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**Abstract**

Sterol composition of dinoflagellates: Different abundance and composition in heterotrophic species and resting cysts.

**Keywords**

Dinoflagellates, Sterols, Heterotrophic, Resting cysts

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Sterol composition of dinoflagellates: Different abundance and composition in heterotrophic species and resting cysts

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INTRODUCTION

Dinoflagellates have been one of the major primary marine producers since the Mesozoic. These microalgae occur throughout the world’s oceans but are often more abundant in coastal areas. A very small number of dinoflagellates are calcareous or siliceous, unlike other major primary producers, such as coccolithophores and diatoms. The motile stage of dinoflagellates is rarely recorded in sediments, because the motile cells of dinoflagellates are labile against biological and chemical degradation during settling and early diagenesis. Thus, organic geochemical techniques are useful in reconstructing paleo-dinoflagellate productivity. The sterol compositions of dinoflagellates are generally dominated by 4α-methyl sterols, including the C30 steroid called dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol; e.g., Withers, 1987; Pirretti et al., 1997). This steroid is rarely found in other algae and therefore has been often used as an indicator of the dinoflagellate contribution to marine sediments and primary productivity. While approximately half of dinoflagellate species are autotrophic, the other half are heterotrophic. Because culturing heterotrophic dinoflagellates is difficult, the sterol compositions of heterotrophic dinoflagellates have been reported in only a few species (Withers et al., 1978; Klein et al., 1999; Leblond and Chapman, 2004; Leblond et al., 2006; Chu et al., 2008), which were found to contain dinosterol. Thus, the dinosterol concentration of the sediment might not reveal the primary productivity of autotrophic dinoflagellates. It is worthwhile to investigate whether sedimentary dinosterols contain a significant proportion from heterotrophic dinoflagellates.

Some dinoflagellate species are able to force themselves into a dormant stage as part of their relatively complicated life cycle. During these dormant stages, resting...
cysts form, typically characterized by a thick and highly specialized cell covering. The resting cysts of some dinoflagellate species are composed of resistant biomacromolecules, which can be preserved in sediments and sedimentary rocks. The geologic record of dinoflagellate evolution, therefore, is based largely on their resting cyst fossils in sedimentary rocks. The rich fossil record of dinoflagellates, which extends back at least 225 million years, is composed almost exclusively of selectively preserved resting cyst cell walls (Evitt, 1961, 1985). Dinosterol has been detected in resting cysts, such as a resting cyst of *Lingulodinium polyedrum* (former *Gonyaulax polyedra*; Kokinos et al., 1998). Sedimentary dinosterols thus do not necessarily provide sufficient information about the motile stage of dinoflagellates. Sterol compositions vary quite considerably between different species of dinoflagellates (Volkman et al., 1999). Some sterols may be potential biomarkers for the motile stage and resting cyst. The objective of the present study was to compare the dinosterol content in autotrophic and heterotrophic dinoflagellates, and to try to identify characteristic sterols of autotrophic and heterotrophic dinoflagellates. We also compared the dinosterol content in motile cells with that in resting cysts and investigated each for biomarkers. Such studies detailing the source of biomarkers, based on culture experience, are necessary for the application of organic geochemical techniques in paleoceanographic reconstruction.

**SAMPLES AND METHODS**

Motile cells of four dinoflagellates (*Akashiwo sanguinea* (Hirasaka), *Scrippsiella tinctoria* Indelicato et Loeblich III, *Prorocentrum micans* Ehrenberg, and *Protoperidinium crassipes* (Kofoid) Balech) were isolated from sea water samples, which were collected at Otaru harbor, Otaru City, Hokkaido, Japan (43°10′ N, 141°01′ E). A motile cell and a resting cyst of *Peridinium umbonatum var. inaequale* were isolated from a pond in Kiritappu Moor, Hamanaka Town, Hokkaido, Japan (43°11′ N, 145°10′ E). Clonal cultures of marine phototrophic dinoflagellates (*S. tinctoria*, *P. micans*, *A. sanguinea*) were established from seawater samples collected along the coast of Japan. The seawater samples were placed in plastic cups, and cells were isolated from these cups by micropipetting individual cells viewed with an inverted microscope (Olympus CK-41,
Tokyo, Japan), followed by several rinses in sterile Provasoli’s Enrichment Seawater (PES) medium (Provasoli, 1968). Isolated cells were individually put into a 24-well tissue culture plate (TPP, Trasadingen, Switzerland), with each well containing the PES medium. Established cultures were grown under a 16/8 h light/dark cycle at a constant temperature of 20°C. Details of the culture procedure for the heterotrophic dinoflagellate P. crassipes have been previously reported by Yamaguchi and Horiguchi (2008). Cells of P. crassipes were isolated from seawater by micropipetting individual cells viewed with an inverted microscope through a series of drops of sterile PES medium, into 24-well tissue culture plates. Each well contained 1–2 ml of the PES medium and received 7–11 mg of rice flour. These plates were incubated in a temperature-controlled room at 20°C, under illumination of 30–40 µmol photons m⁻²s⁻¹ with 16:8 light:dark (L:D) light regime.

