A Novel Monogalactosylacylglycerol with Inhibitory Effect on Platelet Aggregation from the Cyanophyceae *Oscillatoria rosea*

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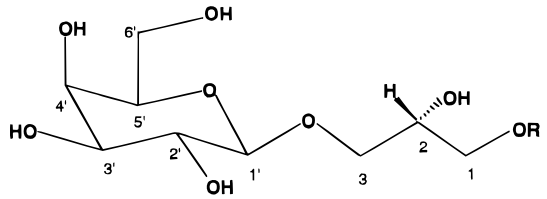
(2*S*)-1-*O*-Palmitoyl-3-*O*-*β*-D-galactopyranosylglycerol, isolated from the marine alga *Oscillatoria rosea*, inhibited platelet aggregation induced by U46619, a thromboxane A_2 analogue. Its structure was elucidated by spectroscopic analysis and chemical evidence.

Though marine microorganisms such as blue-green algae[1] and dinoflagellates[2] have been attractive as a valuable new source of pharmacologically useful compounds,[3−5] their metabolites have not been well studied because of the difficulty in the isolation and cultivation of these microorganisms.[6] As part of our search for pharmacologically active compounds, we have devoted our attention to substances with inhibitory effects on platelet aggregation from marine organisms, since these compounds are useful for basic physiological research and therapeutic applications. In this paper, we report the successful cultivation of *Oscillatoria rosea* (NIES-208) and the isolation of a new monogalactosylacylglycerol possessing platelet aggregation inhibitory activity.

The Cyanophyceae *Oscillatoria rosea* (NIES-208) was mass cultured in ESM medium at 25 °C under illumination on a 16L:8D cycle in our laboratory. From the EtOAc solubles of the MeOH extracts of the harvested cells, a metabolite was isolated by repeated silica gel chromatography and by preparative HPLC directed by platelet aggregation inhibitory activity. The active compound **1** showed a quasimolecular ion peak at *m*/*z* 515 ($C_{25}H_{48}O_9Na$) in FABMS. Its IR spectrum displayed absorption bands at 3400 and 1740 cm^{-1}, indicating the presence of OH and ester functionalities. In its ^1H NMR spectrum, there was a triplet methyl signal at *δ* 0.87 (3H, t, *J* = 7.0 Hz), a mass of oxymethylene and oxymethine hydrogen signals between *δ* 3.4 and 4.3, and methylene hydrogen signals between *δ* 1.2 and 1.6. No signals for olefinic protons were observed. These spectral features are characteristic for glycolipids bearing saturated fatty acids. A signal at *δ* 4.21 (1H, d, *J* = 7.6 Hz) indicated the presence of a *β*-glycosidic linkage.[7] Analysis of the $^1H-^1H$ COSY spectrum of **1** gave assignment of all the ^1H NMR signals for sugar and glycerol moieties as listed in the Experimental Section. Acid hydrolysis of **1** with 2 M TFA yielded galactose. Alkaline hydrolysis of **1** with NaOMe in MeOH yielded methyl palmitate from the *n*-hexane solubles. In the COLOC (correlation spectroscopy via long-range couplings) spectrum, long-range connectivities were observed between the ester carbonyl carbon (*δ* 175.6) and the H-1 protons (*δ* 4.12 and *δ* 4.13), indicating that the compound is acylated at C-1. If the actual natural product were acylated at C-2 and 1−2 acyl migration occurred during isolation, some 2-*O*-palmitoylgalactopyranosylglycerol might be observed, but this compound was not obtained. Uzawa *et al.* reported that ^1H 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-galactopyranosylglycerol regardless of acyl substituents (Table 1).[8] Typically, in the 2*S*, the chemical shifts of 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). In the 2*S*, the coupling constant value between H-2 proton and H-3a (4.5 Hz) is smaller than H-3b (5.2 Hz), but in the 2*R*, H-3a (6.5 Hz) is larger than H-3b (4.3 Hz). The ^1H NMR spectrum of **1** was characteristic of the 2*S* type, and thus, **1** was assigned the *S*-configuration. The structure of the new monogalactosylacylglycerol was therefore assigned as (2*S*)-1-*O*-palmitoyl-3-*O*-*β*-D-galactopyranosylglycerol (**1**).



R = palmitoyl

1

The effects of **1** on platelets were examined, and it caused a concentration-dependent inhibition of platelet aggregation induced by U46619, a thromboxane A_2 analogue, with an IC_{50} value of 6.0×10^{-5} M. We examined the effects of **1** on U46619- (3 *μ*M), thrombin- (0.25 unit/mL), or ionomycin-induced (5 *μ*M) platelet aggregation compared with lysophosphatidylcholine (lyso-PC), which is well known as a detergent. Lyso-PC (100 *μ*g/mL) completely inhibited U46619-, thrombin-, and ionomycin-induced platelet aggregation. However, **1** (100 *μ*g/mL) only markedly inhibited platelet aggregation induced by U46619 and not that induced by thrombin or ionomycin. It is therefore unlikely that the effect of **1** on platelets is due to detergent action. In addition, **1** (>50 *μ*g/mL) exhibited cytotoxic activity against P388 cell culture.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a JASCO DIP-360 digital polarimeter, and the IR spectrum was taken on a Shimadzu IR-408 spectrometer. The FABMS or EIMS spectra were obtained on a JEOL JMS AX-500 spec-

