Marine Natural Products. XXIX.[1] Heterosigma-glycolipids I, II, III, and IV, Four Diacylglyceroglycolipids Possessing ω3-Polyunsaturated Fatty Acid Residues, from the Raphidophycean Dinoflagellate *Heterosigma akashiwo*

Motomasa KOBAYASHI, Kozo HAYASHI, Kazuyoshi KAWAZOE, and Isao KITAGAWA*

Faculty of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565, Japan. *Received December 9, 1991*

Heterosigma-glycolipids I (1), II (2), III (3), and IV (4), four new Diacylglyceroglycolipids possessing ω3-polyunsaturated fatty acid residues, were isolated from the cultured raphidophycean dinoflagellate *Heterosigma akashiwo*.

Based on enzymatic partial hydrolysis using lipase and physicochemical evidence, the structures of heterosigma-glycolipids I (1), II (2), III (3), and IV (4) have been determined as (2′*S*)-2′,3′-di-*O*-(6,9,12,15-octadecatetraenoyl)glyceryl β-D-galactopyranoside, (2′*S*)-2′-*O*-(6,9,12,15-octadecatetraenoyl)-3′-*O*-(5,8,11,14,17-eicosapentaenoyl)glyceryl β-D-galactopyranoside, (2′*S*)-2′-*O*-(6,9,12,15-octadecatetraenoyl)-3′-*O*-(3,6,9,12,15-octadecapentaenoyl)glyceryl β-D-galactopyranoside, and (2′*S*)-2′-*O*-(5,8,11,14,17-eicosapentaenoyl)-3′-*O*-(6,9,12,15-octadecatetraenoyl)glyceryl 6-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside, respectively.

Keywords heterosigma-glycolipid; dinoflagellate raphidophycean; *Heterosigma akashiwo*; marine phytoplankton; fatty acid ω3-polyunsaturated; EPA; eicosapentaenoic acid; galactosyldiacylglycerol

The low incidence of acute myocardial infarction among Eskimos in Greenland has been ascribed to the high content of 5,8,11,14,17-eicosapentaenoic acid (EPA) in their diet fish on the basis of an epidemiological investigation.[2] In recent years, EPA has been shown to exhibit various biological activities,[3] some of which are consistent with the epidemiological evidence. The photosynthetic raphidophycean dinoflagellate *Heterosigma akashiwo* is a wall-less unicellular alga that is widely distributed in coastal waters, where it may cause "red-tide" phenomena and can be responsible for extensive mortality of cultured fish and other marine organisms.[4,5]

As part of our search for new biologically active marine natural products,[6] we have been investigating the metabolites of the cultured dinoflagellate *H. akashiwo*, which was collected from the coastal water of Suma-ura, Osaka Bay. Recently, we have found four new galactosyldiacylglycerols comprising ω3-polyunsaturated fatty acid residues, which are designated heterosigma-glycolipids I (**1**), II (**2**), III (**3**), and IV (**4**).[7] This paper presents a full account of the structure elucidation of these glyceroglycolipids.[8]

The raphidophycean dinoflagellate *H. akashiwo* was cultured in 1 : 1 mixed media of ASP-2[9] and ES[10] in 20-liter glass bottles at 21 °C. The cultivation was carried out in a 16 : 8 light to dark cycle using Homolux lamps FL40S-PG (National) for 40 d. The combined 60-liter culture was sonicated and extracted with ethyl acetate (AcOEt). The AcOEt-soluble portion was then evaporated under reduced pressure to give 1.2 g of the extractive. Silica gel column chromatography of the extractive furnished a monogalactosyl diacylglycerol fraction (A) (120 mg), a digalactosyl diacylglycerol fraction (B) (70 mg), and a 6-sulfoquinovosyl diacylglycerol fraction (C) (130 mg) as the major con-

