Constituents of Crinoidea. 5. Isolation and Structure of a New Glycosyl Inositolphosphoceramide-Type Ganglioside from the Feather Star *Comanthina schlegeli*

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A new glycosyl inositolphosphoceramide-type ganglioside, CSP2, was obtained from the polar lipid fraction of the chloroform/methanol extract of the feather star *Comanthina schlegeli* together with a known same type of ganglioside CJP2. The structure of this ganglioside has been determined on the basis of chemical and spectroscopic evidence to be 9-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-inositolphosphoceramide, which contains C_{16}-sphingosine and $C_{22:0}$-, $C_{24:0}$-fatty acid as major component. This is the first report on the isolation and structural elucidation of a glycosyl inositolphosphoceramide-type ganglioside possessing *N*-acetyl-neuraminic acid (NeuAc) residue.

Key words glycosphingolipid; ganglioside; feather star; *Comanthina schlegeli*

In our continuing research on biologically active glycosphingolipids (GSLs) from echinoderms, a series of studies on the isolation and structural elucidation of biologically active GSLs have been performed in our laboratory.[1—3] In the study of the GSLs of the crinoidea, we reported on the isolation and structural elucidation of cerebrosides,[4] inositolphosphoceramide,[5] and the glycosyl inositolphosphoceramide-type gangliosides[6,7] from the feather star *Comanthus japonica*. Continuing the preceding studies, isolation and characterization of GSLs from the feather star *Comanthina schlegeli* (*Hanaumishida* in Japanese) was conducted. In this paper, we report on the isolation and structure of a new and a known glycosyl inositolphosphoceramide-type ganglioside from the whole bodies of *C. schlegeli*.

The polar lipid fraction, which was obtained from the chloroform/methanol extract of the whole bodies of *C. schlegeli*, was subjected to repeated column chromatography followed by preparative thin-layer chromatography (TLC) to give two polar compounds, **1** and **2**, each showing a single spot on silica gel TLC.

Compounds **1** and **2** show strong hydroxy, amide and phosphate absorptions in the IR spectrum and exhibit a positive reaction to the Dittmer–Lester reagent,[8] which indicates the presence of a phosphate group. The negative-ion FAB-MS of **1** and **2** exhibits quasimolecular ion peaks $[M-H]^-$ at *m/z* 1139, 1167 in **1** and 1155, 1183 in **2** together with fragment ion peaks at *m/z* 834, 862 and 672, 700 in both **1** and **2** as shown in Fig. 2. The loss of 305 and 321 mass units from the molecular ions suggested the existence of a monomethylated *N*-acetyl- and *N*-glycolyl-neuraminic acid residues in **1** and **2**, respectively. Furthermore, **1** and **2** were hydrolyzed with 5% acetic acid to yield L-*myo*-inositol-1-*O*-phosphoceramide (**3**) which was previously reported.[7] Therefore **1** and **2** are suggested to be monomethylated *N*-acetyl- and *N*-glycolyl-neuraminosyl inositolphosphoceramide (Fig. 1).

The structure of the ceramide moiety of **1** and **2** was verified as follows. When **1** and **2** were subjected to mild alkaline hydrolysis with 1 M KOH, the ceramide part (**4**) was obtained with sugar part (**5**). Compound **4** was methanolyzed with methanolic hydrochloric acid, and a mixture of fatty acid methyl esters (FAM) and a long-chain base (LCB) was obtained. The FAM mixture was analyzed using GC-MS, which revealed the presence of $C_{22:0}$ and $C_{24:0}$ normal fatty acids as the major components. On the other hand, the LCB was characterized as C_{16}-sphingosine, based on GC-MS analysis of its TMS derivative. The stereochemistry of **4** must be (2*S*,3*R*,4*E*), since the ^{1}H-NMR spectrum and the optical rotation of **4** (-5.9) were in good agreement with that of the ceramide (-5.8) obtained from the gorgonian *Acabaria undulata*[9] which has the 2*S*, 3*R*, 4*E* configuration.

The structures of the sugar part of **1** and **2** were established as follows. Compound **1** and **2**, respectively, was methylated with CD_3I according to the Hakomori method[10] and yielded the perdeuteriomethylated product **6** and **7**. Upon methanolysis followed by acetylation of **6** and **7**, respectively, the partially trideuteriomethylated sialic acid derivatives (S-1 and S-2) originated from the terminal 9-*O*-Me-NeuAc and the terminal 9-*O*-Me-NeuGc were detected in GC-MS analysis, which indicated the presence of terminal 9-*O*-Me-NeuAc and NeuGc moiety in **1** and **2**.

