Isolation and Structure of a Monomethylated Ganglioside Possessing Neuritogenic Activity from the Ovary of the Sea Urchin *Diadema setosum*

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Received January 18, 2008; accepted February 18, 2008; published online February 19, 2008

A new monomethylated ganglioside, DSG-A (3), was obtained, together with four known gangliosides, compounds (1, 2, 4, 5), from the lipid fraction of the chloroform/methanol extract of the ovary of the sea urchin *Diadema setosum*. The structures of the new ganglioside was determined on the basis of chemical and spectroscopic evidence to be 1-*O*-[9-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-ceramide (3). The ceramide moiety of 3 was composed of C_{18}-phytosphingosine base, and 2-hydroxy and nonhydroxylated fatty acid units. These gangliosides showed neuritogenic activity toward the rat pheochromocytoma cell line PC-12 in the presence of nerve growth factor, in which compound 3 showed the most potent activity.

Key words ganglioside; sea urchin; *Diadema setosum*; neuritogenic activity

In our continuing research on biologically active gangliosides from echinoderms, a series of studies on the isolation and structural elucidation of biologically active gangliosides from starfish, sea cucumber and feather star have been performed in our laboratory.[1—5] Continuing the previous studies, we conducted the investigation of the biologically active gangliosides from sea urchin. Regarding the ganglioside constituents of sea urchin, several kinds of gangliosides have been obtained from *Anthocidaris crassipina*,[6,7] *Echinocardium cordatum*,[8] *Strongylocentrotus intermedius*,[9] and *Hemicentrotus pulcherrimus*.[10] In this paper, we report the isolation and characterization of gangliosides from the ovary of the sea urchin *Diadema setosum* (Gangaze in Japanese). The biological activities of the gangliosides are also reported.

The lipid fraction, which was obtained from the chloroform/methanol extract of the ovary of *D. setosum*, was subjected to repeated silica gel column chromatography to yield five compounds (**1**—**5**), each showing a single spot on TLC. Compounds **1**, **2**, **4** and **5** (Fig. 2) were identified as the same type of gangliosides, previously obtained from the sea urchin *H. pulcherrimus*[10] and the brittle star *Ophiocoma scolopendrina*[11] on the basis of spectroscopic evidence (Fig. 3, Table 1) and the similar chemical degradations (data not shown) of compound **3** described below.

Compound **3** exhibits characteristic signals due to the phytosphingosine-type ceramide,[11] with a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ 70.3 (C-1), 51.2 (C-2), 76.1 (C-3), 72.2 (C-4), 175.8 (C-1′) and 72.2 (C-2′)] in its ^{13}C-NMR spectrum (Fig. 1, Table 1). The ^{13}C-NMR spectrum of **3** also shows two anomeric carbon signals at δ 104.8 and 100.1, one of which (δ 100.1) is a quaternary carbon signal derived from one sialic acid moiety (Table 1). Accordingly, **3** is suggested to be a phytosphingosine-type ganglioside, with 2-hydroxy fatty acid groups and two monosaccharide units. Furthermore, **3** has normal-type fatty acids and long-chain base (LCB), since the carbon signals for the terminal methyl groups are observed at δ 14.1 (Fig. 1, Table 1).[3]

The structure of the ceramide moiety was examined first. When **3** was methanolyzed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAMs) and an LCB was obtained, together with methyl glucoside. The FAM mixture was analyzed using GC-MS, which revealed the presence of eight components (see Experimental section), and the major one was characterized as methyl 2-hydroxyoctadecanoate. The LCB was found to be 2-amino-1,3,4-trihydroxyoctadecane based on GC-MS analysis of its trimethylsilyl (TMS) derivative.

The structure of the disaccharide moiety of **3** was established as follows. The presence of glucose (Glc) was obvious from the results of the methanolysis of **3** (*vide supra*). A detailed analysis of the ^{13}C-NMR spectrum of **3** revealed the

Table 1. ^{13}C-NMR Spectral Data (δ Values) of Compounds **1**, **2**, **3**, and **5** in C_5D_5N

