Isolation and Structure of Monomethylated GM_3-Type Ganglioside Molecular Species from the Starfish *Luidia maculata*

Satoshi KAWATAKE,[a] Masanori INAGAKI,[a] Ryuichi ISOBE,[b] Tomofumi MIYAMOTO,[a] and Ryuichi HIGUCHI*,[a]

[a] *Faculty of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan: and* [b] *Department of Industrial Chemistry, Faculty of Engineering, Towa University; 1–1–1 Chikusigaoka, Minami-ku, Fukuoka 815–0036, Japan.* Received April 25, 2002; accepted June 20, 2002

Two monomethylated GM_3-Type ganglioside molecular species, 1 and 2, have been obtained from the polar lipid fraction of the chloroform/methanol extract of the starfish *Luidia maculata*. The structures of these gangliosides have been determined on the basis of chemical and spectroscopic evidence as 1-*O*-[8-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-ceramide (1) and 1-*O*-[8-*O*-methyl-(*N*-glycolyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-ceramide (2). The ceramide moieties were composed of heterogeneous unsubstituted fatty acid, 2-hydroxy fatty acid, sphingosine and phytosphingosine units. Compound 1, designated as LMG-3, represents new ganglioside molecular species. Compound 2 was a known ganglioside molecular species.

Key words glycosphingolipid; ganglioside; starfish; *Luidia maculata*

In the course of our continuing research on biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structure elucidation of the GSLs from starfish have been performed in our laboratory.[1—6] In the study of the GSLs of the starfish *Luidia maculata* (Yatsudesunahitode in Japanese), we reported on the isolation and structure of a sulfatide and a ganglioside molecular species, LMG-1[7] and LMG-2.[8] Continuing the previous studies, further isolation and characterization of gangliosides from *L. maculata* was conducted. In this paper, we report on the isolation and structure of two ganglioside molecular species from the whole bodies of *L. maculata*.

The polar lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *L. maculata*, was subjected to repeated column chromatography to give two ganglioside molecular species, **1** and **2**, each showing a single spot on silica gel thin-layer chromatography (TLC).

In its ^{13}C-NMR spectrum (Fig. 1, Table 1), **1** exhibits the characteristic signals of a sphingosine-type ceramide, possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ: 69.6 (C-1), 54.1 (C-2), 72.5 (C-3), 131.7 (C-4), 132.8 (C-5), 175.5 (C-1′), 72.5 (C-2′)]. The ^{13}C-NMR spectrum of **1** also features signals due to three anomeric carbons at δ: 106.0, 105.3 and 99.9, one of which (δ: 99.9) is a quaternary carbon signal, indicating the presence of a sialic acid residue. The negative FAB-MS exhibits a series of quasi-molecular ion peaks $[M-H]^-$ at *m/z*: 1230—1330. Therefore, **1** is suggested to be a molecular species of a sphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and three monosaccharide units. It is further presumed to have mainly normal-type fatty acids and normal and *ante*-iso type long-chain bases, since the carbon signals for the terminal methyl groups are observed at δ: 14.3 (normal form) and δ: 11.6 and 19.4 (*ante*-iso form) in the ^{13}C-NMR spectrum (Fig. 1, Table 1).

The structure of the ceramide moiety was examined first. When **1** was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAM) and long-chain bases (LCB) was obtained, together with methyl glycosides. The FAM mixture was analyzed by GC-MS, which revealed the presence of three components. These were characterized as methyl octadecanoate, methyl 2-hydroxydocosanoate (major) and methyl 2-hydroxytricosanoate. The LCB mixture was found to be composed of 2-amino-1,3-dihydroxy-4-octadecene, 2-amino-1,3-dihydroxy-4-nonadecene (major), 2-amino-1,3,4-trihydroxy-heptadecane and 2-amino-1,3,4-trihydroxy-nonadecane, based on GC-MS analysis of its TMS derivative (Fig. 1).

