Structure of Novel Gangliosides, Deaminated Neuraminic Acid (KDN)-Containing Glycosphingolipids, Isolated from Rainbow Trout Ovarian Fluid†

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ABSTRACT: Two acidic glycosphingolipids were isolated and purified from rainbow trout ovarian fluid. They were designated as ovarian fluid gangliosides ofg-2a and ofg-2b. Both of these glycolipids were found to contain glucose, galactose, and *N*-acetylgalactosamine in a molar ratio of 1:2:1, but they differ by the presence of 2 mol of deaminated neuraminic acid (KDN; 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) in ofg-2a and 1 mol each of KDN and 9-*O*-acetyl-KDN in ofg-2b. On the basis of composition analysis, methylation analysis, mild acid hydrolysis, fast atom bombardment mass spectrometry (FABMS), 400-MHz ^{1}H nuclear magnetic resonance spectroscopy, and immunochemical analysis using a monoclonal antibody (mAb.kdn3G), the complete structures of these gangliosides were determined to be KDNα2→3Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer for ofg-2a [(KDN)G_{D1a}] and 9-*O*-AcKDNα2→3Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer for ofg-2b [(KDN)G_{D1a}-(*O*Ac+)]. The ceramide moieties (Cer) in both ofg-2a [(KDN)G_{D1a}] and ofg-2b [(KDN)G_{D1a}(*O*Ac+)] were found by combining of the results from fatty acid analysis and FABMS measurements to be made up of 4-sphingenine and mainly a C24:1 fatty acyl chain (nervonate). The structures of ofg-2a and ofg-2b are novel, and they represent the second example of naturally occurring KDN-gangliosides. Mild acid hydrolysis of both ofg-2a and ofg-2b resulted in formation of (KDN)G_{M1a}.

The first KDN-containing[1] glycoconjugate, polysialoglycoprotein (PSGP), from the unfertilized eggs of rainbow trout, was described in 1986 (Nadano et al., 1986). In PSGP, KDN residues were found to cap the nonreducing termini of oligo-/polysialyl chains (Nadano et al., 1986). Subsequently, an increasing number of KDN-containing glycoconjugates have been reported to occur in nature (Iwasaki et al., 1987, 1990; Inoue et al., 1988; Kanamori et al., 1989, 1990; Yu Song et al., 1991; Knierel et al., 1989; Strecker et al., 1992a,b). In 1991, we first demonstrated the occurrence in rainbow trout sperm of KDN-containing glycosphingolipid ("KDN-ganglioside"), and its complete structure was determined as KDNα2→3Galβ1→4Glcβ1→Cer, designated (KDN)G_{M3} (Yu Song et al., 1991). Availability of (KDN)G_{M3} has enabled us to generate antibodies against the KDN-glycan unit, and most recently we have produced and characterized a monoclonal antibody IgG3 subclass, obtained by a fusion of spleen cells from a mouse immunized with (KDN)G_{M3} and P3-X63 Ag8.U1 (P3U1) mouse myeloma cells (Yu Song et al., 1993). A monoclonal antibody thus obtained was designated mAb.kdn3G and found to specifically bind to the disaccharide sequence KDNα2→3Galβ1→. Such monoclonal antibodies with high specificity should have much potential applicability, and in particular such probes will be of general use in searching for yet undiscovered KDN-glycoconjugates. Indeed, the utility of mAb.kdn3G is addressed in searching and identifying KDN-gangliosides which contain the KDNα2→3Galβ1→ epitope structure.

We have continued our search for KDN-gangliosides, and this paper describes the discovery and structural elucidation of the two di-KDN-gangliosides present as a minor component in the ovarian fluid of rainbow trout.

MATERIALS AND METHODS

Ovarian Fluid of Rainbow Trout. Genital fluid of female rainbow trout, *Oncorhynchus mykiss*, was obtained from mature fish by courtesy of the Gunma Prefectural Experimental Station at Kawaba and the Okutama fish farm, Department of Fishery, Tokyo Metropolitan Government.

Extraction of Crude Glycolipids. Rainbow trout ovarian fluid (10 L) was concentrated to 1 L by evaporation at 35 °C and dialyzed against distilled water at 4 °C for 4 days. The

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[1] Abbreviations: KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid, or naturally occurring deaminoneuraminic acid; KDN-ganglioside, KDN-containing glycosphingolipid; (KDN)G_{M3}, KDNα2→3Galβ1→4Glcβ1→Cer; (Neu5Ac)G_{M3}, Neu5Acα2→3Galβ1→4Glcβ1→Cer; (KDN)G_{M1} or (KDN)G_{M1a}, Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer; (KDN)G_{D1a}, KDNα2→3Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer; (KDN)G_{D1a}(*O*Ac+), 9-*O*-AcKDNα2→3Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer; (Neu5Ac)-G_{D1a}, Neu5Acα2→3Galβ1→3GalNAcβ1→4(Neu5Acα2→3)-Galβ1→4Glcβ1→Cer; Hex, hexose residue; HexNAc, *N*-acetylhexosamine residue; PSGP, polysialoglycoprotein; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PVP, poly(vinylpyrrolidone); TBA, thiobarbituric acid; TFA, trifluoroacetic acid; mAb.kdn3G, a monoclonal antibody that specifically binds to the KDNα2→3Galβ1→ element; GLC, gas–liquid chromatography; HPTLC, high-performance thin-layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; 1D HOHAHA, one-dimensional homonuclear Hartmann–Hahn spectroscopy; FABMS, fast atom bombardment mass spectrometry.

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dialysate was lyophilized after evaporation to about 300 mL. The crude glycolipids were obtained by procedures previously described (Yu Song et al., 1991). In brief, lyophilized powder (124 g) was extracted first with 2.5 L of chloroform/methanol (2:1, v/v) and then with 2.5 L of chloroform/methanol (1:2, v/v) at room temperature for 2 h each. The combined extract was dried by rotary evaporation at 37 °C and subjected to the partition procedure of Folch et al. (Folch et al., 1957). The dried extract was dissolved in 1250 mL of chloroform/methanol (2:1, v/v) and partitioned by addition of an equal volume of 0.88% aqueous KCl. The organic lower phase was washed twice with chloroform/methanol/0.88% aqueous KCl (3:48:47, v/v) and chloroform/methanol/water (3:48:47, v/v). The combined glycolipid-containing aqueous upper phase was concentrated and dialyzed against distilled water at 4 °C for 2 days. The crude glycolipid fraction (22 g) was obtained by lyophilization of the dialysate.

