Glycoconj J (2006) 23:489–499
DOI 10.1007/s10719-006-6562-1

Unique disialosyl gangliosides from salmon kidney: Characterization of $V^3\alpha$Fuc, $IV^3\beta$GalNAc, $II^3(\alpha NeuAc)_2$-Gg_4Cer and its analogue with 4-*O*-acetyl-*N*-acetylneuraminic acid

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Received: 1 November 2005 / Revised: 5 December 2005 / Accepted: 7 December 2005
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Abstract Four unidentified acidic glycolipids (X3-X6) were isolated from the kidney of the Pacific salmon on an anion exchange column and by high performance liquid chromatography using a silica bead (Iatrobeads) column. Based on methylation analysis, chemical and enzymatic degradation, proton nuclear magnetic resonance spectroscopy and mass spectrometry, the glycon structure of X5 and X6 was identified as a unique disialosyl fucosyl-*N*-acetylgalactosaminyl ganglio-*N*-tetraose:

Fucα3GalNAcβ3Galβ3GalNAcβ4[NeuAcα8NeuAcα3] Galβ4Glcβ1Cer. NMR showed that X3 and X4 were analogues of X5 and X6 and contained *O*-acetyl groups on C4 of the outer *N*-acetylneuraminic acid, first disialosyl gangliosides containing 4-*O*-acetyl-*N*-acetylneuraminic acid. The ceramides of X3 and X5 contained predominantly C24: 1, and X4 and X6 contained saturated fatty acids (C14: 0, C16: 0 and C18: 0), whereas the long chain base was exclusively sphingenine. The concentrations of X3 and X4 were 0.13 and 0.16 nmol/g of kidney respectively and those of X5 and X6, were 0.07 nmol/g each.

Keywords Fucosyl gangliosides · Disialosyl gangliosides · 4-*O*-acetyl-*N*-acetylneuraminic acid · Salmon kidney

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Abbreviations

The nomenclature system for lipids follows the recommendation of the Nomenclature Committee, International Union of the Pure and Applied Chemistry [1]

GalNAc	*N*-acetylgalactosamine
Fuc	fucose
NeuAc	*N*-acetylneuraminic acid
Cer	ceramide
d18:1	4-sphingenine
d18:0	sphinganine
t18:0	4-hydroxysphinganine
TLC	thin layer chromatography
HPTLC	high performance TLC
HPLC	high performance liquid chromatography
GLC	gas-liquid chromatography
GC-MS	gas chromatography-mass spectrometry
^{1}H-NMR	proton nuclear magnetic resonance
1-D	one-dimensional
2-D	two-dimensional
COSY	chemical shift correlated spectroscopy
LSIMS	liquid secondary ion mass spectrometry
Me_2SO	dimethyl sulfoxide
BSTFA	bis-(trimethylsilyl)trifluoroacetamide

Introduction

The function of the acidic lipids present in mammalian renal tubules has been pursued since it was reported that the levels of sulfated glycolipids increased under conditions of high osmolality in organs related to ion transport [2,3]. Concurrently, sulfated glycolipids with a variety of carbohydrate chains have been isolated from mammalian kidneys [3], and studies on the metabolism of these lipids support the hypothesis that sulfoglycolipids act as an ion barrier on the

renal tubular membrane [4–7]. During a survey of acidic glycolipids in the kidneys of lower vertebrates, we found that the novel monosialosyl ganglioside, fucosyl-GalNAc-GM1a, comprised a major portion of the monosialosyl gangliosides in the kidney of the Pacific salmon, *Oncorhynchus keta* [8]. We have also studied the glycolipids present in the disialosyl ganglioside fractions of the salmon kidney. The present paper describes the characterization of a novel disialosyl ganglioside and its *O*-acetyl analogue.

Materials and methods

Materials

Kidneys were freshly prepared from Pacific salmons as described [8]. GD3 (NeuAc-NeuAc) and fucosyl GM1a were prepared from dolphin kidney [9] and boar testis [10] respectively. Other standard glycosphingolipids, long chain bases and partially *O*-methylated saccharides, chemicals and reagents have been descried previously [8].

Thin-layer chromatography

TLC was performed on Silicagel 60 HPTLC plates (Art. 5641, Merck) with the following solvent systems: I, chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v); II, 2-propanol/3.5 M ammonium hydroxide (7:3, v/v). Glycolipids were visualized and quantified by spraying the plate with orcinol/H_2SO_4 reagent and heating for 5 min at 120°C. TLC densitometric analysis of resorcinol-stained gangliosides [11] was performed at 580 nm using a Shimadzu Flying-Spot Scanner CS-9000 [8].

Lipid extraction and purification of gangliosides

Lipids were extracted according to Bligh and Dyer [12] as described previously [8]. We also used an alternative procedure in the present study. Kidneys (0.9 kg) were homogenized with 12 l of chloroform/methanol (2:1, v/v). After filtration, the extract was partitioned by addition of water (three liters). The residue was stirred with six liters of chloroform/methanol/0.88% KCl (1:2:0.8, v/v), and then filtered. The filtrate was partitioned by addition of chloroform/water (1:1, v/v, three liters). After concentration, the combined upper phases of these partitions were dialyzed, lyophilized, redissolved in chloroform/methanol/water (30:60:8, v/v), and chromatographed on a DEAE-Sephadex A-25 (Figure 1. legend) column. After washing the column with four liters of the same solvent, acidic glycolipids were eluted with a gradient of chloroform/methanol/water (30:60:8, v/v, 1.5 l), chloroform/methanol/1 M ammonium acetate (30:60:8, v/v, 1.5 l) and chloroform/methanol/2.5 M ammonium acetate (30:60:8, v/v, 1.5 l) at the rate of 2 ml/min. Fractions

