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A New Screening Method for Antimitotic Substances and Isolation of Glycolipids as Stimulators of Tubulin Polymerization from Okinawan Sponge *Pseudoceratina* sp.

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A new screening method to detect antimitotic substances utilizing purified porcine brain microtubule proteins was developed. This method observes the inhibitory and stimulatory activities on microtubule polymerization and inhibitory activity on depolymerization in sequence. Two glycolipids, 1-*O*-*β*-D-galactopyranosyl-2,3-di-*O*-acylglycerol and 1-*O*-tetrahydroxycyclopentyl-2-*O*-acyl-3-*O*-alkylglycerol were isolated from Okinawan marine sponge *Pseudoceratina* sp. by this screening method. These compounds stimulated the microtubule polymerization at 10°C.

Inhibitors of microtubules are important compounds as antitumor agents[1] and also as biochemical tools to study the structures and functions of microtubules and tubulins[2~4]. We recently reported an effective screening method for antimitotic substances, which observes deformations of mycelia germinated from conidia of *Pyricularia oryzae* P-2b[5]. Phomopsidin, a potent inhibitor of microtubule assembly, and paecilospirone, which possess a unique structure, were obtained utilizing this screening method from marine fungi *Phomopsis* sp. collected in Pohnpei[6] and *Paecilomyces* sp. isolated in Yap[7], respectively.

The above screening method detects the inhibition of microtubule polymerization as well as other antifungal and cytotoxic activities[5,8,9]. We, therefore, developed a new screening method to observe directly the inhibition and stimulation of microtubule polymerization and inhibition of the depolymerization in sequence using purified porcine brain microtubule proteins. Two glycolipids (**1** and **2**, Fig. 1) were obtained from the Okinawan marine sponge *Pseudoceratina* sp. as stimulators of microtubule polymerization by this screening method. We describe here the details of the new screening method and the isolation, characterization, and bioactivity of glycolipids **1** and **2**.

Materials and Methods

Microtubule Proteins

Preparation of microtubule proteins was performed as described previously[9]. In brief, fresh porcine brains were homogenized at 0°C in a buffer solution (100 mM 4-morpholinoethansulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 1 mM 2-mercaptoethanol (2-ME), pH 6.5) and centrifuged at 50,000 *g* at 4°C. A glycerol buffer (8 M glycerol in the above buffer solution, pH 6.5) was added to the supernatant, and the mixture was incubated at 37°C for 30 minutes and centrifuged at 100,000 *g* to afford the precipitate (microtubule). The cycle of depolymerization and polymerization was further performed twice to purify the microtubule proteins. The concentration of proteins was quantified using the Coomassie Protein Assay Kit® (Pierce).

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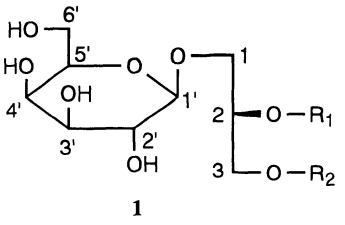
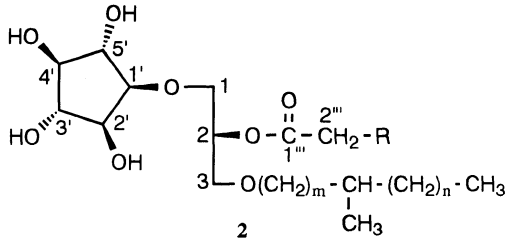
Fig. 1. Structures of 1-*O*-*β*-D-galactopyranosyl-2,3-di-*O*-acylglycerol (**1**) and 1-*O*-tetrahydroxycyclopentyl-2-*O*-acyl-3-*O*-alkylglycerol (**2**) isolated from *Pseudoceratina* sp.



1

R_1, R_2 = myristoyl (14:0), pentadecanoyl (15:0), palmitoyl (16:0), palmitoleoyl (16:1), and oleoyl (18:1) (2:1:30:24:2)



2

m + n = 13 and 14 (1:7)

R = $C_{12}H_{25}$, $C_{13}H_{27}$, $C_{14}H_{29}$, and $C_{15}H_{31}$ (1:13:5:2)

$C_{13}H_{27}$ = four components (11:17:2:1)

$C_{14}H_{29}$ = three components (3:2:1)

Screening Assay (Fig. 2)

Polymerization and depolymerization were observed by the turbidity at 400 nm in a glass UV cell with a Shimadzu model U-3000 spectrophotometer equipped with an electronic temperature controller.

