Isolation and Structure of Four New Ceramides from the Starfish *Luidia maculata*

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A new sphingosine-type ceramide LMCer-1-1 (1) and three new phytosphingosine-type ceramides, LMCer-2-1 (2), LMCer-2-6 (3), and LMCer-2-7 (4), were isolated from the anti-hyperglycemic active ceramide molecular species LMCer-1 and LMCer-2, obtained from the less polar fraction of the chloroform–methanol extract of the whole bodies of *Luidia maculata*. The structures of these ceramides were determined on the basis of chemical and spectroscopic evidence as: (2*S*,3*R*,4*E*,2′*R*)-2-(2-hydroxyhexadecanoylamino)-16-methyl-4-octadecene-1,3-diol (1), (2*S*,3*S*,4*R*,2′*R*)-2-(2-hydroxyhexadecanoylamino)-16-methyl-octadecane-1,3,4-triol (2), (2*S*,3*S*,4*R*,2′*R*)-2-(2-hydroxydocosanoylamino)-hexadecane-1,3,4-triol (3), and (2*S*,3*S*,4*R*,2′*R*)-2-(2-hydroxydocosanoylamino)-14-methyl-hexadecane-1,3,4-triol (4).

Key words sphingolipid; ceramide; starfish; *Luidia maculata*; anti-hyperglycemic activity

A series of studies on the isolation and structure elucidation of the sphingolipids from several starfish species have been performed in our laboratory. From the starfish *Luidia maculata*, we have isolated and characterized one sulfatide molecular species,[1] six cerebrosides,[2] four ceramide lactosides,[3] and three ganglioside molecular species.[4—6] Continuing the previous studies, the isolation and characterization of ceramides from *L. maculata* were carried out because of the considerable interest and importance connected with determination of the composition of the mixture of sphingolipids and in the hope of discovering the biologically active compounds from marine natural products.

In this paper, we report on the isolation and structure determination of four ceramides, LMCer-1-1 (**1**), LMCer-2-1 (**2**), LMCer-2-6 (**3**), and LMCer-2-7 (**4**) from the starfish *L. maculata*.

The MeOH extract, obtained from the less polar fraction of the $CHCl_3$–MeOH extract of the whole bodies of *L. maculata*, was separated by normal phase silica gel column chromatography to give two substances, LMCer-1 and LMCer-2, which appear as a single spot on the normal phase TLC, and revealed anti-hyperglycemic activity.[7]

Structure of Ceramide Molecular Species LMCer-1 The positive ion FABMS of LMCer-1 exhibited a series of $[M+Na]^+$ ion peaks at *m/z* 590, 616, 630, 644, 646, 656, 660, 670, 674, and 688. In the ^{13}C-NMR spectrum, the characteristic signals of a sphingosine-type ceramide possessing 2-hydroxy fatty acid were observed (Table 1). Therefore, LMCer-1 must be a molecular species of a sphingosine-type ceramide containing 2-hydroxy fatty acid. Furthermore, LMCer-1 was thought to possess normal and *ante*-iso[8] type side chains since the carbon signals for the terminal methyl groups were observed at δ_C=14.2 (normal) and δ_C=11.4, 19.2 (*ante*-iso) in the ^{13}C-NMR (Table 1). The ^{1}H-NMR signals of the basic structure of LMCer-1 were in good agreement with those of known synthetic ceramide 1b,[9] which is composed of (2*S*,3*R*,4*E*)-sphingosine and (*R*)-2-hydroxy fatty acid. The above fact and the optical rotation of the LMCer-1 series, LMCer-1-1 (+3.3) (vide infra) and the synthetic ceramide (+7.4)[9] suggested that LMCer-1 has the same absolute configuration as that of the synthetic one for the core structure, the 2,3,2′ part. The component of LMCer-1 was examined. When LMCer-1 was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAM) was obtained. A gas chromatography-mass spectrometry (GC-MS) analysis of the FAM mixture showed the existence of six components, which were characterized as methyl 2-hydroxypentadecanoate (FAM-1), methyl 2-hydroxyhexadecanoate (FAM-2), 2-hydroxyheneicosanoate (FAM-3), methyl 2-hydroxydocosanoate (FAM-4), methyl 2-hydroxytricosanoate (FAM-5), and methyl 2-hydroxytetracosanoate (FAM-6). The major FAM was methyl 2-hydroxydocosanoate (FAM-4). Therefore, the structure of the ceramide molecular species LMCer-1 was determined to be that shown in Fig. 1.

