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Isolation and characterization of acidic glycosphingolipids from the gill of the Pacific Salmon (*Oncorhynchus keta*): A novel hybrid-type ganglioside with *iso*globo- and *neo*lacto-Series

Yukio Niimura

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Abstract Monosialosyl gangliosides and sulfoglycolipids in the gill of pacific salmon, *Oncorhynchus keta*, have been prepared by solvent extraction and DEAE-Sephadex column chromatography. Acidic glycolipid bands (M1-M13) detected by thin layer chromatography were separated by Iatrobeads column chromatography and 13 components were characterized by TLC, compositional analysis, methylation analysis, chemical and enzymatic degradation, liquid secondary ion mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy. In addition to the acidic glycolipids with known structures (SM4s, SM3, GM3, LM1, GM1b and $V^3\alpha$Fuc,$IV^3\beta$GalNAc-GM1a), two fractions (M11 and M13) of unknown monosialosyl gangliosides with TLC mobility slower than GM1a were isolated and characterized as having the following structure with a hybrid of *iso*globo- and *neolacto*-series.

$$\begin{array}{l} \text{NeuAc}\alpha 2-3\text{Gal}\beta-4\text{GlcNAc}\beta-3\text{Gal}\alpha-3\text{Gal}\beta-4\text{Glc}\beta-\text{Cer} \\ \quad\quad 3 \\ \quad\quad | \\ \quad\quad \text{Fuc}\alpha 1 \end{array}$$

Analysis of fatty acid indicated predominance of C24:1 fatty acid in the upper band (M11) and shorter chain saturated fatty acids in the lower band (M13). The tissue concentrations of M11 and M13 were 1.15 and 0.96 μmol/kg wet weight, respectively.

Keywords Fucoganglioside · Salmon gill · Isoglobo- and neolacto-series · Sulfoglycolipids

Abbreviations

GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
Hex	hexose
HexNAc	*N*-acetylhexosamine
NeuAc	*N*-acetylneuraminic acid
Cer	ceramide
LacCer	Galβ1-4Glcβ1-1Cer
Gg_3Cer	GalNAcβ1-4Galβ1-4Glcβ1-1Cer
Gg_4Cer, asialo GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer
SM4s	GalCer I^3-sulfate
SM3	LacCer II^3-sulfate
GM3	$II^3\alpha$NeuAc-LacCer
GM1a	$II^3\alpha$NeuAc-Gg^4Cer
GM1b	$IV^3\alpha$NeuAc-Gg^4Cer
LM1	IV3αNeuAc-nLc
d18:1	4-sphingenine
d18:0	4-sphinganine
t18:0	4-hydroxysphinganine
TLC	thin layer chromatography
HPTLC	high performance TLC
HPLC	high performance liquid chromatography
TEAB	triethanolamine carbonate
GLC	gas–liquid chromatography
GC-MS	gas chromatography-mass spectrometry

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

Y. Niimura (✉)
Research Center of Biomedical Analysis and Radioisotope,
Teikyo University School of Medicine,
2-11-1, Kaga Itabashi-ku,
Tokyo 173-8605, Japan
e-mail: yniimura@med.teikyo-u.ac.jp

LSIMS	liquid secondary ion mass spectroscopy
NMR	nuclear magnetic resonance
COSY	chemical shift correlated spectroscopy
Me_2SO	dimethyl sulfoxide
BSTFA	bis-(trimethylsilyl)trifluoroacetamide
TMS	tetramethylsilane

Introduction

Glycolipids serve for various functions of cell membranes [2, 3]. Glycolipids in actively transporting organs such as the kidney of mammals [4, 5] have been studied in detail. The roles of glycolipids in transport function of kidney were investigated using some renal cell lines [6–10]. However, there have been only a few works on the glycolipids of the major osmoregulatory organs of aqueous vertebrates except for the report that the metabolism of sulfatide in the gill of eel was activated when eels were transferred to sea water from fresh water [11]. A unique ganglioside, fucosyl-GalNAc-GM1a, was characterized from the kidney of the salmon [12]. Recently we reported the related disialosyl gangliosides containing 4-*O*-acetyl-*N*-acetylneuraminic acid from salmon kidney [13]. The glycosphingolipids of teleost gill have not been studied to date, although the glycosphingolipids of the teleost have been reported from the brain [14], milt [15], liver [16, 17] and roe [18, 19]. The teleost gill consists of branched or feathery tissue richly supplied with blood vessels, and the structure of the gill surface is well developed as a respiratory organ for facilitating the exchange of oxygen and carbon dioxide. Another significant function of the gill is the osmoregulation which maintains the constant level in body-ions under the various osmotic conditions. Because a salmon is able to adapt to both sea water and fresh water, the cell surface of the salmon gill is considered to be a unique membrane structure containing special components. Glycosphingolipids as amphiphiles are the candidates for supporting elements of the ion-barrier function of the gill cell surface. For this purpose we investigated the profile of acidic glycosphingolipids in the gill of Pacific salmon, *Oncorhynchus keta*. In the present paper a unique ganglioside with a hybrid type glycon was demonstrated in addition to known four gangliosides and two sulfated glycolipids.

Materials and methods

Materials

The gills were freshly prepared from pacific salmons captured offshore Sanriku Coast in Japan in December. The material was frozen at −40°C until use. Standard glycosphingolipids, compounds, chemicals and reagents were described as previously [12]. β-Galactosidase (EC 3.2.1.23, grade VII) and β-*N*-acetylhexosaminidase (EC 3.2.1.30) from jack bean, α-galactosidase (EC 3.2.1.22,) from green beans and neuraminidase (EC 3.2.1.18, type V) from *Clostridium perfringens* were the products of Sigma, St. Louis, U.S.A. Reagents for derivatization or NMR spectroscopy were previously described [20].

Thin layer chromatography

TLC was performed on Silicagel 60 HPTLC plates (Merck) with the following solvent systems: I, chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v); II, chloroform/methanol/water (60:35:8, v/v); and III, chloroform/methanol/3.5 M ammonium hydroxide (55:45:10, v/v). Glycolipids were visualized by spraying the plate with orcinol/H_2SO_4 reagent or resorcinol reagent [21] and heating for 5 min at 120°C. Sulfoglycolipids were detected by staining with azure A solution [22].

