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A MONOGALACTOPYRANOSYL ACYLGLYCEROL FROM *OLTMANNSIELLOPSIS UNICELLULARIS* (NIES-359)

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Key Word Index—*Oltmannsiellopsis unicellularis*; Chlorophyceae; marine alga; glycolipid; monogalactopyranosyl acylglycerol; platelet aggregation.

Abstract—(2*S*)-1-*O*-Hexadeca-4″,7″,10″,13″-tetraenoyl-3-*O*-β-D-galactopyranosyl glycerol, isolated from the marine alga *Oltmannsiellopsis unicellularis* (NIES-359), inhibited platelet aggregation induced by U46619, a thromboxane A_2 analogue. Its structure was elucidated by spectroscopic analysis and chemical evidence.

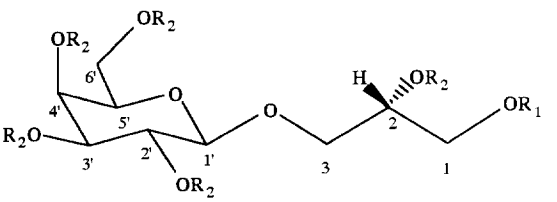
INTRODUCTION

Marine microorganisms such as blue–green algae and dinoflagellates [1, 2] have been reported as valuable new sources of pharmacologically active compounds [3–5]. However, their metabolites have not been studied extensively because of difficulties in the isolation and cultivation of these microorganisms [6].

As part of our studies on bioactive compounds from marine microorganisms, we have found that some show powerful excitable effects on platelet and actomyosin system [3–5]. In this paper, we report the successful cultivation of *Oltmannsiellopsis unicellularis* and the isolation of a new monogalactosyl acylglycerol possessing platelet aggregation inhibitory activity.

RESULTS AND DISCUSSION

The Chlorophyceae *O. unicellularis* was mass-cultured in enriched seawater medium (ESM) at 25° under illumination on a 16L-8D cycle in our laboratory. From the *n*-hexane solubles of the MeOH extracts of the harvested cells, a metabolite was isolated by repeated silica gel column chromatography and by preparative HPLC, based on platelet aggregation inhibitory activity. The active compound (**1**) showed a quasi-molecular ion peak at *m/z* 507 $[C_{25}H_{40}O_9Na]^+$ in its FAB-mass spectrum. Its IR spectrum displayed absorption bands at 3390 and 1736 cm^{-1}, indicating the presence of OH and ester functionalities. In its ^1H NMR spectrum, signals were observed corresponding to a methyl triple at δ 1.01 (3H, *t*, *J* = 7.3 Hz), numerous oxymethylenes and oxymethyne hydrogens between δ 3.5 and 4.3, methylenic hydrogens between δ 2.1 and 2.9, and olefinic hydrogens between δ 5.3 and 5.5; these spectral features are characteristic for glycolipids bearing an unsaturated fatty acid. A signal at δ 4.26 (1H, *d*, *J* = 7.7 Hz) indicated the presence of a β-glycosidic linkage [7]. Analysis of the ^1H–^1H COSY spectrum of **1** permitted the assignment of all the ^1H signals for the sugar and glycerol moieties. The presence of a significant IR absorption at 715 cm^{-1} and the absence of any at 965 cm^{-1} indicated that all the double bonds of the hexadeca-4″,7″,10″,13″-tetraenoic acid unit in **1** had a *cis*-geometry [7, 8]. Alkaline hydrolysis of **1** with NaOMe in MeOH yielded methyl hexadeca-4″,7″,10″,13″-tetraenoate from the *n*-hexane solubles. Acetylation of **1** with Ac_2O in pyridine yielded **2**. In the ^1H NMR spectrum of **2**, the H-2 proton appeared at δ 5.20, indicating that compound was acylated at



