Gangliosides from the starfish *Evasterias echinosoma*: identification of a disialoganglioside containing 8-*O*-methyl-*N*-acetylneuraminic acid and *N*-formylgalactosamine

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Gangliosides were isolated from the starfish *Evasterias echinosoma* and their structures were elucidated by chemical and physicochemical methods. Two major gangliosides were found to be disialogangliosides, whose carbohydrate chain is based on the trisaccharide β-*N*-acylgalactosaminyl-(1→3)-β-galactosyl-(1→4)-β-glucose (acyl is formyl or acetyl), both residues of 8-*O*-methyl-*N*-acetylneuraminic acid being attached to the *N*-acylgalactosamine residue at positions 3 and 6. The minor components are disialogangliosides with linear carbohydrate chains in which the terminal sialic acid residue is attached to the penultimate *N*-acetylneuraminic acid residue at positions 4, 8, or 9. The lipid part of the gangliosides consists of sphingenine and unsubstituted fatty acids (mainly, palmitic and stearic acids).

Key words: gangliosides, starfish, *Evasterias echinosoma*, 8-*O*-methyl-*N*-acetylneuraminic acid, *N*-formylgalactosamine.

It was shown previously that the carbohydrate chains of starfish gangliosides have no common structural type, not only for the whole class of *Asteroidea* but also for individual orders.[1–3] Even among the representatives of one family, gangliosides can differ in the type of the oligosaccharide structure. Thus gangliosides of the ganglio series[4–7] and hematosides[8–10] have been found in the pedicellarian starfishes, belonging to the same family. Apparently, gangliosides with identical structures of the carbohydrate chains can be found only in animals that are closely related taxonomically, *i.e.*, those belonging to the same genus. It has been shown that oligosaccharide chains of the major gangliosides in three starfish species of the genus *Asterias* (*A. amurensis*, *A. rubens*, and *A. rathbuni*) have the same structure. A distinctive feature of this structure is the presence of the terminal branched trisaccharide fragment 8-O-Me-Neu5Gc-α2→3-(8-O-Me-Neu5Gc-α2→6)-Gal*N*Ac,[4–7] in which two sialic acid residues are attached to one amino sugar residue. In order to find out whether this structural similarity exists in the gangliosides in other starfish genera, we continued investigation along this line. Previously, we showed[4] that the disialoganglioside Neu5Ac-α2→9-Neu5Ac-α2→3-Gal*N*Ac-β1→3-Gal-β1→4-Glc-β1→1-Cer (Cer is ceramide) is the principal ganglioside in the liver of the starfish *Evasterias retifera*. In this work, we present data on the structure of the gangliosides from another representative of the genus *Evasterias*, the starfish *E. echinosoma*.

The preparation of polar lipids obtained from the total lipid extract of the animal liver as described previously[11] included, according to TLC, three sialo-containing glycolipids, one major and two minor components, together with neutral glycolipids and phospholipids. The sialo-containing lipids were isolated from the mixture by ion exchange chromatography on DEAE-cellulose. The major component was eluted from the column as disialoganglioside, one of the minor components was eluted as monosialoganglioside, and the other one was eluted as the most polar ganglioside.

The structure of the major ganglioside was determined using partial and total acid hydrolysis, methylation, oxidation with chromic anhydride, methanolysis, and enzymatic hydrolysis by neuraminidase and was confirmed by ^{1}H NMR spectroscopy.

The total acid hydrolysis of the ganglioside gave glucose, galactose, and galactosamine in 1 : 1 : 1 molar ratio. Mild acid hydrolysis gave sialic acid, whose content was twice as high as that of any of the neutral sugars, and asialoglycolipid, whose TLC mobility was identical to that of trihexosylceramide Gal*N*Ac-β1→3-Gal-β1→4-Glc-β1→1-Cer, which we had isolated previously upon partial acid hydrolysis of the ganglioside from the starfish *E. retifera*.[4] In addition, a small amount of dihexosylceramide, containing glucose and galactose, was detected. No oligosaccharides containing sialic acid were found in the products of mild acid hydrolysis.

To determine the monosaccharide sequence and the positions of linkages in the oligosaccharide chain of a ganglioside, the structure of the *N*-acyl substituents in amino sugars, and the presence of *O*-methylated sialic acids, methylation and trideuteriomethylation were used.

Analysis of the acetates of partially methylated hexitols, prepared from methylated dihexosylceramide,

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showed that the galactose residue occupies a terminal position and that the glucose residue is substituted at position 4 (the presence of glucose in the furanose form substituted at position 5 can be ruled out because the glucoside bond in the glycolipid is stable under conditions of partial acid hydrolysis). Thus, the glucose residue is located in the beginning of the ganglioside carbohydrate chain.