Lipid extraction and purification of gangliosides

Gills, 550 g, were extracted by homogenization using a Warring blender with 4.5 l of chloroform/methanol (2:1, v/v). After filtration, the second and the third extraction were performed with chloroform/methanol/0.88% KCl (60:120:8, v/v, 1.9 l) and chloroform/methanol/0.4 M sodium acetate (30:60:8, v/v, 1.9 l), respectively. The extracts were combined, concentrated with rotary evaporator, and partitioned by the addition of chloroform/methanol/0.88% KCl (8:4:3, v/v, 1.5 l). The upper phase was concentrated, dialyzed and lyophilized. The lower phase was concentrated to dryness and treated with 0.1 M NaOH in methanol at 37°C for 1 h, then neutralized with 1-M acetic acid and partitioned as above. The upper phase was dialyzed and lyophilized and the lower phase was concentrated. The resultant two upper phase fractions and the lower phase fraction were combined with chloroform/methanol/water (30:60:8, v/v, 170 ml), and the aliquot was subjected to DEAE-Sephadex A-25 (hydrogen carbonate form, 1.2 × 45 cm) column chromatography. After washing the column with 4 l of the same solvent, acidic glycolipids were separated with a linear gradient of chloroform/methanol/water (30:60:8, v/v, 500 ml) to chloroform/methanol/0.8 M TEAB (triethylamine carbonate) (30:60:8, v/v, 500 ml) at the flow rate of 1 ml/min. The eluates (10 ml/tube) were monitored by HPTLC. The glycolipid fractions in the monosialosyl glycolipids were further purified by HPLC in a Shimadzu LC 4A apparatus using a column (1 × 30 cm) of Iatrobeads (6RS-8005) with chloroform/methanol/water (60:40:2, v/v) at the flow rate of 1 ml/min.

Chemical and spectral analyses

Monosaccharides, fatty acids and sphingoids were analyzed by gas chromatography as described [12]. Gangliosides were determined by resorcinol reagent using *N*-acetyl neuraminic acid as a standard. For the measurement of ^{1}H nuclear magnetic resonance spectroscopy the purified glycolipids were treated repeatedly with 0.5 ml portions of $CH_3O[^2H]$, followed by desiccation over P_2O_5 *in vacuo* to exchange the labile protons with deuteron. The dried glycolipids were redissolved in 0.5 ml of a mixture of $[^2H]Me_2SO/[^2H]_2O$, (98:2, v/v). The spectra were recorded on a GX-400 400 MHz spectrometer of Japan Electron Optical Laboratory (JEOL) at 60°C. The operation conditions for one-dimensional spectrum were as follows: frequency, 400 MHz; sweep width, 4 kHz; sampling points, 16 k. All the two-dimensional spectra were recorded with 512 × 2,048 data points and a spectral width of 2,500 Hz as previously described [20]. Chemical shifts were indicated by ppm from the signal of TMS as an internal standard. Negative-ion LSIMS was performed on a Concept IH mass spectrometer (Shimadzu/Kratos) fitted with a cesium ion gun [23].

Methylation study

A portion (20–50 μg) of each glycolipid was methylated [24, 25], acetolyzed [26], reduced with $NaB[^2H]_4$ [4], and acetylated according to the published procedures [27]. The acetates of partially methylated, 6-deoxyhexitol, hexitol, and hexosaminitol were analyzed by gas chromatography-electron impact mass spectrometry as described [12]. A mass range from 45 to 450 atomic mass units was scanned every 6 s. Peaks were identified by retention times and characteristic fragment ions. Mass fragmentograms of terminal sugar ions were used to calculate the yields of methylated glycolipids. For cerebroside, *m/z* 187 (Hex-MeOH) was measured at an ion-source temperature of 250°C, *m/z* 344 (NeuAc-MeOH) for GM3 at 280°C, and *m/z* 260 (HexNAc) for globoside at 285°C.

Limited degradation of glycolipid

Purified glycolipid was treated with 1% trichloroacetic acid at 100°C for 1 h. Treatment of glycolipids with β-galactosidase from Jack beans (Sigma Co.) was performed