1 R_1 = hexadeca-4″,7″,10″,13″-tetraenoyl R_2 = H

2 R_1 = hexadeca-4″,7″,10″,13″-tetraenoyl R_2 = Ac

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Table 1. ^{1}H NMR chemical shifts and coupling constants of glycerol part of the (2*R*)-, (2*S*)-1-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerol and (2*S*)-1-*O*-hexadeca-4″, 7″, 10″, 13″-tetraenoyl-3-*O*-β-D-galactopyranosyl glycerol (**1**) in CD_3OD

	Chemical shift values (ppm)		
^{1}H	(2*R*)-AGG*	(2*S*)-AGG†	**1**
H-1a (*J* = Hz)	4.08 (11.6, 6.4)	4.18 (12.0, 6.0)	4.18 (11.0, 5.9)
H-1b (*J* = Hz)	4.16 (11.6, 4.0)	4.19 (12.0, 5.0)	4.21 (11.0, 4.8)
H-2	4.0	3.99	4.02
H-3a (*J* = Hz)	3.57 (10.0, 6.5)	3.65 (11.0, 4.5)	3.69 (10.6, 4.4)
H-3b (*J* = Hz)	3.93 (10.0, 4.3)	3.94 (11.0, 5.2)	3.95 (10.5, 5.1)

*(2*R*)-1-*O*-Acyl-3-*O*-β-D-galactopyranosyl glycerol.
†(2*S*)-1-*O*-Acyl-3-*O*-β-D-galactopyranosyl glycerol.

C-1. Uzawa *et al.* reported that ^{1}H NMR chemical shifts and coupling constants in CD_3OD were significantly different between (2*R*)- and (2*S*)-1-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerol regardless of acyl substituents (Table 1) [9]. Typically, in the 2*S* form, the chemical shifts of the H-1 methylene protons are very close (δ 4.18 and 4.19) but not in the 2*R* (δ 4.08 and 4.19). The value of the coupling constant $J_{2,3a}$ (4.5 Hz) is smaller than the value of the $J_{2,3b}$ (5.2 Hz) for the (2*S*)-form, while the reverse is true for the (2*R*)-form. The ^{1}H NMR spectrum of **1** was characteristic of the (2*S*)-type and thus **1** was assigned the *S*-configuration (Table 1). The structure of new monogalactosyl acylglycerol was therefore assigned as (2*S*)-1-*O*-hexadeca-4″,7″,10″,13″-tetraenoyl-3-*O*-β-D-galactopyranosyl glycerol (**1**).

The effect of **1** on platelets was examined. It caused a concentration-dependent inhibition of platelet aggregation induced by U46619, a thromboxane A_2 analogue, with an IC_{50} value of 1.9×10^{-4} M (Fig. 1).

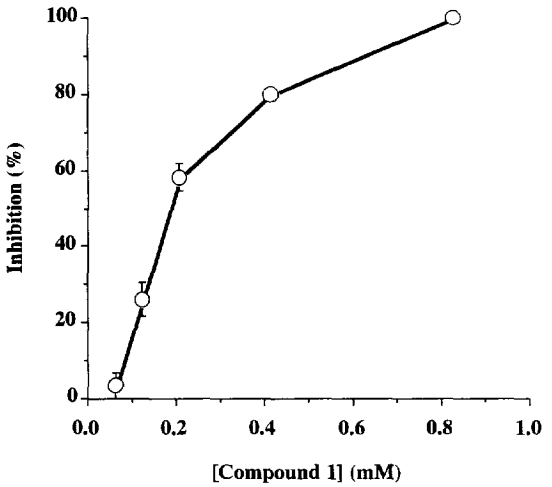

Fig. 1. Inhibitory effects of (2*S*)-1-*O*-hexadeca-4″,7″,10″,13″-tetraenoyl-3-*O*-β-D-galactopyranosyl glycerol on U46619 (3 μM)-induced platelet aggregation. Samples were added 5 min before addition of U46619 in the presence of 1 mM Ca^{2+}. U46619 (3 μM)-induced platelet aggregation in the presence of 1 mM Ca^{2+} was taken as 100% (control). Values are given as mean standard error (n = 3).