Marine organisms (sponges, tunicates, and soft corals) were collected by scuba diving at Iriomote Island in Okinawa, Japan in 1999 and extracted with CH_2Cl_2-MeOH (1 : 1). The screening assay was performed with the crude extract at 100 ppm. The extract was dissolved in DMSO at the concentration of 5 mg/ml, and 20 μl of the solution was poured in a UV cell and diluted with the suspension of microtubule proteins in the buffer solution (1.3 mg in 1 ml) at 0°C to make the final concentration of 100 ppm. Control experiments were done with 2% DMSO.

Inhibitory and stimulatory activities were determined by the following equations.

$$\text{Stimulatory activity (\%) on polymerization} = \frac{S_{10}}{C_{37}} \times 100 \quad (1)$$

$$\text{Inhibitory activity (\%) on polymerization} = \frac{C_{37} - I_{37}}{C_{37}} \times 100 \quad (2)$$

$$\text{Inhibitory activity (\%) on depolymerization} = \left(1 - \frac{S_{ca}}{S_{37}} \div \frac{C_{ca}}{C_{37}}\right) \times 100 = \left(1 - \frac{S_{ca} \times C_{37}}{S_{37} \times C_{ca}}\right) \times 100 = \frac{S_{37} \times C_{ca} - C_{37} \times S_{ca}}{S_{37} \times C_{ca}} \times 100 \quad (3)$$

S: Test sample showed stimulation of polymerization/inhibition of depolymerization.

I: Test sample showed inhibition of polymerization.

C: Control.

S_{10}: (turbidity at 10°C) − (turbidity at 0°C).

S_{37}, I_{37}, and C_{37}: (turbidity at 37°C) − (turbidity at 0°C).

S_{ca} and C_{ca}: (turbidity at 37°C) − (turbidity after addition of Ca^{2+}).

Test samples were dissolved in DMSO and diluted with the suspension of microtubule proteins in the buffer solution at 0°C in a UV cell. The turbidity was observed immediately after the sample preparation, and the sample was incubated at 10°C for 10 minutes. The stimulation of assembly is detected by the increase in turbidity at 10°C, and the activity (%) to control (0%) is calculated by the equation (1). The sample was then incubated at 37°C for 15 minutes to complete the polymerization of microtubule proteins. The inhibitory activity (%) was calculated by the equation (2). A solution of $CaCl_2$ was added to the sample cell (final concentration of 4 mM) after measurement of turbidity at 37°C to detect the inhibitory activity on microtubule disassembly. The activity (%) to control (0%) was calculated by the equation (3).

Isolation of Glycolipids **1** and **2** from *Pseudoceratina* sp.

Pseudoceratina sp. was collected by scuba diving at −3 m in the coral reef at Iriomote Island, Okinawa. The sponge (355 g) was extracted with CH_2Cl_2-MeOH (1 : 1, 500 ml), and the extract was evaporated to remove organic solvents. The aqueous residue was diluted with water and extracted with EtOAc to give 2.5 g of the organic extract. The EtOAc extract (2.25 g) was chromatographed on silica gel with

$CHCl_3$-MeOH (gradient elution), and the bioactivity was detected in the 10% MeOH-$CHCl_3$ fraction (96.6 mg). The bioactive fraction (66.8 mg) was further separated by an LH-20 column with $CHCl_3$-MeOH (1 : 1) to afford 19.2 mg of bioactive fraction, which was then subjected to silica gel column chromatography with benzene-acetone to afford **1** (3.2 mg) and **2** (1.3 mg). TLC analysis (benzene-acetone, 1 : 1) showed each one spot for **1** (Rf=0.35) and **2** (Rf=0.28). Compound **1**: ESI-MS, *m/z* 751 and 765 $[(M+Na)^+]$; ^1H and ^{13}C NMR data are listed in Table 1. Compound **2**: ESI-MS, *m/z* 695, 709, and 723 $[(M+Na)^+]$; ^1H and ^{13}C NMR data are listed in Table 2.

Spectral Analysis

NMR spectra were measured on a Varian Unity Inova-500 spectrometer in $CDCl_3$. Mass spectra were obtained by a JEOL HX-110 mass spectrometer (FAB mode) or a Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode).