The linkage site of the 9-*O*-Me sialic acid moiety to the *myo*-inositol portion was elucidated by methylation linkage analysis combined with ammonolysis.[11] Compound **8**, the permethylated **1** and **2**, was hydrolyzed with aqueous NH_3 to give the partially methylated inositol derivative, S-3. S-3 was identified as 2,4,5,6-tetramethylated *myo*-inositol by comparison of EI-MS spectrum of its TMS derivative with that of the *myo*-inositol derivative.[6] Consequently, the sialic acid residues in **1** and **2** must be linked at the C3-OH group of the inositol part, as shown in Fig. 1.

The configurations of the sialic acids are believed to be α on the basis of their H-3_{eq} signals (δ 2.73 ppm)[12] in the ^{1}H-NMR spectrum of **5**.

In general NeuAc and NeuGc are thought to be D-series, then **1** and **2** are 9-*O*-methyl-(*N*-acetyl-α-D-neuraminosyl)-(2→3)-inositolphosphoceramide and 9-*O*-methyl-(*N*-glycolyl-α-D-neuraminosyl)-(2→3)-inositolphosphoceramide, which contain C_{16}-sphingosine and $C_{22:0}$, $C_{24:0}$ fatty acids as

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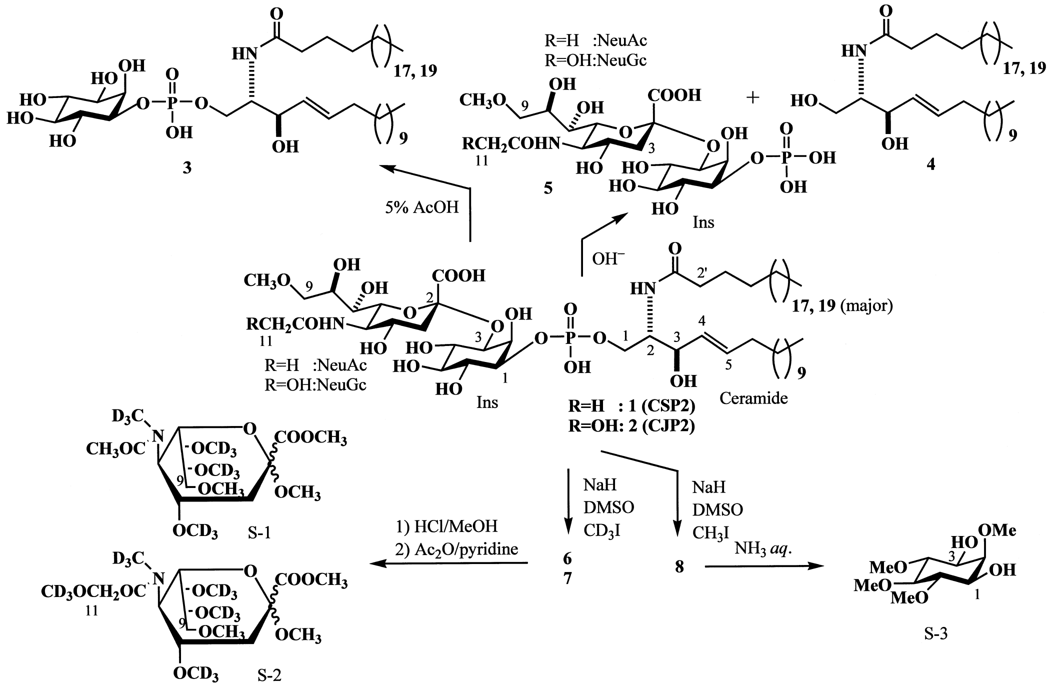

Fig. 1. Structure of **1** (CSP2) and **2** (CJP2)

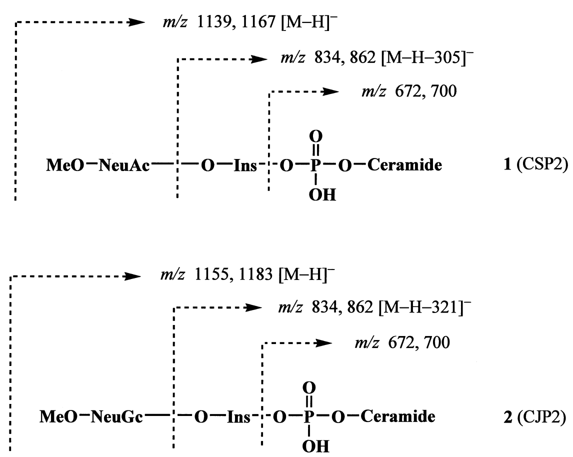

Fig. 2. Negative-Ion FAB-MS Fragmentation of **1** (CSP2) and **2** (CJP2)

major fatty acyl components, as shown in Fig. 1.

To the best of our knowledge, **1** represents a new glycosyl inositolphosphoceramide-type ganglioside found to contain a 9-*O*-Me-NeuAc moiety, and designated as CSP2. Compound **2** was identified as CJP2[6] obtained from the feather star *Comanthus japonica*. Since CJP2 exhibited neuritogenic activity toward the rat pheochromocytoma PC12 cells in the presence of nerve growth factor,[7] CSP2 is expected to have the same biological activity.

