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DISTRIBUTION OF BETAINE LIPIDS IN MARINE ALGAE

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Abstract—DGCC(1,2-diacylglyceryl-3-(*O*-carboxyhydroxymethylcholine)) is a betaine lipid derived from *Pavlova lutheri*. The separation of this lipid from other polar lipids by HPLC was improved in order to survey the distribution of DGCC, other betaine lipids and PC (phosphatidylcholine) in marine algae. DGCC was found to be one of the common constituents of Haptophyceae; it was interesting to note that PC was not detected even as a minor component. DGTA(1,2-diacylglyceryl-*O*-2′-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine) was detected in five out of 16 species of Haptophyceae, whereas DGTS(1,2-diacylglyceryl-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine) was not detected. DGCC was also detected in four strains of Dinophyceae and a strain of Bacillariophyceae. Acyl moieties of DGCC in Haptophyceae were 16:0, 18:0 or 18:1 and C_{20} or C_{22} polyunsaturated fatty acids.

INTRODUCTION

Betaine lipids are complex lipids which have a positively charged trimethyl ammonium group and constitute a group of polar lipids in plants, together with phospholipids and glycolipids. Three types of betaine lipids have been reported up to now (Fig. 1). DGTS(1,2-diacylglyceryl-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine) has been detected in most species of Pteridophytes [1], Bryophytes [2] and green algae [2, 3]. However, no species of vascular plants contain DGTS. The presence of DGTA(1,2-diacylglyceryl-*O*-2′-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine) in algae has been investigated and reported to occur only in a limited number of algal species [4–7] because DGTA had not been identified until 1990 [4]. The occurrence of DGTA is mostly restricted to algae which contain chlorophylls *a* and *c*. DGCC(1,2-diacylglyceryl-3-(*O*-carboxyhydroxymethylcholine)) was discovered in *Pavlova lutheri* (Haptophyceae) by our group [8]. The distribution of this lipid in plants has yet to be clarified. The aim of the present work was to improve the separation system of DGCC from other polar lipids and to survey the distribution of DGCC in some marine algae.

One of the major problems encountered during the investigation was the difficulty in separating DGCC from phosphatidylcholine (PC) on silica gel TLC. In this paper, the presence of DGCC was examined in several species of marine algae by HPLC which was suitable for the separation of DGCC and PC. In addition, the fatty acid composition of DGCC in same species of Haptophyceae was also investigated.

RESULTS AND DISCUSSION

We succeeded in separating DGCC from PC using an HPLC equipped with a CN-silica gel column. The classes of lipids were identified by the characteristic mass fragment ion peaks at *m/z* 104 and 132 for DGCC and *m/z* 184 for PC, respectively. The complete identification of these lipids was performed with these characteristic fragmentations by HPLC-mass spectrometry in addition to their retention times.

Table 1 shows the distribution of DGCC and other lipids in 16 species of Haptophyceae. DGCC was discovered originally in *P. lutheri* [8]. In order to ascertain whether DGCC was a lipid specific to *P. lutheri* or not, other Haptophyceae species from all four orders were examined; DGCC was detected in all species examined. It was interesting to note that PC could be detected in only two species of Haptophyceae. PC was a very minor component in polar lipids of these algae and the content, which was estimated by the area of HPLC peaks detected at 210 nm, was much lower than that of DGCC. No species contained DGTS and lyso-DGTS; a few species contained DGTA.

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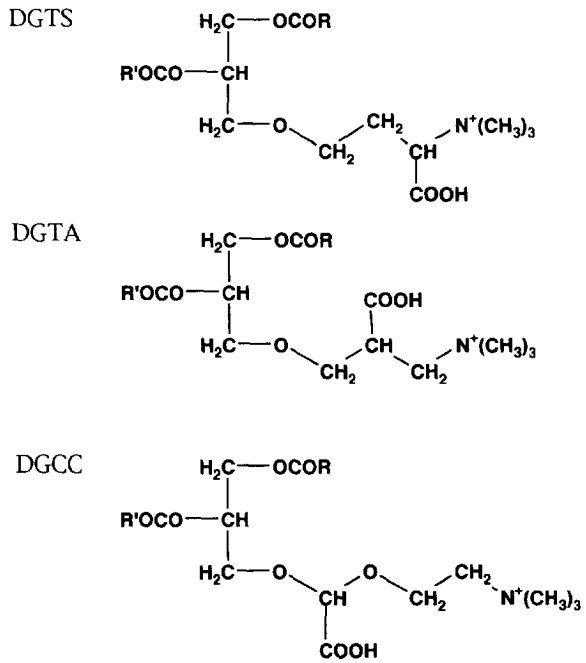

Fig. 1. Structures of betaine lipids (R,R′ = an acyl group).

