Sterol Chemotaxonomy of Marine Pelagophyte Algae

by **José-Luis Giner***[a]), **Hui Zhao**[a]), **Gregory L. Boyer**[a]), **Michael F. Satchwell**[a]), and **Robert A. Andersen**[b])

[a]) Department of Chemistry, State University of New York-ESF, Syracuse, NY 13210, USA (phone: +1-315-470-6895; fax: +1-315-470-6856 ; e-mail: jlginer@syr.edu)

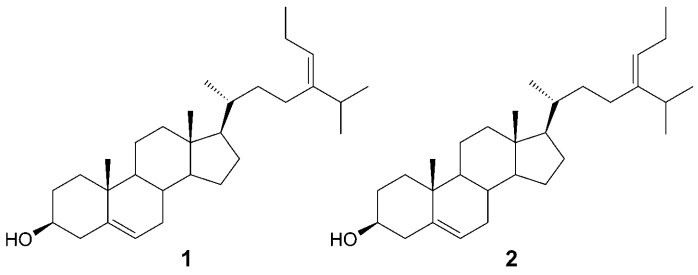
[b]) Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575, USA

Dedicated to Professor *Carl Djerassi* on the occasion of his 85th birthday

Several marine algae of the class Pelagophyceae produce the unusual marine sterol 24-propylidenecholesterol, mainly as the (24*E*)-isomer. The (24*Z*)-isomer had previously been considered as a specific biomarker for *Aureococcus anophagefferens*, the 'brown tide' alga of the Northeast coast of the USA. To test this hypothesis and to generate chemotaxonomic information, the sterol compositions of 42 strains of pelagophyte algae including 17 strains of *Aureococcus anophagefferens* were determined by GC analysis. A more comprehensive sterol analysis by HPLC and ^{1}H-NMR was obtained for 17 selected pelagophyte strains. All strains analyzed contained 24-propylidenecholesterol. In all strains belonging to the order Sarcinochrysidales, this sterol was found only as the (*E*)-isomer, while all strains in the order Pelagomonadales contained the (*Z*)-isomer, either alone or together with the (*E*)-isomer. The occurrence of Δ^{22} and 24α-sterols was limited to the Sarcinochrysidales. The first occurrence of Δ^{22}-24-propylcholesterol in an alga, CCMP 1410, was reported. Traces of the rare sterol 26,26-dimethyl-24-methylenecholesterol were detected in *Aureococcus anophagefferens*, and the (25*R*)-configuration was proposed, based on biosynthetic considerations. Traces of a novel sterol, 24-propylidenecholesta-5,25-dien-3β-ol, were detected in several species.

Introduction. – The C_{30} marine sterol, 24-propylidenecholesterol, is generated in an unusual and mechanistically interesting biosynthetic reaction that involves a protonated cyclopropane [1][2]. The first isolation of this sterol was from the scallop *Placopecten magellanicus*, where it was found as a mixture of the (24*E*)- and (24*Z*)-isomers (**1** and **2**, resp.) [3–5]. Its presence in this and other marine invertebrates is thought to reflect dietary input. The (*E*)-isomer **1** was subsequently detected in two marine algae, *Chrysoreinhardia* sp. [6], and *Nematochrysopsis roscoffensis* [7]. Later, a mixture of **1** and **2** was found in *Aureococcus anophagefferens* [8], the species responsible for the 'brown tides' that have decimated the scallop fisheries of the Northeast coast of the USA [9]. Another harmful alga, *Aureoumbra lagunensis*, the cause of the 'Texas brown tide' along the coast of the Gulf of Mexico, was found to contain **1** [10].

All of the algae that make 24-propylidenecholesterol belong to a single class, the Pelagophyceae, which is comprised of the orders Pelagomonadales and Sarcinochrysidales [11–14]. These small unicellular marine algae were formerly grouped in the Chrysophyceae, and include species that grow on the surface of substrates in shallow waters (*Sarcinochrysis marina* and *Chrysoreinhardia* sp.), algae that form harmful



blooms in coastal waters (*A. anophagefferens* and *A. lagunensis*), and open-ocean picoplanktonic species that are important to global carbon fixation (*Pelagomonas calceolata* and *Pelagococcus subviridis*) [15–17]. Sterols have long been recognized as useful characteristics for distinguishing algal groups [18][19], and it was proposed that all pelagophyte algae contain 24-propylidenecholesterol [8]. It was also proposed that the (*Z*)-isomer **2** was specific to *Aureococcus anophagefferens* and could be regarded as a biomarker for this species [8]. To test these hypotheses, a survey of the sterol compositions of 42 strains of Pelagophyte algae including 17 strains of *Aureococcus anophagefferens* was carried out.

Results and Discussion. – 1. *GC Analysis.* Quantitative gas-chromatographic analysis was carried out for the sterols of 17 strains of *Aureococcus anophagefferens* (*Table 1*), twelve strains of other members of the Pelagomonadales (*Table 2*), and 13 strains from the Sarcinochrysidales (*Table 3*). The sterol identities were assigned by comparison of their retention times with those of known standards.

To assist in the interpretation of the large amount of information, the method to display the data graphically was developed based on biosynthetic parameters (*Fig. 1*). In the biosynthetic modification of sterol side chains [2], successive methylation reactions of *S*-adenosylmethionine (SAM)-dependent sterol methyltransferases convert C_{27} sterols to C_{28} sterols, C_{28} sterols to C_{29} sterols, and C_{29} sterols to C_{30} sterols (*Scheme 1*). These sterols can then undergo subsequent modification by redox enzymes whereby the side-chain C=C bond is first hydrogenated by a sterol side-chain reductase, and a new C=C bond is introduced at the 22-position by a Δ^{22} sterol desaturase. Two parameters were calculated for the total sterols of each organism, the methylation degree (MD) and reduction-oxidation degree (ROD). These parameters were defined according to the equations:

$$\text{Methylation degree (MD)} = 1 \times \%\ C_{28}\ \text{sterols} + 2 \times \%\ C_{29}\ \text{sterols} + 3 \times \%\ C_{30}\ \text{sterols}$$

$$\text{Reduction–oxidation degree (ROD)} = 1 \times \%\ \text{sterols with saturated side chains} + 2 \times \%\ \text{sterols with } \Delta^{22}\text{-side chains}$$

1.1. *Aureococcus anophagefferens.* For the brown tide alga, *Aureococcus anophagefferens*, the major sterols found by GC analysis were 24-methylenecholesterol (**19**), 24-methylcholesterol (**20**/**21**), (*E*)-24-propylidenecholesterol (**1**), and (*Z*)-24-propyli-

Table 1. *Sterol Compositions of Algae of the Pelagomonadales by GC Analysis.* Part I, *Aureococcus anophagefferens.*

Sterol[a])	RRT[b])	Percentage of Total Sterols																
		1706[c]) Aa[d])	1707 Aa	1708 Aa	1784 Aa	1785 Aa	1789 Aa	1790 Aa	1791 Aa	1794 Aa	1847 Aa	1848 Aa	1849 Aa	1850 Aa	1851 Aa	1852 Aa	1853 Aa	1854 Aa
24MC	1.14	5.0	4.7	50.0			9.8			35.5		78.6	48.5		6.3	35.4	65.2	43.8
24Me	1.15	36.6	30.5	20.2	30.6	46.0	13.1	36.2	51.0	27.0	51.2		24.4	31.1	35.6	32.3	12.4	31.6
24Et	1.32	1.5	4.0		2.4	4.0	12.6	7.0	7.2		3.2			12.8	6.7		7.7	
24EC	1.35	1.5	3.1		1.8		23.8	3.5		3.0	3.3	4.2	3.8	3.6	5.3	3.4	6.3	2.3
24EP	1.44	42.3	43.5	20.2	52.8	40.6	25.5	41.8	33.8	19.6	31.6	13.4	17.1	40.3	36.7	22.5	8.4	17.4
24ZP	1.50	13.1	14.2	9.6	12.4	9.4	15.2	11.5	8.0	14.9	10.7	3.8	6.2	12.2	9.4	6.4		4.9
Total sterol [μg]		15.9	15.7	7.4	18.2	2.9	35.2	11.4	2.7	7.5	4.6	20.2	10.5	21.9	13.0	17.0	3.3	19.6
MD[e])[f])		2.14	2.23	1.60	2.35 (2.14)	2.04 (2.29)	2.18 (2.03)	2.17	1.91	1.72	1.91 (2.21)	1.39 (2.05)	1.50	2.22 (2.29)	2.04	1.61	1.31 (1.98)	1.47
ROD[e])[f])		0.38	0.35	0.2 (0)	0.33 (0.18)	0.50 (0.14)	0.26 (0.25)	0.43	0.58	0.27	0.54 (0.21)	0.00 (0.06)	0.24	0.44 (0.21)	0.42	0.32	0.20 (0.03)	0.32
(*E*)/(*Z*)[e])[f])		3.2	3.1	2.1	4.3 (5.8)	4.3 (4.5)	1.7 (3.4)	3.6	4.2	1.4	3.0 (3.4)	3.5 (3.7)	2.8	3.3 (4.4)	3.9	3.5	∞ (2.9)	3.6

[a]) Abbreviations of sterols: 24MC = 24-methylenecholesterol (**19**); 24Me = 24-methylcholesterol (**20**/**21**); 24Et = 24-ethylcholesterol (**14**/**15**); 24EC = 24-ethylidenecholesterol (**12**/**13**); 24EP = (*E*)-24-propylidenecholesterol (**1**); 24ZP = (*Z*)-24-propylidenecholesterol (**2**). [b]) RRT = GC Retention time relative to cholesterol. [c]) CCMP Strain number (*Provasoli–Guillard* National Center for Culture of Marine Phytoplankton). [d]) Species of algae: Aa = *Aureococcus anophagefferens.* [e]) MD = Methylation degree (see text). ROD = Reduction–oxidation degree (see text). (*E*)/(*Z*) = Ratio of the (24*E*)- *vs.* (24*Z*)-isomers of 24-propylidenecholesterol (**1** *vs.* **2**). [f]) Values in parentheses were obtained from HPLC analysis (see *Table 5*).