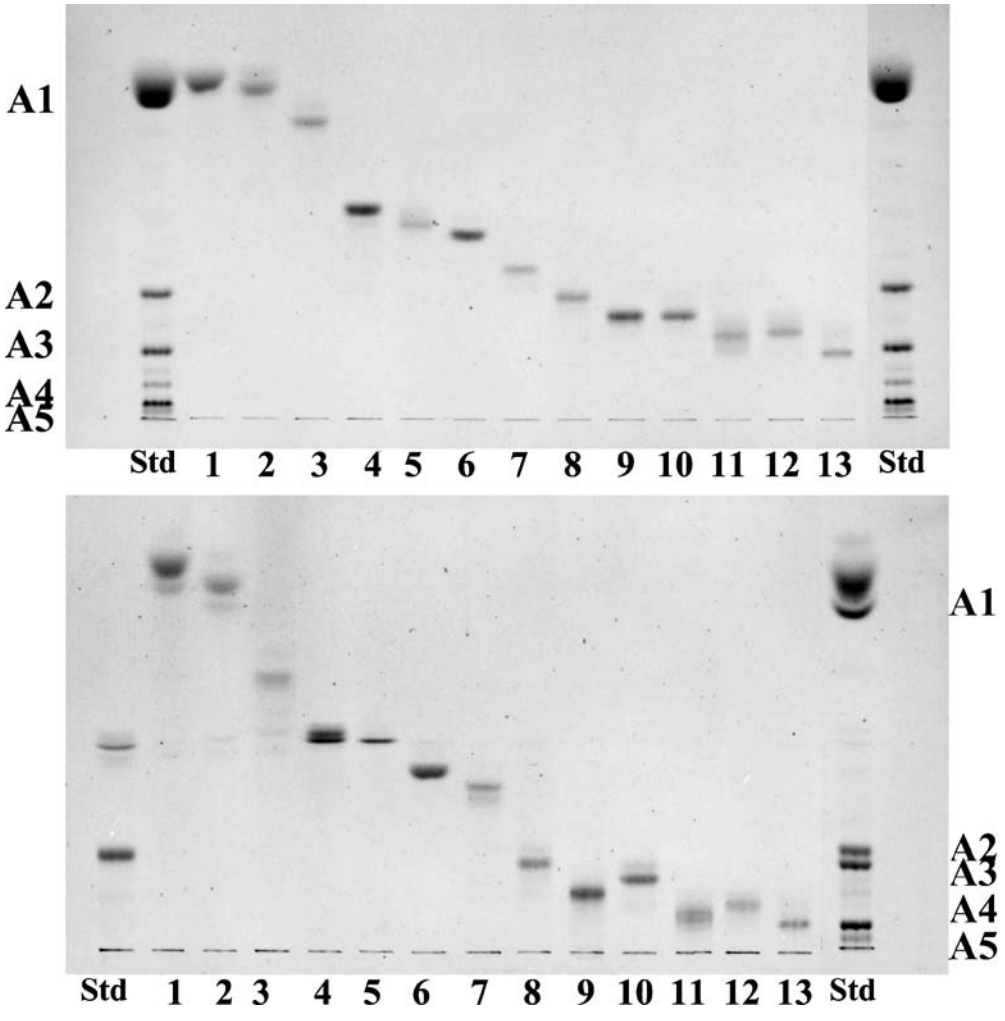

Fig. 1 HPTLC pattern of gangliosides purified from salmon gill. TLC was performed with solvent system I (chloroform/methanol/0.2% $CaCl_2$, 55:45:10, v/v) in the *upper panel* and III (chloroform/methanol/3.5 N ammonium hydroxide, 55:45:10, v/v) in the *lower panel*, respectively. *Lanes std in panels A and B* (*right side*) are rat brain acidic glycosphingolipids for reference (A1, SM4s; A2, GM3; A3, GM1a; A4, GD1a; A5, GD1b; A6, GT1b + GQ1b) and *left lane std in panel B* is standard gangliosides, GM3 (*upper band*) and GM1a (*lower band*). *Lanes 1–13* correspond to M1–M13, respectively. Orcinol reagent was sprayed for detection

Table 1 Carbohydrate composition of acidic glycolipids

Fraction	Glc	Gal	GalNAc	GlcNAc	Fuc	NeuAc
M1	–	1.00	–	–	–	–
M2	–	1.00	–	–	–	–
M3	1.00	0.84	–	–	–	–
M4	1.00	0.68	–	–	–	0.71
M5	1.00	0.79	–	–	–	0.71
M7	1.00	1.44	–	0.75	–	0.67
M8	1.00	1.80	0.98	–	–	0.61
M9	1.00	1.85	1.83	–	0.68	0.57
M10	1.00	1.97	0.98	–	–	0.76
M11	1.00	2.89	–	0.56	0.69	0.60
M13	1.00	2.69	–	0.60	0.44	0.53

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

as described [12]. In the case of β-*N*-acetylhexosaminidase from Jack beans the sodium citrate buffer of pH 5.0 was used and α-galactosidase from green beans (Sigma Co.) was used with sodium taurocholate (1 mg/ml) in the sodium citrate buffer of pH 4.0. The reaction mixture was adjusted to chloroform/methanol/water (30:60:8, v/v), and the product purified as described [12].

Results

Preparation of monosialosyl gangliosides and sulfoglycolipids from gill of salmon

Acidic glycolipids were eluted from DEAE-Sephadex A-25 by increasing the concentration of salt (triethylamine carbonate). Monosialosyl fraction contained 11.5 μmol gangliosides per kg wet weight. More than 13 bands (M1–M13) were detected in the region of monosialosyl fraction by TLC. These glycolipids were further purified by HPLC on an Iatrobeads column to single bands on TLC plates with solvent system II and I respectively (Fig. 1). The glycon structures of 11 glycolipids except for M6 and M12 were further characterized.

Identification of sulfoglycolipids

All these acidic glycolipids reacted with orcinol/H_2SO_4, and acidic glycolipids except for M1, M2 and M3 were found to be sialosylglycolipids by the reaction with resorcinol/HCl. By staining with azure A solution on TLC plate, three glycolipids (M1–M3) were found to be sulfoglycolipids. M1 and M2 comigrated with standard SM4s and M3 migrated slower than standard SM3. Carbohydrate analysis showed that M1 and M2 contained only Gal, while M3 contained Glc and Gal at the ratio of 1 to 1 (Table 1).

Methylation study yielded 2,4,6-tri-*O*-methylgalactitol in M1 and M2. Methylation analysis of M3 was not performed due to the scarcity of the compound. Based on these results, M1 and M2 were identified as SM4s and M3 was supposed as SM3. Predominant fatty acid was C24:1 in M1 and M3, and C16:0 in M2 (Table 2). The major long chain base in M1 and M2 was d18:1.

Gangliosides M4 and M5

Gangliosides M4 and M5 were characterized as GM3 ganglioside by the migration behavior on TLC, carbohydrate composition and methylation studies. Desialylation of these gangliosides by mild acid treatment resulted in a glycolipid comigrating with LacCer on HPTLC. The major fatty acid component was C24:1 in M4 and C16:0 in M5, respectively. Only NeuAc was detected by GLC as sialic acid of these gangliosides.

Ganglioside M7

Ganglioside M7 was composed of Glc, Gal, GlcNAc and sialic acid in the ratio of 1:2:1:1. NeuAc was detected by GLC as a sole species of sialic acid. This ganglioside was susceptible to neuraminidase from *Clostridium perfringens* and the desialylated glycolipid was sequentially degraded by β-galactosidase, β-*N*-acetylhexosaminidase and β-galactosidase to a monohexosylceramide (data not shown). Methylation analysis yielded 2,3,6-tri-*O*-methyglucitol, 2,4,6-tri-*O*-methylgalactitol, and 3,6-di-*O*-methyl-*N*-acetyl-glucosaminitol at an approximate ratio of 1:2:1 (Table 3).

^{1}H NMR spectrum showed anomeric protons of Glc (I) at 4.164 ppm (J = 7.8 Hz), Gal (II) at 4.260 ppm (J = 6.8 Hz), Gal (IV) at 4.191 ppm (J = 7.8 Hz) and GlcNAc (III) at 4.639 ppm (J = 8.3 Hz) (Table 4). Based on these results, the structure of M7 was characterized as LM1.