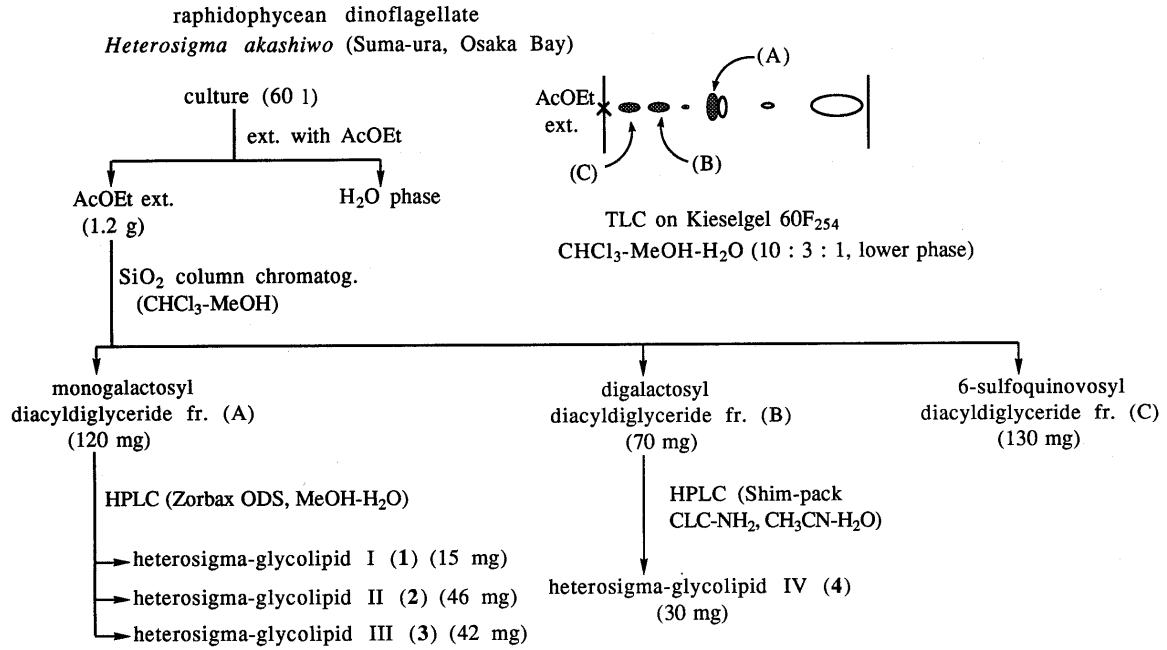


Fig. 1. Isolation Procedure for Glyceroglycolipids

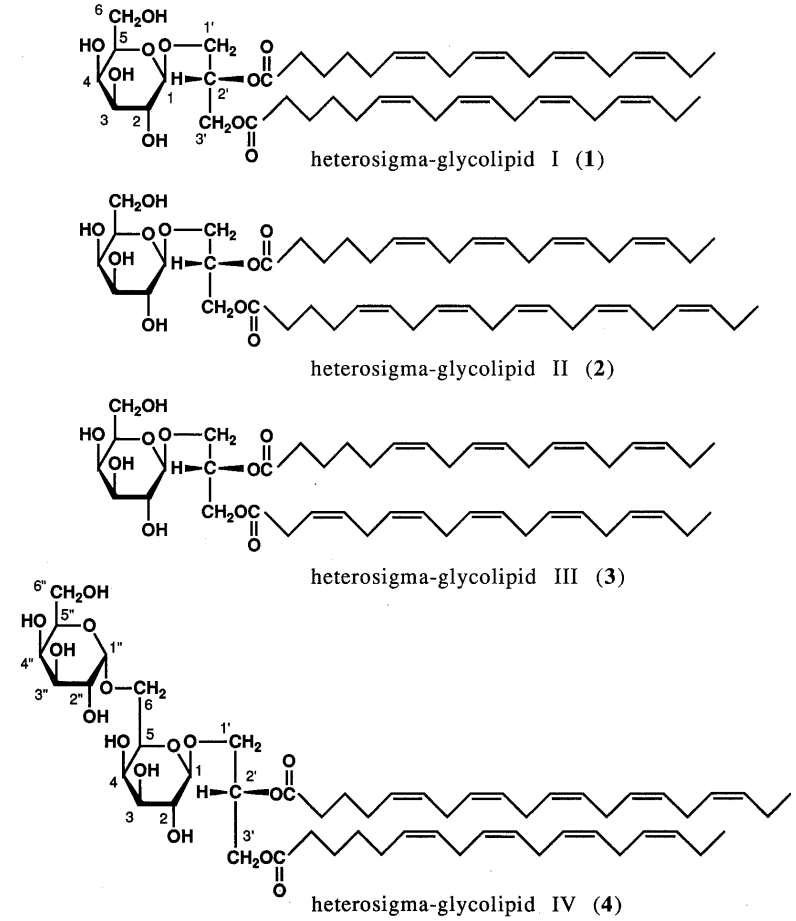


Chart 1

TABLE I. ^{1}H-NMR Data for **1**, **2**, **3**, **5**, **6**, and **7**[a]

^{1}H	1	2	3	5	6	7
1	4.74 (d, J=7.9)	4.75 (d, J=7.6)	4.74 (d, J=7.9)	4.88 (d, J=7.6)	4.82 (d, J=7.6)	4.79 (d, J=7.6)
2	4.32[b]	4.36[b]	4.32[b]	4.48 (dd, J=7.6, 9.5)	4.42[b]	4.39 (dd, J=7.6, 9.2)
3	4.06 (dd, J=9.5, 3.4)	4.07 (dd, J=9.6, 2.9)	4.06 (dd, J=9.8, 3.3)	4.13 (dd, J=9.5, 3.4)	4.11 (dd, J=9.5, 3.3)	4.09[b]
4	4.46 (d, J=3.4)	4.49 (d, J=2.9)	4.45 (d, J=3.3)	4.52 (d, J=3.4)	4.50 (d, J=3.3)	4.47 (d, J=3.1)
5	3.98 (dd, J=5.8, 5.5)	4.00[c]	3.99[c]	4.04 (dd, J=6.4, 5.5)	4.03[c]	3.99 (dd, J=6.1, 6.1)
6	4.32 (2H, m)[b]	4.36 (2H, m)[b]	4.32 (2H, m)[b]	4.40 (2H, m)[b]	4.36 (2H, m)[b]	4.34 (2H, m)[c]
1′	4.29 (dd, J=10.7, 5.2)	4.29 (dd, J=10.8, 5.3)	4.28 (dd, J=10.8, 5.3)	4.44 (dd, J=10.1, 6.2)	4.37[b]	4.34[c]
	4.01 (dd, J=10.7, 5.0)	4.00[c]	3.99[c]	4.22 (dd, J=10.1, 4.3)	4.04 (dd, J=10.4, 5.2)[c]	4.12 (dd, J=11.6, 4.7)[b]
2′	5.58 (m)	5.59 (m)	5.58 (m)	4.40 (m)[b]	4.42 (m)[b]	5.54 (m)
3′	4.62 (dd, J=11.9, 3.1)	4.62 (dd, J=11.9, 3.4)	4.62 (dd, J=12.1, 3.2)	4.10 (d, J=4.6)	4.51 (d, J=5.5)	4.42 (dd, J=10.8, 5.2)
	4.45 (dd, J=11.9, 5.2)	4.46 (dd, J=11.9, 6.6)	4.46 (dd, J=12.1, 5.6)	4.09 (d, J=5.5)	4.50 (d, J=3.1)	4.17 (dd, J=10.8, 5.0)

a) All compounds were measured in C_5D_5N (containing 1 drop of D_2O) at 500 MHz. *b, c*) Signals within the same column overlapped.

stituents, each of which gave a single spot on an ordinary thin-layer chromatogram (TLC) (Fig. 1). The monogalactosyl diacylglycerol fraction was further purified by high-performance liquid chromatography (HPLC) to afford three major components named heterosigma-glycolipids I (**1**), II (**2**), and III (**3**). Furthermore, HPLC purification of the digalactosyl diacylglycerol fraction provided heterosigma-glycolipid IV (**4**) as the major component.

Heterosigma-glycolipid I (**1**) gave a quasimolecular ion $(M+Na)^+$ peak at m/z 793 in the fast atom bombardment mass spectrum (FAB-MS). The infrared (IR) spectrum of **1** showed the presence of hydroxyl groups and ester functions (3425, 1725 cm^{-1}), while the proton nuclear magnetic resonance (^{1}H-NMR) spectrum and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** showed signals which were characteristically attributable to a monogalactosyl diacylglycerol (Tables I and II).

Treatment of heterosigma-glycolipid I (**1**) with sodium methoxide (NaOMe) in methanol furnished a glycerol galactoside (**5**) and methyl 6,9,12,15-octadecatetraenoate (**8**), which was identified by analysis of its MS, ^{1}H-NMR and ^{13}C-NMR spectra. The glycerol galactoside, $[\alpha]_D$ −8° (H_2O), was shown to be identical with (2′*R*)-1′-*O*-glyceryl β-D-galactopyranoside (**5**), which was obtained by NaOMe treatment of an anti-inflammatory galactolipid M-5[11] previously isolated by us from the Okinawan marine sponge

TABLE II. ^{13}C-NMR Data for **1**, **2**, **3**, and **5**

	1[a]	2[a]	3[a]	5[b]
1	106.1 d	106.1 d	106.1 d	104.9 d
2	73.2 d	73.2 d	73.2 d	72.7 d
3	75.7 d	75.7 d	75.6 d	74.6 d
4	71.0 d	71.0 d	71.0 d	70.4 d
5	77.6 d	77.6 d	77.5 d	76.9 d
6	63.2 t	63.2 t	63.2 t	62.8 t
1′	69.5 t	69.5 t	69.5 t	72.3 t
2′	72.6 d	72.6 d	72.5 d	72.5 d
3′	64.8 t	64.9 t	65.2 t	64.2 t
COO	175.7 s	175.6 s	175.4 s	—
	175.4 s	175.4 s	173.8 s	—
Me	22.3 q	22.3 q	22.3 q	—

a) Measured in CD_3OD at 125 MHz. *b*) Measured in CD_3OD–D_2O (1:2) at 125 MHz.