Experimental

Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 25 °C. IR spectra were obtained on a Jasco FT/IR-410 infrared spectrophotometer. 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, TEA). GC-MS were recorded on a Shimadzu QP-5050A [EI mode; ionizing potential, 70 eV; column, NEUTRA BOND-5 (0.25 mm×30 m); carrier gas, He].

Separation of 1 and 2 Whole bodies of the feather star *C. schlegeli* (14.1 kg), collected in 2000 at Hedo cape, Okinawa Prefecture, Japan, were chopped and extracted with $CHCl_3$–MeOH (1 : 2, 22.5, 13.5 and 10.5 l). The combined extracts were concentrated *in vacuo* to give an extractive (694 g), which was partitioned between H_2O (4.5 l) and AcOEt–*n*-BuOH (3 : 1, 4 l) (three times). The aqueous layer was washed with *n*-BuOH saturated with H_2O, dialyzed followed by lyophilized to give a residue (27.3 g). The residue (polar lipid fraction, 7.4 g) was chromatographed over Cosmosil 140C18-PREP (reversed-phase) [solvent 50%, 80%, 100% MeOH, and $CHCl_3$–MeOH (3 : 7)]. The crude ganglioside fraction (100% MeOH eluate) was chromatographed on silica gel [solvent $CHCl_3$–MeOH–H_2O (7 : 3 : 0.35→5 : 5 : 1)] to give six fractions. Fraction 2 of the six fractions was further purified by using preparative TLC [solvent $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5)] to yield compounds **1** (3.4 mg) and **2** (3.9 mg). They were detected with 5% H_2SO_4–MeOH and Dittmer–Lester reagent on silica gel TLC [solvent $CHCl_3$–MeOH–H_2O (6.5 : 3.5 : 0.75)], *Rf*=0.38 (**1**), 0.35 (**2**).

Compound **1** (CSP2): Amorphous powder. IR (KBr) cm^{-1}: 3389 (OH), 1645, 1556 (amide), 1220 (phosphate). Negative-ion FAB-MS *m/z*: 1139, 1167 $[M-H]^-$, 834, 862 $[M-H-305]^-$, 672, 700 (see Fig. 2). ^{1}H-NMR (DMSO-d_6) δ: 0.84 (6H, t, J=6.5 Hz, terminal methyl groups).

Compound **2** (CJP2): Amorphous powder. IR (KBr) cm^{-1}: 3389 (OH), 1645, 1556 (amide), 1220 (phosphate). Negative-ion FAB-MS *m/z*: 1155, 1183 $[M-H]^-$, 834, 862 $[M-H-321]^-$, 672, 700 (see Fig. 2). ^{1}H-NMR (DMSO-d_6) δ: 0.84 (6H, t, J=6.1 Hz, terminal methyl groups).

Partial Hydrolysis of 1 and 2 Compounds **1** and **2** were heated with 5% aqueous AcOH at 90 °C for 4 h to give **3**.

Alkaline Hydrolysis of 1 and 2 Compounds **1** and **2** were hydrolyzed with 1 M KOH at 35 °C for 28 h. The hydrolyzate was diluted with H_2O and extracted with $CHCl_3$. The organic layer was concentrated *in vacuo*, and the residue was purified by preparative TLC [solvent $CHCl_3$–AcOEt (1 : 3)] to give **4**. The aqueous layer was neutralized with Dowex 50W-X8 (H^+ form), lyophilized and the residue was purified by reversed-phase column chromatography to give **5**.

Compound **4**: $[\alpha]_D$ −5.9° (c=0.06, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.86 (6H, t, J=6.9 Hz, terminal methyl groups), 2.21 (2H, t, J=7.7 Hz, H_2-2′), 3.69 (1H, dd, J=11.0, 3.4 Hz, H-1), 3.89 (1H, m, H-2), 3.94 (1H, dd, J=11.0, 3.7 Hz, H-1), 4.31 (1H, br s, H-3), 5.52 (1H, dd, J=15.1, 6.4 Hz, H-4), 5.77 (1H, dt, J=15.1, 6.8 Hz, H-5), 6.17 (1H, d, J=7.6 Hz, NH).

Compound **5**: ^{1}H-NMR (D_2O) δ: 1.83 (1H, t, J=12.4 Hz, H-3_{ax} of sialic acid), 2.73 (1H, dd, J=12.4, 4.7 Hz, H-3_{eq} of sialic acid).

Methanolysis of 4 Compound **4** was heated with 5% HCl in MeOH at 70 °C for 2 h in a small-volume sealed vial. 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 LCB.

