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Sterols of four dinoflagellates from the genus *Prorocentrum*

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Abstract

The compositions of 4-desmethyl sterols and 4-methyl sterols in four species of marine dinoflagellates of the genus *Prorocentrum* (viz., *P. micans* Ehrenberg, *P. minimum* (Pavillard) Schiller, *P. balticum* (Lev.) Lemm and *P. mexicanum* Tafall) were identified by capillary gas chromatography–mass spectrometry as part of a study to identify signature lipids for dinoflagellates in marine organic matter. Complex mixtures were found in each species with over 20 sterols identified in all. All species contained the same core group of sterols, but there were significant differences in the proportions of the various sterols. Two distinct groupings could be discerned in the sterol patterns. The 4-methyl sterol 4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol (dinosterol), which is common in many dinoflagellates, predominated in *P. balticum* and in *P. minimum* whereas in the closely related species *P. micans* and *P. mexicanum* the major sterol was cholesterol. A novel monounsaturated C_{23} sterol having a much shortened side-chain was found in *P. balticum* and *P. minimum* and both *P. balticum* and *P. minimum* contained peridinosterol (4α,23,24-trimethyl-5α-cholest-17(20)-en-3β-ol). 24-Methylenecholesterol was only found in *P. minimum*, where it comprised over one-third of the sterols. The steroid ketone dinosterone occurred in *P. balticum*, but none of the other species contained steroid ketones. Although all the sterol distributions were broadly similar, the presence or absence of specific components might be a useful chemotaxonomic tool for distinguishing between closely related species.

Keywords: Dinoflagellates; Dinosterol; Dinosterone; Dinophyceae; Ketones; 4-Methylsterols; Microalgae; Peridinosterol; *Prorocentrum*; Stanols

1. Introduction

A wide diversity of sterol distributions have been found in microalgae (Withers, 1983, 1987; Volkman, 1986; Volkman, Barrett, Dunstan & Jeffrey, 1993; Piretti et al., 1997), which has prompted a number of studies to test the usefulness of these compounds as signature lipids (biomarkers) for the different microalgal classes. The sterol compositions of most dinoflagellates are dominated by 4α-methyl sterols including the C_{30} sterol dinosterol (4α,23,24-trimethyl-5α-cholest-22*E*-en-3β-ol) (Withers, 1983, 1987). This sterol is rarely found in other algae (Volkman et al., 1993) and hence has been used as an indicator of dinoflagellate contribution of organic matter to marine sediments (Boon et al., 1979; Robinson, Eglinton, Brassell & Cranwell, 1984). While this sterol has proved to be a useful biomarker, some dinoflagellates have high contents of 4-desmethyl sterols and a few lack dinosterol. For example, species of the genus *Amphidinium* synthesize amphisterol (4α,24-dimethyl-5α-cholesta-8(14),24(28)-dien-3β-ol) as their major sterol (Withers, Goad & Goodwin, 1979), whereas several *Gymnodinium* species have 4α,24-dimethylcholestanol as a major sterol and contain little dinosterol (Withers, 1987). A freshwater dinoflagellate, *Ceratium furcoides*,

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apparently contains no 4-methyl sterols at all (Robinson, Cranwell, Eglinton & Jaworski, 1987).

Sterols with a fully saturated ring system (i.e. 5α(H)-stanols) often occur in dinoflagellates, but are only rarely abundant in other classes of marine microalgae (Nishimura & Koyama, 1977; Volkman, Kearney & Jeffrey, 1990). Hence, dinoflagellates can be a major direct input of 5α(H)-stanols in some marine sediments (Robinson et al., 1984), adding to the pool of 5α(H)-stanols produced by bacterial reduction of unsaturated sterols. A few dinoflagellates also contain steroidal ketones which usually show a predominance of dinosterone (Withers et al., 1978; Kokke, Fenical & Djerassi, 1982; Robinson et al., 1987). *Scrippsiella trochoidea* is unusual in that it contains a complex mixture of 21 steroidal ketones (Harvey, Bradshaw, O'Hara, Eglinton & Corner, 1988).

Sterols can exist in a variety of biochemical forms. Most sterols occur mainly in the extractable free (i.e. nonesterified) form, but one can also find smaller amounts of sterols esterified to fatty acids or sugars and in some species steryl sulfates can be found. Most studies report either free sterols or total extractable (free plus esterified) sterols. Data for both types of sterol distribution are reported here.

Prorocentrum species are important components of both phytoplanktonic and benthic microalgal communities. *P. balticum* is planktonic and neritic with a worldwide distribution. *P. micans* and *P. minimum* are both planktonic; *P. micans* is neritic and estuarine, but found in oceanic waters. *P. minimum* is mostly estuarine, but also neritic. Both are cosmopolitan in cold temperate to tropical waters. *P. minimum* and *P. balticum* are morphologically very similar and have been confused in literature records.