Purification of Gangliosides. The crude glycolipid fraction thus obtained was subjected to DEAE-Sephadex column chromatography as reported by Momoi et al. (1976) and Iwamori and Nagai (1978). To a column of DEAE-Sephadex A-25 (0.9 × 10.5 cm), preequilibrated with chloroform/methanol/water (30:60:8, v/v), was applied each of the three portions of the crude glycolipids (7.1 mg), dissolved in 24 mL of chloroform/methanol (1:1, v/v). The column was eluted every time with 3 column volumes of chloroform/methanol/water (30:60:8, v/v) and then with 1 column volume of methanol to remove neutral glycosphingolipids. Acidic glycosphingolipids were fractionated by a gradient of ammonium acetate in methanol produced by connecting two reservoirs containing 150 mL each of 0 and 0.5 M salt in methanol. The elution profile was monitored by the modified TBA method (Uchida et al., 1977) with prehydrolysis of the fractions with 0.1 N TFA. The fractions were examined by HPTLC on silica gel 60 plates (Merck, Darmstadt) for purity by spotting a 10–20-μL sample for each fraction and developing in the solvent system chloroform/methanol/0.2% $CaCl_2$ (55:45:10). Acidic glycosphingolipids were visualized by spraying the plate with 10% H_2SO_4 in ethanol (Kitazume et al., 1992). The diacidic fractions obtained by DEAE-Sephadex A-25 chromatography of the three portions of the crude glycolipid fraction were combined and further purified by chromatography on an Iatrobeads column. The combined diacidic fraction (1.3 mg as KDN) was concentrated, desalted, dissolved in chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v), and applied to a column (0.9 cm × 95 cm) containing 35 g of 6RS 8060 Iatrobeads. The column was eluted with 200 mL of the same solvent. The elution profile was monitored by the TBA method and HPTLC as described above. Each of the fractions comprising single components was further purified by preparative thin-layer chromatography on silica gel 60 plates (Merck). Freeze-dried ganglioside mixtures were dissolved in chloroform/methanol (1:1, v/v) and applied to TLC plates (7 × 20 cm) as streaks 2 cm from the edges. The plates were developed in chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v) at room temperature and dried under air. A strip 2.2 cm wide was cut from each side of the plate, and gangliosides were visualized with 1% orcinol in 50% H_2SO_4 (Iwasaki & Inoue, 1985; Kitazume et al., 1992). By comparison with these stained side strips, the positions of the ganglioside bands in 2.6-cm-wide unstained zones of the plates were accurately estimated. Silica gel areas corresponding to selectively visualized bands were scraped off. The gangliosides were then extracted twice from the silica gel by sonication in chloroform/methanol (1:1, v/v) for 1 min followed by vigorous agitation in the same solvent for 30 min at room temperature, and were centrifuged at 3000 rpm for 5 min. The clear supernatant containing the individual gangliosides was evaporated and desalted by passage through a Sephadex G-50 column (1.3 × 42 cm; eluted with water). The isolated individual ganglioside samples (ofg-2a and ofg-2b) were freeze-dried and used for analysis.

HPTLC of Isolated Gangliosides. HPTLC of gangliosides was performed on silica gel 60 plates (No. 5641; Merck, Darmstadt) with chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v). The ganglioside bands were visualized by spraying the plates with 1% orcinol in 50% H_2SO_4 and heating at 100 °C for 1 h.

Mild Alkaline Hydrolysis of Acetylated Di-KDN-ganglioside. The acetylated di-KDN-ganglioside (10 μg) was treated in 20 μL of 2.5 N NH_4OH at 37 °C for 1 h (Iwasaki et al., 1990). The reaction mixture was applied to HPTLC plates, developed, and visualized as described above.

Mild Acid Hydrolysis of ofg-2a and ofg-2b. To remove the KDN residues from ofg-2a and ofg-2b, each of the samples was treated with 0.1 N TFA at 80 °C for 30 and 90 min. The oligosaccharide products were purified by preparative TLC followed by Sephadex G-50 chromatography.

Carbohydrate Composition Analysis. Sugar components in the gangliosides (10 μg in KDN) were analyzed following methanolysis, re-*N*-acetylation, and trimethylsilylation as described previously (Nadano et al., 1986; Nomoto et al., 1982) using a Shimadzu GC-14A gas chromatograph with a glass column (3 mm × 1 m) packed with 1.5% OV-17 on Chromosorb W (temperature from 128 to 230 °C; 4 °C/min). KDN was quantitated by the TBA method (Kitajima et al., 1992).

Fatty Acid Analysis. Fatty acid composition was determined by methanolysis of gangliosides (about 10 μg) in 0.4 *N* methanolic HCl at 100 °C for 3 h, and separation of the methylesters, obtained by extraction of the methanolysates with hexane, on a 2% OV-101 glass column (3 mm × 2 m) with a temperature gradient from 180 to 230 °C at 2.5 °C/min using a Shimadzu GC-14A gas chromatograph was essentially based on the method previously described (Ando & Yu, 1979). A mixture of standard fatty acid methylesters was obtained from Nu-Chek-Prep, Inc.

Methylation Analysis. Permethylation of the intact KDN-gangliosides was carried out according to a simple methylation procedure (Larson et al., 1987; Mansson et al., 1986) with modification (Iwasaki & Inoue, 1985). Dry samples (50 μg) were sonicated in 0.1 mL of dimethyl sulfoxide (DMSO) for 1 min. Methyl iodide (50 μL) and finely powdered NaH (5 mg) were then added, and the mixture was sonicated at 25 °C for 30 min. After reaction, 0.5 mL of distilled water was added, and then 0.5 mL of chloroform was added. The mixture was stirred and centrifuged. After centrifugation, the clear chloroform phase was washed with water (5 × 0.5 mL) until the aqueous phase became neutral. The chloroform phase was concentrated and applied to an Iatrobeads column (packed with 100 mg of 6RS 8060 Iatrobeads) to eliminate DMSO and salts. The sugar fraction, visualized with 1% orcinol in 50% H_2SO_4 on a silica gel plate developed with chloroform/methanol (9:1, v/v), was hydrolyzed (0.5 *N* H_2SO_4/95% acetic acid), reduced, and acetylated (Nomoto et al., 1982). Partially methylated alditol acetates thus produced were analyzed by GLC using a capillary column (CBJ5, 30 m × 0.32 mm; Shimadzu) and a temperature gradient from 180 to 260 °C at 2 °C/min with a Shimadzu GC-14A gas chromatograph (Taguchi et al., 1993).

Identification of the Proximal Monosaccharide Residue in a Di-KDN-ganglioside (ofg-2b). To identify the sugar residue directly attached to the ceramide moiety and to examine if this ganglioside is susceptible to *Rhodococcus* sp. endoglycoceramidase (Seikagaku Kogyo Co., Tokyo), ofg-2b (27 μg) was dissolved in 20 μL of 0.4% Triton X-100/10 mM sodium acetate buffer, pH 5.5, and treated with the enzyme (about 0.5 milliunit) at 37 °C for 16 h (Shimamura et al., 1988). The digests were examined by HPTLC on silica gel plates as previously reported (Shimamura et al., 1988), and the released oligosaccharide was isolated and subjected to reduction with 0.5 mL of 1 M $NaBH_4$ at 4 °C overnight. The oligosaccharide alditol was then analyzed for the component sugars by GLC.

