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HAEMOLYTIC GLYCOGLYCEROLIPIDS FROM *GYMNODINIUM* SPECIES

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Key Word Index—*Gymnodinium mikimotoi*; *Gymnodinium* cf. *nagasakiense*; toxic dinoflagellates; lipids; fatty acids; haemolytic activity.

Abstract—Glycoglycerolipids derived from microalgae can be a source of biologically active substances including toxins. Such glycolipids were analysed in two isolates of toxic marine dinoflagellates from European waters. The lipids of *Gymnodinium mikimotoi* contained 17% of monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), while in *Gymnodinium* sp. the proportion was 35%. MGDG and DGDG from both species were haemolytic. The major unsaturated fatty acid in both algal glycolipids was 18:5ω3.

INTRODUCTION

There is currently considerable interest in both intracellular and extracellular glycolipids derived from microalgae as a source of biologically active substances [1, 2]. In particular, unsaturated glycolipids in microalgae have been studied for their toxicity [3–7] and as a source of health-promoting ω3 fatty acids [8]. This may at first seem contradictory, but the stimulatory and cytotoxic effects of polyunsaturated fatty acids (PUFA) and their oxidation products are concentration-dependent [9].

Yasumoto *et al.* [7] showed that a free polyunsaturated fatty acid and a digalactosyl monoacylglycerol derived from a marine dinoflagellate, *Gyrodinium aureolum*, were responsible for fish-kills. The origin of both of these haemolysins could have been a more common type of glycolipid, a glycosyl diacylglycerol. Hydrolysis of this type of glycolipid would yield a free fatty acid and a glycosyl monoacylglycerol. In the present study, we sought to identify and quantify glycolipids in two toxic *Gymnodinium* species that are closely related to *Gyrodinium aureolum*.

RESULTS AND DISCUSSION

Examination of the chromatograms (Fig. 1) and a comparison of the lipid class proportions (Table 1) suggests the two *Gymnodinium* species cultured here are not closely related. In addition to there being different proportions of the various classes, *Gym-*

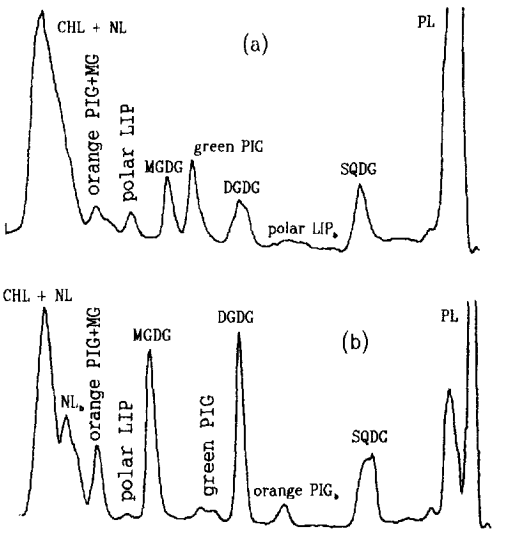

Fig. 1. TLC/FID chromatograms of glycolipids in dinoflagellate extracts: (a) *Gymnodinium mikimotoi* extract; and (b) *Gymnodinium* sp. cultured from a sample taken in the Adriatic Sea. CHL + NL, chlorophyll *a* + neutral lipid; NL_b, neutral lipid; orange PIG + MG, fucoxanthin + monoacylglycerol; polar LIP, polar lipid; MGDG, monogalactosyl diacylglycerol; green PIG, chlorophyll *c*; DGDG, digalactosyl diacylglycerol; orange PIG_b, light orange pigment; polar LIP_b, polar lipid; SQDG, sulphoquinovosyl diacylglycerol; PL, phospholipid.

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Table 1. Lipid class composition (% total lipid) of *Gymnodinium mikimotoi* (mean ± s.d., $n = 8$) and *Gymnodinium* sp. ($n = 3$). Abbreviations as in caption to Fig. 1

Lipid class	*G. mikimotoi*	*Gymnodinium* sp.
CHL + NL	23.7 ± 2.8	13.5 ± 2.2
NL_b	—	10.2 ± 1.8
Orange PIG + MG	5.1 ± 1.4	5.8 ± 0.9
Polar LIP	4.5 ± 1.3	0.7 ± 0.1
MGDG	7.6 ± 1.1	14.2 ± 0.2
Green PIG	5.8 ± 1.7	2.7 ± 0.7
DGDG	8.9 ± 2.1	20.8 ± 5.0
Orange PIG_b	—	4.1 ± 0.3
Polar LIP_b	6.1 ± 1.5	—
SQDG	11.8 ± 2.6	14.1 ± 0.4
PL	26.4 ± 4.3	13.8 ± 2.4

nodinium sp. is different from *G. mikimotoi* (Table 1), *Isochrysis galbana* and spinach [10] in that it has a light orange pigment (orange PIG_b) at a scan time of 0.39 ± 0.01 min (mean ± s.d., $n = 3$). Similarly *G. mikimotoi* is different from the other plant samples analysed in this manner in that it has a polar lipid peak (polar LIP_b) at 0.40 ± 0.02 min (mean ± s.d., $n = 8$).

