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Myrmekiosides A and B, Novel Mono-*O*-alkyl-diglycosylglycerols Reversing Tumor Cell Morphology of *ras*-Transformed Cells from a Marine Sponge of *Myrmekioderma* sp.

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Abstract: Novel mono-*O*-alkyl-diglycosylglycerols named myrmekiosides A (**1**) and B (**2**) were isolated from a marine sponge of *Myrmekioderma* sp. and their absolute stereostructures were elucidated on the basis of chemical and physicochemical evidence. Myrmekiosides A (**1**) and B (**2**) reversed the phenotype of melanoma H-*ras* transformed NIH3T3 cells at 5 μg/ml.

Keywords: Glyceroglycolipid; *ras* Transformed cell; Marine metabolite; Marine sponge

Introduction

Ideal anticancer agents, which show activity against tumor cells but not against normal cells, have been sought for successful cancer chemotherapy. Therefore, it is important to evaluate the potential of an anticancer agent using both tumor cells and normal cells in a screening program. Since *ras* oncogenes have been activated in a wide variety of human tumors,[1] selective inhibitors of the function of *ras* oncogene have been expected to be a new type of anticancer agent. In the course of our study of bioactive substances from marine organisms[2], we focused on a search for selective inhibitors of the function of activated *ras* oncogene and isolated novel mono-*O*-alkyl-diglycosylglycerols named myrmekiosides A (**1**) and B (**2**) from a marine sponge of *Myrmekioderma* sp. This paper describes the elucidation of the absolute stereostructures of myrmekiosides A (**1**) and B (**2**).

Results and Discussion

The acetone extract of the titled frozen sponge collected at Ashizuri Cape, Kochi Prefecture, Japan, was partitioned into an AcOEt-water mixture to provide the AcOEt soluble portion. The water phase was further partitioned with *n*-BuOH to give the *n*-BuOH soluble portion. The AcOEt soluble portion, which exhibited weak cytotoxic activity against KB cells, was subjected to bioassay-guided separation. Repeated SiO_2 column chromatography of the AcOEt soluble portion furnished the known phenolic sesquiterpene, (+)-curcuphenol ($[\alpha]_D$ +26.8) as a cytotoxic substance (IC_{50} = 2 μg/ml against KB cells). (+)-Curcuphenol[3] has been isolated from another marine sponge (*Epipolasis* sp.) as a H^+-K^+ ATPase inhibitor, and also (-)-curcuphenol,[4] an epimer at C-7, was also isolated as an antibacterial substance from a gorgonian soft coral. We further investigated the

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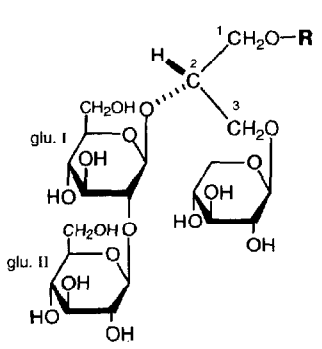
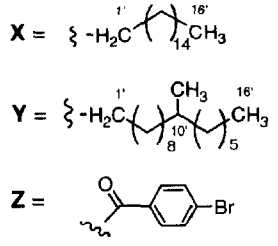
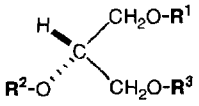


myrmekioside A (1) : R = X

myrmekioside B (2) : R = Y





	R¹	R²	R³
3 :	X	H	H
4 :	X	Z	Z
5 :	Y	H	H
6 :	Y	Z	Z

chemical constituents of the *n*-BuOH soluble portion, which showed the reversing activity of the phenotype of melanoma H-*ras* transformed NIH3T3 cells. The *n*-BuOH soluble portion was subjected to repeated SiO_2 column chromatography and ODS HPLC to furnish active substances, myrmekiosides A (**1**) and B (**2**).