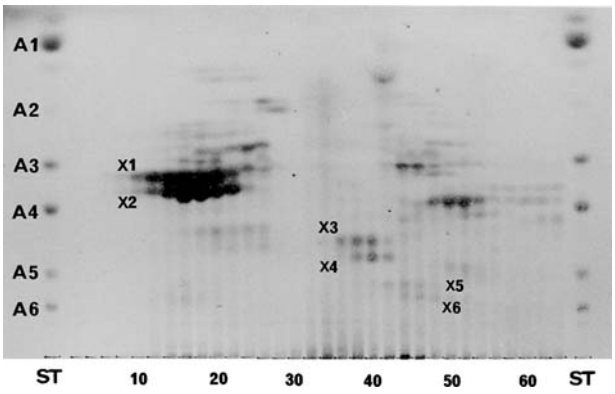

Fig. 1 Fractionation of salmon kidney gangliosides by DEAE-Sephadex column chromatography. Acidic glycolipids were separated on a 2 × 37 cm DEAE-Sephadex (acetate form) column and every other fraction was monitored by HPTLC with solvent system I, chloroform/methanol/0.2% $CaCl_2$, 55:45:10, v/v. Glycolipid bands were visualized by orcinol/H_2SO_4 spray. Lane ST, a mixture of rat brain acidic glycosphingolipids (A1, galactosyl sulfatide; A2, GM3; A3, GM1; A4, GD1a; A5, GD1b; A6, GT1b + GQ1b).

(18 ml/tube) of the eluate were collected and monitored by HPTLC. The fractions in tube numbers 31–40 and 41–50 respectively were combined and further purified by HPLC (Shimadzu LC 4A) on an Iatrobeads (6RS-8005) column (1 × 30 cm) with a gradient from chloroform/methanol (8:2, v/v, one liter) to chloroform/ methanol/water (60:40:10, v/v, 1.1 l) at the flow rate of 1 ml/min. After monitoring by HPTLC, the relevant fractions for X3 (192–209), X4 (210–230), X5 (236–246) and X6 (247–254) were collected and concentrated.

Gas chromatography

Derivatives of monosaccharides and fatty acids prepared from 20 nmol of glycolipids [8] were separated on CBP-1 (Shimadzu) (0.2 mm × 25 m, temperature program, 150–250°C at 4°C/min) and CBP-20 (Shimadzu) capillary columns (temperature program, 160–220°C at 4°C/min) respectively using a Shimadzu GC-14A gas chromatograph. Derivatives of sphingoid bases were prepared from 20 nmol of glycolipids according to the method of Sweeley and Moscatelli [13] and analyzed isothermally at 260°C on a CBP-1 capillary column.

Methylation studies

A portion (20–50 μg) of the glycolipids was methylated [14] (R-OH in glycoside→R-O-CH_3), acetolyzed (R_1-O-R_2 in glycoside linkage→R_1-OAc + R_2-OAc), reduced with NaB$[^2H]_4$ [15] (-C_1-O-C_5- in glycoside→-$C_1H[^2H]$-O$[^2H]$), and acetylated according to published procedures [16](-$C_1H[^2H]$-O$[^2H]$→-$C_1H[^2H]$-OAc). The acetates of partially methylated 6-deoxyhexitol, hexitol, and hexosaminitol were analyzed by gas chromatography under similar conditions to

the monosaccharides. For the analysis of the linkage between sialic acids, permethylated gangliosides were hydrolyzed with 0.3 M HCl in methanol (0.5 ml) for 18 h at 75°C [9]. After fatty acids were removed by extraction with hexane (1 ml × 2), the methanolic solution was concentrated and dried over P_2O_5 and trimethylsilylated using BSTFA/pyridine (9:1, v/v) at room temperature for 30 min [17].

Limited hydrolysis of gangliosides

Sialic acids were removed from gangliosides (50 nmol) by incubation with 10 μg neuraminidase (EC 3.2.1.18, type V, Sigma) from *Clostridium perfringens* at 37°C for 30 min in 50 mM sodium acetate buffer (pH 5.0, 60 μl). After the addition of chloroform/methanol (2:1, v/v, 1.2 ml), the mixture was desalted using a Sephadex G-25 column (0.5 × 1 cm). To split the sialic acid from the *O*-acetyl gangliosides, X3 and X4 were pretreated with 0.1 M NaOH in methanol at 37°C for 1 h, then neutralized with acetic acid. The reaction products were concentrated, redissolved in chloroform/methanol/water (120:60:9, v/v) and desalted using a Sephadex G-25 column.

Negative-ion LSIMS and ^{1}H-NMR spectroscopy

Negative-ion LSIMS was performed on a Concept IH mass spectrometer (Shimadzu/Kratos) fitted with a cesium ion gun [18]. About 0.5 nmol of underivatized glycolipid was mixed with 1 μl triethanolamine as the matrix. Spectra were recorded at an accelerating voltage of 8 kV, with a scan rate of 5s/decade, and at a resolution of 1000 to 2000. For NMR spectroscopy, approximately 1 mg of glycolipid was lyophilized twice in a small amount of $[^2H]_2O$ to exchange labile protons with deuterium, and redissolved in 0.5 ml of a mixture of $[^2H]$ $Me_2SO/[^2H]_2O$, (98:2, v/v) [19]. The one and two dimensional spectra were recorded by a GX-400 spectrometer from Japan Electron Optical Laboratory (JEOL) at 60°C. Chemical shifts were indicated by ppm from the signal of Me_4Si as an internal standard.

Results

Chemical analysis of gangliosides X3, X4, X5 and X6

Acidic glycolipids of salmon kidney were eluted from a DEAE-Sephadex column and analyzed by TLC (Figure 1).

After two major monosialosyl gangliosides, fucosyl-GalNAc-GM1as [8] were eluted, four gangliosides (X3, X4, X5 and X6 in Figure 1) were eluted in the earlier part of disialosyl ganglioside fractions. They were purified by HPLC on an Iatrobeads column to single bands on TLC developed with solvent systems I and II (Figure 2).

