A Ceramide and Cerebroside from the Starfish *Asterias amurensis* Lütken and Their Plant-Growth Promotion Activities

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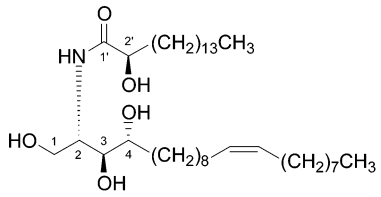
The new phytosphingosine-type ceramide asteriaceramide A (**1**) and glucocerebroside asteriacerebroside G (**2**), together with two known cerebrosides, asteriacerebrosides A and B, were isolated from lipophilic fractions of the whole bodies of the Northern Pacific starfish *Asterias amurensis* Lütken. The water-soluble fraction afforded two known asterosaponins, glycoside B_2 and asterosaponin-1. The structures of **1** and **2** were determined on the basis of chemical and spectroscopic evidence as (2*S*,3*S*,4*R*,13*Z*)-2-[(2′*R*)-2-hydroxyhexadecanoylamino]-13-docosene-1,3,4-triol (**1**) and 1-*O*-(*β*-D-glucopyranosyl)-(2*S*,3*R*,4*E*,13*Z*)-2-[(2′*R*)-2-hydroxytetradecanoylamino]-4,13-docosadiene-1,3-diol (**2**). Compounds **1**, **2**, and asteriacerebrosides A and B promoted plant growth in sprouts of *Brassica campestris*.

A large number of studies on the isolation and structure elucidation of glycosphingolipids and saponins from starfish have been reported.[1,2] These compounds are the predominant metabolites of starfish and include a broad variety of biological activities.[3,4]

The starfish *Asterias amurensis* Lütken is widely distributed in the North Pacific. Asteroids in the genus *Asterias* are notoriously efficient predators, attacking mollusks and other echinoderms.[5] Any outbreak of this starfish causes severe damage to the fishery and aquacultural grounds for benthic shellfish.[6,7] Although such starfish are a nuisance to fishermen, the collected starfish can be utilized as fertilizer.[8] Thus, we investigated *A. amurensis* as a source of plant-growth-regulating compounds. A bioassay-guided chromatographic separation of the methanol extract afforded a new ceramide, asteriaceramide A (**1**), and a new glucocerebroside, asteriacerebroside G (**2**), together with two known compounds, asteriacerebrosides A and B. In this paper, we report the isolation and characterization of these compounds and the evaluation of their biological activities.



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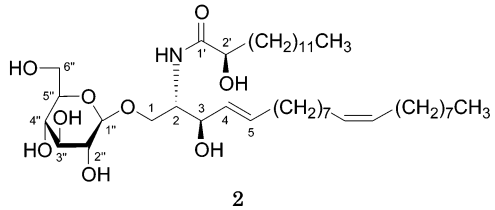

2

The starfish *A. amurensis* was collected in January 2004 off the eastern coast of Hokkaido and extracted with methanol. The methanol extract was partitioned between EtOAc and H_2O. The aqueous layer potently inhibited growth of the sprout *Brassica campestris*, while the hydrophobic layer had a promotive effect in this biological model. The EtOAc-soluble fraction was separated by Si gel column chromatography to obtain two active fractions. These fractions were further subjected to preparative TLC and reversed-phase HPLC to yield two new compounds, asteriaceramide A (**1**) and asteriacerebroside G (**2**), along with two known compounds, asteriacerebrosides A and B.[9] Conversely, the aqueous fraction, which showed growth inhibitory activity, was separated using a combination of Amberlite XAD-2 column, ODS open column, and RP8-HPLC to give glycoside B_2 and asterosaponin-1. These known compounds were identified by comparing their spectroscopic data with those reported in the literature.[9−12]

