Biologically Active Glycosides from Asteroidea, 43.1) Isolation and Structure of a New Neuritogenic-Active Ganglioside Molecular Species from the Starfish Linckia laevigata

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A ganglioside molecular species, LLG-5 (1), has been obtained from the water soluble lipid fraction of the CHCl3/MeOH extract of the starfish Linckia laevigata. On the basis of chemical and spectroscopic findings, the structure of 1 has been elucidated. Negative ion FAB-MS provided important information both on the structure of the sugar moiety and on the molecular mass of the ganglioside. 1 is a new ganglioside molecular species possessing a 2→11 linked linear-type trisialosyl moiety. Moreover, 1 exhibited neuritogenic activity in rat pheochromoeytoma PC-12 cells in the presence of nerve growth factor.

Key words glycosphingolipid; ganglioside; starfish; Linckia laevigata

During the course of our search for biologically active glycosphingolipids from starfish, we have isolated cerebrosides, ceramide-lactosides, sulfatides, and gangliosides having some biological activities.2,3) As a continuation of our previous studies, we recently carried out the isolation and structure elucidation of a disialo-ganglioside molecular species, LLG-5 (1), i.e., 8-O-Me-NeuAc2→11 NeuGcα2→3Galβ1→4Glcβ1→1ceramides, from the starfish Linckia laevigata (Aohitode in Japanese).4) In this paper, we report on the isolation and characterization of the newly isolated trisialo-ganglioside molecular species LLG-5 from the starfish L. laevigata. The biological activity of the gangliosides is also reported.

Results and Discussion

A water soluble lipid fraction, obtained from the CHCl3/MeOH extract of the whole bodies of L. laevigata, was subjected to reversed-phase followed by normal-phase column chromatography, and sephadex LH-20 column chromatography to give a ganglioside molecular species, LLG-5 (1). Compound 1 showed a single spot on normal-phase thin-layer chromatography (TLC) and exhibited a positive reaction with resorcinol-HCl reagent, which indicate the presence of a sialic acid moiety. In the IR spectrum, strong hydroxyl and amide absorptions were observed. The negative ion FAB-MS exhibited a series of molecular ion peaks due to anionized cluster ions [M—H]− at m/z 1890—1940, and the fragment ion peaks arising from cleavage of the glycosidic linkages were observed at m/z 1570—1620, 1260—1310, 950—1000, 790—840, and 630—680. Therefore, 1 was suggested to be a ganglioside molecular species, which contains the pentasaccharide moiety of NeuGc→NeuGc→NeuGc→Hexose→Hexose.

The structure of the ceramide moiety was examined first. When 1 was methanolysed with methanolic hydrochloric acid, a mixture of fatty acid methyl esters (FAM) was obtained together with a mixture of long-chain base (LCB) and methyl glycosides. Gas chromatography-mass spectrometry (GC-MS) analysis of the FAM mixture showed the existence of three components, which were characterized as methyl 2-hydroxydocosanoate (FAM-1), methyl 2-hydroxytricosanoate (FAM-2), and methyl 2-hydroxytetracosanoate (FAM-3). The major FAM was methyl 2-hydroxydocosanoate (FAM-1). On the other hand, in the GC-MS analysis of the TMS derivative of the LCB mixture, the LCB components were suggested to be 2-amino-1,3,4-heptadecanetriol (LCB-1) and 2-amino-1,3,4-octadecanetriol (LCB-2). LCB-2 was the major sphingoid.

The stereochemistry of the ceramide moiety was determined as follows. Compound 1 was hydroylysed with 5% AcOH to give ceramide dihexoside (2) (Fig. 1). Compound 2 showed the characteristic signals of phytosphingosine-type ceramide dihexoside possessing a 2-hydroxy fatty acid and a sugar moiety at C-1 in its 13C-NMR spectrum (Table 1) [δ 70.2 (C-1), 51.6 (C-2), 75.8 (C-3), 72.4 (C-4), 175.5 (C-1′) and 72.5 (C-2′)]. Furthermore, the 1H- and 13C-NMR spectra of 2 were in good agreement with those of the synthetic lactosyl ceramide, (2S,3S,4R)-1-O-[O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]-2-[2(R)-2-hydroxytetraicosanoylamin]-1,3,4-hexadecanetriol (3) (Table 1). The above fact and the optical rotations of 2 (+11.9) and 3 (+8.0) suggest that 2 must be ceramide lactoside and has the same absolute configuration as 3 for the core structure (C-2, C-3, C-4, C-2′, and lactose). Therefore, the absolute configuration of the ceramide part of the parent ganglioside 1 must be 2S, 3S, 4R, 2′R (Fig. 1). Furthermore, 1 is thought to possess the normal type of side chain, mainly, since the carbon atom signals due to the terminal methyl groups were observed at δ 14.2 in the 13C-NMR spectrum of 2. However, the existence of a small amount of the ante–iso type of terminal methyl groups is conceivable since the terminal methyl groups were observed, although to a low degree, at δ 11.5 and 19.3.

