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Sterols and fatty acids of three harmful algae previously assigned as *Chattonella*

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ABSTRACT

Sterol and fatty acid compositions were determined for three harmful algal species previously classified in the genus *Chattonella* (Raphidophyceae): the new genus *Chloromorum toxicum* (ex *Chattonella* cf. *verruculosa*), *Verrucophora farcimen* (Dictyochophyceae), previously *Chattonella* aff. *verruculosa*, and *Verrucophora verruculosa* (=*Pseudochattonella verruculosa*) previously *Chattonella verruculosa*. The major fatty acids of *C. toxicum* were 14:0, 16:0, 18:1*n*−9, 18:4*n*−3 and 20:5*n*−3, and those of the *Verrucophora* strains were. 14:0, 16:0, 18:0, 18:4*n*−3, 18:5*n*−3 and 22:6*n*−3. *C. toxicum* contained the 24β-ethyl sterols, poriferasterol and clionasterol, as its major sterols. For comparison, the stereochemistry of the 24-ethyl sterols of two raphidophytes, *Chattonella marina* and *Heterosigma akashiwo*, was determined to be 24α and 24β, respectively. Both *Verrucophora* strains contained the 27-nor sterol occelasterol as the only detected sterol. This was the first time occelasterol has been found in algae.

1. Introduction

The chemical diversity of marine lipids, especially sterols (Kerr and Baker, 1991), has long been recognized as a valuable resource for biomarkers and the chemotaxonomy of marine algae (Volkman et al., 1998). We have recently isolated novel sterols from a number of harmful algal bloom (HAB) species (Giner and Boyer, 1998; Giner et al., 2001, 2003; Giner and Li, 2000) and have proposed that these sterols promote bloom events by interfering with the nutritional requirements of invertebrate predators (Giner et al., 2003). As a continuation of these studies, the sterol and fatty acid analyses of three HAB species previously classified in the genus *Chattonella* (Raphidophyceae) are reported herein.

Many fish-kills throughout the world have been associated with blooms of *Chattonella* species. A causative role has been proposed for brevetoxins, usually associated with the dinoflagellate *Karenia brevis*, but which have also been identified by Japanese workers in *Chattonella marina*, *Chattonella antiqua*, as well as in the related raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica* (Onoue et al., 1990; Khan et al., 1995a,b, 1996, 1997). Much higher levels of brevetoxins were found in an alga associated with fish-kills in coastal Delaware, initially considered to be a raphidophyte, which was tentatively called *Chattonella* cf. *verruculosa* (Bourdelais et al., 2002). Pigment analysis of this species, as well as the Japanese ictyotoxic species *Chattonella verruculosa*, and the closely related *Chattonella* aff. *verruculosa* responsible for massive blooms and fish-kills in the North Sea, showed the two latter strains to closely resemble one another, but not the American species (Tomas et al., 2004). Molecular taxonomic studies and pigment analysis confirm *Chattonella* cf. *verruculosa* to be a raphidophyte, although there are affinities to the Xanthophyceae. This species has now been renamed *Chloromorum toxicum* (Tomas et al., submitted for publication). Phylogenetic studies of *C. verruculosa* and *Chattonella* aff. *verruculosa* showed these to be members of the Dictyochophyceae (silicoflagellates), and they have been named *Verrucophora verruculosa* (=*Pseudochattonella verruculosa*) and *Verrucophora farcimen*, respectively (Edvardsen et al., 2007; Hosoi-Tanabe et al., 2007; Mostaert et al., 1998).

2. Results and discussion

2.1. Fatty acids

The fatty acid compositions of *C. toxicum*, *V. farcimen*, and *V. verruculosa* (=*P. verruculosa*) are listed in Table 1, together with published data for *C. marina*. For *C. toxicum*, the fatty acid composition of was very similar to that of *Chattonella* and related raphidophytes, containing high proportions of eicosapentaenoic acid (EPA, 20:5*n*−3) and stearidonic acid (18:4*n*−3) (Marshall et al.,

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Table 1
Fatty acid compositions of "*Chattonella*" species