The positive ion FAB MS of myrmekioside A (**1**) gave a quasimolecular ion [(M+Na)$^+$] peak at *m/z* 795 and the molecular formula was determined as $C_{36}H_{68}O_{17}$ by HR-positive ion FAB MS. The IR spectrum of **1** showed a strong absorption band due to polyhydroxyl groups (3418 cm^{-1}). The ^{1}H-NMR spectrum of **1** showed a triplet methyl signal (δ 0.87, 3H, *J*= 6.8 Hz), long alkyl chain signals (δ ca 1.3, ca 26H), many oxylmethine and oxylmethylene signals (δ 3.0 - 4.5), and three doublet signals (δ 5.20, *J*=7.3 Hz; δ 5.24, *J*=7.5 Hz; δ 4.82, *J*=7.3 Hz) which were assignable to anomeric protons of the sugar moieties. The ^{13}C-NMR and DEPT spectra of **1** also disclosed the presence of three anomeric carbons (each δc 102.3, 105.9, 104.9), fifteen oxylmethine carbons and six oxylmethylene carbons. All the proton and carbon signals of **1** were assigned by 2D-NMR (COSY, HMQC, HOHAHA) analysis of **1** (Table 1), and five partial structures [an alkyl ether (A), a glycerol portion (B), two hexoses (C, D) , and a pentose (E)] were elucidated (Fig. 1). Methanolysis of **1** furnished two moles of glucose and one mole of xylose as saccharide moieties, which were confirmed by GLC-analysis, and 1-*O*-alkylglycerol **3**. The absolute stereostructure of each sugar was determined as *D*-form by the HPLC analysis of thiazolidine derivatives[5] which were obtained by condensation of the aqueous acidic hydrolysate of **1** with *L*-cysteine methyl ester. The positive ion FAB MS of 1-*O*-alkylglycerol **3** gave a quasimolecular ion [(M+Li)$^+$] peak at *m/z* 323 ($C_{19}H_{40}O_3Li$ from HR-positive ion FAB MS) which was shown to have a 16-carbon chain (= 1-*O*-hexadecylglycerol). Adjacencies among five partial structures were revealed by the HMBC spectrum of **1**. An alkyl chain moiety (A), glucose (C), and xylose (E) were attached to C-1, C-2, and C-3 of glycerol (B), respectively, and glucose (D) attached to C-2 of glucose (C) (Fig. 1). The glycosidation shifts observed for C-2 and C-1 signals of glucose (C) also supported the connectivity between glucose (C) and glucose (D). The coupling constant of the anomeric proton in each sugar indicated the β-glycosidic linkage. In order to determine the absolute configuration at C-2 in the glycerol portion, the benzoate chirality method[6] was applied to 1-*O*-hexadecyl-2,3-di-*O*-*p*-bromobenzoyl-*sn*-glycerol **4** which was prepared

Table 1 ^{1}H- and ^{13}C-NMR Data for Myrmekioside A (**1**) and Myrmekioside B (**2**). (500 MHz in C_5D_5N)

myrmekioside A (**1**)			myrmekioside B (**2**)		
No.	^{13}C δc (mult.)	^{1}H δ (mult., *J* (Hz))	No.	^{13}C δc	^{1}H δ (mult., *J* (Hz))
glycerol			glycerol		
1	70.8 (t)	4.05 (2H, m)	1	70.8 (t)	4.05 (2H, m)
2	77.6 (d)	4.51 (quintet-like, 4.9)	2	77.5 (d)	4.50 (quintet-like, 4.9)
3	69.8 (t)	4.17 (m)	3	69.8 (t)	4.16 (m)
		4.43 (m)			4.43 (m)
glu. I			glu. I		
1	102.3 (d)	5.20 (d, 7.3)	1	102.3 (d)	5.21 (d, 7.3)
2	83.6 (d)	4.07 (m)	2	83.6 (d)	4.07 (m)
3	77.8 (d)	4.26 (m)	3	77.9 (d)	4.27 (m)
4	71.2 (d)	4.14 (m)	4	71.2 (d)	4.13 (m)
5	78.2 (d)	3.85 (m)	5	78.3 (d)	3.85 (m)
6	62.5 (t)	4.27 (m)	6	62.4 (t)	4.28 (m)
		4.43 (m)			4.43 (m)
glu. II			glu. II		
1	105.9 (d)	5.24 (d, 7.5)	1	105.9 (d)	5.26 (d, 7.5)
2	76.2 (d)	4.08 (m)	2	76.2 (d)	4.09 (m)
3	78.0 (d)	4.18 (m)	3	78.0 (d)	4.17 (m)
4	71.8 (d)	4.15 (m)	4	71.8 (d)	4.15 (m)
5	78.6 (d)	3.98 (m)	5	78.6 (d)	3.97 (m)
6	62.9 (t)	4.33 (m)	6	62.9 (t)	4.33 (m)
		4.54 (dd, 11.5, 2.5)			4.55 (dd, 11.5, 2.5)
xyl			xyl		
1	104.9 (d)	4.82 (d, 7.3)	1	104.9 (d)	4.80 (d, 7.3)
2	74.6 (d)	4.00 (m)	2	74.6 (d)	4.00 (m)
3	77.7 (d)	4.09 (m)	3	77.7 (d)	4.10 (m)
4	70.9 (d)	4.17 (m)	4	70.9 (d)	4.16 (m)
5	66.8 (t)	3.64 (dd, 11.0, 9.8)	5	66.9 (t)	3.63 (dd, 11.0, 9.8)
		4.30 (m)			4.30 (m)
O-alkyl			O-alkyl		
1'	71.8 (t)	3.55 (t, 6.7)	1'	71.8 (t)	3.52 (t, 6.8)
2'	30.1 (t)	1.61 (m)	2'	30.2 (t)	1.60 (m)
3'-14'	26.5-32.1 (12C, t)	1.20-1.40 (24H, m)	3'-8'	23.0-32.2 (6C, t)	1.20-1.40 (12H, m)
15'	22.9 (t)	1.28 (m)	9'	37.4 (t)	1.32 (m)*
16'	14.2 (q)	0.87 (t, 6.8)	10'	33.1 (d)	1.38 (m)
			11'	37.4 (t)	1.10 (m)*
			12'-14'	23.0-32.2 (3C, t)	1.20-1.40 (6H, m)
			15'	22.9 (t)	1.28 (m)
			16'	14.2 (q)	0.87 (t, 6.8)
			10'CH_3	19.9 (q)	0.89 (d, 6.5)