C		**1**	**2**	**3**	**5**
Ceramide					
1	(t)	70.7	70.7	70.3	70.5
2	(d)	54.4	54.1	51.2	51.0
3	(d)	76.5	76.0	76.1	75.7
4	(d)	72.3	72.1	72.2	72.2
1′	(s)	176.5	175.7	175.8	175.8
2′	(d)			72.2	72.2
CH=	(d)	130.0			
CH_3	(q)	14.1	14.1	14.1	14.1
Glc					
1	(d)	105.4	104.7	104.8	104.9
2	(d)	75.0	74.3	74.5	74.7
3	(d)	78.0	77.2	77.3	78.0
4	(d)	70.2	69.8	69.9	69.2
5	(d)	76.5	76.0	76.1	77.1
6	(t)	70.7	70.2	70.3	70.0
NeuAc					
1	(s)	173.6	173.7	173.8	173.2
2	(s)	101.0	—	100.1	—
3	(t)	42.7	42.6	42.6	40.9
4	(d)	68.8	68.6	68.6	68.4
5	(d)	52.1	51.2	51.2	53.9
6	(d)	72.8	72.5	72.6	71.6
7	(d)	71.1	70.6	70.7	70.8
8	(d)	75.5	74.9	75.0	80.6
9	(t)	64.4	63.8	69.8	63.5
10	(s)	173.5	173.7	173.8	173.2
11	(q)	22.9	22.7	22.7	22.7
OCH_3	(q)			54.2	

—: These signals were not observed.

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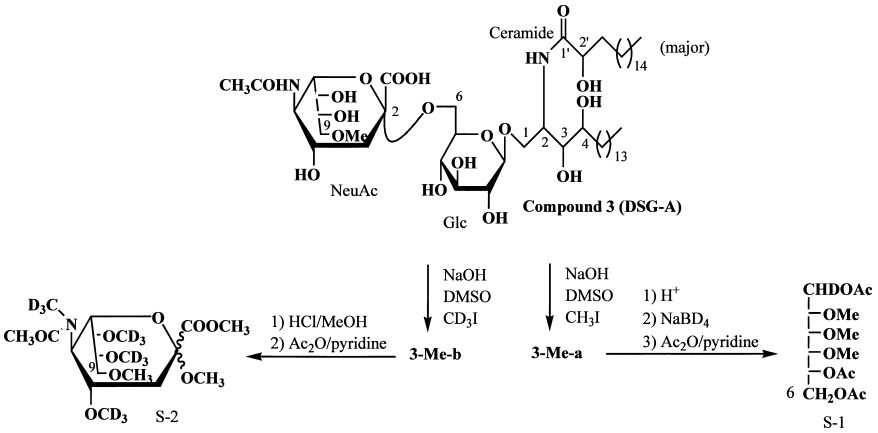

Fig. 1. Structure of Compound **3**

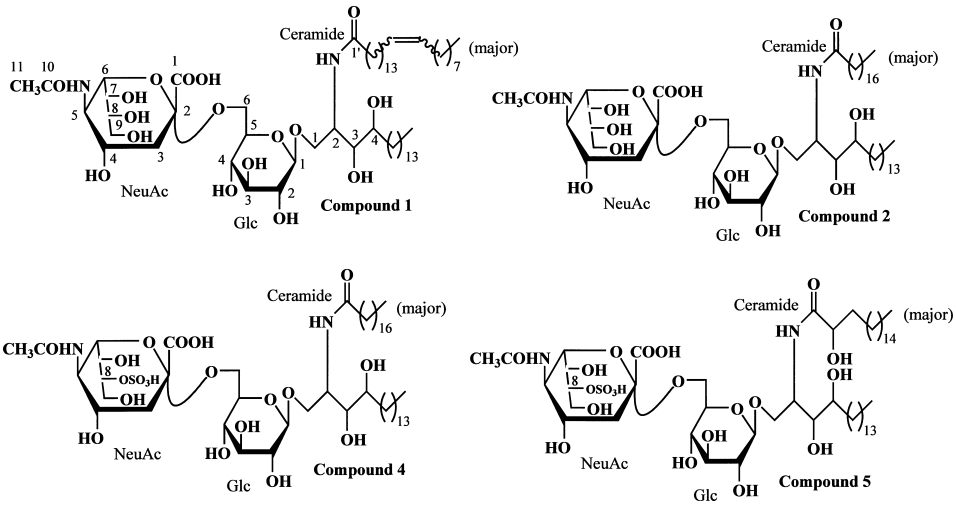

Fig. 2. Structure of Compounds **1**, **2**, **4** and **5**

characteristic signals [δ 173.8 (C-1), 100.1 (C-2), 42.6 (C-3), 51.2 (C-5), 173.8 (C-10), 22.7 (C-11)] of an *N*-acetylneuraminic acid (NeuAc) residue together with the signal due to a methoxy group (δ 54.2) (Table 1). In the negative FAB-MS of **3**, the molecular ion and fragment ion peaks arising from cleavage of the glycosidic linkages of the major component are observed at *m/z* 1065, 760, and 598, indicating the presence of a disaccharide moiety, NeuAc (Me)→hexose, as shown in Fig. 3.