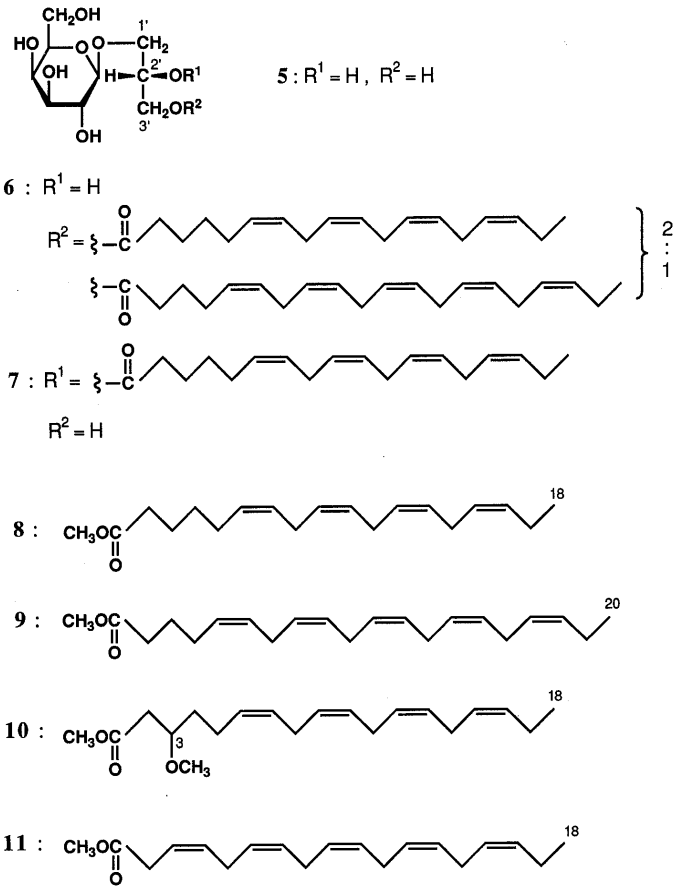


Chart 2

Phyllospongia foliascens. Detailed comparisons of the ^{1}H-NMR and ^{13}C-NMR spectra of **1** with those of **5** have shown that the fatty acid residues of **1** are attached to C-2′ and C-3′ in the glycerol moiety (Tables I and II). Thus, the carbon signal due to C-1′ of the glycerol moiety of **1** is observed at higher field (*ca.* 3 ppm) as compared with that of **5**, while the signals ascribable to C-2′ and C-3′ of **1** and **5** are observed at similar chemical shifts.[11] Consequently, the chemical structure of heterosigma-glycolipid I has been determined as (2′*S*)-2′,3′-di-*O*-(6,9,12,15-octadecatetraenoyl)glyceryl β-D-galactopyranoside (**1**).

The FAB-MS of heterosigma-glycolipid II (**2**) gave a quasimolecular ion $(M+Na)^+$ peak at *m/z* 819. The ^{1}H-NMR and ^{13}C-NMR spectra of **2** closely resembled those of heterosigma-glycolipid I (**1**) except for the signals due to the fatty acid moieties. Treatment of **2** with NaOMe–MeOH as carried out for the hydrolysis of **1** furnished the same glyceryl β-D-galactopyranoside (**5**) together with a mixture of fatty acid methyl esters. The fatty acid composition in **2** was determined by gas-liquid chromatographic (GLC) analysis of these liberated methyl esters to be a 1:1 mixture of methyl 6,9,12,15-octadecatetraenoate (**8**) and methyl 5,8,11,14,17-eicosapentaenoate (**9**). The latter **9** was isolated in a pure form by HPLC separation and found to be identical with an authentic sample (Sigma) by GLC, HPLC, and ^{1}H-NMR comparisons. Furthermore, the ^{1}H-NMR and ^{13}C-NMR analyses of heterosigma-glycolipid II (**2**) in comparison with heterosigma-glycolipid I (**1**) indicated that the fatty acid residues in **2** are attached to 2′-OH and 3′-OH of the glycerol moiety.

In order to define the locations of these fatty acid residues in heterosigma-glycolipid II (**2**), we next attempted reductive partial hydrolysis of the acyl moieties in **2**. Thus, treatment of **2** with LiAlH(O-*tert*-Bu)$_3$ in benzene at 40 °C for 2 h appeared to provide a 3′-*O*-deacylated galactolipid, as expected. However, the ^{1}H-NMR spectrum of the galactolipid thus obtained showed that the signal assignable to 2′-H was observed at δ 4.42 (m), which was at distinctly higher field than the corresponding proton signal observed in the case of **2**. Furthermore, treatment of this galactolipid with NaOMe–MeOH provided a 2:1 mixture of methyl 6,9,12,15-octadecatetraenoate (**8**) and methyl 5,8,11,14,17-eicosapentaenoate (**9**). Consequently it became apparent that the LiAlH(O-*tert*-Bu)$_3$ reduction of **2** proceeded less regioselectively, partly resulting in: i) reductive hydrolysis at the 2′-*O*-acyl moiety and ii) reductive hydrolysis at the 2′-*O*-acyl (less) and 3′-*O*-acyl (more) moieties accompanied by acyl migration of the acyl residue from the 2′-*O*- to the 3′-*O*-moiety.

We next applied enzymatic hydrolysis, anticipating partial hydrolysis of the acyl moieties in **2**. On enzymatic hydrolysis using lipase type XIII (prepared from *Pseudomonas* sp.) in dioxane–H_2O (1:1) at 37 °C for 4 h, heterosigma-glycolipid II (**2**) furnished quantitatively a 2′-*O*-acylated galactolipid **7** formed by selective partial hydrolysis at the 3′-*O*-acyl moiety and EPA as a single acidic product, which was identified as the methyl ester **9** after CH_2N_2 treatment. As shown in Table I, the ^{1}H-NMR spectrum of **7** showed the signals due to 3′-H_2 at higher fields than those in **2**. Furthermore, treatment of **7** with NaOMe–MeOH liberated methyl 6,9,12,15-octadecatetraenoate (**8**) as a single product from the 2′-*O*-acyl moiety.