The lipophilic moiety of this ganglioside may be predominantly the combination of C24:1 and d18:1.

Gangliosides M8 and M10

Carbohydrate composition of both gangliosides M8 and M10 was shown to be Glc, Gal, GalNAc and sialic acid in the approximate proportion of 1:2:1:1. Only NeuAc was

Table 2 Compositional analyses of fatty acids and long chain bases

	M1	M2	M3	M4	M5	M7	M8	M9	M10	M11	M13
(Fatty acid)											
C14:0	–	2.9	6.1	3.7	13.4	–	–	–	4.8	10.6	33.4
C16:0	10.4	33.7	38.8	12.2	25.0	16.1	9.3	15.9	35.0	22.9	44.7
C16:0(h)	2.5	1.7	–	–	–	–	–	–	–	–	–
C18:0	4.2	11.8	21.2	4.2	19.9	12.3	7.7	12.0	26.5	14.9	21.9
C18:1	4.2	6.5	10.7	2.2	5.2	3.2	2.7	3.4	8.9	9.8	–
C18:0(h)	1.6	1.7	–	–	–	–	–	–	–	–	–
C20:0	1.0	1.4	5.1	0.4	–	–	–	–	3.6	–	–
C20:0(h)	0.4	1.2	–	0.9	2.9	–	–	–	–	–	–
C22:0	7.4	7.4	5.9	2.2	–	–	–	–	–	–	–
C22:0(h)	0.2	5.1	–	–	–	–	–	–	–	–	–
C24:0	4.2	1.5	1.5	3.1	1.7	–	4.9	–	3.5	–	–
C24:1	60.9	21.9	7.3	65.3	25.8	59.4	65.2	58.5	10.1	41.9	–
Others	2.9	3.3	3.4	5.6	6.2	8.9	10.3	10.1	7.5	–	–
(Long chain base)											
d18:1	78	80		74	84	100	36	64	4	30	
d18:0	22	–	nd	–	16	–	26	–	–	4	nd
t18:0	–	20		26	–	–	38	36	96	66	

Each component was identified from the retention time of GLC. Data were obtained from peak areas.
nd Not determined.

detected as the sialic acid species in both gangliosides. Methylation study of both gangliosides yielded 2,3,6-tri-*O*-methyglucitol, 2,3,6-tri-*O*-methylgalactitol, 2,4,6-tri-*O*-methygalactitol and 4,6-di-*O*-methyl-*N*-acetylgalactosaminitol in a equimolar proportion (Table 3). Ganglioside M8 was degraded by neuraminidase to a neutral glycolipid, which comigrated on HPTLC with Gg_4Cer from bovine brain GM1. Stepwise degradation of this glycolipid by β-galactosidase, β-*N*-acetylhexosaminidase and β-galactosidase yielded Gg_3Cer, LacCer and GalCer, respectively, on HPTLC (data not shown). Ganglioside M10 was also degraded similarly to M8 by a set of neuraminidase and glycosidases, and the corresponding products migrated slower than those of M8. ^{1}H-NMR spectrum of M8 showed anomeric protons ascribed to Glc (I) at 4.177 ppm (J = 7.8 Hz), Gal (II) at 4.198 ppm (J = 8.8 Hz), Gal (IV) at 4.238 ppm (J = 7.8 Hz) and GalNAc (III) at 4.522 ppm (J = 7.8 Hz) (Table 4). Spectrum similar to that of M8 was obtained from M10. The difference between M10 and M8 was only in the content of unsaturated fatty acids. Fatty acids in M8 were predominantly C24:1 in contrast to C16:0 and C18:0 in M10. Long chain base was d18:1, d18:0 and

Table 3 Partially-*O*-methylated hexitol and hexosaminitol acetates identified in the permethylated intact gangliosides

	2,3,6-Glc[a]	2,3,6-Gal[b]	2,4,6-Gal[c]	2,6-Gal[d]	4,6-GalNAc[e]	3,6-GlcNAc[f]	6-GlcNAc[g]	2,3,4-Fuc[h]
M7	1.00	–	2.39	–	–	0.82	–	–
M8	1.00	0.83	0.88	–	0.80	–	–	–
M9	1.00	–	0.92	1.25	1.85	–	–	1.05
M10	1.00	0.78	0.90	–	0.89	–	–	–
M13	1.00	–	2.59	–	–	–	0.99	0.63

Values are determined by GLC on a CBP-1 capillary column.
[a] 2,3,6-tri-*O*-methylglucitol.
[b] 2,3,6-tri-*O*-methylgalactitol.
[c] 2,4,6-tri-*O*-methylgalactitol.
[d] 2,6-di-*O*-methylgalactitol.
[e] 4,6-di-*O*-methyl-*N*-acetylgalactosaminitol.
[f] 3,6-di-*O*-methyl-*N*-acetylglucosaminitol.
[g] 6-*O*-methyl-*N*-acetylglucosaminitol.
[h] 2,3,4-tri-*O*-methyl-6-deoxygalactitol.

Table 4 Chemical shifts and coupling constants of gill gangliosides

	V	IV	III	II	I				
	NeuAcα–Galβ–HexNAcβ–Galβ–Galβ–Cer								
	V-3eq	IV-1	III-1	II-1	I-1				
M7	2.746	4.191	4.639	4.260	4.164				
	(7.8)	(8.3)	(6.8)	(7.8)					
M8	2.770	4.238	4.522	4.198	4.177				
	(7.8)	(7.8)	(8.8)	(7.8)					
M10	2.745	4.297	4.597	4.222	4.183				
	(7.3)	(7.3)	(7.3)	(7.8)					
	VI	V	IV	III	VII	II	I		
	Fucα–3GalNAcβ–3Galβ–3GalNacβ–4[NeuAcα2–3]Galβ–4Glcβ–1Cer								
	VII-3eq	VI-1	V-1	IV-1	III-1	II-1	I-1		
M9	2.570	4.761	4.661	4.276	4.884	4.284	4.155		
	(3.9)	(8.3)	(7.8)	(8.3)	(6.4)	(7.8)			
				VI	V	IV	III	II	I
				NeuAcα2–3Galβ–4GlcNAcβ–3Galα–3Galβ–4Glcβ–Cer					
						3			
						|			
						Fucα1			
						VII			
	VII-6	VII-5	VII-1	VI-3eq	V-1	IV-1	III-1	II-1	I-1
M11	1.008	4.618	4.872	2.735	4.311	4.715	4.872	4.311	4.192
	(6.4)					(6.8)			(7.3)
M13	1.009	4.625	4.881	2.757	4.311	4.732	4.854	4.311	4.191
	(6.4)		(3.9)		(7.3)	(7.3)	(3.4)	(7.3)	(7.3)

t18:0 in M8, while t18:0 was predominant in M10 (Table 2). Based on these results both gangliosides were identified as GM1b with different ceramide species.