Asteriaceramide A (**1**) was obtained as an optically active amorphous solid. The molecular formula of **1** was revealed to be $C_{38}H_{75}NO_5$ by HRFABMS [*m*/*z* 648.5543, $(M + Na)^+$, Δ +0.0 mmu]. The ^1H and ^{13}C NMR spectra of **1** exhibited the characteristic signals of a phytosphingosine-type ceramide containing a 2-hydroxy fatty acid (Table 1). Compound **1** possessed a normal acyl chain (straight chain), since the methyl groups were only two terminal methyl carbons observed at 14.4 ppm (Table 1).[9] Analysis of the DQF-COSY and HMBC spectra confirmed the existence of a phytosphingosine-type molecule. Furthermore, ^1H and ^{13}C NMR shift values were in good agreement with those of a known normal phytosphingosine-type ceramide, AC-1-6, possessing a normal 2-hydroxy fatty acid, which had been isolated from the starfish *Acanthaster planci*.[13] Methanolysis of **1** afforded a fatty acid methyl ester (FAME), which was identified as methyl 2-hydroxyhexadecanoate ($[M]^+$ 286) by GC-MS analysis, as well as the presence of a hydroxy group in the 2-position by identification of its specific fragments at *m*/*z* 227 $[M - COOCH_3]^+$ and at *m*/*z* 90 $[C_3H_6O_3]^+$, resulting from the McLafferty rearrangement. The intense fragment ion (*m*/*z* 394) obtained from the positive-ion FABMS (Figure S5, Supporting Information) indicated that the long-chain base (LCB) moiety of **1** was a docosene derivative.[14] Both the location and geometry of the double bond in the LCB moiety were determined as follows. The mass spectrum of the dimethyl disulfide (DMDS) derivative[15,16] of **1** showed a characteristic fragment-ion peak at *m*/*z* 173 due to cleavage between the carbons bearing the methylthio groups (Figure S6, Supporting Information). Therefore, a double bond in the LCB moiety of **1** was located at C-13 rather than C-9 in the case of AC-1-6. Moreover, the *cis*-geometry (*Z*) of the double bond was determined from the δ value (27.7) of the allylic carbon, since allylic carbon signals of *Z*- and *E*-isomers were observed at δ ca. 27 and ca. 32, respectively.[16] On the basis of the above data, the gross structure of **1** was determined to be a phytosphingosine-type ceramide. The absolute configuration of **1** is proposed to be

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Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data for **1** and **2**[a]

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
ceramide				
NH	8.59 (d, J = 8.8 Hz)		8.36 (d, J = 8.7 Hz)	
1a	4.52 (dd, J = 10.9, 4.8 Hz	62.2 (CH_2)	4.25 (m)	70.2 (CH_2)
1b	4.43 (dd, J = 10.9, 4.8 Hz)		4.72 (m)	
2	5.13 (m)	53.1 (CH)	4.80 (m)	54.6 (CH)
3	4.36 (m)	76.9 (CH)	4.80 (m)	72.4 (CH)
4	4.29 (m)	73.1 (CH)	5.98 (m)	131.8 (CH)
5			5.90 (m)	132.8 (CH)
6				32.8 (CH_2)
13	5.49 (m)	130 (CH)*[b]	5.50 (t-like)	130.3 (CH)
14		130 (CH)*		130.3 (CH)
1′		175 (qC)		175.7 (qC)
2′	4.63 (dd, J = 7.6, 3.7 Hz)	72.6 (CH)	4.58 (m)	72.6 (CH)
3′				35.7 (CH_2)
CH_3	0.86 (m, 6H)	14.4 (CH_3)	0.86 (m, 6H)	14.3 (CH_3)
glucose				
1″			4.92 (d, J = 8.0 Hz)	105.7 (CH)
2″			4.04 (dd, J = 8.3, 8.0 Hz)	75.2 (CH)
3″			4.21 (m)	78.5 (CH)
4″			4.23 (m)	71.6 (CH)
5″			3.91 (m)	78.6 (CH)
6″a			4.36 (dd, J = 11.7, 5.4 Hz)	62.7 (CH_2)
6″b			4.51 (m)	

[a] Measured in pyridine-d_5. [b] Chemical shifts marked with an asterisk (*) may be interchanged.