Several species of *Prorocentrum* are known to produce toxins and other bioactive compounds (Grzebyk, Denardou, Berland & Pouchus, 1997) and at least two primarily benthic species have been cultured and shown to produce DSP toxins (Hallegraeff, 1991). A Caribbean species, *P. concavum* Fukuyo, was cultured and shown to be a producer of okadaic acid (Dickey, Borzin, Faulkner, Bencsath & Andrzejewski, 1990). Two potent toxins were isolated from cultures of *P. lima* and identified as okadaic acid and 5-methylene-6-hydroxy-2-hexen-1-okadate (Yasumoto, Seino, Murakami & Murata, 1987). Indeed, *P. lima* is believed to be a source of DSP toxins in Spanish coastal waters and harvesting of molluscs is restricted when it is present (Shumway, 1990). *P. minimum* and *P. micans* produce an extracellular β-diketone norcarotenoid which has been found to exhibit antibacterial and antifungal activity in both the laboratory and in the field (Trick, Andersen & Harrison, 1984).

In view of the importance of species of *Prorocentrum* as bloom forming species (Hallegraeff, 1991, 1993) and as potentially important sources of organic matter in marine environments, research groups at CSIRO and NIOZ independently undertook investigations of the sterol compositions of several of the commonly found species. Our main aim was to see whether these microalgae might have characteristic sterols that could be used as biomarkers for identifying dinoflagellate-derived organic matter in marine sediments. Although the methods used in our two laboratories are not identical (for example, at NIOZ data were obtained for extractable free sterols while at CSIRO information was obtained on total extractable free plus esterified sterols), we felt that there was value in combining our data sets and thus presenting a more comprehensive overview of the sterol distributions of this algal genus.

2. Results

Compositional data on the sterols identified in the *Prorocentrum* species are shown in Table 1. Information is also provided for 2 different strains of *P. micans* and 2 analyses of the same strain of *P. balticum*. Compositional data for total extractable sterols for other strains of *P. micans* reported by Yamaguchi, Ito and Hata (1986) and by Piretti et al. (1997), as well as *P. cordatum* analysed by Nichols, Jones, de Leeuw and Johns (1984) are provided for comparison. Sterol structures are shown in Fig. 2.

2.1. Prorocentrum minimum

This species contained 3 major sterols in the free sterol fraction, which collectively represented over 85% of the sterols present; 5 minor sterols were also identified (Table 1). The major sterol was 24-methylcholesta-5,24(28)-dien-3β-ol (38.3%), followed in abundance by dinosterol and the unusual 4-desmethyl sterol 23,24-dimethylcholesta-5,22*E*-dien-3β-ol. Cholesterol was not detected in this species, which sets it apart from most other dinoflagellates. Rather, in this species there is efficient production of sterols alkylated in the side-chain.

2.2. Prorocentrum balticum

Two cultures of *P. balticum* were analyzed and both had very similar free sterol profiles consisting of 13 identified components (Table 1). The major 4-methyl sterol was dinosterol (46.6 and 42.2%), while the major 4-desmethyl sterol was the C_{28} sterol 24-methylcholesta-5,22*E*-dien-3β-ol (16.9 and 15.0%). Small amounts of a monounsaturated C_{23} sterol (1.7 and 0.7%) were found in both samples (see below).

Table 1
Comparison of sterol composition of *Prorocentrum* species (as % of total sterols) analysed here with those reported previously