Isolation and Structure of Ceramides from LMCer-1 LMCer-1 was separated by reversed-phase HPLC into twelve components, which were recovered to give the twelve fractions LMCer-1-1 to LMCer-1-12. One of the twelve fractions, LMCer-1-1 (**1**), revealed the single quasi-molecular

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of LMCer-1 and LMCer-2 [δ Values in $CDCl_3$ (LMCer-1), C_5D_5N (LMCer-2)]

Position	LMCer-1		LMCer-2	
	^{1}H	^{13}C	^{1}H	^{13}C
NH	7.21 (d, *J* = 7.9 Hz)		8.56 (d, *J* = 9.2 Hz)	
1a	3.74[a)]	62.2 (t)	4.40 (dd, *J*=10.7, 5.1 Hz)	62.2 (t)
1b	3.87[a)]		4.49 (dd, *J*=10.7, 4.5 Hz)	
2	3.91[a)]	54.6 (d)	5.09[a)]	52.9 (d)
3	4.27[a)]	74.3 (d)	4.34 (dd, *J*=6.6, 4.6 Hz)	76.9 (d)
4	5.49 (dd, *J*=15.3, 6.1 Hz)	128.6 (d)	4.26 (t, *J*=6.6, 6.2 Hz)	73.2 (d)
5	5.77 (dt, *J*=15.3, 7.9 Hz)	134.4 (d)		
1′		175.1 (s)		175.4 (s)
2′	4.11 (dd, *J*=7.8, 3.8 Hz)	72.5 (d)	4.60 (dd, *J*=7.6, 4.0 Hz)	72.5 (d)
–CH_3	0.88 (m)	14.2 (q)	0.88 (m)	14.2 (q)
		11.4 (q)		11.4 (q)
		19.4 (q)		19.4 (q)

a) *J* values could not be observed because they overlapped with other signals.

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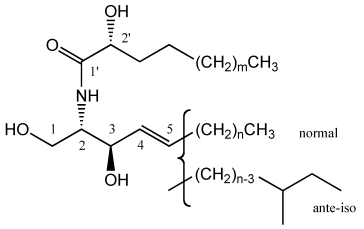
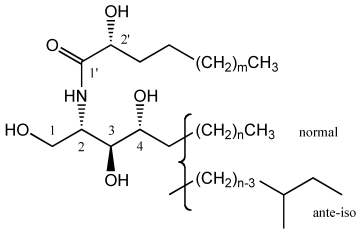


LMCer-1 : m = 10, 11, 16, 17, 18, 19 n = not identified
LMCer-1-1(**1**) : m = 11 n = 13 (ante-iso)



LMCer-2 : m = 11, 16, 17, 18, 19 n = not identified
LMCer-2-1(**2**) : m = 11 n = 13 (ante-iso)
LMCer-2-6(**3**) : m = 17 n = 10 (normal)
LMCer-2-7(**4**) : m = 17 n = 11 (ante-iso)

Fig. 1

ion peak $[M+Na]^+$ in the positive ion FAB-MS, but the other eleven fractions exhibited plural molecular ion peaks. Upon methanolysis, **1** afforded only FAM-2 and **1** also showed the signals of normal and *ante*-iso type[8] of terminal methyl groups in its ^{13}C-NMR spectrum. On the basis of the above data and the molecular mass of **1** (*m/z* 590 $[M+Na]^+$), the structure of **1** was determined to be (2*S*,3*R*,4*E*)-2-[(*R*)-2-hydroxyhexadecanoylamino]-16-methyl-4-octadecene-1,3-diol (Fig. 1).