Alkaline Methanolysis of **1**

A solution of **1** (560 μg) in dry MeOH (112 μl) was hydrolyzed with 1 M NaOMe in MeOH (56 μl) at room temperature until the reaction was completed (monitored by TLC). The reaction mixture was treated with ion-exchange resin (Amberite IR-120) and filtered. The filtrate was extracted with hexane, and the extract was concentrated under reduced pressure to yield a mixture of fatty acid methyl esters. The methyl esters of fatty acids were identified by GC-MS.

Alkaline Methanolysis of **2**

Compound **2** (260 μg) was treated with 1 M NaOMe in MeOH (52 μl) as above. The hexane extract of the reaction mixture was subjected to GC-MS to analyze fatty acid methyl esters. The lower layer obtained after hexane extraction of the reaction mixture was separated by TLC ($CHCl_3$-MeOH, 9 : 1) to give 1-*O*-tetrahydroxycyclopentyl-3-*O*-acylglycerol: FAB-MS, *m/z* 463 and 449 $[(M+H)^+]$; ^1H NMR (500 MHz, CD_3OD), δ 3.93 (1H, m, H-2), 3.87 (1H, m, H-1′), 3.84 (1H, m, H-2′), 3.76 (1H, m, H-3′), 3.74 (1H, m, H-1a), 3.57 (1H, m, H-4′), 3.55 (1H, m, H-5′), 3.55 (1H, m, H-1b), 3.50 (1H, m, H-3a), 3.47 (1H, m, H-3b), 3.47 (2H, m, H-1″), 1.58 (2H, m, H-2″), 1.30 (aliphatic chain), and 0.98~0.84 (2×d and 2×t); ^{13}C NMR (125 MHz, CD_3OD), δ 73.2 (C-1), 70.8 (C-2), 73.0 (C-3), 75.0 (C-1′), 80.0 (C-2′), 81.8 (C-3′), 81.5 (C-4′), 84.4 (C-5′), 72.7 (C-1″), 20.1 (CH_3), 14.5 (CH_3), 14.4 (CH_3), and 11.4 (CH_3).

GC-MS Analysis of Fatty Acid methyl Esters

GC-MS was carried out with a Hewlett Packard G1800A GCD SYSTEM gas chromatograph-mass spectrometer. Fatty acids were methylated with (trimethylsilyl)-diazomethane (Sigma-Aldrich) in hexane at room temperature for 30 minutes. The methyl esters were analyzed by GC-MS with a TC 70 capillary column (GL Science, 0.25 μm thick of fused silica, 0.25 mm×30 m): column temperature at 80°C for 5 minutes, raised to 200°C at 5°C/minute and at 200°C for 10 minutes; injection temperature, 200°C; detector temperature, 230°C; carrier gas, He at 1.0 ml/minute. Total ions generated by an electron impact (EI) mode were monitored to obtain the chromatograms. Mass spectra of peaks appeared in the total ion chromatograms (TICs) were measured from 45 to 425 mass units. Mass spectra of the test sample were compared with those of authentic fatty acid methyl esters to identify the components.

Fatty acid methyl esters in the sample were quantified by a selected ion monitor (SIM) mode. The calibration curve of each fatty acid methyl ester was calculated by linear squares regression from a calibration table created by the authentic specimen. The sample was injected and the chromatograms were integrated in terms of concentration.

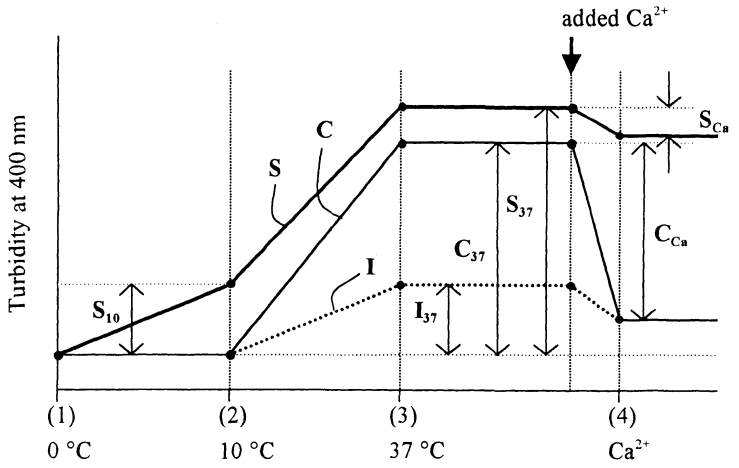
Results and Discussion

Screening Assay

Inhibitors of microtubule polymerization, such as phomopsidin[6], rhizoxin, and colchicine, inhibit tubulin polymerization under the standard condition for assembly *in vitro*. On the other hand, paclitaxel-like strong stimulators of microtubule polymerization induce tubulin assembly under conditions where the polymerization usually dose not occur. The assembly of microtubule proteins can occur at low temperatures with these stimulators, and the microtubule formed is resistant to disassembly by calcium ion or low temperature.

