Unusual Tetraene Sterols in Some Phytoplankton

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Sterols were analyzed from four phytoplankton strains which are under investigation as possible sources of food for oysters in culture. One strain of *Pyramimonas* contained only 24-methylenecholesterol as a major sterol component. *Pyramimonas grossii, Chlorella autotrophica* and *Dunaliella tertiolecta* each contained a complex mixture of C_{28} and C_{29} sterols with Δ^7, $\Delta^{5,7}$ and $\Delta^{5,7,9(11)}$ nuclear double bond systems. Sterols were found both with and without the C-22 side chain double bond. Ergosterol and 7-dehydroporiferasterol were the principal sterols in each of the latter three species, which also contained the rare tetraene sterols, 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol and 24-ethylcholesta-5,7,9(11),22-tetraen-3β-ol.
***Lipids* 27, 154–156 (1992).**

Most marine invertebrates examined have a complex sterol composition attributed to lack of sterol synthesis in the animal and incorporation of a wide variety of dietary sterols into the animal tissue (1). The oyster (*Crassostrea virginica*) has been shown to contain over forty sterols (2–4), but it cannot synthesize any of them from acetate (5–7). Recent research shows a strong correlation between oyster growth rate and the type and amount of dietary sterol present (8).

Approximately 60% of total oyster sterol is cholesterol, 24-methylenecholesterol and 24-methyl-22-dehydrocholesterol (2). The latter two sterols are found in specific diatoms (9) occurring in the estuarine environments, and several diatoms are known to provide a good food source for oysters (10). The source of the oyster's cholesterol has not been identified and most phytoplankton do not contain more than trace quantities of it (9).

The work described here is part of an effort to improve our knowledge of the effect of dietary sterol on oyster growth rates by examining a wide range of phytoplankton with varied sterol composition which are within the size range utilized by the oyster.

MATERIALS AND METHODS

Axenically cultured algae (strains 580, De, and 78) obtained from the Milford microalgal culture collection were prepared for analysis as previously described (11). Pyram 2 was a gift from Jeff Johanson of the Solar Energy Research Institute (Golden, CO). Dry algal samples (0.3–1.0 g) were extracted with $CHCl_3$/MeOH and sterols were isolated from the non-saponifiable lipid (11). Sterols were analyzed on a 30 m × 0.25 μm fused silica capillary column with a 0.25 mm film of SPB-1 (Supelco, Bellefonte, PA) at 255°C in a Varian Model 3500 chromatograph equipped with a Varian Model 8100 autosampler, an on-column injector, and a hydrogen flame detector (Varian Associates, Palo Alto, CA). Each unknown was injected alone and with a cholesterol standard for relative retention time (RRT) determination. Sterol identity was confirmed by electron impact gas chromatography/mass spectrometry (GC/MS) with a 30 m × 0.32 mm i.d. fused silica capillary column with a 0.25 mm film of DB-1.

RESULTS AND DISCUSSION

Pyramimonas sp. had a simple sterol composition of 99% 24-methylenecholesterol and 1% cholesterol (Table 1). However, the compositions of *Pyramimonas grossii, Chlorella autotrophica* and *Dunaliella tertiolecta* were complex. The principal sterols of *D. tertiolecta* and *C. autotrophica* have been identified previously as ergosterol and 7-dehydroporiferasterol (7,12), but detailed sterol analyses were not reported in either case. In *D. tertiolecta* the side chain methyl and ethyl of these compounds were determined by ^{13}C nuclear magnetic resonance spectrometry to be oriented in the β position (12). Side chain stereochemistry of *C. autotrophica* was not determined, but in other *Chlorella* species where it was determined, the C-24 methyl and ethyl groups were also β-oriented (9,13).

In this work, capillary gas chromatography confirmed the presence of major peaks for the above two sterols but

TABLE 1

Abundance of Sterols in Four Cultured Algae[a]

Sterol	RRT	Species[b] 1	2	3	4
Cholesterol	1.00	—	—	—	1
24-Methylenecholesterol	1.20	—	—	—	99
9(11)-Dehydroergosterol	1.08	3	1	1	—
Ergosterol	1.18	36	22	31	—
5-Dihydroergosterol	1.23	1	5	3	—
Ergost-5,7-dienol	1.35	3	—	—	—
Ergost-7-enol	1.41	20	8	4	—
Stigmasta-5,7,9(11),22-tetraenol	1.33	3	2	2	—
7-Dehydroporiferasterol	1.46	23	46	53	—
Stigmasta-7,22-dienol	1.51	1	9	4	—
Stigmasta-5,7-dienol	1.66	1	—	—	—
Stigmast-7-enol	1.71	6	4	1	—

[a]Abundances are expressed as % of total sterol. RRT are relative to cholesterol. C-24 orientation was not determined for any compound.

[b]Cultures were obtained from the Milford Culture Collection (strain No. in parentheses) except for *Pyramimonas* sp., which was from the Solar Energy Research Center.