Immunostaining on TLC Plates of (KDN)G_{D1a}, (KDN)-G_{D1a}(OAc+), and a Mono-KDN-ganglioside, (KDN)G_{MX}, Obtained by Mild Acid Hydrolysis of (KDN)G_{D1a}(OAc+). Gangliosides (2.5 μg each for visualization by H_2SO_4/ethanol and 50 ng each for immunostaining) were first applied to a silica gel TLC plate (Polygram SilG, Machery-Nagel, Germany) (Higashi et al., 1984), which was developed with chloroform/methanol/0.2% aqueous $CaCl_2$ solution (55:45:10, v/v). The ganglioside bands were visualized either by spraying the plates with 10% H_2SO_4/ethanol and heating at 120 °C for 30 min or by the immunostaining procedure. TLC immunostaining was carried out as follows (Higashi et al., 1984; Ye et al., 1993): After the plate was air-dried, it was incubated at 37 °C for 1 h with blocking buffer [1% poly(vinylpyrrolidone) (PVP), 1% BSA, and 0.02% NaN_3 in PBS] and then with the monoclonal antibody, mAb.kdn3G (Yu Song et al., 1993) (7.5 μg/mL as IgG), at 37 °C for 2 h. The plate was washed 3 times with the washing buffer (0.05% Tween 20 in PBS), incubated at room temperature for 30 min with a biotin-conjugated horse anti-mouse IgG antibody (Vector Laboratories, Inc.) under gentle shaking, and washed 3 times with the washing buffer. Then the plate was incubated with avidin-biotinylated horseradish peroxidase complex solution (Vectastain Elite ABC Kit, Vector Laboratories, Inc.) at room temperature for 30 min under gentle shaking. The enzyme activity remaining on the plate was visualized by incubation with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.009% H_2O_2, 0.8 mg/mL 3,3′-diaminobenzidine (DAB), and 0.4 mg/mL $NiCl_2$ at room temperature.

400-MHz ^1H NMR Spectroscopy. For NMR measurements, two samples of di-KDN-gangliosides (ofg-2a, 0.45 mg; ofg-2b, 0.65 mg) were exchanged repeatedly with 99.8% D_2O and dissolved finally in dimethyl sulfoxide-d_6/D_2O (98:2, v/v). One-dimensional ^1H NMR spectra were measured at 37 °C on a Bruker WM-400 spectrometer. Chemical shifts were referenced to internally added tetramethylsilane and expressed in ppm.

Fast Atom Bombardment Mass Spectrometry. Negative-ion FAB-MS spectra were determined with a Finnigan TSQ70 mass spectrometer. The native di-KDN-gangliosides (10 μg each) were dissolved in chloroform/methanol (1:1, v/v), mixed with a target matrix of triethanolamine, and bombarded with a xenon beam source at 8 kV.

RESULTS

Isolation and Purification of Gangliosides from Rainbow Trout Ovarian Fluid. About 22 mg of the crude polar lipid fraction obtained by the partition procedure of Folch et al. (1957) from 10 L of ovarian fluid was chromatographed on a DEAE-Sephadex column (Figure 1a). The TBA-positive dianionic fraction (ofg-2) was further purified by Iatrobeads column chromatography, and the elution pattern monitored by HPTLC is shown in Figure 1b. Examination by HPTLC revealed the presence of two dianionic gangliosides, which were denoted as ofg-2a and ofg-2b. The yields of ofg-2a and ofg-2b from 10 L of ovarian fluid were 0.86 and 1.2 mg, respectively.

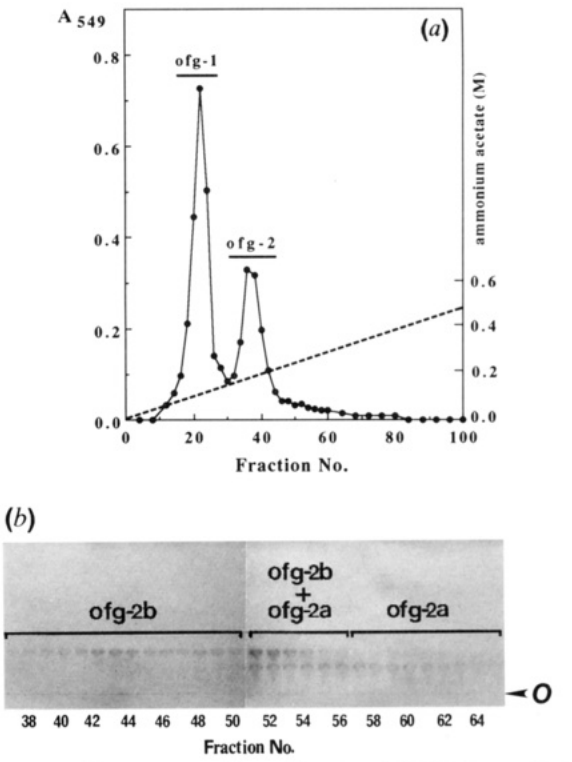

FIGURE 1: Chromatographic purification of di-KDN-gangliosides obtained from the ovarian fluid of rainbow trout. (a) DEAE-Sephadex A-25 chromatography of the crude polar lipid fraction (about 22 mg) obtained by the Folch partition procedure (Folch et al., 1957) from 10 L of ovarian fluid. (b) The TBA-positive dianionic fraction (ofg-2 in panel a) was further purified by Iatrobeads column chromatography, and the elution pattern was monitored by HPTLC. *O*, origin.

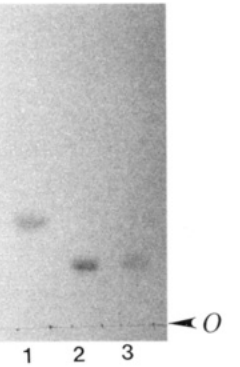

FIGURE 2: HPTLC of di-KDN-gangliosides ofg-2a and ofg-2b. HPTLC was developed and visualized as described under Materials and Methods. Lane 1, ofg-2b; lane 2, ofg-2b after mild alkaline treatment; lane 3, ofg-2a. *O*, origin.

Structural Determination of Di-KDN-gangliosides ofg-2a and ofg-2b. HPTLC of Di-KDN-gangliosides ofg-2a and ofg-2b. The orcinol/H_2SO_4 patterns observed on HPTLC of the di-KDN-gangliosides ofg-2a and ofg-2b isolated from ovarian fluid of rainbow trout are shown in Figure 2 (lanes 1 and 3). For each sample, only a single band was observed, and the faster moving band (ofg-2b) disappeared on mild alkaline treatment with the simultaneous appearance of a band identical to ofg-2a (lane 2). On the basis of the known data

Table I: Carbohydrate Composition of Rainbow Trout Ovarian Fluid Gangliosides ofg-2a and ofg-2b

	Gal	Glc	GalNAc	KDN
ofg-2a	2.0	1.0[a]	0.9	2.1
ofg-2b	1.9	1.0[a]	1.1	2.1

[a] Molar ratios are relative to Glc taken as 1.0.