Other aspects of the pigment distribution were also quite different between the species. A yellow band was clearly visible in the *G. mikimotoi* samples just at the bottom of the CHL + NL band but not in the *Gymnodinium* sp. samples. This yellow band is probably diadinoxanthin [11, 12], although the elution order suggests the possibility of neodinoxanthin as well [13]. Riley and Wilson [13] describe the silica gel elution order for pigments as chlorophyll *a*, neodinoxanthin, fucoxanthin, diadinoxanthin and finally chlorophyll *c*; however, they used a very different solvent system to the one used here. Diadinoxanthin has already been shown to be an important contributor to the pigments of *Gymnodinium nagasakiense* [14], as well as to those of the toxic dinoflagellates, *Gymnodinium catenatum* [15] and *Gyrodinium aureolum* [16].

An orange band, likely from fucoxanthin [12], was present in both species, but in *G. mikimotoi* there are clearly two components to this band (Fig. 1). This could be due to the presence of monoacylglycerol (MG) or it could indicate the presence of another pigment, 19′-hexanoyloxyfucoxanthin, which is spectrally similar to fucoxanthin but which runs at a different R_f [12]. 19′-Hexanoyloxyfucoxanthin has already been shown to be an important contributor to the pigments of *G. nagasakiense* [14], as well as to those of *G. aureolum* [16]. The second green pigment band observed about half-way down the chromatogram is probably chlorophyll c_2 [17], but again there is evidence for two components in this band (Fig. 1), this time in *Gymnodinium* sp., perhaps because of the co-occurrence of chlorophylls c_1 and c_2.

The proportion of phospholipid (PL) for *G. mikimotoi* determined in this experiment is similar to the proportion determined previously over the course of a batch culture grown at 18° and 75 μE m^{-2} s^{-1} [18] or during the stationary phase of a culture grown at 13° and 75 μE m^{-2} s^{-1} [19]. The proportion of PL in the *Gymnodinium* sp. is closest to that determined previously over the course of the *G. mikimotoi* culture grown at 18° and 35 μE m^{-2} s^{-1} [18].

The proportions of MGDG and DGDG for the two species bracket the values given for the marine dinoflagellate *Glenodinium* sp. [20] which had 12% MGDG and 17% DGDG as a proportion of the total polar lipids. The ratio of MGDG to DGDG in the two *Gymnodinium* species again bracket the ratio of 0.71 for *Glenodinium* sp. Surprisingly, the proportions of galactosyl diacylglycerols in the *Gymnodinium* sp. were more similar to those in *Glenodinium* than has been reported for *Gymnodinium microadriaticum* [21]. Galactosyl diacylglycerols in *G. microadriaticum* constituted *ca* 50% of the lipids; the MGDG to DGDG ratio was 1.29.

The haemolytic activity of the glycolipids was examined by applying 250 μg of lipids from the two *Gymnodinium* species to TLC plates and developing them in a solvent system designed to optimize the separation of glycolipid bands [10]. After development, the plates were sprayed with a suspension of horse red blood cells. Haemolytic activity was confined to the MGDG and DGDG bands (detected using α-naphthol reagent) and was much stronger in the *Gymnodinium* sp. extract.

The neutral lipid fraction was also examined by TLC and by GC after separation on silica gel cartridges [22]. Neutral lipids were separated by plate TLC and compared with free fatty acid (FFA) and triacylglycerol (TAG) standards. After spraying with a suspension of horse red blood cells, the haemolytic effect was observed only in the polyunsaturated FFA bands and at a similar intensity in each algal species. The intensity was lower than that observed in the glycolipid bands. FFA are present in lower amounts than glycolipids. FFA proportions range from 3.8–7.5% of the total lipids in *G. mikimotoi* cultures grown at 75 μE m^{-2} s^{-1} [18, 19]. The major fatty acid in the neutral lipid fraction was 16:0 in *G. mikimotoi* (25 ± 0.2 wt%, mean ± s.d., $n = 4$) and in *Gymnodinium* sp. (24 ± 4%). The major unsaturated fatty acid in *G. mikimotoi* neutral lipids was 18:5ω3 (14 ± 0.2%), while in *Gymnodinium* sp. it was 22:6ω3 (21 ± 4%).