Fatty acid	RRT of FAME[a]	Percentage of total fatty acids			
		Chattonella marina[b]	*Chloromorum toxicum*	*Verrucophora verruculosa*	*Verrucophora farcimen*
14:0	0.58	9.1	13.8	15.6	13.8
16:0	0.73	20.1	14.7	6.2	9.5
16:1*n*−9	0.77			2.0	3.2
16:1*n*−7	0.80	8.8	1.4		
16:1*n*−5	0.82			5.2	7.8
18:0	1.00	1.2	5.7	3.2	12.3
18:1*n*−9	1.11	7.2	9.0		
18:1*n*−7	1.12	1.7	2.6		
18:2*n*−6	1.30	2.0	2.4	2.4	2.0
18:3*n*−6	1.46	0.2	1.9		
18:3*n*−3	1.58		4.8	3.2	2.9
18:4*n*−3	1.80	15.1	11.6	27.5	21.9
18:5*n*−3	2.11	0.6		12.8	6.5
20:4*n*−6	2.48	2.8	3.7		
20:4*n*−3	2.82	0.7	1.5	0.9	
20:5*n*−3	3.14	21.2	19.0	1.1	0.8
22:6*n*−3	5.84	3.3		12.7	12.9
SFA		30.4	34.2	25.0	35.6
MUFA		17.7	13.0	7.2	11.0
PUFA		45.9	44.9	60.6	47.0
Other[c]		6.0	7.9	7.2	6.4

[a] RRT = GC retention time relative to 18:0 (methyl stearate) using GC system 1.
[b] Average of published values (Marshall et al. 2002).
[c] Includes minor and unidentified components (each less than 2% of the total).

2002; Mostaert et al., 1998; Nichols et al., 1987). In contrast, docosahexaenoic acid (DHA, 22:6*n*−3), reported as about 2–3% of the total fatty acids in other raphidophyte algae (Marshall et al., 2002; Mostaert et al., 1998; Nichols et al., 1987), was not detected. Stearic acid (18:0) represented 5.7% of the total fatty acids of *C. toxicum* but was not found in *Chattonella* (Marshall et al., 2002; Mostaert et al., 1998; Nichols et al., 1987). Fewer data are available for comparison with the Xanthophyceae. Two reports come from freshwater species (Patil et al., 2007; Mercer et al., 1974), and only one from a marine xanthophyte (Hu et al., 1999). All of these reported 16:0, 16:1 and 20:5 to be the major fatty acids. Although the high proportions of EPA (20:5) in both *C. toxicum* and the reported xanthophytes suggest they may be contaxic, this fatty acid is widely distributed among marine algae including the raphidophytes.

The fatty acids of *V. farcimen* and *V. verruculosa* (Table 1) were significantly different from those of *Chattonella* and *C. toxicum*, but were very similar to one another, differing mainly in the relative amounts of stearic acid (18:0) and octadecapentaenoic acid (18:5*n*−3). These organisms contained high levels of stearidonic acid (18:4*n*−3), DHA (22:6*n*−3), and 18:5*n*−3. The latter is considered a rare polyunsaturated fatty acid which has been found in *Prorocentrum spp.* (Dinophyceae) (Mansour et al., 1998), *Isochrysis sp.* (Prymnesiophyceae) (Renaud et al., 1999), and *H. akashiwo* (Raphidophyceae) (Marshall et al., 2002; Nichols et al., 1987; Bell et al., 1997). However, the fatty acid compositions of the *Verrucophora* strains diverge from those of *H. akashiwo* and other raphidophytes as they contain a large percentage of DHA and not of EPA (Marshall et al., 2002; Mostaert et al., 1998; Nichols et al., 1987). The fatty acid compositions of *Verrucophora* most closely resemble that of *Isochrysis sp.*), and were quite different from the only other dictyochophyte to be reported, a *Pseudopedinella* species that was found to contain 16:0, 16:1 and 20:5 as its major fatty acids (Yongmanitchai and Ward, 1991).

