Isolation and Structure Determination of Cerebrosides from Garlic, the Bulbs of *Allium sativum* L.

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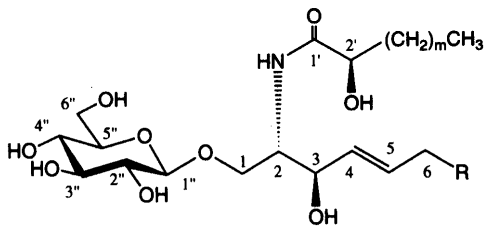
Five cerebrosides, AS-1-1 (1), AS-1-2 (2), AS-1-3 (3), AS-1-4 (4), and AS-1-5 (5) were obtained from the $CHCl_3$–MeOH extract of Garlic, the bulbs of *Allium sativum* L. (Liliaceae). On the basis of spectroscopic results, structures of 1—5 have been elucidated. Compounds 1, 2, and 5 were new cerebrosides. Compounds 3 and 4 were identified with the known glucocerebroside soya-cerebroside I and II, respectively, which have been previously obtained from soybean with ionophoretic activity. Positive ion FAB-MS/MS of the $(M+Na)^+$ ion gave important information on the length of the fatty acyl chain of the ceramide moiety.

Key words *Allium sativum*; glycosphingolipid; FAB-MS/MS; cerebroside; garlic

Garlic, the bulbs of *Allium sativum* L. (Liliaceae), has been used worldwide as a tonic, a bactericide, and a popular remedy for various ailments. In the course of investigating the biologically-active components of garlic, we have investigated some bioactive glycosides.[1] In a continuation of these studies, the isolation and characterization of glycosphingolipids from garlic has been conducted in the hope of discovering new bioactive compounds. In this paper, the isolation and structure determination of three new, and two known cerebrosides are reported.

Structure of Cerebroside Molecular Species AS-1 The acetone insoluble fraction, obtained from $CHCl_3$–MeOH extraction of garlic, was separated by silica gel chromatography, followed by reversed phase (C_{18}) column chromatography, to afforded AS-1 as a single spot on silica gel TLC.

In the IR and positive ion FAB mass spectra of AS-1, strong hydroxyl and amide absorptions and a series of $(M+Na)^+$ ion peaks were observed. The ^{13}C-NMR spectrum of AS-1 exhibited characteristic signals due to sphingosine-type cerebrosides possessing 2-hydroxy fatty acid and β-glucopyranose moieties (Fig. 1 and Table 1). Therefore, AS-1 was expected to be a sphingosine-type cerebroside having 2-hydroxy fatty acids and β-glucopyranose moieties. Furthermore, AS-1 was thought to possess normal[2] types of side chains, since the carbon atom signals due to the terminal methyl groups were observed at δ 14.2 in the ^{13}C-NMR spectrum (Table 1). The ^{1}H-NMR spectrum of AS-1 was in good agreement with that of the known glucocerebroside S-1,[3] which was obtained from the starfish *Stellaster equestris*, suggesting that AS-1 was composed of (2*S*,3*R*,4*E*)-sphingosine, (2*R*)-2-hydroxy fatty acid, and β-D-glucopyranose (Table 2). The above fact and the optical rotations of



AS-1 ; m, R = not identified

AS-1-1 (**1**) ; m = 11, R = 7 8 9 10 $(CH_2)_7CH_3$

AS-1-2 (**2**) ; m = 12, R = $(CH_2)_7CH_3$

AS-1-3 (**3**) ; m = 13, R = $(CH_2)_7CH_3$

AS-1-4 (**4**) ; m = 13, R = $(CH_2)_7CH_3$

AS-1-5 (**5**) ; m = 13, R = $(CH_2)_7CH_3$

Fig. 1. Structures of **1**—**5**

Table 1. ^{13}C-NMR Spectral Data for AS-1, AS-1-1, AS-1-2, AS-1-3, AS-1-4, and AS-1-5, in Pyridine-d_5