The stereochemistry of the ceramide moiety is presumed to be (2*S*, 3*R*, 4*E*, 2′*R*), since the aforementioned ^{13}C-NMR signals assignable to C-1, 2, 3, 4, 5 and 2′ of **1** are in good agreement with those of the sphingosine-type glucocerebroside molecular species possessing (2*S*, 3*R*, 4*E*, 2′*R*) configurations.[9]

The structure of the trisaccharide moiety of **1** was established as follows. The GLC analysis of the TMS derivatives of the methyl glycosides, which was obtained by methanolysis of **1** (*vide supra*), showed the existence of one mole each of glucose (Glc) and galactose (Gal). A detailed analysis of the ^{13}C-NMR spectrum of **1** revealed the characteristic signals [δ: 173.0 (C-1), 99.9 (C-2), 42.2 (C-3), 54.1 (C-5), 62.3 (C-9), 172.9 (C-10), and 22.9 (C-11)] of an *N*-acetylneuraminic acid (NeuAc) derivative residue together with the signal due to a methoxy group (δ: 58.7) (Table 1). The existence of the methoxy group in the NeuAc residue is also supported by the ^{1}H-NMR spectrum of **1** showing a singlet signal at δ: 3.78. In the negative FAB-MS of **1**, molecular ion and fragment ion peaks arising from cleavage of the glycosidic linkages are observed at *m/z*: 1230—1330, 930—1030, 770—850, and 600—650, indicating the presence of the trisaccharide moiety, NeuAc (OMe)→Hexose→Hexose, as shown in Fig. 3.

Methylation of **1**, according to the Ciucanu–Kerek method,[10] afforded the permethylated product **3a**. Partially methylated alditol acetates prepared from **3a** were analyzed by GC-MS and identified as the alditols derived from 3-linked hexopyranose (S-1) and 4-linked hexopyranose (S-2). The structure of the sialic acid moiety was established as follows. Since the sialic acid residue has a methoxy group, per-

* To whom correspondence should be addressed. e-mail: rhiguchi@ phar.kyushu-u.ac.jp

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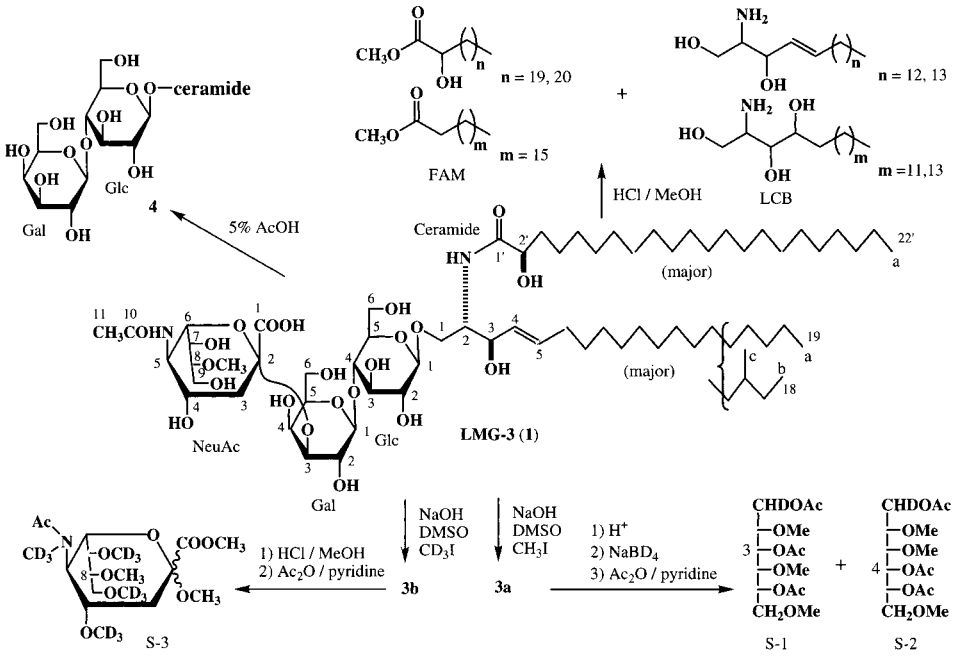

Fig. 1. Structure of Compound **1** (LMG-3)

