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

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The motile cells of the autotrophic dinoflagellates *Peridinium umbonatum* var. *inaequale*, *Akashiwo sanguinea*, *Scrippsiella tinctoria*, and *Prorocentrum micans* commonly contain five major sterols: cholesterol, 4-methylcholestan-3-ol, 4,24-dimethylcholestan-3-ol, dinosterol, and dinostanol. The motile cell of the heterotrophic dinoflagellate *Protoperidinium crassipes* contains cholesterol, 4,24-dimethylcholestan-3-ol, dinosterol, dinostanol, and 4-tetramethylcholestan-3-ol as major free sterols. The dinosterol concentrations of heterotrophic dinoflagellates are about 4–12 times higher than those of autotrophic species, suggesting that the heterotrophic dinoflagellate is an important source of dinosterol in some sediments. 4-Tetramethylcholestan-3-ol has not been reported in cultured samples of other heterotrophic dinoflagellates. The difference in 4-methyl sterol composition in heterotrophic dinoflagellates may be related to the feeds used during the culture experiment. The resting cyst of *P. umbonatum* var. *inaequale* contains 4-methylcholestan-3-ol, 4,24-dimethylcholestan-3-ol, dinosterol, dinostanol, and $C_{31}\Delta^{5,22}$-sterol as major free sterols. The $C_{31}\Delta^{5,22}$-sterol has not been reported in cultured samples of motile cells from dinoflagellates. This compound might be produced during the resting stage, and thus may serve as a potential biomarker for the resting cysts of dinoflagellates.

Keywords: 4α-methyl sterol, autotrophic dinoflagellate, heterotrophic dinoflagellate, resting cyst, motile cell

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 C_{30} sterol called dinosterol ($4\alpha,23,24$-trimethyl-5α-cholest-22E-en-3β-ol; e.g., Withers, 1987; Pirretti *et al.*, 1997). This sterol 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

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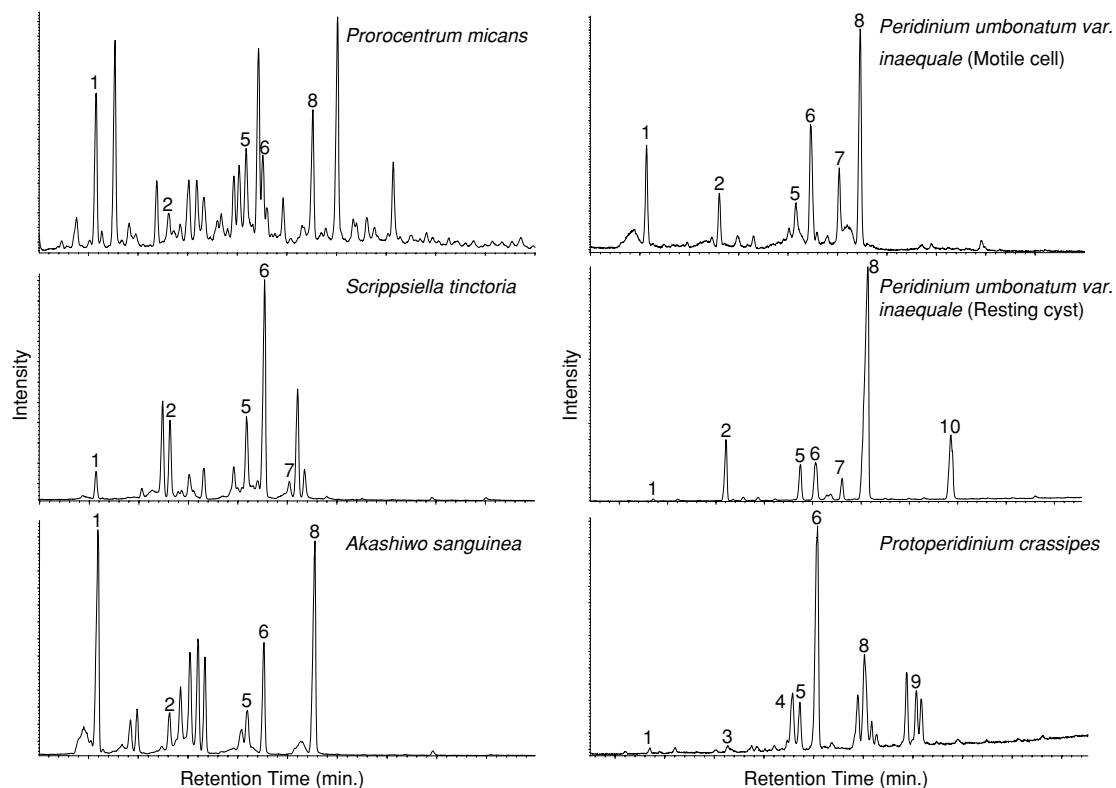[‡]Present address: Institute for East China Sea Research, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521, Japan.