Table 2. *Sterol Compositions of Algae of the Pelagomonadales by GC Analysis.* Part II, *Pelagomonas* and *Pelagococcus.*

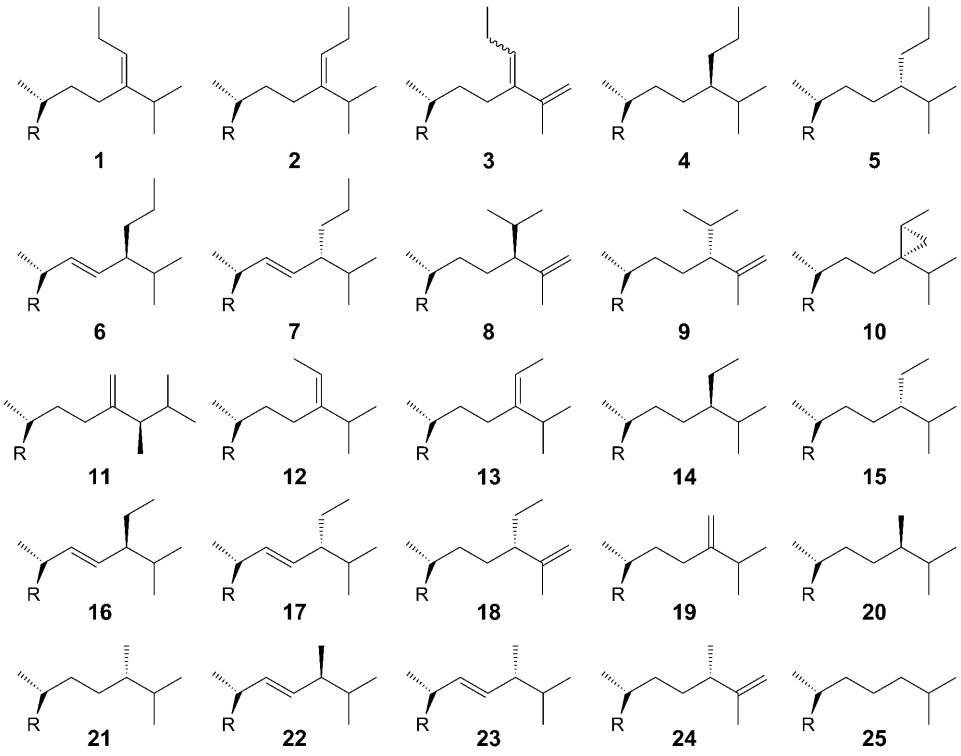
Sterol[a])	RRT[b])	Percentage of Total Sterols											
		1602[c]) Pc[d])	1603 Pc	1682 Pc	1756 Pc	1864 Pc	1865 Pc	1954 Pc	2779 Pc	1145 Pcc	1429 Ps	1252 Psp	1395 Psp
Ch	1.00									2.3			
Δ^{22}-24Me	1.06							34.4					
24MC	1.14	58.8	60.2	62.9	57.1	80.2	66.0	48.0	60.5			17.1	
24Me	1.15									50.4	46.3		15.7
Δ^{22}-24Et	1.21							2.2			3.2		2.4
24Et	1.32	2.1	2.2		1.9					31.8	34.1	14.6	70.3
24EC	1.35	4.4	7.7	6.0	18.1	4.3	4.6	7.7	5.5			20.3	3.4
24EP	1.44									12.9	11.3	38.0	4.9
24ZP	1.50	34.7	29.9	31.1	22.9	15.5	29.4	7.7	34.0	2.6	5.1	10.0	3.3
Total sterol [μg]		5.0	15.4	14.6	7.0	10.5	9.9	3.4	30.0	12.1	3.7	1.6	3.9
MD[e])[f])		1.76	1.70 (1.41)	1.68	1.66 (1.87)	1.35	1.63	1.25 (1.33)	1.74 (1.57)	1.61 (1.54)	1.70 (2.02)	2.31 (1.41)	1.93 (1.92)
ROD[e])[f])		0.02	0.02 (0.01)	0.00	0.02 (0.02)	0.00	0.00	0.73 (0.00)	0.00 (0.00)	0.84 (0.68)	0.87 (0.71)	0.15 (0.01)	0.91 (0.88)
(*E*)/(*Z*)[e])[f])		0.0	0.0 (0.04)	0.0	0.0 (0.01)	0.0	0.0	0.0 (0.0)	0.0 (0.0)	5.0 (4.1)	2.2 (5.0)	3.8 (3.3)	1.5 (2.0)

[a]) Abbreviations of sterols: Ch = cholesterol (**25**); Δ^{22}-24Me = Δ^{22}-24-methylcholesterol (**22/23**); 24MC = 24-methylenecholesterol (**19**); 24Me = 24-methylcholesterol (**20/21**); Δ^{22}-24Et = Δ^{22}-24-ethylcholesterol (**16/17**); 24Et = 24-ethylcholesterol (**14/15**); 24EC = 24-ethylidenecholesterol (**12/13**); 24EP = (*E*)-24-propylidenecholesterol (**1**); 24ZP = (*Z*)-24-propylidenecholesterol (**2**). [b]) RRT = GC Rretention time relative to cholesterol. [c]) CCMP strain number (*Provasoli–Guillard* National Center for Culture of Marine Phytoplankton). [d]) Species of algae: Pc = *Pelagomonas calceolata*; Pcc = *Pelagomonas* cf. *calceolata*; Ps = *Pelagococcus subviridis*; Psp = *Pelagococcus* sp. [e]) MD = Methylation degree (see text). ROD = Reduction–oxidation degree (see text). (*E*)/(*Z*) = Ratio of the (24*E*)- *vs.* (24*Z*)-isomers of 24-propylidenecholesterol (**1** *vs.* **2**). [f]) Values in parentheses were obtained from HPLC analysis (*Table 5*).

Table 3. *Sterol Compositions of Algae of the Sarcinochrysidales by GC analysis*

Sterol[a])	RRT[b])	Percentage of Total Sterols												
		1502[c]) Al[d])	1503 Al	1504 Al	1505 Al	1507 Al	1508 Al	1509 Al	1510 Al	1681 Al	1410 Co	292 Csp	770 Sm	1664 Sm
Ch	1.00	13.7	12.5	11.1	13.4	11.4	15.0	14.5	17.0	15.8	11.7			
Δ^{22}-24Me	1.06	1.8					2.4	3.2	2.2	1.4	5.0	20.9	6.9	36.3
24MC	1.14							1.1						
24Me	1.15	3.4				4.2	3.8	6.4	2.8	2.3	2.3	11.2	26.7	6.6
22Et	1.21	35.6	23.7	18.5	25.8	34.2	31.9	29.5	41.8	32.2	33.7	6.0	5.8	2.5
24Et	1.32	9.0	14.0	13.4	12.0	19.4	7.9	10.2	6.6	11.2	5.5	7.6	25.1	3.0
24EC	1.35										2.1			
24EP	1.44	22.8	39.7	46.6	34.8	19.5	25.5	25.9	16.7	26.9	33.8	54.3	32.4	51.6
24Pr	1.48	13.7	10.1	10.4	14.0	11.3	13.5	9.2	12.9	10.2	5.9		3.1	
Total sterol [μg]		145.4	166.3	287.3	158.2	268.2	82.2	84.4	65.4	77.3	32.9	1.9	2.3	5.4
MD[e])[f])		2.04	2.25	2.35	2.22	2.04	2.03	1.95	1.91	2.02	2.09 (2.13)	2.22	2.0	2.09
ROD[e])[f])		1.15	0.84	0.72	0.91	1.15	1.09	1.06	1.27	1.07	1.03 (0.94)	0.73	0.80	0.87