So it has been concluded that the 6,9,12,15-octadecatetraenoyl and 5,8,11,14,17-eicosapentaenoyl residues in **2** are attached respectively to the 2′-OH and 3′-OH functions of the glycerol moiety in **2**. As a result, the chemical structure of heterosigma-glycolipid II has been determined as (2′*S*)-2′-*O*-(6,9,12,15-octadecatetraenoyl)-3′-*O*-(5,8,11,14,17-eicosapentaenoyl)glyceryl β-D-galactopyranoside (**2**). It should be noted here that the enzymatic hydrolysis of heterosigma-glycolipid II (**2**) using lipase in dioxane-water proceeded quantitatively and regioselective partial hydrolysis occurred at the 3′-*O*-acyl function of the glycerol moiety without acyl migration.

The FAB-MS of heterosigma-glycolipid III (**3**) gave a quasimolecular ion $(M+Na)^+$ peak at *m/z* 791. The ^{1}H-NMR and ^{13}C-NMR spectra of **3** closely resembled those of heterosigma-glycolipids I (**1**) and II (**2**) and showed signals which were characteristic of a monogalactosyl-2′,3′-diacylglycerol. Treatment of **3** with NaOMe–MeOH as carried out for **1** provided the same glyceryl β-D-galactopyranoside (**5**) as obtained from **1** and **2**, and a mixture of fatty acid methyl esters. The mixture of fatty acid methyl esters was further subjected to HPLC separation to afford methyl 6,9,12,15-octadecatetraenoate (**8**) and methyl 3-methoxy-6,9,12,15-octadecatetraenoate (**10**) in 1 : 1 ratio. The structure of **10** was determined by ^{1}H-NMR, ^{13}C-NMR, and MS analyses.

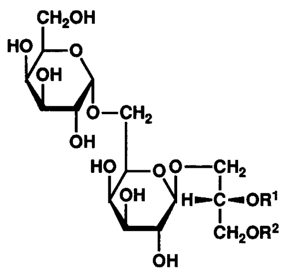
The enzymatic partial hydrolysis of **3** using lipase type XIII in dioxane–H_2O (1 : 1) furnished quantitatively the 2′-*O*-acyl galactolipid **7** obtained by similar enzymatic hydrolysis of **2** and 3,6,9,12,15-octadecapentaenoic acid as a single fatty acid, which was isolated as the methyl ester **11** after CH_2N_2 treatment. The structure of **11** was elucidated as methyl 3,6,9,12,15-octadecapentaenoate by ^{1}H-NMR, ^{13}C-NMR, and MS analyses. So, we concluded that the 6,9,12,15-octadecatetraenoyl and 3,6,9,12,15-octadecapentaenoyl residues in **3** are respectively attached to the 2′-OH and 3′-OH functions of the glycerol moiety. It was presumed therefore that methyl 3-methoxy-6,9,12,15-octadecatetraenoate (**10**), obtained above by methanolysis of heterosigma-glycolipid III (**3**), was formed from methyl 3,6,9,12,15-octadecapentaenoate (**11**), which was initially liberated by methanolysis of **3**, through the double bond migration from Δ^3 to Δ^2 and subsequent 1,4-addition of methanol to the resulting αβ-unsaturated ester moiety. Consequently, the chemical structure of heterosigma-glycolipid III has been determined as (2′*S*)-2′-*O*-(6,9,12,15-octadecatetraenoyl)-3′-*O*-(3,6,9,12,15-octadecapentaenoyl)glyceryl β-D-galactopyranoside (**3**).

Finally, the digalactosyl diacylglycerol designated heterosigma-glycolipid IV (**4**) gave a quasimolecular ion $(M+Na)^+$ peak at *m/z* 981 in FAB-MS. The ^{1}H-NMR and ^{13}C-NMR spectra of **4** showed signals which were characteristically attributable to a digalactosyl diacylglycerol. Treatment of **4** with NaOMe–MeOH furnished a glycerol digalactoside **12** and a mixture of fatty acid methyl esters. The glycerol digalactoside thus obtained was shown to be identical with (2′*R*)-1′-*O*-glyceryl 6-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside, which was obtained by NaOMe–MeOH treatment of a digalactosyl diacylglycerol previously isolated by us from North American alfalfa, *Medicago sativa*,[12] on the basis of ^{13}C-NMR and $[\alpha]_D$ comparisons. On the other hand, the mixture of fatty acid methyl esters obtained above was determined by GLC analysis to be a 1 : 1 mixture of methyl 6,9,12,15-octadecatetraenoate (**8**) and methyl 5,8,11,14,17-eicosapentaenoate (**9**). Detailed comparisons of the ^{1}H-NMR and ^{13}C-NMR data for **4** and **12** have shown that the fatty acid residues are attached to C-2′ and C-3′, respectively, in the glycerol moiety of **4**.

In order to determine the locations of these fatty acid residues in heterosigma-glycolipid IV (**4**), the glycolipid was subjected to enzymatic partial hydrolysis using lipase type XIII in dioxane–H_2O as above. It was found that **4** provided quantitatively a 2′-*O*-acylated digalactolipid **13** with liberation of 6,9,12,15-octadecatetraenoic acid, which was identified after CH_2N_2 treatment as the methyl ester **8**. The ^{1}H-NMR spectrum of **13** showed the signals due to 3′-H_2 at higher fields than those observed in the case of the parent **4**, and further treatment of **13** with NaOMe–MeOH liberated methyl 5,8,11,14,17-eicosapentaenoate (**9**) as a single fatty acid methyl ester. We concluded therefore that the 6,9,12,15-octadecatetraenoyl and 5,8,11,14,17-eicosapentaenoyl residues are attached respectively to the 3′-OH and 2′-OH functions in the glycerol moiety of **4**. Based on the above-mentioned evidence, the chemical structure of **4** has been determined as (2′*S*)-2′-*O*-(5,8,11,14,17-eicosapentaenoyl)-3′-*O*-(6,9,12,15-octadecatetraenoyl)-glyceryl 6-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside. Here again it is noteworthy that enzymatic partial hydrolysis is a convenient and reliable method to define the locations of fatty acid residues in galactosyl diacylglycerol glycolipids.

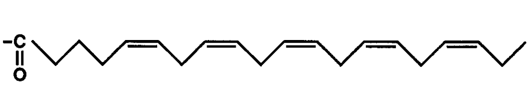
Methanolic alkaline hydrolysis of the 6-sulfoquinovosyldiacylglycerol fraction furnished a glycerol sulfonoglycoside and a mixture of fatty acid methyl esters. The glycerol sulfonoglycoside was found to be identical with glyceryl α-D-6-sulfoquinovopyranoside (**14**), which was obtained by similar alkaline treatment of a sulfonoglycolipid Ant-1[13] previously isolated by us from the sea urchin *Anthocidaris crassispina*. On the other hand, the major component of the fatty acid methyl esters was elucidated by FAB-MS analysis to be a 5 : 3 mixture of methyl palmitate (16 : 0) and methyl hexadecaenoate (16 : 1), while no polyunsaturated fatty acid methyl ester was detected. Therefore, we concluded that the 6-sulfoquinovosyldiacylglycerol fraction comprises sulfonoglycolipids having palmitoyl and hexadecaenoyl residues as the major components.

