J. Biochem. 125, 923-930 (1999)

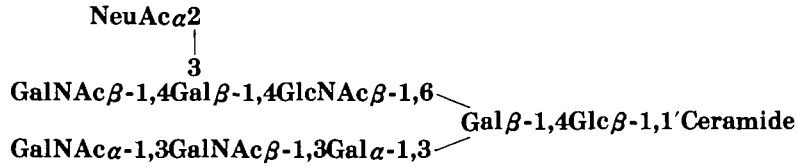
Isolation and Characterization of a Novel Forssman Active Acidic Glycosphingolipid with Branched Isoglobo-, Ganglio-, and Neolacto-Series Hybrid Sugar Chains

Harumi Yamamoto,* Naoko Iida-Tanaka,† Takeshi Kasama,‡ Ineo Ishizuka,† Yasunori Kushi,* and Shizuo Handa*,[1]

*Department of Biochemistry, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519; ‡Instrumental Analysis Research Center for Life Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510; and †Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605

Received January 26, 1999; accepted February 6, 1999

Equine kidney and spleen contain a Forssman active glycosphingolipid, and the structure of this glycolipid has been reported to be that of a globopentaosylceramide (GalNAcα-1, 3GalNAcβ-1,3Galα-1,4Galβ-1,4Glcβ-1,1'Ceramide). We found that equine kidney contains several other anti-Forssman antibody-reactive glycosphingolipids. One of these acidic Forssman active glycosphingolipids was isolated and characterized by means of NMR, mass spectrometry, permethylation studies, and TLC-immunostaining. This glycolipid contains three moles of galactose, one mole of glucose, three moles of *N*-acetylgalactosamine, one mole of *N*-acetylglucosamine, and one mole of *N*-acetylneuraminic acid, and is stained on TLC with anti-Forssman antibodies and anti-GM2 ganglioside antibodies. HOHAHA and ROESY experiments and permethylation studies showed this glycolipid oligosaccharide to be branched at the innermost galactose; one chain has an isoglobo structure with a terminal Forssman disaccharide and the other chain is branched through the linkage of *N*-acetylglucosamine β-1,6 to the inner galactose. The nonreducing end of the GM2 trisaccharide is linked to this glucosamine. The structure of the oligosaccharide of the glycolipid presented here is a novel type, having branched isoglobo-, ganglio-, and neolacto-series oligosaccharides. Mass spectrometric analyses indicated the ceramide moiety of the glycolipid to be composed predominantly of hydroxy fatty acids (C20:0, C22:0, C23:0, C24:0, and C25:0) and hydroxysphinganine.



Key words: Forssman, glycosphingolipid, GM2, isoglobo, neolacto.

Glycosphingolipids are biological membrane components whose distribution patterns reflect tissue and species specificity. It is also known that the distribution of glycosphingolipids change during cell growth, development, and malignant transformation. Today, several series of basic oligosaccharide structures (*globo, isoglobo; lacto, neolacto; ganglio*, and *gala*) have been found in various glycosphingolipids in mammals (*1*).

Forssman antigen was first reported by Forssman in 1911 as a so-called heterophile antigen (*2*). The well-known typical chemical structure of Forssman active glycolipids is that of a globo-series pentaglycosylceramide (GalNAcα-1,3GalNAcβ-1,3Galα-1,4Galβ-1,4Glcβ-1,1'Cer) (*3*). In this glycolipid, the Forssman antigen determinant disaccharide GalNAc-1,3GalNAc-1,3R is linked to a globo-series glycolipid.

[1] To whom correspondence should be addressed. Tel: +81-3-5803-5164, Fax: +81-3-5803-0121

Abbreviations: anti-SRBC, anti-sheep red blood cell stroma; C18:0h, hydroxy stearic acid; Cer, ceramide; CID, collision-induced dissociation; CMH, ceramide monohexoside; DQF-COSY, double quantum filtered correlated spectroscopy; GC/EI-MS, gas chromatography electron ionization mass spectrometry; GD1a, NeuAcα-2,3Galβ-1,3GalNAcβ-1,4(NeuAcα-2,3)Galβ-1,4Glcβ-1,1'Cer; GD1b, Galβ-1,3GalNAcβ-1,4(NeuAcα-2,8NeuAcα-2,3)Galβ-1,4Glcβ-1,1'Cer; GM1, Galβ-1,3GalNAcβ-1,4(NeuAcα-2,3)Galβ-1,4Glcβ-1,1'Cer; GM2, GalNAcβ-1,4(NeuAcα-2,3)Galβ-1,4Glcβ-1,1'Cer; Hex, hexose; HexNAc, *N*-acetylhexosamine; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; HPTLC, high-performance thin layer chromatography; MALDI, matrix-assisted laser desorption ionization; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; ROESY, rotating frame Overhauser enhancement spectroscopy; SIMS, secondary ion mass spectrometry; t18:0, hydroxysphinganine; TMS, trimethylsilyl; TOF-MS, time-of-flight mass spectrometry.

In this report, we show that the novel structure of an acidic Forssman active glycolipid is a branched hybrid of isoglobo-, ganglio-, and neolacto-series structures as shown in Fig. 7. The Forssman antigen determinant disaccharide is linked to one non-reducing end of the branched sugar chain and the GM2 terminal trisaccharide is linked to the other non-reducing end of the sugar chain of this glycolipid.

MATERIALS AND METHODS

Materials—DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Iatrobeads 6RS-8060 (particle size, 60 μm diameter) were purchased from Iatron (Tokyo). Silica gel 60 HPTLC plates were purchased from Merck (Darmstadt, Germany). Bovine brain gangliosides, as reference acidic glycolipids, were prepared in our laboratory. Anti-sheep red blood cell stroma antibodies were purchased from Sigma (St. Louis, MO, USA). The anti-Forssman glycolipid rabbit IgG fraction was from Dr. Tatsuji Yasuda (Okayama University, Okayama). The anti-GM2 and anti-GM_2(NeuGc) monoclonal antibodies were from Drs. Ikuo Kawashima and Tadashi Tai (Tokyo Metropolitan Institute of Medical Science, Tokyo). The peroxidase conjugated goat anti-rabbit antibodies and peroxidase conjugated rabbit anti-mouse antibodies were from ICN Pharmaceuticals (Aurora, OH, USA). The Konica immunostain kit was purchased from Konica (Tokyo). Polyisobutylmethacrylate, NaB^2H_4, and ^2H_2O were purchased from Aldrich Chem. (Milwaukee, WI, USA). CMH from a tapeworm, *Spirometra erinacei*, with Cer (t18:0, C18:0h) was donated by Dr. Yasushi Kawakami (Azabu University, Kanagawa). Endoglycoceramidase II (1 mU/μl, from *Rhodococcus* sp. with Activator II) was purchased from Takara Shuzo (Shiga). $(C^2H_3)_2SO$ was purchased from Isotec (Miamisburg, OH, USA). All the chemicals obtained from commercial sources were of analytical grade.