(2*S*,3*S*,4*R*,13*Z*)-2-[(2′*R*)-2-hydroxyhexadecanoylamino]-13-docosene-1,3,4-triol on the basis of similarities in optical rotation ($[\alpha]^{28}_D$ +20.5, *c* 0.2, pyridine) with AC-1-6, (2*S*,3*S*,4*R*,9*Z*)-2-[(2′*R*)-2-hydroxyhexadecanoylamino]-9-docosene-1,3,4-triol (+11.5).[13]

Asteriacerebroside G (**2**) was also obtained as an optically active amorphous solid. The molecular formula of **2** was revealed to be $C_{42}H_{79}NO_9$ by HRESIMS [*m*/*z* 764.5658, $(M + Na)^+$, Δ +0.5 mmu]. Compound **2** showed signals ascribable to a bis-unsaturated normal sphingosine-type cerebroside, bearing a normal 2-hydroxy fatty acid and a β-glucopyranose unit by ^{1}H and ^{13}C NMR analysis (Table 1) in comparison with known glucocerebrosides.[9,17] The overall structure of **2** was confirmed with the aid of 2D NMR experiments, and the FAME obtained by methanolysis of **2** was identified as methyl 2-hydroxytetradecanoate by GC-MS. As the retention time by ODS-HPLC using isocratic methanol as the eluate has been reported to reflect alkyl chain length of this type of cerebroside,[9] compound **2** (n = 11, t_R 22.1 min), asteriacerebroside A (n = 12, t_R 25.2 min), and asteriacerebroside B (n = 13, t_R 28.3 min) were sequentially eluted, supporting the chain length of **2**. The positive-ion FAB mass spectra of **2** exhibited a characteristic fragment-ion peak $[514 + H + Na]^+$ at *m*/*z* 538 due to cleavage of the amide bond, indicating that the LCB moiety of **2** was a docosadiene derivative (Figure S5, Supporting Information).[18] The mass spectra of the DMDS derivative of **2** showed a diagnostic fragment ion at *m*/*z* 173.1356 ($C_{10}H_{21}S$), indicating that the double bond in the LCB moiety was located at C-13 (Figure S6, Supporting Information). The geometry of the C-13 double bond was again characterized as being *Z* from the δ value (27.6) of the allylic carbon. In contrast, the C-4 double bond was *E* as deduced from the olefin carbon chemical shifts (δ_C 131.8 and 132.8).[9] In conclusion, we propose that the planer structure for **2** was the β-glucosyl ceramide. Because the optical rotation of **2** (+12.8) was similar to that of asteriacerebroside A, 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,13*Z*)-2-[(2′*R*)-2-hydroxypentadecanoylamino]-1,3-dihydroxy-4,13-docosadiene (+8.5),[8] we propose the structure of **2** as 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,13*Z*)-2-[(2′*R*)-2-hydroxytetradecanoylamino]-1,3-dihydroxy-4,13-docosadiene.