Fig. 1. Total ion chromatograms of sterol fractions from all dinoflagellates samples. 1: cholesterol, 2: 4α-methylcholestan-3β-ol, 3: campesterol, 4: β-sitosterol, 5: 4α,24-dimethyl-5α-cholestan-3β-ol, 6: dinosterol, 7: 4α-23,24-trimethylcholest-17(20)-en-3β-ol, 8: 4α,23R,24R-trimethyl-5α-cholestan-3β-ol, 9: 4α-tetramethycholestan-3β-ol, 10: unknown C_{31} sterol.

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* (formerly *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* (Lemmermann) 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^{-2}s^{-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 Na$_2$SO$_4$ 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 (C$_{24}$D$_{50}$) 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 × 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 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 (MS) 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 fragment 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
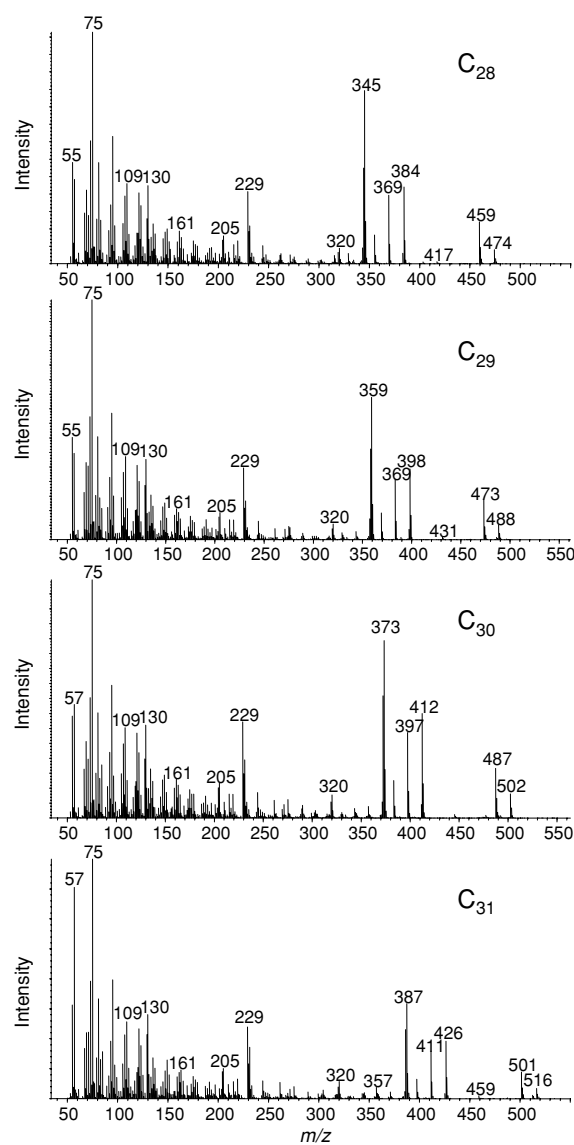

Fig. 2. Mass spectra of (a) TMSi-ether of 4α-methylcholestan-3β-ol (C$_{28}$), (b) TMSi-ether of 4α,24-dimethyl-5α-cholestan-3β-ol (C$_{29}$), (c) TMSi-ether of 4α,23R,24R-trimethyl-5α-cholestan-3β-ol (C$_{30}$) from the resting cyst of Peridinium umbonatum var. inaequale, and (d) TMSi-ether of 4α-tetramethylcholestan-3β-ol (C$_{31}$) from Protoperidinium crassipes.

peak area of specific compounds with that of the internal standard (C$_{24}$D$_{50}$) in gas chromatograms.

RESULTS AND DISCUSSION

Sterol compositions of motile cells of autotrophic and heterotrophic dinoflagellates

Motile cells of the autotrophic dinoflagellates *P. umbonatum* 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), stigmast-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 C_{28}, C_{29}, and C_{30} 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
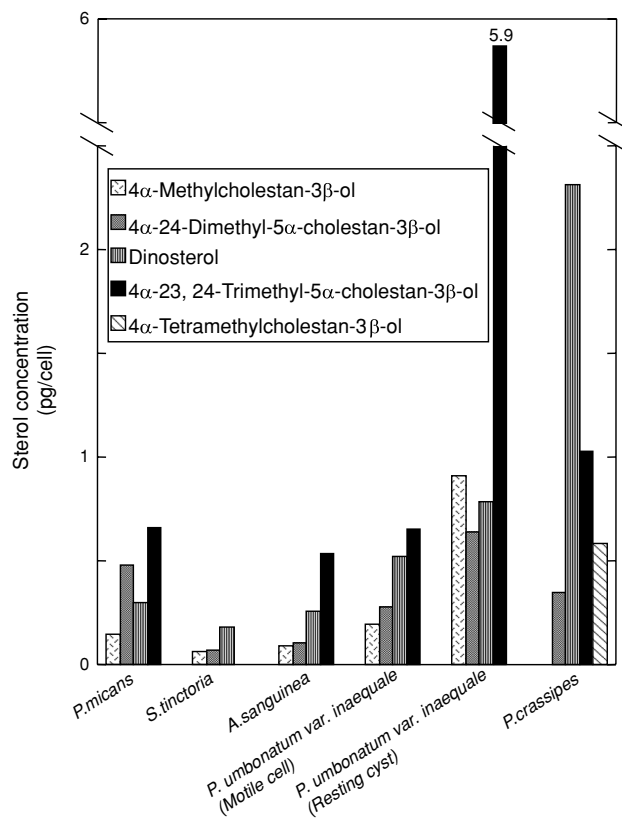