Sterol	Code[a]	*P. minimum*, NIOZ free	*P. cordatum*[b], Australia total	*P. balticum*, NIOZ #1 free	*P. balticum*, NIOZ #2 free	*P. micans*, NIOZ free	*P. micans*, CSIRO total	*P. micans*[c], Japan total	*P. micans*[d], Adriatic free	*P. mexicanum*, CSIRO total
$C_{23:1}$ (unknown)	Ia?	2.2		1.7	0.7					
$C_{27:2}$ ($\Delta^{5,22}$)	If			3.8	2.4					
$C_{27:1}$ (Δ^{5})(cholesterol)	Ib		4.2	2.0	1.7	32.4	44.2	28.8		48.0
$C_{28:2}$ ($\Delta^{5,22}$/24Me)	Ic		2.5	16.9	15.0	0.7	1.9	8.6		
$C_{28:2}$ ($\Delta^{5,24(28)}$/24Me)	Ig	38.3	35.4							
$C_{28:0}$ (4α,24diMe)	IIIi					1.1				
$C_{29:2}$ ($\Delta^{5,22}$/23,24diMe)	Id	18.8	12.5	4.6	3.6	17.4	12.4			35.2
$C_{29:2}$ ($\Delta^{5,22}$/24Ethyl)	Im							14.2		
$C_{29:2}$ ($\Delta^{7,22}$/23,24diMe)	IVd									0.7
$C_{29:1}$ (Δ^{22}/23,24diMe)	IId	2.3	1.5			2.0	13.0			2.5
$C_{29:1}$ (Δ^{22}/4α,24diMe)	IIIc	2.7	1.4	2.9	2.5	4.0	2.2		6.3	1.7
$C_{29:1}$ (Δ^{22}/4α,23diMe)	IIIl							20.9		
$C_{29:1}$ ($\Delta^{24(28)}$/4α,24diMe)	IIIg	3.6	5.2	3.8	1.5					
$C_{29:1}$ (Δ^{5}/24Ethyl)	Ij					2.8				
$C_{29:1}$ (Δ^{5}/23,24diMe)	Ie						2.0			
$C_{29:0}$ (5α/24Ethyl)	IIj					1.0				
$C_{29:0}$ (5α/23,24diMe)	IIe						2.2			
$C_{30:1}$ (Δ^{22}/4α,23,24triMe)	IIId	28.4	28.7	46.6	42.2	14.5	4.6	14.5	45.6	7.1
$C_{30:2}$ (unknown)				4.5	9.9					
$C_{30:1}$ ($\Delta^{17(20)}$/4α,23,24triMe)	IIIn	3.2		4.5	2.1					
$C_{30:1}$ ($\Delta^{24(28)}$/4α,23,24triMe)[e]	IIIh		8.6							
$C_{30:1}$ ($\Delta^{8(14)}$/4α,23,24triMe)	VIe			2.8	2.2					
$C_{30,0}$ (dinostanol isomer - 23*S*)	IIIe			4.2	10.3					
$C_{30:0}$ (dinostanol isomer - 23*R*)	IIIe			1.7	5.3	24.1	17.5	10.9		1.2
$C_{30:0}$ (4Me,24Ethyl isomer)	IIIj								48.1	
Others		0.5			0.6					3.6
Total		100.0	100.0	100.0	100.0	100.0	100.0	97.9	100.0	100.0

[a] 'Code' refers to structures shown in Fig. 2.
[b] Data from Nichols et al. (1984) for the species *P. cordatum* (Ostenfeld) Dodge obtained from the Marine Science Laboratories, Queenscliff, Vic. as strain DINO-EX.
[c] Data from Yamaguchi et al. (1986) for a strain of *P. micans* of unknown origin.
[d] Data from Piretti et al. (1997) for a strain of *P. micans* from the northwestern Adriatic Sea from red tide blooms.
[e] Probable misidentification, likely to be peridinosterol (IIIn).

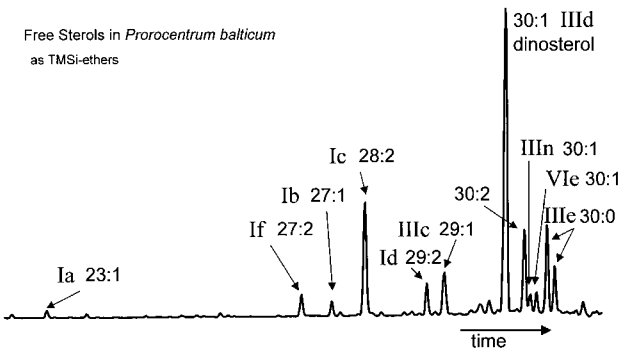

Fig. 1. Partial capillary gas chromatogram of free sterols (as TMSI-ethers) in *Prorocentrum Balticum*.

Cholesterol was also a relatively minor component (2.0 and 1.7%). An interesting feature of the distributions was the presence of a monounsaturated C_{30} 4-methyl sterol tentatively identified as either 4α,23,24-trimethyl-5α-cholest-8(14)-en-3βol (labeled VIe in Fig. 1) or 4α-methyl,24-ethyl-5α-cholest-8(14)-en-3β-ol (VIm in Fig. 2) based on its mass spectrum (base peak at *m*/*z* 500 with other characteristic ions at *m*/*z* 227, 243, 269, 395, 410 and 485). This mass spectrum is very similar to that presented by Harvey et al. (1988) for a sterol with the same retention index in *Scrippsiella trochoidea* which they identified as VIe. However, it is also possible that this sterol is 4α-methyl,24-ethyl-5α-cholest-8(14)-en-3β-ol (VIm) since this is also found in several dinoflagellates (Piretti et al., 1997 and Refs. therein) and the two sterols would have very similar mass spectra and retention time indices. The mass spectrum of the Δ^7 isomer (Ve in Fig. 2) would also be similar, but this sterol should elute after dinostanol rather than before it.

A minor sterol eluting after dinosterol was identified as peridinosterol (4α,23,24-trimethyl-5α-cholest-17(20)-en-3β-ol by its close match with the mass spectrum reported by Robinson et al. (1986) and retention index reported by Harvey et al. (1988). This sterol also occurs in *P. minimum*. In *P. balticum*, this sterol eluted just before an unidentified C_{30} sterol having 2 double bond equivalents (major ions at *m*/*z* 498, 408, 369, 129) as shown in Fig. 1. This sterol eluted much earlier than gorgosterol (Ik) and is likely to have a Δ^5 double bond based on the presence of *m*/*z* 129 and M^+-129 ions. Further research is required to obtain a full structural identification.