Compound **1** (100 μg/ml^{-1}) only markedly inhibited platelet aggregation induced by U46619, but not that induced by thrombin (0.25 unit ml^{-1}) or ionomycin (5 μM). In addition, **1** (30 μg/ml^{-1}) exhibited cytotoxic activity against P388 cell culture.

EXPERIMENTAL

General experimental procedures. The optical rotation was measured on a Jasco DIP-360 digital polarimeter, and the IR spectrum was recorded on a Shimadzu IR-408 spectrometer. The FAB-MS or EI-MS was obtained on a JEOL JMS AX-500 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM GX-500 spectrometer.

Cultivation. The *O. unicellularis* (NIES-359) was supplied by the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Environmental Agency, Japan). Uni-algal cultures of *O. unicellularis* were grown in 3 l glass bottles containing 2 l of sea water medium enriched with modified ESM supplement [10] which consisted of the following elements in 1 l of sea-water: $NaNO_3$, 120 mg; K_2HPO_4, 5 mg; Fe-EDTA, 259 μg; Mn-EDTA, 332 μg; vitamin B_{12}, 1 μg; thiamin hydrochloride (vitamin B_1), 100 μg; D-biotin (vitamin H), 1 μg; tris(hydroxymethyl)aminomethane, 1 g. The pH of the supplement was adjusted to 8.0 with 6 N HCl, prior to mixing with sea-water, which was sterilized by autoclaving. Cultures were incubated statically at 25° in an apparatus where illumination from a fluorescent light source was supplied in a cycle of 16 hr light and 8 hr darkness. After 7 days the cultured cells (pH 8.3) were harvested with a glass filter (GF/F, Whatman) to yield the cells.

Isolation of (2S)-1-O-*hexadeca-4″,7″,10″,13″-tetraenoyl*-3-O-β-D-*galactopyranosyl glycerol* (**1**). The harvested cells (100 g, wet weight) from 1000 l of culture were extracted with MeOH (10 l) to give a MeOH extract which was partitioned with *n*-hexane/H_2O. The *n*-hexane extract was partitioned with *n*-hexane–MeOH. The MeOH extract (2.04 g) was subjected to silica gel column chromatography (Kieselgel 60, Merck) repeatedly eluted with $CHCl_3$–MeOH to give

a monogalactosyl acylglycerol mixture (110 mg). The fr. was sepd by HPLC {column: Senshu Pak Pegasil ODS (250 mm × 20 i.d.); pressure: 900 kgf cm^{-2}; flow rate: 9 ml min^{-1}; solvent: H_2O–MeCN (50:50); detection: UV (215 nm)} to give monogalactosyl acylglycerol (**1**) (16.5 mg) as an amorphous powder, $[\alpha]_D^{23}$ −18.67° (MeOH; *c* 1.6); IR (Film) cm^{-1}: 3390, 1736; FAB MS *m/z*: 507 $[M+Na]^+$; ^1H NMR (500 MHz, CD_3OD); δ 1.01 (3H, *t*, *J* = 7.3 Hz), 2.12 (2H, *m*), 2.44 (4H, *m*), 2.86 (2H, *m*), 2.89 (4H, *m*), 4.26 (1H, *d*, *J* = 7.7 Hz, H-1′), 3.57 (1H, *dd*, *J* = 7.7, 9.5 Hz, H-2′), 3.50 (1H, *dd*, *J* = 3.3, 9.5 Hz, H-3′), 3.85 (1H, *dd*, *J* = 1.2, 3.3 Hz, H-4′), 3.55 (1H, *m*, H-5′), 3.75 (1H, *dd*, *J* = 5.5, 11.0 Hz, H-6′a), 3.79 (1H, *dd*, *J* = 6.7, 11.0 Hz, H-6′b), 4.18 (1H, *dd*, *J* = 5.9, 11.0 Hz, H-1a), 4.21 (1H, *dd*, *J* = 4.8, 11.0 Hz, H-1b), 4.02 (1H, *m*, H-2), 3.69 (1H, *dd*, *J* = 4.4, 10.6 Hz, H-3a), 3.95 (1H, *dd*, *J* = 5.1, 10.6 Hz, H-3b), 5.30–5.46 (8H, *m*); ^{13}C NMR (125 MHz, CD_3OD); δ_c 106.11 (*d*, C-1′), 73.37 (*d*, C-2′), 75.65 (*d*, C-3′), 71.08 (*d*, C-4′), 77.57 (*d*, C-5′), 63.28 (*t*, C-6′), 67.47 (*t*, C-1), 70.42 (*d*, C-2), 72.69 (*t*, C-3), 175.54 (*s*, C-1″), 35.73 (*t*, C-2″), 24.54 (*t*, C-3″), 128.99 (*d*), 129.71 (*d*), 129.86 (*d*), 129.88 (*d*), 130.04 (*d*), 130.28 (*d*), 131.10 (*d*), 133.61 (*d*), 27.21 (*t*), 27.29 (*t*), 27.31 (*t*), 22.28 (*t*, C-15″), 15.45 (*q*, C-16″).