Table II: Partially Methylated Alditol Acetates Derived from the Carbohydrate Residues Present in ofg-2a and ofg-2b in Methylation Analysis

	ofg-2a	ofg-2b
3-substituted Gal	0.9	0.8
3-substituted GalNAc	1.2	1.2
3,4-disubstituted Gal	0.9	1.0
4-substituted Glc	1.0[a]	1.0[a]

[a] The data are expressed in molar ratios relative to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol set to 1.0.

on the relative mobilities of ganglioside derivatives, it would seem reasonable that the fast-moving spot (ofg-2b) has a structure identical to that of ofg-2a except that ofg-2b is possibly *O*-acetylated KDN.

Carbohydrate Composition. Monosaccharide analysis of the two ovarian fluid gangliosides revealed that both ofg-2a and ofg-2b had the same carbohydrate composition, consisting of Gal, Glc, GalNAc, and KDN in the ratio of 2:1:1:2 (Table I).

Nature of the Ceramide Portion. The structures of the ceramide moieties of ofg-2a and ofg-2b were determined by GLC analysis of methyl esters of the fatty acids obtained upon methanolysis of the gangliosides. The results demonstrated that both ofg-2a and ofg-2b contained a C24:1 fatty acid (nervonic acid) and a C18:1 long base chain (data not shown). The dominant species of sphingoid base detected was C18:1 (4-sphingenine) as judged from the FABMS data (see below).

Methylation Analysis of ofg-2a and ofg-2b. The results of methylation analysis are summarized in Table II. The analysis of the partially methylated alditol acetates derived from ofg-2a and ofg-2b revealed the presence of 1 mol each of 3-*O*-substituted Gal, 4-*O*-substituted Glc, 3,4-di-*O*-substituted Gal, and 3-*O*-substituted GalNAc residues.

Mild Acid Hydrolysis of ofg-2a and ofg-2b. About 80% of the KDN and *O*-acetyl-KDN residues were removed from ofg-2a and ofg-2b by mild acid hydrolysis (0.1 N TFA, 80 °C, 90 min), and the KDN-depleted ofg-2a and ofg-2b were isolated from the hydrolysates by preparative TLC and Sephadex G-50 chromatography. The products were found to be identical in carbohydrate composition (Gal/Glc/GalNAc = 2:1:1) and on TLC behavior. Methylation analysis of the KDN-depleted ofg-2b showed the presence of the terminal Gal, 4-*O*-substituted Gal, 4-*O*-substituted Glc, and 3-*O*-substituted GalNAc residues in a ratio of 1.1:1.1:1.0:1.1. These results demonstrated that in ofg-2a and ofg-2b one KDN residue resided at O-3 of the nonreducing terminal Gal residue and the other KDN residue was attached at O-3 of the internal Gal residue. After mild acid hydrolysis (0.1 N TFA, 80 °C, 30 min) of ofg-2b, in addition to a few other spots, a spot with mobility relative to KDN of 1.9 was seen on a silica gel 60 HPTLC plate (1-propanol/25% aqueous ammonia/water = 6:1:2.5, v/v, at 15 °C for 2 h; the final concentration of ammonia was 2.6%, and the pH was about 10.5) visualized with 10% H_2SO_4/ethanol. On the basis of direct comparison with the relative mobility of the authentic 9-*O*-acetyl-KDN that was isolated from kokanee salmon polysialoglycoprotein and identified by 400-MHz ^1H NMR spectroscopy (Iwasaki et al., 1990), the fast-moving nonulosonic acid on TLC was tentatively identified as 9-*O*-acetyl-KDN. Although mild acid hydrolysis and TLC showed the presence of 9-*O*-acetyl-KDN in ofg-2b, we were not able to determine at this stage which KDN residue was specifically acetylated.

Partial acid hydrolysis of ofg-2a in 0.1 N TFA at 80 °C for 30 min yielded a single major mono-KDN-ganglioside [designated (KDN)G_{MX}]. (KDN)G_{MX} was purified first by preparative TLC and second by gel chromatography on Sephadex G-50 and was subsequently analyzed for carbohydrate composition and interglycosidic linkage. (KDN)-G_{MX} thus obtained was shown to be homogeneous by TLC, and the carbohydrate compositional analysis showed Gal/Glc/GalNAc/KDN = 1.9:1.0:1.1:0.8. Methylation analysis revealed (KDN)G_{MX} to be composed of 1 mol each of unsubstituted Gal, 3,4-di-*O*-substituted Gal, 4-*O*-substituted Glc, and 3-*O*-substituted GalNAc (1.1:1.0:1.0:0.92). Mild acid hydrolysis of ofg-2b (in 0.1 N TFA for 30 min at 80 °C) also gave a mono-KDN-ganglioside which was completely identical with (KDN)G_{MX} as judged from the results of sugar composition and methylation analyses (data not shown). These findings for (KDN)G_{MX} combined with the carbohydrate sequence data and information on the anomeric configuration (see below) were compatible with the sequence Galβ1→3GalNAcβ1→4(KDNα2→3)Galβ1→4Glcβ1→Cer. Namely, (KDN)G_{MX} was indeed (KDN)G_{M1}.

Carbohydrate Sequence. Two di-KDN-gangliosides, ofg-2a and ofg-2b, were analyzed by FABMS to obtain information about their carbohydrate sequences and *O*-acetyl substitution in KDN and to confirm the structure of the ceramide portion. The FAB mass spectra, measured in the negative-ion mode, of the native di-KDN-gangliosides (ofg-2a and ofg-2b) are reproduced in Figure 3. The molecular ion $(M-H^+)^-$ observed at m/z 1835 in Figure 3a for ofg-2a revealed that the major molecular species contained two KDN and three hexose residues and one *N*-acetylhexosamine residue as the carbohydrate moiety and that the ceramide moiety was composed of a C18:1 sphingoid base and a major C24:1 fatty acid. The corresponding peak observed at m/z 1877 in Figure 3b for ofg-2b was consistent with the $(M - H^+)^-$ ion for the *O*-acetylated form of ofg-2a. Diagnostic sequence ions are prominent in the negative FAB mass spectra of ofg-2a and ofg-2b.

ofg-2a. (i) The fragment ions of analytical importance were observed at m/z 632 $(M - Cer - 2Hex - KDN - H^+)^-$, which was composed of KDN, Hex, and HexNAc, and at m/z 1423 $(M - KDN - Hex - H^+)^-$. When these data were combined with the results of methylation analysis for ofg-2a (Table II) and those for (KDN)G_{MX} (see above), the sequence of the nonreducing trisaccharide was established to be KDN2→3Gal1→3GalNAc. (ii) Figure 3a displayed a peak at m/z 1220 $(M - KDN - Hex - HexNAc - H^+)^-$, indicating that this fragment was composed of two Hex residues, one KDN residue, and a ceramide residue. This finding in combination with the results of methylation analysis and endoglycoceramidase digestion followed by borohydride reduction (see below) revealed the sequence features of the proximal portion of the glycan: →4(KDN2→3)-Gal1→4Glc1→Cer. (iii) Additional fragment ions $(M - KDN - H^+)^-$ at m/z 1585, $(M - 2KDN - H^+)^-$ at m/z 1335, $(M - 2KDN - Hex - H^+)^-$ at m/z 1173, $(M - 2KDN - Hex - HexNAc - H^+)^-$ at m/z 970, $(M - 2KDN - 2Hex - HexNAc - H^+)^-$ at m/z 808, and $(M - 2KDN - 3Hex - HexNAc - H^+)^-$ at m/z 646 for ofg-2a in Figure 3a are also compatible