Ganglioside M9 and M12

The *Rf* of M9 on HPTLC was close to the upper band of fucosyl-*N*-acetylgalactosaminyl-GM1a from salmon kidney. From carbohydrate composition analysis ganglioside M9 contained Glc, Gal, GalNAc, Fuc and sialic acid at the approximate proportion of 1:2:2:1:1. Only NeuAc was detected as sialic acid. Methylation analysis yielded 2,3,6-tri-*O*-methylglucitol, 2,4,6-tri-*O*-methylgalactitol, 2,6-di-*O*-methylgalactitol, 2,3,4-tri-*O*-methyl-6-deoxygalactitol and 4,6-di-*O*-methyl-*N*-acetylgalactosaminitol at an approximate proportion of 1:1:1:1:2. The ^{1}H-NMR spectrum showed doublets of H-1 of GalNAc (III), Fuc (VI), GalNAc (V), and Glc (I) at 4.884 ppm (J = 8.3 Hz), 4.761 ppm (J = 3.9 Hz), 4.661 ppm (J = 8.3 Hz), and 4.155 ppm (J = 7.8 Hz), respectively. Two doublets of H-1 of Gal (II and IV) at 4.284 ppm (J = 6.4 Hz) and 4.276 ppm (J = 7.8 Hz), a quartet of H-5 of Fuc at 4.048 ppm were also identified. Since these NMR data were similar to those of fucosyl-*N*-acetylgalactosaminyl-GM1a from salmon kidney [12], M9 was characterized as follows:

$$\begin{aligned}&\text{Fuc}\alpha-3\text{GalNAc}\beta-3\text{Gal}\beta-3\text{GalNAc}\beta\\&\quad-4[\text{NeuAc}\alpha2-3]\text{Gal}\beta-4\text{Glc}\beta-1\text{Cer}\end{aligned}$$

C24:1 fatty acid was shown to be predominant and d18:1 and t18:0 were detected in M9 as long chain bases. Negative ion LSIMS of M12 demonstrated the above glycon structure of M9 with ceramide of d18: 1/C14:0 (data not shown).

New gangliosides M11 and M13

Both M11 and M13 contained Glc, Gal, GlcNAc, Fuc and sialic acid at the approximate proportion of 1:3:1:1, respectively. Only NeuAc was detected as sialic acid. Alditol acetates of 2,3,6-*O*-methylglucitol, 2,4,6-*O*-methylgalactitol, 2,3,4-*O*-methyl-6-deoxygalactitol, and 6-*O*-methyl-*N*-acetylglucosaminitol in a proportion of 1:3:1:1 were detected by methylation analysis of M13 (Fig. 2a,b).