After cultivation, cell density was estimated by counting the number of cells in 10-µl samples. The cultured samples were immediately collected in GF/F filters. After filtration, lipids were immediately extracted by ultrasonication with methanol/dichloromethane (3:1), methanol/dichloromethane (1:1), and dichloromethane and then concentrated and passed through a short bed of Na₂SO₄ to remove water. The combined lipid extract was saponified with 0.5 mol KOH/methanol and added to distilled water. The neutral fraction was extracted with diethylether/n-hexane (1:9) and then fractionated into four fractions (F1: 3 ml of hexane; F2: 3 ml of hexane:toluene (3:1); F3: 4 ml of toluene; F4: 3 ml of toluene:methanol (3:1)) by silica gel column chromatography. The F4 was converted to trimethylsilyl esters using bis(trimethylsilyl)-trifluoroacetamide (BSTFA); an internal standard (C₂₄D₅₀) was added prior to injection for component quantification. The F4 was analyzed using a Hewlett Packard 6890 series gas chromatograph (GC) equipped with a splitless injector, fused silica column DB-5 (30 m x 0.32 mm i.d.; film thickness 0.25 µm), and flame ionization detector. The injection volume of the samples was 1 µl. The carrier gas was He, at a flow rate of 3.6 ml/min. The oven temperature program was as follows: isothermal at 40°C for 2 min, followed by temperature increase from 40 to 300°C at 4°C/min (hold 20 min). Components were identified from analysis of mass spectra obtained using GC-mass spectrometry with a Hewlett Packard HP6890 chromatograph and HP5973 spectrometer. The GC column was as above, and the temperature program was as follows: isothermal at 50°C for 4 min, 50 to 300°C at 4°C/min (hold 20 min). The carrier gas was He with a flow rate of 1.5 ml/min. Sterols were identified by comparison with fragmentation ions of dinoflagellate sterols reported by Bohlin et al. (1981), Kokke et al. (1981), and Nichols et al. (1984). Quantification was performed by comparing the peak area of specific compounds with that of the internal standard (C₂₄D₅₀) in gas chromatograms.

**RESULTS AND DISCUSSION**

Sterol compositions of motile cells of autotrophic and heterotrophic dinoflagellates

Motile cells of the autotrophic dinoflagellates *P. umbonatumin var. inaequale*, *A. sanguinea*, *S. tinctoria*, and *P. crassipes* have been previously reported by Yamaguchi and Horiguchi (2008). Cells of *P. crassipes* were isolated from seawater by micropipetting individual cells viewed with an inverted microscope through a series of drops of sterile PES medium, into 24-well tissue culture plates. Each well contained 1–2 ml of the PES medium and received 7–11 mg of rice flour. These plates were incubated in a temperature-controlled room at 20°C, under illumination of 30–40 µmol photons m⁻²s⁻¹ with 16:8 light:dark (L:D) light regime.

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**RESULTS AND DISCUSSION**

Sterol compositions of motile cells of autotrophic and heterotrophic dinoflagellates

Motile cells of the autotrophic dinoflagellates *P. umbonatumin var. inaequale*, *A. sanguinea*, *S. tinctoria*, and
**P. micans** commonly contained five major sterols in the free sterol fraction (Fig. 1). These major sterols were cholest-5-en-3β-ol (cholesterol), 4-methylcholestan-3β-ol, 4,24-dimethylcholestan-3β-ol, dinosterol, and 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol). The major sterols of **P. micans** were the same as those reported by Volkman et al. (1999).

A motile cell of the heterotrophic dinoflagellate **P. crassipes** contained cholesterol, 4,24-dimethylcholestan-3-ol, dinosterol, campest-5-en-3β-ol (campesterol), stigmaster-5-en-3β-ol (β-sitosterol), dinostanol, and 4-tetramethylcholestan-3-ol as major free sterols (Fig. 1). The 4-tetramethylcholestan-3-ol was identified from comparison with the mass spectra of C28, C29, and C30 4-methyl sterol (Fig. 2). A series of mass spectra of 4-methyl sterols were similar, and the number of diagnostic ions increased by 14 as the carbon number increased.