Although generally thought of as essential nutrients, polyunsaturated fatty acids (PUFAs) have been proposed to be responsible for the ictyotoxic effects of many HAB species. The rare fatty acid 18:5*n*−3 was shown to be toxic to sea urchin eggs (Sellem et al., 1999). Stearidonic acid (18:4*n*−3) was found through bioassay guided isolation to be an allelopathic agent of the brown alga *Cladosiphon okamuranus* (Kakisawa et al., 1988) and, together with EPA and arachidonic acid (20:4*n*−6), a hemolytic agent from the raphidophyte *F. japonica* (Fu et al., 2004). A synergistic effect between PUFAs and superoxide anion has been proposed for the fish-killing effects of *C. marina* (Marshall et al., 2003, 2005). These toxic effects are mediated by the fatty acids in their free, unesterified forms. No attempt was made in this study to analyze for free fatty acids.

2.2. Sterol analysis

The detailed sterol compositions of *C. toxicum*, the two species of *Verrucophora*, and the raphidophytes *C. marina* and *H. akashiwo* were determined using reversed phase HPLC separation and 600 MHz ^{1}H NMR spectrometric analysis. In contrast, the majority of published data on algal sterols have been obtained by GC–MS analysis. While GC–MS is a sensitive technique, it is generally insufficient to specify the configuration at C-24 of many of the most common phytosterols, and of limited value in situations where rare or unusual sterols might be present (Gerst et al., 1997).

Six sterol fractions were isolated by HPLC from *C. toxicum*. The major sterols were determined by ^{1}H NMR spectrometric analysis to be poriferasterol (**4**) and clionasterol (**6**) (Table 2, Fig. 1). These are both 24β-ethyl sterols and account for 83% and 11% of the total sterols, respectively. The dominant sterol in *Chattonella spp.* was previously reported to be 24-ethylcholesterol, but the configuration at C-24 was not specified (Marshall et al., 2002; Mostaert et al., 1998; Nichols et al., 1987). Reinvestigation of the sterols of *C. marina* using ^{1}H NMR spectrometry indicated the 24α-configuration (sitosterol, **7**). This suggested that *C. toxicum* is not closely related to *Chattonella*. For comparison the sterols of another raphidophyte, *H. akashiwo* (Marshall et al., 2002; Mostaert et al., 1998; Nichols et al., 1987) were also reinvestigated. In this case, however, the 24β-configuration was found (**5** and **6**). It is noteworthy that *C. marina* and *H. akashiwo*, although considered closely related species, display opposite configurations in their 24-alkyl

Table 2
Sterol composition of algal species

Sterols	RRT[a]	Percentage of total sterols		
		Chloromorum toxicum	*Chattonella marina*	*Heterosigma akashiwo*
Desmosterol (**1**) (cholesta-5,24-dien-3β-ol)	0.81	1[b]		
Brassicasterol (**2**) (24(*R*)-ergosta-5,22-dien-3β-ol)	0.93	2[b]		
Dihydrozymosterol (cholest-8-en-3β-ol)	0.95		4	
Cholesterol (**3**) (cholest-5-en-3β-ol)	1.00	2[b]	18	
Poriferasterol (**4**) (24(*R*)-stigmasta-5,22-dien-3β-ol)	1.09	83		
Dihydrobrassicasterol (**5**) (24(*S*)-ergost-5-en-3β-ol)	1.12	1[b]		14
Clionasterol (**6**) (24(*S*)-stigmast-5-en-3β-ol)	1.22	11		86
Sitosterol (**7**) (24(*R*)-stigmast-5-en-3β-ol)	1.22		78	

[a] RRT = HPLC retention time relative to cholesterol.
[b] These sterols were identified based on retention time.