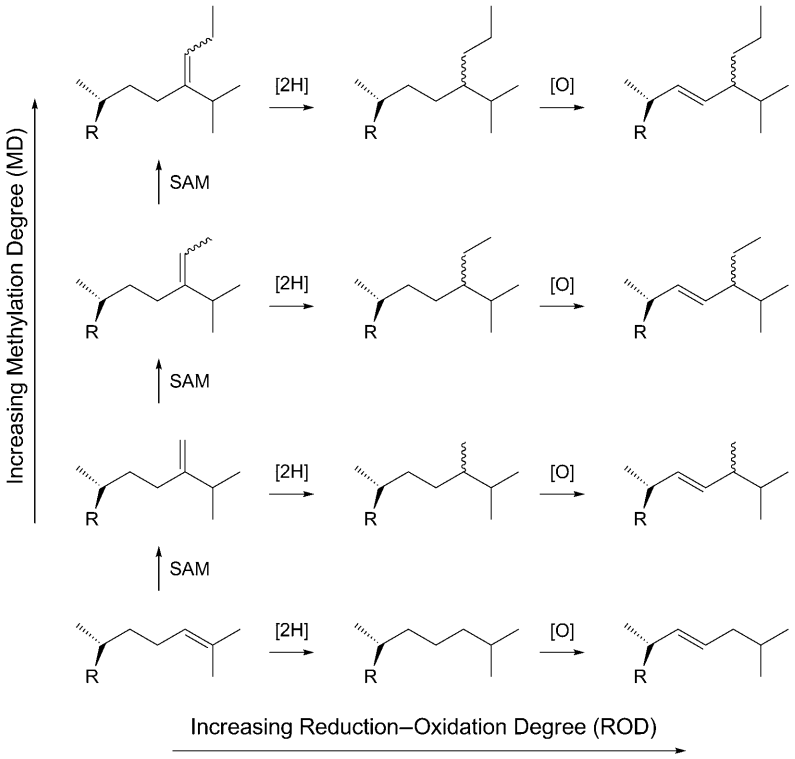
[a]) Abbreviations of sterols: Ch = cholesterol (**25**); Δ^{22}-24Me = Δ^{22}-24-methylcholesterol (**22**/**23**); 24MC = 24-methylenecholesterol (**19**); 24Me = 24-methylcholesterol (**20**/**21**); Δ^{22}-24Et = Δ^{22}-24-ethylcholesterol (**16**/**17**); 24Et = 24-ethylcholesterol (**14**/**15**); 24EC = 24-ethylidenecholesterol (**12**/**13**); 24EP = (*E*)-24-propylidenecholesterol (**1**); 24Pr = 24-propylcholesterol (**4**/**5**). [b]) RRT = GC Retention time relative to cholesterol. [c]) CCMP Strain number (*Provasoli–Guillard* National Center for Culture of Marine Phytoplankton). [d]) Species of algae: Al = *Aureoumbra lagunensis*; Co = unidentified coccoid; Csp = *Chrysoreinhardia* sp.; Sm = *Sarcinochrysis marina*. [e]) MD = Methylation degree (see text). ROD = Reduction–oxidation degree (see text). [f]) Values in parentheses were obtained from HPLC analysis (*Tables 5* and *6*).



denecholesterol (**2**; *Table 1*). Except for CCMP 1853, for which **2** was not detected, the (*E*)/(*Z*)-ratio of 24-propylidenecholesterol (**1** *vs.* **2**) varied from 1.4 to 4.3, with an average of 3.2. The methylation degree (MD) calculated for these strains ranged from 1.3 to 2.4, with a cluster centered at *ca.* 2.1 and another centered at *ca.* 1.6 (*Fig. 1*). C_{28} and C_{30} sterols were predominant, with no C_{27} sterols and only small amounts of C_{29} sterols typically being present. Unusually high amounts of C_{29} sterols were found in CCMP 1789 (36.4%), CCMP 1850 (16.4%), and CCMP 1853 (14.0%). The ROD was low in this species, ranging between 0 and 0.6. This variation was largely due to the amount of 24-methylcholesterol (**20**/**21**) present. However, because the retention times of 24-methylcholesterol (**20**/**21**) and 24-methylenecholesterol (**19**) were close, the ratios of these two sterols could not be accurately determined by GC.

The observed variation in sterol composition between the different strains within this species could be due to genetic factors, and such traits as a high ratio of **1** *vs.* **2** or a low ROD might be characteristic for particular strains. Alternatively, the variation in MD or ROD could reflect the availability of the cofactors for the biosynthetic enzymes (SAM and NADPH), which might be influenced by the metabolic state of the cells. It was unknown to what extent the sterol composition changes during the growth cycle of a culture. To test this, a culture of CCMP 1708 was sampled, and the sterols were

Scheme 1. *Sterol Side-Chain Biosynthesis*



analyzed at different stages during its growth (*Table 4*). Again, the proportions of 24-methylenecholesterol (**19**) and 24-methylcholesterol (**20**/**21**) were difficult to evaluate. Despite this, the sterol compositions at different time points proved to be fairly constant. However, it is worth noting that this strain showed significantly larger amounts of 24-propylidenecholesterol in the growth study (*Table 4*) than in the GC analysis (*Table 1*), and that this strain would, therefore, be relocated into the upper cluster of *Aureococcus* strains in the biosynthetic graph (*Fig. 1*). Every strain that was re-examined by HPLC (see below), also mapped into the upper cluster.

1.2. *Pelagomonas and Pelagococcus.* Besides *Aureococcus*, two other genera of the order Pelagomonadales were analyzed (*Table 2*). Both of these, *Pelagomonas* and *Pelagococcus*, are picoplanktonic organisms that grow in the open ocean. The former is a flagellate occurring in tropical and warm temperate waters, and the latter a coccoid species found in colder waters. *Pelagomonas calceolata* is the basis for the order Pelagomonadales and the class Pelagophyceae [11].

The predominant sterol of *Pelagomonas calceolata* was shown to be 24-methylenecholesterol (**19**), which accounted for 48–80% of total sterols. The calculated biosynthetic parameters clustered very closely for this species with a MD of *ca.* 1.7 and a ROD of *ca.* 0 (*Fig. 1*). Like *Aureococcus*, C_{29} sterols were relatively minor

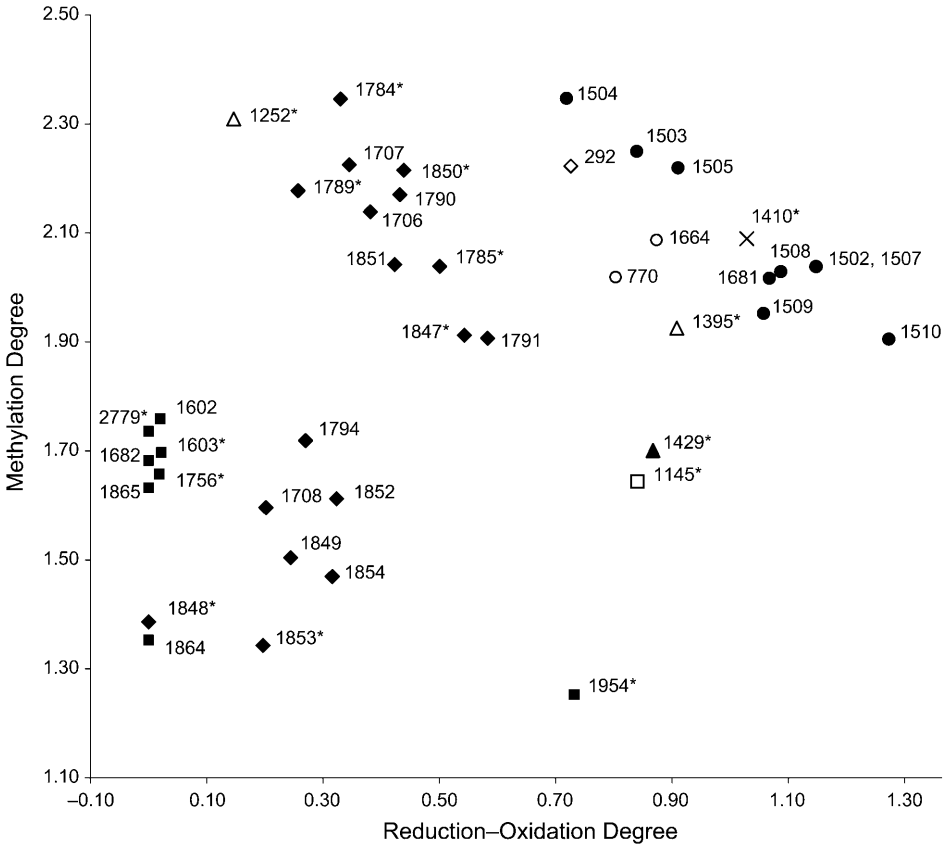


Fig. 1. *Biosynthetic mapping of the sterol compositions of pelagophyte algae based on GC analysis.* ◆: *Aureococcus anophagefferens*, ●: *Aureoumbra lagunensis*, ×: unidentified coccoid species, ■: *Pelagomonas calceolata*, □ *Pelagomonas* cf. *calceolata*, ▲: *Pelagococcus subviridis*, △: *Pelagococcus* sp., ◇: *Chrysoreinhardia* sp., ○: *Sarcinochrysis marina.* *: MD and ROD were re-evaluated by HPLC and NMR analysis (see *Tables 1–3*).

components. However, in contrast to *Aureococcus*, the (*E*)-isomer of 24-propylidenecholesterol (**1**) was absent, and only the (*Z*)-isomer (**2**) was found. The strain CCMP 1954 was substantially different from the other strains because of the presence of sterols containing a Δ^{22} C=C bond. This was regarded suspicious because of the typical ROD = 0 found in this species. Also, although Δ^{22}-24-methylcholesterol (**22**/**23**) was present, none of its biosynthetic precursor, 24-methylcholesterol (**20**/**21**), was detected in this or any of the other strains of this species (see *Scheme 1*). Contamination during culture or analysis was, therefore, considered likely. In the HPLC analysis (see below), Δ^{22}-24-methylcholesterol (**22**/**23**) was not detected.