* The assignments are interchangeable.

from **3**. The CD spectrum of **4** gave the CD maxima at 252 nm (Δε= -7.0) and 235 nm (Δε= +1.7) showing a negative exciton couplet CD. Thus, the absolute configuration in the glycerol portion of **4** was determined as 2*R*. Consequently, the chemical structure of myrmekioside A was elucidated to be **1**.

The positive ion FAB MS of myrmekioside B (**2**) gave a quasimolecular ion [$(M+Na)^+$] peak at *m/z* 809 and the molecular formula was determined as $C_{37}H_{70}O_{17}$ by HR-positive ion FAB MS. The ^{1}H- and ^{13}C-NMR spectra of **2** showed closely similar signals with those of **1** except for an additional secondary methyl signal (δ 0.89, 3H, d, *J*= 6.5 Hz; δc 19.9). All the proton and carbon signals were assigned by 2D-NMR (COSY, HMQC, HOHAHA) analysis of **2** (Table 1), and the HMBC spectrum of **2** revealed the plane structure of **2** having a branched alkyl chain. So as to determine the position of the branched methyl in the alkyl moiety,

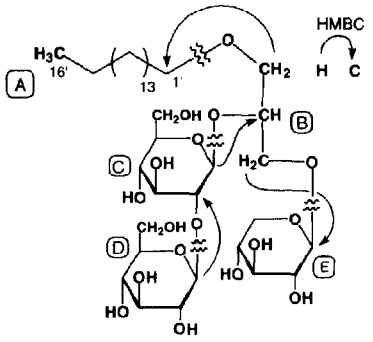


Fig. 1 The HMBC Correlations among Five Partial Structures in **1**

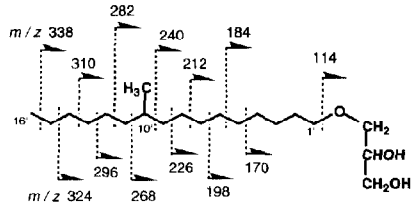


Fig. 2 The MIKES Cleavage Patterns of **5**

MIKES (Mass-analyzed Ion Kinetic Energy Spectrometry)[7] of the quasimolecular ion peak [*m/z* 353 $(M+Na)^+$] of **5**, which was prepared by methanolysis of **2**, was analyzed. As shown in Fig. 2, the characteristic fragmentation pattern indicated that the branched methyl was attached at C-10' in the alkyl chain. The absolute configuration in the glycerol portion was also determined as 2*R* using the same method as in the case of **1**. Consequently, the chemical structure of myrmekioside B was elucidated to be **2**.

Myrmekiosides A (**1**) and B (**2**) altered the tumor cell morphology of H-*ras* transformed NIH3T3 fibroblasts to the normal one of the parental NIH3T3 cells at 5 μg/ml concentration. Furthermore, **1** was found to arrest NIH3T3 cells at G_1 point in the cell cycle. Further mechanistic study is under way.