Fig. 3. Cell concentrations of 4-methyl sterols from Prorocentrum micans, Scrippsiella tinctoria, Akashiwo sanguinea, Peridinium umbonatum var. inaequale (motile cell), Peridinium umbonatum var. inaequale (resting cyst), Protoperidinium crassipes.

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-

3β-ol (4α-methylgorgostanol), which has been reported in extant species of dinoflagellates. In the present study, 4α-methylgorgostanol was not detected in the living dinoflagellate *P. crassipes*. Thus, some dinoflagellate species may biosynthesize 4-tetramethylcholestan-3-ol. In this study, the heterotrophic dinoflagellate *P. crassipes* was fed rice flour during the culture experiment. In general, *Protoperidinium* sp. feed on diatoms in the natural environment. 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, C_{29} β-sitosterol was the dominant sterol. Many diatoms contain a great deal of C_{27} and C_{28} sterols, such as cholesterol and brassicasterol (Volkman *et al.*, 1998). Differences in sterol compositions of the feed might affect the 4-methyl sterol compositions of heterotrophic dinoflagellates. Further sterol analyses of other *Protoperidinium* sp. are necessary to clarify that the 4-tetramethylcholestan-3-ol is indigenous to *Protoperidinium* 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.

Although the 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 dinostanol in the sterol fraction. Mass spectra of the trimethylsilylated (TMS) derivative of the unknown compound are shown in Fig. 4. The fragment 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
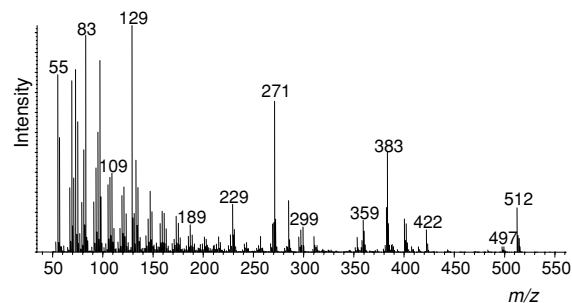

Fig. 4. Mass spectra of unknown C_{31} sterol from resting cysts of Peridinium umbonatum var. inaequale.

was 440. This compound showed ions at *m/z* 129 and 383 (M-129), characteristic fragment ions of the TMS derivatives of Δ^5-sterols. It also had an intense MS fragmentation peak at *m/z* 271, a typical fragment ion of the Δ^{22}-sterols. These results suggested that the unknown compound was $C_{31}\Delta^{5,22}$-sterol ($C_{31}H_{52}O$). 4α-Methylgorgostanol is known to be a typical C_{31} sterol produced by dinoflagellates (Goad 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 $C_{31}\Delta^{5,22}$-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 C_{30} 4α-demethylsterol, gorgosterol (22,23-methylene-23,24-dimethylcholest-5-en-3β-ol), in the sterol fraction of a resting cyst of *Ligulodinium 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 C_{31} sterol in the present study was not gorgosterol because the molecular weight of gorgosterol is 428. Several $C_{31}\Delta^5$-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 $C_{31}\Delta^5$-sterols in sponges show fragment ions at M-43, M-61, and M-112 (M-C_3H_7, M-(C_3H_7 + H_2O) and M-C_8H_{16}), resulting from allylic cleavage at C-24. These fragment ions were not present in the mass spectrum of the $C_{31}\Delta^{5,22}$-sterol detected in this study. None of the motile cells in this study contained the $C_{31}\Delta^{5,22}$-sterol. The $C_{31}\Delta^{5,22}$-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-

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 content in each dinoflagellate, and to try to identify the characteristic sterols of each dinoflagellate. The dinosterol concentrations of heterotrophic dinoflagellates are approximately 4-12 times higher than those of autotrophic species, suggesting that heterotrophic dinoflagellates can be an important source of dinosterol in some sediments. 4-Tetramethylcholestan-3-ol was detected only in the heterotrophic dinoflagellate, *P. crassipes*. This compound has not been reported in cultured samples of other heterotrophic dinoflagellates. The difference in the 4-methyl sterol compositions of heterotrophic dinoflagellates might be related to the fact that the heterotrophic dinoflagellate, *P. crassipes*, was fed rice flour during the culture experiment.

The dinosterol concentration of the resting cyst of *P. umbonatum* var. *inaequale* was higher than 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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