Next, the structure of the sugar moiety of 1 was examined. Since the presence of lactosyl ceramide moiety was already mentioned, the linear carbohydrate sequence of 1 must be NeuGc→NeuGc→NeuGc→β-Galp→(1→4)→β-GlcP. Methylation of 1 according to the Hakomori method afforded the permethylated product 4. Partially methylated alditol acetates prepared from 4 were analyzed by GC-MS and identified as the alditoles derived from 4-linked hexopyranose (S-1) and 3-linked hexopyranose (S-2) (Fig. 1). The structure of the siaec...
The acid part was characterized as follows. Since 1 was thought to possess a methoxy group in its sialic acid residue like co-existing ganglioside LLG-3, pertrideuteromethylated product 5 was prepared and 5 was methanolyzed and then acetylated, and the acetates of partially trideuteromethylated sialic acid derivatives (S-3 and S-4) were analyzed by GC-MS to give characteristic fragment ion peaks (Fig. 2), indicating the presence of terminal 8-O-Me NeuGc and 11-linked NeuGc at the ratio of ca. 1:2.

On the basis of the above evidence, the pentasaccharide moiety of 1 must be 8-O-Me-NeuGc-(2→11)-NeuGc-(2→11)-NeuGc-(2→3)-β-Galp-(1→4)-β-Glcpc. The configurations of the sialic acids were determined as follows. Compound 1 was converted to a methyl ester derivative (6), because 1 was difficult to dissolve in the solvents and revealed very low resolution in the measurement of NMR spectra. The 13C-NMR spectrum of 6 showed five anomeric carbons at δ = 99.6, 100.0, 105.0, and 105.7, three of which (δ = 99.6, 100.0, 100.0) were quaternary carbon signals arising from the sialic acids. The configurations of the sialic acids were regarded to be α, on the basis of their anomeric carbon signals.

Consequently, if NeuGc is assumed to belong to the most commonly found D series, then LLG-5 (1) is O-8-O-methyl-(N-glycolyl-α-D-neuraminosyl)-(2→11)-O-(N-glycolyl-α-D-neuraminosyl)-(2→11)-O-(N-glycolyl-α-D-neuraminosyl)-(2→3)-O-β-D-galactopyranosyl-(1→4)-O-β-D-glucopyranoside of a ceramide composed of heterogeneous phosphatidylcholine and n-2-hydroxy fatty acid units. The major components of the fatty acid and long-chain base moiety of 1 are n(2R)-2-hydroxydocosanoic acid and (2S,3S,4R)-2-amino-1,3,4-octadecanetriol, respectively (Fig. 1).

The effects of LLG5 (1) and LLG-3 on the neuritogenesis of a rat pheochromocytoma cell line (PC-12) have been investigated. The results showed 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 1 and LLG-3 at a concentration of 10 μM was 59.3 and 63.1% when compared with the control (NGF, 5 ng/ml: 20.6%). Furthermore, their effect was greater than that of the mammalian ganglioside GM1 (47.0%).

