Isolation and Structure of Hematoside-Type Ganglioside from the Starfish *Linckia laevigata*

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A hematoside-type ganglioside, LLG-1 (1), has been obtained from the polar lipid fraction of the chloroform/methanol extract of the starfish *Linckia laevigata*. The structure of the ganglioside has been determined on the basis of chemical and spectroscopic evidence as 1-*O*-[*N*-glycolyl-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 naked hematoside-type ganglioside from echinoderms.

Key words glycosphingolipid; ganglioside; hematoside; starfish; *Linckia laevigata*

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] In the study of the GSLs of the starfish *Linckia laevigata* (Aohitode in Japanese), we reported on the isolation and structure of cerebrosides and gangliosides.[2—4] Continuing the previous studies, further isolation and characterization of gangliosides from *L. laevigata* was conducted. In this paper, we report on the isolation and structure of newly isolated ganglioside LLG-1 (**1**) from the whole bodies of *L. laevigata*.

The polar lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *L. laevigata*, was subjected to repeated column chromatography to give a ganglioside **1** 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 phytosphingosine-type ceramide,[5] possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ: 70.5 (C-1), 51.4 (C-2), 75.7 (C-3), 72.5 (C-4), 175.9 (C-1′) and 72.5 (C-2′)]. The ^{13}C-NMR spectrum of **1** also features signals due to three anomeric carbons at δ: 105.5, 104.5 and 100.8, one of which (δ: 100.8) is a quaternary carbon signal, indicating the presence of a sialic acid residue. Therefore, **1** is suggested to be a phytosphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and three monosaccharide units. It is further presumed to have normal-type fatty acids and normal- and iso-type long-chain bases, since the carbon signals for the terminal methyl groups are observed at δ 14.1 (normal form) and 22.8 (iso form)[6] 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 five components. These were characterized as methyl 2-hydroxyhexadecanoate, methyl 2-hydroxyheptadecanoate, methyl 2-hydroxydocosanoate (major), methyl 2-hydroxytricosanoate, methyl 2-hydroxytetracosanoate. The LCB mixture was found to be composed of 2-amino-1,3,4-trihydroxy-hexadecane, 2-amino-1,3,4-trihydroxy-heptadecane (major), 2-amino-1,3,4-trihydroxy-octadecane, 2-amino-1,3,4-trihydroxy-nonadecane, based on GC-MS analysis of its trimethylsilyl (TMS) derivative.

The stereochemistry of the ceramide moiety is presumed to be (2*S*,3*S*,4*R*,2′*R*), since the aforementioned ^{13}C-NMR signals assignable to C-1, 2, 3, 4 and 2′ of **1** are in good agreement with those of the synthetic phytosphingosine-type β-lactosyl ceramide, (2*S*,3*S*,4*R*)-1-*O*-[β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-2-[(2*R*)-2-hydroxytetracosanoylamino]-1,3,4-trihydroxy-hexadecane (compound **3**)[5] as shown in Table 1.

The structure of the trisaccharide moiety of **1** was estab-

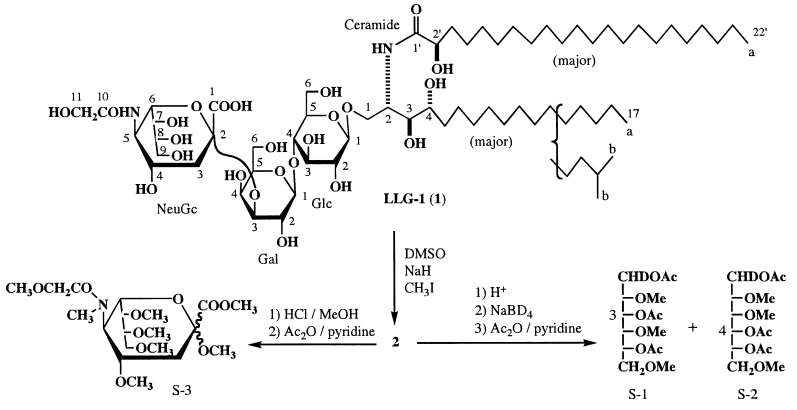

Fig. 1. Structure of Compound **1** (LLG-1)