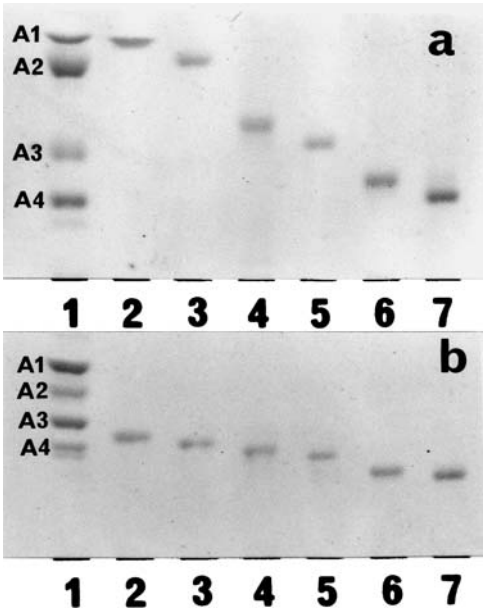

Fig. 2 HPTLC of gangliosides purified from salmon kidney. The plate was developed in solvent system I (chloroform/methanol/0.2% $CaCl_2$, 55:45:10, v/v) and II (isopropylalcohol/3.5 M ammonium hydroxide, 7:3, v/v) in panel a and b respectively and stained with orcinol reagent. Lane 1, rat brain gangliosides (A1, GM1; A2, GD1a; A3, GD1b; A4, GT1b + GQ1b); lanes 2–6, purified gangliosides: lane 2, X1; lane 3, X2; lane 4, X3; lane 5, X4; lane 6, X5; lane 7, X6.

GLC of trimethylsilyl methylglycosides indicated that the component monosaccharides of X3 – X6 were Glc, Gal, GalNAc, Fuc and sialic acid in the proportion of 1:2:2:1:2 (Table 1).

The partially methylated alditol acetates of these gangliosides were separated on a CBP-1 capillary column and identified by the GC-MS retention times and mass spectra as 2,3,4-tri-*O*-methyl-6-deoxygalactitol, 2,4,6-tri-*O*-methylgalactitol, 2,3,6-tri-*O*-methylglucitol, 2,6-di-*O*-methylgalactitol and 4,6-di-*O*-methyl-*N*-acetylgalactosaminitol in the approximate ratio of 1:1:1:1:2 (Table 2).

From the retention time and the peak area of GLC the major fatty acids were 24:1 for X3 and X5, and 14:0 and 16:0 for X4 and X6. Long chain bases of X4 and X5 were predominantly d18:0 and t18:0, whereas X3 and X6 contained mainly d18: 1 (Table 3).

Table 1 Carbohydrate composition of gangliosides X3–X6

	Fuc	Gal	Glc	GalNAc	NeuAc
X3	0.88	1.82	1.00	1.97	2.15
X4	0.86	1.81	1.00	1.86	1.80
X5	0.77	1.78	1.00	1.92	2.05
X6	0.81	1.85	1.00	1.81	1.83

Molar ratios were calculated based on the GLC peak areas of methylated glycosides using methylated fucosyl GM1a as the calibration standard.

Table 2 Partially *O*-methylated alditol acetates from gangliosides X3, X4 and X5

	2,3,4-Fuc[a]	2,4,6-Gal[b]	2,3,6-Glc[c]	2,6-Gal[d]	4,6-GalNAc[e]
X3	0.94	0.99	1.00	1.22	1.40
X4	0.40	1.43	1.00	1.01	1.83
X5	0.31	1.11	1.00	1.07	2.06

Molar ratios were determined by GLC.
[a]2,3,4-tri-*O*-methyl-6-deoxygalactitol
[b]2,4,6-tri-*O*-methylgalactitol
[c]2,3,6-tri-*O*-methylglucitol
[d]2,6-di-*O*-methylgalactitol
[e]4,6-di-*O*-methyl-*N*-acetylgalactosaminitol
Values for 4,6-di-*O*-methyl-*N*-acetylgalactosaminitol were calibrated using the standard ganglioside GM1a.

Table 3 Fatty acids and long chain bases of gangliosides X3–X6

	X3	X4	X5	X6
Fatty Acid	Composition (%)			
14:0	8.3	45.5	4.8	26.0
16:0	10.7	19.3	19.1	25.9
18:0	12.8	11.5	13.2	14.5
18:1	2.8	8.6	6.6	12.8
18:2	—	—	1.3	2.2
24:0	—	4.4	5.1	2.1
24:1	65.5	10.8	49.9	16.6
Long Chain Base				
d18:1	81	19	30	83
d18:0	11	48	43	11
t18:0	8	33	27	6

Each component was identified from the retention time of GLC. Data were obtained from peak areas.

Degradation of the gangliosides by alkali and neuraminidase treatment

Gangliosides, X3 and X4, were resistant to neuraminidase from *Clostridium perfringens* (lane 3 in Figures 3 and 4) and migrated similarly to X5 and X6 respectively on TLC after treatment with 0.1 M NaOH (lane 4 in Figures 3 and 4).

These deacylation products, as well as X5 and X6, were susceptible to neuraminidase to yield the compounds comigrating with X1 (one fucosyl-GalNAc-GM1a, upper band) and X2 (another fucosyl-GalNAc-GM1a, lower band) [8] respectively (lane 6 in Figures 3 and 4) (Figure 5).

Based on the above results, gangliosides, X5 and X6, were identified as disialosyl gangliosides with the fucosyl-GalNAc-GM1a core structure, and gangliosides, X3 and X4, were thought to be the derivatives of X5 and X6 respectively, with an additional alkali-susceptible group.

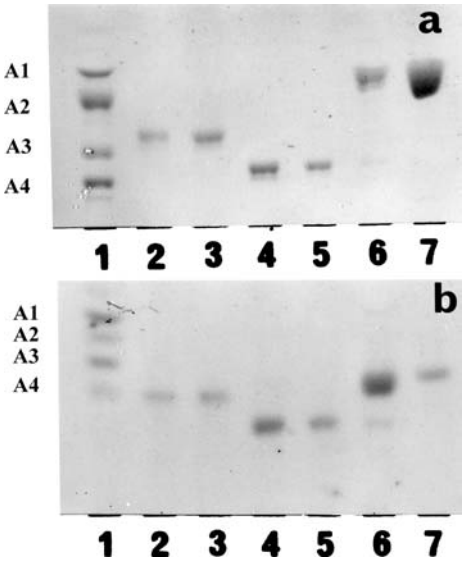

Fig. 3 Hydrolysis of X3 by mild alkali and neuraminidase. Lane 1, rat brain gangliosides (cf. Figure 2); lane 2, X3; lane 3, X3 treated with neuraminidase; lane 4, the product from X3 treated with 0.1 M NaOH in methanol; lane 5, X5; lane 6, the ganglioside of lane 4 treated with neuraminidase; lane 7, X1. The plates were developed with solvent systems I (panel a) and II (panel b), and stained with orcinol reagent.