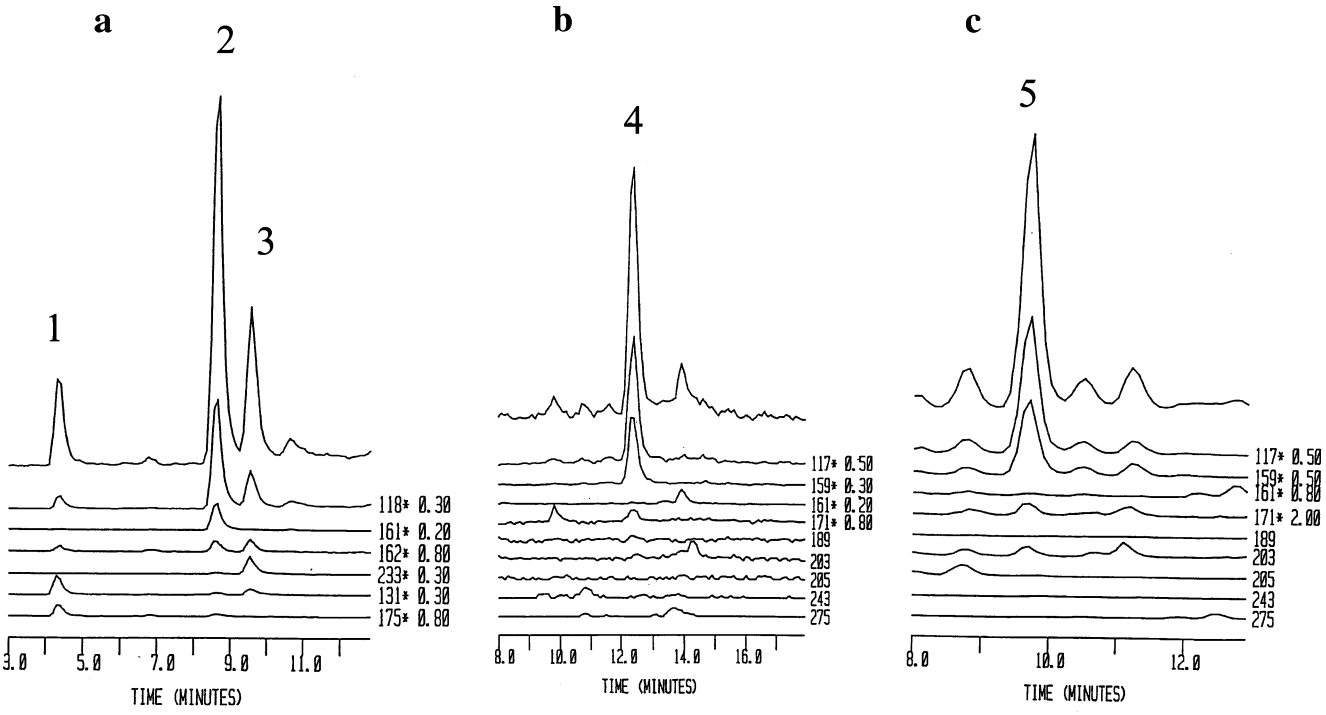

Fig. 2 Mass-chromatography of partially methylated alditol acetates of glycosides prepared from ganglioside M13. (*Panel A*) Partially-*O*-methylated hexitol acetates of M13 were separated on a SP-2340 column. Hexosaminitol derivatives were not detected on this column. (*Panel B*) Partially-*O*-methyl-*N*-methyl-*N*-acetylhexosaminitol acetate was separated on an OV-17 column and was identified from the *m/z* 117, 159 and 171. (*Panel C*) Acetate of partially methylated hexosaminitol from desialylated and defucosylated M13 was separated on OV-17 column and was identified by the retention time of 3,6-di-*O*-methyl-*N*-acetylglucosaminitol from standard sialosylparagloboside. Detailed conditions are described in Materials and Methods section. Peaks are numbered as follows: (1) 2,3,4-tri-*O*-methyl-6-deoxygalactitol; (2) 2,4,6-tri-*O*-methylgalactitol; (3) 2,3,6-tri-*O*-methylglucitol; (4) 6-*O*-methyl-*N*-acetylglucosaminitol; (5) 3,6-di-*O*-methyl-*N*-acetylglucosaminitol

M13 was treated with neuraminidase from *Clostridium perfringens*, and the desialylation product was then defucosylated by trichloroacetic acid (Fig. 3). The desialylation and defucosylated M13 could be sequentially degraded by β-galactosidase, β-*N*-acetylhexosaminidase, α-galactosidase and β-galactosidase to a monohexosylceramide (Fig. 3).

Based on these results the structure of M13 was proposed to be:

$$\begin{aligned}&\text{NeuAc}\alpha 2-3\text{Gal}\beta-(4\text{ or }3)[\text{Fuc}\alpha-3\text{ or }4]\text{GlcNAc}\beta\\&\quad-3\text{Gal}\alpha-3\text{Gal}\beta-4\text{Glc}\beta-1\text{Cer}\end{aligned}$$

In order to elucidate the linkage of Gal-GlcNAc, the desialylated and defucosylated derivative was prepared and analyzed by methylation study. Since 3,6-*O*-methyl-*N*-acetylglucosaminitol was detected from this compound using sialosylpalagloboside as a standard, the linkage of Gal1-4GlcNAc could be confirmed (Fig. 2c). One-dimensional ^{1}H-NMR spectroscopy of M13 showed doublets of H-1 of Fuc, Gal (III), GlcNAc, Gal (II and V), and Glc at 4.881 ppm (J = 3.9 Hz), 4.854 ppm (J = 3.4 Hz), 4.732 ppm (J = 7.3 Hz), 4.311 ppm (J = 7.3 Hz), and 4.191 ppm (J = 7.3 Hz), respectively. A quartet of H-5 of Fuc at 4.625 ppm, a quartet of H-3eq of NeuAc at 2.757 ppm and a doublet of H-6 of Fuc at 1.009 ppm (J = 6.4 Hz) also supported the proposed structure (Table 4). *N*-Acetylmethyl protons of NeuAc and GlcNAc were observed at 1.885 and 1.811 ppm, respectively, as a singlet. Two-dimensional COSY spectrum also revealed cross peaks of H-1/H-2, H-1/H-3 and others (Fig. 4).

H-2 of Gal (V) was observed in the lower field than that of Gal (II) because of the attachment of NeuAc to position 3. NMR spectrum of M11 also showed signals of the saccharide chain similar to M13 (Table 4). In agreement to the fatty acid analysis, the signals of *cis*-olefin methines of unsaturated fatty acid in M11 were much larger than those in M13, and d18:1/C24:1, respectively.

Negative ion LSIMS analysis of M13 (Fig. 5a) demonstrated molecular ions $(M\text{-}H)^-$ at *m/z* 1,814 and 1,796 corresponding to the ceramide species (t18:0/C14:0) and (d18:1/C14:0), respectively. Furthermore, characteristic fragment ions, *m/z* 1,523$(M\text{-}H\text{-}NeuAc)^-$, 1,361$(M\text{-}H\text{-}NeuAc\text{-}Hex)^-$,

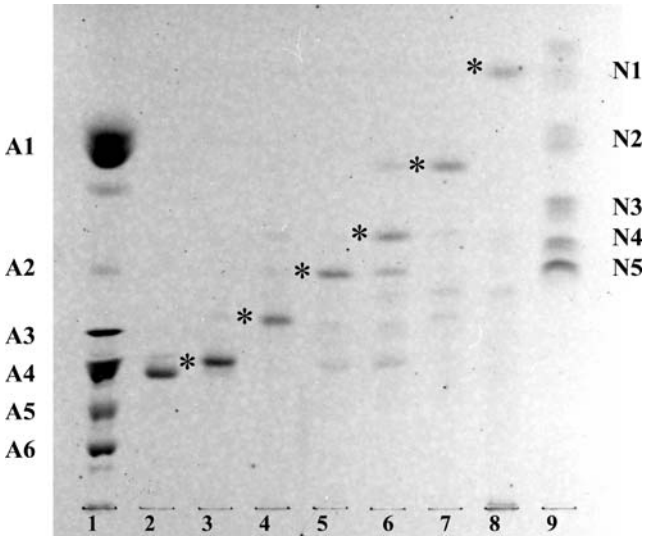

Fig. 3 Sequential degradation of ganglioside M13 by exoglycosidases. *Lane 1*, rat brain acidic glycolipid (cf. Fig. 1): A1, SM4s; A2, GM3; A3, GM1a; A4, GD1a; A5, GD1b; A6, GT1b + GQ1b. *Lane 2*, purified M13. *Lane 3*, product of M13 by neuraminidase. *Lane 4*, product of *lane 3* treated with trichloroacetic acid. *Lane 5*, product of *lane 4* incubated with β-galactosidase from jack bean. *Lane 6*, product of *lane 5* incubated with β-*N*-acetylhexosaminidase from jack bean. *Lane 7*, product of *lane 6* incubated with α-galactosidase from jack bean. *Lane 8*, product of *lane 7* incubated with β-galactosidase from jack bean. *Lane 9*, neutral glycolipid mixture from horse kidney as standards: *N*1 Monohexosylceramide, *N*2 dihexosylceramide, *N*3 Gb_3Cer, *N*4 Gb_4Cer, *N*5 Gb_5Cer. The HPTLC plate was developed with solvent system (II)