Isolation of the Acidic Forssman Active Glycolipid—A batch of equine kidneys was extracted successively with 2-3 volumes of chloroform/methanol (1:1, by volume), chloroform/methanol (2:1, by volume), and isopropanol/hexane/water (55:20:25, by volume). Following Folch's partition (*4*), the upper phase glycolipids were subjected to DEAE-Sephadex column chromatography. The acidic glycolipids were eluted with a linear gradient of chloroform/methanol/water (30:60:8, by volume) to chloroform/methanol/2 M CH_3COONa (30:60:8, by volume) using two glass reservoirs. Monosialoganglioside fractions were further purified by Iatrobeads column chromatography eluted with a linear gradient of chloroform/methanol/water from 80:20:2 to 30:70:2 (by volume). Rechromatography was performed on an Iatrobeads column with a linear gradient of chloroform/methanol/water from 55:45:2 to 30:70:2 (by volume).

Thin-Layer Chromatography—TLC was carried out with a developing solvent mixture of chloroform/methanol/water containing 0.2% $CaCl_2$ (55:45:10, by volume). Glycolipids with sialic acids were visualized by spraying the plate with resorcinol/HCl reagent (*5*).

TLC-Immunostaining—Glycolipids were separated by TLC. After development, the plate was dried and then soaked in chloroform/*n*-hexane (20/105, by volume) containing 0.4% polyisobutylmethacrylate for 1 min. The plate was dried and then incubated with a 1:150 (or 200) dilution of anti-Forssman antibodies (or anti-sheep red blood cell stroma antibodies) in PBS containing 1% bovine serum albumin at room temperature for 2 h. After washing with PBS, sequential incubations were performed with 1:2,000 diluted peroxidase conjugated goat anti-rabbit antibodies in PBS containing 1% bovine serum albumin at room temperature for 2 h. After washing with PBS, the Forssman active glycolipids were visualized using the Konica immunostain kit. TLC-immunostaining with anti-monoclonal GM2 (*6*) and anti-monoclonal GM2 (NeuGc) antibodies (*7*) was performed in the same way except that the dilution of the first antibody was 1:10 and that of the secondary antibody (peroxidase conjugated anti-mouse IgG) was 1:250.

NMR Spectroscopy—The glycolipid was deuterium-exchanged and lyophilized two times in ^2H_2O and then dried over P_2O_5 under high vacuum before dissolution in $(C^2H_3)_2SO/^2H_2O$ (98:2, by volume). The final concentrations were approx. 200 μM. Spectra were obtained at 400 MHz using a JEOL GX-400 spectrometer with a probe temperature of 60°C. Chemical shifts were referenced to tetramethylsilane. All 2D-HOHAHA experiments were performed in the phase-sensitive mode. In DQF-COSY and 2D-HOHAHA experiments, data matrices of 256 ($t1$)×4,096 ($t2$) points, acquired with a spectral width of 2,500 Hz, were zero-filled to 1,024×4,096 points. The mixing time of 2D-HOHAHA was 155 ms. 2D-ROESY spectra were acquired with a 200 ms spin-lock period with a spectral width of 5 kHz. The 2D matrix consisting of 512 ($t1$)×4,096 ($t2$) points was zero-filled to 1,024×4,096 points.

Mass Spectrometry—MALDI mass spectra of the glycolipid were obtained with a TOF-mass spectrometer (Voyager DE-STR; PerSeptive Biosystems, Framingham, MA, USA) with Delayed Extraction™. The intact and permethylated glycolipids were measured in the positive ion reflection mode. The MALDI matrix was 2,5-dihydroxybenzoic acid. The accelerating voltage was 20 kV. The grid voltage and delay time for Delayed Extraction™ were 14 kV and 200 ns, respectively.

Permethylation Analysis—The glycolipid was permethylated according to the modified procedure (*8*) of Ciucanu and Kerek (*9*). Fifty micrograms of the glycolipid was dissolved in 150 μl DMSO containing NaOH (40 mg/ml), 30 μl of methyl iodide was added, and the mixture was sonicated in an ultrasonic bath for 60 min. This step was repeated for complete methylation of the glycolipid. The permethylated glycolipid was extracted from the reaction mixture by Folch's partition, and the lower phase was evaporated to dryness. Aliquots of the permethylated glycolipid were analyzed by MALDI-TOF-MS, after which the remainder of the permethylated samples was hydrolyzed with 0.5 M HCl-80% acetic acid at 75°C for 18 h according to the method of Ohashi *et al.* [Ohashi, M., Uchida, K., and Yamakawa, Y. (1984) *Proc. Jpn. Conf. Biochem. Lipids* **26**, 75-77 (in Japanese)], reduced with NaB^2H_4-10 mM NaOH for several hours, adjusted to pH 4 or 5 with 1 M acetic acid, and then peracetylated with an acetic anhydride-pyridine mixture (1:1, by volume) at 100°C for 30 min (*10*). The acetates of the partially methylated alditols were analyzed by GC/EI-MS (5973 MSD; Hewlett Packard, Palo Alto, CA, USA) with a splitless injector. For GC, an HP-5MS column (0.25 mm

i.d. × 30 m; Hewlett Packard) was used. The oven temperature was programmed at 50°C-1 min-50°C-30 C/min-160°C-1°C/min-200 C. The carrier gas (He) flow rate was 1 ml/min.

Identification of Sialic Acid—The sialic acid component of the glycolipid was determined according to the method of Yu and Ledeen (*11*). The glycolipid (30-60 μg) was methanolyzed with 0.05 N methanolic HCl at 80°C for 1 h. The methanolyzed sample was dried under an air stream and then derivatized with 50 μl of TMS reagent (pyridine/trimethylchlorosilane/1,1,1,3,3,3-hexamethyldisilazane, 1:1.3:0.8, by volume). The TMS derivatives were analyzed by GC/EI-MS (5973 MSD; Hewlett Packard) with a split injector. For GC, an HP-5MS column (0.25 mm i.d. × 30 m; Hewlett Packard) was used. The oven temperature was programmed from 240°C to 255°C at 1°C/min. The carrier gas (He) flow rate was 1 ml/min.