C		AS-1	AS-1-1	AS-1-2	AS-1-3	AS-1-4	AS-1-5
1	(t)	70.1	70.0	70.0	70.0	70.1	70.1
2	(d)	54.6	54.6	54.6	54.6	54.6	54.7
3	(d)	72.3[a]	72.3[a]	72.3[a]	72.3[a]	72.3[a]	72.4[a]
4	(d)	131.9[b]	131.9[b]	131.9[b]	132.0	131.9[b]	131.7[b]
5	(d)	132.0[b]	132.0[b]	132.0[b]	132.0	132.0[b]	132.0[b]
6	(t)		32.9[c]	32.8	32.9	33.0[c]	
7	(t)		32.0	32.0	27.5[b]	32.1	
8	(d)		129.9[c]	129.9[c]	129.4[c]	129.9[d]	
9	(d)		131.1[d]	131.1[c]	130.6[c]	131.1[d]	
10	(t)		32.8[c]	32.8	27.3[b]	32.9[c]	
1′	(s)	175.7	175.6	175.6	175.6	175.7	175.7
2′	(d)	72.5[a]	72.5[a]	72.5[a]	72.4[a]	72.5[a]	72.6[a]
$-CH_3$	(q)	14.2	14.2	14.2	14.2	14.2	14.2
1″	(d)	105.6	105.6	105.6	105.5	105.6	105.6
2″	(d)	75.1	75.0	75.0	75.0	75.1	75.1
3″	(d)	78.4	78.4	78.4	78.4	78.4	78.5
4″	(d)	71.6	71.6	71.6	71.6	71.6	71.7
5″	(d)	78.4	78.4	78.4	78.4	78.4	78.5
6″	(t)	62.7	62.7	62.7	62.7	62.7	62.8

a—d) Assignment may be reversed in each vertical column.

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the AS-1 series, AS-1-1 (**1**)—AS-1-5 (**5**) (+5.2°, +8.8°, +7.9°, +9.2°, and +8.3° respectively) (*vide infra*) and S-1 (+9.5°) suggested that AS-1 has the same absolute configuration as that of S-1 for the core structure. Accordingly, the structure of the cerebroside molecular species AS-1 was estimated as shown in Fig. 1.

Isolation and Structure of Cerebrosides from AS-1 AS-1 was separated by reversed phase HPLC into five major components, AS-1-1 (**1**)—AS-1-5 (**5**). The ^{13}C-NMR spectra of **1**—**5** were essentially identical with that of AS-1 (Table 1). This proves that these five compounds were cerebroside components of AS-1. The positive ion FAB mass spectra of **1**—**5** revealed a single $(M+Na)^+$ ion peak at *m/z* 708, 722, 736, 736, and 738, respectively. We have already reported[4] that collision-induced dissociation (CID) spectra of $(M+Na)^+$ ions produced by FAB-MS of glycosphingolipids indicate the length of the fatty acyl chain of ceramide moieties. When the CID spectra of the $(M+Na)^+$ ions of **1**—**5** were measured, prominent fragment ions were observed at *m/z* 482 (**1**—**4**) and 484 (**5**), which originated from the cleavage of the amide bond (Fig. 2). Taking into account the molecular mass of **1**—**5** and the characteristic fragment ions obtained from the CID spectra, we may regard the long chain base and 2-hydroxy fatty acid component of **1**—**5** as shown in Table 3.

Table 2. ^{1}H-NMR Spectral Data for S-1 and AS-1, in Pyridine-d_5

H	S-1[a]	AS-1
1a	4.23 (m)	4.21 (m)
1b	4.75 (dd, J=12, 6 Hz)	4.76 (dd, J=12, 7 Hz)
2	4.80 (m)	4.80 (m)
3	4.80 (m)	4.80 (m)
4	6.02 (dd, J=15, 6 Hz)	6.00 (dd, J=16, 6 Hz)
5	5.91 (dt, J=15, 6 Hz)	5.92 (dt, J=16, 6 Hz)
2′	4.59 (dd, J=8, 4 Hz)	4.56 (dd, J=8, 4 Hz)
CH_3	0.88 (m)	0.88 (t, J=6 Hz)
1″	4.92 (d, J=8 Hz)	4.90 (d, J=8 Hz)
2″	4.04 (t, J=8 Hz)	4.01 (t, J=8 Hz)
3″	4.23 (m)	4.21 (m)
4″	4.23 (m)	4.21 (m)
5″	3.91 (m)	3.89 (m)
6″a	4.36 (dd, J=12, 5 Hz)	4.33 (dd, J=12, 5 Hz)
6″b	4.52 (dd, J=12, 2 Hz)	4.49 (dd, J=12, 2 Hz)
NH	8.37 (d, J=8 Hz)	8.32 (d, J=8 Hz)

a) Data from reference 2.

Next, the location and geometry of the double bond in the side chains of the long chain base of **1**—**4** were determined as follows. In the positive ion FAB-MS spectra, the dimethyl disulfide (DMDS) derivatives of each compound showed a remarkable fragment ion peak at *m/z* 187 due to the cleavage of the bond between the carbons bearing the methylthio group (Fig. 3).[5] These data indicated that the double bond is located at C-8 in these cerebrosides.