Linkage analysis of sialic acids

Ratios of the two types of sialic acids in X3 and X5 each were found to be one to one individually by the GC-MS of equimolar mixtures of their respective methyl esters of 4, 7, 8, 9-tetra-*O*-methylglycosides and 8-*O*-TMS-4, 7, 9-

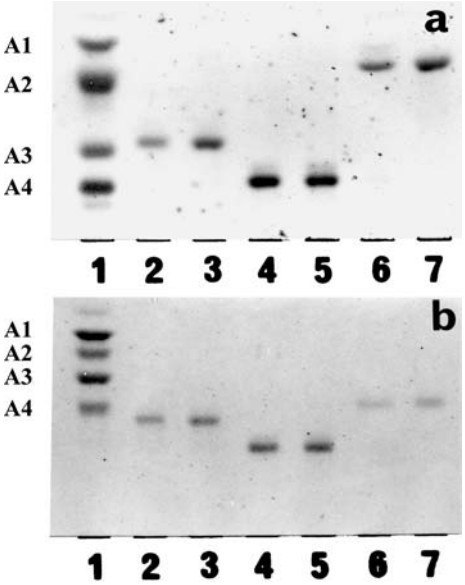

Fig. 4 Hydrolysis of X4 by mild alkali and neuraminidase. Lane 1, rat brain gangliosides (cf. Figure 2); lane 2, purified X4; lane 3, X4 treated with neuraminidase; lane 4, X4 treated with 0.1 M NaOH in methanol; lane 5, X6; lane 6, alkali-treated X4 incubated with neuraminidase; lane 7, X2. The HPTLC plates were developed with solvent systems I (panel a) and II (panel b).

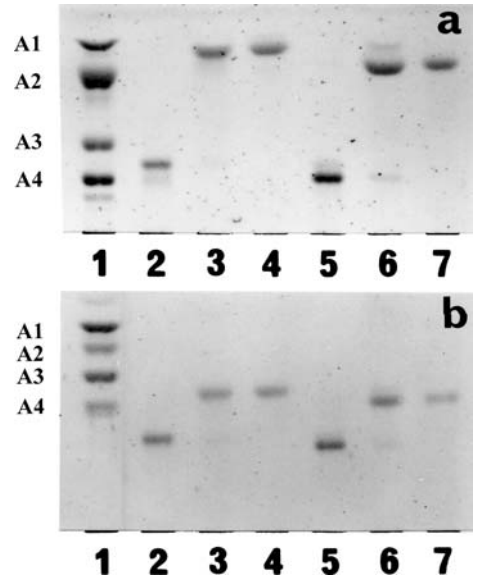

Fig. 5 Hydrolysis of X5 and X6 by neuraminidase. Lane 1, rat brain gangliosides (cf. Figure 2); lane 2, X5; lane 3, X5 treated with neuraminidase; lane 4, X1; lane 5, X6; lane 6, X6 treated with neuraminidase; lane 7, X2. The plates were developed and stained as described in Figure. 4.

trimethylglycosides of NeuAc using GD3 as the standard (Figure 6).

As the control, the methyl ester of 4,7,8,9-tetra-*O*-methylglycoside of NeuAc of fucosyl-GalNAc-GM1a was also measured by GC-MS. Based on these results, the sialic acid linkage of X3 and X5 was assigned to NeuAc2-8 NeuAc.

^{1}H NMR

The anomeric and ring proton regions of the one-dimensional spectra indicated that X3 (Figure 7) and X5 (Figure 8) contained three β-hexoses and two β-*N*-acetylhexosamines in addition to an α-Fuc as was found for Fuc-GalNAc-GM1a [8]. The chemical shifts of H-1 to H-4 and coupling constants (^3J) assessed on the two dimensional spectra (Figures 7 and 8) indicated that the glycosides are a β-Glc, two β-Gal and two β-GalNAc. The β-Glc is linked to ceramide, which is supported by the coupling of doublet signals of H-1 at 4.16 ppm (Table 4) to the coupling of a triplet signal of H-2 at 3.04 ppm.