Analysis of the Ceramide Moiety—Ten nanomoles of glycolipid was dissolved in 50 μl of 50 mM sodium acetate buffer (pH 5.0, 0.4% Triton X-100) to which 5 μl (5 mU) of endoglycoceramidase (*12*) was added. The mixture was incubated at 37°C for 2 days and then evaporated to dryness under an air stream. A negative SIMS spectrum of the released ceramide was obtained with a triple quadrupole mass spectrometer (TSQ 70; Finnigan MAT, San Jose, CA, USA) equipped with a Cs ion gun and a 20 kV conversion dynode. The SIMS matrix was triethanolamine. The energy of the Cs^+ primary beam was 20 keV. CID spectra of the ceramide were obtained by means of collision with Ar in Q2 under 0.4 Pa. The deprotonated ceramide molecule ($[M-H]^-$) was selected as the precursor ion. The collision energy (E_{Lab}) was 15 eV. CMH obtained from a tapeworm (*13*) was used as a reference glycolipid containing ceramide with t18:0 and C18:0h.

RESULTS

Isolation of Equine Kidney Acidic Glycolipids—Figure 1 shows the TLC pattern of total acidic glycolipids separated by DEAE-Sephadex column chromatography. Anti-Forssman rabbit serum reacted strongly with the monosialoganglioside fraction, and this fraction was further purified by Iatrobeads column chromatography. The elution pattern of the monosialoganglioside fraction from Iatrobeads column chromatography as visualized by resorcinol reagent is shown in Fig. 2A. On HPTLC, this acidic Forssman glycolipid migrated somewhat more slowly than GD1b (Fig. 2B). Some fractions (No. 31-42) reacted with anti-Forssman antibodies suggesting the presence of the terminal Forssman epitope structure, GalNAcα-1,3GalNAcβ (Fig. 2B). Figure 3 shows the TLC of fr. No. 39 with half the plate stained with resorcinol reagent and the other half stained with anti-SRBC and anti-GM2 antibodies. The same resorcinol positive spot was stained with both antibodies. This indicates that this glycolipid has the structures of both the terminal Forssman and terminal GM2 epitope oligossacharides. This glycolipid was not stained with anti- GM2 (NeuGc), suggesting the sialic acid is NeuAc. This glycolipid from fr. No. 39 was named AF and used for further analyses.

NMR Spectroscopy—Figure 4A shows the 2D-HOHAHA spectrum of AF in the region of the sugar proton resonances with the one dimensional ^{1}H-spectrum. The chemical shifts of H1 to H4 and the coupling constants determined by DQF-COSY and HOHAHA experiments indicated that eight anomeric signals at 4.23, 4.29, 4.31, 4.40, 4.63, 4.74,

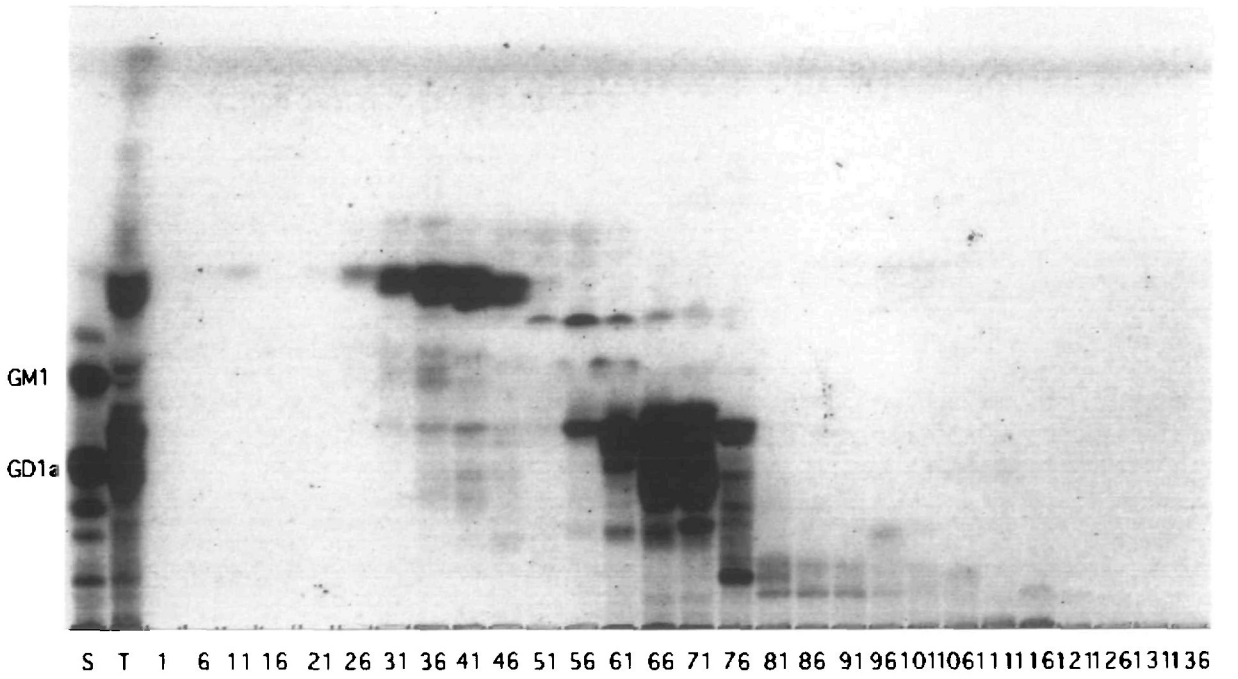
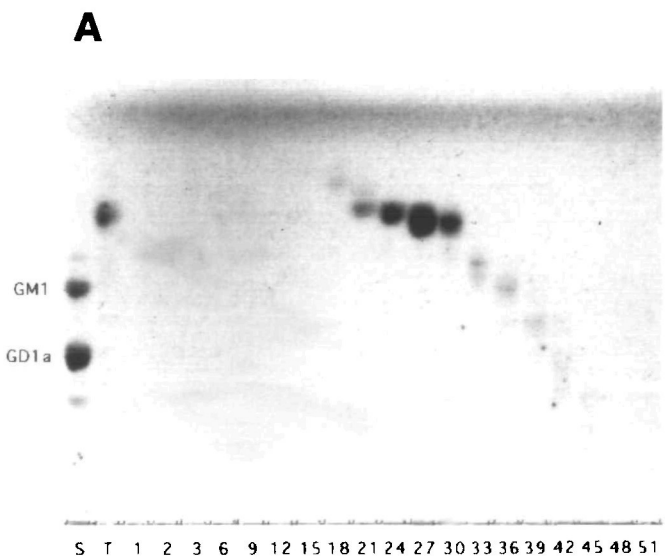


Fig. 1. **Elution pattern of equine kidney acidic glycolipids from a DEAE-Sephadex column.** The column was eluted with a linear gradient of chloroform/methanol/water (30:60:8, by volume) with increasing salt concentrations in water to 2 M CH_3COONa. The eluate was checked by HPTLC as described in "MATERIALS AND METHODS." Lane S, bovine brain gangliosides; lane T, total glycosphingolipids from Folch's upper phase of the equine kidney extract.