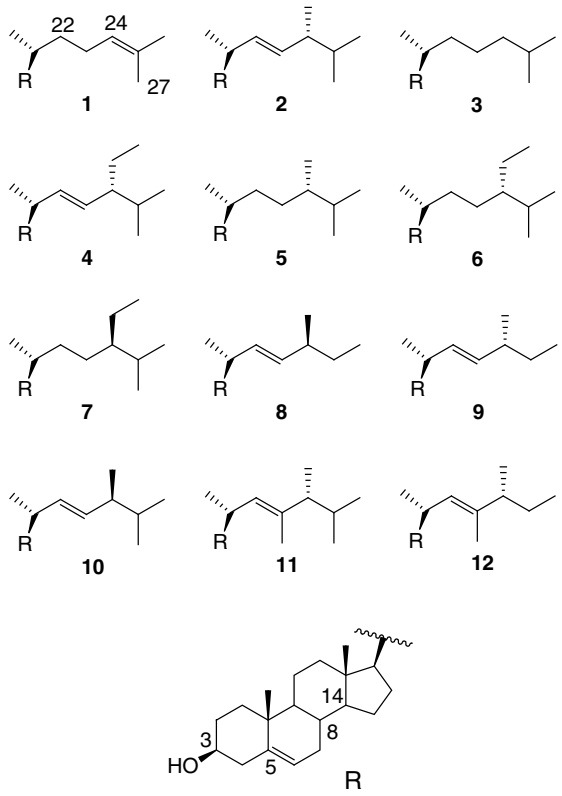

Fig. 1. Structures of sterols.

sterols. A similar situation has been described in the genus *Isochrysis* (Prymnesiophyceae) (Patterson et al., 1994). In contrast to *C. marina* and *H. akashiwo*, *C. toxicum* contained Δ^{22} sterols. These are virtually unknown in raphidophytes, but have been reported on one occasion in *Olisthodiscus luteus* (Marshall et al., 2002). Therefore, affinity between *C. toxicum* and other raphidophytes is supported on the basis of sterol composition. For comparison with the Xanthophyceae, detection of clionasterol (**6**) as the major sterol in three freshwater species supports the affinity of *C. toxicum* to this class (Mercer et al., 1974). Too much emphasis, however, is unwarranted since this is the only published report of xanthophyte sterols.

In contrast, both *Verrucophora* species contained the rare occelasterol (**8**) as their only detected sterol. Until now, no algal source was known for this sterol. Occelasterol (**8**) has long been known at higher trophic levels and was first isolated as a minor sterol from an annelid (Kobayashi and Mitsuhashi, 1974). A stanol with the same side chain was later detected as a minor component of the sterols of a scallop (Kobayashi and Mitsuhashi, 1975). Its structure was originally determined by NMR and MS analysis and later confirmed by stereospecific synthesis (Hirano and Djerassi, 1982). Since its discovery, this sterol has been reported in marine shellfish from Labrador to New Zealand (Copeman and Parrish, 2004; Murphy et al., 2002), Antarctic pteropods (Phleger et al., 2001), and marine sediments from the Arctic (Belicka et al., 2002). The C-24 epimer of occelasterol (epioccelasterol, **9**) was found to be a major sterol in two other dinoflagellate species, *Gymnodinium simplex* (Goad and Withers, 1982), and the symbiont of *Orbulina universa* (Kokke and Spero, 1987). A 27-nor sterol of undetermined stereochemistry was found in the Antarctic species *Polarella glacialis* (Thomson et al., 2004). Based on the prevalence of the 24β stereochemistry in dinoflagellates, this is likely to be epioccelasterol (**9**). The 24β-27-nor side chain is also found in brevesterol (24(*R*)-4α-methyl-27-norergosta-8(14), 22-dienol), which was recently shown to be a major sterol in the toxic dinoflagellate *Karenia brevis* (Giner et al., 2003; Leblond and Chapman, 2002). The finding of occelasterol (**8**) in *Verrucophora* is the first time any sterol with this side chain has been found in algae and it is the first time a 27-nor sterol has been found in a non-dinoflagellate alga.

Only one report exists on the sterols of a dictyochophyte alga, *Dictyocha fibula*, in which 24-methylenecholesterol and ergosta-5,22-dienol were found. The stereochemical configuration of the later sterol was not determined, although it was argued to have the 24α-configuration (i.e. 24-epibrassicasterol **10**) (Patterson and Van Valkenburg, 1990). This would be the same configuration as that of occelasterol (**8**), and **10** may represent its precursor in a