GC-MS Analysis of FAM from 4 A FAM mixture from **4** was subjected to GC-MS [column temperature 150—300 °C (rate of temperature increase 4 °C/min)]. The results were as follows: methyl octadecanoate, t_R [min] (ratio of peak areas)=21.7 (7.9), *m/z*: 298 (M^+), 255 $(M-43)^+$; methyl docosanoate, t_R=30.3 (35.6), *m/z*: 354 (M^+), 311 $(M-43)^+$; methyl tricosanoate, t_R=32.3 (17.6), *m/z*: 368 (M^+), 325 $(M-43)^+$; methyl tetracosenoate, t_R=33.8 (6.1), *m/z*: 380 (M^+), 337 $(M-43)^+$; methyl tetracosanoate, t_R=34.2 (32.8), *m/z*: 382 (M^+), 339 $(M-43)^+$.

GC-MS Analysis of TMS Ether of LCB from 4 The LCB from **4** was heated with 1-(trimethylsilyl) imidazole–pyridine (1 : 1) for 20 min at 70 °C and the reaction mixture (TMS ether) was analyzed using GC-MS [column temperature 180—250 °C (rate of temperature increase 5 °C/min)]. The results were as follows: 2-amino-hexadec-4-ene-1,3-diol, t_R [min]=15.5, *m/z*: 312 $(M-103)^+$, 283 $(M-132)^+$, 132.

Methylation of 1 and 2 (Hakomori Method) Compounds **1** and **2** were treated with NaH and CD_3I (or CH_3I) in DMSO according to the Hakomori method. The reaction mixture was 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 **6** and **7** (pertrideuteriomethylated **1** and **2**) and **8** (permethylated **1** and **2**).

Preparation and GC-MS Analysis of Partially Trideuteriomethylated Sialic Acid Derivatives from 6 and 7 Compounds **6** and **7** were heated with 5% HCl in MeOH at 70 °C for 15 h in a small-volume sealed vial. The reaction mixture was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated *in vacuo*. The residue (methanolysate) was heated with Ac_2O–pyridine (1 : 1) at 70 °C for 2 h, concentrated *in vacuo* and the residue was subjected to GC-MS [column temperature 150—300 °C (rate of temperature increase 5 °C/min)]: S-1 (sialic acid derivative from **6**), 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)]; S-2 (sialic acid derivative from **7**), t_R=25.7, *m/z*: 168, 293, 313, 337, 360, 393, 421 [methyl *N*-glycolyl-*N*-trideuteriomethyl-2,9-di-*O*-methyl-4,7,8,11-tetra-*O*-trideuteriomethyl-neuraminate (derived from terminal 9-*O*-Me-NeuGc)].

Ammonolysis of 8 Compound **8** was heated with 28% aqueous NH_3 at 150 °C for 16 h in a sealed pressure tube. The reaction mixture was evaporated and dried with an air stream to give the residue containing the partially methylated inositol derivative. The residue was chromatographed on silica gel [solvent $CHCl_3$–acetone (6.5 : 3.5)] to give S-3, which was identified as 2,4,5,6-tetramethylated *myo*-inositol by comparison of EI-MS spectrum of its TMS derivative with that of reported data.[6]

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References

1) Higuchi R., Inagaki K., Natori T., Komori T., Kawajiri S., *Liebigs Ann. Chem.*, **1991**, 1—10 (1991).
2) Higuchi R., Inukai K., Jhou J.-X., Honda M., Komori T., Tsuji S., Nagai Y., *Liebigs Ann. Chem.*, **1993**, 359—366 (1993).
3) Kaneko M., Yamada K., Miyamoto T., Inagaki M., Higuchi R., *Chem. Pharm. Bull.*, **55**, 462—463 (2007).
4) Inagaki M., Oyama A., Arao K., Higuchi R., *Chem. Pharm. Bull.*, **52**, 1307—1311 (2004).
5) Arao K., Inagaki M., Higuchi R., *Chem. Pharm. Bull.*, **47**, 687—689 (1999).
6) Arao K., Inagaki M., Higuchi R., *Chem. Pharm. Bull.*, **49**, 695—698 (2001).
7) Arao K., Inagaki M., Miyamoto T., Higuchi R., *Chem. Pharm. Bull.*, **52**, 1140—1142 (2004).
8) Dittmer J. C., Lester R. L., *Lipid Res.*, **5**, 126—127 (1964).
9) Shin J., Seo Y., *J. Nat. Prod.*, **58**, 948—953 (1995).
10) Hakomori S., *J. Biochem.* (Tokyo), **55**, 205—208 (1964).
11) Barr K., Lester R. L., *Biochemistry*, **23**, 5581—5588 (1984).
12) Dabrowski U., Friebolin H., Brossmer R., Supp M., *Tetrahedron Lett.*, **48**, 4637—4640 (1979).