

Constituents of *Holothuroidea*. 9.¹⁾ Isolation and Structure of a New Ganglioside Molecular Species from the Sea Cucumber *Holothuria pervicax*

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A new ganglioside molecular species, HPG-7 (**1**) was obtained from the polar fraction of the chloroform/methanol extract of the sea cucumber, *Holothuria pervicax*. On the basis of chemical and spectroscopic evidence, the structure of **1** was determined, and the major component was 1-*O*-[α -L-fucopyranosyl-(1 \rightarrow 4)-(N-acetyl- α -D-neuraminosyl)-(2 \rightarrow 11)-(N-glycolyl- α -D-neuraminosyl)-(2 \rightarrow 4)-(N-acetyl- α -D-neuraminosyl)-(2 \rightarrow 6)- β -D-glucopyranosyl]-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetacosanoylamino]-14-methyl-hexadecane-1,3,4-triol. This is the first report on the isolation and structure elucidation of trisialo-ganglioside from sea cucumber. **1** showed neuritogenic activity toward the rat pheochromocytoma cell line, PC-12 cell.

Key words glycosphingolipid; ganglioside; sea cucumber; *Holothuria pervicax*; neuritogenic activity

In the course of our continuing search for biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structure elucidation of the GSLs from sea cucumber species have been performed in our laboratory.²⁾ In the study of the GCLs of the sea cucumber *Holothuria pervicax* (Torafunamako in Japanese), we reported on the isolation and structure of three new ganglioside molecular species, HPG-1, HPG-3, and HPG-8.^{2d)} Continuing the preceding studies, the isolation and characterization of the more polar ganglioside from *H. pervicax* was conducted. In this paper, we report on the isolation and structure of a new ganglioside molecular species from the whole bodies of *H. pervicax*. The biological activity of the ganglioside is also reported.

The water-soluble lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *H. pervicax*, was subjected to reversed-phase followed by silica gel column chromatography to give a ganglioside molecular species HPG-7 (**1**), showing a single spot on silica gel thin-layer chromatography (TLC).

In its ¹³C-NMR spectrum, **1** exhibits the characteristic signals of a phytosphingosine-type ceramide possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 [δ : 71.2 (C-1), 52.2 (C-2), 75.9 (C-3), 72.3 (C-4), 176.1 (C-1'), 72.3 (C-2')]. The ¹³C-NMR spectrum of **1** also features signals due to five anomeric carbons at δ : 105.1, 101.3 and 101.0, three of which δ : 101.0 are quaternary carbon atom signals, indicating the presence of three sialic acid residues. The negative FAB-MS exhibits a series of quasi-molecular ion peaks [M-H]⁻ at m/z : 1850–1900. Therefore, **1** is suggested to be a molecular species of a phytosphingosine-type ganglioside, possessing 2-hydroxy fatty acid groups and five monosaccharide units. Furthermore, **1** is presumed to have mainly normal type fatty acids and *ante*-iso type long-chain bases, since the carbon atom 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 ¹³C-NMR spectrum.

The structure of the ceramide moiety was examined first. When **1** was methanolized with methanolic hydrochloric acid, a mixture of fatty acid methyl ester (FAM) and long-

chain bases (LCB) was obtained, together with methyl glucopyranoside and fucopyranoside. The FAM mixture was analyzed by GC-MS, which revealed the presence of four components. They were characterized as methyl 2-hydroxydocosanoate, methyl 2-hydroxytricosanoate, methyl 2-hydroxytetacosanoate, and methyl 2-hydroxytetacosanoate (major). The LCB mixture was found to be composed of 2-amino-1,3,4-trihydroxy-14-methylhexadecane (major), -15-methylheptadecane, and -16-methyloctadecane, based on GC-MS analysis of its trimethylsilyl (TMS) derivative (Chart 1).

The relative stereochemistry of the ceramide moiety is presumed to be (2*S*,3*S*,4*R*,2'*R*), since the aforementioned ¹³C-NMR signals ascribable to C-1, 2, 3, 4, 1' and 2' of **1** are in good agreement with those of the co-existing phytosphingosine-type ganglioside molecular species possessing (2*S*,3*S*,4*R*,2'*R*) configurations.^{2d)}

The structure of the pentasaccharide moiety of **1** was established as follows. The existence of one mole each of glucose (Glc) and fucose (Fuc), and three moles of sialic acids is obvious from the ¹³C-NMR spectrum and the results of the methanolysis of **1** (*vide supra*). 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 : 1865, 1719, 1428, 1121, 830 and 668, indicating the presence of the pentasaccharide moiety, deoxyhexose (fucose, Fuc) \rightarrow NeuAc \rightarrow NeuGc \rightarrow NeuAc \rightarrow hexose (glucose, Glc), as shown in Fig. 1.