It is noteworthy that polyunsaturated fatty acids produced by the present phytoplankton *H. akashiwo* are contained in the forms of galactosyldiacylglycerols such as heterosigma-glycolipids I (**1**), II (**2**), III (**3**), and IV (**4**), and are characterized by their ω3-type polyunsaturated struc-



12 : R^1 = H , R^2 = H

13 : R^1 =



R^2 = H

14

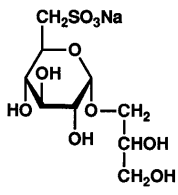


Chart 3

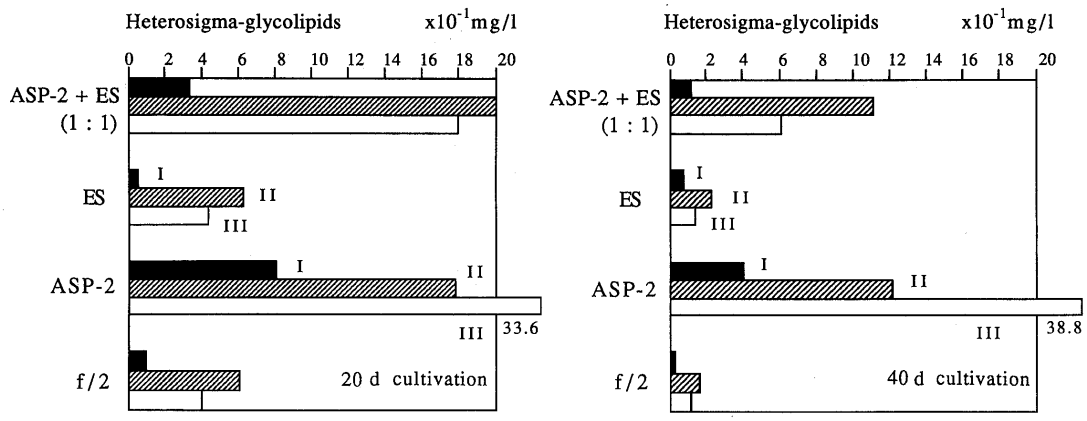


Fig. 2. Productivity of Heterosigma-glycolipids in Various Culture Media

tures: 18 : 4(ω3), 18 : 5(ω3), and 20 : 5(ω3) (=EPA). The structural correlation of these ω3-type polyunsaturated fatty acids is also interesting, since they may form part of a sequential biosynthetic chain.[14] EPA at the final stage, which is contained in the dietary fish oil, has been suggested to be responsible for the low incidence of myocardial infarction and for the decreased platelet aggregability among Eskimos in Greenland. As for the source of EPA in the fish oil, the present findings indicate that one possible source may be these galactosyldiacylglycerols initially biosynthesized by phytoplankton on which fish feed.[15]

In order to improve the productivity of EPA in the cultivation of the phytoplankton *H. akashiwo*, we investigated the effect of the culture medium, *e.g.*, ES, ASP-2, ASP-2 : ES (1 : 1), or f/2.[16] Thus, *H. akashiwo* was inoculated into 1 liter each of these media at a density of 1.0×10^3 cells/ml and the whole was cultivated for 40 d. The cell number of each culture was counted every week and the content of heterosigma-glycolipids was quantified after 20 and 40 d. It was found that the time course of the cell growth was similar (*ca.* 5×10^5 cells/ml at 35th day) in each medium, while the productivity of heterosigma-glycolipids I (**1**), II (**2**), and III (**3**) varied depending upon the kind of culture medium (Fig. 2). As shown in Fig. 2, *H. akashiwo* displayed good productivity of **1**, **2**, and **3** in the ASP-2 medium, which was prepared in artificial sea-water, and 3.88 mg of **3** was obtained from the 1 l of culture.

Next, we investigated the effect of the additon of D-galactose in the ASP-2 : ES (1 : 1) medium. D-Galactose (none, 62.5 mg, 125 mg or 250 mg) was added to 1-l cultures of *H. akashiwo* (2.5×10^3 cells/ml) and the whole was cultivated for 40 d. The time course of the cell growth was again similar in every case, though it was found that the addition of D-galactose did not improve the productivity of heterosigma-glycolipids in 20 days' cultivation, but instead decreased the productivity of heterosigma-glycolipids during 40 days' cultivation. Furthermore, we investigated the effect of the addition of α-linolenic acid [18 : 3(ω3)], which has been presumed to be a precursor in the biosynthesis of EPA. The cultivation was carried out under the same conditions as described above, and it was found that α-linolenic acid added in the form of either the ammonium salt or Na^+ salt or added as free acid greatly inhibited the cell growth of *H. akashiwo*.[17]

It is interesting that the phytoplankton *H. akashiwo* produces several galactosyl diacylglycerols possessing ω3-polyunsaturated fatty acid residues as major secondary products, even though some of these polyunsaturated fatty acids are known to be very toxic for the growth of *H. akashiwo* itself. The physiological functions of these galactosyl diacylglycerols [*e.g.* **1**—**4**] would be an interesting subject for future investigation.

Experimental

The instruments used to obtain physical data and experimental conditions for chromatography were the same as described in our previous paper.[18]

Cultivation of *H. akashiwo* The phytoplankton *H. akashiwo* was isolated from the coastal water of Suma-ura, Osaka Bay, 1988, by means of the sterile micropipette method. Cells were cultured aseptically at 21 °C in autoclaved ASP-2 : ES (1 : 1) medium, initially in 2-ml vials then in 2-l Erlenmeyer flasks or in the ASP-2 : ES (1 : 1) medium sterilized by passing it through 0.1 μm membrane filters (Advantec Toyo) in 20-l glass bottles. The cultivation was carried out in a 16 : 8 light-to-dark cycle using Homolux lamps FL40S-PG (National) for 40 d.

ASP-2: NaCl 18 g, $MgSO_4 \cdot 7H_2O$ 5 g, KCl 0.6 g, $CaCl_2$ 0.1 g, $NaNO_3$ 50 mg, K_2HPO_4 5 mg, $Na_2SiO_3 \cdot 10H_2O$ 150 mg, Tris 1 g, vitamin B_{12} 2 μg, S3 solution 10 ml, Na_2EDTA 30 mg, $FeCl_3$ 0.8 mg, $ZnCl_2$ 150 μg, $MnCl_2$ 1.2 mg, $CoCl_2$ 3 μg, $CuCl_2$ 1.2 μg, H_3BO_3 6 mg, distilled water 1 l (pH 7.8). (S3 solution: thiamine hydrochloride 50 mg, nicotinic acid 10 mg, calcium pantothenate 10 mg, *p*-aminobenzoic acid 1 mg, biotin 0.1 mg, inositol 0.5 g, folic acid 0.2 μg, thymine 0.3 g, distilled water 1 l).