P. balticum also contained two C_{30} stanols (labeled IIIe in Fig. 1) which had very similar mass spectra (characteristic ions at *m*/*z* 502, 487, 412, 397, 373, 372, 261, 229, 75 base peak). The retention indices and mass spectral features matched data presented by Harvey et al. (1988) for the two dinostanol isomers 4α,23*S*,24*R*-trimethyl-5α-cholestan-3β-ol and 4α,23*R*,24*R*-trimethyl-5α-cholestan-3β-ol found in *S. trochoidea*. Mass spectra obtained from the Fisons MD-800 GC–MS system show a characteristic difference in the abundance of ions at *m*/*z* 229 and 261. In the first eluting 23*S*,24*R* isomer the ratio of *m*/*z* 229:261 is 1.31 whereas in the second 23*R*,24*R* isomer it is only 0.96 (although different ratios may be found using other MS systems). This feature, plus retention index data, was used to identify the particular dinostanol isomer in the other species.

2.3. Prorocentrum micans

Two strains of this species were analysed. The NIOZ strain was analysed for free sterols, while data for the CSIRO strain refer to total 'free plus esterified' sterols. Both contained the same suite of major sterols, but a few minor sterols were detectable in only one of the strains. 4-Desmethyl sterols predominated in both strains, but there were some quantitative differences in major components. The major sterols were cholesterol (32.4 and 44.2% in the NIOZ and CSIRO strains respectively), 23,24-dimethylcholesta-5,22*E*-dien-3β-o1 (17.4 and 12.4%), dinosterol (14.5 and 4.6%) and dinostanol (24.1% and 17.5%).

2.4. Prorocentrum mexicanum

The total extractable sterols of this species contained a very high proportion of 4-desmethyl sterols (90%) in a simple distribution which consisted mainly of cholesterol (48%) and 23,24-dimethylcholesta-5,22*E*-dien-3β-ol (35.2%). The only major 4-methyl sterol was dinosterol (7.1%).

2.5. Novel sterols

In each species all of the major sterols were assigned, but a few minor sterols remain to be identified. In gas chromatograms of the sterols in *P. minimum* and *P. balticum*, a minor compound (2.1 and 1.7% of total sterols) was observed which eluted well before the other sterols (Fig. 1). The mass spectrum of this compound (as the TMSi-ether) showed a molecular ion at *m*/*z* 402 and base peak at *m*/*z* 75 with major ions in decreasing abundance at *m*/*z* 73, 273 (M^+-129), 135 (C/D rings), 129, 387 (M^+–CH_3), 255, 269, 297 M^+-90–15), 359 (M^+-43), 312 (M^+-90) and 345 (M^+-57). The presence of ions at M^+-129 and *m*/*z* 129 suggests a Δ^5 unsaturated sterol and the ions at M^+-15, M^+-43 and M^+-57 are consistent with cleavage of alkyl groups from the side-chain. The molecular ion indicates a C_{23} monounsaturated sterol. These data are consistent with the identification of the sterol as an analogue of cholesterol (i.e. with an androst-5-en-3β-ol skeleton; Ia in Fig. 2), but one in which the side-chain