A new screening method we developed observes the inhibition and stimulation of microtubule polymerization and inhibition of depolymerization in sequence using purified porcine microtubule proteins. Microtubule proteins polymerize *in vitro* at 37°C in a buffer solution consisted of 100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 1 mM 2-ME (pH 6.5). The polymerization can be detected in terms of the turbidity measured by a spectrophotometer, and the process of polymerization is monitored by incubating the microtubule proteins at 37°C[9,10]. The suspension of the proteins is poured into a UV cell with or

Fig. 2. Schematic outline of screening assay.



Turbidity was measured at 0°C (1), 10°C (2), and at 37°C (3) and after addition of $CaCl_2$ (4): S, with stimulator of assembly/inhibitor of disassembly of microtubule; I, with inhibitor of microtubule assembly; C, control.

without an inhibitor to measure the change in turbidity. This experiment takes a time to measure a sample and is difficult to detect the stimulatory activity of polymerization.

We, therefore, modified the procedure to assay many samples in a day and to observe stimulation and inhibition of microtubule assembly and inhibition of disassembly in one pot.

A schematic outline of the screening assay is shown in Fig. 2. The turbidity at 400 nm was detected: 1) immediately after addition of a sample and microtubule proteins at 0°C, 2) 10 minutes after incubation of the sample at 10°C, 3) 15 minutes after incubation of the above sample at 37°C, and then 4) after addition of a $CaCl_2$ solution (final concentration of 4 mM) to the sample (Fig. 2). Inhibitory and stimulatory activities were determined by the equations shown in the Materials and Methods section.

Although microtubule proteins do not assemble at 10°C, stimulators, such as taxol, polymerize the proteins. The activity (%) on stimulation of assembly is calculated by the equation (1). Inhibitors of assembly, such as colchicine and rhizoxin, gave lower turbidity than that of control. The inhibitory activity (%) was calculated by the equation (2). The negative control shows 0%, and stronger inhibitors give higher figures. The inhibitory activity (%) on microtubule disassembly was calculated by the equation (3).

The screening assay, thus developed, detected the activities of taxol (stimulator of assembly and inhibitor of disassembly), colchicine, rhizoxin, vinblastine, and phomopsidin (inhibitors of assembly). Extracts of marine organisms (sponges, tunicates, and soft colals) collected at Iriomote Island in Okinawa, Japan, Pohnpei, and Palau were tested by the screening method, and the extract of the marine sponge *Pseudoceratina* sp. collected at Iriomote Island showed stimulation of assembly (25.3% at 100 ppm) and inhibition of disassembly (27.2%) of microtubule proteins.

Isolation and Structure Assignment of Glycolipids **1** and **2** from *Pseudoceratina* sp.

Pseudoceratina sp. was collected by scuba diving at Iriomote Island, Okinawa and extracted with CH_2Cl_2-MeOH. The extract was partitioned between water and EtOAc, and the EtOAc extract was subjected to bioassay-guided separation to give two stimulators of microtubule polymerization, **1** and **2**. These compounds showed each one spot on TLC.

Compound **1** was suggested to be a mixture of homologs, since the ESI-MS of **1** showed two molecular ions at *m/z* 751 and 765, which were later assigned as $(M+Na)^+$ ion peaks. The ^1H NMR spectrum of **1** showed three methyl triplets between δ 0.84 and 0.88, a number of methylene protons at δ 1.30, signals due to oxymethylene and oxymethine protons between δ 3.55 and 5.32, and olefin proton signals at δ 5.34. ^{13}C and ^1H NMR data for **1** (Table 1) were characteristic of those for glycolipids possessing

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for **1** in $CDCl_3$.