1, *Chlorella autotrophica* (580); 2, *Dunaliella tertiolecta* (De); 3, *Pyramimonas grossi* (78); 4, *Pyramimonas* sp. (Pyram 2).

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Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; RRT, relative retention time.

also detected the presence of a number of additional compounds. GC/MS enabled the identification of 7(8)-monoene sterols and 7,22-diene sterols with 28 and 29 carbon atoms (Table 1). In each case mass spectra were identical with those of reference compounds isolated from *Chlorella emersonii* (14). A small amount of 14α-methyl-5α-stigmast-9(11)-enol was identified in *Chlorella autotrophica* by comparison of its mass spectra with those reported by the Akihisa group (15,16). The 24α-epimer of this sterol (all sterols of *Chlorella* to date are 24β) has been reported in *Cucumis sativa* (15), and 14-methyl-5α-ergost-9(11)-enol has been reported recently in *Chlorella vulgaris* (16). Also detected were C_{28} and C_{29} tetraene sterols with GC retention times shorter than for the corresponding trienols ergosterol and 7-dehydroporiferasterol.

Tetraene sterols are not common, but a number of them are known to occur in living organisms. Cholesta-5,7,22,24-tetraenol has been isolated from *Tribolium confusum*, where it is an intermediate in the dealkylation of stigmasterol to cholesterol (17). Known tetraethenoid C_{28} sterols include 14-dehydroergosterol from a strain of *Aspergillus niger* (18), 24-dehydroergosterol from yeast (19), 25(27)-dehydroergosterol (protothecasterol) from the colorless alga, *Prototheca* (20), and 9(11)-dehydroergosterol from *Giberella fujikuroi*, *Candida lipolytica* and *Chlorella vulgaris* (16,21,22). The tetraene sterol reported here from *Chlorella*, *Dunaliella* and *Pyramimonas* has gas chromatographic characteristics (Table 1) and mass spectra identical to those of a synthetic sample of 9(11)-dehydroergosterol provided by Nes (20). Major mass spectra peaks were observed for the free sterol at *m/z* (rel. abundance), 394(8), 376(17), 361(3), 251(100) and 69(100).

Each of the algae containing 9(11)-dehydroergosterol also contained a C_{29} tetraene. Gas chromatographic retention times were 1.23 times those of the C_{28} tetraene in each case (Table 1), which is the increase expected for the addition of an extra carbon at C-28. Mass spectra of the tetraene from each alga were identical and were very similar to those of the C_{28} tetraene, especially with respect to the dominant base peak at *m/z* 251. This compound is identified as 24-ethylcholesta-5,7,9(11),22-tetraenol, a compound to our knowledge not previously reported to occur in nature. Species previously shown to produce C_{28} tetraenes did not produce C_{29} sterols (16,18–22). *Chlorella autotrophica*, *D. tertiolecta* and *P. grossii* each produces a mixture of C_{28} and C_{29} monoenes, dienes, trienes and tetraenes.

Gibberella produced 9(11)-dehydroergosterol only in stationary phase cultures (19). It should be pointed out that the cultures examined here also were harvested from the stationary phase. Detailed analyses from log phase cultures of these algae are not available. Such data may be important in the culture of algae for oyster rearing, because recent data from our laboratories indicate a correlation between algal sterol composition and the growth rates of oysters fed unialgal diets (8).

Neither the metabolic origin nor the role of 9(11)-dehydroergosterol is known. Its accumulation in stationary phase cultures prompts speculation of a new pathway *via* parkeol (lanosta-9(11),24-dien-3β-ol) or alternately by the dehydrogenation of ergosterol. The sterol composition of *D. tertiolecta* determined here is in agreement with that reported in previous work (12). It should be noted that while two other *Dunaliella* species have been reported to contain principally $\Delta^{5,7}$-sterols (23,24), *D. minuta* has 24-methylenecholesterol and 24-methylcholesterol as principal sterols (25), and *D. acidophila* contains 24-ethylcholesterol and 24-ethylidenecholesterol as its major sterols (26). An unialgal diet of *D. tertiolecta* supports moderate growth of oysters (27). Because sterol composition varies widely from species to species, the comparative nutritional value of these species is of great interest.

In our studies, oysters grew more poorly on *P. grossii* than on *D. tertiolecta* (G.H. Wikfors, unpublished data). We have no nutritional data on *Pyramimonas* sp. It is interesting to note that (as is the case with *Dunaliella*), sterol composition varies widely with species in *Pyramimonas*. In contrast with the species of *Pyramimonas* studied here, Volkman (28) has found isofucosterol to be the principal sterol of *Pyramimonas gelidicola*. In many phytoplankton taxa, names and taxonomic affinities of species are in a constant state of flux. More work, especially on taxonomy, will be necessary to determine whether these three genera have the variety of sterols they now appear to have.

ACKNOWLEDGMENT

This is Contribution No. 8412 of the Maryland Agricultural Experiment Station.

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[Received October 21, 1991; Revision accepted January 3, 1992]