The fatty acid composition of the haemolytic glycolipids (Table 2) indicates, again, differences between the two species. 18:5ω3 is prominent in the glycolipids of both, but it is nearly twice the proportion in the *Gymnodinium* sp. extract where it is by far the major fatty acid in both glycolipids. The second most prominent polyunsaturated fatty acid in *Gymnodinium* sp. glycolipids was 22:6ω3, while in *G. mikimotoi*, 16:4ω3 and 18:2ω6 were present in higher proportions than 22:6ω3. In comparison with the neutral lipids, the glycolipids had nearly the same or much lower pro-

Table 2. Major fatty acids (>5% in at least one fraction) in the haemolytic glycolipids of *Gymnodinium mikimotoi* and *Gymnodinium* sp. Glycolipid fractions were obtained by TLC. Data are mean wt% ± s.d., $n = 3$

Fatty acid	*G. mikimotoi* MGDG	DGDG	*Gymnodinium* sp. MGDG	DGDG
14:0	8.9±1.2	12.5±3.0	1.8±0.3	0.9±0.2
16:0	20.1±1.9	22.6±3.0	12.9±0.7	8.0±0.3
16:1ω7	3.8±3.4	5.3±1.5	0.3±0.05	<0.1
16:4ω3	7.5±0.4	10.9±0.2	0.2±0.1	—
18:1ω9	5.3±1.2	7.8±2.0	3.6±0.3	4.3±0.4
18:2ω6	5.2±0.4	12.4±2.4	2.2±0.2	1.6±0.2
18:4ω3	0.8±0.1	1.0±0.2	9.3±0.7	30.0±1.5
18:5ω3	26.4±0.2	13.9±1.6	51.1±3.3	40.4±5.4
20:2ω6	5.6±1.9	0.7±0.4	0.3±0.2	1.4±0.2
22:6ω3	3.4±0.9	2.9±1.0	11.6±1.4	5.9±2.0

Fatty acids are given as the ratio of carbon atoms to double bonds. ω designates the location of the first double bond from the methyl end of the molecule.

portions of total trienes but much higher proportions of total tetraenes. Among the major fatty acids, 16:0 and 22:6ω3 were present at nearly the same or in much lower proportions in the glycolipids than in the neutral lipids, while 18:5ω3 and 20:2ω6 were present at nearly the same or in much higher proportions in the glycolipids.

Yasumoto *et al.* [7] indicated that the strongest haemolytic activity against mouse red blood cells in *G. aureolum* was associated with free 18:5. Free unsaturated fatty acids in extracts from scallops [23], anchovies [24] and mussels [25] have been shown to be toxic in mouse assays. Preparations of pure individual polyunsaturated fatty acids have also been shown to be toxic [23, 26]. Arzul *et al.* [26] showed that 18:5ω3 was haemolytic against sheep red blood cells as well and that it was an even more potent repressor of diatom growth than 20:5ω3 or 22:6ω3.

The fraction containing the second strongest haemolytic activity in *G. aureolum* was a glycolipid containing a single polyunsaturated fatty acid esterified to the glycerol residue, a digalactosyl monoacylglycerol containing 20:5 [7]. Here, however, the major haemolytic activity was associated with the MGDG and DGDG fractions and 20:5ω3 was not a major component (<2% of the fatty acids). 18:5ω3 was the major unsaturated fatty acid, especially in *Gymnodinium* sp.

The origin of both the haemolysins identified by Yasumoto *et al.* [7] could have been a more common type of glycolipid, a glycosyl diacylglycerol containing two polyunsaturated fatty acids. Such a glycolipid was found to be the major polar lipid in a freshwater diatom [8]. Hydrolysis of this type of glycosyl diacylglycerol would yield a polyunsaturated lysoglycoglycerolipid and a polyunsaturated free fatty acid.

EXPERIMENTAL

Cultures. Two isolates of toxic marine dinoflagellates were grown in batch culture. *G. mikimotoi* (strain GATIN87; alias *G. nagasakiense*) was isolated from Brest Bay, France, during a toxic bloom, and *Gymnodinium* sp. was isolated from a sample taken in the north Adriatic Sea. Both species are cultured routinely at IFREMER in France. Cells of *Gymnodinium* sp. are 16–24 μm long and 13–21 μm wide; confirmation of their identity awaits further analyses. The unialgal *Gymnodinium* sp. were grown without agitation in sterilized Guillard's f/2 medium in 6 l flasks at 20±1° under a 12 hr light:dark cycle at 17 μE m^{-2} s^{-1}. Samples were taken from several batch cultures of each species at the end of the exponential phase.