Structure of Ceramide Molecular Species LMCer-2 LMCer-2 exhibited a series of $[M+Na]^+$ ion peaks at *m/z* 608, 622, 650, 664, 678, 692, 706, and 720 in its positive ion FAB-MS. In the ^{13}C-NMR spectrum, the characteristic signals of phytosphingosine-type ceramide possessing 2-hydroxy fatty acids were observed. Therefore, LMCer-2 must be a molecular species of phytosphingosine-type ceramide containing 2-hydroxy fatty acids. Furthermore, LMCer-2 was thought to possess normal and ante-iso[8] types side chains from the carbon signals for the terminal methyl groups the same as those of LMCer-1 (Table 1). The ^{1}H-NMR signals of the basic structure of LMCer-2 were in good agreement with those of known synthetic ceramide,[10] which is composed of (2*S*,3*S*,4*R*)-phytosphingosine and (*R*)-2-hydroxy fatty acid. The above fact and the optical rotations of the LMCer-2 series, LMCer-2-1, LMCer-2-6, LMCer-2-7 (+10.1 to +12.8) (vide infra) and the synthetic ceramide (+9.1)[10] suggested that LMCer-2 has the same absolute configuration as that of the synthetic one for the core structure, the 2,3,4,2′ part. When LMCer-2 was methanolyzed with methanolic hydrochloric acid, a mixture of FAM was obtained. GC-MS analysis of the FAM mixture shows the existence of five components, which were characterized as FAM-2, FAM-3, FAM-4, FAM-5, and FAM-6. The major FAM was FAM-4. Therefore, the structure of the ceramide molecular species LMCer-2 was determined to be that shown in Fig. 1.

Isolation and Structure of Ceramides from LMCer-2

Table 2. ^{13}C-NMR Spectral Data of **1**—**4** (δ Values in C_5D_5N)

Position	**1**	**2**	**3**	**4**
1 (t)	62.0	62.0	62.0	62.0
2 (d)	56.1	53.0	53.0	53.0
3 (d)	73.1	76.8	76.8	76.8
4 (d)	132.0	73.1	73.0	73.0
5 (d)	135.5			
1′ (s)	175.4	175.3	175.3	175.3
2′ (d)	72.5	72.4	72.4	72.4
$-CH_2\underline{C}H_2\underline{C}H_3$	14.2	14.2	14.2	14.2
$-CH(CH_3)CH_2\underline{C}H_3$	11.5	11.5		11.5
$-CH(\underline{C}H_3)CH_2CH$	19.3	19.3		19.3

By means of reversed-phase HPLC, LMCer-2 was separated into thirteen components and they were recovered to give the thirteen fractions LMCer-2-1 to LMCer-2-13. Three of the thirteen fractions, LMCer-2-1 (**2**), LMCer-2-6 (**3**), and LMCer-2-7 (**4**), revealed the single quasi-molecular ion peak $[M+Na]^+$ in the positive ion FAB-MS, and afforded homogeneous FAMs, FAM-2, FAM-4, and FAM-4, respectively, upon methanolysis. Compounds **2** and **4** showed the signals of normal and *ante*-iso type[8] of terminal methyl groups in its ^{13}C-NMR spectra while **3** revealed the signals of normal type[8] terminal methyl groups. On the basis of the above facts and the molecular mass of **2** (*m/z* 608 $[M+Na]^+$), **3** (*m/z* 650 $[M+Na]^+$) and **4** (*m/z* 664 $[M+Na]^+$), the structures of **2**, **3**, and **4** were determined to be (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxyhexadecanoylamino]-16-methyl-octadecane-1,3,4-triol, (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxydocosanoylamino]-hexadecane-1,3,4-triol, and (2*S*,3*S*,4*R*)-2-[(*R*)-2-hydroxydocosanoylamino]-14-methyl-hexadecane-1,3,4-triol.

To our knowledge, isolation and structure determination of the compounds **1**, **2**, **3**, and **4** has been made for the first time, although **1** and **2** have been reported as major components of ceramide molecular species from the viscera of a Pulmonate *Euhadra hickonis*.[11] Anti-hyperglycemic activity of the compounds **1**—**4** will be examined.

Experimental

Melting points were determined on a micro melting point apparatus (Yanako MP-3) without correction. Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 25 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on Jeol GX-270 (270, 67.8 MHz) and Varian Unity-500 spectrometers (500, 125 MHz) with the internal standard (pyridine-d_5 or chloroform-d). Positive ion FAB-MS spectra were acquired with a Jeol SX102A mass spectrometer [xenon atom beam; matrix, *m*-nitrobenzyl alcohol]. GC-MS were taken with a Shimadzu QP-5050A [EI mode; ionization potential, 70 eV; separator and ion-source temperature 300 °C; column, NEUTRA BOND-5 (ϕ 0.25 mm×30 m) (GL Science); carrier gas, He]. Column chromatography was performed on columns of either Silica gel 60 (Merck) or Silica gel BW-300 (Fuji Davison Co., Ltd.). TLC was performed on Silica gel $60F_{254}$ (Merck). HPLC was performed with PU-980 and RI-930 (Jasco) as a pump and RI detector, respectively.