The alga CCMP 1145 (*Pelagomonas* cf. *calceolata*) was an anomaly. Unlike the other strains of *Pelagomonas calceolata*, it is a coccoid species, but it is clearly closely related as shown by an identical 18S rRNA gene sequence as CCMP 1214 [20]. The

Table 4. *Sterol Composition of a Culture of* Aureococcus anophagefferens (CCMP 1708) *at Different Points in the Growth*

Sterols[a])	RRT[b])	Percentage of Total Sterols				
		Day 9	Day 11	Day 12	Day 14	Day 16
24MC	1.14	28.6	26.8	28.9	31.6	33.3
24Me	1.15	5.6	6.2	5.6	6.1	9.3
24EP	1.44	48.9	51.8	50.1	48.5	45.2
24ZP	1.50	16.9	15.2	15.4	13.8	12.2
MD[c])		2.32	2.34	2.31	2.25	2.15
ROD[c])		0.06	0.06	0.06	0.06	0.09
(*E*)/(*Z*)[c])		2.9	3.4	3.3	3.5	3.7
Cells/ml		1.85×10^6	4.20×10^6	4.80×10^6	4.43×10^6	2.18×10^6

[a]) Abbreviations of sterols: 24MC = 24-methylenecholesterol (**19**); 24Me = 24-methylcholesterol (**20**/**21**); 24EP = (*E*)-24-propylidenecholesterol (**1**); 24ZP = (*Z*)-24-propylidenecholesterol (**2**). [b]) RRT = GC Retention time to relative cholesterol. [c]) MD = Methylation degree (see text). ROD = Reduction–oxidation degree (see text). (*E*)/(*Z*) = Ratio of the (24*E*)- *vs.* (24*Z*)-isomers of 24-propylidenecholesterol (**1** *vs.* **2**).

sterol profile it displayed, however, was very different, since its major sterols, 24-methyl- and 24-ethylcholesterol (**14**/**15** and **20**/**21**, resp.), bear saturated side chains. Also in contrast to most strains of *P. calceolata*, the (*E*)-isomer **1** of 24-propylidenecholesterol was present, and predominated over the (*Z*)-isomer **2**. Based on the graph of biosynthetic parameters (*Fig. 1*), and its ratio of **1** and **2** (*Table 2*), this alga was found to be very similar to CCMP 1429 (*Pelagococcus subviridis*).

Three strains of the genus *Pelagococcus*, CCMP 1429 (*Pelagococcus subviridis*), and two unnamed species (CCMP 1395 and CCMP 1252), were also analyzed by GC (*Table 2*). These three strains contained both isomers of 24-propylidenecholesterol, **1** and **2**, at ratios between 1.5 and 3.8. The sterol composition of *Pelagococcus subviridis* (CCMP 1429) was quite similar to that of the *Pelagococcus* species CCMP 1395, which also contained more than 80% sterols with saturated side chains, in this case predominantly as 24-ethylcholesterol (**14**/**15**). However, the *Pelagococcus* species CCMP 1252 contained mainly unreduced sterols and more closely resembled *A. anophagefferens*.

1.3. *Sarcinochrysidales.* A total of 13 strains were analyzed from the order Sarcinochrysidales, representing four different species including the 'Texas brown tide' alga, *Aureoumbra lagunensis*, *Chrysoreinhardia* sp., *Sarcinochrysis marina*, and an unidentified coccoid CCMP 1410 (*Table 3*). The (*Z*)-24-propylidenecholesterol (**2**) was not detected in any of these strains. Unlike the Pelagomonadales, all members of the Sarcinochrysidales contained Δ^{22}-sterols.

The nine strains of the 'Texas brown tide', *Aureoumbra lagunensis*, showed very similar sterol compositions. The MD ranged between 1.9 and 2.4, and the ROD from 0.7 to 1.3 (*Fig. 1*). The major sterols were Δ^{22}-24-ethylcholesterol (**16**/**17**; average 30.4%), (*E*)-24-propylidenecholesterol (**1**; average 28.7%), cholesterol (**25**; average 13.8%), 24-propylcholesterol (**4**/**5**; average 11.7%), and ethylcholesterol (**14**/**15**; average

11.5%). This species is unusual among the pelagophytes because of its relatively large content of cholesterol (**25**) and 24-propylcholesterol (**4**/**5**). We previously suggested **4**/**5** could be a useful biomarker for the 'Texas brown tide' [10][21], and this study confirms its presence in all strains of *A. lagunensis*.

Four other strains from the Sarcinochrysidales were analyzed. Two of these represent the type species for this order, *Sarcinochrysis marina*, which forms colonies on rocks in the splash zone (superlitoral) in temperate waters. In the literature, the sterol composition of this species is mentioned twice in reference to prior studies [22][23], however, the citations referred to in those publications do not deal with this species, and it is likely to have been confused with CCMP 292, which was extensively investigated chemically before it was securely classified biologically. CCMP 292, which, like *Sarcinochrysis marina*, also forms colonies on substrates in shallow water, was previously referred to either as an unidentified chrysophyte [6][22], *Chrysoderma mucosa* [1], or *Pulvinaria* sp. [8], and has now been identified as *Chrysoreinhardia* sp. Our analysis showed the sterol compositions of *Chrysoreinhardia* sp. and *Sarcinochrysis marina* to be quite similar (*Table 3*). Like *Aureoumbra*, both algae contained sterols predominantly with saturated side chains and Δ^{22} C=C bonds. However, the C_{30} sterols were largely unmodified, indicating that the side chain reductase apparently does not function well with the 24-propylidene group, probably due to its steric bulk. The sterol composition of CCMP 1410 (an unidentified coccoid strain collected from the Sargasso Sea at a depth of 84 m) very closely resembled that of *Aureoumbra lagunensis*.

2. *Analysis by HPLC and NMR.* Although GC analysis is sensitive and requires only small amounts, it often does not provide adequate information for molecular identification, even when coupled to MS (mass spectrometry) detection, especially for stereoisomers or in situations where rare and unusual sterols might be present [24]. Also, at the high temperatures required for volatilization, selective decomposition of the less stable sterols may occur. To achieve greater confidence in our data, a detailed analysis was undertaken of selected strains using reversed-phase (RP) HPLC to separate the sterol mixtures, and 600-MHz NMR to provide definite identities of the components.

Several algal strains were chosen for reinvestigation, including some obvious outliers that were evident from the graph of biosynthetic descriptors, *e.g.*, CCMP 1954 and CCMP 1848 (*Fig. 1*). Larger sample sizes (8-l cultures *vs.* 200 ml for GC analysis) and the sensitivity of the NMR instrument allowed the detection of even minor sterols. In total, 17 were examined, and 23 sterols were identified including a new C_{30} sterol, **3**. All of the sterols had the Δ^5-sterol nucleus.

2.1. *Aureococcus anophagefferens.* Seven strains of *A. anophagefferens* were reinvestigated (*Table 5*). The results were generally consistent with the GC analysis, although there were some differences. All strains of *A. anophagefferens* contained both (*E*)- and (*Z*)-24-propylidenecholesterol (**1** and **2**, resp.) as a high percentage of the total sterols (48–67%). The (*E*)/(*Z*) ratios (**1**/**2**) were somewhat higher for the HPLC analysis than for GC analysis (*Table 1*). The strain CCMP 1853, for which **2** was not detected by GC analysis, was found to have similar amounts of **2** as the other *A. anophagefferens* strains.