To the best of our knowledge, this is the first isolation and characterization of a trisialo-ganglioside from Asteroidea. Furthermore, 1 is a new ganglioside molecular species containing a 2→11 linked trisialosyl moiety. The isolation and characterization of such neuritogenically active gangliosides is attracting considerable attention with regard to the manufacture of new medicines from marine natural products.
Experimental

IR spectra: Jasco IR-700 infrared spectrophotometer. Optical rotations: were measured with a Jasco DIP-370 digital polarimeter at 25 °C. NMR spectra: 1H-NMR spectra were recorded on a Varian Unity-400 spectrometer (400 MHz), and 13C-NMR spectra on a Varian Unity-500 spectrometer (125 MHz) with an internal standard (pyridine-d6 or chloroform-d). MS: Jeol SX102A mass spectrometer [xenon atom beam, 5 kV; ion-source temperature, 250 °C; ionization potential, 70 eV; separator and ion-source temperature 300 °C; column, GL Science NEUTRA BOND-5 (0.25 mm×30 m; carrier gas, He).  

Separation of LLG-5 (1) Whole bodies of the starfish Linckia laevigata (wet weight 18 kg), collected at Okinawa, Japan, in May 1995, were chopped to give a mixture of LCB and methyl glycoside. The mixture was extracted with CHCl3/MeOH (1 : 2, 12 l, two times). The combined extracts were concentrated in vacuo to give a mixture of FAM for GC-MS analysis. The MeOH layer was concentrated in vacuo to give a brown-colored syrup. The residue was heated with Ac2O/C5H5N (1 : 1) (0.5 ml) at 70 °C for 30 min, and the mixture was concentrated in vacuo to give a mixture of LCB and methyl glycoside. The crude polar glycosphingolipid fraction (100% MeOH and CHCl3/MeOH (6 : 4 : 0.5)) followed by sephadex LH-20 (CHCl3/MeOH/H2O (6 : 4 : 1)) chromatography afforded ca. 1 mg of LCB-5 (–OH), 1650, 1780, 2850, 2930 cm⁻¹ (IR). It showed a single spot on silica gel TLC (solvent CHCl3/MeOH/H2O (6 : 4 : 1)), Rf = 0.32.  

LCG-5 (1) Amorphous powder. IR (KBr): ν = 3400 cm⁻¹ (–OH), 1650, 1560 (amide): Negative ion FAB-MS: m/z 1941, 2127, 1913, 1899 [M–H]⁻, 1592, 1285, 978, 816, 654 (fragment ions of major component).  

Methylation of 1 Compound 1 (1.0 mg) was heated with 5% HCl in MeOH (1 ml) at 70 °C for 12 h in a sealed small-volume vial. The reaction mixture was extracted with n-hexane, and the hexane layer was concentrated in vacuo to give a mixture of FAM for GC-MS analysis. The MeOH layer was concentrated in vacuo to give a mixture of LCB and methyl glycoside.  

GC-MS Analysis of FAM from 1 The FAM mixture from 1 was subjected to GC-MS [column temp. 100–250 °C (rate of temp. increases 5 °C/min)]. The results were as follows: FAM-1 (methyl 2-hydroxytricosanoate), tR [min] (ratio of peak areas) = 37.5 (47), EI-MS m/z: 370 [M]⁺, 311 [M–59]⁺. FAM-2 (methyl 2-hydroxytricosanoate), tR = 40.6 (40), EI-MS m/z: 384 [M]⁺, 325 [M–59]⁺. FAM-3 (methyl 2-hydroxytriacontanoate) tR = 44.5 (13), EI-MS m/z: 398 [M]⁺, 339 [M–59]⁺.  

GC-MS Analysis of TMS Ethers of LCB from 1 The LCB mixture from 1 was heated with 1-trimethylsilyl) imidazole/pyridine (1 : 1) for 10 min at 70 °C and then the reaction mixture [trimethylsilyl (TMS) ethers] was analyzed by GC-MS [column temp. 180–250 °C (rate of temp. increases 5 °C/min)]. The results were as follows: LCB-1 (1,3,4-tri-O-trimethylsilyl-2-amino-1,3,4-heptadecanetriol), tR [min] (ratio of peak areas) = 16.0 (35), EI-MS m/z: 326 [M–193]⁺, 285 [M–234]⁺. LCB-2 (1,3,4-tri-O-trimethylsilyl-2-amino-1,3,4-octadecanetriol), tR [min] = 17.6 (65), EI-MS m/z: 340 [M–193]⁺, 299 [M–234]⁺.  

