30 column was operated at 230 C. The ionizing energy was 70 eV.

RESULTS AND DISCUSSION

Analysis of the isolates belonging to the genus *Chlorella* showed 6 different patterns of sterol synthesis. Isolates belonging to each of these 6 groups produced component sterols in similar proportions.

Chlorella Group IA

Five of the analyzed isolates (see Table 1) synthesize sterols characterized by a single nuclear unsaturation at C-5. A small but consistent amount of cholesterol was found in these isolates, but the other component sterols were alkylated at C-24, producing both C_{28} and C_{29} sterols. The major sterol produced in this group was the diunsaturated C_{29} sterol, poriferasterol, followed by the C_{28} sterol, 5-ergostenol and the C_{29} sterol clionasterol. These sterols are the 24β epimers of the commonly found series of higher plant sterols, campesterol, stigmasterol and sitosterol.

Group IB

The 2 isolates classified as *Chlorella* IB show a related composition, but one that differs in 2 aspects. The major product of sterol synthesis was 5-ergostenol (69-73% of total sterol) and significant amounts of the C_{29} homolog of ergosterol, 7-dehydroporiferasterol, was also found.

Chlorella Group II

The remaining 28 isolates lack, to some extent, the biosynthetic capacities of the previously discussed isolates. Seven isolates synthesize a series of sterols that are the Δ^7 isomers of the Δ^5-sterols of group IA. There is no Δ^7 equivalent of cholesterol found, however. These isolates alkylate at C-24 exclusively rather than reducing the 24(25) bond of precursor sterols. The Δ^7 sterols produced by these isolates are found in similar proportions to the Δ^5 isomers of group IA, with the major sterol a Δ^{22}, C_{29} sterol, chondrillasterol. The major biosynthetic difference between the group II isolates and those of group I is the inability of group II to introduce the Δ^5 bond, presumably due to the absence of a Δ^5-dehydrogenase.

Chlorella Group IIIA

The largest group, 15 isolates, produced the group IIIA series of sterols. Inspection of their structures shows 2 major biosynthetic differences between these algae and groups I and II. These organisms lack the Δ^7 reductase for the removal of the double bond at C-7 after the introduction of the bond at C-5, the accepted series of biosynthetic events (7). In addition, these isolates lack the ability to introduce a second alkyl group at C-24 resulting in the production, exclusively, of the C_{28} sterols, ergosterol and its mono- and diunsaturated companion sterols, in which ergosterol is the major sterol produced. Biosynthetic studies using the hypocholesteremic drugs Triparanol and AY-9944 result in a build-up of precursors in these isolates. Analysis of AY-9944 inhibition products of treated *C. sorokiniana* (UTEX #1230) showed no 24-methylene intermediates that are believed to be precursors of the 24-ethyl sterols in algae (8). Evidence for the intermediacy of these 24-methylene sterols has been provided by Tomita et al. (9,10) and Tsai and Patterson with the use of labeled compounds in *Chlorella* (11) and by Goad et al. (12) with *Scenedesmus* and *Trebouxia*. However, 24-methylene intermediates have been demonstrated in treated cultures of the C-29-producing *Chlorella* group I and II isolates, *C. emersonii* and *C. ellipsoidea* (13-15). Moreover, the treated *C. sorokiniana* cultures produced 4α,14α-dimenthyl-5α-ergosta-8,25-dienol, indicating the role of 25-methylene sterols in the production of C-28 sterols (8).

Group IIIB

Group IIIB isolates lack the same Δ^7 reductase as group IIIA, resulting again in the production of ergosterol and 5,7-ergostadienol. However, they do have the ability to introduce the