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Table 1. ^{13}C-NMR Spectral Data (δ Values) of Compounds **1** and **3** in C_5D_5N

C	**1**	**3**
Ceramide		
1 (t)	70.5	70.3
2 (d)	51.4	51.6
3 (d)	75.7	75.8
4 (d)	72.5	72.6
1′ (s)	175.9	175.6
2′ (d)	72.5	72.6
CH_3[a] (q)	14.1	14.3
CH_3[b] (q)	22.8	
Glc		
1 (d)	104.5	105.0
2 (d)	74.7	74.6
3 (d)	76.2	76.5
4 (d)	81.8	81.7
5 (d)	77.0	77.2
6 (t)	61.5[c]	62.1[d]
Gal		
1 (d)	105.5	105.8
2 (d)	69.8	72.4
3 (d)	77.7	75.2
4 (d)	68.9	70.1
5 (d)	76.2	76.6
6 (t)	62.0[c]	62.0[d]
NeuGc		
1 (s)	174.0	
2 (s)	100.8	
3 (t)	42.9	
4 (d)	68.2	
5 (d)	53.7	
6 (d)	74.0	
7 (d)	70.0	
8 (d)	73.0	
9 (t)	64.0	
10 (s)	176.8	
11 (t)	62.5[c]	

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

lished 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 1 mol each of glucose (Glc) and galactose (Gal). A detailed analysis of the ^{13}C-NMR spectrum of **1** revealed the characteristic signals [δ: 174.0 (C-1), 100.8 (C-2), 42.9 (C-3), 53.7 (C-5), 64.0 (C-9), 176.8 (C-10), and 62.5 (C-11)] of an *N*-glycolylneuraminic acid (NeuGc) derivative residue (Table 1). In the negative FAB-MS of **1**, the molecular ion and fragment ion peaks arising from cleavage of the glycosidic linkages of the major component are observed at *m*/*z* 1271, 964, 802, and 640, indicating the presence of a trisaccharide moiety, NeuGc→hexose→hexose, as shown in Fig. 2.

When the signals due to the sugar moieties of **1** and **3** (ceramide β-lactoside) in their ^{13}C-NMR spectra were compared, they were nearly identical except for the signals ascribable to NeuGc and C-3 of Gal (Table 1). The downfield signal for C-3 (δ 77.7) of the Gal unit resulting from glycosylation[7,8] in the ^{13}C-NMR spectrum of **1** indicates that the NeuGc residue is located at C-3 of the Gal unit. Therefore the trisaccharide moiety of **1** must be NeuGc-(2→3)-β-Gal-(1→4)-β-Glc. Furthermore, chemical degradation of **2**, permethylated **1** prepared by the Hakomori method,[9] yielded the alditols derived from the 3-linked and 4-linked hexopyranoses (S-1 and S-2) and the permethylated NeuGc (S-3) derived from the terminal NeuGc, and which confirmed the structure of the trisaccharide (Fig. 1).

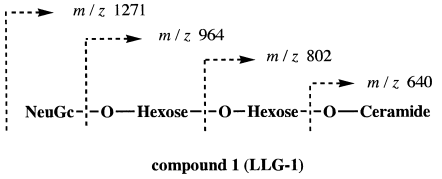

Fig. 2. Negative FAB Mass Fragmentation of the Major Component of Compound **1**

The configuration of NeuGc is believed to be α on the basis of its anomeric carbon signal (δ 100.8)[10] in the ^{13}C-NMR spectrum of **1**. In addition, the absolute configurations of Glc, Gal and NeuGc were verified as being the D-form using the methods of Hara *et al.*[11] and Kisa *et al.*[12]

Consequently, compound **1** is the (*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, as shown in Fig. 1, and designated as LLG-1.