Experimental Section

Isolation from a marine sponge of *Myrmekioderma sp.* The frozen sponge (3 kg, wet weight) collected in July, 1996 at Ashizuri Cape in Kochi Prefecture was extracted with acetone (5 L) at room temperature 3 times for 12 h each. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt-water mixture (1:1), and the AcOEt layer was evaporated to give the AcOEt-soluble portion (14 g). The remaining water phase was further partitioned into an equal volume of *n*-BuOH, and the *n*-BuOH layer was evaporated to give the *n*-BuOH-soluble portion (8.5 g). The AcOEt-soluble portion showed cytotoxicity with 59 % growth inhibition at 10 μg/ml against KB cells and was subjected to bioassay-guided separation (cytotoxicity against KB cells). The SiO_2 column (eluted with *n*-hexane : AcOEt=40:1 → 10:1 → 5:1 → 2:1 → AcOEt → acetone) gave (+)-curcuphenol (1150 mg, 8.2 % from the AcOEt extract) as a cytotoxic compound (IC_{50} = 2 μg/ml against KB cells).

The *n*-BuOH-soluble portion was also subjected to SiO_2 column (eluted with AcOEt : MeOH : H_2O=40:3:1 → 10:3:1 → 6:3:1 → 3:3:1 → MeOH) to give seven fractions (Fr. A ~ Fr. H). The Fr. D (1.96 g) was separated by SiO_2 column (eluted with $CHCl_3$: MeOH : H_2O=10:3:1 → 7:3:1 → 6:4:1 → MeOH) to give six fractions (Fr. D-1 ~ Fr. D-6). The Fr. D-3 (408 mg) was further separated by Sephadex LH-20 column (eluted

with $CHCl_3$: MeOH = 4:1) to give three fractions (Fr. D-3-1 – Fr. D-3-3). Finally, the Fr. D-3-2 (216 mg) was purified by HPLC (ODS, Mightysil RP-18, Kanto Chemical Co., Inc., 10φ x 250 mm, MeOH:H_2O= 8:1) to give myrmekiosides A (**1**, 23 mg, 0.27 % from *n*-BuOH ext.) and B (**2**, 27 mg, 0.31 %). Myrmekioside A (**1**): amorphous solid. $[\alpha]_D$ -19.8° (*c* = 0.50, MeOH, 22 °C). IR ν max. (KBr): 3418 cm^{-1}. Positive ion FAB MS (nitrobenzyl-alcohol or glycerol): *m/z* 795 $(M+Na)^+$. HR-FAB MS *m/z* : Calcd for $C_{36}H_{68}O_{17}Na$: 795.4364. Found: 759.4391. ^{1}H-NMR (500 MHz, C_5D_5N, δ) and ^{13}C-NMR (125 MHz, C_5D_5N, δc): as shown in Table 1. Myrmekioside B (**2**): amorphous solid. $[\alpha]_D$ -19.5° (*c* = 0.50, MeOH, 22 °C). IR ν max. (KBr): 3356 cm^{-1}. Positive ion FAB MS : *m/z* 809 $(M+Na)^+$. HR-FAB MS *m/z* : Calcd for $C_{37}H_{70}O_{17}Na$: 809.4511. Found: 809.4567. ^{1}H-NMR (C_5D_5N, δ) and ^{13}C-NMR (C_5D_5N, δc): as shown in Table 1.

Methanolysis of myrmekiosides A (1) and B (2). A solution of **1** (2.0 mg) in anhydrous 10% HCl-MeOH (1.0 ml) was heated under reflux for 1.5 h. The reaction mixture was neutralized with Ag_2CO_3 and poured into water and then extracted with AcOEt. The AcOEt layer was concentrated under reduced pressure to yield 1-*O*-alkylglycerol **3** (0.6 mg). A solution of **2** (2.1 mg) in anhydrous 10% HCl-MeOH (1.0 ml) was also treated the same as in the case of **1** to obtain 1-*O*-alkylglycerol **5** (0.7 mg). 1-*O*-Alkylglycerol **3**: colorless oil. IR ν max. (KBr): 3418 cm^{-1}. Positive ion FAB MS : *m/z* 323 $(M+Li)^+$. HR-FAB MS *m/z* : Calcd for $C_{19}H_{40}O_3Li$: 323.3138. Found: 323.3129. 1-*O*-Alkylglycerol **5**: colorless oil. IR ν max. (KBr): 3418 cm^{-1}. Positive ion FAB MS : *m/z* 337 $(M+Li)^+$. HR-FAB MS *m/z* : Calcd for $C_{20}H_{42}O_3Li$: 337.3294. Found: 337.3266.