On the other hand, it is known[6] that the geometry of the double bond in a long chain alkene can be determined from the ^{13}C-NMR chemical shift of the methylene carbon adjacent to the olefinic carbon. Thus, the carbon signal is observed at δ *ca.* 27 ppm in (*Z*)-olefins and at δ *ca.* 32 ppm in (*E*)-olefins. The geometry is also inferred from the ^{1}H-NMR signals of the olefinic proton, appearing as triplet-like in the (*Z*)-type and as a multiplet in the (*E*)-type.[7] When the ^{13}C- and ^{1}H-NMR spectra of **1**—**4** were assigned in detail, the signals of the three methylene

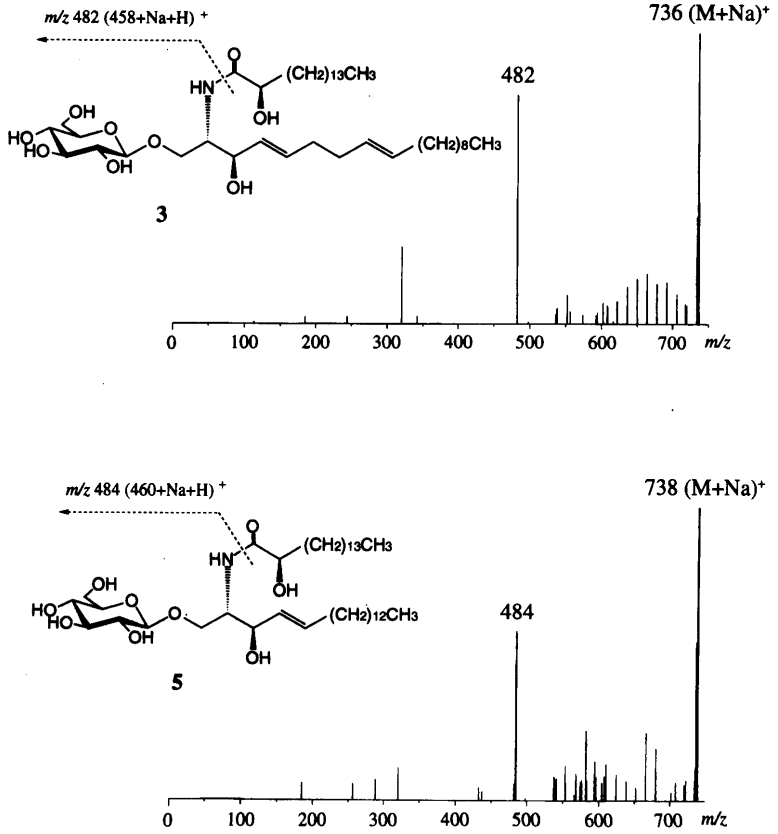

Fig. 2. CID Spectra of $[M+Na]^+$ Ions Obtained from the Positive Ion FAB-MS of **3** and **5**

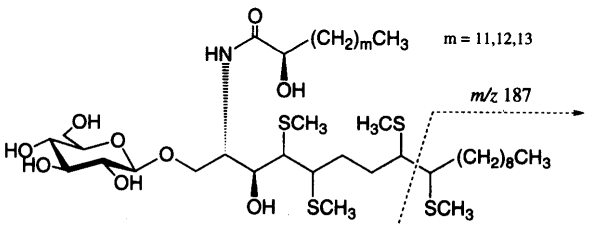

Fig. 3. Fragmentation of the DMDS Derivatives of **1**—**4** in the Positive Ion FAB-MS

Table 3. Fatty Acid and Long Chain Base Composition of AS-1 Series

	Fatty Acid	Long Chain Base
AS-1-1	α-OH ($C_{14:0}$)	Sphingosine ($C_{18:2}$)
AS-1-2	α-OH ($C_{15:0}$)	Sphingosine ($C_{18:2}$)
AS-1-3	α-OH ($C_{16:0}$)	Sphingosine ($C_{18:2}$)
AS-1-4	α-OH ($C_{16:0}$)	Sphingosine ($C_{18:2}$)
AS-1-5	α-OH ($C_{16:0}$)	Sphingosine ($C_{18:1}$)