ES: $NaNO_3$ 3.5 g, sodium β-glycerophosphate $\cdot 5H_2O$ 0.5 g, Fe as EDTA 250 ml, PII metal 250 ml, vitamin B_{12} 0.1 mg, thiamine mononitrate 5 mg, biotin 50 μg, Tris 5 g, distilled water 1 l, sea water 75 l (pH 7.8). (Fe as EDTA: $Fe(NH_4)_2(SO_4) \cdot 6H_2O$ 351 mg, Na_2EDTA 330 mg, distilled water 500 ml; PII metal: H_3BO_3 3.4 g, $FeCl_3 \cdot 6H_2O$ 147 mg, $MgSO_4 \cdot 7H_2O$ 492 mg, $ZnSO_4 \cdot 7H_2O$ 66 mg, $CoSO_4 \cdot 7H_2O$ 14 mg, Na_2EDTA 3 g, distilled water 3 l).

f/2: $NaNO_3$ 75 mg, $NaH_2PO_4 \cdot 2H_2O$ 6 mg, vitamin B_{12} 0.5 μg, biotin 0.5 μg, thiamine hydrochloride 100 μg, $NaSiO_3 \cdot 9H_2O$ 10 mg, f/2 metal 1 ml, sea water 1 l. (f/2 metal: Na_2EDTA 4.4 g, $FeCl_3 \cdot 6H_2O$ 3.2 g, $CoSO_4 \cdot 7H_2O$ 12 mg, $ZnSO_4 \cdot 7H_2O$ 21 mg, $MnCl_2 \cdot 4H_2O$ 180 mg, $CuSO_4 \cdot 5H_2O$ 7 mg, $NaMoO_4 \cdot 2H_2O$ 7 mg, distilled water 1 l).

Isolation of Galactosyldiacylglycerols The combined 60-l culture of *H. akashiwo* was sonicated for 10 min and then partitioned with AcOEt. The AcOEt-soluble portion was evaporated under reduced pressure to give 1.2 g of the extractive. The extractive was then purified by column chromatography [Silica gel 60, 60—230 mesh (Merck), 100 g, $CHCl_3$–MeOH (20 : 1)] to furnish a monogalactosyl diacylglycerol fraction (A) (120 mg), a digalactosyl diacylglycerol fraction (B) (70 mg), and a 6-sulfoquinovosyl diacylglycerol fraction (C) (130 mg). The monogalactosyldiacylglycerol fraction was further subjected to HPLC [Zorbax ODS, MeOH–H_2O (20 : 1)] to isolate heterosigma-glycolipids I (**1**, 15 mg), II (**2**, 46 mg), and III (**3**, 42 mg).

Heterosigma-glycolipid I (**1**): A colorless oil, $[\alpha]_D$ −4.5° (c = 1.3, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3425, 2900, 2845, 1725. ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: as given in Table I and 0.86 (6H, t, J = 7.5 Hz, Me), 5.40 (16H, m, olefinic H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: Table II. FAB-MS m/z: 793 $(M+Na)^+$. High-resolution FAB-MS:

Obsd.: 793.4860. Calcd for $C_{45}H_{70}NaO_{10}$: 793.4869.

Heterosigma-glycolipid II (**2**): A colorless oil, $[\alpha]_D$ −4.1° (c=1.3, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420, 2998, 2905, 2845, 1720. ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: as given in Table I and 0.87 (6H, t, J=7.5 Hz, Me), 5.42 (18H, m, olefinic H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: Table II. FAB-MS m/z: 819 $(M+Na)^+$. High-resolution FAB-MS: Obsd.: 819.5023. Calcd for $C_{47}H_{72}NaO_{10}$: 819.5027.

Heterosigma-glycolipid III (**3**): A colorless oil, $[\alpha]_D$ −4.5° (c=1.3, $CHCl_3$, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3460, 2910, 2845, 1740. ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: as given in Table I and 0.85 (6H, t, J=7.5 Hz, Me), 5.40 (18H, m, olefinic H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: Table II. FAB-MS m/z: 791 $(M+Na)^+$. High-resolution FAB-MS: Obsd.: 791.4649. Calcd for $C_{45}H_{68}NaO_{10}$: 791.4713.

Purification of the digalactosyl diacylglycerol fraction by HPLC [Shim-pack (CLC-NH_2), CH_3CN–H_2O (10 : 1)] afforded **4** (30 mg).

4: A colorless oil, $[\alpha]_D$ +21° (c=0.4, pyridine, 25 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3404, 2924, 2850, 1727. ^{1}H-NMR (500 MHz, C_5D_5N) δ: 4.76 (d, J=7.9 Hz), 4.69 (m), 4.57—3.43 (m), 4.18 (dd, J=6.1, 5.8 Hz), 4.12 (dd, J=3.1, 9.8 Hz), 4.04 (dd, J=5.5, 10.7 Hz), 0.95 (6H, t, J=7.6 Hz, Me), 5.52 (18H, m, olefinic H). ^{13}C-NMR (125 MHz, C_5D_5N) δ_C: 173.0, 172.9 (each s, CO), 105.1 (d, C_1), 101.3 (d, $C_{1''}$), 75.0 (d, C_3), 74.5 (d, C_5), 72.8 (d, $C_{5''}$), 72.1 (d, C_2), 71.6 (d, $C_{3''}$), 71.0 (t, C_6), 71.0 (d, $C_{4''}$), 70.6 (d, $C_{2'}$), 69.8 (d, $C_{2''}$), 68.1 (t, $C_{1'}$), 68.0 (d, C_4), 63.4 (t, $C_{3'}$), 62.5 (t, $C_{6''}$), 14.4 (q, Me). FAB-MS m/z: 981 $(M+Na)^+$. High-resolution FAB-MS: Obsd.: 981.5522. Calcd for $C_{53}H_{82}NaO_{15}$: 981.5549.

Alkaline Treatment of Heterosigma-glycolipid I (1) A solution of **1** (35 mg) in MeOH (2 ml) was treated with 2.8% NaOMe–MeOH (2 ml) and the whole solution was stirred at 20 °C for 30 min. The reaction mixture was neutralized with Dowex 50W × 8 (H^+ form) and partitioned into an n-hexane–MeOH mixture. Evaporation of the solvent under reduced pressure from the MeOH phase yielded a residue, which was purified by SiO_2 column chromatography ($CHCl_3$–MeOH) to furnish **5** (7 mg). The n-hexane phase was evaporated under reduced pressure to give methyl 6,9,12,15-octadecatetraenoate (**8**, 18 mg), which showed a single peak on gas-liquid chromatography (GLC) [5% Silicone SE-52, Uniport HP 80/100 mesh (3 mm × 1 m); column temperature, 160 °C; N_2 flow rate, 50 ml/min] and on HPLC [Zorbax ODS, MeOH–H_2O (10 : 1)].