Alkaline hydrolysis of **1**. A solution of **1** (5.0 mg) in MeOH (0.5 ml) was treated with 3% NaOMe–MeOH (0.5 ml) at room temp for 20 min. The reaction mixt. was neutralized with Dowex 50W (H^+ form) and partitioned between *n*-hexane and MeOH. The *n*-hexane solubles were evapd at red. pres. to give methyl hexadeca-4″,7″,10″,13″-tetraenoate as an oil, EI-MS; *m/z* 262 $[M]^+$.

Acetylation of **1**. A 1.0 mg portion of **1** dissolved in pyridine (0.5 ml) was treated with acetic anhydride (0.5 ml) at room temperature for 12 hr. The reaction mixt. was evapd at red. pres. to give 2,2′,3′,4′,6′-pentaacetyl-(2*S*)-1-*O*-hexadeca-4″,7″,10″,13″-tetraenoyl-3-*O*-β-D-galactopyranosyl glycerol (**2**): FAB-MS *m/z*: 717 $[M+Na]^+$; ^1H NMR (500 MHz, CD_3OD); δ 4.69 (1H, *d*, *J* = 7.9 Hz, H-1′), 5.11 (1H, *dd*, *J* = 7.9, 10.4 Hz, H-2′), 5.16 (1H, *dd*, *J* = 3.1, 10.4 Hz, H-3′), 5.38–5.43 (1H, *overlap*, H-4′), 4.13–4.19 (4H, *m*, H-5′, H-6′a, H-6′b, H-1a), 4.35 (1H, *dd*, *J* = 3.7, 12.2 Hz, H-1b), 5.21 (1H, *m*, H-2), 3.77 (1H, *dd*, *J* = 5.5, 11.0 Hz, H-3a), 3.96 (1H, *dd*, *J* = 5.5, 11.0 Hz, H-3b).

Pharmacological tests. Washed platelets were prepared by the method of Rho *et al.* [3]. Platelet aggregation was determined by a turbidometric method [11] using an aggregometer (PAM-6C, Merbanix, Tokyo, Japan). Platelet aggregation was expressed as an increase in light transmission. The levels of light transmission were calibrated as 0% for a platelet suspension and 100% for the Tyrode–HEPES solution. Platelet suspension (0.3 ml) in the aggregometer cuvette was preincubated for 5 min at 37° under stirring at 1000 rpm and then $CaCl_2$ was added to 1 mM (final concentration). After 5 min, various concns of samples were added, and 5 min after, U46619 (3.0 μM) was added and platelet aggregation was monitored for 20 min.

Cytotoxicity against P388 cell *in vitro* was performed by the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} colorimetric method [12, 13].

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