4.82, and 4.88 ppm can be attributed to Glcβ(I), Galβ(VII), Galβ(II), GlcNAcβ(VI), GalNAcβ(IV), GalNAcα(V), GalNAcβ(VIII), and Galα(III), respectively (Table I). The chemical shifts of H1 to H4 of GalNAcα(V) were in good agreement with the corresponding chemical shifts in the Forssman glycolipid (*14*). The chemical shifts of the protons of Galα(III) and Galβ(II), however, were similar to the corresponding chemical shifts in isoglobo-series glycosphingolipids (*8, 15*) rather than globo-series glycosphingolipids (*14, 16*). These results suggest that AF contains the Forssman-like isoglobo core, GalNAcα(V)-1,3GalNAcβ-(IV)-1,3Galα(III)-1,3Galβ(II)-. The chemical shifts of H1 to H3 of GalNAcβ(IV), which constitute GalNAcα(V)-1,3GalNAcβ(IV)-, are apparently different from those in the Forssman glycolipid (the chemical shift displacements are in the range of 0.05–0.1 ppm) (*14*), which also suggests that AF contains an isoglobo core instead of a globo core.

The H1 to H4 resonances of another GalNAcβ residue [GalNAcβ(VIII)] are similar to the corresponding resonances of the terminal GalNAcβ in GM2 ganglioside (*17*). The signals of Galβ(VII) also have chemical shifts similar to GM2. An H2 signal resonating at a higher field than 3.2 ppm, which is characteristic of H2 of Galβ(VII) in the GalNAcβ(VIII)-1,4(NeuAcα-2,3)Galβ(VII)-structure, was also observed. The above results suggest that in addition to the existence of the GlcNAcβ(VI) residue, AF has a unique carbohydrate structure that is a hybrid of the isoglobo-, ganglio-, and lacto- (or neolacto-) series.

The linkages between the carbohydrate residues were determined by 2D-ROESY experiments. Figure 4B shows the ROESY spectrum in the region of the sugar proton resonances. Interresidual ROE's observed were GalNAcα(V)-1/GalNAcβ(IV)-3, GalNAcα(V)-1/GalNAcβ(IV)-4, GalNAcβ(IV)-1/Galα(III)-3, Galα(III)-1/Galβ(II)-3, Galα(III)-1/Galβ(II)-4, Galβ(II)-1/Glcβ(I)-4, GalNAcβ(VIII)-1/Galβ(VII)-4, and Galβ(VII)-1/GlcNAcβ(VI)-4. The observation of cross-peaks of both Galα(III)-1/Galβ(II)-3 and Galα(III)-1/Galβ(II)-4 confirmed the isoglobo-



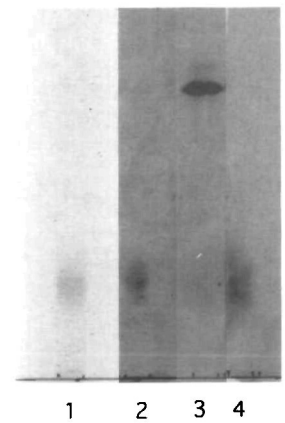
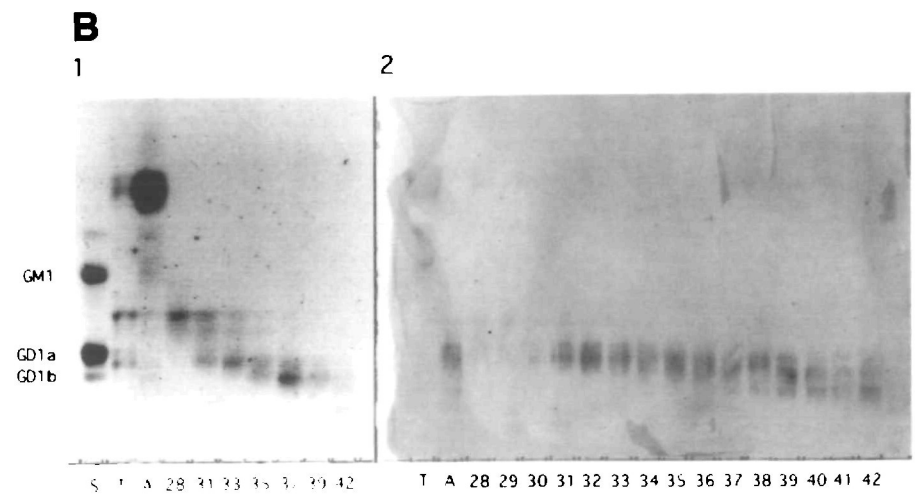


Fig. 3. **Immunostaining of AF.** The purified AF was applied to a HPTLC plate and then developed with chloroform/methanol/water (55:45:10, by volume) containing 0.02% $CaCl_2$. Lane 1, purified AF stained with resorcinol reagent; lane 2, purified AF stained with anti-GM2 antibodies; lane 3, reference GM2 stained with anti-GM2 antibodies; lane 4, purified AF stained with anti-SRBC antibodies.

Fig. 2. **A: Elution pattern of equine kidney monosialogangliosides from an Iatrobeads column.** Monosialogangliosides were eluted with a linear gradient of chloroform methanol water from 80:20:2 to 30:70:2 (by volume). The eluate was checked by HPTLC as described in "MATERIALS AND METHODS." Glycolipids were visualized with resorcinol reagent. Lane S, bovine brain gangliosides; lane T, equine kidney monosialogangliosides. **B: Elution pattern of acidic Forssman glycolipids from an Iatrobeads column, and immunostaining of the acidic Forssman glycolipids.** B-1 shows acidic Forssman glycolipids eluted with a linear gradient of chloroform methanol water from 55:45:2 to 30:70:2 (by volume). The eluate was checked as in A. Lane S, bovine brain gangliosides; lane T, total glycosphingolipids from Folch's upper phase of the equine kidney extract; lane A, equine kidney monosialogangliosides. B-2 shows the immunostaining of acidic Forssman glycolipids stained with anti-SRBC antibodies.