Separation of LMCer-1 and LMCer-2 Whole bodies of the starfish *Luidia maculata* (wet weight 57 kg, collected in Hakata Bay in Fukuoka, Japan in May 1995) were homogenized and extracted with $CHCl_3$/MeOH (1 : 3, 80 l) followed by further extraction with $CHCl_3$/MeOH (1 : 2, 24 l, two times). The combined extracts were concentrated *in vacuo* to give a condensed extract (2 l). The extract was added to H_2O (43 l) and this aqueous suspension extracted with AcOEt/*n*-BuOH (2 : 1, 40 l) for separation of less polar lipids. The organic layer was concentrated *in vacuo*, and residue was washed with cold acetone (600 ml, four times) to give an acetone-insoluble fraction (337 g). A portion of the acetone-insoluble material (230 g) was partitioned between MeOH and *n*-hexane (1 : 1, 2 l, two times). The MeOH layer was concentrated *in vacuo* to give a MeOH extract (191.5 g). A portion

of the MeOH extract (103.8 g) was chromatographed on silica gel [solvent: $CHCl_3$/MeOH (97 : 3→95 : 5) for the first chromatography and $CHCl_3$/MeOH (100 : 0→98 : 2→95 : 5→90 : 10→85 : 15→70 : 30→6 : 4) for the second chromatography] and BW-300 [solvent: $CHCl_3$/EtOAc (1 : 1→2 : 3)] to afford LMCer-1 (390 mg), and LMCer-2 (411 mg). LMCer-1 and LMCer-2 each showed a single spot on silica gel TLC ($CHCl_3$/MeOH, 95 : 5); *Rf*=0.22 (LMCer-1), 0.14 (LMCer-2).

LMCer-1: Amorphous powder. IR (KBr) cm^{-1}: 3413 (hydroxyl), 1646, 1540 (amide). Positive ion FAB-MS *m/z*: 590, 616, 630, 644, 646, 656, 660, 670, 674, 688 $[M+Na]^+$ series. ^{1}H- and ^{13}C-NMR: See Table 1.

LMCer-2: Amorphous powder. IR (KBr) cm^{-1}: 3410 (hydroxyl), 1646, 1540 (amide). Positive ion FAB-MS *m/z*: 608, 622, 650, 664, 678, 692, 706, 720 $[M+Na]^+$ series. ^{1}H- and ^{13}C-NMR: See Table 1.

Methanolysis of LMCer-1 LMCer-1 (0.5 mg) was heated with 5% HCl in MeOH (0.5 ml) at 80 °C for 1 h in a sealed small-volume vial. The reaction mixture was extracted with *n*-hexane, and the hexane layer was concentrated to give a mixture of FAM for GC-MS analysis.

GC-MS Analysis of FAM from LMCer-1 The FAM mixture from LMCer-1 was subjected to GC-MS [column temperature 180—320 °C (rate of temperature increased 4 °C/min)]. The results were as follows: FAM-1 (methyl 2-hydroxypentadecanoate), t_R [min] (ratio of peak areas)=10.3 (2), *m/z* 272 (M^+), 213 $(M-59)^+$; FAM-2 (methyl 2-hydroxyhexadecanoate), t_R=12.3 (12), *m/z* 286 (M^+), 227 $(M-59)^+$; FAM-3 (methyl 2-hydroxyheneicosanoate), t_R=22.5 (3), *m/z* 356 (M^+), 297 $(M-59)^+$; FAM-4 (methyl 2-hydroxydocosanoate), t_R=24.5 (49), *m/z* 370 (M^+), 311 $(M-59)^+$; FAM-5 (methyl 2-hydroxytricosanoate), t_R=26.5 (27), *m/z* 384 (M^+), 325 $(M-59)^+$; FAM-6 (methyl 2-hydroxytetracosanoate), t_R=28.3 (7), *m/z* 398 (M^+), 339 $(M-59)^+$.

Isolation of Ceramides from LMCer-1 The ceramide molecular species LMCer-1 showed 12 peaks in the reversed phase HPLC [column, cosmosil $5C_{18}$ AR-II (10 mm×250 mm, Nacalai Tesque); solvent, MeOH; flow rate, 3 ml/min]. Using these conditions, 100 mg of LMCer-1 was separated to give 12 compounds: LMCer-1-1 (**1**) (4.2 mg, t_R=15.0 min), LMCer-1-2 (2.9 mg, t_R=16.5 min), LMCer-1-3 (9.8 mg, t_R=19.0 min), LMCer-1-4 (1.8 mg, t_R=20.5 min), LMCer-1-5 (4.1 mg, t_R=22.0 min), LMCer-1-6 (10.6 mg, t_R=24.0 min), LMCer-1-7 (6.0 mg, t_R=25.2 min), LMCer-1-8 (6.0 mg, t_R=27.5 min), LMCer-1-9 (8.6 mg, t_R=29.5 min), LMCer-1-10 (2.5 mg, t_R=31.5 min), LMCer-1-11 (9.6 mg, t_R=34.0 min), LMCer-1-12 (4.8 mg, t_R=39.5 min).