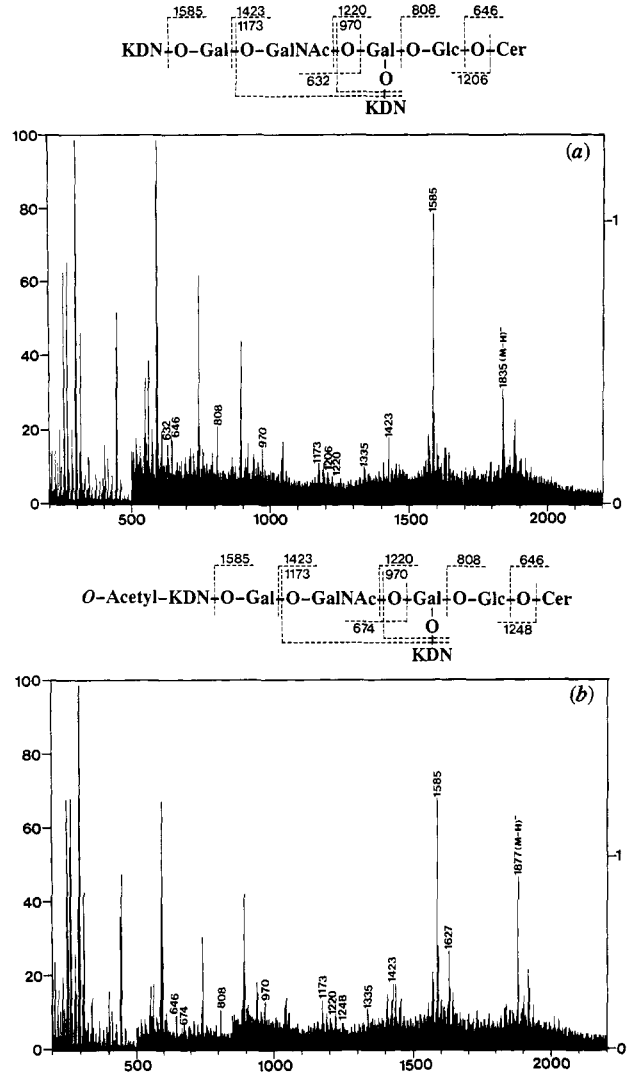

FIGURE 3: Negative-ion FABMS spectra of di-KDN-gangliosides ofg-2a (a) and ofg-2b (b). Proposed fragmentation patterns in FABMS are shown, using the finally established formulae for (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+).

with the sequence deduced from the above findings. Consequently, the carbohydrate sequence of the di-KDN-ganglioside, ofg-2a, appeared most likely to be KDN2→3Gal1→3GalNAc1→4(KDN2→3)-Gal1→4Glc1→Cer. These results were further confirmed by the positive-ion FABMS measurements of the permethylated derivative of ofg-2a (data not shown).

It should be noted that the unambiguous assignment of the proximal hexose residue to glucose was made by GLC analysis of the component sugars obtained from the oligosaccharide alditol derived from ofg-2b by treatment with endoglycoceramidase followed by reduction with $NaBH_4$.

ofg-2b. (i) The negative FABMS spectrum of ofg-2b (Figure 3b) was again characterized by the appearance of the diagnostically important fragment ions, detected at m/z 674 $(M - Cer - 2Hex - KDN - H^+)^-$, which were composed of *O*-AcKDN, Hex, and HexNAc, and at m/z 1423 $(M - AcKDN - Hex - H^+)^-$. These results in conjunction with methylation analysis (Table II) led to the nonreducing terminal trisaccharide sequence AcKDN2→3Gal1→3GalNAc. The failure to observe any of the peaks at m/z 632, 1465 $(M - KDN - Gal - H^+)^-$, and 1262 $(M - KDN - Gal - GalNAc - H^+)^-$ strongly supports that the nonreducing trisaccharide sequence is not KDN→Gal→GalNAc but *O*-AcKDN→Gal-GalNAc, namely, that the *O*-acetyl group is substituted only on the distal KDN residue. (ii) The molecular ion and the fragment mass ions, detected at m/z 1877 $(M - H^+)^-$, 1627 $(M - KDN - H^+)^-$, 1585 $(M - AcKDN - H^+)^-$, 1335 $(M - AcKDN - KDN - H^+)^-$, 1173 $(M - AcKDN - KDN - Hex - H^+)^-$, 970 $(M - AcKDN - KDN - Hex - HexNAc - H^+)^-$, and 646 $(M - AcKDN - KDN - 3Hex - HexNAc - H^+)^-$, were all compatible with the structure for ofg-2b shown here: AcKDN2→3Gal1→3GalNAc1→4(KDN2→3)Gal1→4Glc1→Cer.

400-MHz ^{1}H NMR Spectroscopy of Di-KDN-gangliosides ofg-2a and ofg-2b. Proton NMR spectra were measured at 400 MHz for ofg-2a and ofg-2b to determine the anomeric configuration of each of the sugar residues and to obtain more information about an *O*-acetyl substitution on the KDN residue (Figure 4). Assignment of signals to the specific protons in ofg-2b was made by one-dimensional homonuclear Hartmann–Hahn (HOHAHA) spectral measurement (data not shown) and by comparison with the assignments reported for (Neu5Ac)G_{D1a} (Inagaki, 1990) and (KDN)G_{M3} (Yu Song et al., 1991). The resonance signals of all anomeric protons thus assigned to specific residues for ofg-2a and ofg-2b are shown in Figure 4, and the anomeric configurations of all the sugar residues in the core glycan chain, Gal1→3GalNAc1→4Gal1→4Glc1→, were determined unequivocally as β on the basis of their chemical shifts and the $J_{1,2}$ values.