Methylation of **1** according to the Ciucanu-Kerek method³⁾ afforded the permethylated product **2**. Partially methylated alditol acetates (S-1, S-2) prepared from **2** were analyzed by GC-MS and identified as the alditols derived from 6-linked hexopyranose and terminal 6-deoxyhexopyranose. On the other hand, **2** was methanolized, the methanolysate was acetylated, and the acetate of partially methylated NeuAc (S-3), derived from 4-linked NeuAc, and of partially methylated NeuGc (S-4), originated from 11-linked NeuGc, were detected in the ratio of 2:1 by GC-MS analysis. On the basis of the above evidence, the pentasaccharide moiety of **1** must be Fuc(p)-(1 \rightarrow 4)-NeuAc-(2 \rightarrow 11)-NeuGc-(2 \rightarrow 4)-NeuAc-(2 \rightarrow 6)-Glc(p). The configurations of

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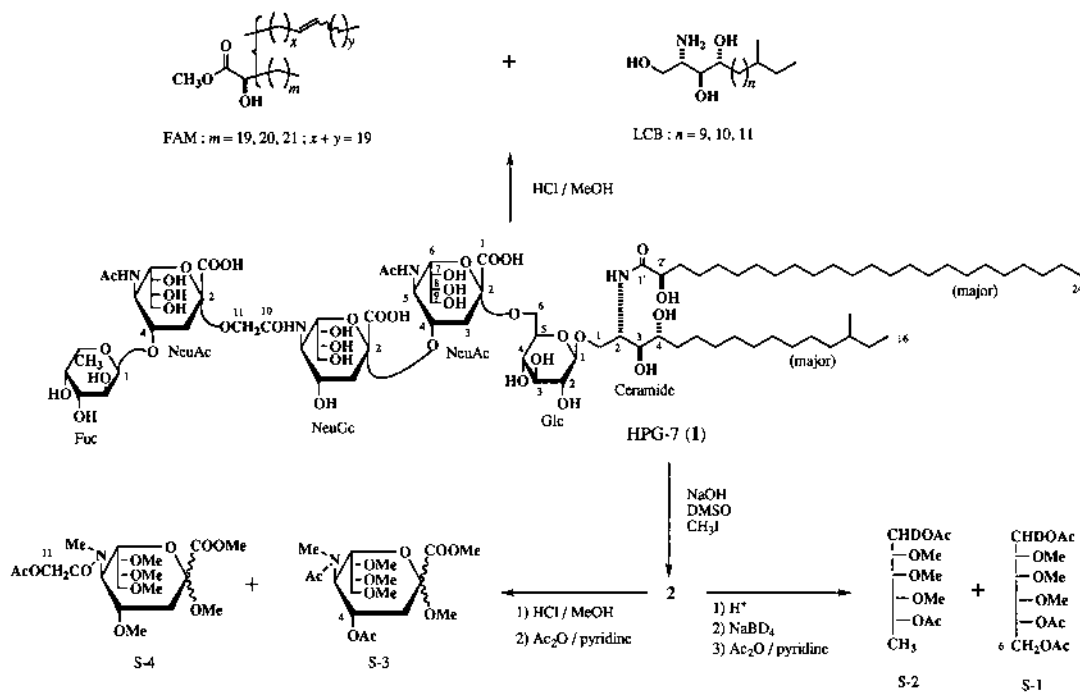


Chart 1

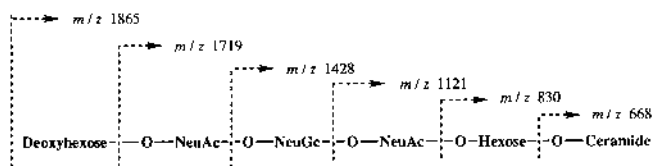


Fig. 1. The Negative Ion FAB-MS Fragmentation of the Major Component of HPG-7

Glc, Fuc, and sialic acids (NeuAc, NeuGc) are believed to be β , α , and α on the basis of their anomeric carbon signals (δ : 105.1, 101.3, and 101.0)^{2d} in the ¹³C-NMR spectrum of **1**.

Consequently, if Glc, Fuc, and sialic acids are assumed to belong to the most commonly found D-, L-, and D-series, then **1** is the α -L-fucopyranosyl-(1 \rightarrow 4)-(N-acetyl- α -D-neuraminosyl)-(2 \rightarrow 11)-(N-glycolyl- α -D-neuraminosyl)-(2 \rightarrow 4)-(N-acetyl- α -D-neuraminosyl)-(2 \rightarrow 6)- β -D-glucopyranoside of a ceramide composed of heterogeneous (2*S*,3*S*,4*R*)-phytosphingosine and (2*R*)-2-hydroxy fatty acid units. The major components of the fatty acid and LCB moiety of **1** are (2*R*)-2-hydroxytetracosanoic acid and (2*S*,3*S*,4*R*)-1,3,4-trihydroxy-2-amino-14-methyl-hexadecane, respectively (Chart 1).