1,012(trihexosyl ceramide)$^-$, 850(dihexosyl ceramide)$^-$, 688 (monohexosyl ceramide)$^-$ and 526(ceramide, t18:0/C14:0)$^-$, were obtained with a sequential cleavage at the glycosidic linkage. Peaks of 1303 and 1141 were also shown as characteristic glycon fragments. Fragment pattern of M11 was similar with M13. A set of ceramide species *m/z* 664 (t18:0/C24:1)$^-$ and 646 (d18:1/C24:1)$^-$ were detected, and their molecular ions and sequential fragments at the glycosidic linkage were shown in Fig. 5b.

Based on these results, the structure of M11 and M13 was characterized as a new hybrid-type ganglioside of *neo*lacto- and *iso*globo-series:

$$\begin{aligned}&\text{NeuAc}\alpha2-3\text{Gal}\beta-4[\text{Fuc}\alpha-3]\text{GlcNAc}\beta-3\text{Gal}\alpha\\&\quad-3\text{Gal}\beta-4\text{Glc}\beta-1\text{Cer}\end{aligned}$$

The tissue concentrations of M11 and M13 were 1.15 and 0.96 μmol/kg wet weight, respectively.

Discussion

This study was motivated by the interest of structure analysis of acidic glycosphingolipids of teleost gill. We have elucidated the structures of 11 acidic glycosphingolipids from the gill of Pacific salmon, *Oncorhynchus keta*, a marine fish known to be back to the river of birthplace in the northeastern Japan for reproduction. Sulfoglycolipids of fish gill have never been characterized though the metabolism of sulfatide in eel gill has been shown to be stimulated in the seawater. In this study SM4s and SM3 were chemically characterized as sulfoglycolipids. Sulfoglycolipids in kidneys [4, 20, 28, 29] and kidney cell lines [4, 6, 7, 30] has been reported and discussed on the possible function of transport [31]. The sulfoglycolipids in salmon gill may play a role in the adaptation of the body from fresh water to seawater as shown in eel gill [11] and salt gland of duck [32]. In fish gill the mitochondorion-rich cells which are interspersed among pavement cells, are important osmoregulatory sites in maintaining ionic balance [33, 34]. It is interesting how these glycolipids are distributed in these cell types and are organized in the cell surface.

In gill of salmon GM3 and LM1 were demonstrated as hematoside-series and neolacto-series gangliosides, respectively. As ganglio-series gangliosides GM1b and fucosyl-GalNAc-GM1a were identified in this study. Fucosyl-GalNAc-GM1a

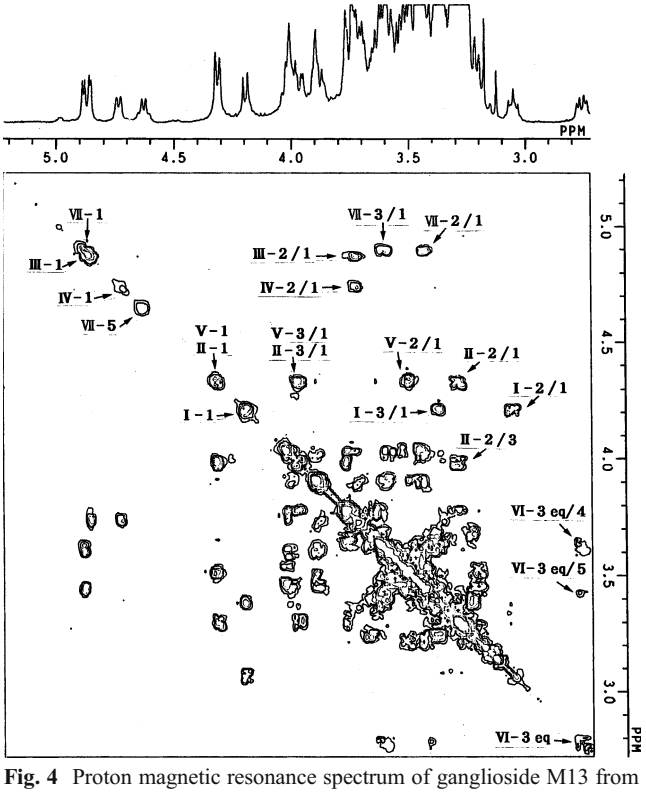

Fig. 4 Proton magnetic resonance spectrum of ganglioside M13 from salmon gill. The purified M13 was treated repeatedly with a few drops of $CH_3O[^2H]$, followed by desiccation over P_2O_5 *in vacuo* to exchange the labile protons with deuterons. Then the dried M13 was redissolved in 0.5 ml of a mixture of $[^2H]$-$Me_2SO/[^2H]_2O$, (98:2, v/v). A spectrum was obtained with JEOL JNM GX-400 400 MHz spectrometer at 60°C. Chemical shifts were indicated by ppm from the signal of TMS as an internal standard. Two-dimensional relayed COSY spectrum of M13