5: A white powder, $[\alpha]_D$ −8° (c=0.7, H_2O, 25 °C). ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: Table I. ^{13}C-NMR (22.5 MHz, CD_3OD–D_2O=1 : 2) δ_C: Table II. FAB-MS m/z: 277 $(M+Na)^+$.

Methyl 6,9,12,15-octadecatetraenoate (**8**): A colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.40 (8H, m, olefinic H), 3.70 (3H, s, OMe), 2.85 (6H, m, allylic H), 2.32 (2H, t, J=7.5 Hz, H_2), 2.10 (4H, m, H_5, H_{17}), 1.65 (2H, tt, J=7.5, 8.8 Hz, H_3), 1.45 (2H, tt, J=8.8, 8.8 Hz, H_4), 1.00 (3H, t, J=7.5 Hz, H_{18}). ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 174.2 (s, C_1), 51.6 (q, OMe), 34.1 (t, C_2), 24.9 (t, C_3), 20.6 (t, C_{17}), 14.4 (q, C_{18}). MS m/z: 290 (M^+).

Alkaline Treatment of Heterosigma-glycolipid II (2) A solution of **2** (30 mg) in MeOH (2 ml) was treated with 2.8% NaOMe–MeOH (2 ml) and the whole was stirred at 20 °C for 30 min. The reaction mixture was neutralized with Dowex 50W × 8 (H^+ form) and the whole solution was then partitioned into an n-hexane–MeOH mixture. The MeOH phase was separated and treated as described above for the alkaline treatment of **1** and the product was purified by SiO_2 column chromatography ($CHCl_3$–MeOH) to furnish **5** (8 mg). Concentration of the n-hexane phase gave a mixture of fatty acid methyl esters, which was purified by HPLC [Zorbax ODS, MeOH–H_2O (10 : 1)] to furnish methyl 6,9,12,15-octadecatetraenoate (**8**, 6 mg) and methyl 5,8,11,14,17-eicosapentaenoate (**9**, 7 mg).

Methyl 5,8,11,14,17-Eicosapentaenoate (**9**): A colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.40 (10H, m, olefinic H), 3.70 (3H, s, OMe), 2.85 (8H, m, allylic H), 2.35 (2H, t, J=7.7 Hz, H_2), 2.15 (4H, m, H_4, H_{19}), 1.70 (2H, tt, J=7.7, 8.6 Hz, H_3), 1.00 (3H, t, J=7.5 Hz, H_{20}). ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 174.1 (s, C_1), 51.5 (q, OMe), 33.5 (t, C_2), 24.9 (t, C_3), 20.6 (t, C_{19}), 14.4 (q, C_{20}). MS m/z: 316 (M^+).

LiAlH(O-*tert*-Bu)$_3$ Reduction of 2 A solution of **2** (10 mg) in benzene (2 ml) was treated with LiAlH(O-*tert*-Bu)$_3$ (25 mg) and then the whole was stirred at 40 °C for 2 h under an N_2 atmosphere. After filtration, the filtrate was partitioned into an AcOEt–H_2O mixture. The AcOEt-soluble portion was washed with brine, dried over $MgSO_4$ and then evaporated under reduced pressure. The residue thus obtained was purified by SiO_2 column chromatography ($CHCl_3$–MeOH) to furnish **6** (4 mg).

6: A colorless oil. ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: as given in Table I and 5.44 (m, olefinic H), 0.87 (t, J=7.5 Hz, Me).

Alkaline Treatment of 6 Compound **6** (2 mg) was treated with NaOMe–MeOH under the same conditions as described for the alkaline treatment of **2**. The resulting n-hexane-soluble portion was analyzed by GLC to identify **8** and **9** in 2 : 1 peak ratio.

Enzymatic Hydrolysis of Heterosigma-glycolipid II (2) A solution of **2** (10 mg) in dioxane–water (1 : 1) (2 ml) was treated with lipase type XIII (from *Pseudomonas* sp., lot 67F-0961, Sigma) (3 mg) and the whole was stirred at 37 °C for 4 h. After evaporation of the solvent under reduced pressure, the residue was partitioned into a mixture of n-hexane–MeOH. The n-hexane phase was concentrated under reduced pressure and the resulting residue was treated with ethereal CH_2N_2 to yield **9** (3 mg). Compound **9** was found to be identical with that obtained above by ^{1}H-NMR, GLC, and HPLC comparisons. The MeOH-soluble portion was concentrated under reduced pressure to give a product, which was purified by SiO_2 column chromatography ($CHCl_3$–MeOH, 10 : 1) to afford **7** (4 mg).

7: A colorless oil. ^{1}H-NMR (500 MHz, C_5D_5N containing 1 drop of D_2O) δ: as given in Table I and 5.44 (8H, m, olefinic H), 0.87 (3H, t, J=7.6 Hz, Me). FAB-MS m/z: 535 $(M+Na)^+$.

Alkaline Treatment of 7 Compound **7** (2 mg) was treated with NaOMe–MeOH under the same conditions as described for the alkaline treatment of **2**. The resulting n-hexane-soluble portion was analyzed by GLC to identify **8** as a single product.

Alkaline Treatment of Heterosigma-glycolipid III (3) Compound **3** (14 mg) was treated with NaOMe–MeOH under the same conditions as described above in the case of **2**. From the MeOH-soluble portion, **5** (5 mg) was obtained. The n-hexane-soluble portion was purified by HPLC to furnish methyl 6,9,12,15-octadecatetraenoate (**8**, 4 mg) and methyl 3-methoxy-6,9,12,15-octadecatetraenoate (**10**, 3 mg).

10: A colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.37 (8H, m, olefinic H), 3.69 (3H, s, C_1-OMe), 3.65 (1H, m, H_3), 3.35 (3H, s, C_3-OMe), 2.56 (1H, dd, J=15.3, 7.0 Hz, H_{2a}), 2.43 (1H, dd, J=15.3, 5.8 Hz, H_{2b}), 2.14 (2H, m, H_5), 2.07 (2H, m, H_{17}), 1.62 (2H, m, H_4), 0.98 (3H, t, J=7.6 Hz, H_{18}). ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 172.2 (s, C_1), 77.3 (d, C_3), 57.1 (q, C_3-OMe), 51.7 (q, C_1-OMe), 14.4 (q, C_{18}). MS m/z: 320 (M^+).