Table 1. ^{13}C-HMR Spectral Data (δ Values) of the Gangliosides in C_5D_5N

C		**1**	**4**	**2**
Ceramide				
1	(t)	69.6	70.0	69.7
2	(d)	54.1	54.5	51.4
3	(d)	72.5	72.6	75.1
4	(d)	131.7	131.8	72.4
5	(d)	132.8	132.8	
1′	(s)	175.5	175.8	175.8
2′	(d)	72.5	72.6	72.4
CH_3[a)]	(q)	14.3	14.3	14.2
CH_3[b)]	(q)	11.6	11.6	11.5
CH_3[c)]	(q)	19.4	19.4	19.3
Glc				
1	(d)	105.3	105.0	104.9
2	(d)	74.6	74.7	74.4
3	(d)	76.3	76.6	76.2
4	(d)	81.8	81.8	82.0
5	(d)	76.4	77.3	76.2
6	(t)	61.7	62.1	61.7
Gal				
1	(d)	106.0	105.7	105.7
2	(d)	70.1	72.4	70.1
3	(d)	78.2	75.2	77.7
4	(d)	69.1	70.1	68.9
5	(d)	76.5	76.5	76.3
6	(t)	61.7	62.0	61.7
NeuAc (Gc)				
1	(s)	173.0[d)]		173.7[e)]
2	(s)	99.9		100.6
3	(t)	42.2		42.4
4	(d)	68.5		68.2
5	(d)	54.1		53.8
6	(d)	75.6		74.4
7	(d)	69.2		69.7
8	(d)	81.8		81.8
9	(t)	62.3		61.7
10	(s)	172.9[d)]		175.5[e)]
11	(q, t)	22.9		62.5
OCH_3	(q)	58.7		58.6

a—c) Terminal methyl groups in the normal and *ante*-iso type of side chain (see Fig. 1). *d*, *e*) Assignments may be interchanged in each vertical column.

trideuteriomethylated product **3b** was prepared. Upon methanolysis followed by acetylation of **3b**, the partially trideuteriomethylated NeuAc (S-3) derived from the terminal 8-*O*-Me-NeuAc was detected by GC-MS analysis. **1** was hydrolyzed with 5% aq. AcOH to give a ceramide dihexoside **4**, which was determined as ceramide lactoside [1-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl ceramide] by comparison of the ^{13}C-NMR spectral data of sugar moiety with a reference compound.[11)]

On the basis of the above evidence, the trisaccharide moiety of **1** must be 8-*O*-Me-NeuAc-(2→3)-β-galactopyranose-(1→4)-β-glucopyranose. The configuration of NeuAc is believed to be α on the basis of its anomeric carbon signal (δ: 99.9)[1,12)] in the ^{13}C-NMR spectrum of **1**. Consequently, if NeuAc, Gal and Glc are assumed to belong to the most commonly found D-series, respectively, then **1** is the 8-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside of a ceramide, composed of heterogeneous fatty acid and long-chain base units. The major components of the fatty acid and long-chain base moiety of **1** are (2*R*)-2-hydroxydocosanoic acid and (2*S*,3*R*,4*E*)-C_{19}-sphingosine, respectively (Fig. 1).

To the best of our knowledge, compound (**1**) represents a new GM_3 type ganglioside molecular species found to contain a monomethylated NeuAc moiety, and designated as LMG-3. Compound **2** was identified as the 8-*O*-methyl-(*N*-glycolyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside of a ceramide composed of (2*S*,3*S*,4*R*)-C_{17}-phytosphingosine and (2*R*)-2-hydroxydocosanoic acid as major components (see Fig. 2), on the basis of chemical and spectroscopic evidence like **1**. Compound (**2**) has already been obtained from the starfish *Aphelasterias japonica*[13)] and *Asterias amurensis versicolor*,[2)] although it differs from them slightly in the ceramide moiety. The biological activity of these gangliosides will be examined in due course.