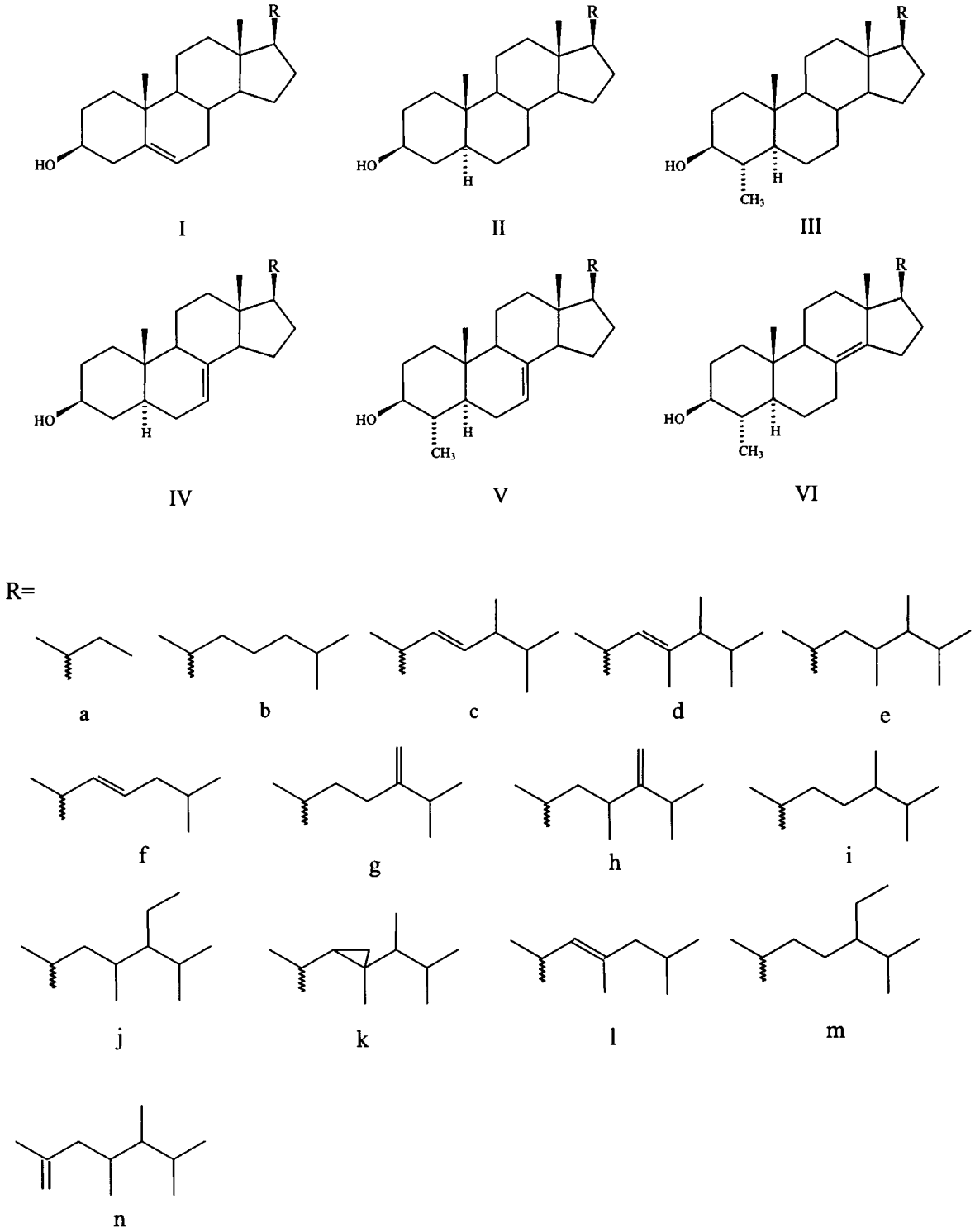

Fig. 2. Structures of sterols identified in the four dinoflagellates. Structures are named in Table 1.

is only 3 carbon atoms long as found in some marine invertebrates (Carlson et al., 1978; Carlson, Tarchini & Djerassi, 1980). A direct comparison with the mass spectral data reported by these authors was not possible since they analysed the sterols as free alcohols whereas our spectrum is that of the TMSi–ether.

3. Discussion

3.1. Taxonomy of the genus Prorocentrum

The genus *Prorocentrum* has been the subject of considerable interest to taxonomists with many revisions

and reassignments. As with other microalgae, assignments have been based mainly on morphology (e.g. Faust, 1991). One group of particular interest has been the morphologically similar genus *Exuviaella* which was initially merged into the Prorocentrales (Dodge, 1975) only to be removed in 1997 (McLachlan, Boalch & Jahn, 1997). Recently, 18S rDNA sequences have been obtained which show that species in the Prorocentrales likely have a common origin, but nonetheless the genus *Prorocentrum* is still very heterogeneous (Grzebyk, Sako & Berland, 1998). Grzebyk et al. (1998) distinguished two groups: one cluster included truly benthic species (such as *P. lima*) while the other consisted of planktonic or bentho-planktonic species. All of the species analysed in our work are planktonic and thus fall into this latter group.

3.2. Previous studies of the sterols in species of *Prorocentrum*

There have been few studies of the lipid composition of species from the genus *Prorocentrum*. Nichols et al. (1984) reported total fatty acid and sterol compositions of *P. cordatum* isolated from marine waters off Victoria, Australia. Their sterol data are shown in Table 1 for comparison with those obtained in our work. The major sterol in *P. cordatum* was dinosterol (28.7%) which co-occurred with small amounts of other 4-methyl sterols. The major 4-desmethyl sterols in *P. cordata* were 24-methylcholesta-5,24(28)-dien-3β-ol (35.4%) and 23,24-dimethylcholesta-5,22*E*-dien-3β-ol (12.5%). The composition of both 4-methyl and 4-desmethyl sterols is very similar to that found by us for *P. minimum* (Table 1), suggesting that the two strains may be very closely related.