The biological activity of each compound toward the plant *B. campestris* was evaluated by a simple test with filter paper (see Experimental Section). Asteriacerebrosides A, B, and G exhibited growth-promoting activity for the whole body of *B. campestris*, while asteriaceramide A showed activity stimulating only root growth. Marine-derived cerebrosides have been reported to have beneficial pharmacological qualities.[19,20] Meanwhile, terrestrial cerebrosides have been known to play an eliciting role in cell suspension in rice cultures, [21] as well as to stimulate the fruiting body formation of fungi.[22] The promotive plant-growth activity of ceramide and cerebroside is reported for the first time here. However, the fraction containing saponins exhibited potent inhibitory effects. Saponins are known to possess various inhibitory properties such as cytotoxic, ichthyotoxic, repellent, and hemolytic activities.[3] Glycoside B_2 and asterosaponin-1 in *A. amurensis* also showed an inhibitory action toward plants, as we speculated. The application of these asterosaponins as natural herbicides is expected. The structure−activity relationship and details of the bioassays will be published elsewhere.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Horiba SEPA-300 high sensitive polarimeter. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz; DEPT) spectra were obtained in C_5D_5N and D_2O (acetone as an internal standard, δ_H 2.05, δ_C 30.5 from DSS) solution using a JEOL JNM-EX-400 spectrometer. Proton-detected heteronuclear correlations were measured using HSQC and HMBC. 2D NMR spectra were recorded on a JEOL JNM-ECA-600 spectrometer. The low- and high-resolution EI mass spectra (70 eV) were recorded on a JEOL JMS-GCmate spectrometer. FABMS were obtained on a JEOL JMS-HX110 [matrix, glycerol and *m*-nitrobenzyl alcohol (NBA)], and ESIMS spectra were acquired with a JEOL JMS-700TZ spectrometer. GC-MS were taken with a Shimadzu QP-5050 [EI mode; ionizing potential, 70 eV; column, DB-5MS 30 m × 0.25 mm i.d. (J&W Scientific Inc., Kyoto, Japan); carrier gas, He]. Silica gel (Merck, Kieselgel 60, 70−230 mesh) was used for open column chromatography. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 60 F_{254}s aluminum sheets. Spots were visualized by UV light or by spraying with a 5% phosphomolybdic acid ethanol solution. Silica gel plates (Merck, Kieselgel 60 F_{254} with a layer thickness of 0.5 mm and concentrating zone) were used for preparative thin-layer chromatography (PTLC). HPLC was carried out using Develosil ODS-HG-5 and Shim-Pack CLC-C8 columns.

Animal Material. The starfish *Asterias amurensis* Lütken (Asteriidae) was collected in January 2004 off the eastern coast of Hokkaido. A voucher specimen is deposited in the Graduate School of Environ-

mental Earth Science, Hokkaido University. Animal material was kept in MeOH until workup.

Extraction and Isolation. The fresh starfish (950 g wet wt) was cut into small pieces, homogenized in methanol, and then soaked in methanol (3 L) at room temperature for 24 h. After centrifugation and filtration, the crude extract was evaporated under reduced pressure and then lyophilized. The methanol extract (31 g) was partitioned between EtOAc and H_2O. The lipophilic fraction (EtOAc-soluble portion, 1.72 g), which exhibited promotive growth activity for the *B. campestris* sprout, was chromatographed on a Si gel column using $CHCl_3$/MeOH/H_2O systems of increasing polarity. The $CHCl_3$/MeOH/H_2O (80:20:1) eluate (72.3 mg) was successively rechromatographed over Si gel eluting with a gradient of increasing MeOH in $CHCl_3$ to afford four fractions. The fraction (32.3 mg) eluted with $CHCl_3$/MeOH (9:1), which showed growth stimulating activity on the root of the *B. campestris* sprout, was further submitted to preparative TLC with the following two solvent systems: (A) *n*-hexane/EtOAc/MeOH/H_2O = 30:20:10:1, (B) toluene/EtOAc/MeOH/H_2O = 30:20:10:1 to yield asteriaceramide A (**1**) (R_f(B) = 0.44, 2.3 mg) and asteriacerebroside B (R_f(B) = 0.27, 1.7 mg). A polar fraction (18.9 mg) eluted with $CHCl_3$/MeOH/H_2O (85:15:1) was subjected to reversed-phase HPLC (Develosil ODS-HG-5) using MeOH as eluent to give pure asteriacerebrosides A (1.5 mg) and G (**2**) (0.8 mg).

