# 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→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→11)-(*N*-glycolyl-α-D-neuraminosyl)-(2→4)-(*N*-acetyl-α-D-neuraminosyl)-(2→6)-β-D-glucopyranosyl]-(2*S*,3*S*,4*R*)-2-[(2*R*)-2-hydroxytetracosanoylamino]-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 $^{13}$C-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 $^{13}$C-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 $[\mathrm{M-H}]^-$ 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 $^{13}$C-NMR spectrum.

The structure of the ceramide moiety was examined first. When **1** was methanolyzed 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-hydroxytetracosenoate, and methyl 2-hydroxytetracosanoate (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 $^{13}$C-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 $^{13}$C-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)→NeuAc→NeuGc→NeuAc→hexose (glucose, Glc), as shown in Fig. 1.

Methylation of **1** according to the Ciucanu–Kerek method[3] 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 methanolyzed, 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→4)-NeuAc-(2→11)-NeuGc-(2→4)-NeuAc-(2→6)-Glc(p). The configurations of

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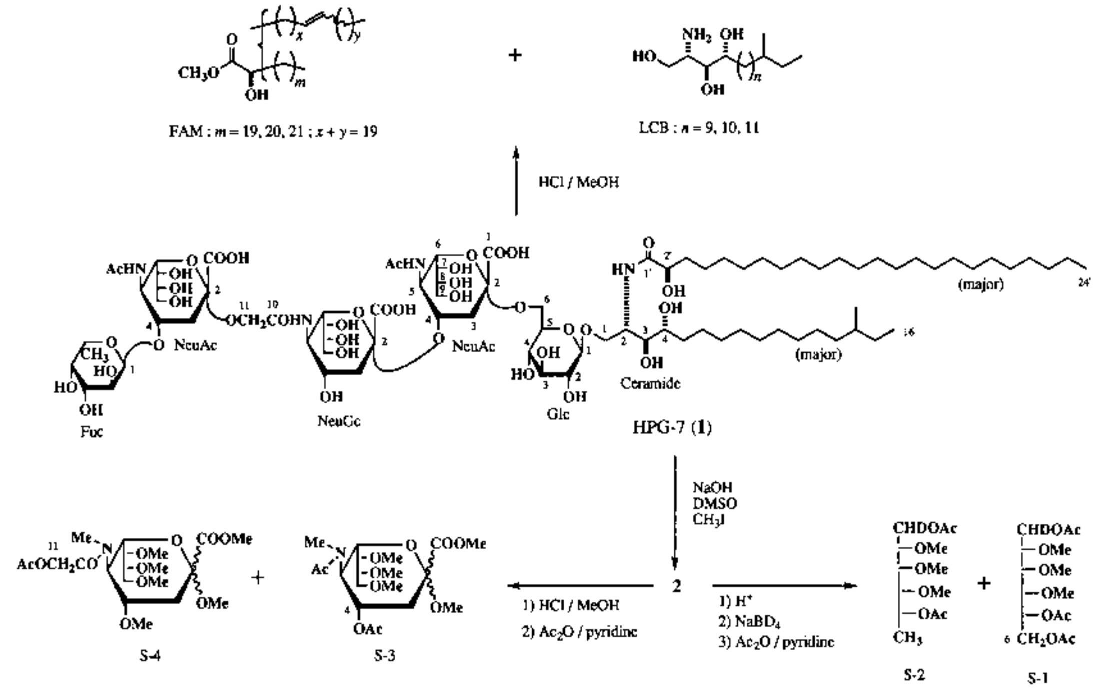

Chart 1

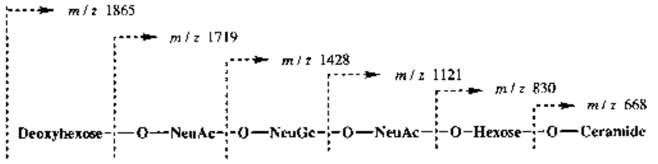

Fig. 1. The Negative Ion FAB-MS Fragmentation of the Major Component of HPG-7

Glc, Fuc, and sialic acids (NeuAc, NeuGc) are believed to be $\beta$, $\alpha$, and $\alpha$ on the basis of their anomeric carbon signals ($\delta$: 105.1, 101.3, and 101.0)[2d] in the $^{13}$C-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 $\alpha$-L-fucopyranosyl-(1→4)-(*N*-acetyl-$\alpha$-D-neuraminosyl)-(2→11)-(*N*-glycolyl-$\alpha$-D-neuraminosyl)-(2→4)-(*N*-acetyl-$\alpha$-D-neuraminosyl)-(2→6)-$\beta$-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 $H_2O$ (control) at a concentration of above 10 $\mu$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. pervicax*,[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. $^1$H- and $^{13}$C-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 °C; column, CBP10-W12-100 (0.53 mm×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×30 m, J & W Scientific); carrier, $N_2$]. 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 pervicax* (126 kg), the preceding paper should be referred to.[2d] The crude ganglioside fraction (13 g) was chromatographed on silica gel (solvent $CHCl_3$–MeOH–$H_2O$, 7 : 3 : 0.5 to 5 : 5 : 1) to give seven fractions. Successive column chromatography of fraction 6 (silica gel, solvent $CHCl_3$–MeOH–$H_2O$, 6.5 : 3.5 : 0.5 to 5 : 5 : 1) to afford HPG-7 (**1**) (14 mg) (*Rf*=0.31) [silica gel TLC, solvent $CHCl_3$–MeOH–$H_2O$ (6 : 4 : 1)]. *Rf* values; HPG-8 (0.57), HPG-3 (0.46), and HPG-1 (0.42).

HPG-7 (**1**) : Amorphous powder, mp 185—190 °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.). $^1$H-NMR ($C_5D_5N$–$D_2O$, 95 : 5) $\delta$: 0.90 (9H, m, Me groups).[6] $^{13}$C-NMR ($C_5D_5N$–$D_2O$, 95 : 5) $\delta$: 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, $-(CH_2)_nCH_3$], 11.6 [q, $-CH(CH_3)CH_2CH_3$], 19.4 [q, $-CH(CH_3)CH_2CH_3$], 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 °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 glycosides.

**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-hydroxytetracosenoate, $t_R$=10.2, *m*/*z*=396 ($M^+$), 337 $(M-59)^+$; methyl 2-hydroxytetracosanoate (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 $\alpha$- and $\beta$-glucopyranoside), 12.6 and 13.0 (methyl $\alpha$- and $\beta$-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 $H_2O$ (15 ml), and 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 **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% $CF_3COOH$ (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 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 hexopyranose)]; 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 $Ag_2CO_3$, 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 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 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*-methylneuraminate (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*-methylneuraminate (derived from 11-linked NeuGc)].

**Biological Assay** Neuritogenic activity of **1** on PC-12 cells was observed according to the method previously reported.2*d*) Cells treated with above 10 $\mu$g/ml of **1** showed neurite outgrowth compared with those treated with $H_2O$ (control).

**Acknowledgements** The authors thank Mr. Y. Tanaka and Ms. Y. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for NMR measurements. This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 08780545, 08680632, 09470486) from the Ministry of Education, Science, Sports and Culture, Japan, which are gratefully acknowledged.

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6) The NMR of HPG-7 showed no analyzable spectra except for the signals described in this section because of their low resolution, the reasons for which are unknown.