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Table 1. ^{1}H NMR Chemical Shifts and Coupling Constants of the Glycerol Part of the (2*R*)- and (2*S*)-1-*O*-Acyl-3-*O*-*β*-D-galactopyranosylglycerol and (2*S*)-1-*O*-Palmitoyl-3-*O*-*β*-D-galactopyranosylglycerol (**1**) in CD_3OD

	chemical shift values (ppm)		
^{1}H (*J*, Hz)	(2*R*)-AGG[a]	(2*S*)-AGG[b]	**1**
H-1a	4.08 (11.6, 6.4)	4.18 (12.0, 6.0)	4.12 (11.0, 6.0)
H-1b	4.19 (11.6, 4.0)	4.19 (12.0, 5.0)	4.13 (11.0, 5.0)
H-2	4.0	3.99	3.97
H-3a	3.57 (10.0, 6.5)	3.65 (11.0, 4.5)	3.63 (10.2, 4.0)
H-3b	3.93 (10.0, 4.3)	3.94 (11.0, 5.2)	3.90 (10.2, 5.0)

[a] (2*R*)-1-*O*-Acyl-3-*O*-*β*-D-galactopyranosylglycerol. [b] (2*S*)-1-*O*-Acyl-3-*O*-*β*-D-galactopyranosylglycerol.

trometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer (600 MHz for ^{1}H NMR) using TMS as an internal standard.

Cultivation. The Cyanophyceae *O. rosea* (NIES-208) was supplied by the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Environmental Agency, Japan). Unialgal cultures of *O. rosea* were grown in 3 L glass bottles containing 2 L of sea water medium enriched with modified ESM supplement[9] 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; thiamine 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 3 N HCl, prior to mixing with sea water which was sterilized by autoclaving. Cultures were incubated statically at 25 °C in an apparatus where illumination from a fluorescent light source was supplied in a cycle of 16 h of light and 8 h of darkness. After 10−14 days the cultured cells (pH 8.1) were harvested with a glass filter (GF/F, Whatmann) to yield the cells.

Isolation of (2*S*)-1-*O*-Palmitoyl-3-*O*-*β*-D-galactopyranosylglycerol (1). The harvested cells (600 g, wet weight) from 500 L of culture were extracted with MeOH (500 mL × 3) to give a MeOH extract which was partitioned with EtOAc/H_2O. EtOAc solubles (5.6 g) were subjected to silica gel column chromatography (Kieselgel 60, Merck) eluted with $CHCl_3$−MeOH to give a monogalactosylacylglycerol mixture (650 mg). The fraction was separated by HPLC [column: Senshu Pak Pegasil ODS (2.0 ϕ × 250 mm); flow rate: 9 mL/min; solvent: H_2O−MeCN (25:75); detection: UV (215 nm)] to give a monogalactosyl acylglycerol (**1**) (24.0 mg): an amorphous powder; $[\alpha]^{31}_D$ +9.25° (*c* = 0.8, MeOH); IR (film) cm^{-1} 3400, 1740; FABMS *m*/*z* 515 $[M + Na]^+$; ^{1}H NMR (600 MHz, CD_3OD) δ 0.87 (3H, t, *J* = 7.0 Hz), 1.25−1.29 (24H, m), 1.60 (2H, m), 2.33 (2H, t, *J* = 7.2 Hz), δ 4.21 (1H, d, *J* = 7.6 Hz, H-1′), 3.54 (1H, dd, *J* = 10.0, 7.6 Hz, H-2′), 3.46 (1H, dd, *J* = 10.0, 3.3 Hz, H-3′), 3.82 (1H, dd, *J* = 3.3, 1.0 Hz, H-4′), 3.49 (1H, ddd, *J* = 6.9, 5.4, 1.0 Hz, H-5′), 3.71 (1H, dd, *J* = 11.4, 5.4 Hz, H-6′), 3.75 (1H, dd, *J* = 11.4, 6.9 Hz, H-6′), 4.12 (1H, dd, *J* = 11.0, 6.0 Hz, H-1), 4.13 (1H, dd, *J* = 11.0, 5.0 Hz, H-1), 3.97 (1H, m, H-2), 3.63 (1H, dd, *J* = 10.2, 4.0 Hz, H-3), 3.90 (1H, dd, *J* = 10.2, 5.0 Hz, H-3).

Acid Hydrolysis of 1. A 2 mg portion of **1** dissolved in a sealed vial in 2 M TFA at 110 °C for 4 h yielded galactose which was identified by TLC on silica gel using *n*-BuOH−Me_2CO−H_2O (4:5:1; saturated chamber).

Alkaline Hydrolysis of 1. A solution of **1** (5 mg) in MeOH (0.5 mL) was treated with 3% NaOMe/MeOH (0.5 mL) at room temperature for 20 min. The reaction mixture was neutralized with Dowex 50W (H^+ form) and partitioned between *n*-hexane and MeOH. The *n*-hexane solubles were evaporated at reduced pressure to give methyl palmitate as a colorless oil, which showed a single peak on GC (SS-4, 40 m; column temperature, 185 °C; N_2 flow rate, 1.4 mL/min), EIMS *m*/*z* 270 $[M]^+$.

Pharmacological Tests. Washed platelets were prepared by the method of Rho *et al.*,[3] and platelet aggregation was determined by a standard turbidometric method[10] 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 °C under continuous stirring at 1000 rpm, and then $CaCl_2$ was added to a final concentration of 1 mM. After 5 min, various concentrations of samples were added 5 min before addition of U46619 (3.0 μM), and platelet aggregation was monitored for 20 min.

Bioassay of cytotoxic activity against P388 cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetic method.[11,12]

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