TABLE 1

Component Sterols and Ranges of Sterol Composition in the 6 *Chlorella* Groups

Systematic name (all 3β-ol)	Trivial name	% Composition		
		Group IA	Group IB	
Cholest-5-enol	Cholesterol	1-2	0-1	
24β-Methylcholesta-5,22-dienol	Brassicasterol	1-5	1	
24β-Methylcholest-5-enol	5-Ergostenol	28-34	69-73	
24β-Ethylcholesta-5,22-dienol	Poriferasterol	55-66	10-12	
24β-Ethylcholest-5-enol	Clionasterol	1-8	a	
24β-Ethylcholesta-5,7,22-trienol	7-Dehydroporiferasterol	–	9[a]	
		Group II		
24β-Methylcholesta-7,22-dienol	7,22-Ergostadienol	1-4		
24β-Methylcholest-7-enol	7-Ergostenol	20-28		
24β-Ethylcholesta-7,22-dienol	Chondrillasterol	45-68		
24β-Ethylcholest-7-enol	7-Chondrillastenol	5-27		
		Group IIIA	Group IIIB	Group IIIC
24β-Methylcholesta-5,7,22-trienol	Ergosterol	32-80	23-41	53
24β-Methylcholesta-5,7-dienol	5,7-Ergostadienol	>32-63[b]	>13-35[b]	>13[b]
24β-Methylcholest-7-enol	7-Ergostenol			
24β-Ethylcholesta-7,22-dienol	Chondrillasterol	–	>29-37[c]	–
24β-Ethylcholesta-5,7,22-trienol	7-Dehydroporiferasterol			
24β-Ethylcholest-7-enol	7-Chondrillastenol	–	3-14	–
24β-Methylcholesta-5,8-dienol	5,8-Ergostadienol	–	–	1
24β-Methylcholesta-5,8,22,-trienol	5,8,22-Ergostatrienol	–	–	30

[a]Combined peak—clionasterol seen as a shoulder on the back side of the 7-dehydroporiferasterol.

[b]Combined peak—difficult to separate due to similar retention times; 7-ergostenol is the major component in most cases.

[c]Combined peak, with 7-dehydroporiferasterol the major component.

second alkyl group at C-24, producing the C-29 homolog of ergosterol, 7-dehydroporiferasterol, plus chondrillasterol and 7-chondrillastenol. The presence of C_{29} sterols in ergosterol synthesizing organisms is a rare occurrence. While 7-dehydroporiferasterol has been found in conjunction with ergosterol in *Euglena gracilis* (16), *Ochromonas danica* (17), and one strain of *Chlamydomonas reinhardii* (18), most ergosterol synthesizing species produce only C_{28} sterols as do *Chlorella* group IIIA (7). This is sufficiently widespread that the possibility has been suggested by Nes and Nes (19) of genetic coupling between production of ergosterol and the inability to introduce the second alkyl group.

Group IIIC

The last type of sterol biosynthetic pattern is encountered in only one strain of *Chlorella*. This previously published work (20) was repeated here and the same unique $\Delta^{5,8}$ and $\Delta^{5,8,22}$ sterols were found along with ergosterol, 5,7-ergostadienol, and 7-ergostenol. No second alkylation step occurs in this strain as only C_{28} sterols are produced. But the presence of the $\Delta^{5,8}$ bonds are difficult to explain because the presence of the double bond at C-7 is considered necessary for the introduction of the double bond at C-5 (7). It is either unnecessary in this strain or, perhaps, one is seeing a high degree of reversibility in the $\Delta^8 \rightarrow \Delta^7$ isomerase.

Sterols and Taxonomy

The analysis of a large number of isolates of *Chlorella* uncovered more patterns of sterol synthesis than previously suspected. This suggested more strongly than before the possibility of the use of sterols as a taxonomic marker. Three groups have worked extensively on the taxonomic classification of this genus. All 3 recognized that members of the genus exhibited morphological and physiological diversity between isolates and variability in the behavior of an individual isolate under differing environmental conditions. The 3 groups of researchers accordingly adopted their own standardized conditions, but approaches differed from this point. Shihira and Krauss combined morphological and physiological criteria with the emphasis on physiological (21). Responses to carbon and nitrogen sources were graded and, at times, small differences were considered justification for creation of separate taxa. Observation of 41

isolates led to the creation of 22 species and 8 varieties (21). Fott and Novakova took a more classical morphological approach (22). Fifteen taxa, 9 species and 6 varieties, were defined on the basis of observations of cellular structure. Aspects of the life cycle and reproductive behavior were studied. They agreed with Shihira and Krauss on the necessity for biochemical markers but disagreed with the choice of markers. Kessler and coworkers initially ignored cellular structure and defined groups of related isolates on the basis of biochemical and physiological characters, e.g., hydrogenase activity, formation of secondary carotenoids, pH tolerance and thermophily (23). Agreement was found between Kessler's groups and Fott and Novakova's species, leading to a more complete definition of the various taxa.