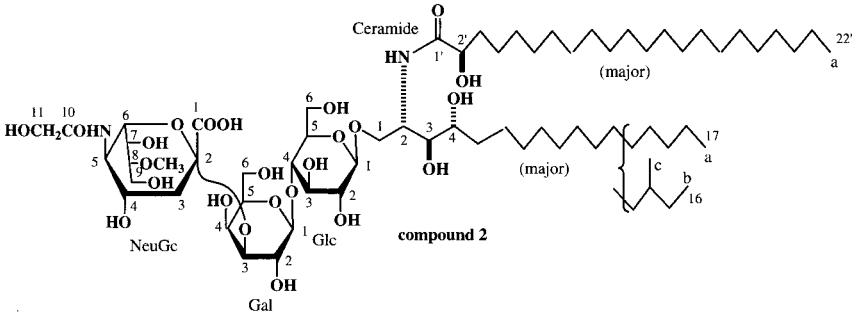

Fig. 2. Structure of Compound **2**

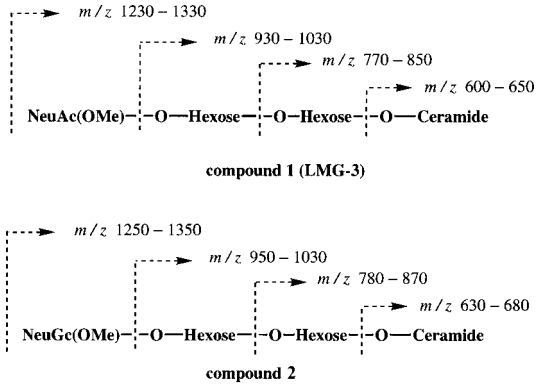

Fig. 3. The Negative Ion FAB-MS Fragmentation of Compounds **1** and **2**

Experimental

Melting points were determined on a micro melting point apparatus (Yanako MP-3) without correction. IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. NMR spectra were recorded on a Varian Unity-500 spectrometer (^{1}H: 500 MHz, ^{13}C: 125 MHz). Negative-ion FAB-MS spectra were acquired with a JEOL SX-102 mass spectrometer (xenon atom beam; matrix, HMPA-TEG). GC-MS was taken with a Shimadzu QP-1000 [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 °C; column (A), TC-1701 (0.25 mm×30 m, GL Science Inc.); column (B), CBP10-W12-100 (0.53 mm×12 m, Shimadzu); carrier gas, He]. GC was run on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.32 mm×30 m, J & W Scientific); carrier, N_2].

Separation of 1 and 2 For the extraction and fractionation of the crude ganglioside fraction from the whole bodies of the starfish *Luidia maculata* (17 kg), the preceding paper should be referred to.[8] The crude ganglioside fraction, the 100% MeOH eluate, was successively separated by chromatography on silica gel (solvent $CHCl_3$–MeOH–7% NH_3 aq., 6 : 4 : 1) to afford **1** (13 mg) (*Rf*=0.38) and **2** (31 mg) (*Rf*=0.36) [silica gel TLC, solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 0.9)].

Compound **1** (LMG-3): Amorphous powder, mp 154—160 °C. IR (KBr) cm^{-1}: 3387 (OH), 1645, 1542 (amide). Negative-ion FAB-MS *m/z*: 1230—1330 $[M-H]^-$ series (see Fig. 3). ^{1}H-NMR (C_5D_5N) δ: 1.94 (3H, s, $COCH_3$), 3.78 (3H, s, OMe). ^{13}C-NMR: See Table 1.

Compound **2**: Amorphous powder, mp 135—140 °C. IR (KBr) cm^{-1}: 3397 (OH), 1647, 1541 (amide). Negative-ion FAB-MS *m/z*: 1250—1350 $[M-H]^-$ series (see Fig. 3). ^{1}H-NMR (C_5D_5N) δ: 3.80 (3H, s, OMe). ^{13}C-NMR: See Table 1.

Methanolysis of 1 Compound **1** (1 mg) was heated with 5% HCl in MeOH (1 ml) at 70 °C for 12 h. The reaction mixture was then extracted with *n*-hexane, and the extract was concentrated *in vacuo* to yield a mixture of FAM. The MeOH layer was concentrated under a stream of N_2 to give a mixture of LCB and methyl glycosides.

GC-MS Analysis of FAM from 1 A FAM mixture from **1** was subjected to GC-MS [column (A), column temp. 180—250 °C (rate of temp. increase 4 °C/min)]. The results were as follows: methyl octadecanoate, t_R [min]=3.6, *m/z*: 298 (M^+), 255 $(M-49)^+$; methyl 2-hydroxydocosanoate (major), t_R=8.6, *m/z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=9.4, *m/z*: 384 (M^+), 325 $(M-59)^+$.