On the basis of the known data on the chemical shifts of *O*-acetyl-KDN (Iwasaki et al., 1990) and the presently observed chemical shifts for the *O*-acetyl methyl proton (2.03 ppm) and the H-9 proton (4.26 ppm) of the AcKDN residue together with the data reported for (9-*O*-acetyl-Neu5Ac)G_{D3} (Thurin et al., 1985), it would seem reasonable that the *O*-acetylated KDN residue found in ofg-2b was most likely to be 9-*O*-acetyl-KDN [for H-9 protons (Iwasaki et al., 1990), δ 4.36 ppm (9-*O*-AcKDN) versus δ 3.83 ppm (KDN); for H-9′ protons (Iwasaki et al., 1990), δ 4.21 ppm (9-*O*-AcKDN) versus δ 3.64 ppm (KDN); for H-9 protons (Thurin et al., 1985), δ 4.23 ppm ((9-*O*-AcNeu5Ac)G_{D3}) versus δ 3.58 ppm ((Neu5Ac)G_{D3})]. The rationale for eliminating the possibility of either 4-*O*- or 7-*O*-acetyl substitution is fourfold: (i) As judged from the reported data for the chemical shift values of H-4 of 4-*O*-AcNeu5Ac (or 4-*O*-AcNeu5Gc) residues and H-7 of 7-*O*-AcNeu5Ac (or 7-*O*-AcNeu5Gc) residues, respectively (Haverkamp et al., 1982; Iwasaki et al., 1990), the corresponding H-4 and H-7 protons for 4-*O*-AcKDN and 7-*O*-AcKDN are expected to resonate at lower field than H-9 of 9-*O*-AcKDN. However, no signal originating from the acetylated KDN residue is seen in the lower field below 4.4 ppm in Figure 4b, strongly indicating the absence of 4-*O*-AcKDN and/or 7-*O*-AcKDN residues in ofg-2b. (ii) The 1D HOHAHA spectral determination of (KDN)G_{D1a}(*O*Ac+) demonstrated that both the H-4 and H-5 proton chemical shifts unambiguously assigned by means of magnetization transfer from each of the two H-3_{eq} protons were identical to H-4 (3.47 ppm) and H-5 (3.26 ppm) observed for the KDN residues of (KDN)G_{D1a} and (KDN)G_{M3}, showing that the possibility of 4-*O*-acetyl- and/or 5-*O*-acetyl-KDN groups in (KDN)G_{D1a}(*O*Ac+) was ruled out. (iii) The relative intensities of the peak areas for KDN H-9 (δ = 4.26 ppm), GalNAc H-1 (δ = 4.83 ppm), and Glc H-1 (δ = 4.15 ppm) were found to be 1:1:1, indicating that the acetylated KDN residue in (KDN)G_{D1a}(*O*Ac+) must be almost all, if not exclusively, 9-*O*-acetyl-KDN. (iv) The bound KDN residue was shown

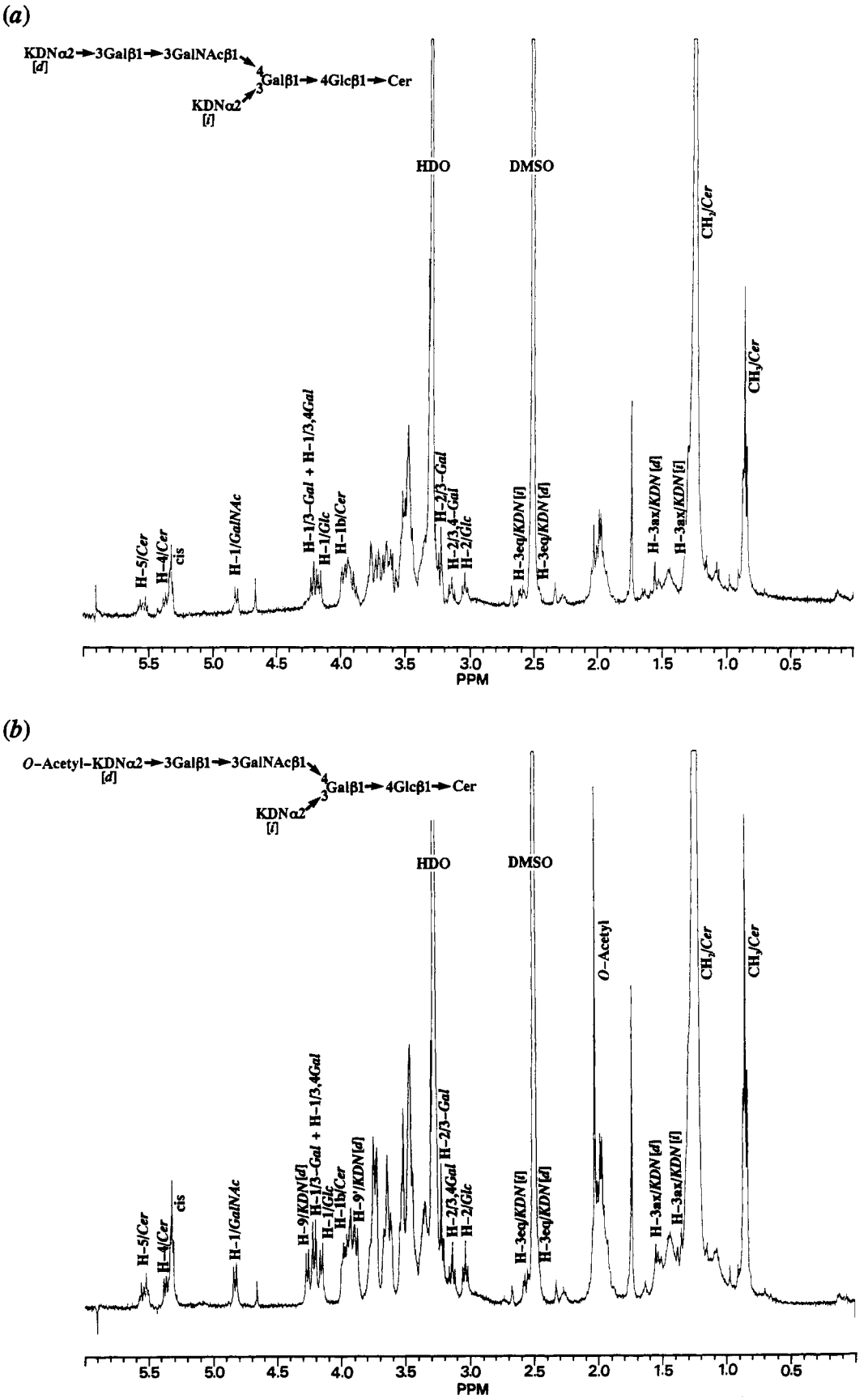

FIGURE 4: One-dimensional 400-MHz ^{1}H NMR spectra of (a) ofg-2a, (KDN)G_{D1a}, and (b) ofg-2b, (KDN)G_{D1a}(*O*Ac+), at 37 °C. Ganglioside samples were dissolved in 0.4 mL of dimethyl-d_6 sulfoxide/D_2O(99.95%) (98:2, v/v) containing tetramethylsilane. Assignments of the resonances to specific protons are indicated. To discriminate the internal and distal KDN residues, the notations [*i*] and [*d*] are used. For further details, see the finally established structures for (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+) shown in Figure 5.

to be positive upon the TBA reaction because the oxidative cleavage at the bond between C-4 and C-5 gave rise to β-formylpyruvate. (KDN)G_{D1a}(*O*Ac+) gave a comparable color yield upon the TBA reaction before and after mild acid