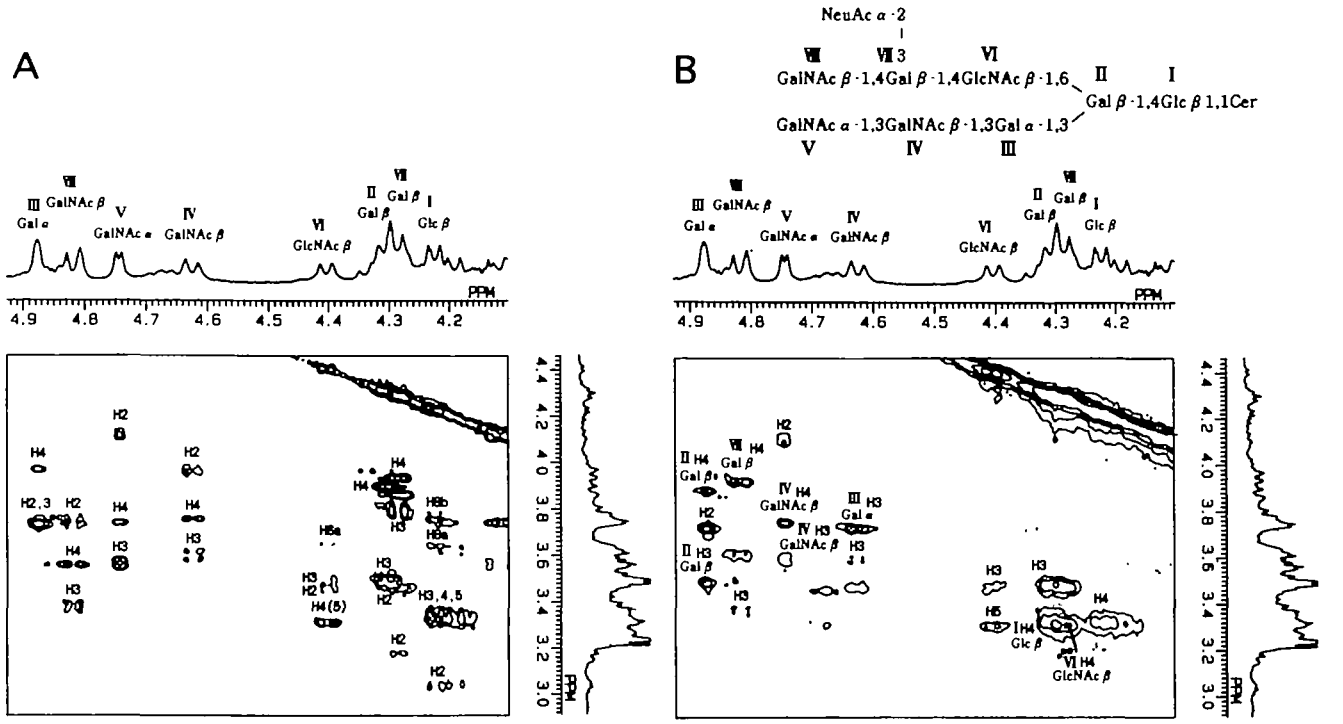


Fig. 4. **2D-HOHAHA (A) and ROESY (B) spectra of AF.** The spectra in the cross peak region between resonances of anomeric protons and other sugar protons are shown. The spectrum in A was obtained with a mixing time of 155 ms and the spectrum of B was acquired with a spin-lock period of 200 ms at 60°C. The abbreviations labeling each cross-peak correspond to suger residue numbering (Roman numerals), followed by the proton assignment (Arabic numerals). The proposed structure of AF is shown with abbreviations of the carbohydrate residues. In the spectrum in B, the alternative assignments for the cross-peak Galβ(II)-1/Glcβ(I)-4 are to Galβ(II)-1/Glcβ(I)-3 or Galβ(II)-1/Glcβ(I)-5, because the H3, H4, and H5 signals of Glcβ(I) are strongly coupled.

TABLE I. **^{1}H-chemical shifts (ppm) and coupling constants (Hz, in parentheses) of the constituent sugar residues in the AF.**

Residue	H1($^3J_{1,2}$)	H2 ($^3J_{2,3}$)	H3 ($^3J_{3,4}$)	H4[b]
I: Glcβ	4.23 (8.3)	3.02[a]	3.36[a]	3.3[a]
VII: Galβ	4.29 (8.8)	3.18 (10.1)	3.79 (2.3)	3.93
II: Galβ	4.31 (8.1)	3.47[a]	3.51 (<3.9)	3.89
VI: GlcNAcβ	4.40 (7.8)	3.44 (10.9)	3.51 (7.0)	3.31
IV: GalNAcβ	4.63 (8.1)	3.96 (10.9)	3.60 (2.7)	3.76
V: GalNAcα	4.74 (3.2)	4.12 (11.0)	3.56 (2.7)	3.74
VIII: GalNAcβ	4.82 (8.5)	3.75 (10.0)	3.38 (2.9)	3.56
III: Galα	4.88 (<3.2)	3.73[a]	3.73 (<3.4)	3.97

[a]The coupling constants could not be determined due to strong coupling. The H3, H4, and H5 signals of Glcβ were also strongly coupled. [b]The coupling constants, $^3J_{3,4}$, were below the digital resolution (1.2 Hz).

structure, -Galα-1,3Galβ-, because the structure of -Galα-1,4Galβ- shows only the Galα-1/Galβ-4 cross peak in the ROESY spectrum (*18*). The results of 2D-HOHAHA and ROESY analyses shows that AF consists of two structures, GalNAcα(V)-1,3GalNAcβ(IV)-1,3Galα(III)-1,3Galβ(II)-1,4Glcβ(I)- and GalNAcβ(VIII)-1,4(NeuAcα-2,3)Galβ(VII)-1,4GlcNAcβ(VI)-. The chemical shifts of the protons of GlcNAcβ(VI) (Table I) suggest a branched structure, -3(GlcNAcβ-1,4)Galβ- or -3(GlcNAcβ-1,6)Galβ- (*19*). In the ROESY spectrum, no detectable cross-peak indicating a linkage between GlcNAcβ(VI) and Galβ(II) was observed. This suggests that the branched structure is -3(GlcNAcβ-1,6)Galβ- rather than -3(GlcNAcβ-1,4)Galβ-, because a 1,6 linkage is less rigid and its ROE may be weaker than a 1,4 linkage. The structure deduced by NMR analyses (see Fig. 4B) is in good agreement with the results of the methylation analysis described below.