Two other sterol analyses are available for various strains of *P. micans* (Yamaguchi et al., 1986; Piretti et al., 1997), in addition to the two strains cultured for the present study (Table 1). Yamaguchi et al. (1986) reported broadly similar results for total extractable sterols to those obtained in the present study (Table 1), but with some important differences. They too found a relatively simple distribution of 6 major sterols (Table 1). Four of these sterols are the same as those reported here and the relative amounts are similar (note the misprint in Table 3 of their paper: 11a should probably read 11c; also 4a is incorrectly referred to as a Δ5,24-sterol in the footnote to Table 2). However, two sterols were reported which we have been unable to identify in our strains. These are 24-ethylcholesta-5,22*E*-dien-3β-ol and the unusual sterol 4α,23-dimethyl-5α-cholest-22*E*-en-3β-ol which has a 23-methyl group rather than the much more usual 24-methyl group. This sterol was also positively identified in *Glenodinium foliaceum* from ^{1}H NMR data (Alam, Martin & Ray, 1979). However, in the study of *P. micans* (Yamaguchi et al., 1986) the identification was based on the mass spectrum of the acetate derivative (their Fig. 2(a) by comparison with data presented by Alam, Schram and Ray (1978) for a sterol isolated from the dinoflagellate *Gonyaulax diagenesis*. However, this mass spectrum is little different from that of 23-desmethyldinosterol and in our view this identification warrants further investigation. Their identification of 24-ethylcholesta-5,22*E*-dien-3β-ol, rather than the more common 23,24-dimethylcholesta-5,22*E*-dien-3β-ol is also unusual and differs from all other strains analysed to date (Table 1). These are readily distinguished from their mass spectra as TMSi–ether derivative (the 23,24-dimethyl isomer gives a base peak at *m*/*z* 69 rather than *m*/*z* 83) and from GC retention indices (Volkman, 1986).

The most recent analysis of the free sterols of *P. micans* was reported by Piretti et al. (1997) for a strain designated P1 isolated from red tide blooms in the northwestern Adriatic Sea. These authors found a very simple distribution of 4-methyl sterols which is quite different from those found by us or others (Yamaguchi et al., 1986). The 3 major sterols were 4α,24-dimethyl-5α-cholest-22*E*-en-3β-ol (6.3%), dinosterol (45.6%) and a C_{30} 4-methyl stanol identified as 4-methyl,24-ethyl-5α-cholestan-3β-ol. The latter eluted after dinostanol which is thus consistent with retention data reported by Withers (1987). However, these two stanols were reported to have very similar mass spectra (their Table 3), whereas in our experience the two isomers are readily distinguished from the abundance of characteristic ions *m*/*z* 261 and 372/373. Unfortunately these data are not reported by Piretti et al. (1997) to allow a more direct comparison.

4α-Methyl,24-ethyl-5α-cholestan-3β-ol has been reported in a few cultured dinoflagellates (Bohlin, Kokke, Fenical & Djerassi, 1981; Kokke, Fenical & Djerassi, 1981a; Kokke, Fenical, Bohlin & Djerassi, 1981b) and at least one species contains sterols having 24-ethyl and 23,24-dimethyl side-chains (Kokke et al., 1981a). We found two C_{30} stanols in some of the *Prorocentrum* species (Table 1), although not in *P. micans*, but we have identified these as isomers of dinostanol having different stereochemistry at C-23 as found in *Scrippsiella trochoidea* (Harvey et al., 1988). This identification was substantiated by coinjection with stanols produced from catalytic hydrogenation of dinosterol which gives both 23*R* and 23*S* isomers. These isomers can be separated on nonpolar capillary GC columns (Harvey et al., 1988) and the presence of two isomers in the alga is readily explained if their biosynthesis proceeds via nonstereospecific reduction of the C-22(23) double bond of dinosterol. Note, however, that in some of the species only a single isomer of dinostanol occurs which is usually the 23*R*,24*R* isomer (Table 1).

3.3. Unusual sterols

We have not been able to find any previous reports of a C_{23} sterol in dinoflagellates or any other microalga. However, a variety of short-chain sterols have been reported in some marine invertebrates from the Porifera and Coelenterata (Carlson et al., 1978, 1980). These sterols all had the androst-5-en-3β-ol skeleton and had from 0 to 6 carbon atoms in the side-chain at C-17. The amounts isolated were quite small (typically each was less than 0.3% of total sterols) suggesting that they did not have a physiological function in the animal as membrane constituents. Carlson et al. (1978) postulated that these sterols arose from natural in vivo autoxidation at physiological temperatures. A similar process might account for the C_{23} sterol found in *P. minimum* and *P. balticum*, but the presence of a single isomer suggests that a specific biosynthetic pathway is involved. Moreover, the absence of similar sterols in the other dinoflagellates analyzed suggest the presence of this sterol is not due to nonspecific autoxidation of the lipids either in the cell or as a laboratory artifact. If this were the case, we would observe a range of short-chain sterols with unsaturated side-chains (as observed in some marine invertebrates), but none were found. Our finding raises the possibility that at least some of the short-chain sterols found in marine animals might have a direct origin from dietary sources or from algal endosymbionts and indeed the wide range of such compounds found in marine animals suggests that other short-chain sterols might occur in microalgae.