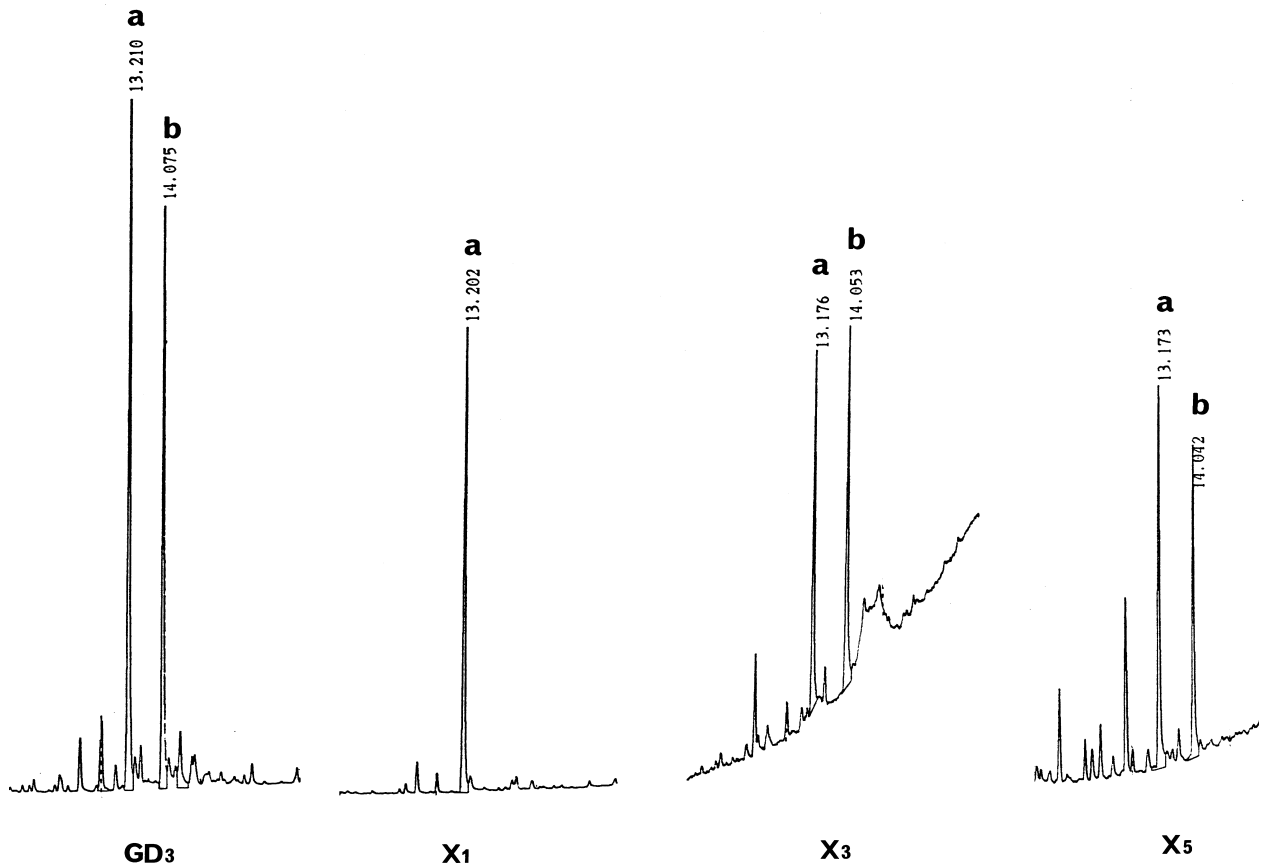

Fig. 6 Gas chromatography of sialic acid derivatives from permethylated gangliosides. a, methyl ester of 4,7,8,9-tetra-*O*-methylglycoside of NeuAc; b, methyl ester of 8-*O*-TMS-4,7,9-tri-*O*-methylglycoside of NeuAc.

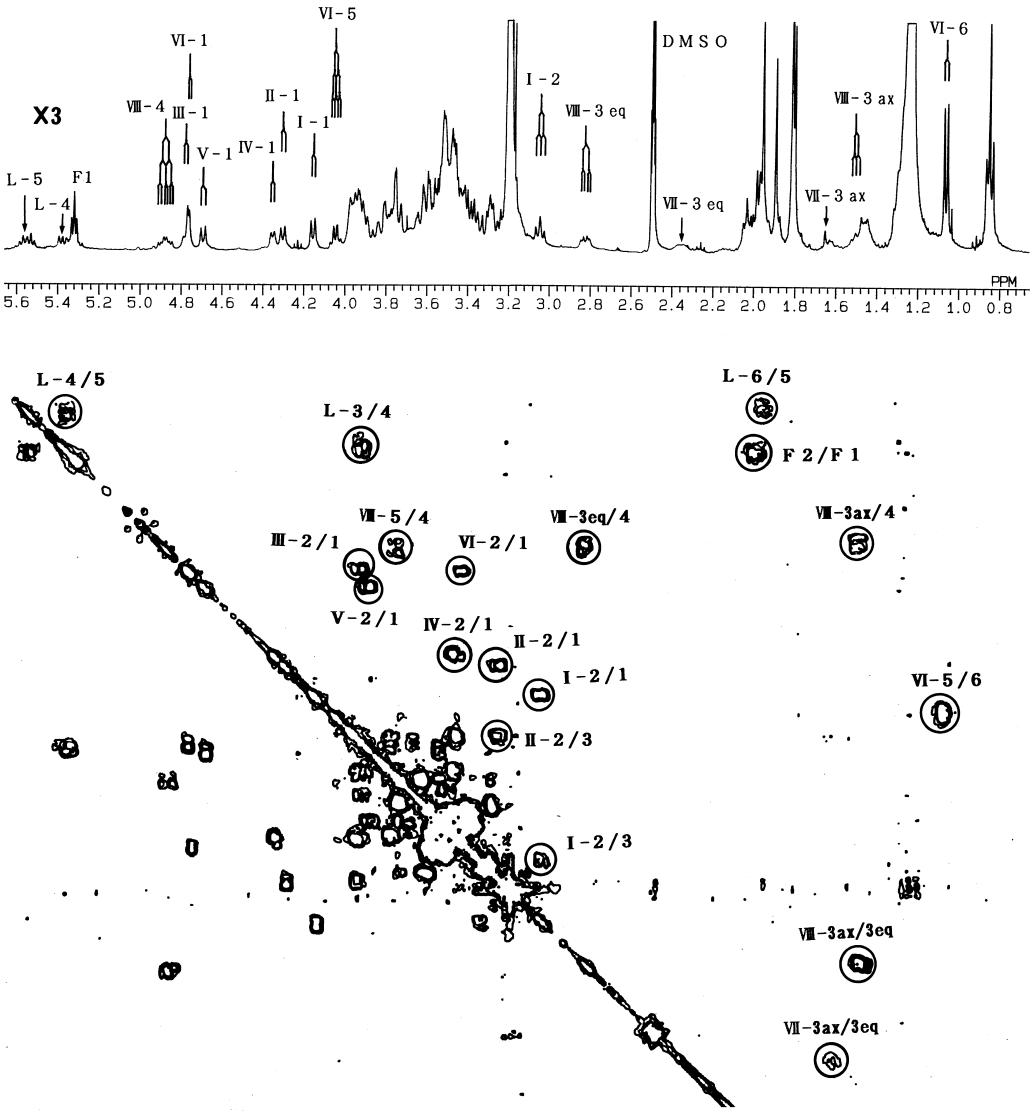

Fig. 7 2-D COSY spectrum of ganglioside X3 from salmon kidney. The conditions for 2-D COSY NMR spectroscopy are described in the text. Arabic numbers refer to the ring protons of sugar residues marked by Roman numerals assigned to the saccharide in Results. Resonances marked by L- are from the sphingoid, while resonances marked by F1 are assigned to *cis*-olefinic methines of unsaturated fatty acids. I-2/1 etc. means the cross peak of e.g. H-2 and H-1 protons in the glucosyl residue.