GC-MS Analysis of TMS Ethers of LCB from 1 The mixture of LCB and methyl glycoside from **1** was heated with 1-(trimethylsilyl) imidazole–pyridine (1 : 1) for 30 min at 70 °C and the reaction mixture (TMS ethers) was analyzed by GC-MS [column (A), column temp. 180—250 °C (rate of temp. increase 4 °C/min)]. The results were as follows: 2-amino-1,3-dihydroxy-4-octadecene, t_R [min]=7.9, *m/z*: 340 $(M-103)^+$, 132; 2-amino-1,3,4-trihydroxy-heptadecane, t_R=8.8, *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3-dihydroxy-4-nonadecene (major), t_R=9.4, *m/z*: 354 $(M-103)^+$, 132; 2-amino-1,3,4-trihydroxy-nonadecane, t_R=11.9, *m/z*: 354 $(M-193)^+$, 313 $(M-234)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycoside from 1 The mixture of TMS ethers of LCB and methyl glycosides was analyzed by GC [column temp.: 100—250 °C (rate of temp. increase 3 °C/min)]: t_R [min]=22.0 and 22.7 (methyl galactopyranosides); t_R=23.8 and 24.0 (methyl glucopyranosides).

Methylation of 1 (Ciucanu–Kerek Method) NaOH–dimethylsulfoxide (DMSO) solution, which was prepared from powdered NaOH (40 mg) and DMSO (1 ml), and CH_3I (or CD_3I) (0.2 ml) were added to **1** (2 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with H_2O (15 ml), extracted with $CHCl_3$ (10 ml×3), and the $CHCl_3$ phases were washed with H_2O, and the solvent was evaporated *in vacuo* to give permethylated (or pertrideuteriomethylated) **1**, denoted **3a** (or **3b**).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 3a Compound **3a** (0.7 mg) was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (1 ml) at 70 °C for 18 h in a small-volume sealed vial, and then the solvents were evaporated *in vacuo*. The residue was dissolved in H_2O (5 ml), and 28% NH_3 (2 drops), and $NaBD_4$ (10 mg) were added. After allowing the mixture to stand at room temp. for 7 h, it was acidified with AcOH to pH=3.5 and concentrated *in vacuo*. H_3BO_3 present in the residue was removed by distillation with MeOH (three times). The residue was heated with Ac_2O–C_5H_5N (1 : 1, 0.3 ml) at 70 °C for 2 h. After dilution with H_2O, the mixture was extracted with $CHCl_3$ (0.2 ml×3). The combined $CHCl_3$ extracts were washed with H_2O, and the solvent was evaporated to give partially methylated alditol acetate. The acetate was subjected to GC-MS [column (A), column temp. 150 °C], with the following results: S-1, t_R [min]=14.3, *m/z*: 45, 118, 161, 234 [1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (derived from 3-linked hexopyranose)]; S-2, t_R=15.3, *m/z*: 45, 118, 233 [1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (derived from 4-linked hexopyranose)].

Preparation and GC-MS Analysis of Partially Trideuteriomethylated NeuAc Derivative from 3b Compound **3b** (0.5 mg) was heated with 5% HCl in MeOH (0.5 ml) at 70 °C for 4 h in a small-volume sealed vial. The reaction mixture was then concentrated *in vacuo*, and the residue (methanolysate) was heated with Ac_2O–C_5H_5N (1 : 1, 0.2 ml) at 70 °C for 2 h. The resulting mixture was diluted with H_2O and extracted with $CHCl_3$ (0.2 ml×3), the combined $CHCl_3$ extracts were washed with H_2O, and the solvent was evaporated *in vacuo*. The residue was subjected to GC-MS [column (B), column temp.: 180—250 °C (rate of temp. increase 4 °C/min)]: S-3, t_R=4.8 min, *m/z*: 135, 280, 327, 360, 371, 388 [methyl *N*-acetyl-*N*-trideuteriomethyl-2,8-di-*O*-methyl-4,7,9-tri-*O*-trideuteriomethylneuraminate (derived from terminal 8-*O*-Me-NeuAc)].

Partial Hydrolysis of 1 Compound **1** (5 mg) was heated with 5% aq. AcOH (3 ml) at 90 °C for 4 h. The mixture was then extracted with AcOEt/*n*-BuOH (2 : 1), the organic layer was concentrated *in vacuo*, and the residue was chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (8 : 2 : 0.1)] to give **4** (2 mg) as an amorphous powder (^{13}C-NMR: Table 1).

Acknowledgements We thank Mr. Y. Tanaka and Ms. Y. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for NMR measurement. This work was supported in part by Grant-in-Aids of Scientific Research Nos. 12045253 (Priority Area A), 13780468, and 13024260 (Priority Area A) from the Ministry of Education, Culture, Science, Sports and Technology, Japan, which are gratefully acknowledged.

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