GLC Analysis of sugar components in 1 and 2. The H_2O layer obtained by methanolysis of **1** or **2** was concentrated under reduced pressure. The residue was dissolved in pyridine (100 μl) and treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (10 μl) at 25 °C for 1 h. The resulting methylglycoside TMS ethers were subjected to GLC analysis. GLC conditions: detection, FID; column, CBP-5 (0.25 mm φ x 25 m); mobile phase, N_2; column temperature, initial 140 °C and +5 °C/min gradient after 5 min. R.t.: methyl glucoside TMS ether = 14.6 and 16.5 min, methyl xyloside TMS ether = 11.1 and 11.5 min. Each R.t. was identical with those of authentic methyl glycoside TMS ethers.

Preparations of 1-*O*-alkyl-2,3-di-*O*-*p*-bromobenzoyl-*sn*-glycerols 4 and 6. A solution of **3** (0.5 mg) in CH_2Cl_2 (0.5 ml) was treated with *p*-bromobenzoic acid (1.0 mg), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI, 1.0 mg), and dimethylaminopyridine (DMAP, 0.5 mg) at 25 °C for 3.5 h under an N_2 atmosphere. The reaction mixture was partitioned into an AcOEt-water mixture and the AcOEt extract was concentrated under reduced pressure to furnish di-*p*-Br-benzoate **4** (0.7 mg). A solution of **5** (0.5 mg) in CH_2Cl_2 (0.5 ml) was similarly treated with *p*-bromobenzoic acid (1.0 mg), EDCI (1.0 mg), and DMAP (0.5 mg) to afford di-*p*-Br-benzoate **6** (0.6 mg). **4**: colorless oil. IR ν max. (KBr): 1764 cm^{-1}. Positive ion FAB MS : *m/z* 705 $(M+Na)^+$. HR-FAB MS *m/z* : Calcd for $C_{33}H_{46}O_5{}^{79}Br^{81}BrNa$: 705.1589. Found: 705.1585. CD (MeOH): 252 nm (Δε= -7.0), 235 nm (Δε= +1.7). **6**: colorless oil. IR ν max. (KBr): 1765 cm^{-1}. Positive ion FAB MS : *m/z* 719 $(M+Na)^+$. HR-FAB MS *m/z* : Calcd for $C_{34}H_{48}O_5{}^{79}Br^{81}BrNa$: 719.1746. Found: 719.1779. CD (MeOH): 252 nm (Δε= -3.5), 235 nm (Δε= +2.2).

HPLC Analysis of sugar components in 1 and 2. A solution of **1** (0.5 mg) in 2*N* aq. HCl (3.0 ml) was heated under reflux for 2 h. The reaction mixture was neutralized with amberlite IRA-400 (OH^- form) and concentrated under reduced pressure after filtration. The residue was dissolved in pyridine (500 μl) and treated with *L*-cysteine methyl ester hydrochloride (1.0 mg) at 60 °C for 1 h. The resulting thiazolidine derivatives were

analyzed by HPLC. HPLC conditions: detection, UV (220 nm); column, Cosmosil $5C_{18}$-AR (4.6 mm ϕ x 250 mm); mobile phase, H_2O; flow rate 1 ml/min. R.t.: methyl 2-(*D*-gluco-pentahydroxypentyl)thiazolidine-4*R*-carboxylate = 15.8 and 29.8 min, methyl 2-(*D*-xylo-tetrahydroxy-butyl)thiazolidine-4*R*-carboxylate = 17.3 and 26.3 min. Each R.t. was identical with that of authentic sample.

Bioassay.

Effect on the morphology of H-*ras* transformed cells; Parental NIH3T3 cells and NIH3T3 cells transformed with human c-H-*ras* gene carrying a point mutation at codon 61 were used for the assay.[8] Both cells were cultured in DMEM supplemented with 0.44 mg/ml of glutamine, 50 μg/ml of kanamycin sulfate, and 10 % newborn calf serum. Equal numbers of cells (2 x 10^3) were inoculated into each well of a 96-well plate with 90 μl of the culture medium, and a testing sample was added to each well as 10 μl of dimethylsulfoxide solution. After 48 h incubation (37 °C, 5 % CO_2), the morphology of cells was observed by phase-contrast microscope.

Cell cycle analysis; NIH3T3 Cells (4 x 10^4) were inoculated into each well of an 8-well plate with 1 ml of the culture medium, and a testing sample was added to each well as 10 μl of dimethylsulfoxide solution. After 48 h incubation (37 °C, 5 % CO_2), the harvested cells with trypsin treatment were washed with PBS, and the nuclei in the cells were stained with DNA-Prep Reagents Kit (Colter Corporation, USA). The distribution of DNA content in the stained cells was analyzed by flow cytometry with FACSCalibur system (Becton Dickinson inc., USA).

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