A factor complicating the ROD calculation from the GC data was that 24-methylenecholesterol (**19**) and 24-methylcholesterol (**20**/**21**) had very close retention

Table 5. *Sterol Compositions of Algae of the Order Pelagomonadales by HPLC and NMR Analysis*

Sterol[a])	RRT[b])	Percentage of Total Sterols[c])																
		1784[d]) Aa[e])	1785 Aa	1789 Aa	1847 Aa	1848 Aa	1850 Aa	1853 Aa	1214 Pc	1603 Pc	1756 Pc	1954 Pc	2779 Pc	1145 Pcc	1429 Ps	1252 Psp	1395 Psp	1395[f]) Psp
Δ^{25}-24Me	0.83		0.2		0.3			0.3										1.0
24MC	0.86	14.0	19.6	9.0	16.8	38.3	6.5	45.2	49.8	70.5	50.6	67.3	62.3	0.9		63.8		2.4
Δ^{25}-24Et	0.94	1.1	1.4	1.2	1.2	1.2	1.3	0.8		1.4	1.4	1.3	1.4	1.2	0.7	2.4	1.2	1.9
Δ^{25}-24Pr	0.94	2.2	0.5	4.1	0.3	0.8	0.3	0.4		0.5		1.3		0.6	3.4		0.2	0.3
Ch	1.00		0.2			0.2		0.2		1.1				0.4		1.4		
E24EC	1.00	2.0	1.7	1.5	1.6	1.1	2.1	1.0	8.1		1.1			0.9	1.2	5.9	1.5	1.9
Z24EC	1.00	0.5	0.6	1.5	1.6	0.6	1.1	1.0	32.1	11.9	7.2	8.8	14.9	1.1	1.2	11.7	2.9	3.8
24EP	1.05	51.8	50.7	42.0	43.8	40.4	54.6	35.9	6.7	0.6	0.2			22.3	19.0	11.3	4.0	2.4
RiPC	1.05				0.1		0.1											
SiPC	1.05	0.3		0.4	0.2		0.3											
24ZP	1.08	8.9	11.2	12.4	12.8	10.9	12.5	12.2	3.3	14.0	39.5	21.3	21.4	5.4	3.8	3.5	2.0	1.2
DMMC	1.08	0.3	0.3	0.4	0.2	0.3	0.4	0.1										
S24Me	1.12	18.9	13.6	25.1	19.8	6.2	20.8	2.9						44.1	25.8		14.1	15.3
RRMS	1.12				0.4													
S24Et	1.22			2.4	0.9									23.1	44.9		74.1	69.8
Total sterol [mg]		0.92	1.48	0.42	2.30	1.14	2.10	1.33	0.60	0.43	1.05	0.19	0.12	0.57	0.38	0.18	2.30	0.90

[a]) Abbreviations of sterols: Δ^{25}-24Me = (24*S*)-Δ^{25}-24-methylcholesterol (**24**); 24MC = 24-methylenecholesterol (**19**); Δ^{25}-24Et = (24*S*)-Δ^{25}-24-ethylcholesterol (**18**); Δ^{25}-24Pr = Δ^{25}-24-propylidenecholesterol (**3**); Ch = cholesterol (**25**); E24EC = (*E*)-24-ethylidenecholesterol (**12**); Z24EC = (*Z*)-24-ethylidenecholesterol (**13**); 24EP = (*E*)-24-propylidenecholesterol (**1**); RiPC = (24*R*)-24-isopropenylcholesterol (**9**); SiPC = (24*S*)-24-isopropenylcholesterol (**8**); 24ZP = (*Z*)-24-propylidenecholesterol (**2**); DMMC = (25*R*)-26,26-dimethyl-24-methylenecholesterol (**11**); S24Me = (24*S*)-24-methylcholesterol (**21**); RRMS = (24*R*,28*R*)-24,28-methylenestigmast-5-en-3-ol (**10**); S24Et = (24*S*)-24-ethylcholesterol (**15**). [b]) RRT = GC Retention time relative to cholesterol. [c]) MD, ROD, and (*E*)/(*Z*)-ratios of 24-propylidenecholesterol are given in *Tables 1* and *2*. [d]) CCMP Strain number (*Provasoli–Guillard* National Center for Culture of Marine Phytoplankton). [e]) Species of algae: Aa = *Aureococcus anophagefferens*; Pc = *Pelagomonas calceolata*; Pcc = *Pelagomonas* cf. *calceolata*; Ps = *Pelagococcus subviridis*; Psp = *Pelagococcus* sp. [f]) Saponified sterol esters.

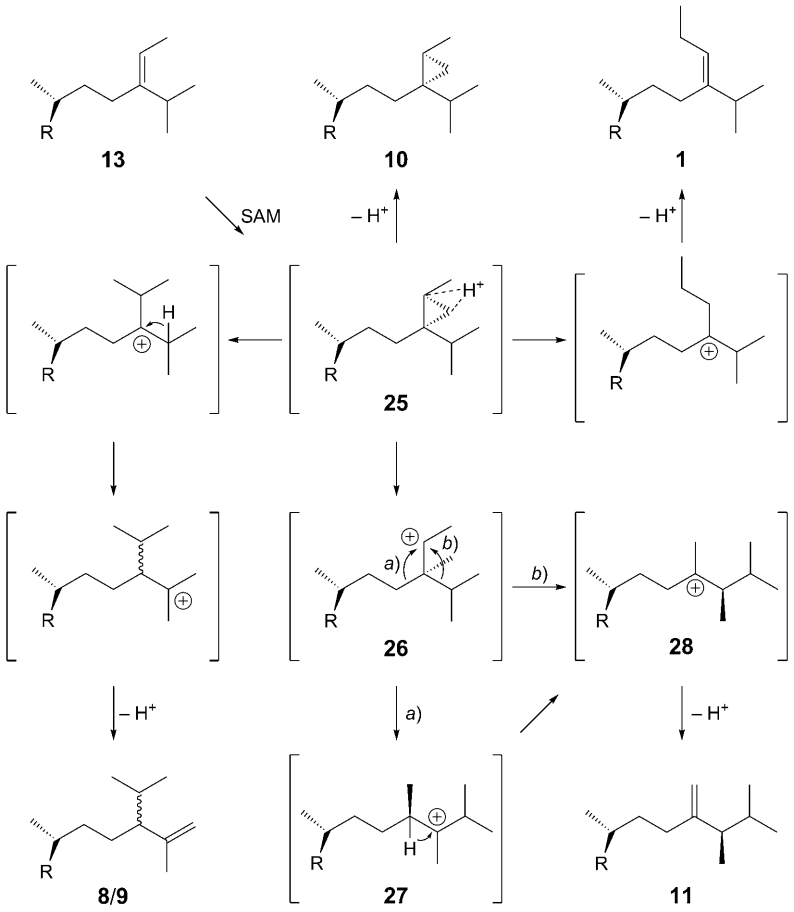
times. In some cases, only one of the two sterols was detected, perhaps because of poor resolution (*Table 1*). HPLC led to a complete separation of these two sterols and showed that both sterols were present in all of the *A. anophagefferens* strains analyzed, although the proportions were highly variable (*Table 5*). Nevertheless, good general agreement was found in the relative amounts of the two sterols as determined by the two methods. All strains, with the exception of CCMP 1785, that had higher amount of one sterol when measured by GC, also had more of that sterol when measured by HPLC. The high variability in the ratios of 24-methylcholesterol (**20**/**21**) to 24-methylenecholesterol (**19**) may be explained by differences in the activities of the biosynthetic enzymes among strains. In some strains, the alkylation of the 24-methylene side chain were faster, while reduction of $\Delta^{24(28)}$ C=C bond was favored in other strains. Whether this is a genetic characteristic of the strains, or it depends on the metabolic conditions, remains to be determined.

Relatively high levels of C_{29} sterols were observed for CCMP 1789, 1850, and 1853 by GC analysis (*Table 1*). By HPLC analysis, however, all strains had low amounts of C_{29} sterols (**8**, **10**, **11**, and **12**), ranging from 2.9–4.4%. While the reason for the high levels of C_{29} sterols as determined by GC is unknown, HPLC analysis is more reliable, and low levels of C_{29} sterols should, therefore, be considered typical of this species.

The high sensitivity of 600-MHz ^{1}H-NMR analysis enabled us to identify several minor sterols in *A. anophagefferens*. From CCMP 1847 and CCMP 1853, two minor compounds were found to co-elute with (*E*)-24-propylidenecholesterol (**1**). Attempts to isolate them were carried out using different HPLC mobile phases and preparative argentic TLC. However, none of these methods was successful. Based on the retention time and by comparison with published ^{1}H-NMR data, the two compounds were identified as the two epimers **8** and **9** of 24-isopropenylcholesterol [25][26]. A cyclopropa-sterol was isolated from the (24*S*)-24-methylcholesterol (**21**) fraction of CCMP 1847, and was shown to be the (24*S*,28*S*)-isomer of 24,28-methylenestigmast-5-en-3β-ol (**10**) based on ^{1}H-NMR data [19][27][28]. Another minor sterol co-eluted with (*Z*)-24-propylidenecholesterol (**2**) in all seven of the strains investigated. This could not be separated using different HPLC mobile phases, but was purified by repeated preparative argentic TLC of the acetate esters. After saponification, the minor sterol was identified as 26,26-dimethyl-24-methylenecholesterol (**11**) by comparison with the published ^{1}H-NMR data [22]. These four minor sterols, **8**–**11**, are C_{30} sterols that are believed to be by-products in the biosynthesis of 24-propylidenecholesterol (*Scheme 2*). The cyclopropa-sterol **10** is product of simple deprotonation of the hypothetical nonclassical carbocationic intermediate **26** [1][2]. Although the configuration of sterol **11** is presently unknown, based on least motion pathways for the two possible biosynthetic alkyl shifts from **27** to either **28** or **29** (*Scheme 2*), we postulate that the configuration of C(25) is (*R*).