Table III: Chemical Shifts for H-3_{ax} and H-3_{eq} Protons of KDN Residues in KDN-Gangliosides and the Relevant NeuAc-Gangliosides

	proton chemical shift (ppm)[a]			
	distal KDN residue		internal KDN residue	
	H_{3a}	H-3_{eq}	H-3_{ax}	H-3_{eq}
(KDN)G_{M3}[b]	1.54	2.48		
(KDN)G_{D1a}	1.56	2.45	1.38	2.59
(KDN)G_{D1a}(*O*Ac+)	1.56	2.42	1.35	2.56
(NeuAc)G_{M3}[c]	1.36	2.75		
(NeuAc)G_{D1a}[d]	1.38	2.72	1.61	2.61

[a] Determined at 400 MHz at 37 °C in $(CD_3)_2SO/D_2O$ (98:2, v/v) for KDN-gangliosides referenced to internal Me_4Si. [b] Taken from Yu Song et al. (1991). [c] Taken from Koerner et al. (1983). [d] Taken from Thurin et al. (1985).

hydrolysis, indicating no *O*-acetyl substituent is present at C-4 or C-5.

The apparent greater height of the *O*-acetyl methyl proton resonance signal as compared to the height of the *N*-acetyl methyl signal of the GalNAc residue in (KDN)G_{D1a} (*O*Ac+) (Figure 4b) must be partly due to the overlapping nature of the former signal and partly due to a difference in the relaxation rate. At any rate, there seems to be no chance to assume more than one *O*-acetyl group per molecule, which is also in support of the failure to detect any sign of the presence of di-*O*-acetylated (KDN)G_{D1a} on TLC (see lane 1 in Figure 2).

The chemical shift values for H-3_{ax} and H-3_{eq} were unambiguously interpreted as those assignable to an α-configuration of the KDN ketosidic linkages (Figure 4). The H-3_{ax} and H-3_{eq} proton signals for the distal KDN residues in ofg-2a and ofg-2b were tentatively assigned by comparison with the corresponding proton signals for (KDN)G_{M3}, and the remaining resonance signals were then assigned to the corresponding H-3 protons of the internally substituted KDN residues as indicated in Table III. The assignment of the H-3 protons of the distal KDN residue in (KDN)G_{D1a} (*O*Ac+) was made by consideration of the fact that the reported chemical shift values of both H-3_{eq} and H-3_{ax} for the distal Neu5Ac residue are nearly identical to the Neu5Acα2→3Galβ1→4Glc sequence in (Neu5Ac)G_{M3} and in the Neu5Acα2→3Galβ1→3GalNAc sequence in (Neu5Ac)G_{D1a}. In our 1D HOHAHA experiments, because of an extremely small spin–spin coupling interaction between the H-6 and H-7 protons, no magnetization transfer was observed beyond H-6. Moreover, because of a shortage of the material, we were not able to carry out COZY analysis. Therefore, we could not make a complete assignment of the proton signals arising from the non-*O*-acetylated KDN residues. It may be interesting to note that the chemical shift values for the distal and internal KDN residues in (KDN)G_{D1a} are uniquely different from those for the corresponding residues in (Neu5Ac)G_{D1a} (Table III). The pairing of two sets of H-3_{ax} and H-3_{eq} protons for the two KDN residues was confirmed by using 1D HOHAHA measurements.

Lack of material prevented further degradative work such as isolation of the *O*-acetylated KDN from ofg-2b by mild acid hydrolysis as conducted on kokanee salmon PSGP (Iwasaki et al., 1990). Nevertheless, an interpretation of the combined data revealed by FABMS and ^1H NMR measurements and the alkali lability of the substituent shown above led us to conclude that ofg-2b ganglioside was characterized by the presence of a 9-*O* acetylated KDN residue. Consequently, the complete structures of a pair of novel KDN-glycosphingolipids, ofg-2a and ofg-2b, were established as depicted in Figure 5. As the complete structures of ofg-2a and ofg-2b have been identified as being the gangliosides, they can be designated (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+), respectively.

TLC Immunostaining on TLC Plates of (KDN)G_{D1a}, (KDN)G_{D1a}(OAc+), and (KDN)G_{M1} Isolated from the Mild Acid Hydrolysate of (KDN)G_{D1a}(OAc+). Three gangliosides were applied to TLC plates, developed, and visualized as described under Materials and Methods. The results are shown in Figure 6. As anticipated from the known epitope specificity of mAb.kdn3G, the monoclonal antibody reacted only with (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+), and no reaction was observed with (KDN)G_{M1} (Figure 6b, lane 3). When the mild acid hydrolysates (0.1 N TFA, 80 °C, 30 min) of (KDN)G_{D1a} were examined, no mAb.kdn3G-reactive band was observed (data not shown), indicating that the KDN residue linked α2→3 to the terminal Gal residue had been completely hydrolyzed, although (KDN)G_{M1} was present as the major reaction product. (KDN)G_{M1} (~20%) was still detected in the hydrolysate (0.1 N TFA, 80 °C, 90 min) of (KDN)G_{D1a}, demonstrating that the KDN residue linked α2→3 to the internal Gal residue was relatively stable to mild acid hydrolysis.

DISCUSSION

The deaminated neuraminic acid (KDN) residue was first discovered, and its structure was first described, by Nadano et al. in 1986. The compound first appeared as an unknown peak in a GLC analysis of a methanolysate of rainbow trout egg PSGP. What do we know about the occurrence of KDN other than in PSGP, and what is the future particularly in mammalian cells and tissues? It has since been shown that KDN residues occur (a) in a unique glycoprotein isolated from the vitelline envelope of rainbow trout eggs (Inoue et al., 1988; Kanamori et al., 1990), in which KDN is the sole nonulosonic acid component; (b) in a lipopolysaccharide of Gram-negative bacteria, *Klebsiella ozaenae* (Knierel et al., 1989); (c) in the O-linked glycan chains of glycoproteins isolated from the egg jelly of two different species of amphibian (Strecker et al., 1992a,b); and (d) in a KDN-ganglioside, (KDN)G_{M3}, found in rainbow trout sperm, the first example of KDN-containing glycosphingolipids (Yu Song et al., 1991). For the present, we can utilize various KDN-glycan chains in developing KDN-glycan-specific antibodies as sensitive probes in searching for KDN-glycoconjugates. We can enquire into the occurrence of KDN-glycoproteins and KDN-gangliosides other than in bacteria, fishes, and amphibians. For the future, although KDN has not yet been found to be a constituent of mammalian glycoconjugates, there is no doubt that KDN-glycan-specific monoclonal antibodies must play a great role in identification of KDN-glycoconjugates.