Peridinosterol was only found in *P. balticum* and *P. minimum* (Table 1). This sterol has an unusual structure containing a double bond at position 17(20). It has previously been found in *P. mariae-lebouriae* (W.C.M.C. Kokke unpublished data in Withers, 1987), which is probably conspecific with *P. minimum* (Grzebyk et al., 1998). This sterol is found in a few other dinoflagellates including species of *Peridinium* (Swenson et al., 1980) and *Scrippsiella* (Harvey et al., 1988) and so cannot be considered to be characteristic of any particular genus. Peridinosterol is probably also present in *P. cordata* (Nichols et al., 1984) and the algal strain FCRG 51 (Nichols, Volkman & Johns, 1983) thought to be closely related to *Prorocentrum* (Nichols et al., 1984), since these algae contained a sterol tentatively identified as either 4α-methyl,24-ethyl or 4α,23,24-trimethyl-5α-cholest-24(28)-en-3β-ol, which has the same retention index and mass spectrum as the compound identified here as peridinosterol. Data for 4α,23,24-trimethyl-5α-cholest-24(28)-en-3β-ol in the compilation of sterol mass spectra data by Jones, Nichols and Shaw (1994) are also likely to be incorrect.

3.4. Stanols and steroidal ketones

Small amounts of dinostanol and the steroid ketone dinosterone were detected in *P. balticum* and in *P. minimum*. In *P. balticum*, the abundance of dinosterone was less than 5% of that of dinosterol. These compounds are not commonly reported in other microalgae although they are found in some species of dinoflagellates (Withers et al., 1978; Kokke et al., 1982; Robinson et al., 1987; Harvey et al., 1988), but not in others (Robinson et al., 1987; Mansour, Volkman, Jackson & Blackburn, 1999). Dinosterone is often found in those dinoflagellates that synthesize dinosterol in high concentration and it seems likely that the biosynthesis of 4-methyl sterols and steroidal ketones is closely linked.

3.5. Variations between strains and effects of culture conditions

The two analyses of *P. balticum* (#1 and #2; Table 1) provided an opportunity to compare the distributions of free sterols of the same strain cultured several months apart. Both samples contained the same suite of sterols and, with a few exceptions, the compositional data are very similar; the main difference is the slightly higher proportion of C_{30} 4-methylsterols in the second culture. The different *P. micans* strains cultured in Hobart (Australia) and Texel (The Netherlands) both contained the same suite of major sterols apart from a few minor components. The relative abundances of most components are quite similar (although interestingly the concentrations of dinosterol and its 4-desmethyl counterpart are reversed in the two strains), despite the fact that the CSIRO results refer to total sterols, whereas the NIOZ results are for free sterols only.

4. Conclusions

The sterol data presented here confirm the presence of the common dinoflagellate sterol dinosterol and other 4-methylsterols in four species from the genus *Prorocentrum*. However, these data suggest diversity in both the suite of sterols present and in the abundance of major components. Some species contain a high content of 4-methyl sterols whereas others contain a high content of cholesterol and other 4-desmethylsterols. Small amounts of unusual and novel sterols were also observed in some of the species. The similarity in sterol compositions for the same species grown in two laboratories suggests that sterol distributions are more strongly determined by genetic factors than by environmental influences. However, a few authors have noted significant changes in the abundances of sterols

in some species at different growth stages (e.g. Piretti et al., 1997), so comparisons are best made using a standard set of culture conditions. *P. minimum*, *P. micans* and *P. mexicanum* have recently been shown to be genetically very similar (Grzebyk et al., 1998), with the latter two species having most recently diverged. Our work shows a close similarity in the sterol distributions of all three, particularly *P. micans* and *P. mexicanum*. However, *P. minimum* also contains large amounts of 24-methylenecholesterol which is not found in the other species. Such compositional differences might provide additional chemotaxonomic tools for distinguishing between closely-related species.

5. Experimental

5.1. Microalgal cultures

The microalgae were cultured either at the Netherlands Institute for Sea Research (NIOZ, Texel) or at CSIRO Marine Research, (Hobart, Tas.). *P. minimum* (also known as *Exuviaella mariae-lebouriae*) was obtained from E.G. Vrieling (Groningen) as stock number W420Mn1 and *P. balticum* was obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (strain CCMP 1260). A non-toxic strain of *P. micans* was obtained from RIKZ-Middelburg (Netherlands Institute for Coast and Sea Research). *P. balticum*, *P. minimum* and *P. micans* were cultured at NIOZ as clonal batch cultures in 1 L Erlenmeyer flasks containing 800 ml, of an artificial seawater medium described in Hansen (1989). Growth was maintained at 15°C under 150 μmol photons m^{-2} s^{-1} of light for 1–2 months until a sufficient cell density was obtained for analysis. The microalgae were then collected by centrifugation at 4500 × *g* for 1 min. (*P. minimum* and *P. balticum*) or through GFF filters (*P. micans*).