The effects of the above, newly isolated ganglioside molecular species **1** on the neuritogenesis of rat pheochromocytoma cell line (PC-12 cells) have been investigated. The results show that **1** displays neuritogenic activity compared with H₂O (control) at a concentration of above 10 μ g/ml same as previously reported for three kinds of ganglioside molecular species HPG-1, HPG-3, and HPG-8.^{2d}

Besides the three kinds of ganglioside molecular species reported from *H. perricax*,^{2d} four kinds of ganglioside molecular species have been obtained and characterized from the sea cucumbers *Cucumaria japonica*,⁴ *Holothuria atra*,⁵ *Telenota ananas*,⁵ and *Cucumaria echinata*.^{2c} However, the ganglioside molecular species HPG-7 isolated in this study

is, to the best of our knowledge, a new ganglioside. Most notably, a ganglioside possessing a trisialoyl residue has been isolated for the first time from a sea cucumber species. The isolation and characterization of such neuritogenically active ganglioside is attracting considerable attention with regard to the manufacture of new medicines from marine natural products.

Experimental

Melting points were determined on a micro melting point apparatus (Yanaco MP-3) without correction. ¹H- and ¹³C-NMR spectra were recorded at 270 and 67.8 MHz, respectively, on a JEOL GX-270 spectrometer. Negative-ion FAB-MS spectra were acquired with a JEOL DX-300 mass spectrometer (xenon atom beam; matrix, HMPA-TEG). GC-MS were taken with a Shimadzu QP-1000 [EI mode; ionizing potential, 70 eV; separator and ion-source temperature 250 $^{\circ}$ C; column, CBP10-W12-100 (0.53 mm \times 12 m, Shimadzu); carrier gas, He]. GC were run on a Shimadzu GC-14B [FID mode; column, Fused Silica Capillary Column DB-17 (0.317 mm \times 30 m, J & W Scientific); carrier, N₂]. Abbreviations: Cer, ceramide; SA, sialic acid.

Separation of HPG-7 (1) For the extraction and fractionation of the crude ganglioside fraction from the whole bodies of the sea cucumber *Holothuria perricax* (126 kg), the preceding paper should be referred to.^{2d} The crude ganglioside fraction (13 g) was chromatographed on silica gel (solvent CHCl₃-MeOH-H₂O, 7:3:0.5 to 5:5:1) to give seven fractions. Successive column chromatography of fraction 6 (silica gel, solvent CHCl₃-MeOH-H₂O, 6.5:3.5:0.5 to 5:5:1) to afford HPG-7 (**1**) (14 mg) (*R*_f=0.31) [silica gel TLC, solvent CHCl₃-MeOH-H₂O (6:4:1)]. *R*_f values; HPG-8 (0.57), HPG-3 (0.46), and HPG-1 (0.42).

HPG-7 (**1**): Amorphous powder, mp 185–190 $^{\circ}$ C. Negative-ion FAB-MS *m/z*: 1850–1900 [M-H]⁻ series, 1865, 1719, 1428, 1121, 830, 668 (molecular and fragment ions of major component) (see Fig. 1). ¹H-NMR (C₅D₅N-D₂O, 95:5) δ : 0.90 (9H, m, Me groups),⁶ ¹³C-NMR (C₅D₅N-D₂O, 95:5) δ : 71.2 (t, C-1 of Cer), 52.2 (d, C-2 of Cer), 75.9 (d, C-3 of Cer), 72.3 (d, C-4 of Cer), 176.1 (s, C-1' of Cer), 72.3 (d, C-2' of Cer), 14.3 [q, -(CH₂)_nCH₃], 11.6 [q, -CH(CH₃)CH₂CH₃], 19.4 [q, -CH(CH₃)CH₂CH₃], 105.1 (d, C-1 of Glc), 101.3 (d, C-1 of Fuc), 16.8 (q, C-6 of Fuc), 174.0 (s, C-1 of SA), 101.0 (s, C-2 of SA).⁶

Methanolysis of 1 Compound **1** (0.4 mg) was heated with 5% HCl in MeOH (0.4 ml) at 70 $^{\circ}$ 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 Ag₂CO₃, filtered, and the filtrate was concentrated *in vacuo* to give a mixture of LCB and methyl glyco-

sides.