Another minor sterol was found in all *Aureococcus* strains analyzed by HPLC. It co-eluted with clerosterol (**18**), and was isolated by changing the HPLC mobile phase to MeCN/MeOH/AcOEt 11:4:4 (*v*/*v*/*v*). Mass spectrometry showed an M^+ ion peak at *m*/*z* 424, suggesting the formula $C_{30}H_{48}O$. The ^{1}H-NMR spectrum of this compound showed signals of four olefinic H-atoms that were consistent with a Δ^5-sterol-triene bearing three olefinic H-atoms in the side chain. In $CDCl_3$, two broad *singlet*s in a pattern typical of an olefinic $=CH_2$ group were found at 4.976 and 4.867 ppm. The third

Scheme 2. *Mechanistic Details of the Biosynthetic Formation of C_{30} Sterols in Pelagophytes*



olefinic signal was a *triplet* at 5.508 ppm, typical of a trisubstituted olefin. Structure determination by 2D-NMR (*Fig. 2*) was performed in C_6D_6, because of overlapping signals in the Me region of the $CDCl_3$ spectrum. In C_6D_6, the chemical shifts of these three H-atoms changed to 5.015, 5.225, and 5.608 ppm, respectively. Correlations were

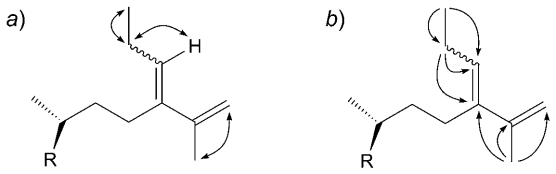


Fig. 2. *Selected 2D-NMR correlations for the novel C_{30} sterol Δ^{25}-24-propylidenecholesterol* (**3**)*:* a) *COSY correlations;* b) *HMBC correlations.*

observed in the COSY spectrum for the olefinic methylene H-atoms and a Me *singlet* at 1.925 ppm, indicating an isopropenyl group. Both the *triplet* olefinic H-atom peak and a Me *triplet* at 1.007 ppm had a correlation with a CH_2 *multiplet* at 2.167 ppm. In the HMBC spectrum, the Me *triplet* (1.007 ppm) had correlations with an olefinic C-atom signal at 129.6 and a signal at 22.5 ppm. The *multiplet* at 2.167 ppm also had correlations with the olefinic C-atom signal at 129.6 and another olefinic C-atom signal at 140.6 ppm, indicating the *multiplet* came from two allylic H-atoms. The olefinic C-atom signal at 140.6 ppm correlated to the Me *singlet* at 1.925 ppm, which also correlated to two other olefinic C-atom signals at 144.1 and 111.2 ppm. All of the information was consistent with Δ^{25}-24-propylidenecholesterol (**3**). An attempt to determine the configuration of the $\Delta^{24(28)}$ C=C bond by ROESY was unsuccessful. This novel compound was isolated as a minor component in all of the Pelagophyte species analyzed by HPLC. Because it is so ubiquitous, it is believed to originate from the decomposition of 24-propylidenecholesterol (**1**/**2**), perhaps *via* an allylic hydroperoxide intermediate. In our experience, 24-propylidenecholesterol (**1**/**2**), like other trisubstituted olefinic sterols, is rather susceptible to decomposition. Precautions were, therefore, taken throughout this study to prevent autoxidation: exposure to light was minimized, especially in the presence of pigments, and tetramethylethylene was added to the solvents as a singlet-oxygen scavenger. It is unknown whether the two isomers of 24-propylidenecholesterol display different stabilities, but we suspect the (*Z*)-isomer **2** is more labile.

2.2. *Pelagomonas.* Of the eight strains of *Pelagomonas calceolata* analyzed by GC, four were reinvestigated by HPLC; strain CCMP 1214 was only analyzed by HPLC. The results (*Table 5*) were generally consistent with the GC data (*Table 2*). The previous detection of Δ^{22}-sterols in CCMP 1954, which raised suspicions of contamination, was not confirmed by HPLC analysis, and this strain looked very similar to the others of the species. All strains displayed RODs close to zero. All of the strains except CCMP 1214 contained almost exclusively only the (*Z*)-isomers of 24-propylidenecholesterol and of 24-ethylidenecholesterol (**2** and **13**, resp.). To rule out the possibility to contamination, the HPLC/NMR analysis of CCMP 1214 was repeated with a fresh culture, giving similar results. The unusual (*E*)/(*Z*) ratio of CCMP 1214 compared to other strains of the same species is further complicated by the fact that CCMP 1214 is the authentic strain for this species [11]. The reasons that most strains of this species favor the (*Z*)-isomers **2** and **13** are unknown. We have previously shown that isofucosterol (**13**) is the biosynthetic precursor of (*E*)-24-propylidenecholesterol (**1**) in *Chrysoreinhardia* [1], and it would be interesting to determine the precursor of the (*Z*)-isomer **2** in *Pelagomonas calceolata*.

The HPLC results confirmed the differences that GC analysis indicated between *Pelagomonas* cf. *calceolata* (CCMP 1145) and the different strains of *Pelagomonas calceolata*. A second HPLC analysis with a fresh culture of CCMP 1145 also gave similar results. As discussed previously, despite an 18S rRNA sequence identical to *Pelagomonas calceolata*, the sterol composition of CCMP 1145 closely resembles that of *Pelagococcus subviridis* (CCMP 1429). This may be related to the fact that CCMP 1145, unlike the other *Pelagomonas calceolata* strains which are flagellate [11], is a nonflagellate, coccoid cell surrounded by a thick, three-layered cell wall. Similarly, *Pelagococcus subviridis* is a coccoid cell with a trilayered wall [29], and it is possible

that similarities in sterol composition may be partly related to the life form. Since the same species may have different life forms (*e.g.*, alternating between a flagellate swimming stage and a nonmotile coccoid stage), the sterol composition could potentially change with the life form, which may account for the anomalous chemotaxonomic affinities of CCMP 1145.

2.3. *Pelagococcus.* Detailed analysis of *Pelagococcus subviridis* (CCMP 1429) and two unnamed *Pelagococcus* species (CCMP 1252 and CCMP 1395) by HPLC and NMR (*Table 5*) gave generally similar results to those obtained by GC analysis (*Table 2*). As discussed previously, the sterol composition of CCMP 1395 was similar to that of the type species, *Pelagococcus subviridis* (CCMP 1429), and like *P. subviridis*, has a similar trilayered cell wall (unpublished results). Of all of the pelagophyte algae investigated, only CCMP 1395 contained significant amounts of sterol esters. After saponification, the composition of these, however, did not differ much from that of the free sterols. The configuration of the 24-alkyl-sterols in this genus was shown by ^{1}H-NMR to be exclusively 24β (**21** and **15**). The Δ^{22}-sterols that had previously been detected by GC analysis were not confirmed by NMR. All *Pelagococcus* strains showed both isomers of 24-propylidenecholesterol, and the (*E*)/(*Z*)-ratios (**1**/**2**) were similar to *Aureococcus*, but the quantities were considerably lower, especially for CCMP 1395.

The sterol composition of CCMP 1252 was quite different from the others because of the lack of side chain reductase activity in this strain as inferred from the lack of reduced sterol side chains (*Scheme 1*). In contrast, CCMP 1395 and CCMP 1429 contained mainly sterols with saturated side chains. In the GC analysis, CCMP 1252 had contained a small amount of sterol with a saturated side chain (*i.e.*, **14**/**15**), but, upon HPLC analysis, no reduced sterols were evident. The HPLC analysis also showed more 24-methylenecholesterol (**19**) than before. From the GC analysis, CCMP 1252 most closely resembled *Aureococcus,* but this similarity was not strong by HPLC analysis. Instead, CCMP 1252 was found to strongly resemble the authentic strain for *Pelagomonas calceolata* (*i.e.*, CCMP 1214).

2.4. *CCMP 1410.* The algal strain CCMP 1410 is an unidentified coccoid species belonging to the Sarcinochrysidales. GC Analysis had shown this species had a sterol profile similar to that of *Aureoumbra lagunensis* (*Table 3*). HPLC and NMR analyses displayed a complex mixture (*Table 6*). In total, 14 sterols were isolated. The predominant sterol of this alga was (*E*)-24-propylidenecholesterol (**1**); none of the (*Z*)-isomer **2** was observed. The other major sterols were Δ^{22}-(24*S*)-24-ethylcholesterol (stigmasterol, **16**) and cholesterol (**25**). Phylogenetic studies based on 18S rRNA and *rbcL* had shown this alga to be closely related to *Aureoumbra lagunensis*, and also related to *Chrysoreinhardia* sp. and *Nematochrysopsis roscoffensis* (*Fig. 3*) [13][14][30]. Comparison of the published sterol compositions of these algae showed that the sterol pattern of CCMP 1410 more closely resembles that of *N. roscoffensis* and *A. lagunensis,* than that of *Chrysoreinhardia* (*Table 6*).