^{1}H COSY spectra demonstrated the spin connectivity between H-3ax/H-3eq, H-3ax/H-4, H-3eq/H-4 and H-5/H-4 of NeuAc. On the spectra of X3 (and X4), the signals corresponding to H-3eq and H-3ax of one of the NeuAc were found at 2.328 (2.322) and 1.642 (1.654) ppm respectively. This agrees with the previous observation that the H-3ax and h-3eq of the inner 8-*O*-sialylated NeuAc "shrinks", i.e. the shift difference between these two signals becomes smaller [20]. Another set of signals from H-3eq, H-3ax and H-4 of the outer NeuAc were observed at 2.826 (2.831), 1.514 (1.515) and 4.879 (4.884) ppm respectively. The signal of H-4 of X3 and X4, resonating at approximately 0.7 ppm lower field than H-4 (4.18 ppm, at 40°C) of the outer NeuAc of GD1b [20] (Table 4), and 1.1 ppm downfield in comparison to the NeuAc of the monosialosyl analogues of this ganglioside [8]. These results suggest that an *O*-acyl group is located at position 4 of NeuAc [21]. The singlet at 1.958 ppm was ascribed to the methyl protons of the 4-*O*-acetyl group in agreement with the similar resonance (1.954 ppm) obtained for 4-*O*-acetyl GM3 [21]. The three singlets between 1.8–1.9 ppm could be assigned to the *N*-acetyl methyl groups (Table 5).

Proton resonances of the saccharide chain in X4 were essentially similar to those of X3 (Table 4). Intense resonances of methine protons in the unsaturated fatty acid were observed at 5.3 ppm in X3, whereas the signal was weak in X4. These data agreed with the results from the compositional analysis of the fatty acid. Based on the above

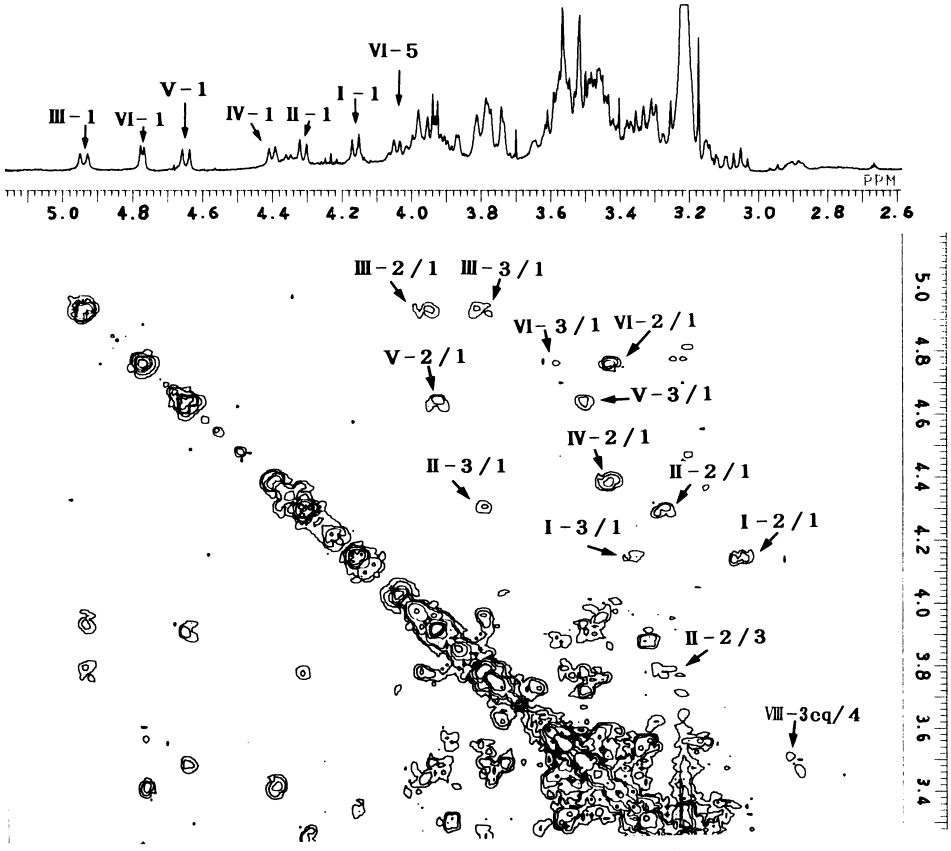

Fig. 8 2-D relayed-COSY spectrum of ganglioside X5. The conditions for the analysis are described in the Methods section.

Table 4 Proton chemical shifts (ppm from Me_4Si) and vicinal coupling constants *J* (Hz) for gangliosides X3–X6

VI V IV III II I
Fucα1-3GalNAcβ1-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer
3
|
(4-*O*-Ac)NeuAcα2-8NeuAcα2
VIII VII