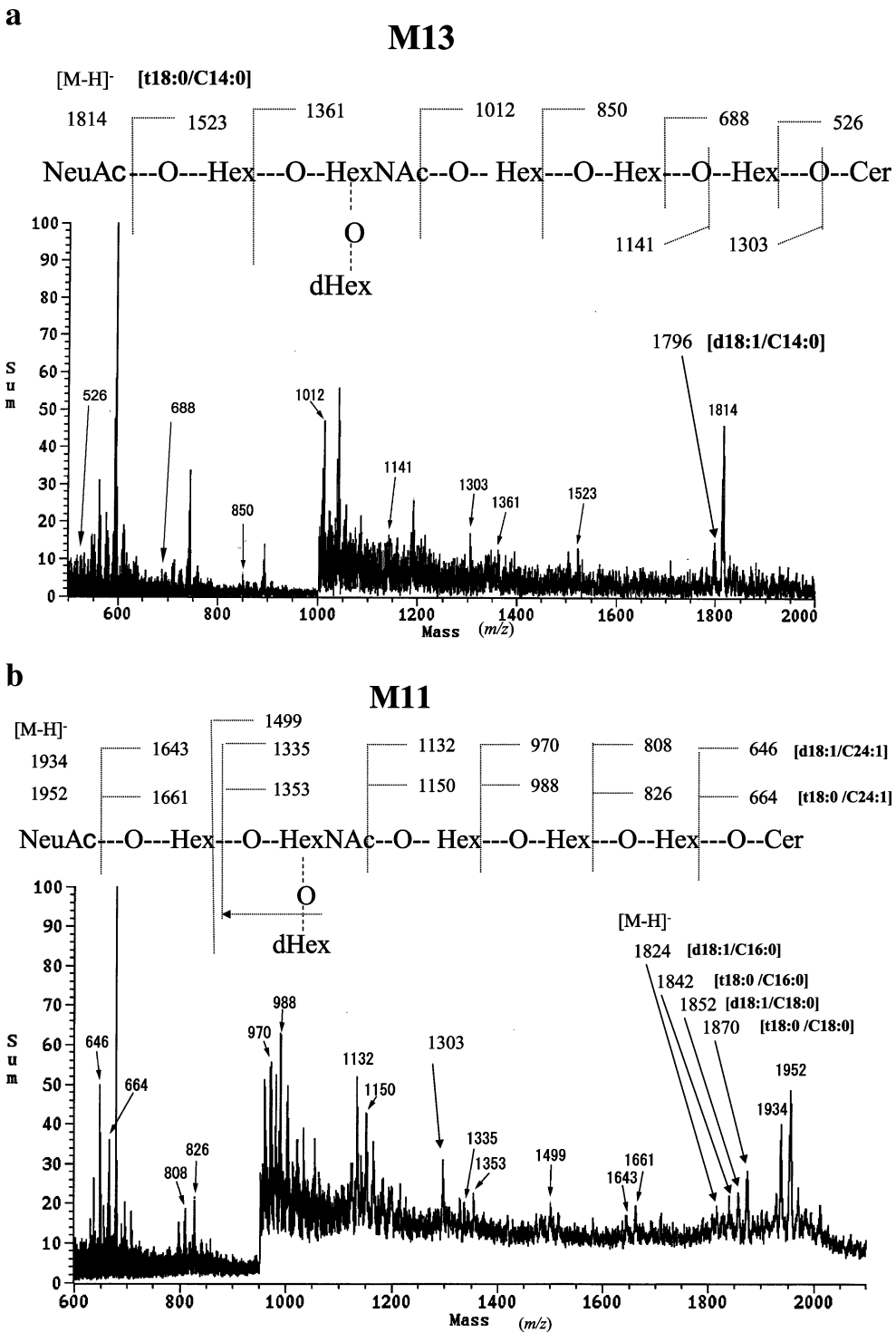

Fig. 5 Negative ion liquid secondary ion mass spectroscopy. Mass numbers indicated in the spectrum are characteristic signals. (a) M13: The peaks in the mass ranges higher than *m/z* 1,000 were amplified tenfold. (b) M11: The peaks in the mass ranges higher than *m/z* 950 were amplified fivefold. The values of *m/z* are represented by nominal masses omitting the decimal fractions

was demonstrated as a major ganglioside of salmon kidney [12]. A unique ganglioside with ganglio-series core and Forssman antigen determinant, GalNAcα1-3GalNAcβ1-3R, in the terminal residue has been elucidated in the liver of English sole of a teleost [16]. It is interested that the gangliosides of ganglio-series were commonly found in the non-neuronal tissues of teleost [16–19]. Glycosphingolipids containing the *isoglobo* structure have been described in fish [17], rat [35], dog [36], pig [37], and horse [38]. The gene of a UDP-galactose: β-galactosyl-1, 4-glucosylceramide α-1, 3-galactosyltransferase for the synthesis of *isoglobo*-glycolipids was cloned from rat as a member of ABO blood type glycosyltransferase family [39]. The existence of other *isoglobo*-glycolipids in the gill and the evolutional relationship of the fish gene to the mammalian gene will be revealed in the future.

A striking feature of gangliosides in salmon gill is the presence of a unique hybrid-type ganglioside with *isoglobo*- and *neolacto*-series. The *isoglobo*-series glycolipid and the novel hybrid glycolipid of *isoglobo*- and *neolacto*-series have been reported as tumor-associated markers in studies of glycosphingolipids from rat colon tumors [40]. Glycolipids of *lacto-ganglio* hybrid structures are expected to be a leukemia markers since these were expressed in the undifferentiated cells of murine leukemia cell line [41]. However, in normal liver of English sole of a teleost a unique hybrid glycolipid of *neolacto*-, *ganglio*- and *isoglobo*-series has been characterized [16, 17] and a novel Forssman active acidic glycosphingolipid with branched *isoglobo*-, *ganglio*-, and *neolacto*-series hybrid sugar chains was also characterized from equine kidney [38]. Glycolipids of (*neo*)*lacto-ganglio* hybrid series were found as normal constituents of gangliosides from striped mullet roe [19]. These results suggest that some glycolipids associated with tumor of higher vertebrate may be expressed in some normal tissues of lower vertebrate.

Large quantities of unsaturated fatty acid, 24:1, and of saturated fatty acids, 18:0, 16:0 and 14:0, were detected as major components. It is interesting to note that a higher degree of unsaturation is present in gill gangliosides as shown in kidney. Since a higher degree of unsaturation was observed in the ganglioside of fish liver [17] and fish brain [42], it may help maintain membrane fluidity at low temperatures in poikilothermic animals. As characteristic long chain base t18:0 was found in gangliosides with longer carbohydrate chain. Because long chain base t18:0 is found generally in plant, this may be due to eating the plant as food in sea or river.

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