Enzymatic Hydrolysis of Heterosigma-glycolipid III (3) A solution of **3** (18 mg) in dioxane–water (1 : 1) (2 ml) was treated with lipase type XIII (5 mg) and the whole was stirred at 37 °C for 4 h. After evaporation of the solvent under reduced pressure, the whole was partitioned into a mixture of n-hexane–MeOH. The n-hexane phase was concentrated under reduced pressure and the resulting residue was treated with ethereal CH_2N_2 to give methyl 3,6,9,12,15-octadecapentaenoate (**11**, 5 mg). The MeOH-soluble portion was purified by SiO_2 column chromatography to give **7** (12 mg).

Methyl 3,6,9,12,15-Octadecapentaenoate (**11**): A colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.58 (2H, m, H_3, H_4), 5.38 (8H, m, olefinic H), 3.69 (3H, s, OMe), 3.13 (2H, d, J=5.8 Hz, H_2), 2.84 (8H, m, allylic H), 2.08 (2H, m, H_{17}), 0.98 (3H, t, J=7.6 Hz, H_{18}). ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 172.3 (s, C_1), 51.9 (q, OMe), 14.3 (q, C_{18}). MS m/z: 288 (M^+).

Alkaline Treatment of 4 Compound **4** (40 mg) was treated with NaOMe–MeOH under the same conditions as described for the alkaline treatment of **2**. The resulting n-hexane-soluble portion was purified by HPLC to furnish **7** (8 mg) and **8** (10 mg). The MeOH-soluble portion was purified by SiO_2 column chromatography ($CHCl_3$–MeOH) to furnish **12** (18 mg).

12: A white powder, $[\alpha]_D$ +81° (c=0.6, H_2O, 25 °C). ^{1}H-NMR (500 MHz, C_5D_5N) δ: 5.50 (1H, d, J=3.7 Hz, $H_{1''}$), 4.82 (1H, d, J=7.6 Hz, H_1), 4.68 (1H, dd, J=3.7, 10.1 Hz, $H_{2''}$), 4.61 (1H, dd, J=10.1, 3.7 Hz, $H_{3''}$), 4.53 (2H, m, H_4, $H_{1'a}$), 4.46 (1H, m, H_2), 4.40 (2H, m, $H_{4''}$, $H_{2'}$), 4.28 (1H, m, $H_{1'b}$), 4.24 (2H, m, $H_{5''}$, H_5), 4.14 (1H, m, H_3), 4.10 (6H, m, $H_{6''}$, H_6, $H_{3'}$). ^{13}C-NMR (125 MHz, C_5D_5N) δ_C: 105.7 (d, C_1), 101.0 (d, $C_{1''}$), 75.1 (d, C_3), 74.5 (d, C_5), 73.0 (d, $C_{5''}$), 72.7 (d, C_2), 72.4 (d, $C_{3''}$), 72.1 (t, C_6), 71.6 (d, $C_{4''}$), 70.8 (d, $C_{2'}$), 70.6 (d, $C_{2''}$), 70.0 (t, $C_{1'}$), 68.2 (d, C_4), 64.3 (t, $C_{3'}$), 62.3 (t, $C_{6''}$).

Enzymatic Hydrolysis of 4 A solution of **4** (10 mg) in dioxane–water (1 : 1) (3 ml) was treated with lipase type XIII (5 mg) and the whole was stirred at 37 °C for 24 h. After evaporation of the solvent under reduced pressure, the residue was partitioned into a mixture of n-hexane–MeOH. The n-hexane-soluble portion was concentrated under reduced pressure and the residue obtained was treated with ethereal CH_2N_2 to give **8** (2 mg). The MeOH-soluble portion was concentrated under reduced pressure to give a product, which was purified by SiO_2 column chromatography ($CHCl_3$–MeOH–H_2O) to give **13** (5 mg).

13: A colorless oil. ^{1}H-NMR (500 MHz, C_5D_5N) δ: 5.51 (1H, d, J=3.7 Hz, $H_{1''}$), 4.81 (1H, d, J=7.6 Hz, H_1), 4.67 (1H, dd, J=3.7, 10.1 Hz, $H_{2''}$), 4.58 (m), 4.53—4.32 (m), 4.23—4.10 (m), 4.04 (2H, dd, J=5.3, 10.4 Hz, $H_{3'}$), 2.97—2.91 (m), 2.38—2.06 (m), 1.65—1.26 (m), 0.91 (3H,

t, $J=7.6$ Hz, Me). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: 173.4 (s, CO), 105.6 (d, C_1), 101.3 (d, $C_{1''}$), 75.0 (d, C_3), 74.5 (d, C_5), 72.7 (d, $C_{5''}$), 72.1 (d, C_2), 71.6 (d, $C_{2'}$), 71.5 (t, C_6), 70.9 (d, $C_{4''}$), 70.6 (d, $C_{3''}$), 69.9 (d, $C_{2''}$), 68.2 (d, C_4), 68.2 (t, $C_{1'}$), 62.4 (t, $C_{6''}$), 61.2 (t, $C_{3'}$), 14.1 (q, Me). FAB-MS m/z: 723 $(M+Na)^+$.

Alkaline Treatment of 13 Compound **13** (2 mg) was treated with NaOMe–MeOH under the same conditions as described above for the alkaline treatment of **4**. The resulting *n*-hexane-soluble portion was analyzed by GLC to identify **9** as a single product.

Alkaline Treatment of 6-Sulfoquinovosyl Diacylglycerol Fraction The 6-sulfoquinovosyl diacylglycerol fraction (20 mg) was treated with NaOMe–MeOH under the same conditions as described above. The resulting *n*-hexane-soluble portion was analyzed by FAB-MS to identify methyl palmitate (16 : 0) and methyl hexadecaenoate (16 : 1) in a 5 : 3 ratio. The MeOH-soluble portion was purified by SiO_2 column chromatography to furnish glycerol α-D-6-sulfoquinovopyranoside, which was identical with the authentic compound[13] obtained from the sea urchin *Anthocidaris crassispina* by ^{1}H-NMR, ^{13}C-NMR, and $[\alpha]_D$ comparisons.

HPLC Analysis of Heterosigma-glycolipids The culture (1 l) of *H. akashiwo* was sonicated for 10 min and then extracted with AcOEt (1 l) 3 times. The AcOEt-soluble portion was evaporated under reduced pressure to give a residue, which was purified by column chromatography [SiO_2 20 g, $CHCl_3$–MeOH (20 : 1)] to furnish a monogalactosyl diacylglycerol fraction. The monogalactosyl diacylglycerol fraction was analyzed by HPLC [Zorbax ODS, 9.4 × 250 mm; MeOH–H_2O (20 : 1); detected by RID-6A (Shimadzu)]; peak area was calculated by means of a Chromatopac C-R6A (Shimadzu).

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