LMCer-1-1 (**1**): Amorphous powder, mp 72—75 °C. $[\alpha]_D$ +3.3° (*c*=0.38, $CHCl_3$). Positive-ion FAB-MS: *m/z* 590 $[M+Na]^+$. ^{13}C-NMR: See Table 2. Compound **1** was methanolyzed using the same method as described for LMCer-1 to yield FAM-2 (methyl 2-hydroxyhexadecanoate).

Methanolysis of LMCer-2 LMCer-2 (0.5 mg) was methanolyzed using the same method as described for LMCer-1 to give a mixture of FAM for GC-MS analysis.

GC-MS Analysis of FAM from LMCer-2 The FAM mixture from LMCer-2 was subjected to GC-MS under the same conditions as that of LMCer-1 to afford FAM-2 (ratio of peak areas, 2), FAM-3 (2), FAM-4 (54), FAM-5 (35), and FAM-6 (7).

Isolation of Ceramides from LMCer-2 The ceramide molecular species LMCer-2 showed 13 peaks in the reversed phase HPLC [column, cosmosil $5C_{18}$ AR-II (10 mm×250 mm, Nacalai Tesque); solvent, MeOH; flow rate, 3 ml/min]. Using these conditions, 100 mg of LMCer-2 was separated to give 13 compounds: LMCer-2-1 (**2**) (1.4 mg, t_R=13.0 min), LMCer-2-2 (0.7 mg, t_R=14.5 min), LMCer-2-3 (N.D., t_R=17.0 min), LMCer-2-4 (N.D., t_R=18.0 min), LMCer-2-5 (1.1 mg, t_R=19.0 min), LMCer-2-6 (**3**) (3.7 mg, t_R=21.0 min), LMCer-2-7 (**4**) (12.0 mg, t_R=22.0 min), LMCer-2-8 (4.3 mg, t_R=24.0 min), LMCer-2-9 (16.7 mg, t_R=25.0 min), LMCer-2-10 (2.2 mg, t_R=27.0 min), LMCer-2-11 (20.6 mg, t_R=29.0 min), LMCer-2-12 (0.9 mg, t_R=31.5 min), LMCer-2-13 (9.6 mg, t_R=34.0 min).

LMCer-2-1 (**2**): Amorphous powder, mp 125—128 °C. $[\alpha]_D$ +10.1° (*c*=0.13, pyridine). Positive-ion FAB-MS: *m/z* 608 $[M+Na]^+$. ^{13}C-NMR: See Table 2. Compound **2** was methanolyzed using the same method as described for LMCer-1 to yield FAM-2 (methyl 2-hydroxyhexadecanoate).

LMCer-2-6 (**3**): Amorphous powder, mp 127—137 °C. $[\alpha]_D$ +12.8° (*c*=0.33, pyridine). Positive-ion FAB-MS: *m/z* 650 $[M+Na]^+$. ^{13}C-NMR: See Table 2. Compound **3** was methanolyzed as above to yield FAM-4 (methyl 2-hydroxydocosanoate).

LMCer-2-7 (**4**): Amorphous powder, mp 135—137 °C. $[\alpha]_D$ +10.4° (*c*=1.1, pyridine). Positive-ion FAB-MS: *m/z* 664 $[M+Na]^+$. ^{13}C-NMR: See Table 2. Compound **4** was methanolyzed as above to yield FAM-4 (methyl 2-hydroxydocosanoate).

Biological Activity Anti-hyperglycemic activity of LMCer-1 and LMCer-2 was observed according to the method previously reported.[12] Statistically significant decrease of blood glucose levels and hemoglobin A1c levels were observed in diabetic mice.

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8) Normal means the straight chain [$-CH_2CH_2CH_2CH_3$], and *ante*-iso means the branched chain possessing a methyl group on the third carbon atom of the terminal methyl group [$-CH_2CH(CH_3)CH_2CH_3$].
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