Methylation of **3**, following the Ciucanu–Kerek method,[12] afforded the permethylated product **3-Me-a**. Partially methylated alditol acetate (S-1) prepared from **3-Me-a** was analyzed by GC-MS and identified as the alditol derived from 6-linked hexopyranose. The structure of the sialic acid moiety was established as follows. Since the sialic acid residue has a methoxy group, pertrideuteriomethylated product **3-Me-b** was prepared. Upon methanolysis followed by acetylation of **3-Me-b**, the partially trideuteriomethylated NeuAc (S-2) derived from the terminal 9-*O*-Me-NeuAc was detected by GC-MS analysis (Fig. 1). The downfield signal for C-9 (δ 69.8) of the NeuAc unit resulting from *O*-methylation[13] in the ^{13}C-NMR spectrum of **3** also supports the presence of the methoxy group at C-9 of the NeuAc unit.

On the basis of the above evidence, the disaccharide moiety of **3** must be 9-*O*-Me-NeuAc-(2→6)-Glc. The configura-

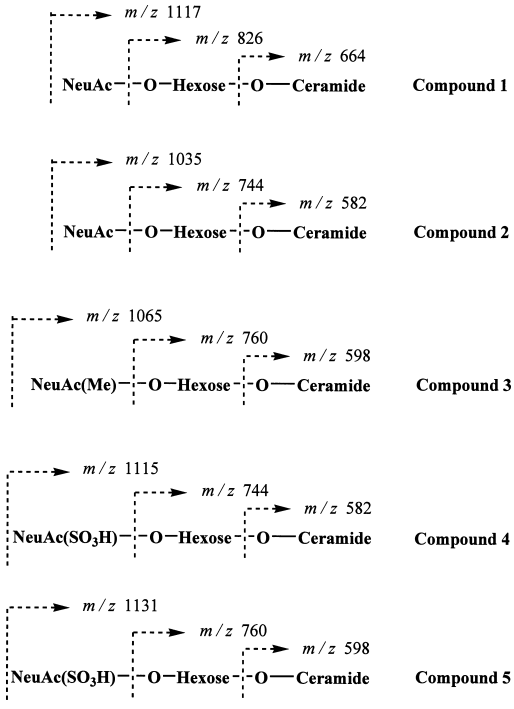

Fig. 3. Negative FAB Mass Fragmentation of the Major Component of Compounds **1**—**5**

tions of NeuAc and Glc are believed to be α and β on the basis of their anomeric carbon signals (δ 100.1[14], 104.8) in the ^{13}C-NMR spectrum of **3**. In addition, the absolute configuration of the glucose unit was verified as being the D-form using the method of Hara *et al*.[15]

Consequently, if NeuAc is assumed to belong to the most commonly found D-series, then compound **3** is the 9-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranoside of a ceramide, composed of C_{18}-phytosphingosine and heterogeneous fatty acids (2-hydroxyoctadecanoic acid as the major component), as shown in Fig. 1.

The effects of the isolated gangliosides (**1**—**5**) on the neuritogenesis of a rat pheochromocytoma cell line (PC-12 cells) were investigated. The results show that they displayed neuritogenic activity in the presence of nerve growth factor (NGF). The proportion of cells with neurites longer than the diameter of the cell body of compounds **1**—**5** at a concentration of 10 μg/ml was 24.9%, 34.0%, 40.8%, 30.0%, and 25.8% when compared with the control (NGF 5 ng/ml: 19.3%). Furthermore, the effects of **2**—**4** were greater than that of the mammalian ganglioside GM_1 (25.4%). Interestingly, compound **3** with a methoxy group at the NeuAc unit showed the most potent activity. In the gangliosides from starfish and sea cucumber, we found that gangliosides having 8-*O*-Me sialic acid showed stronger activity than the other gangliosides.[5] These facts indicate that *O*-methylated sialic acid plays an important role in the neuritogenic activity of gangliosides from echinoderms.