Partial Hydrolysis of 1 Compound 1 (15 mg) was heated with 5% AcOH (8 ml) at 90 °C for 4 h. The reaction mixture was extracted with AcOEt/ν-BuOH (3 : 1), the organic layer was concentrated in vacuo to give a brown-colored syrup. The residue was chromatographed on silica gel [CHCl3/MeOH/H2O (8 : 2 : 0.1)] to give four fractions. The crude polar glycosphingolipid fraction (100% MeOH and CHCl3/MeOH eluate, 3.9 g) was chromatographed on silica gel [CHCl3/MeOH/H2O (6 : 4 : 0.5 → 6 : 4 : 0.7 → 6 : 4 : 1)] followed by sephadex LH-20 [CHCl3/MeOH/H2O (6 : 4 : 1)] to afford 1 (110 mg).  

Preparation and GC-MS Analysis of Partially Methylated Alditol Acetates from 4 Compound 4 (1 mg) was heated with 90% HCOOH/10% CF3COOH (1 : 1) (1 ml) at 90 °C for 12 h in a small-volume sealed vial, and the mixture was concentrated in vacuo. The residue was dissolved in H2O (2 ml), then 28% NH3 aq. (two drops) and NaBH4 (10 mg) were added to the solution. After standing at room temperature for 2 h, the reaction mixture was acidified with AcOH to pH 3.5 and concentrated in vacuo. The residue was heated with Ac2O/H2O (1 : 1) (0.5 ml) at 70 °C for 30 min, and the mixture was concentrated in vacuo. The residue was extracted with CHCl3, and the CHCl3 layer was washed with H2O dried (Na2SO4), and the CHCl3 solution was evaporated to give partially methylated alditol acetates. These acetates were subjected to GC-MS [column...
temp. 175 °C constant]. The results were as follows: \( t_R \) [min] = 15.6, \( m/z \): 117, 161, 233 [1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitol (S-1, derived from 4-linked hexopyranose)]; \( t_R \) [min] = 16.0, \( m/z \): 117, 161, 233 [1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol (S-2, derived from 3-linked hexopyranose)].

**Preparation and GC-MS Analysis of Partially Trideuteromethylated Neuraminic Acid Derivatives from 5**

Compound 5 (1 mg) was heated with 5% HCl–MeOH (1 ml) at 70 °C for 4 h in a small-volume sealed vial. The reaction mixture was concentrated in vacuo, and the residue was heated with Ac₂O/C₅H₅N (1 : 1) (1 ml) at 70 °C for 1 h, then the mixture was concentrated in vacuo, and the residue was subjected to GC-MS [column temp. 180—250 °C (rate of temp. increase 4 °C/min): \( t_R \) [min] = 20.3, \( m/z \): 168, 293, 313, 334, 360, 393, 421 [methyl N-glycolyl-N-trideuteromethyl-2,8-di-O-methyl-4,7,9,11-tetra-O-trideuteromethylneuraminic (S-3, from terminal 8-O-Me NeuGc)]; \( t_R \) [min] = 26.3, \( m/z \): 193, 318, 338, 362, 385, 421, 449 [methyl N-glycolyl-11-O-acetyl-N-trideuteromethyl-2-O-methyl-4,7,8,9-tetra-O-trideuteromethylneuraminic (S-4, from 11-linked NeuGc)].

**Methyl Esterification of 1**

10 mg of 1 was dissolved in 1 ml of DMSO and 0.2 ml of MeI was added to this solution. After stirring for 1 h at room temperature, the reaction mixture was diluted with 10 ml of 50% MeOH and applied to RP-CC (cosmosil 140C₁₈ PREP) prewashed with 50% MeOH. The column was washed with 30 ml of 50% MeOH to remove CH₃I and DMSO and then the esters were eluted with 30 ml of CHCl₃–MeOH (1 : 1). The eluate was concentrated and purified by Si-CC (CHCl₃ : MeOH : H₂O/6 : 4 : 0.5) to give \( \delta ^{13} C \)-NMR data (Table 1).

**Biological Assay**

Neuritogenic activity of 1 and LLG-3 in PC-12 cells was observed according to a method previously reported.⁹)

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### References and Notes

6) Normal means straight chain (...CH₂CH₂CH₂CH₃), ante–iso means branched chain possessing a methyl group on the third carbon from the terminal methyl group [...CH₂CH(CH₃)CH₂CH₃].