Table 2 shows the distribution of DGCC and other lipids in 29 species of marine algae other than Haptophyceae. DGCC was detected in four species of Dinophyceae and a species of Bacillariophyceae. PC was also detected in these species, but the critical difference between these algae and Haptophyceae was that the content of PC in these algae was much higher than that of DGCC. DGCC was absent in *Corallina* sp., *Amphiroa* sp. and *Halimeda opuntia* which are calcareous algae. All species of algae in which DGCC was detected were restricted to those that contained chlorophylls *a* and *c*, which was similar to the distribution of DGTA, as was indicated by Sato [9]. However, DGCC was not detected in two species of Cryptophyceae which contained DGTA.

DGTS was detected in Chlorophyceae, but was not detected in algae containing chlorophylls *a* and *c*. PC was detected in all species of algae except the three species of Chlorophyceae. These algae contained lyso-DGTS as a major polar lipid in place of PC. We could not distinguish lyso-DGTS from PC by two-dimensional TLC. The presence of lyso-DGTS, however, could be ascertained by use of HPLC-mass spectrometry. Some species of Chlorophyceae, for example *Chlamydomonas reinhardtii* [10] and *Volvox carteri* [11], which accumulate high levels of DGTS, have been

Table 1. Distribution of betaine lipids and PC in Haptophyceae

Order	Species		DGCC	PC	DGTS	Lyso-DGTS	DGTA
Isochrysidales	*Isochrysis galbana*	(TKBIS6)	+	+	n.d.	n.d.	n.d.
	Isochrysis sp.	(CS177)	+	n.d.	n.d.	n.d.	n.d.
	Emiliana huxleyi	(MBIJ06)	+	+	n.d.	n.d.	n.d.
	Pleurochrysis haptonemofera	(TKBPL1)	+	n.d.	n.d.	n.d.	n.d.
	Pleurochrysis carterae	(TKBPL2)	+	n.d.	n.d.	n.d.	n.d.
	Isochrysis sp.	(TKBIS1)	+	n.d.	n.d.	n.d.	n.d.
	Isochrysis sp.	(TKBIS2)	+	n.d.	n.d.	n.d.	n.d.
Coccosphaerales	*Calyptrosphaera sphaeroidea*	(TKBHC1)	+	n.d.	n.d.	n.d.	—
	Cruciplacolithus neohelis	(TKBCR1)	+	n.d.	n.d.	n.d.	n.d.
	Helladosphaera	(TKBHC3)	+	n.d.	n.d.	n.d.	n.d.
Prymnesiales	*Chrysochromulina hirta*	(TKB8602)	+	n.d.	n.d.	n.d.	+
Pavlovales	*Pavlova lutheri*	(CS23)	+	n.d.	n.d.	n.d.	+
	Pavlova salina	(TKB8708)	+	n.d.	n.d.	n.d.	+
	Pavlova pinguis	(CCMPIG7)	+	n.d.	n.d.	n.d.	+
	Pavlova sp.	(TKBPA2)	+	n.d.	n.d.	n.d.	—
	Pavlova sp.	(TKBPA3)	+	n.d.	n.d.	n.d.	+

+, Detected; n.d., not detected; −, not clear.