carbons (C-6, C-7 and C-10) adjacent to the olefinic carbons were observed at δ 32.0—33.0, and the signals of olefinic protons (8-H and 9-H) appeared at δ 5.47 as a multiplet, in compounds **1**, **2**, and **4**. Thus, the olefinic group in the long-chain base of **1**, **2**, and **4** was characterized as (*E*)-type. Meanwhile, in the ^{13}C-NMR spectrum of **3**, the signals of the three methylene carbons (C-6, C-7 and C-10) adjacent to the olefinic carbons were observed at δ 32.9, 27.5, and 27.3. When the heteronuclear multiple bond correlation (HMBC) spectrum of **3** was measured, remarkable correlations were observed between the signals of the olefinic protons (8-H and 9-H) at δ 5.48 and the signals for the methylene carbons at δ 27.3 and 27.5. Therefore, the olefinic group in the side chain of the long chain part of **3** must be (*Z*)-type. The *Z* configuration was also verified from the fact that the olefinic protons (8-H and 9-H) appeared at δ 5.48 as a triplet-like signal.

Since the absolute configuration of the parent molecular species AS-1 was already determined (*vide supra*), the structures of **1**—**5** were deduced to be 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxytetradecanoylamino]-4,8-octadecadiene-1,3-diol (**1**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxypentadecanoylamino]-4,8-octadecadiene-1,3-diol (**2**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*Z*)-2-[(2*R*)-2-hydroxyhexadecanoylamino]-4,8-octadecadiene-1,3-diol (**3**), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxyhexadecanoylamino]-4,8-octadecadiene-1,3-diol (**4**), and 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*E*)-2-[(2*R*)-2-hydroxyhexadecanoylamino]-4-octadecene-1,3-diol (**5**), respectively as shown in Fig. 1.

To our knowledge, isolation and structure determination of AS-1-1 (**1**), AS-1-2 (**2**), and AS-1-5 (**5**) were made for first time, although **5** has been obtained as a synthetic product.[8] AS-1-3 (**3**) and AS-1-4 (**4**) were found to be identical with the known glucocerebroside soya-cerebroside I and II,[9] which were obtained from soybean with ionophoretic activity.

Experimental

Melting points were determined on a micro melting point apparatus (Yanaco MP-3) without correction. Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 28 °C. IR spectra were obtained on a Jasco IR-700 infrared spectrophotometer. ^{13}C-NMR spectra were recorded at 67.8 MHz on a Jeol GX-270 spectrometer. ^{1}H- and HMBC spectra were recorded at 500 MHz on a Varian Unity-500 spectrometer. Chemical shifts were referenced to the solvent signal (pyridine-d_5: δ_H 7.19, δ_C 123.5). FAB-MS and FAB-MS/MS spectra were acquired with a Jeol JMS-SX/SX102A four sector type tandem mass spectrometer of BE / BE geometry. The FAB mass spectra were obtained by using only the first spectrometer. $CHCl_3$–MeOH (1 : 1, v/v) solution of the sample was prepared at a concentration of *ca.* 10 μg/μl, and the solution (1 μl) was mixed with 1 μl of *m*-nitrobenzyl alcohol (*m*-NBA) saturated by NaCl on a stainless-steel tip. Ions were produced by bombardment with neutral xenon atoms at 5 kV. The $(M+Na)^+$ ions were selected as precursor ions and then achieved high energy (10 kV) by collision with argon molecules in the third field-free region. The argon pressure was sufficient to attenuate the primary ion beam by 50%. The fragment ions were dispersed by the second spectrometer and the spectra were recorded as CID spectra. HPLC was carried out with Jasco PU-980 HPLC pump and RI-930 refractive index (RI) detector.

Separation of AS-1 Ten kilograms of frozen, freshly skinned garlic (collected in Hokkaido, Japan), were homogenized and extracted with $CHCl_3$–MeOH (1 : 3) (16 l), followed by further extraction with $CHCl_3$–MeOH (1 : 2) (9 l), and the combined $CHCl_3$–MeOH solutions were concentrated *in vacuo* to give a residue (700 g). The residue (400 g) was partitioned between H_2O (2 l) and AcOEt–1-BuOH (2 : 1) (1 l, twice), and the organic layer was concentrated *in vacuo* to give the less polar fraction (15 g), which was washed with cold acetone (100 ml, twice). The acetone-insoluble part (11 g) was chromatographed over silica gel [solvent AcOEt–MeOH (9 : 1→8 : 2→1 : 1) (for the first chromatography) and $CHCl_3$–MeOH H_2O (19 : 1 : 0→9 : 1 : 0→9 : 1.5 : 0.05→1 : 1 : 0) (for the second chromatography)] to afford the crude cerebroside fraction. This fraction was refined by reversed phase column chromatography [Cosmosil 140C_{18} PREP, solvent MeOH] to give AS-1 (120 mg) which showed a single spot on silica gel TLC [solvent $CHCl_3$–MeOH–H_2O (9 : 1.5 : 0.05)], Rf=0.32.