Compound **3** is, to the best of our knowledge, the first ganglioside with a methoxy group from sea urchin, and designated as DSG-A. The isolation and characterization of such neuritogenically active ganglioside are attracting considerable attention with regard to the development of new medicines from natural marine products.

Experimental

IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. ^{13}C-NMR spectra were recorded on a Jeol GX-270 spectrometer (67.8 MHz) or a Varian Unity-500 spectrometer (125 MHz). Negative-ion FAB-MS spectra were acquired with a Jeol JMS-SX-102 mass spectrometer (xenon atom beam; matrix, triethanolamine). GC-MS were recorded with a Shimadzu QP-5050A [EI mode; ionizing potential, 70 eV; column, TC-1701 (0.25 mm×30 m, GL Science Inc.); carrier gas, He].

Separation of Compounds 1—5 The ovary (215 g) of the sea urchin *D. setosum*, which was collected from the sea coast of Kagoshima prefecture, Japan, in 1999, was extracted with CHC_3–MeOH [1 : 4 (500 ml), 1 : 2 (500 ml)×3]. The combined extracts were concentrated *in vacuo* to give an aqueous solution (50 ml), which was diluted with 200 ml of H_2O and extracted three times with *n*-hexane (100 ml). The *n*-hexane phase was concentrated *in vacuo* to give a residue (11.2 g). The residue was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5 to 6 : 4 : 1) to give twelve fractions. Successive column chromatography of fraction 11 (silica gel, solvent $CHCl_3$–MeOH–H_2O, 5 : 5 : 1) afforded compound **1** (14.2 mg) (*Rf*=0.33). On the other hand, fraction 12 was further chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5 to 65 : 35 : 5) to give compound **2** (12.5 mg) (*Rf*=0.31), compound **3** (24.6 mg) (*Rf*=0.29), compound **4** (4.6 mg) (*Rf*=0.10), and compound **5** (34.5 mg) (*Rf*=0.08) [silica gel TLC, solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5].

Compound **1**: Amorphous powder. IR (KBr) cm^{-1}: 3346 (OH), 1635, 1556 (amide). Negative-ion FAB-MS: see Fig. 3. ^{13}C-NMR: see Table 1.

Compound **2**: Amorphous powder. IR (KBr) cm^{-1}: 3377 (OH), 1634, 1554 (amide). Negative-ion FAB-MS: see Fig. 3. ^{13}C-NMR: see Table 1.

Compound **3** (DSG-A): Amorphous powder. IR (KBr) cm^{-1}: 3375 (OH), 1637, 1542 (amide). Negative-ion FAB-MS: see Fig. 3. ^{13}C-NMR: see Table 1.

Compound **4**: Amorphous powder. Negative-ion FAB-MS: see Fig. 3. ^{13}C-NMR: not obtained because of lack of the sample.

Compound **5**: Amorphous powder. IR (KBr) cm^{-1}: 3375 (OH), 1638, 1547 (amide), 1257 (sulfate). Negative-ion FAB-MS: see Fig. 3. ^{13}C-NMR: see Table 1.

Methanolysis of 3 Compound **3** (0.7 mg) was heated with 10% HCl in MeOH (0.5 ml) at 70 °C for 18 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 glycoside.

GC-MS Analysis of FAM from 3 A FAM mixture from **3** was subjected to GC-MS [column temperature: 150—250 °C (rate of temperature increase 5 °C/min)]. The results were as follows: methyl tetradecanoate, t_R [min] (ratio of peak areas)=5.2 (8.2), *m/z*: 242 (M^+), 199 $(M-43)^+$; methyl pentadecanoate, t_R=6.4 (3.2), *m/z*: 256 (M^+), 213 $(M-43)^+$; methyl hexadecenoate, t_R=7.6 (3.2), *m/z*: 268 (M^+), 225 $(M-43)^+$; methyl hexadecanoate, t_R=7.8 (27.4), *m/z*: 270 (M^+), 227 $(M-43)^+$; methyl heptadecanoate, t_R=9.3 (3.2), *m/z*: 284 (M^+), 241 $(M-43)^+$; methyl octadecenoate, t_R=10.7 (3.2), *m/z*: 296 (M^+), 253 $(M-43)^+$; methyl octadecanoate, t_R=10.9 (10.0), *m/z*: 298 (M^+), 255 $(M-43)^+$; methyl 2-hydroxyoctadecanoate, t_R=13.8 (41.6), *m/z*: 314 (M^+), 255 $(M-59)^+$.