C#	^{13}C	^{1}H (J in Hz)	COSY (H#)
1	68.0	(a)3.75, dd (11.8, 6.6)	1b, 2
		(b)3.91, dd (11.8, 5.2)	1a, 2
2	69.9	5.32, m	1a, 1b, 3a, 3b
3	62.4	(a)4.21, dd (11.8, 6.8)	2, 3b
		(b)4.40, dd (11.8, 3.5)	2, 3a
1'	103.7	4.28, d (7.0)	2'
2'	71.2	3.65, m	1', 3'
3'	73.0	3.60, dd (9.5, 2.8)	2', 4'
4'	69.1	4.02, br d (2.5)	3', 5'
5'	74.1	3.55, br t	4', 6'a, 6'b
6'	62.4	(a)3.86, m	5', 6'b
		(b)3.98, m	5', 6'a
2'', 2'''	34.0	2.31, dd	
3'', 3'''	24.5	1.62, m	
=CH-	129.0	5.34, m	
=CH-CH_2-	26.9	2.01, m	
CH_3	22.4	0.86	
CH_3	19.2	0.84	
CH_3	13.8	0.88	

unsaturated fatty acid(s). The ^{1}H and ^{13}C NMR signals due to the sugar and glycerol units were assigned by the ^{1}H-^{1}H COSY, HMQC, and HMBC experiments.

The structure of **1** was, therefore, assigned as a monoglycosyl diacylglycerol derivative, which was very similar to the structure of M-5 isolated from the Okinawan marine sponge *Phyllospongia foliascens*[11]. Comparison of ^{1}H and ^{13}C NMR data for **1** (Table 1) with the reported values for M-5 revealed the stereochemistry of the galactose and glycerol units. However, the signals due to the fatty acid units were slightly different between **1** and M-5.

Components of the acyl units were assigned by alkaline methanolysis of **1** with NaOMe in MeOH. Structures and the ratio of fatty acid methyl esters were assigned by GC-MS using authentic specimens as shown in Fig. 1.

The ESI mass spectrum of **2** showed quasimolecular ion peaks $(M+Na)^+$ at *m/z* 695, 709, and 723, suggesting that **2** was also a mixture of homologs which was not further separated. The structure of **2** was elucidated base on ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMBC, and HMQC spectral data. ^{1}H-^{1}H COSY data for **2** (Table 2) revealed the presence of glycerol and cyclopentanepentol units. HMBC correlations were detected between H-2 of the glycerol unit at δ_H 5.18 and an acyl carbonyl (C-1''') at δ_C 174.1 and between H-1'' of an alkyl group (δ_H 3.46 and 3.42) and C-3 of the glycerol unit (δ_C 69.2). These data suggested that the acyl group was connected to C-2 of the glycerol unit through an ester bond and the alkyl group to C-3 *via* an ether bond. The signal due to H-1' (δ_H 3.64) of the cyclopentanepentol unit showed the HMBC correlation to one of two oxymethylene carbons at δ_C 69.3 (C-1) of the glycerol unit. The cyclopentanepentol unit was, therefore, attached to the C-1 position of the glycerol unit through an ether linkage. Thus, the structure of **2** was assigned as 1-*O*-tetrahydroxycyclopentyl-2-*O*-acyl-3-*O*-alkylglycerol.

The structure of **2** resembled those of crasserides isolated from the Caribbean sponge *Pseudoceratina crassa*[12] and of keruffaride from the Okinawan sponge *Luffariella* sp.[13]. ^{13}C and ^{1}H NMR data for **2** (Table 2) ascribed to the cyclitol and glycerol units were identical to those of the reported values for these compounds. The stereochemistry of the cyclitol and glycerol units in **2** was assigned as shown in Fig. 1.

Table 2. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data for **2** in $CDCl_3$.

C#	^{13}C	^1H (J in Hz)	COSY (H#)
1	69.3	(a)3.77, dd (10.7, 4.3)	2
		(b)3.71, dd (10.7, 6.4)	2
2	71.5	5.18, m	1, 3
3	69.2	(a)3.59, dd (11.2, 5.6)	2
		(b)3.56, dd (11.2, 4.8)	2
1'	82.0	3.64, dd (6.0, 6.3)	2', 5'
2'	73.0	3.92, dd (6.0, 6.0)	1', 3'
3'	80.0	3.80, dd (6.0, 6.1)	2', 4'
4'	78.9	3.65, dd (6.1, 6.6)	3', 5'
5'	78.5	3.88, dd (6.3, 6.6)	1', 4'
1''	71.9	(a)3.46, dt (9.6, 6.7)	2''
		(b)3.42, dt (9.6, 7.0)	2''
2''	29.5	1.53, m	1'', 3''
ω-CH_3	14.1	0.87, t (7.0)	
CH-CH_3	19.7	0.83, d (6.5)	
1'''	174.1	-----	-----
2'''	34.4	2.33, t (7.0)	3'''
3'''	25.0	1.60, m	2''', 4'''
CH_3	22.6	0.87, t (7.0)	