The isolation of monomethylated hematoside-type gangliosides from starfish has already been reported.[13—16] To the best of our knowledge, this is the first report on the isolation and structure elucidation of naked hematoside-type ganglioside, NeuGcα(2→3)Galβ(1→4)Glcβ(1→1)Cer, from echinoderms. The isolation and characterization of such a ganglioside are attracting considerable attention with regard to the development of new medicines from natural marine products.

Experimental

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-5050A [EI mode; ionizing potential, 70 eV; column, NEUTRABOND-D (0.25 mm×30 m, GC Science); carrier gas, He].

Separation of 1 For the extraction and fractionation of the crude polar glycosphingolipid fraction from the whole bodies of the starfish *Linckia laevigata* (15 kg), collected at Okinawa, Japan, in 2000, the preceding paper should be referred to.[4] The crude lipid fraction was successively separated by chromatography on silica gel [solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 0.3→6 : 4 : 0.5→6 : 4 : 0.7→6 : 4 : 1)] to afford **1** (12 mg) (*Rf*=0.50) together with known compounds LLG-3[3] (42 mg) (*Rf*=0.37) and LLG-5[4] (17 mg) (*Rf*=0.23) [silica gel TLC, solvent $CHCl_3$–MeOH–H_2O (6 : 4 : 1)].

Compound **1** (LLG-1): Amorphous powder. Negative-ion FAB-MS: see Fig. 2. ^{13}C-NMR: see Table 1.

Methanolysis of 1 Compound **1** (1 mg) was heated with 5% HCl in MeOH (1 ml) at 90 °C for 20 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 neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo* 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 temperature: 180—320 °C (rate of temperature increase 4 °C/min)]. The results were as follows: methyl 2-hydroxyhexadecanoate, t_R [min] (ratio of peak areas)=12.3 (13.5), *m*/*z*: 286 (M^+), 227 $(M-59)^+$; methyl 2-hydroxyheptadecanoate, t_R=14.4 (8.4), *m*/*z*: 300 (M^+), 241 $(M-59)^+$; methyl 2-hydroxydocosanoate, t_R=24.7 (36.5), *m*/*z*: 370 (M^+), 311 $(M-59)^+$; methyl 2-hydroxytricosanoate, t_R=26.6 (27.9), *m*/*z*: 384 (M^+), 325 $(M-59)^+$; methyl 2-hydroxytetracosanoate, t_R=28.5 (13.7), *m*/*z*: 398 (M^+), 339 $(M-59)^+$.

GC-MS Analysis of TMS Ethers of LCB from 1 The mixture of LCB and methyl glycosides from **1** was heated with 1-(trimethylsilyl) imida-

zole–pyridine (1 : 1) for 15 min at 70 °C and the reaction mixture (TMS ethers) was analyzed by GC-MS [column temperature: 180—320 °C (rate of temperature increase 8 °C/min)]. The results were as follows: 2-amino-1,3,4-trihydroxy-hexadecane, t_R [min] (ratio of peak areas)=13.7 (17.4), *m/z*: 312 $(M-193)^+$, 271 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-heptadecane, t_R=14.4 (45.0), *m/z*: 326 $(M-193)^+$, 285 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-octadecane, t_R=15.3 (30.9), *m/z*: 340 $(M-193)^+$, 299 $(M-234)^+$, 132; 2-amino-1,3,4-trihydroxy-nonadecane, t_R=16.4 (6.7), *m/z*: 354 $(M-193)^+$, 313 $(M-234)^+$, 132.

GC Analysis of TMS Ethers of Methyl Glycosides from 1 The mixture of TMS ethers of LCB and methyl glycosides from **1** was analyzed using GC-MS [column temperature: 150—320 °C (rate of temperature increase 4 °C/min)]: t_R [min]=16.2, 17.5, 18.6 (methyl Gal); 19.5, 20.2 (methyl Glc).