Mass Spectrometry—The positive MALDI-TOF mass spectrum of AF showed some molecular related ions ([M−H+2Na]$^+$) such as *m/z* 2,452.0, 2,466.0, 2,480.1, and 2,494.0 (Fig. 5A). The negative SIMS spectrum of the ceramide obtained from AF (by treatment with endoglycoceramidase) showed deprotonated molecules ([M−H]$^-$) such as *m/z* 626.5, 654.5, 668.6, 682.6, and 696.6 (Fig. 6). The difference in molecular weight between the intact AF and the ceramide is 1,751.4, which is equal to the total mass of tetra-hexose, tetra-*N*-acetylhexosamine, and an *N*-acetylneuraminic acid. This sugar composition is supported by the results of TMS-sugar and sialic acid analyses involving GC/EI-MS and NMR analysis with DQF-COSY and 2D-HOHAHA. The results of the TLC-immunostaining experiment with anti-GM2 antibodies also support the notion that AF contains only *N*-acetylneuraminic acid. The positive MALDI-TOF mass spectrum of the permethylated AF shows some sodium adducted ions ([M+Na]$^+$) such as *m/z* 2,906.8, 2,920.7, 2,934.8, and 2,948.7 (Fig. 5B). The difference in molecular weights between intact AF and permethylated AF is 477.9, which corresponds to 34 methylated function groups. This amount suggests that the ceramide consists of a hydroxysphinganine and a saturated hydroxy fatty acid. In accordance with this suggestion, the glycosphingolipid at *m/z* 2,452.0 in Fig. 5A may contain ceramide with t18:0 and C22:0h. The ceramide CID spectra were compared between the glycosphingolipid and CMH from tapeworm. The two spectra were the same

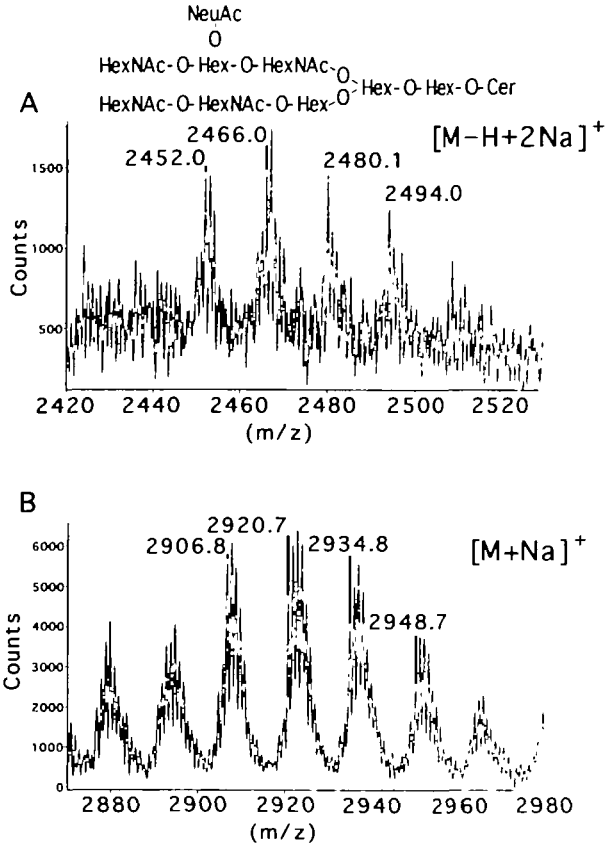


Fig. 5. **Positive ion TOF-MS of native (A) and permethylated (B) AF.**

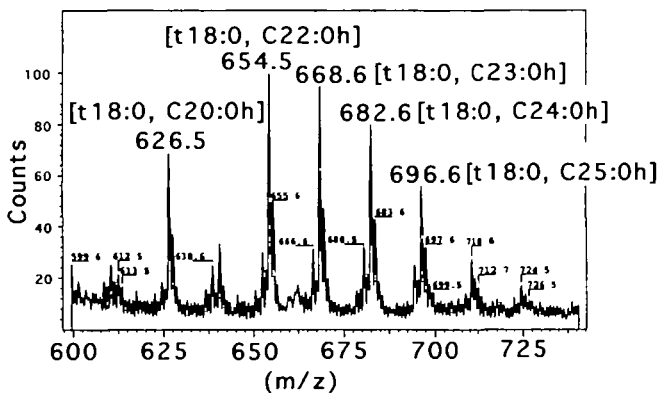


Fig. 6. **Negative ion SIMS of the ceramide of AF.**

except for a difference due to the hydrocarbon chain length of the fatty acid, which supports the above expectation.

Permethylation Analysis—Permethylation analysis indicated the presence of 7 peaks on GC-MS, which were identified as the acetates of 2,3,6-tri-*O*-methylglucitol(-4Glc), 2,4,6-tri-*O*-methylgalactitol(-3Gal), 2,6-di-*O*-methylgalactitol(-3,4Gal), 2,4-di-*O*-methylgalactitol(-3,6Gal), 3,4,6-tri-*O*-methyl-2-*N*-methylacetamidogalactitol(GalNAc-), 3,6-di-*O*-methyl-2-*N*-methylacetamidogluctitol(-4GlcNAc), and 4,6-di-*O*-methylacetamidogalactitol(-3GalNAc). These results confirm the presence of isoglobo, neolacto, and GM2 terminal sugar chains branched at the

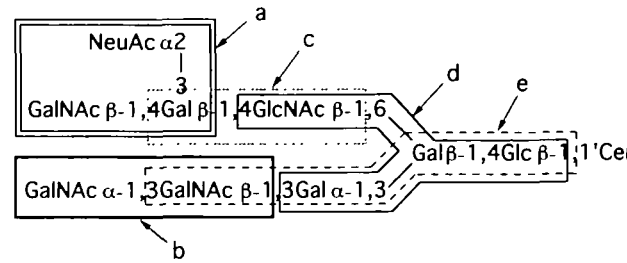


Fig. 7. **Structure of AF.** AF consists of a novel Forssman active acidic glycosphingolipid with branched isoglobo-series isogloboside (indicated by ---- and marked as e), Forssman epitope disaccharide (indicated by — and marked as b), ganglio-series GM2 trisaccharide (indicated by ▬ and marked as a), and neolacto-series disaccharide (indicated by · · and marked as c) hybrid sugar chains. This glycolipid contains a novel branched linkage (indicated by — and marked as d).