Table 2. Distribution of betaine lipids and PC in marine algae

Order	Species		DGCC	PC	DGTS	Lyso-DGTS	DGTA
Dinophyceae	*Prorocentrum micans*	(NIES 12)	+	+	n.d.	n.d.	n.d.
	Crypthechodinium cohnii	(ATCC 30021)	+	+	n.d.	n.d.	n.d.
	Gymnodium sp.	(MBI H05-1)	+	+	n.d.	n.d.	n.d.
	Symbiodinium microadriaticum		+	+	n.d.	n.d.	n.d.
Cryptophyceae	*Rhodomonas* sp.	(MBI B38-X)	n.d.	+	n.d.	n.d.	+
	Chroomonas salina	(CCAP 978/24)	n.d.	+	n.d.	n.d.	+
Rhodophyceae	*Schizymenia dubyi*		n.d.	+	n.d.	n.d.	n.d.
	Gracilaria verrucosa		n.d.	+	n.d.	n.d.	n.d.
	Corallina sp.		n.d.	+	n.d.	n.d.	n.d.
	Amphiroa sp.		n.d.	+	n.d.	n.d.	n.d.
	Porphyridium purpureum	(IAM R-1)	n.d.	+	—	n.d.	n.d.
	Cyanidium caldarium	(NIES 551)	n.d.	+	n.d.	n.d.	n.d.
Phaeophyceae	*Undaria pinnatifida*		n.d.	+	n.d.	n.d.	n.d.
	Padina crassa		n.d.	+	n.d.	n.d.	+
Bacillariophyceae	*Chaetoceros calcitrans*	(CS 178)	−	+	n.d.	n.d.	n.d.
	Skeletonema costatum	(NIES 16)	n.d.	+	n.d.	n.d.	n.d.
	Phaeodactylum tricornutum	(IAM B-14)	n.d.	+	n.d.	n.d.	n.d.
Eustigmatophyceae	*Nannochloropsis salina*	(CCAP 849/2)	n.d.	+	—	n.d.	n.d.
Chlorophyceae	*Chlorococcum* sp.	(MBI 26-A)	n.d.	+	+	n.d.	n.d.
	Chlorococcum littorale	(MBI)	n.d.	n.d.	+	+	n.d.
	Chlorella saccharophila	(UTEX 2469)	n.d.	n.d.	+	+	n.d.
	Halochlorococcum saccatum	(UTEX LB2072)	n.d.	n.d.	+	+	n.d.
	Nannochloris atomus	(CCAP 251/4B)	n.d.	+	+	n.d.	n.d.
	Chlorosarcinopsis halosphila	(UTEX 2073)	n.d.	+	+	n.d.	n.d.
	Dunaliella tertiolecta	(CS 175)	n.d.	+	+	+	n.d.
	Halimeda opuntia		n.d.	+	+	n.d.	n.d.
	Stichococcus bacillaris	(CCAP 379/36)	n.d.	+	−	n.d.	n.d.
Prasinophyceae	*Tetraselmis chui*	(CS 26)	n.d.	+	n.d.	n.d.	−
	Pycnococcus provasolii	(CS 185)	n.d.	+	+	n.d.	n.d.

+, Detected; n.d., not detected; −, not clear.

reported not to contain PC. But the occurrence of lyso-DGTS as a major component in place of PC was not known in any algae.

Table 3 shows the fatty acid composition of total lipids from ten species of Haptophyceae. 16:0 was one of the common major fatty acids in all species; however, 16:1, 18:1, 18:3 (n3), 18:4, 20:5 and 22:6 are major constituents in same species of algae. The average in the percentage (C_{20} and C_{22} polyunsaturated fatty acids) of the total in 10 species was 17.8%. 18:5, which was reported to be a characteristic fatty acid in Haptophyceae [12], was detected in seven species. Species belonging to the Pavlovales did not contain 18:5; on the other hand, 14:0 and 16:0 accounted for 20% of the total fatty acids, respectively.

Table 4 shows the fatty acid composition of DGCC in Haptophyceae. 16:0 and one of C_{18} fatty acids or C_{20} fatty acids are the major fatty acids. C_{20} and/or C_{22} polyunsaturated fatty acids are also major, the average of which in 10 species was 28.9%. This is in agreement with our previous data on *P. lutheri* [13]. Polyunsaturated fatty acids were also reported to be major constituents of DGTS and DGTA in other algae [7, 9, 14]. In particular, the localization of polyunsaturated fatty acids in DGTA was clearly demonstrated [6, 7, 9, 14]. Some species of algae which contain DGTS lack C_{20} and C_{22} polyunsaturated fatty acids [15, 16]. On the contrary, all algae in which DGTA or DGCC was present contain C_{20} and C_{22} polyunsaturated fatty acids. The biosynthetic pathway of polyunsaturated fatty acids conjugated to betaine lipids remains to be clarified.