Alkaline methanolysis of **2** with NaOMe gave a polyol compound and a mixture of fatty acid methyl esters. The ^1H NMR spectrum of the polyol showed the signals due to the cyclitol and glycerol units. In the high-field region of the ^1H NMR spectrum of this compound, two methyl triplets and two methyl doublets were detected between δ 0.98 and 0.84, indicating the presence of each one branch-methyl group in two different alkyl chains. The ESI-MS of this compound showed $(M+H)^+$ ions at *m/z* 463 and 449 (intensity ratio of 7 : 1). The alkyl chains were, therefore, the mixture of $C_{17}H_{35}$ (main component, m+n=14) and $C_{16}H_{33}$ (minor component, m+n=13). This polyol compound was identical to the hydrolyzate obtained from keruffaride[14].

The GC-MS of the fatty acid methyl esters showed the molecular ion (M^+) peaks at *m/z* 228, 242, 256, and 270, indicating the presence of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, and $C_{17:0}$ fatty acids (intensity ratio of 1 : 13 : 5 : 2). The peak in the TIC, showed the molecular (M^+) ion at *m/z* 228, was assigned as methyl myristate. The same molecular (M^+) ion at *m/z* 242 was observed by four peaks in the TIC (intensity ratio of 11 : 17 : 2 : 1), which were assigned to one linear and three mono-methyl branched fatty acids. There were also three peaks (3 : 2 : 1) in the TIC, ascribable to one linear and two mono-methyl-branched fatty acids, gave the (M^+) ion at *m/z* 256. The last peak in the TIC showed the molecular ion at *m/z* 270.

Table 3. Bioactivity of compounds **1** and **2** and taxol.

Compound	conc.	stimulation of assembly (%)	inhibition of disassembly (%)
1	100 ppm	95.6	70.2
1	25 ppm	24.8	37.6
2	100 ppm	42.2	48.3
Taxol	10 ppm	35.2	114.0

Keruffaride contained four different linear acyl groups ($C_{15:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$) at C-2 of the glycerol unit[13]. Crasserides consisted of six different acyl groups at C-2: one *n*- $C_{14:0}$ acyl group, three mono-methyl-branched $C_{15:0}$ ($C_{14:0}$+one methyl) acyl groups, one mono-methyl-branched $C_{16:0}$ ($C_{15:0}$+one methyl) acyl group, and one mono-methyl-branched $C_{17:0}$ ($C_{16:0}$+one methyl) acyl group[12]. Therefore, compound **2** contained both the same components as keruffaride and crasserides and the different combinations of the acyl and alkyl groups from them.

Biological Activity

The activity of compounds **1** and **2** on microtubule polymerization was summarized in Table 3. The stimulatory activity of **1** on microtubule assembly was 24.8% at 25 ppm, which was a similar activity to that of taxol at 10 ppm (35.2%). Compound **2** was, however, less active than **1** and showed the stimulatory activity (42.2%) at 100 ppm. The activity of **2** was remarkably decreased when measured at lower concentration. Compounds **1** and **2** showed moderate inhibition of microtubule disassembly by Ca^{2+} (Table 3).

A few bioactivities have been reported for monogalactosyl diacylglycerols, such as a weak anti-tumor-promoting effect[15], a weak growth inhibition to cyanobacterium *Phormidium tenue*[16], and a weak inhibitory effect on the reverse transcriptase of HIV-1[17]. Interestingly, *P. teune* produces monogalactosyl diacylglycerol, which showed the antialgal effect to the producing organism[16]. Only one bioactivity has been reported for cyclopentanepentol glycolipids. Crasserides showed antifeedant activity on a fish *Carassius auratus*[12]. This is, therefore, the first report of the stimulatory activity

on microtubule assembly and inhibitory activity on microtubule disassembly *in vitro* for monogalactosyl diacylglycerols (compound **1**) and cyclopentanepentol glycolipids (compound **2**).

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