It would thus be useful to develop immunochemical probes for KDN residues which permit screening for KDN-containing glycoconjugates in nature. Identification of KDN-transferases and clarification of the biosynthetic mechanism of KDN-glycoconjugate synthesis would also create great interest in future research on KDN-glycoconjugates. Recently, using (KDN)G_{M3} as antigen, we succeeded in generating a monoclonal antibody (mAb.kdn3G) which reacted specifically with (KDN)G_{M3} but not at all with (Neu5Ac)G_{M3} (Yu Song et al., 1993). The antigenic determinant of mAb.kdn3G was established on the basis of binding experiments to be KDNα2→3Galβ1→ (Yu Song et al., 1993). In isolation procedures in the present study we utilized mAb.kdn3G and demonstrated that this monoclonal antibody was useful as a

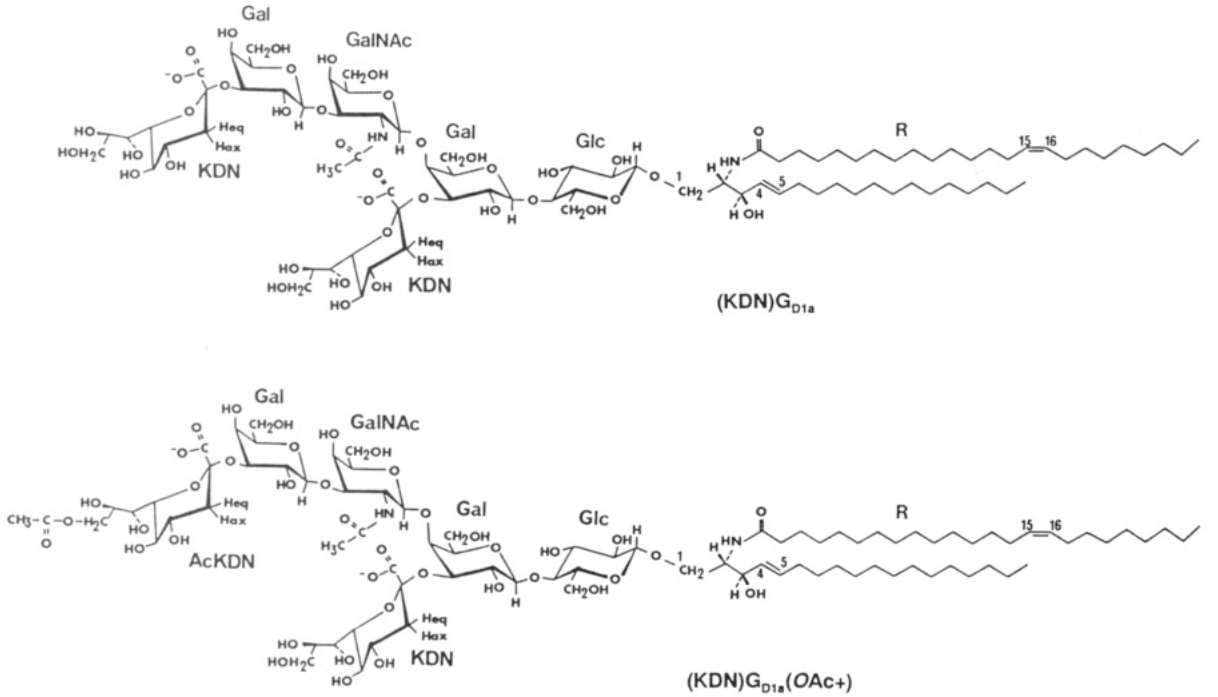

FIGURE 5: The established structures of rainbow trout ovarian fluid di-KDN-gangliosides (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+). The sugar residues are depicted in the $^4C_1$ conformational form for Glc, Gal, and GalNAc and in the $^2C_5$ form for KDN.

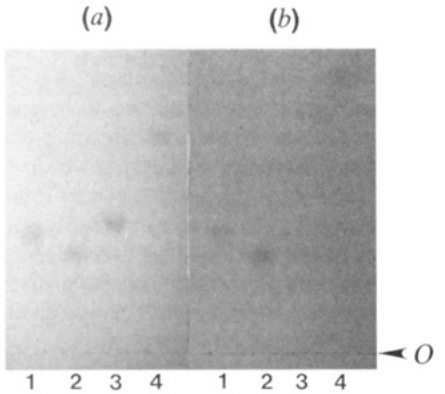

FIGURE 6: Immunostainability of (KDN)G_{D1a}, (KDN)G_{D1a}(*O*Ac+), and (KDN)G_{M1a} on TLC plates with a monoclonal antibody (mAb.kdn3G). In (a), the plate was stained with 10% H_2SO_4/ethanol to reveal (KDN)G_{D1a}(*O*Ac+) (lane 1), (KDN)G_{D1a} (lane 2), (KDN)-G_{M1a} (lane 3), and KDN-depleted (KDN)G_{D1a} (lane 4). (KDN)-G_{M1a} and KDN-depleted (KDN)G_{D1a} were obtained by partial acid hydrolysis of (KDN)G_{D1a}(*O*Ac+). To test the binding of mAb.kdn3G to these KDN-gangliosides, in (b) the plate was immunostained with this monoclonal antibody. mAb.kdn3G-positive bands were observed for (KDN)G_{D1a}(*O*Ac+) (lane 1) and (KDN)G_{D1a} (lane 2), but not for (KDN)G_{M1a} (lane 3) and KDN-depleted (KDN)G_{D1a}(*O*Ac+) (lane 4). *O*, origin.

sensitive probe in searching for KDN-glycoconjugates having the structural element KDNα2→3Galβ1→, as was found in (KDN)G_{D1a} and (KDN)G_{M3}. In the ELISA of (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+), which contains a 9-*O*-acetyl-KDNα2→3Galβ1→ unit, no significant difference was detected, demonstrating that the monoclonal antibody mAb.kdn3G does not distinguish 9-*O*-acetylated KDNα2→3Galβ1→ from the non-*O*-acetylated form. This result is similar to the previous observations for anti-poly(Neu5Ac) antibodies by Ørskov et al. (1979) and Frosch et al. (1985). We can anticipate that the KDN residues might be of widespread occurrence in animals and certain microorganisms, and their identification should be made feasible by development of sensitive probes such as monoclonal antibodies specific to various types of KDN linkages. In this regard, the availability of (KDN)G_{D1a} and (KDN)G_{M1} now allows us to generate a monoclonal antibody specific to the structural element GalNAcβ1→4(KDNα2→3)Galβ1→.

The ^{14}C-labeled sugar nucleotide cytidine 5′-KDN-phosphate (CMP-[^{14}C]KDN) was thought to be required as a donor substrate for the biosynthetic study of KDN-glycan chains. It should be noted here that, most recently, we were also successful in identification and characterization of CMP–KDN synthetase, a novel enzyme which is responsible for synthesis of CMP–KDN from KDN and CTP (Terada et al., 1993).

Although, at present, we have no definite information about the origin of (KDN)G_{D1a} and (KDN)G_{D1a}(*O*Ac+), which were found as minor components in rainbow trout ovarian fluid (0.2 μg/mL of ovarian fluid), they may likely be derived from follicle cells.

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