The dinosterol concentrations of autotrophic dinoflagellates varied within a range of 0.18 to 0.52 pg/cell (Fig. 3). The dinosterol concentration of the heterotrophic dinoflagellate **P. crassipes** was 2.3 pg/cell, which is about 4–12 times higher than the range for the autotrophic species (Fig. 3). Regarding the 4-methyl sterol compositions of heterotrophic dinoflagellates, several studies previously reported that dinosterol was detected in the heterotrophic dinoflagellates **C. cohnii** (Withers et al., 1978), **P. piscicida** (Leblond and Chapman, 2004), **Amoebophrya** sp. (Leblond et al., 2006), and **G. dominans** (Chu et al., 2008). Although dinosterol is a minor sterol in **Amoebophrya** sp. and **G. dominans**, it is the dominant sterol in **P. piscicida** dinosterol (Leblond and Chapman, 2004; Leblond et al., 2006; Chu et al., 2008). The genus *Protoperidinium* currently includes more than 200 species, and its members are some of the most common organisms in neritic plankton (Yamaguchi and Horiguchi, 2005). These results suggest that the heterotrophic dinoflagellate could be an important source of dinosterol in some sediments. Thus, dinosterol content in sediment might not reflect primary production.

Although 4-methyl sterols were major sterols in the motile cells of five dinoflagellates, the 4-methyl sterol compositions of four autotrophic dinoflagellates were different from that of the heterotrophic dinoflagellate **P. crassipes**. 4-Methylcholestan-3-ol was commonly detected in autotrophic dinoflagellates, but not in the heterotrophic dinoflagellate in the present study (Fig. 1). In contrast, 4-tetramethylcholestan-3-ol was detected only in the heterotrophic dinoflagellate, **P. crassipes** (Fig. 1). Other sterols detected in only **P. crassipes** were campesterol and β-sitosterol. Rice flour was fed to **P. crassipes** during the culture experiment. Sterol analysis of the rice flour showed that its major sterols were campesterol and β-sitosterol. This suggests that the campesterol and β-sitosterol may have originated from the rice flour mixed in the culture medium used for **P. crassipes**. However, no 4-tetramethylcholestan-3-ol was detected in the rice flour. The 4-tetramethylcholestan-3-ol detected in **P. crassipes** has not been reported in cultured samples of other heterotrophic dinoflagellates, such as **C. cohnii** (Withers et al., 1978), **O. marina** (Klein et al., 1999), **P. piscicida** (Leblond and Chapman, 2004), **Amoebophrya** sp. (Leblond et al., 2006), and **G. dominans** (Chu et al., 2008). In contrast, 4-methylcholestan-3-ol was detected in the heterotrophic dinoflagellates **P. piscicida**, **Amoebophrya** sp., and **G. dominans** (Leblond and Chapman, 2004; Leblond et al., 2006; Chu et al., 2008). Although 4-tetramethylcholestan-3-ol has not been reported in any extant marine organism, Thomas et al. (1993) and Hou et al. (1999) reported the presence of tentatively assigned 4,22,23,24-tetramethyl cholestanes in brackish and marine sediments. Thomas et al. (1993) speculated that the presence of these abundant components was attributed to a diagenetic cleavage of the cyclopropyl ring in the side chain of 4α-methylgorgostan-
Analyses of other (Volkman C27 and C28 sterols, such as cholesterol and brassicasterol) dominant sterol. Many diatoms contain a great deal of dinoflagellate in extant species of dinoflagellates. In the present study, species may biosynthesize 4-tetramethylcholestan-3-ol. Although we tried to culture P. crassipes by feeding it various diatoms, phytoplanktons, and plant flours, such as wheat flour, P. crassipes grew only when rice flour was used. In rice flour, C29 β-sitosterol was the dominant sterol. Many diatoms contain a great deal of C27 and C28 sterols, such as cholesterol and brassicasterol (Volkman et al., 1998). Differences in sterol compositions in the feed might affect the 4-methyl sterol compositions of heterotrophic dinoflagellates. Further sterol analyses of other Protoperinidium sp. are necessary to clarify that the 4-tetramethylcholestan-3-ol is indigenous to Protoperinidium sp.