GC-MS Analysis of FAM from 1 A FAM mixture from **1** was subjected to GC-MS [column temperature 180–250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: methyl 2-hydroxydocosanoate, t_R [min]=7.5, m/z : 370 (M^+), 311 ($M-59$)⁺; methyl 2-hydroxytricosanoate, t_R =9.0, m/z 384 (M^+), 325 ($M-59$)⁺; methyl 2-hydroxytetra-cosanoate, t_R =10.2, m/z =396 (M^+), 337 ($M-59$)⁺; methyl 2-hydroxytetra-cosanoate (major), t_R =10.7, m/z =398 (M^+), 339 ($M-59$)⁺.

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 10 min at 60 °C and the reaction mixture (TMS ethers) was analyzed by GC-MS [column temperature 180–250 °C (rate of temperature increase 4 °C/min)]. The results were as follows: 2-amino-1,3,4-trihydroxy-14-methylhexadecane (major), t_R [min]=4.2, m/z : 326 ($M-193$)⁺, 285 ($M-234$)⁺, 132; 2-amino-1,3,4-trihydroxy-15-methylheptadecane, t_R =5.3, m/z : 340 ($M-193$)⁺, 299 ($M-234$)⁺, 132; 2-amino-1,3,4-trihydroxy-16-methyloctadecane, t_R =6.6, 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 glycoside was analyzed by GC [column temperature 100–250 °C (rate of temperature increase 5 °C/min)]: t_R [min]=17.9 and 18.1 (methyl α - and β -glucopyranoside), 12.6 and 13.0 (methyl α - and β -fucopyranoside).

Methylation of 1 (Ciucanu–Kerek Method)³⁾ NaOH–dimethyl sulfoxide (DMSO) solution, which was prepared from powdered NaOH (40 mg) and DMSO (1 ml), and MeI (0.2 ml) were added to **1** (1 mg), and the mixture was stirred for 30 min. The reaction mixture was then diluted with H₂O (15 ml), and extracted with CHCl₃ (10 ml×3), the CHCl₃ phases were washed with H₂O, and the solvent was evaporated *in vacuo* to give permethylated **1**, denoted **2** (0.7 mg).

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 2 **2** (0.2 mg) was heated with 90% HCOOH–10% CF₃COOH (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 H₂O (5 ml), and 28% NH₃ (2 drops) and NaBD₄ (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*. H₃BO₃ present in the residue was removed by distillation with MeOH (three times). The residue was heated with Ac₂O–C₅H₅N (1:1, 0.3 ml) at 70 °C for 2 h. After dilution with H₂O, the mixture was extracted with CHCl₃ (0.2 ml×3). The combined CHCl₃ extracts were washed with H₂O, and the solvent was evaporated to give partially methylated alditol acetates. The acetates were subjected to GC-MS [column temperature: 170–230 °C (rate of temperature increase 3 °C/min)]. The results were as follows: S-1, t_R [min]=7.1, m/z : 118, 162, 189, 233 [1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (derived from 6-linked hexopy-

ranose)]; S-2, t_R [min]=3.4, m/z : 118, 162, 175, 131 [1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol (derived from terminal 6-deoxyhexopyranose)].

Preparation and GC-MS Analysis of Acetates of Partially Methylated Sialic Acids from 2 **2** (0.3 mg) was heated with 5% HCl in MeOH (0.5 ml) at 75 °C for 6 h in a small-volume sealed vial. The reaction mixture was then neutralized with Ag₂CO₃, filtered, and the filtrate was concentrated *in vacuo*. The residue (methanolysate) was heated with Ac₂O–C₅H₅N (1:1, 0.2 ml) at 70 °C for 2 h. The resulting mixture was diluted with H₂O and extracted with CHCl₃ (0.2 ml×3), the combined CHCl₃ extracts were washed with H₂O, and the solvent was evaporated *in vacuo*. The residue was subjected to GC-MS [column temperature: 180–250 °C (rate of temperature increase 4 °C/min)]: S-3 (2 mol), t_R =16.7 min, m/z : 157, 346, 376 [methyl *N*-acetyl-4-*O*-acetyl-*N*-methyl-2,7,8,9-tetra-*O*-methylneuraminic acid (derived from 4-linked NeuAc)]; S-4 (1 mol), t_R =19.7 min, m/z : 187, 201, 376, 406 [methyl *N*-glycolyl-11-*O*-acetyl-*N*-methyl-2,4,7,8,9-penta-*O*-methylneuraminic acid (derived from 11-linked NeuGc)].

Biological Assay Neuritogenic activity of **1** on PC-12 cells was observed according to the method previously reported.^{2d)} Cells treated with above 10 µg/ml of **1** showed neurite outgrowth compared with those treated with H₂O (control).

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