Determination of Absolute Configuration of Glc and Gal Moieties of 1 (Method of Hara *et al.*[11]) Compound **1** (0.7 mg) was heated with 2 N HCl (1 ml) at 90 °C for 24 h. The reaction mixture was then extracted with *n*-hexane, and the acidic aqueous phase was concentrated under N_2 stream. The residue (sugar fraction) was heated with L-cysteine methyl ester hydrochloride (0.5 mg) and pyridine (0.3 ml) at 70 °C for 1 h. Then, 0.1 ml of 1-(trimethylsilyl) imidazole was added and the mixture was heated at 60 °C for a further 30 min to yield trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivatives. The derivatives were analyzed using GC-MS [column temperature: 180—250 °C (rate of temperature increase 2.5 °C/min)]; t_R [min]=27.9 and 28.5 (derivative of D-Glc, 27.9; L-Glc, 28.3; D-Gal, 28.5; L-Gal, 29.6).

Determination of Absolute Configuration of NeuGc of 1 (Method of Kisa *et al.*[12]) Compound **1** (2 mg) was heated with 10% HCl in MeOH (0.5 ml) at 80 °C for 24 h and the reaction mixture was then extracted with *n*-hexane. The MeOH layer was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in DMF (0.5 ml), and acetone (0.5 ml) and 10% HCl in MeOH (20 μl) were added. The mixture was heated at 80 °C for 6 h, neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*. To the solution of the product in H_2O (0.5 ml), $NaIO_4$ (1.0 mg) was added and stirred at room temperature for 1 h. The reaction mixture was diluted with H_2O (1 ml) and a small amount of ethylene glycol, and extracted with *n*-BuOH (1 ml×3). The organic layer was concentrated *in vacuo*, the reaction product was heated with 1 N HCl (1 ml) at 100 °C for 20 h, and the reaction mixture was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*. The arabinose from NeuGc, contained in the reaction product, was induced to the thiazolidine derivative using the above mentioned method of Hara *et al.*, and the derivative was analyzed using GC-MS in the same manner as in Glc and Gal; t_R [min]=22.0 (derivative of D-arabinose, 22.0, L-arabinose, 20.6).

Methylation of 1 (Hakomori Method[9]) Compound **1** (1 mg) was treated with NaH (40 mg) and CH_3I (1 ml) in DMSO (1 ml) according to the Hakomori method. The reaction mixture was then diluted with H_2O, extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with H_2O, dried with Na_2SO_4, and the solvent evaporated *in vacuo* to give permethylated **1**, denoted **2**.

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 2 Compound **2** (0.5 mg) was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (1 ml) at 90 °C for 20 h in a small-volume sealed vial, and then the solvents were evaporated *in vacuo*. The residue was dissolved in H_2O (1 ml), and 28% NH_3 (2 drops), and $NaBD_4$ (40 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, 1 ml) at 80 °C for 2 h. After dilution with H_2O, the mixture was extracted with $CHCl_3$. The $CHCl_3$ extract was dried with Na_2SO_4, and the solvent was evaporated to give partially methylated alditol acetates. The acetates were subjected to GC-MS [column temperature: 100—300 °C (rate of temperature increase 8 °C/min)], with the following results: S-1, t_R [min]=18.6, *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=18.5, *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 Permethylated NeuGc Derivative from 2 Compound **2** (0.5 mg) was heated with 5% HCl in MeOH (1 ml) at 80 °C for 20 h in a small-volume sealed vial. The reaction mixture was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*, and the residue (methanolysate) was heated with Ac_2O–C_5H_5N (1 : 1, 1 ml) at 80 °C for 2 h. The resulting mixture was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ extract was dried with Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was subjected to GC-MS [column temperature: 200—300 °C (rate of temperature increase 4 °C/min)]: S-3, t_R [min]=16.9, *m/z*: 159, 348, 378, 406 [methyl *N*-glycolyl-*N*-methyl-2,4,7,8,9,11-hexa-*O*-methylneuraminate (derived from terminal NeuGc)].

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