3,6 position of galactose.

Identification of the Sialic Acid—The sialic acid was identified as NeuAc by GC/MS, after methanolysis and derivatization to TMS derivatives. This was also confirmed by the results of TLC-immunostaining with anti-GM2 and anti-GM2 (NeuGc) antibodies, as described in the previous section.

These results show that the structure of AF is a novel Forssman active acidic glycosphingolipid with branched isoglobo-, ganglio-, and neolacto-series hybrid sugar chains as shown in Fig. 7.

DISCUSSION

Animals can be classified into Forssman-positive (guinea pig, horse, cat, mouse, chicken, tortoise, and carp) and -negative (pig, ox, rabbit, rat, goose, pigeon, and frog) groups (*20*). The well-known typical chemical structure of Forssman active glycolipids is that of globo-series pentaglycosylceramide, GalNAcα-1,3GalNAcβ-1,3Galα-1,4Galβ-1,4Glcβ-1,1'Cer (*3*), distributed in the erythrocytes (*21*) of sheep and goats and the intestine (*22*) and kidney of dogs. However, other Forssman active glycolipid structures have been reported. Forssman active glycolipids with isoglobo type sugar chains have been reported in rat adenocarcinoma (*23*). Structural variants of Forssman active glycolipids with tetrasaccharides (*24*) and hepta- and octa-saccharides (*25, 26*) have also been reported. The presence of Forssman active glycolipids corresponding to ceramide di- or tri-saccharides has also been suggested (*27*). Ostrander *et al.* reported the chemical structure of the acidic Forssman active glycolipid in the liver of English sole (*Paroprys vetulus*), in which the Forssman antigen determinant disaccharides is linked to the terminal Gal of the GM1 ganglioside. It reacted very faintly with anti-Forssman antibodies until it was desialylated (*28*).

In this report, we present the novel structure of an acidic Forssman active glycolipid in equine kidney. The sugar chain of this glycolipid is a branched hybrid of isoglobo-, ganglio-, and neolacto-series structures (Fig. 7) and contains a novel linkage, a -GlcNAcβ-1,6·Gal- and a -Galα-1,3·Gal- branching point, as shown in Fig. 7d. The non-reducing ends of the branched sugar chain of this glycolipid responds to Forssman epitope disaccharide (Fig. 7b) and

GM2 trisaccharide (Fig. 7a), respectively. Although there have been several reports about the structures of glycolipids with hybrid sugar chains (*29-32*), the combination of an oligosaccharide series with a Forssman antigen determinant disaccharide as in this novel glycolipid has not previously been reported. The major Forssman active glycolipid in equine kidney (*33*) and spleen (*3*) is a globo-type pentaglycosylceramide, but in this novel glycolipid the Forssman antigen determinant disaccharide is linked to an isoglobo-series sugar chain. Ito and Yamagata reported that globo-series glycolipids are resistant to the action of endoglycoceramidase (*12*). In our experiments, the neutral globo-series Forssman active glycolipid obtained from equine kidney was resistant to this enzyme, while the novel acidic Forssman active nanoglycosylceramide was susceptible, and the ceramide portion can be cleaved from the glycolipid. The ceramide of this glycolipid is composed mainly of hydroxysphinganine and hydroxy fatty acids. Hara and Taketomi reported the ceramide composition of an equine kidney globo-series Forssman active glycolipid (*33*), and Gasa and Makita reported those for gangliosides (*34*). The finding of this novel glycolipid with unique sugar chains suggests a new research in the field of glycolipid metabolism, especially regarding the specificity and regulation of glycosyltransferases and glycohydrolases.

We wish to thank Dr. Yoshiyuki Azuma (Kitasato University, Akita) and Dr. Hideharu Taira (Iwate University, Iwate) for supplying the equine kidneys. We also thank Dr. Tatsuji Yasuda (Okayama University, Okayama) for the anti-Forssman antibody IgG fraction and Drs. Ikuo Kawashima and Tadashi Tai (Tokyo Metropolitan Institute of Medical Science, Tokyo) for the anti-GM2 and anti-GM_3(NeuGc) monoclonal antibodies. We also thank Dr. Yasushi Kawakami (Azabu University, Kanagawa) for the CMH with Cer (t18:0, C18:0h). Thanks are also due to Dr. Kazuo Nakamura (Kitasato University, Kanagawa) for analyzing the samples by GC/EI-MS.