In a previous study, 24α-sterols, but no 24β-sterols were isolated from *Aureoumbra lagunensis* [10]. Because of this, it was suggested that 24α-sterols were a characteristic of chemotaxonomic value for the Sarcinochrysidales, and it was predicted that, if an algal source were found for Δ^{22}-24-propylcholesterol (**6**/**7**), it would contain the 24α-configuration (**6**) [21]. While the 24α-sterols predominated in CCMP 1410, and the 24α-configuration was the only configuration of the C_{29} sterols **14** and **16**, 24β-sterols

Table 6. *Sterol Composition of CCMP 1410 Compared to Other Algae in the Sarcinochrysidales*

Sterols[a])	RRT[b])	Percentage of Total Sterols			
		1410[c])	1681[d])	292[e])	Nr[f])
24-Methylenecholesterol (**19**)	0.86	0.5	0.4	0.9	4[g])
(24*S*)-Δ^{22}-24-Methylcholesterol (**22**)	0.90	1.3	0.9	0.2	–
(24*R*)-Δ^{22}-24-Methylcholesterol (**23**)	0.93	4.7	–	9.8	2
(24*S*)-Δ^{25}-24-Ethylcholesterol (**18**)	0.94	0.3	1.1	0.8	
Δ^{25}-24-Propylidenecholesterol (**3**)	0.94	0.3	–	–	
Cholesterol (**25**)	1.00	11.5	14.1	0.8	12
(*E*)-24-Propylidenecholesterol (**1**)	1.05	38.9	35.7	46.5	40
(24*S*)-Δ^{22}-24-Ethylcholesterol (**16**)	1.07	27.2	22.2	4.3	25
(24*S*)-24-Methylcholesterol (**21**)	1.12	2.9	0.7[h])	9.5	4[g])[h])
(24*R*)-Δ^{22}-24-Propylcholesterol (**7**)	1.17	0.4	–	–	
(24*S*)-Δ^{22}-24-Propylcholesterol (**6**)	1.17	0.7	–	–	
(24*R*)-24-Ethylcholesterol (**14**)	1.21	6.2	19.2	10.2	6
(24*S*)-24-Propylcholesterol (**5**)	1.30	1.7	–	0.6[h])	9[h])
(24*R*)-24-Propylcholesterol (**4**)	1.30	3.4	5.2		

[a]) MD and ROD values for CCMP 1410 are given in *Table 3*. [b]) RRT = GC Retention time relative to cholesterol. [c]) HPLC Analysis; total sterol: 2.3 mg. [d]) *Aureoumbra lagunensis*; data from [10]. [e]) *Chrysoreinhardia* sp.; data from [18]. [f]) Nr = *Nematochrysopsis roscoffensis*; data from [7]. [g]) Identified only as either 24-methylenecholesterol or 24-methylcholesterol [6]. [h]) The configuration at C(24) was not determined.

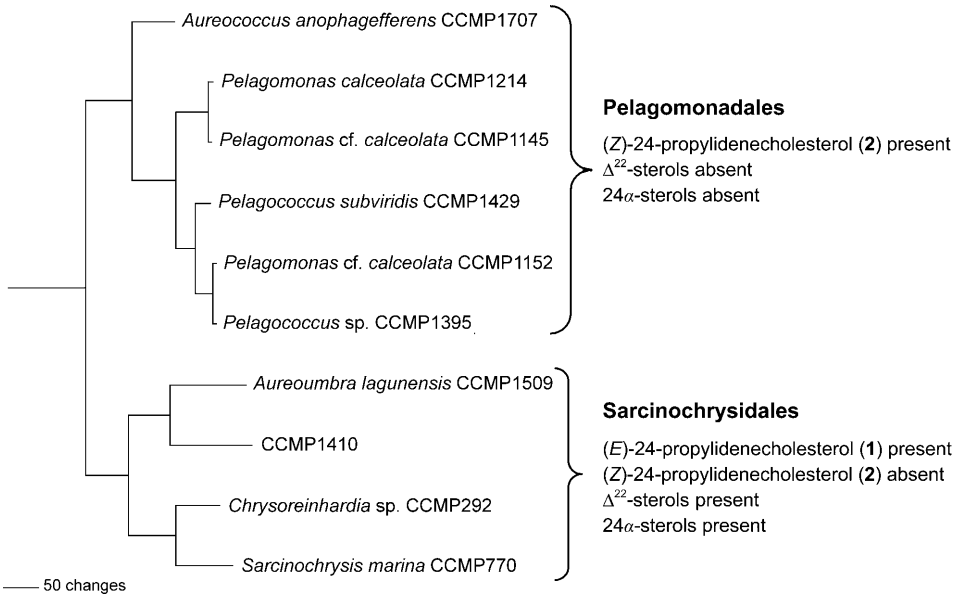


Fig. 3. *Distribution of sterol characteristics within the class Pelagophyceae, shown with the single most parsimonious tree* (phylogram) *derived from a branch-and-bound cladistic analysis using concatenated 18S rRNA and* rbcL *genes* (tree length, 1931; 687, parsimony-informative characters). The outgroup (not shown) was four species of the closely related Dictyochophyceae.

were also present, and dominated the C_{28} sterols **21** and **23**. The C_{30} sterols included both stereoisomers of 24-propylcholesterol (*i.e.*, **4** and **5**) and Δ^{22}-24-propylcholesterol (*i.e.*, **6** and **7**). These were identified using ^{1}H-NMR of reference compounds obtained through chemical synthesis [21]. In the C_{30} sterols, the 24α-configuration (*i.e.*, **4** and **6**) was found to predominate by a factor of 2:1. This is the first time Δ^{22}-24-propylcholesterol has been isolated from an alga.

Conclusions. – All pelagophyte algae analyzed contained one or both of the two isomers of 24-propylidenecholesterol (*i.e.*, **1** and **2**). While this sterol has been detected in other marine organisms such as shellfish, its presence there is thought to derive from dietary sources. It has not been detected in other algae, and the ability to produce this sterol appears to be limited to the Pelagophyceae, where it can be regarded as a defining characteristic of this class.

The sterol compositions correlated well with the biological classification based on 18S *r*RNA and *rbc*L analyses (*Fig. 3*), and are, therefore, of high chemotaxonomic value. All species from order Pelagomonadales contained the (*Z*)-24-propylidenecholesterol (**2**), either alone or together with the (*E*)-isomer **1**. On the other hand, **2** was absent in all species of the order Sarcinochrysidales. Other differences between these two orders are that Δ^{22}-sterols and 24α-sterols were found in all Sarcinochrysidales investigated, but were absent in all algae of the Pelagomonadales.

Within the Pelagomonadales, *Pelagomonas calceolata* contained only sterols with nonreduced side chains (ROD = 0), and, with the exception of CCMP 1214, did not contain the (*E*)-isomer **1** of 24-propylidenecholesterol. In all other algae in this order, both isomers were present, with the (*E*)-isomer **1** predominating. *Aureococcus anophagefferens* had both isomers, with an average (*E*)/(*Z*)-ratio (**1**/**2**) of 4.0 based on HPLC analysis. A similar ratio was found in *Pelagococcus subviridis*, but this species had much lower amounts of the 24-propylidenecholesterols than *Aureococcus*, where they represented greater than 50% of the total sterols. Another difference between these two species was that *Aureococcus* had low amounts ($< 5\%$) of C_{29} sterols, while, in *P. subviridis*, these represented nearly 50% of the total sterols. Two strains that belong to genus *Pelagococcus* also had relatively low amounts of the 24-propylidenecholesterols. CCMP 1395 closely resembled *P. subviridis*, but contained significant amounts of sterol esters. CCMP 1252 had low amounts of the 24-propylidenecholesterols, but differed from the other *Pelagococcus* strains, because it did not contain sterols with reduced side chains (ROD = 0) and had a lower MD. Unlike the other *Pelagococcus* algae, CCMP 1145 is a nonflagellate, coccoid cell, much like *Pelagococcus subviridis*. The fact that its sterol composition more closely resembles the latter species than examples from its own genus suggests that the sterol composition may depend upon the life form of the alga.

Because of the actions of oxidoreductases on the sterol side chain, the sterol compositions in the order Sarcinochrysidales are much more complex than in the Pelagomonadales. The species *Aureoumbra lagunensis* showed high amounts of (24*S*)-Δ^{22}-24-ethylcholesterol (stigmasterol, **16**), (24*R*)-24-ethylcholesterol (sitosterol, **14**), cholesterol (**1**), and 24-propylcholesterol (**4**/**5**). These represent characteristic sterols of this species. However, because of the prevalence of the first three sterols in terrestrial plants and animals, only 24-propylcholesterol (**4**/**5**) would be a useful biomarker.

CCMP 1410, an unidentified coccoid species, displayed a nearly identical sterol composition to *Aureoumbra*, as did the previously reported analysis of *Nematochrysopsis roscoffensis* [7]. The sterol composition of *Sarcinochrysis marina* was reported for the first time and was found to be similar to that of *Chrysoreinhardia* sp. [6][22]. Compared to *Aureoumbra*, these did not contain nearly as much (24*S*)-Δ^{22}-24-ethylcholesterol (stigmasterol, **16**), cholesterol (**25**), or 24-propylcholesterol (**4**/**5**).