EXPERIMENTAL

Microalgae. These were obtained from the Centre for Microbial and Microalgal Research, University of Tokyo, Japan (IAM), Microbial Culture Collection, National Institute for Environmental Studies, Japan (NIES), CSIRO Culture Collection of Microalgae, Australia (CS), American Type Culture Collection, U.S.A. (ATCC), the Culture Collection of Algae at the University of Texas at Austin, U.S.A. (UTEX), Provasoli-Guillard Center for Culture of Marine Phytoplankton, U.S.A. (CCMP), and Culture Collection of Algae and Protozoa, Natural Environment Research Council, U.K. (CCAP). Those with MBI strain numbers were isolated from the Pacific Ocean and maintained at the Marine Biotechnology Institute (MBI). All strains in the Haptophyceae (Table 1), except for those obtained from the institutes listed above, were provided by Dr I. Inouye, University of Tsukuba, Japan. *Symbiodinium microadriatcum* was collected from *Tridacna derasa.*

Macroalgae. Undaria pinnatifida, Schizymenia dubyi and *Gracillaria verrucosa* were harvested at sites on

Table 3. Fatty acid composition (mol %) of total lipids in 10 species of Haptophyceae

	1	2	3	4	5	6	7	8	9	10
14:0	25.5	21.9	0.3	0.9	0.8	0.8	16.3	1.7	1.8	7.6
16:0	18.1	15.7	20.8	30.6	25.6	29.0	19.3	31.9	32.3	27.5
16:1	23.2	21.3	6.8	4.9	4.3	2.3	18.8	2.8	2.8	19.4
16:2	0.4	0.1	0.6	0.3	0.3	0.3	0.1	0.0	0.0	0.1
18:0	0.5	0.4	1.5	1.8	2.8	2.3	0.4	1.6	3.4	0.6
18:1	1.3	1.0	14.3	11.0	14.6	26.6	1.7	28.1	28.5	5.4
18:2	2.5	1.5	5.9	7.6	2.6	4.5	0.8	9.7	11.4	5.7
18:3 (n6)	0.9	0.4	0.2	1.0	0.3	0.4	0.4	1.8	1.6	1.0
18:3 (n3)	0.3	1.9	30.1	9.6	11.6	9.0	1.2	4.7	3.0	0.5
18:4	3.4	4.2	11.0	16.3	24.4	15.0	8.2	6.1	3.7	2.8
18:5	0.0	0.0	0.3	6.5	0.0	0.1	0.7	0.1	1.5	0.1
20:4	1.4	1.6	0.0	0.2	0.0	0.7	1.3	1.2	0.8	1.0
20:5	13.6	16.5	2.8	2.8	5.2	2.4	20.4	2.9	1.3	12.4
22:5	5.9	10.4	0.2	0.4	0.9	0.8	6.9	2.2	2.9	5.3
22:6	3.1	2.9	5.3	6.0	6.6	5.7	3.7	5.1	4.9	10.7
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Strains were as follows; 1, *Pavlova* sp. (TKBPA3); 2, *Pavlova* sp. (TKBPA2); 3, *Pleurochrysis carterae* (TKBPL2); 4, *Pleurochrysis haptonemofera* (TKBPL1); 5, *Isochrysis* sp. (TKBIS2); 6, *Isochrysis* sp. (TKBIS1); 7, *Isochrysis* sp. (CS177); 8, *Calyptrosphaera sphaeroidea* (TKBHC1); 9, *Cruciplacolithus neohelis* (TKBCR1); 10, *Chrysochromulina hirta* (TKB8602).

16:1 is a mixture of isomers 16:1(9) and 16:1(3t); 18:1 is a mixture of isomers 18:1(9) and 18:1(11).

the Omaezaki coast (Shizuoka Prefecture, Japan) at low tide in May 1992. *Padina crassa*, *Corallia* sp. and *Amphiroa* sp. were harvested at sites on Noto (Ishikawa Prefecture, Japan) in September 1990. *Halimeda opuntia* was harvested at sites on Palau in December 1991.

Culture conditions. Microalgae (Table 2) were cultured in 1-l glass bottles for 1–2 weeks with air bubbling, except for *S. microadriaticum* and *C. cohnii*, which were too fragile to place under physical stress. Continuous illumination (50–100 mmol photons $m^{-2} s^{-1}$) was provided by fluorescent lamps. *C. caldarium* NIES 551 was grown at 45° with modified Allen's medium [17] supplemented with EDTA. *P. purpureum* IAM R-1 was cultured with MK medium [18]. *Pavlova pinguis* was cultured with K medium [19]. *P. tricornutum* was cultured with ASP_2 medium [20]. *C. cohnii* were grown in 2% (w/v) glucose and 0.2% (w/v) yeast extract in sea water without aeration at 25° in the dark. All other species were grown at room temp. using ESM medium [21].