Sterol compositions of dinoflagellate motile cells and resting cysts of Peridinium umbonatum var. inaequale

Resting cysts of Peridinium umbonatum var. inaequale contained 4-methylcholestan-3-ol, 4,24-dimethylcholestan-3-ol, dinosterol, and dinostanol as major free sterols (Fig. 3). The dinosterol concentration of the resting cysts of P. umbonatum var. inaequale was higher than that of its motile cells. Lipid biomarkers are generally almost completely degraded by the time of arrival on the bottom sediments. Prahl et al. (2000) showed that the dinosterol concentration normalized to Al could be calculated for the average sediment particles and bottom sediments in the Arabian Sea. Comparisons with the concentrations in average sediment trap particles suggested that each was sensitive to significant (~99%) degradation. Resting cysts are generally found on the seabed, so the dinosterol produced from the resting cyst is not degraded to a greater degree than that from motile cells in the water column. Dinoflagellate resting cysts may contribute to sedimentary dinosterol levels in some sediments. Thus, it is necessary to check whether sedimentary dinosterol contains a significant proportion of dinoflagellate resting cysts.

Algal sterol distribution of resting cysts was closely similar to that of the motile cells, the unknown compound occurred only in the resting cysts (Fig. 1, compound No. 10). This compound was the major compound present, in addition to the dinosterol in the sterol fraction. Mass spectra of the trimethylsilylated (TMS) derivative of the unknown compound are shown in Fig. 4. The fragmentation ions of 497 and 512 show that the molecular weight of the TMS derivatives of the unknown compound was 512. That is, the molecular weight of this compound was 440. This compound showed ions at m/z 129 and 383 (M-129), characteristic fragment ions of the TMS derivatives of Δ5-sterols. It also had an intense MS fragmentation peak at m/z 271, a typical fragment ion of the Δ22-sterols. These results suggested that the unknown compound was C31Δ5-22-sterol (C31H49O). 4α-Methylgorgostanol is known to be a typical C31 sterol produced by dinoflagellates (Good and Goodwin, 1972). The 4α-methylgorgostanol was originally detected in zooxanthellae of Briareum asbestinum and the dinoflagellate Kryptoperidinium (=Glenodinium) foliaceum (Alam et al., 1979; Steudler et al., 1977). However, mass spectral analysis showed that the C31Δ5,22-sterol in the present study was clearly different from 4α-methylgorgostanol. The 4α-sterol did not show ions at m/z 129. Additionally, the molecular weight of 4α-methylgorgostanol is 442. Kokinos et al. (1998) tentatively identified C30 4α-demethylsterol, gorgosterol (22,23-methylene-23,24-dimethylcholesterol-5-en-3β-ol), in the sterol fraction of a resting cyst of Liguodinium polyedrum (formerly Gonyaulax polyedra). Gorgosterol has been reported in a number of marine invertebrates and animals. Recently, it was detected in the pennate diatom genus Delphineis (Rampen et al., 2009). The C31 sterol in the present study was not gorgosterol because the molecular weight of gorgosterol is 428. Several C31Δ5-sterols have been detected in marine sponges (Li and Djerassi, 1983; Ha et al., 1985; Shubina et al., 1985; Calderon et al., 2004). Their sterols have a 24-isopropyl constituent. Thus, the C31Δ5 sterols in sponges show fragmentation ions at M-43, M-61, and M-112 (M-C3H7, M-(C3H7 + H2O) and M-C6H13), resulting from allylic cleavage at C-24. These fragmentation ions were not present in the mass spectrum of the C31Δ5,22-sterol detected in this study. None of the motile cells in this study contained the C31Δ5,22-sterol. The C31Δ5,22-sterol detected in resting cysts of P. umbonatum var. inaequale has not been reported in cultured samples of motile cells of dinoflagellates. This sterol might have been produced during the resting stage. Fur-

Sterol composition of dinoflagellates
thermore, this compound may be a potential biomarker of dinoflagellate resting cysts. Further study of this C_{31}$\Delta^{5,22}$-sterol will provide clues for identifying biomarkers of dinoflagellate resting cysts.

**Conclusions**

The motile stage of four autotrophic and one heterotrophic dinoflagellate and the cysts of an autotrophic dinoflagellate were studied to compare the dinosterol concentration of the resting cyst of P. umbonatum var. inaequale with that of motile cell. Dinoflagellate resting cysts may contribute to sedimentary dinosterol in some sediments because resting cysts are generally found on the seabed. C_{31}$\Delta^{5,22}$-sterol was discovered in the resting cysts of P. umbonatum var. inaequale, and this sterol was not detected in the five types of motile cells analyzed in this study. This compound has not been reported in cultured samples of motile cells of dinoflagellates. This compound might have been produced during the resting stage, and may be a potential biomarker of dinoflagellate resting cysts.

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**References**


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