The CSIRO cultures *P. micans* (CS-293 from Western Australia) and *P. mexicanum* (CS-292 from Wilson Inlet) were obtained from the CSIRO Collection of Living Microalgae (Blackburn, Bolch, Brown, LeRoi & Volkman, 1998). They were grown as clonal batch cultures in 2 L Erlenmeyer flasks containing 1 L of GSe medium, which is a modification of the GP medium of Loeblich (1975), with selenium added as selenite at 10^{-8} M and salinity adjusted to 28. Growth was maintained at 18.5°C under 80 μmol photons m^{-2} s^{-1} of cool white fluorescent light (measured with a Biospherical Optics light meter) on a 12:12 h L:D cycle. Towards the end of logarithmic phase (7 days), the cells were counted from 2 ml samples with a Sedgwick–Rafter slide or a Neubauer haemocytometer depending on the cell size. The microalgae were then harvested by centrifugation at 3000 × *g* for 10 min. Cell damage was avoided by monitoring using light microscopy and checking for the visible appearance of pigment in the medium. The algal pellets were stored in polypropylene cryotubes (5 ml) under liquid nitrogen prior to extraction.

5.2. Lipid extraction

At CSIRO, total lipids were extracted using a modification of the method of Bligh and Dyer (1959). On removal from liquid N_2, the filters containing the microalgae were extracted successively using successive ultrasonication (15 min) and centrifugation (5–6 times) with small volumes of a one-phase mixture of chloroform–methanol–water (1:2:0.8, 7 × 5 ml). To initiate phase separation, chloroform and purified water (Milli-Q® system) were added to the combined extracts to give a final chloroform–methanol–water ratio of 1:1:0.9. The upper aqueous phase containing salts and water-soluble material was discarded and the lipids were recovered in the lower chloroform phase. The solvents were then removed under vacuum and the lipids were stored under nitrogen at −20°C. An aliquot of the total solvent extract was evaporated to near dryness under N_2 and saponified in 5% KOH in methanol–water (4:1) under N_2 at 80°C for 2 h. The resulting neutral lipids were extracted with hexane:-chloroform (4:1) and stored under nitrogen at −20°C. This fraction was treated with *N,O-bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) immediately before GC analysis to convert compounds containing free hydroxyl groups to their trimethylsilylether (TMS) derivatives.

At NIOZ, lipids were extracted ultrasonically with methanol, methanol–dichloromethane (1:1) and dichloromethane (×3) in succession to obtain a total lipid extract. This was methylated using diazomethane (to convert fatty acids to methyl esters) and the free sterols and alcohols in this methylated extract were then eluted from silica gel using ethyl acetate. These were treated with BSTFA for GC analysis.

5.3. Analyses by gas chromatography (GC)

At CSIRO, samples were analysed with a Varian High Temperature Series 5410 gas chromatograph with a Series 8100 autosampler and a septum-equipped programmable injector (SPI) using nonpolar methyl silicone fused-silica capillary columns (50 m × 0.32 mm i.d., HP1 and HP5, Hewlett Packard). The initial column temperature of 45°C as held for 1.5 min, after which the oven temperature was raised to 140°C at 30°C/min and then to 310°C at 3°C/min. Hydrogen was used as the carrier gas and the detector temperature was 315°C. Compounds were identified from their relative retention time, coinjection with previously

identified compounds and authentic standards and from interpretation of mass spectra obtained using gas chromatography–mass spectrometry (GC–MS). At NIOZ, samples were analysed with a Hewlett Packard 5890 gas chromatograph equipped with an on-column injector. A fused silica capillary column (25 m × 0.32 mm) coated with CP-Sil-5 (film thickness 0.15 μm) was used with helium as the carrier gas. The samples were injected at 70°C and the oven temperature raised at 20°C/min. to 130°C and then at 4°C/min to 320°C and held there for 10 min. The detector temperature was 330°C.

5.4. Analyses by gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses of the total extractable sterol fractions at CSIRO were performed with a Fisons Instruments MD800 and Fisons AS800 autosampler equipped with a Carlo Erba on-column injector. The nonpolar column and chromatography conditions were similar to those described above except that the second temperature ramp was 4°C/min and helium was used as the carrier gas. Electron impact mass spectra were acquired and processed with Fisons Masslab software on a PC. Typical mass spectrometer operating conditions were: transfer line, 310°C electron impact energy, 70 eV; 0.8 scans/s; mass range, 40–650. At NIOZ, GC–MS analysis used a Hewlett-Packard 5890 gas chromatograph interfaced to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of 40–800 and a cycle time of 1.7 s (resolution 1000). GC conditions were as described above.

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