Isolation and Structure of a GD_3-Type Ganglioside Molecular Species Possessing Neuritogenic Activity from the Starfish *Luidia maculata*

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A GD_3-type ganglioside molecular species, LMG-4 (1), has been obtained from the polar lipid fraction of the chloroform/methanol extract of the starfish *Luidia maculata*. The structure of this ganglioside has been determined on the basis of chemical and spectroscopic evidence to be 1-*O*-[(*N*-acetyl-α-D-neuraminosyl)-(2→8)-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-ceramide. The ceramide moiety was composed of heterogeneous 2-hydroxy fatty acid and phytosphingosine units. This is the first report on the isolation and structure elucidation of GD_3-type ganglioside from echinoderms. Moreover, 1 exhibited neuritogenic activity toward the rat pheochromocytoma PC12 cells in the presence of nerve growth factor.

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 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, structure and biological activities of cerebrosides,[7] ceramide lactosides,[8] sulfatide,[9] and gangliosides.[10,11] Continuing the previous studies, further isolation and characterization of the more polar ganglioside from *L. maculata* were conducted. In this paper, we report on the isolation and structure of a ganglioside molecular species from the whole bodies of *L. maculata*. The biological activity of the ganglioside is also reported.

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 a polar compound, designated LMG-4 (**1**), showing a single spot on silica gel thin-layer chromatography (TLC).

Compound **1** shows strong hydroxy and amide absorptions in the IR spectrum and has a positive reaction against resorcinol reagent.[12] The negative FAB-MS exhibits a series of quasimolecular ion peaks $[M-H]^-$ at *m*/*z*: 1500—1620. Therefore **1** is suggested to be a molecular species of ganglioside.

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 (LCBs) was obtained. The FAM mixture was analyzed by GC-MS, which revealed the presence of three components. These were characterized as methyl 2-hydroxydocosanoate (major), methyl 2-hydroxytricosanoate, and methyl 2-hydroxytetracosanoate. The LCB mixture was found to be composed of such phytosphingosines as 2-amino-1,3,4-trihydroxy-heptadecane (major), 2-amino-1,3,4-trihydroxy-octadecane, and 2-amino-1,3,4-trihydroxy-nonadecane, based on GC-MS analysis of its TMS derivative (Chart 1).

The structure of the sugar moiety of **1** was established as follows. The GC analysis of hexitol acetate derivatives of the neutral sugars, which were obtained by hydrolysis, reduction, and acetylation of **1**, showed the existence of one mole each of glucose (Glc) and galactose (Gal). 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*: 1500—1620, 1220—1320, 950—1020, 780—860, and 630—680, indicating the presence of the tetrasaccharide moiety, NeuAc→NeuAc→Hexose→Hexose, as shown in Fig. 1.

Methylation of **1**, according to the Ciucanu–Kerek method,[13] afforded the permethylated product **2**. Partially methylated alditol acetates (S-1, S-2) prepared from **2** were analyzed using GC-MS and identified as the alditols derived from 3-linked hexopyranose (S-1) and 4-linked hexopyranose (S-2). Compound **2** was subsequently methanolyzed and acetylated, and the permethylated NeuAc (S-3) derived from the terminal NeuAc and the acetate of partially methylated NeuAc (S-4) originating from 8-linked NeuAc, were detected in the ratio of 1 : 1 in GC-MS analysis. On the other hand, **1** was hydrolyzed with 5% aqueous AcOH to give a ceramide lactoside by comparison with a reference compound.[8]

On the basis of the above evidence, the tetrasaccharide moiety of **1** must be NeuAc-(2→8)-NeuAc-(2→3)-β-galactopyranose-(1→4)-β-glucopyranose. The configuration of NeuAc is believed to be α, which is usual in gangliosides.[14] Consequently, if NeuAc, Gal, and Glc are assumed to belong to the most commonly found D-series, then **1** is the (*N*-acetyl-α-D-neuraminosyl)-(2→8)-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside of a ceramide, composed of heterogeneous fatty acid and LCB units. The major components of the fatty acid and LCB moiety of **1** are 2-hydroxydocosanoic acid and C_{17}-phytosphingosine, respectively, as shown in Chart 1.