	I	II	III	IV	V	VI	VII			VIII		
	H-1	H-1	H-1	H-1	H-1	H-1	H-3eq	H-3ax	H-4	H-3eq	H-3ax	H-4
GM1a	4.158	4.242	4.882	4.279			2.580	1.630				
	(7.8)	(7.1)	(8.8)	(7.8)								
GD1b*	4.15	4.59	4.85	4.18			2.38	1.53	4.18	2.35	1.30	4.18
X1	4.158	4.279	4.871	4.292	4.657	4.765	2.580	1.634	3.75			
	(7.8)	(7.8)	(8.5)	(6.8)	(8.5)	(3.9)						
X2	4.158	4.278	4.871	4.291	4.657	4.763	2.581	1.632	nd			
	(7.8)	(7.8)	(8.7)	(6.8)	(8.6)	(4.0)						
X3	4.156	4.302	4.778	4.353	4.693	4.766	2.328	1.642	nd	2.826	1.514	4.879
	(7.8)	(7.8)	(6.6)	(6.1)	(8.3)	(3.4)						
X4	4.155	4.295	4.765	4.353	4.695	4.765	2.322	1.654	nd	2.831	1.515	4.884
	(7.8)	(7.8)	(6.6)	(6.1)	(8.3)	(3.4)						
X5	4.160	4.310	4.937	4.399	4.647	4.771	2.10	1.75	nd	2.90	1.50	nd
	(7.8)	(7.8)	(8.3)	(6.8)	(8.3)	(3.9)						
X6	4.159	4.309	4.937	4.402	4.649	4.771	nd	nd	nd	2.905	nd	nd
	(7.8)	(7.8)	(8.3)	(6.8)	(8.3)	(3.9)						

nd; not determined.
*Data from Fronza et al. [20], determined at 40°C.

Table 5 Proton chemical shifts of methyl groups in *N*-acetyl or *O*-acetyl group

	III	V	VII	VIII	
	N-Ac	N-Ac	N-Ac	N-Ac	O-Ac
GM1a	1.769	—	1.878	—	—
GD1b*	1.88	—	1.91	1.88	—
X1	1.780	1.812	1.879	—	—
X2	1.779	1.811	1.878	—	—
X3	1.805	1.817	1.897	1.817	1.958
X4	1.806	1.817	1.900	1.817	1.957
X6	1.767	1.811	1.879	1.879	—

*Data from Fronza et al. [20], determined at 40°C.

results, the structures of X5 and X6 are proposed to be as follows:

VI V IV III II I
Fucα1-3GalNAcβ1-3Galβ1-3GalNAcβ1-4Galβ1-4 Glcβ-Cer
3
|
NeuAcα2-8NeuAcα2
VIII VII

The structures of X3 and X4 were proposed to be the derivatives of X4 and X5 containing additional *O*-acetyl groups on C-4 of the outer NeuAc as depicted below:

VI V IV III II I
Fucα1-3GalNAcβ1-3Galβ1-3 GalNAcβ1-4 Galβ1-4 Glcβ-Cer
3
|
(4-*O*-Ac)NeuAcα2-8NeuAcα2
VIII VII

Negative-ion LSIMS analysis

To confirm the structures proposed above, X4 and X5 were analyzed by negative-ion LSIMS (Figure 9).

In the spectrum of X4 (Figure 9A), a deprotonated molecule $[M-H]^-$ together with its intense sodium salt, corresponding to the ceramide species of d18: 1/C14: 0, were detected at *m/z* 2170 and 2192 respectively. Furthermore, characteristic fragment ions were observed at *m/z* 2128 $[M-H-CH_2=C=O]^-$, 1837 [M-H-Ac-NeuAc]$^-$, 1546 [M-H-(Ac-NeuAc-NeuAc)]$^-$, 1677, 1400, 1197, 1326, 1035, 832, 670 and 508 [Cer(d18: 1/C14: 0)-H]$^-$ as shown in Figure 9A. Similarly, a deprotonated molecule and its sodium salt of X5 were detected at *m/z* 2266 and 2288, respectively, = which corresponded to the ceramide species of d18: 1/C24: 1 (Figure 9B). Characteristic fragment ions at *m/z* 1975 [M-H-NeuAc]$^-$, 1684 [M -H- NeuAc-NeuAc]$^-$, 1626, 1464,1261, 1173, 970, 808 and 646 [Cer(d18: 1/C24: 1)-H]$^-$ were assigned as in the scheme of Figure 9B.

Concentration of gangliosides

Pooled fractions (No. 31–40 for X3 and X4) and (No. 41–50 for X5 and X6) from DEAE-Sephadex column chromatography were used for the determination of these gangliosides. After the gangliosides in the fractions were separated on TLC, each spot was determined by resorcinol reagent. The tissue concentrations of X3 and X5 were similar to X4 and X6 respectively, although Fuc-GalNAc-GM1a with longer-chain fatty acids was more abundant than that with shorter-chain fatty acids (Table 6). The acetylated Fuc-GalNAc-GD1a analogues (X3 and X4) were approximately two-fold more enriched than the nonacetylated analogues (X5 and X6). The tissue concentration of disialosyl gangliosides corresponded

Table 6 Structures and concentration of gangliosides X1–X6

Structure	(nmol/g tissue)	
X1 and X2:	X1	X2
Fucα1-3GalNAcβ1-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer 3 \| NeuAcα2	3.68	2.79
X3 and X4:	X3	X4
Fucα1-3GalNAcβ1-3Galβ1-3GalNAcβ1-4 Galβ1-4Glcβ-Cer 3 \| (4-*O*-Ac)NeuAcα2-8 NeuAcα2	0.13	0.16
X5 and X6:	X5	X6
Fucα1-3GalNAcβ1-3Galβ1-3GalNAcβ1-4 Galβ1-4Glcβ-Cer 3 \| NeuAcα2-8 NeuAcα2	0.07	0.07

Data of X1 and X2 were obtained from the reference [8].