Lipid analysis. Cells or tissue were extracted with $CHCl_3$–MeOH (1:2) according to the method of ref. [22]. The extract was evapd to dryness. The residue was separated by 2D silica gel TLC (Merck 5715) with Me_2CO–benzene–MeOH–H_2O (8:3:2:1) in the 1st dimension and with $CHCl_3$–MeOH–aq.–NH_4OH (13:7:1) in the 2nd dimension. The spot which corresponded to PC or/and DGCC was scraped off and

Table 4. Fatty acid composition of (mol %) DGCC in 10 species of Haptophyceae

	1	2	3	4	5	6	7	8	9	10
14:0	27.5	14.9	1.3	2.3	17.0	5.7	13.5	6.8	21.7	0.8
16:0	28.8	28.4	44.2	46.0	16.7	19.0	39.3	33.4	27.7	67.5
16:1	3.1	0.0	0.1	2.4	1.3	0.8	2.6	0.0	0.0	0.0
16:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18:0	9.2	22.2	8.3	10.2	16.0	7.1	7.3	10.4	8.7	3.6
18:1	3.7	6.3	2.7	5.1	9.5	12.6	13.7	4.8	12.6	3.9
18:2	0.0	0.0	0.6	1.4	1.6	1.7	0.7	1.7	0.0	0.7
18:3 (n6)	0.0	0.0	0.1	0.6	0.0	7.6	0.0	0.0	0.0	0.0
18:3 (n3)	0.0	0.0	0.5	1.1	0.0	4.4	0.0	0.0	4.3	2.9
18:4	0.0	0.0	1.2	4.3	8.8	7.1	0.0	1.1	5.6	0.0
18:5	0.0	0.0	0.0	1.0	0.0	0.8	0.0	0.0	0.0	0.0
20:4	0.0	0.0	0.6	0.7	0.0	2.2	0.0	1.7	0.0	1.2
20:5	24.6	25.7	17.3	8.5	5.6	5.5	7.8	2.9	5.4	6.6
22:5	0.0	0.0	0.4	4.4	8.0	3.6	5.0	23.5	6.4	1.6
22:6	3.1	2.5	22.6	11.8	15.5	21.8	10.1	13.6	7.7	11.3
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Species as shown in Table 3.

16:1 is a mixture of isomers 16:1(9) and 16:1(3t); 18:1 is a mixture of isomers 18:1(9) and 18:1(11).

extracted with $CHCl_3$–MeOH (2:1). The extract, which was filtered to remove silica gel, was concd under a N_2 stream and then purified by HPLC on a column (4 mm × 250 mm) of Nucleosil 100-5CN (GL Science). The solvent system was MeCN–HOAc (pH 5.16) (4:1) and the flow rate was 1 ml min^{-1}. Compounds were detected with a UV detector at 210 nm. The R_fs (min) were as follows: DGTS (3.4), lyso-DGTS (3.6), PC (3.8), DGCC (7.0), lyso-DGCC (9.0), DGTA (12).

LC/MS conditions. HPLC was performed with equipment fitted with a 1.7 ml micro cell photodiode array detector. The column and solvent system were as described above. UV spectra were obtained in the range 200–500 nm. Mass chromatograms and spectra were using a JEOL JMS-SX102 mass spectrometer equipped with a Frit-FAB ion source and FAB gun. XeO was used as a primary beam at 5 mA emission current. Primary and secondary accelerating voltages were 6 kV and 8 kV, respectively. LC/MS analyses were carried out using a frit-FAB system of a continuous-flow FAB LC/MS interface. The FAB matrix (3% soln of *m*-nitrobenzylalcohol in MeOH) was mixed with eluent after the HPLC detector at a flow rate of 1.6 ml hr^{-1} through a T-joint for capillary tubing. The mixed soln was split in a ratio of 100:1 using a vacuum splitting apparatus and introduced into the mass spectrometer.

Fatty acid analysis. Spots of DGCC on TLC plates were visualized under UV light after spraying with 0.01% (w/v) primulin in 80% (v/v) Me_2CO, scraped off, then heated at 90° for 2 hr with 2 ml of 5% (w/w) HCl–MeOH. The MeOH soln was extracted ×2 with 2 ml of *n*-hexane and the resultant upper layer was concd to min. vol. GC was conducted in a fused-silica capillary column (DB-23, 0.25 mm × 30 m, J & W), the oven temp. being prog. from 150° to 210 at 3° min^{-1}. To identify the fatty acid Me esters, GC-MS analysis was performed, using an ionization energy of 70 eV with an electron-multiplier voltage of 1.5 kV.

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