In Table 2, the analyzed isolates are grouped according to their sterol composition. Table 3 summarizes the characteristic sterol pattern encountered in those *Chlorella* species defined by Fott and Novakova. Representatives of 6 of the 9 Fott and Novakova species were examined in this study. The *Chlorella* IB and IIIC strains were not studied by Fott and Novakova. The sterol data for these strains, along with other physiological and biochemical work on these isolates, suggest that these strains may comprise yet additional taxa (24 and E. Kessler, personal communication). A subsequent paper will deal in more detail with taxonomic aspects of *Chlorella*. The DNA hybridization studies of Kerfin and Kessler have suggested that *Chlorella* is a genus comprised of biochemically and genetically diverse species united by a common simple morphology (25). The variation in sterol synthetic capacity encountered in this genus underscores this diversity. Sterol composition can serve as a stable marker in the taxonomic definition of an isolate or can serve to set it apart from other defined strains.

TABLE 2

Chlorella Isolates Arranged According to Sterol Composition

	Isolate #
Group IA	
C. saccharophila (Kruger) Nadson[a]	27[b]
C. ellipsoidea Gerneck	247[b]
C. variegata Beij.	257[b]
C. saccharophila (Kruger) Nadson	211/1a[c]
C. saccharophila (Kruger) Nadson	211/1d[c]
Group IB	
C. anitrata S. & K.	1798[b]
C. anitrata var. *minor* S. & K.	1799[b]
Group II	
C. emersonii S. & K.	2[d]
C. pyrenoidosa Chick	251[b]
C. pyrenoidosa Chick[e]	343[b]
C. glucotropha S. & K.	1802[b]
C. pyrenoidosa var. *chick* S. & K.	1806[b]
C. regularis var. *minima* S. & K.	1807[b]
C. emersonii var. *rubescens* Fott et al.	232/1[c]
Group IIIA	
C. pyrenoidosa Chick	26[b]
C. vulgaris var. *viridis* Chodat	30[b]
C. vulgaris Beij.	259[b]
C. vulgaris Beij.	261[b]
C. vulgaris Beij.	265[b]
C. vulgaris var. *viridis* Chodat	396[b]
C. pyrenoidosa Chick	1230[b]
C. infusionum var. *acetophila* S. & K.	1803[b]
C. parva S. & K.	1805[b]
C. vulgaris f. *tertia* F. & N.	211/31[f]
C. salina Butcher	1809[b]
C. sorokiniana var. *pacificiensis* S. & K.	1810[b]
C. vaniellii S. & K.	1811[b]
C. vulgaris f. *tertia* F. & N.	211/40a[f]
C. vulgaris f. *tertia* F. & N.	1/9/30[g]
Group IIIB	
C. vulgaris Beij.	262[b]
C. vulgaris Beij.	397[b]
C. vulgaris Beij.	398[b]
C. vulgaris Beij.	263[b]
C. sp.	580[b]
Group IIIC	
C. ellipsoidea Gerneck	246[b]

[a]Culture names are those assigned by the culture collections.

[b]UTEX # University of Texas at Austin, Culture Collection of Algae.

[c]CCAP # Cambridge Collection of Algae and Protozoa.

[d]MCC # Maryland Culture Collection.

[e]Fott et al. removed this strain from the genus *Chlorella* and suggested it be considered a species of the genus *Scenedesmus* (27).

[f]Gottingen Collection.

[g]High Temperature Strain of Sorokin (26).

TABLE 3

Sterol Composition and *Chlorella* Taxonomy

Sterol biosynthetic pattern	Fott & Novakova species
Group IA	*C. saccharophila*
	C. luteoviridis
Group II	*C. emersonii* var. *vacuolata* (*C. fusca* var. *vacuolata*)[a]
	C. emersonii var. *rubescens* (*C. fusca* var. *rubescens*)[a]
Group IIIA	*C. vulgaris*
	C. sorokiniana (*C. vulgaris* f. *tertia*)[b]
Group IIIB	*C. Kessleri*

[a]Species designation of Shihira and Krauss and adopted by Fott and Novakova in their monograph (22). Species designation determined invalid in 1975 (27).

[b]Species designation assigned by Fott and Novakova later changed.

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