To characterize sialic acids, the trideuteriomethylated ganglioside was subjected to methanolysis and the resulting trideuteriomethylated derivatives of the methyl esters of methyl ketosides of sialic acids were analyzed by the GLC/MS method. The analysis showed the presence of one major compound, whose mass spectrum corresponded to the methyl ester of the methyl ketoside of 4,7,9-tri-*O*-trideuteriomethyl-8-*O*-methyl-*N*-trideuteriomethyl-*N*-acetylneuraminic acid. In addition, the corresponding derivatives of the terminal residues of *N*-glycolyl- and 8-*O*-methyl-*N*-glycolylneuraminic acids and nonterminal residues of *N*-acetylneuraminic acid monosubstituted at position 4, 8, or 9 were detected in small amounts. The content of all the minor components did not exceed 10—12%.

Neutral sugars and galactosamine were analyzed as partially trideuteriomethylated polyol acetates, which were obtained by acetolysis of the trideuteriomethylated ganglioside followed by hydrolysis, reduction of sugar derivatives to polyols, and acetylation. GLC/MS analysis showed that the glucose residue is substituted at position 4 and the galactose residue is substituted at position 3. Two galactosamine derivatives, present in equal amounts, were detected. Both were substituted at positions 3 and 6 and differ only by *N*-acyl substituents. One of these compounds, whose GLC retention time is longer, is a derivative of *N*-acetylgalactosamine (**1**), and the other compound having the shorter retention time is a *N*-formylgalactosamine (GalNFor) (**2**) derivative. In the mass spectrum of the latter component, the *m/z* values of the fragments incorporating the substituent at the nitrogen atom are shifted by 14 units toward lower masses compared to those for the *N*-acetyl derivative (Scheme 1; the dashed line denotes the pathways to the primary fragments, the arrows show the subsequent fragmentation, and the numbers correspond to the mass numbers of the fragments).

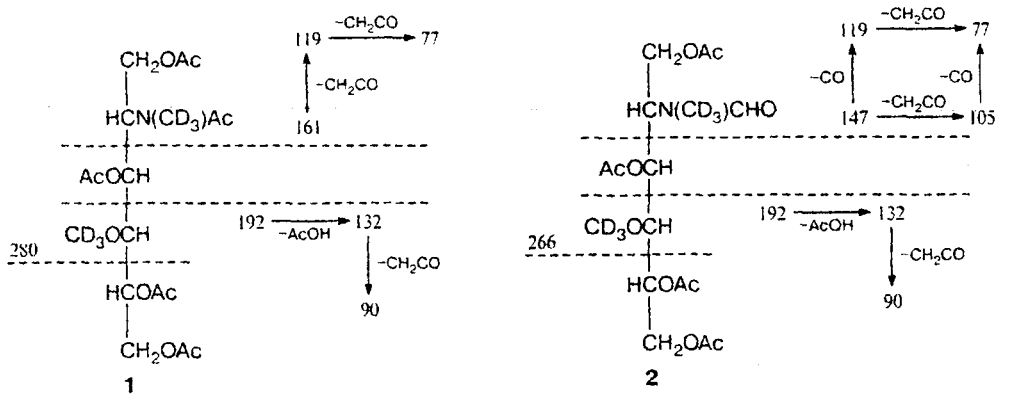
A similar fragmentation pattern has been observed previously for 1,2,5-tri-*O*-acetyl-3-*O*-methyl-4-(*N*-methyl-*N*-formylamino)-4,6-dideoxymannitol, formed during the analysis of the polysaccharide chain in the lipopolysaccharide from *Yersinia enterocolitica* carried out by methylation.[12]

Thus, the specimen of the major ganglioside isolated from the starfish *E. echinosoma* liver includes several compounds, among which two disialoglycolipids containing 8-*O*-methyl-*N*-acetylneuraminic acid predominate. Both residues of this acid are terminal and are attached to the same *N*-acylgalactosamine residue at positions 3 and 6. In one ganglioside, the galactosamine amino group is acylated by an acetyl group, while in the other one, it carries a formyl group. Disialogangliosides with a linear oligosaccharide chain in which the terminal residue of the sialic acid is attached to the penultimate residue of *N*-acetylneuraminic acid at positions 4, 8, or 9 are present in minor amounts.