The effect of **1** on the neuritogenesis of the rat pheochromocytoma cell line (PC 12 cells) has been investigated. The results showed that **1** displayed neuritogenic activity in the presence of nerve growth factor (NGF). The proportion of the neurite-bearing cells of **1** at a concentration of 10 μM was 47.7% when compared with the control (NGF, 5 ng/ml: 20.6%). Furthermore, the effect of **1** was the same as that of

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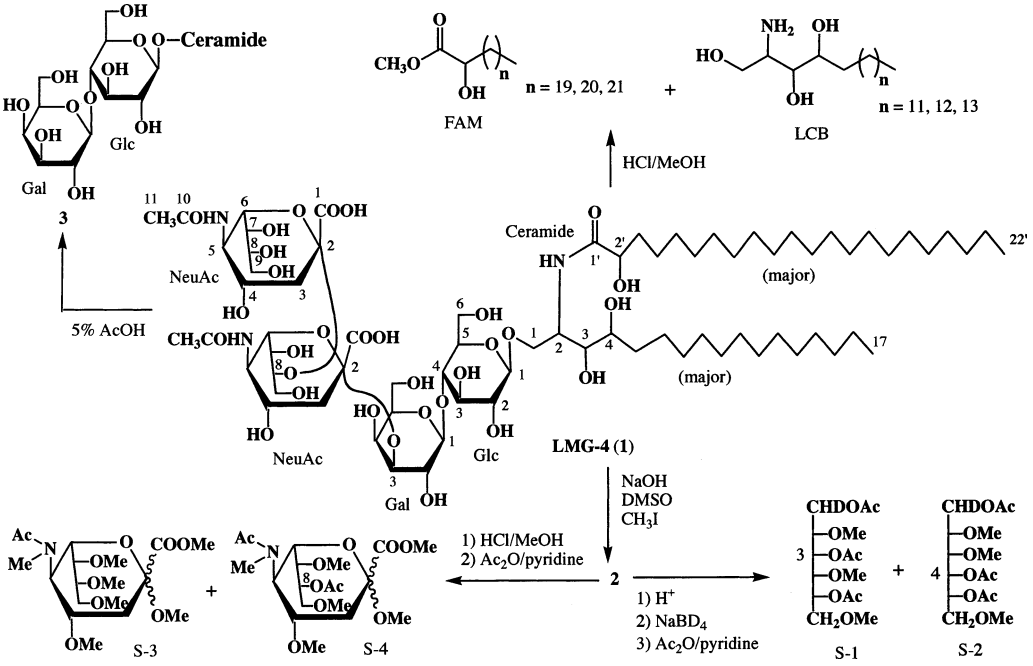

Chart 1

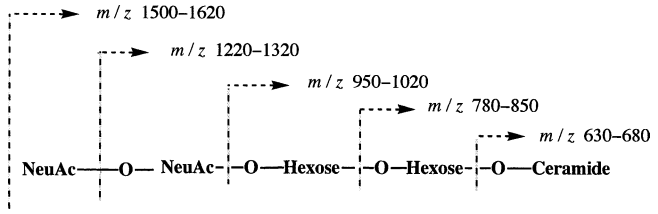

Fig. 1. Negative Ion FAB-MS Fragmentation of Compound **1** (LMG-4)

the mammalian ganglioside GM_1 (47.0%).

To the best of our knowledge, LMG-4 (**1**) is the first GD_3-type ganglioside reported from echinoderms. The isolation and characterization of such a neuritogenically active ganglioside is attracting considerable attention with regard to the manufacture of new medicines from marine natural products.

Experimental

IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. Negative-ion FAB-MS spectra were acquired with a JEOL SX-102 mass spectrometer (xenon atom beam; matrix, HMPA-TEG). GC-MS were recorded with a Shimadzu QP-5050A and 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.) for QP-5050A; column (B), CBP10-W12-100 (0.53 mm×12 m, Shimadzu) for QP-1000; 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]. For the NMR measurement, fine spectra could not be obtained for reasons not understood.