GC-MS Analysis of TMS Ether of LCB from 3 The mixture of LCB and methyl glycoside from **3** was heated with 1-(trimethylsilyl) imidazole–pyridine (1 : 1) for 20 min at 70 °C and the reaction mixture (TMS ethers) was analyzed using GC-MS [column temperature: 180—250 °C (rate of temperature increase 5 °C/min)]. The results were as follows: 2-amino-1,3,4-trihydroxy-octadecane, t_R [min]=18.0, *m/z*: 340 $(M-193)^+$, 299 $(M-234)^+$, 132.

GC Analysis of TMS Ether of Methyl Glycoside from 3 The mixture of TMS ethers of LCB and methyl glycoside from **3** was analyzed using GC-MS [column temperature: 150—200 °C (rate of temperature increase 2.5 °C/min), 200—250 °C (rate of temperature increase 10 °C/min)]: t_R [min]=15.4 and 16.0 (methyl Glc).

Determination of Absolute Configuration of Glc Moiety of 3 (Method of Hara *et al.*[15]) Compound **3** (0.6 mg) was heated with 2 N HCl (0.5 ml) at 100 °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 (1.0 mg) and pyridine (0.2 ml) at 60 °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 derivative. The derivative was analyzed using GC-MS [column temperature: 180—250 °C (rate of temperature increase 2.5 °C/min)]; t_R [min]=25.3 (derivative of D-Glc, 25.3; L-Glc, 26.1).

Methylation of 3 (Ciucanu–Kerek Method[12]) 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 **3** (1.2 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with water (15 ml), extracted with $CHCl_3$ (10 ml×3), the $CHCl_3$ phases were washed with water, and the solvent was evaporated *in vacuo* to give permethylated (or pertrideuteriomethylated) **3**, denoted **3-Me-a** (or **3-Me-b**).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetate from 3-Me-a Compound **3-Me-a** (0.5 mg) was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (1.0 ml) at 70 °C for 24 h in a small-volume sealed vial, and then the solvents were evaporated *in vacuo*. The residue was alkalified with 2% NH_3 aq. and $NaBD_4$ (10 mg) was added. After allowing the mixture to stand at room temperature for 7 h, it was acidified with AcOH 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 (0.6 ml), the mixture was extracted with $CHCl_3$ (0.3 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 temperature 150—250 °C (rate of temperature increase 5 °C/min)]. The results were as follows: S-1, t_R [min]=15.1, *m/z*: 118, 162, 189 [1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (derived from 6-linked hexopyranose)].

Preparation and GC-MS Analysis of Partially Trideuteriomethylated Sialic Acid Derivative from 3-Me-b Compound **3-Me-b** (0.5 mg) was heated with 10% HCl in MeOH (0.5 ml) at 70 °C for 18 h in a small-volume sealed vial. The reaction mixture was then neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*. The residue (methanolysate) was heated with Ac_2O–C_5H_5N (1 : 1, 0.2 ml) at 70 °C for 2 h. The reaction mixture was diluted with H_2O (0.3 ml) and 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 the residue, and the residue was subjected to GC-MS [column temperature 200—250 °C (rate of temperature increase 2.5 °C/min)]: S-2, t_R [min]=23.6, *m/z*: 135, 260, 280, 304, 327, 360, 388 [methyl *N*-acetyl-*N*-trideuteriomethyl-2,9-di-*O*-methyl-4,7,8-tri-*O*-trideuteriomethyl-neuraminate (derived from terminal 9-O-Me-NeuAc)].

Biological Assay The neuritogenic activity of compounds **1**, **2**, **3**, **4**, and **5** on PC-12 cells was observed according to a method previously reported.[3)]

Acknowledgments We thank Mr. Y. Tanaka and Ms. Y. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for the NMR measurements. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 13024260, Priority Area A) from the Ministry of Education, Culture, Science, Sports and Technology, Japan, and a grant (No. 16510163, 18510187) from the Japan Society for the Promotion of Science, which are gratefully acknowledged.

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