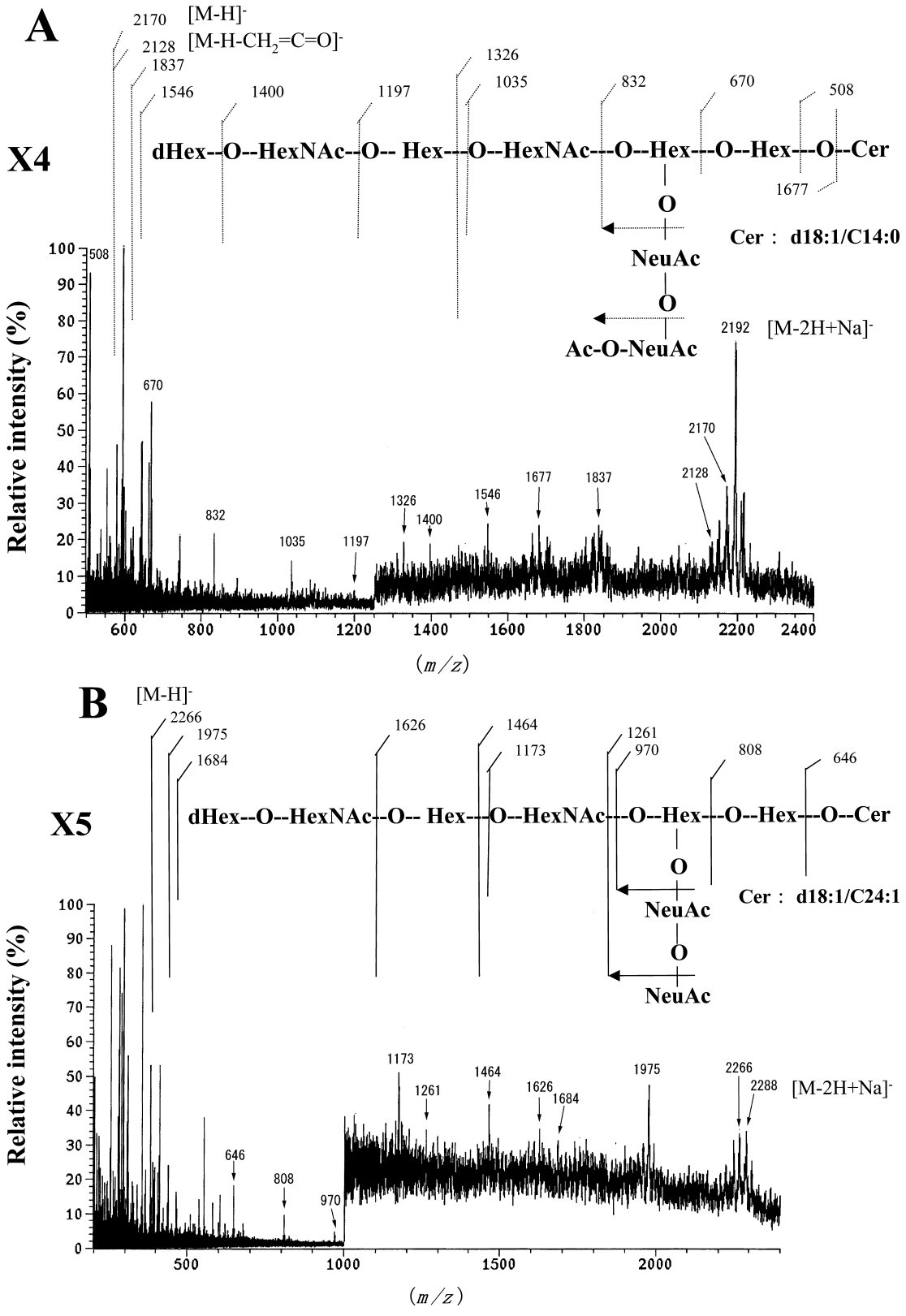

Fig. 9 Negative-ion LSIMS of X4 and X5. A, X4. The peaks in the mass range higher than *m/z* 1250 were amplified three fold. B, X5. The peaks in the mass range higher than *m/z* 1000 were amplified 20-fold. *Hex*, hexose; *HexNAc*, *N*-acetylhexosamine; *dHex*, deoxy hexose; *Ac*, acetyl; *Cer*, ceramide; *d18:1*, 4-sphingenine; *[M-H]*$^{-}$, the deprotonated molecule. The values of *m/z* in the higher mass regions are represented by nominal masses omitting the decimal fractions.

to approximately 6.6% of the total concentration of monosialosyl Fuc-GalNAc-GM1a.

Discussion

It has been shown that salmon kidneys contain a novel fucosyl-GalNAc-GM1a as a major acidic glycolipid [8]. In the present paper, disialosyl fucogangliosides (X3-X6) with a similar core saccharide structure were characterized. X5 and X6 are proposed to be Fuc-GalNAc-GM1a sialylated at position eight of the neuraminic acid residue. NMR spectroscopy unequivocally demonstrated that position four of the terminal neuraminic acid residue of gangliosides X3 and X4 was acetylated. The $[M\text{-}2H + Na]^-$ ion, typical for a acidic glycosphingolipid [3], was detected in the negative-ion LSIMS spectra of both disialosyl gangliosides, X4 and X5. The $[M\text{-}2H+Na]^-$ ion was also predominant in the fast-atom bombardment mass spectra of 9-*O*-acetyl-GD3 [22] and 7-*O*-acetyl-GD3 [23]. While GM3 containing 4-*O*-acetyl-*N*-glycolylneuraminic acid [21] and its derivatives have been characterized in equine erythrocytes [24], the *O*-acetyl disialofucosyl ganglioside of the salmon kidney is the first disialosyl ganglioside containing 4-*O*-acetyl-*N*-acetylneuraminic acid confirmed. As indicated in a recent review on the biological function of *O*-acetylated sialic acids in glycoconjugates [25], *O*-acetylation of fish kidney gangliosides, concomitant with unsaturation of fatty acids [26], may reduce the polarity of gangliosides in fishes, and help them to acclimate to low temperatures in the ocean by maintaining the fluidity of cell membranes. At the same time, *O*-acetyl substitutions of sialic acids in glycoconjugates may protect the kidney from the action of bacterial and viral sialidases [27], in a similar manner to that reported for influenza virus binding [28]. A novel polysialoglycoprotein containing 9-*O*-acetyl-deaminated neuraminic acid was reported in salmon eggs [29]. The physiological role of these *O*-acetyl gangliosides in the salmon kidney is open to further studies.

Acknowledgements We wish to thank Mrs. Michiko Ogawa for technical assistance, Dr. Keiko Tadano-Aritomi for measurement of Negative-ion LSIMS and Dr. Naoko Tanaka for ^{1}H-NMR spectroscopy. This work was supported in part by a grant-in-aid for Co-operative Research (A) (No. 61304034) from the ministry of Education, Science and Culture of Japan.

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