An algal source for Δ^{22}-24-propylcholesterol (**6**/**7**) was identified for the first time in the unidentified alga CCMP 1410. The rare C_{30} sterol 26,26-dimethyl-24-methylenecholesterol (**11**) was isolated from *Aureococcus anophagefferens*, and the (25*R*)-configuration was proposed based on biosynthetic considerations. Traces of a new sterol, Δ^{25}-24-propylidenecholesterol (**3**) were detected and identified. This is thought to be an autoxidation product of 24-propylidenecholesterol (**1**/**2**).

While (*Z*)-24-propylidenecholesterol (**2**) can no longer be regarded as a specific biomarker for the 'brown tide' alga *Aureococcus anophagefferens,* this species was shown to have a typical (*E*)/(*Z*)-ratio (**1**/**2**) of *ca.* 4. Similar mixtures were only found in *Pelagococcus* and a *Pelagomonas* species (CCMP 1145). However, because these species do not occur in coastal waters, mixtures of these two isomers at ratios in the range of 3–5 represent a useful biomarker for the coastal harmful algal blooms caused by *A. anophagefferens.*

Financial support was provided by *New York Sea Grant*, and the *National Oceanographic and Atmospheric Administration's Coastal Ocean Program*. *R. A. A.* was supported by *New York Sea Grant* R/XG-5 and *National Science Foundation* grants 0206590, 0444418, and 0629654.

Experimental Part

General. All solvents were obtained commercially and were further purified by distillation. As a trap for singlet O_2, 2,3-dimethylbut-2-ene was added to all solvents, except the HPLC solvents, at 0.5% (*v*/*v*). All extraction and purification steps were carried out under low light conditions, especially before the pigments had been removed, to minimize autoxidation. Anal. TLC: aluminum-backed plates coated with a 0.25-mm layer of silica gel *60 F254*. Prep. TLC: glass-backed plates coated with a 0.25-mm layer of silica gel *60 F254*. Argentic TLC plates were prepared by wetting prep. and anal. TLC plates with sat. $AgNO_3$/MeOH, allowing the solvent to evaporate in the dark, and then repeating the process a second time. HPLC: *Waters 6000A* pump, *Waters 410* differential refractometer, and two *Altex Ultrasphere ODS* columns (5 μm, 10 × 250 mm) in series, using either MeOH or MeCN/AcOEt/MeOH 11:4:4 as the mobile phase at a flow rate of 3 ml/min. GC: *Hewlett-Packard 6890* gas chromatograph with a *HP-5* column (cross-linked 5% methyl(phenyl)siloxane, film thickness 0.25 μm; 30 m × 0.25 mm), a splitless injector at 250°, He carrier gas at 1.2 ml/min (133 kPa), a temp. gradient of 150 to 260° at 10°/min and a FID detector at 300°. NMR Spectra: *Bruker Avance 600* MHz instrument; $CDCl_3$ was the solvent unless otherwise specified; referenced to residual $CHCl_3$ signals (^{1}H: 7.26 ppm, ^{13}C: 77.0 ppm); only selected ^{1}H-NMR peaks (Me groups and isolated signals) are reported. MS: *Hewlett-Packard 5989B* mass spectrometer operating at 70 eV.

Acetylation of sterols were preformed by treatment with Ac_2O/pyridine 1:4 at 60° for 2 h. The mixture was evaporated with a stream of N_2. The crude product was purified by prep. TLC with hexane/AcOEt 9:1. Saponification of sterol esters was carried out by refluxing with 10% KOH/EtOH for 1 h. The solvent was partially removed with a stream of N_2, and the residue was extracted with Et_2O and H_2O. After evaporation of the org. layer with a stream of N_2, the residue was purified by prep. TLC (hexane/AcOEt 2:1).

Algal culture. Cultures of pelagophyte algae for GC analysis were cultured at 22° in L1 medium using fluorescent light at *ca.* 100 μE m^{-2} s^{-1}. The 200-ml cultures were harvested by filtration on glass fiber discs, and the cells were shipped frozen with liq. N_2.

The culture of *Aureococcus anophagefferens* (CCMP 1708) for the growth curve experiment (see *Table 4*) was grown at 18° in a 8-l glass carboy containing ASP artificial seawater and modified f/2 seawater nutrients [31] using a 14:10 h light/dark cycle at 50–75 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cell growth was measured using *in vivo* chlorophyll-*a* fluorescence and by visual cell counts under phase contrast. Volumes containing 3×10^8 cells were collected at different times on the growth curve by filtration on glass fiber discs.

Cultures of algae for analysis by HPLC were grown at 18 or 21° with aeration in 12-l glass carboys as described above. Each culture was inoculated (10%) with cells in exponential growth phase. Cell growth was measured using *in vivo* chlorophyll-*a* fluorescence and by visual cell counts under phase contrast. Cells were harvested in late exponential or stationary phase by use of a continuous flow centrifuge.

Strains 1429, 1507, 1509, 1510, and 1756 were axenic. The remaining strains contained uncharacterized bacterial species, but no other eukaryotes.

Sterol Analysis. GC Analysis. Algae were extracted twice at r.t. with 1 ml portions of AcOEt using mechanical shaking (*Tony Microtube Mixer*) for 45 min each time. The org. extracts were combined and concentrated with a stream of N_2. The contaminating pigments were removed by chromatography on a pipet column containing *Florisil* (1 g; 60–100 mesh), eluting with hexane/AcOEt 19:1 (5 ml), followed by hexane/AcOEt 4:1 (5 ml). The hexane/AcOEt 4:1 fractions were concentrated to dryness with a stream of N_2, and the residue was trimethylsilylated by reacting with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60° for 1 h to give a mixture of sterol TMS ethers. The samples were analyzed by GC, and the sterols were identified by comparison of their retention times with standards. The identities of all sterols were confirmed by GC/MS through comparison of their mass spectra with standards. The relative proportions were determined from integration of the GC FID traces. The absolute amounts were estimated by comparison with a known amount of an external standard (cholesterol).

HPLC Analysis. The algal pellets were extracted with AcOEt (4 ml) in 20-ml glass vials with vigorous shaking by hand for 5 min. Additional extractions were repeated until no more sterols were detected by anal. TLC. The solvents were evaporated with a stream of N_2, and the pigments were removed by *Florisil* chromatography (see above). The sterols were obtained by prep. TLC with hexane/AcOEt 2:1. Only strain CCMP 1395 had significant amounts of sterol esters which were saponified to give free sterols. The sterols were fractionated by reversed-phase (RP) HPLC with MeOH. The isolated sterol fractions were evaporated with a stream of N_2 and characterized by ^{1}H-NMR. The relative proportions of sterols were determined from the HPLC integrals. The absolute amount of sterols in each sample was estimated from the NMR spectrum by comparison of the signals of H–C(3) and H–C(6) with that of a known concentration of stigmasterol using the residual $CHCl_3$ solvent peak as a reference.

24-Propylidenecholesta-5,25-dien-3β-ol (**3**). ^{1}H-NMR ($CDCl_3$): 5.508 (*t*, *J* = 7.3, 1 H); 5.353 (*m*, 1 H); 4.976 (*s*, 1 H); 4.867 (*s*, 1 H); 3.524 (*m*, 1 H); 2.123 (*quint.*, *J* = 7.5, 2 H); 1.879 (*s*, 3 H); 1.018 (*s*, 3 H); 1.018 (*t*, *J* = 7.4, 3 H); 1.015 (*d*, *J* = 6.5, 3 H); 0.696 (*s*, 3 H). ^{1}H-NMR (C_6D_6): 5.608 (*t*, *J* = 7.3, 1 H); 5.350 (*m*, 1 H); 5.224 (*s*, 1 H); 5.015 (*s*, 1 H); 3.394 (*m*, 1 H); 2.167 (*quint.*, *J* = 7.3, 2 H); 1.925 (*s*, 3 H); 1.081 (*d*, *J* = 6.6, 3 H); 1.007 (*t*, *J* = 7.6, 3 H); 0.952 (*s*, 3 H); 0.670 (*s*, 3 H). EI-MS: 424 (2, M^+, $C_{30}H_{48}O$), 409 (1), 391(1), 381(2), 314 (2), 271 (12), 159 (10), 145 (17), 105 (51), 95 (98), 93 (62), 91(64), 81(94), 79 (74), 67(96), 57 (57), 55 (100). For 2D-NMR results, see the text.

Phylogenetic Analysis. The phylogenetic analysis was conducted using a combined matrix of 18S rRNA and *rbcL* gene sequences. There were ten ingroup taxa belonging to the Pelagophyceae and four outgroup taxa (not shown in the figure) belonging to the Dictyochophyceae (*Dictyocha speculum, Apedinella radians*, *Pseudopedinella elastica, Rhizochromulina* cf. *marina*). There were 3336 total characters (unordered, equal weights), gaps were treated as missing characters, and there were 687 parsimony-informative characters. The analysis was carried out using PAUP* Version 4.0b10, and a branch-and-bound exhaustive search was conducted. A single most parsimonious tree was recovered (*Fig. 3*), and it had a length of 1931, a consistency index of 0.673, and a retention index of 0.674.

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Received October 22, 2008