AS-1: Amorphous powder, $[\alpha]_D$ +8.0° (c=1.00, 1-PrOH). IR (KBr) cm^{-1}: 3350 (OH), 1640, 1540 (amide). Positive ion FAB-MS *m/z*: 750, 738, 736, 722, 708 $(M+Na)^+$ series. ^{13}C- and ^{1}H-NMR: see Tables 1 and 2.

Isolation of Cerebrosides, AS-1-1 (1)—AS-1-5 (5) HPLC of AS-1 showed five major peaks [column, WAKOSIL-II 5C_{18} (300 × 10 mm, i.d.); solvent, MeOH; flow rate, 2.0 ml/min]. t_R [min] (ratio of peak areas): 20.0 (3.3), 22.8 (2.0), 24.8 (14.0), 25.6 (78.5), 29.2 (2.2). AS-1 (60 mg) was separated by HPLC into the above mentioned five fractions by using the conditions described above: fraction 1 (AS-1-1, 2.9 mg), fraction 2 (AS-1-2, 1.6 mg), fraction 3 (AS-1-3, 7.2 mg), fraction 4 (AS-1-4, 37.7 mg), fraction 5 (AS-1-5, 1.8 mg). Positive ion FAB-MS of **1**—**5**: $(M+Na)^+$ at *m/z* 708 (**1**), 722 (**2**), 736 (**3**), 736 (**4**), 738 (**5**). Positive ion FAB-MS/MS [$(M+Na)^+$ ion was selected as a precursor ion]: **1**—**5** revealed single fragment ion peaks due to fission of the amide bond at *m/z* 482 (**1**—**4**) and 484 (**5**) respectively.

AS-1-1 (**1**): Amorphous powder, mp 197—200 °C, $[\alpha]_D$ +5.2° (c=0.25, 1-PrOH). ^{1}H-NMR (pyridine-d_5) δ: olefinic H, 5.47 (2H, m, 8-H, 9-H). ^{13}C-NMR: see Table 1. High resolution positive ion FAB-MS: Calcd for $C_{38}H_{72}NO_9$ $(M+H)^+$: 686.5207. Found: 686.5203.

AS-1-2 (**2**): Amorphous powder, mp 195—200 °C, $[\alpha]_D$ +8.8° (c=0.13, 1-PrOH). ^{1}H-NMR (pyridine-d_5) δ: olefinic H, 5.47 (2H, m, 8-H, 9-H). ^{13}C-NMR: see Table 1. High resolution positive ion FAB-MS: Calcd for $C_{39}H_{74}NO_9$ $(M+H)^+$: 700.5364. Found: 700.5356.

AS-1-3 (**3**): Amorphous powder, mp 168—170 °C, $[\alpha]_D$ +7.9° (c=0.64, 1-PrOH). ^{1}H-NMR (pyridine-d_5) δ: olefinic H, 5.48 (2H, t-like, 8-H, 9-H). ^{13}C-NMR: see Table 1. High resolution positive ion FAB-MS: Calcd for $C_{40}H_{76}NO_9$ $(M+H)^+$: 714.5520. Found: 714.5494.

AS-1-4 (**4**): Amorphous powder, mp 193—197 °C, $[\alpha]_D$ +9.2° (c=0.60, 1-PrOH). ^{1}H-NMR (pyridine-d_5) δ: olefinic H, 5.47 (2H, m, 8-H, 9-H). ^{13}C-NMR: see Table 1. High resolution positive ion FAB-MS: Calcd for $C_{40}H_{76}NO_9$ $(M+H)^+$: 714.5520. Found: 714.5535.

AS-1-5 (**5**): Amorphous powder, mp 205—210 °C, $[\alpha]_D$ +8.3° (c=0.15, 1-PrOH). ^{13}C-NMR: see Table 1. High resolution positive ion FAB-MS : Calcd for $C_{40}H_{78}NO_9$ $(M+H)^+$: 716.5677. Found: 716.5695.

DMDS Derivative of 1—4 Each compound (0.1 mg) was dissolved in carbon disulfide (0.1 ml) and DMDS (0.1 ml) and iodine (1 mg) added to the solution. The obtained mixture was kept at 60 °C for 40 h in a small sealed vial. The reaction was quenched with aqueous $Na_2S_2O_3$

(5%), and extracted with *n*-hexane (0.3 ml). The extract was concentrated and the residue (DMDS derivative) was subjected to positive ion FAB-MS.

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References and Notes

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