REFERENCES

1. Makita, A. and Taniguchi, N. (1985) Glycosphingolipids in *New Comprehensive Biochemistry* (Wiegandt, H., ed.) Vol. 10, pp. 1-99, Elsevier, Amsterdam
2. Forssman, J. (1911) Die Herstellung hochwertiger spezifischer Schaf-hämolysine ohne Verwendung von Schafblut. *Biochem. Z.* **37**, 78-115
3. Siddiqui, B. and Hakomori, S. (1971) Revised structure for the Forssman glycolipid hapten. *J. Biol. Chem.* **246**, 5766-5769
4. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509
5. Svennerholm, L. (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta* **24**, 604-611
6. Kotani, M., Ozawa, H., Kawashima, I., Ando, S., and Tai, T. (1992) Generation of one set of monoclonal antibodies specific for a-pathway ganglio-series gangliosides. *Biochim. Biophys. Acta* **1117**, 97-103
7. Kawashima, I., Ozawa, H., Kotani, M., Suzuki, M., Kawano, T., Gomibuchi, M., and Tai, T. (1993) Characterization of ganglioside expression in human melanoma cells: immunological and biochemical analysis. *J. Biochem.* **114**, 186-193
8. Tadano-Aritomi, K., Kasama, T., Handa, S., and Ishizuka, I. (1992) Isolation and characterization of a mono-sulfated isoglobotetraosylceramide, the first sulfoglycosphingolipid of the isoglobo-series, from rat kidney. *Eur. J. Biochem.* **209**, 305-313
9. Ciucanu, I. and Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131**, 209-217
10. Levery, S.B. and Hakomori, S. (1987) Microscale methylation analysis of glycolipids using capillary gas chromatography-chemical ionization mass fragmentography with selected ion monitoring. *Methods Enzymol.* **138**, 13-25
11. Yu, R.K. and Ledeen, R.W. (1970) Gas-liquid chromatographic assay of lipid-bound sialic acids: measurement of gangliosides in brain of several species. *J. Lipid Res.* **11**, 506-516
12. Ito, M. and Yamagata, T. (1989) Purification and characterization of glycoshingolipid-specific endoglycosidases (endoglycoceramidases) from a mutant strain of *Rhodococcus* sp. *J. Biol. Chem.* **264**, 9510-9519
13. Kawakami, Y., Kojima, H., Nakamura, K., Suzuki, M., Uchida, A., Murata, Y., and Tamai, Y. (1995) Monohexosylceramides of larval and adult forms of the tapeworm, *Spirometra erinacei*. *Lipids* **30**, 333-337
14. Dabrowski, J., Hanfland, P., and Egge, H. (1980) Structural analysis of glycoshingolipids by high-resolution ^{1}H nuclear magnetic resonance spectroscopy. *Biochemistry* **19**, 5652-5658
15. Tadano-Aritomi, K., Okuda, M., Ishizuka, I., Kubo, H., and Ireland, P. (1994) A novel mono-sulfated pentaglycosylceramide with the isoglobo-series core structure in rat kidney. *Carbohydr. Res.* **265**, 49-59
16. Nagai, K., Roberts, D.D., Toida, T., Matsumoto, H., Kushi, Y., Handa, S., and Ishizuka, I. (1989) Mono-sulfated globopentaosylceramide from human kidney. *J. Biol. Chem.* **27**, 16229-16237
17. Koerner, T.A.W., Prestégard, J.H., Demou, P.C., and Yu, R.K. (1983) High-resolution proton NMR studies of gangliosides. 1. Use of homonuclear two-dimensional spin-echo *J*-correlated spectroscopy for determination of residue composition and anomeric configurations. *Biochemistry* **22**, 2676-2687
18. Poppe, L., Dabrowski, J., von der Lieth, C.-W., Koike, K., and Ogawa, T. (1990) Three-dimensional structure of the oligosaccharide terminus of globotriaosylceramide and isoglobotriaosylceramide in solution. A rotating-frame NOE study using hydroxyl groups as long-range sensors in conformational analysis by ^{1}H-NMR spectroscopy. *Eur. J. Biochem.* **189**, 313-325
19. Dabrowski, J. and Hanfland, P. (1982) Structure determination of a ceramide pentadecasaccharide by two-dimentional *J*-resolved and *J*-correlated NMR spectroscopy. *FEBS Lett.* **142**, 138-142
20. Tanaka, N. and Leduc, E.H. (1956) A study of the cellular distribution of Forssman antigen in various species. *J. Immunol.* **77**, 198-212
21. Koizumi, N., Hara, A., Uemura, K., and Taketomi, T. (1988) Glycosphingolipids in sheep liver, kidney, and various blood cells. *Jpn. J. Exp. Med.* **58**, 21-31
22. Sung, S.J., Esselman, W.J., and Sweeley, C.C. (1973) Structure of a pentahexosylceramide (Forssman hapten) from canine intestine and kidney. *J. Biol. Chem.* **248**, 6528-6533
23. Falk, P., Holgersson, J., Jovall, P.-Å., Karlsson, K.-A., Strömberg, N., Thurin, J., Brodin, T., and Sjögren, H.-O. (1986) An antigen present in rat adenocarcinoma and normal colon nonepithelial stroma is a novel Forssman-like glycolipid based on isoglobotetraosylceramide. *Biochim. Biophys. Acta* **878**, 296-299
24. Gahmberg, C.G. and Hakomori, S. (1975) Surface carbohydrates of hamster fibroblasts. I. Chemical characterization of surface-labeled glycosphingolipids and a specific ceramide tetrasaccharide for transformants. *J. Biol. Chem.* **250**, 2438-2446
25. Slomiany, A. and Slomiany, B.L. (1977) Water-soluble glycosphingolipids of dog gastric mucosa. Characterization of a branched ceramide heptasaccharide. *Eur. J. Biochem.* **76**, 491-498
26. Slomiany, B.L. and Slomiany, A. (1978) Forssman glycolipid variants of dog gastric mucosa. Structure of a branched ceramide octasaccharide. *Eur. J. Biochem.* **83**, 105-111
27. Yokota, M., Warner, G.A., and Hakomori, S. (1981) Blood group A-like glycolipid and a novel Forssman antigen in the hepatocarcinoma of a blood group O individual. *Cancer Res.* **41**, 4185-4190
28. Ostrander, G.K., Levery, S.B., Hakomori, S., and Holmes, E.H. (1988) Isolation and characterization of the major acidic glycosphingolipids from the liver of the Englisb sole (*Parophrys*

vetulus). Presence of a novel ganglioside with a Forssman antigen determinant. *J. Biol. Chem.* **263**, 3103-3110
29. DeGasperi, R., Koerner, T.A.W., Quarles, R.H., Ilyas, A.A., Ishikawa, Y., Li, S.-C., and Li, Y.-T. (1987) Isolation and characterization of gangliosides with hybrid neolacto-ganglio-type sugar chains. *J. Biol. Chem.* **262**, 17149-17155
30. Ostrander, G.K., Levery, S.B., Eaton, H.L., Salyan, M.E., Hakomori, S., and Holmes, E.H. (1988) Isolation and characterization of four major neutral glycosphingolipids from the liver of the Einglish sole (*Parophrys vetulus*). Presence of novel branched lacto-ganglio-*iso*-globo hybrid structure. *J. Biol. Chem.* **263**, 18716-18725
31. Thurin, J., Brodin, T., Bechtel, B., Jovall, P.-Å., Karlsson, H., Strömberg, N., Teneberg, S., Sjögren, H.O., and Karlsson, K.-A. (1989) Novel isoglobo-neolacto-series hybrid glycolipid detected by a monoclonal antibody is a rat colon tumor-associated antigen. *Biochim. Biophys. Acta* **1002**, 267-272
32. Fredman, P., Månsson, J.-E., Wikstrand, C.J., Vrionis, F.D., Rynmark, B.-M., Bigner, D.D., and Svennerholm, L. (1989) A new ganglioside of the lactotetraose series, GalNAc-3′-isoLM1, detected in human meconium. *J. Biol. Chem.* **264**, 12122-12125
33. Hara, A. and Taketomi, T. (1975) Long chain base and fatty acid compositions of equine kidney sphingolipids. *J. Biochem.* **78**, 527-536
34. Gasa, S. and Makita, A. (1980) Characterization of gangliosides from equine kidney and spleen. *J. Biochem.* **88**, 1119-1128