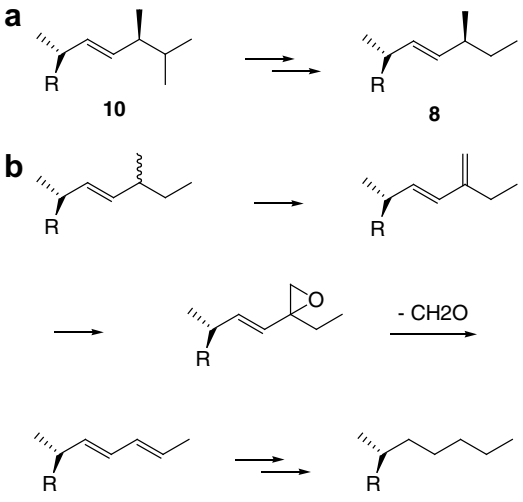

Fig. 2. Hypothetical biological transformations. (a) 27-demethylation pathway and (b) 24-dealkylation pathway applied to 27-nor sterols.

hypothetical biosynthetic dealkylation (Fig. 2). Although there is currently no experimental data to support this, co-occurrence of the dinosterol side chain and its 27-nor analog in the desmethyl sterol fraction (**11** and **12**), and of the brassicasterol (**2**) side chain and its 27-nor analog (**9**) in the 4α-methyl sterol fraction of *K. brevis* suggests dealkylation without the intermediacy of a diene (Giner et al., 2003). We previously proposed that modifications to the sterol side chain would benefit algae by interfering with the nutritional requirements of their invertebrate predators (Giner et al., 2003). Application of the invertebrate 24-dealkylation pathway to 27-nor sterols (if mechanistically compatible with such substrates) would generate a sterol without branching in the side chain (Fig. 2). It is unlikely that such a sterol could substitute for cholesterol in the growth requirements of zooplanktonic grazers. This may help to explain how *V. farcimen* is been able to produce massive blooms such as the 2000 bloom in the North Sea.

3. Conclusions

While the lipid composition of *C. toxicum* was consistent with the little that is known from the Xanthophyceae, it was very similar to that of the better characterized Raphidophyceae. In particular, the high proportions of eicosapentaenoic acid (EPA, 20:5*n*−3) and stearidonic acid (18:4*n*−3) resembled what had previously been found in other raphidophytes. The sterol composition also showed similarities to that of the raphidophytes in its predominance of 24-ethyl sterols. However, the C-24 stereochemistry of these sterols was shown to have limited chemotaxonomic value since two raphidophytes, *C. marina* and *H. akashiwo*, displayed opposite configurations. The chemotaxonomic value of 22-desaturation is uncertain since there is only one report of this feature in a raphidophyte.

Like the Xanthophyceae, very little data is available for the sterols and fatty acids of the Dictyochophyceae (silicoflagellates) for comparison with the two *Verrucophora* species. However, the sterol and fatty acid compositions clearly showed these two species to be very similar to one another, and very different from the Raphidophyceae. The discovery that both *Verrucophora* species contain the rare 27-nor sterol occelasterol solves the puzzle of its origin in the marine environment and provides a chemotaxonomic marker for this taxon.

4. Experimental

4.1. General methods

NMR spectra were acquired using a Bruker Avance-600 instrument using $CDCl_3$ as the solvent. GC analyses were carried out using either: (1) a HP 5790A instrument with a Supelco SP-2330 column (id 0.32 mm, 30 m), an injector temperature of 250 °C, a flow rate of 1 mL/min of He, isothermal column temperature of 180 °C, and FID detector temperature of 250 °C; or (2) Shimadzu GC-17 with a HP-5 column (id 0.25 mm, 30 m), an injector temperature of 280 °C, a flow rate of 0.52 mL/min of He, a temperature gradient from 60 °C to 141 °C at a rate of 15 °C/min and from 141 °C to 300 °C at a rate of 5 °C/min, and FID detector temperature of 300 °C. GC–MS data were obtained using HP 5890 series II gas chromatograph with same column and injector conditions of GC (1) and a HP 5989B mass spectrometer as detector, using both EI (70 eV) and CI mode (gas:methane). HPLC was carried out with a Waters 6000A pump, Waters 410 differential refractometer, and two Altex Ultrasphere ODS 5 μm 10 × 250 mm columns in series using a flow rate of 3 ml/min MeOH. Analytical TLC was performed on aluminum backed plates coated with a 0.25 mm layer of silica gel 60 F254. Preparative TLC was performed on glass backed plates (10 cm in length) coated with a 0.25 mm layer of silica gel 60 F254.