Separation of 1 For the extraction and fractionation of the crude ganglioside fraction from the whole bodies of the starfish *L. maculata* (40 kg), the previous paper should be referred to.[10] The crude ganglioside fraction, the 90% MeOH eluate, was successively separated by chromatography on silica gel (solvent $CHCl_3$–MeOH–7% NH_3 aq, 6 : 4 : 1) to afford **1** (30 mg) (*Rf*=0.26) [silica gel TLC, solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 0.9)].

Compound **1** (LMG-4): Amorphous powder. IR (KBr) cm^{-1}: 3399 (OH), 1637 (amide). Negative-ion FAB-MS *m/z*: 1500—1620 $[M-H]^-$ series (see Fig. 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.

GC-MS Analysis of FAM from 1 A FAM mixture from **1** was subjected to GC-MS [column (A), column temperature 180—250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: methyl 2-hydroxydocosanoate (major), t_R [min]=7.2, *m/z*, 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=8.7, *m/z*, 384 (M^+), 325 $(M-59)^+$, methyl 2-hydroxytetracosanoate, t_R=10.2, *m/z*, 398 (M^+), 339 $(M-59)^+$.

GC-MS Analysis of TMS Ethers of LCB from 1 The mixture of LCB 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 temperature 180—250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: 2-amino-1,3,4-trihydroxy-heptadecane (major), t_R [min]=3.9, *m/z*, 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-octadecane, t_R=4.6, *m/z*, 340 $(M-193)^+$, 299 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-nonadecane, t_R [min]=5.6, *m/z*, 354 $(M-193)^+$, 313 $(M-234)^+$, 132.

GC Analysis of Neutral Sugars from 1 Compound **1** (1 mg) was heated with 2 N HCl (1 ml) at 90 °C for 22 h in a small-volume sealed vial. The reaction mixture was extracted with *n*-hexane and the aqueous layer concentrated *in vacuo*. The residue was dissolved in H_2O (1 ml), and then 28% NH_3 (2 drops) and $NaBH_4$ (40 mg) were added to the solution. After standing at room temperature for 5 h, the reaction mixture was acidified with AcOH to pH 3.5 and concentrated *in vacuo*. H_3BO_3 contained 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, and the mixture was concentrated *in vacuo*. The residue was extracted with $CHCl_3$ (1 ml), the $CHCl_3$ solution was washed with H_2O, dried (Na_2SO_4), and the solvent evaporated to give alditol acetates. These acetates were subjected to GC (column temperature 200 °C). The results were as follows: t_R [min]=20.6 (1 mol) (1,2,3,4,5,6-hexa-*O*-acetyl galactitol); t_R=21.3 (1 mol) (1,2,3,4,5,6-hexa-*O*-acetyl glucitol).

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 (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), the $CHCl_3$ phases were washed with H_2O, and the solvent was evaporated *in vacuo* to give permethylated **1**, denoted as **2**.

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 2 Compound **2** (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 temperature 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 (B), column temperature 150 °C], with the following results: S-1, t_R [min]=18.5, *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=19.5, *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 Acetate of Partially Methylated NeuAc Derivative from 2 Compound **2** (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 (QP-1000) [column, glass column (3 mm×1.2 m) packed with 2% OV-1 on Chromosorb W (60—80 mesh); column temperature, 180—250 °C (rate of temperature increase 4 °C/min)] ; S-3, t_R [min]=9.4 (1 mol), *m*/*z*, 129, 201, 254, 298, 318, 348 [methyl *N*-acetyl-*N*-methyl-2,4,7,8,9-tetra-*O*-methylneuraminate (derived from terminal NeuAc)]; S-4, t_R=11.3 (1 mol), *m*/*z*, 129, 201, 254, 318, 376 [methyl *N*-acetyl-8-*O*-acetyl-*N*-methyl-2,4,7,9-tetra-*O*-methylneuraminate (derived from 8-linked NeuAc)].

Partial Hydrolysis of 1 Compound **1** was heated with 5% aqueous AcOH 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 **3**. Compound **3** was identified as ceramide lactoside by comparison with a reference compound[8] [silica gel TLC, *Rf*=0.68, solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 0.9)].

Biological Assay Neuritogenic activity of **1** in PC12 cells was observed according to the method previously reported.[16]

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