Lipid extraction. Gymnodinium samples were gravity-filtered onto pre-combusted Whatman GF/F glassfibre filters and stored at −20° in *iso*-PrOH until extraction. Particulate material was extracted ×3 by sonication with $CHCl_3$–MeOH (2:1). The combined extracts were washed with H_2O, dried (Na_2SO_4) and evapd at 40° to near dryness in a rotary evaporator. The samples were immediately dissolved in 1–2 ml of $CHCl_3$ and stored under N_2 at < −20° until analysis.

Silica gel cartridge separations. Each of the samples was sepd on silica gel cartridges according to ref. [22]. In *Phaeodactylum tricornutum* samples, this procedure provides frs containing: (1) triacylglycerols and pigments; (2) MGDG; (3) DGDG and some sulphoquinovosyl diacylglycerols (SQDG); (4) SQDG; (5) an unknown acyl lipid and phosphatidyl ethanolamine; (6) phosphatidyl choline; and (7) lysophospholipids. Using the Chromarod-Iatroscan TLC/FID system we were able to confirm the level of purity indicated [22] of most of these frs after applying their procedure to our samples. The first two frs were

pure. Subsequent frs, however, showed significant amounts of contamination with other frs. In our case, the DGDG fr. also contained more polar and less polar lipid classes. The SQDG fr. contained large amounts of more polar lipids (phospholipids?). As in the case of *P. tricornutum*, we found that the fifth fr. was also a mixt. which included phospholipids. In contrast, the most polar frs also included significant amounts of less polar material, which might include SQDG or phospholipid decomposition products as indicated in ref. [27].

Thin-layer chromatography. TLC sepn of extractable lipids or silica gel cartridge frs was carried out on silica gel 60 plates with concn zones. The plates were pre-eluted with Me_2CO, dried at 70° for 1 hr and stored in a desiccator prior to use. Development with $CHCl_3$–MeOH–H_2O (25:10:1) gave a series of clearly sepd polar lipid bands. Triacylglycerol together with other non-polar lipids, chlorophyll and carotenoids were grouped together at the top of the plate. Below this were bands of MGDG, a pigment, DGDG and sulphoquinovosyl diaclyglycerol (SQDG). Phospholipids and lysophospholipids remained near the origin. Most of each glycolipid band was scraped off and extracted ×3 by sonicating in $CHCl_3$–MeOH (2:1); the remainder was sprayed with α-naphthol reagent [28]. Glycolipids appeared as dark blue bands on the TLC plates after spraying with α-naphthol and heating the plate at 110° for 10 min. The purity of each fr. was checked using TLC/FID. The neutral lipid fr. of the algal extract was also examined by TLC. After development in hexane–Et_2O–HCOOH (40:10:1), part of the plate was used to test for haemolytic activity. Lipid bands were visualized on the remainder of the plate by spraying with a soln of 10% $CuSO_4$ in 8% aq. H_3PO_4 and heating to 150° for 15 min.

TLC/FID. Cultures were analysed using the systems described in ref. [10]. This procedure focuses on the quite difficult sepn of glycolipids from other lipids, thus phospholipids are grouped at one end of the chromatogram and neutral lipids, including sterols, are grouped with chlorophyll *a* at the other end of the chromatogram. Peaks were quantified using standards purchased from Sigma (Table 1). Chlorophyll *a* was used for CHL+NL, green PIG, and orange PIG_b, 1-monopalmitoyl glycerol for NL_b, orange PIG+MG and polar LIP, galactosyl diglyceride for MGDG and SQDG, digalactosyl diglyceride for DGDG and polar LIP_b, and dipalmitoyl phosphatidyl ethanolamine for PL.

GC. Fatty acid composition of methylated samples was determined on a 60 m capillary column as described in ref. [10]. Individual peaks were identified by comparison with authentic standards and natural extracts of known composition. Quantitation was performed using 19:0 as int. standard.

Haemolytic test. Sterile horse red blood cells obtained from the Sanofi Diagnostics Pasteur, France, were conditioned [29] and a 10% suspension prepd in *Tris* buffer (with added $CaCl_2$). This was used as a spray reagent to detect haemolytic activity on TLC plates after lipid class development. Bands that caused cytolysis of the blood cells turned white while the rest of the plate remained red after drying at room temp.

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