In contrast, the water-soluble fraction (28.8 g), which demonstrated potent inhibitory properties, was separated using a column of Amberlite XAD-2 eluted with a solvent gradient of H_2O to MeOH. The active fraction eluted with 75% methanol and was successively fractionated by column chromatography on ODS with a stepwise gradient (H_2O and MeOH). The fraction (131 mg) eluting with MeOH/H_2O (75:25) showed strong inhibitory activity and was subjected to reversed-phase HPLC (Shim-Pack CLC-C8) with MeCN/H_2O (24:76) (flow rate 1.5 mL/min) to afford glycoside B_2 (10.7 mg) and asterosaponin-1 (19.5 mg).

Asteriaceramide A (1): amorphous white solid; $[\alpha]^{28}_D$ +20.5 (*c* 0.2, pyridine); ^{1}H NMR and ^{13}C NMR data, see Table 1; LR-FABMS *m*/*z* 648 $[M + Na]^+$, 394 $[370 + Na + H]^+$; HRMSFAB (*m*/*z*) $[M + Na]^+$ calcd for $C_{38}H_{75}NO_5Na$, 648.5543; found, 648.5543.

Asteriacerebroside G (2): amorphous white solid; $[\alpha]^{26}_D$ +12.8 (*c* 0.2, *n*-PrOH); ^{1}H NMR and ^{13}C NMR data, see Table 1; LRFABMS *m*/*z* 764 $[M + Na]^+$, 538 $[514 + Na + H]^+$; LRESIMS *m*/*z* 764 $[M + Na]^+$, 562 $[(M + H) - H_2O -$ hexose$]^+$; HRMSESI (*m*/*z*) $[M + Na]^+$ calcd for $C_{42}H_{79}NO_9Na$, 764.5653; found, 764.5658.

Methanolysis of 1 and 2. Compounds **1** and **2** (ca. 1 mg) were heated with 10% HCl in MeOH (1 mL each) at 70 °C for 18 h. The reaction mixture was then extracted with *n*-hexane and concentrated in vacuo. The residue was subjected to Si gel column chromatography [*n*-hexane/EtOAc (7:3)] to yield a FAME. The FAME from **1** and **2** was subjected to GC-MS [column: J&W Scientific DB-5MS 30 m, i.d. 0.25 mm, column temp 150−300 °C (rate of temp increase = 10 °C/min), injection volume 1 μL, splitless]. FAME from **1** (methyl 2-hydroxyhexadecanoate): t_R [min] = 13.0, *m*/*z* 286 (M^+), 227 $(M - COOCH_3)^+$. FAME from **2** (methyl 2-hydroxytetradecanoate): t_R [min] = 10.8, *m*/*z* 258 (M^+), 199 $(M - COOCH_3)^+$.

DMDS Derivatives of 1 and 2. Each compound (0.5 mg) was dissolved in CS_2 (0.2 mL) with the addition of dimethyl disulfide (DMDS) (0.2 mL) and 20 μL of iodine solution (60 mg of I_2 in 1 mL of diethyl ether). After stirring for 48 h at 60 °C in a 10 mL tube sealed with a Teflon-lined cap, the reaction mixture was diluted with 0.2 mL of *n*-hexane and the excess iodine was quenched with 0.2 mL of $Na_2S_2O_3$ solution (5% in distilled water). The organic phase was removed and the aqueous phase extracted twice with 0.2 mL of *n*-hexane. The organic extracts were concentrated, and the residue was purified by Si gel CC [$CHCl_3$/acetone (85:15)] to afford the pure DMDS derivative as an amorphous powder. Finally, the DMDS derivatives were analyzed by EIMS, with both showing a single major ion at *m*/*z* 173.

Bioassay. The seeds of *B. campestris* were placed on the filter paper including sample solution (2 mL) in a Petri dish and incubated at 25 °C for 1 week under a 12 h/12 h light−dark cycle condition. The growth of their shoots and roots was estimated by weight and length.

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Supporting Information Available: NMR spectra and MS fragmentations for asteriaceramide A (**1**), asteriacerebroside G (**2**), and their DMDS derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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