All reagents were obtained commercially and were purified by redistillation. In order to protect the lipids from autoxidation, 2,3-dimethyl-2-butene was added (0.5%) to the solvents in all procedures (except FAME formation) prior to the HPLC analysis. All extraction and purification steps were carried out under low light conditions to minimize the autoxidation.

4.2. Cultures

Cultures of *C. toxicum* was grown at 24 °C in DYV media of 20 practical salinity units (PSU) (Andersen, 2005) using a 12:12 h light/dark cycle of cool white fluorescent light (50–60 μmol quanta m^{-2} s^{-1}). The two *Verrucophora* species, *C.* marina, and *H. akashiwo* were grown at 20 °C in enriched natural seawater (ES) sea water media of 33 PSU (Andersen, 2005) using a 12:12 h light/dark cycle of cool white fluorescent light (70–100 μmol quanta m^{-2} s^{-1}). All cultures were kept in EGS (Environmental Growth System) incubators that maintained the culture conditions mentioned above. Cells were harvested by centrifugation and the pellets were stored frozen at −80 °C until the analysis.

4.3. Extraction of lipids

Solvent extractions were carried out by adding 1 ml of CH_2Cl_2/CH_3OH 1:1 (v/v) to the algae pellets in 1.5 ml polypropylene microfuge tubes and shaking vigorously with a Mini-Beadbeater at room temperature for 5 min. After microfuge centrifugation, the supernatants were removed, and the extraction of the pellets was repeated until there was no green color in the supernatants. The final extraction was carried out using EtOAc (1 ml). The organic extracts were combined and concentrated to dryness with a stream of N_2. The extracts were divided into two equal portions for sterol and for fatty acid analysis.

4.4. Fatty acid analysis

To prepare FAMEs, crude lipid extracts were heated with 200 μl of BF_3–Et_2O/MeOH 1:8 (v/v), 400 μl of methanol, and 150 μl of benzene (final concentration = 3% BF_3–Et_2O) in a glass reaction vial at 100 °C for 1 h. After cooling to room temperature, the reaction mixtures were partitioned between H_2O (1 ml) and hexane (1 ml). The aqueous layer was extracted with more hexane (2 × 1 ml). The combined organic layers were concentrated to dryness with a stream of N_2 and the residue dissolved with hexane (100 μl). The FAMEs of *C. toxicum* were analyzed by GC (1), *V. farcimen* FAMEs were analyzed using GC (2), and those of *V. verruculosa* (=*P. verruculosa*) were analyzed on both instruments. The identities of the FAMEs of 14:0, 16:0, 16:1*n*−7, 18:0, 18:1*n*−9, 18:1*n*−7, 18:2*n*−6, 18:3*n*−6, 18:3*n*−3, 18:4*n*−3, 20:4*n*−6, 20:5*n*−3, and 22:6*n*−3 were assigned by comparison of the retention times with standards and confirmed by GC–MS. Identification of the FAMEs of 16:1*n*−9, 16:1*n*−5, 18:5*n*−3, and 20:4*n*−3 were based on mass spectrometry and retention times. The relative proportions of the fatty acids were determined from the integrals of the GC FID signal.

4.5. Sterol analysis

Saponification of the crude extracts was accomplished with 5% KOH/CH_3OH at reflux for 1 h. The reaction mixtures were partitioned between H_2O and Et_2O, and the organic layers were concentrated to dryness with a stream of N_2. The total sterols were isolated by preparative TLC using hexane/EtOAc 2:1; and were further purified by preparative TLC using two developments with benzene/EtOAc 9:1. Reversed-phase HPLC was used to isolate the

individual components of the sterol fractions. After evaporation of the HPLC solvent with a stream of N_2, the sterols characterized by ^1H NMR spectrometric analyses. The structures of sterols were assigned by comparison of NMR spectra with authentic standards. The relative proportions of the sterols were determined from the integrals of the HPLC differential refractometer signal.

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