The anomeric configurations in the glucose, galactose, and galactosamine residues were determined using oxidation of acetylated derivatives of trihexosylceramides, resulting from mild acid hydrolysis of the gangliosides, on treatment with chromic anhydride.[13] The fact that this treatment had destroyed all sugars led to the conclusion that all the anomeric centers have the β-configuration. The glycosidic configurations in sialic acids were determined by enzymatic hydrolysis. Sialic acid was split off almost completely on treatment of the gangliosides with neuraminidase from *Vibrio cholerae*; thus, both sialic acid residues are attached by the α-ketosidic bond.

The data on the structures of the oligosaccharide chains in gangliosides, obtained by chemical methods, were confirmed by NMR spectroscopy. The ^{1}H NMR spectrum of gangliosides recorded in DMSO-d_6 without preliminary exchange of active hydrogens with deute-

Scheme 1



1 **2**

rium exhibits in the anomeric region doublets for the H(1) protons of the β-Glc*p* (δ 4.21), β-Gal*p* (δ 4.28), and β-GalN*p* (δ 4.55) residues. The pyranose form of these monosaccharide residues is confirmed by the positions of the anomeric proton signals, while the β-configuration of the glycoside centers is indicated by the magnitudes of the coupling constants, $^3J_{H(1),H(2)} \approx 7$ Hz. The lower-field region contains the signals for the NHCO-group protons of galactosamine (δ 6.75), ceramide (δ 7.28), and two sialic acid residues (δ 7.71 and 7.75) and a signal with δ 7.35, which was assigned to the proton of the formyl group. This signal is retained in the spectrum of ganglioside treated preliminarily with D_2O when the signals of the amide protons disappear. It is known from studies dealing with the structures of bacterial O-antigenic polysaccharides, which incorporate *N*-formyl derivatives of amino sugars, that in the ^{1}H NMR spectra recorded in D_2O, the proton of the formyl group is responsible for two signals located in a lower field (δ 7.93—8.01 and 8.19—8.25), which are assigned to *E*- and *Z*-*N*-formyl groups, respectively.[14—16] However, in the spectrum of O-antigenic polysaccharide from *Pseudomonas aeruginosa*, containing *N*-formylgalactosaminuronic acid, recorded in DMSO-d_6, these signals proved to be in a higher field (δ 7.35 and 8.01) and the signal of the *E*-isomer coincided with the signal of the nonexchangeable proton in the ganglioside. Apparently, the *N*-formyl group of the GalNFor residue in the ganglioside exists only in the *E*-form.

It is of interest that in the ^{1}H NMR spectrum of the ganglioside, the proton of the NHCO-group of galactosamine (δ 6.75) resonates in a substantially higher field than is normally observed in the spectra of galactosamine-containing neutral and acidic glycolipids (δ 7.10—7.79).[17] In the spectrum of trihexosylceramide, obtained from the *E. echinosoma* ganglioside after mild acid hydrolysis, this signal is shifted downfield and has a typical position at 7.55 ppm. A similar shift of the proton signal for the amide group of the GalNAc residue in the ^{1}H NMR spectra of glycolipids has been noted for tetrahexosylceramide GalNAc-β1→4-(GlcNAc-β1→3)-Gal-β1→4-Glc-β1→1-Cer. Its chemical shift is 6.93 ppm, whereas for a related trisaccharide containing no *N*-acetylglucosamine, GalNAc-β1→4-Gal-β1→4-Glc-β1→1-Cer, it is equal to 7.76 ppm.[18] Using calculations for the conformations of the terminal fragments of the oligosaccharide chains in these glycolipids, determination of the spin—spin coupling constants for the amide protons $J_{H(2),NH}$, and study of the rates of the deuterium exchange of the amide protons, this change in the chemical shift has been explained[18] by the close arrangement of the GalNAc and GlcNAc acetamide groups in the tetrasaccharide, which results in the amide proton of the GalNAc residue being cryptic and falling into the area of shielding of the GlcNAc residue by the acetamide carbonyl. In addition, it has been assumed[18] that the proton of the amide group of *N*-acetylgalactosamine and the carbonyl oxygen atom of the *N*-acetyl group in *N*-acetylglucosamine might be linked by a hydrogen bond. Apparently, in the disialoganglioside from *E. echinosoma*, the amide proton of the galactosamine is located in the area influenced by the oxygen atom of the carboxy group of the sialic acid that occupies position 3 in galactosamine; this also induces an upfield shift of the ^{1}H NMR signal of this proton. The analysis of the molecular model for the Neu5Ac-α2→3-GalNAc disaccharide fragment confirms this assumption.

To characterize the lipid moiety of the disialogangliosides from *E. echinosoma*, we used methanolysis, which gave sphingosine bases and methyl esters of fatty acids. TLC analysis of the methyl esters of fatty acids showed that the gangliosides contain only unsubstituted acids, whose composition was determined by GLC. The major components of the mixture of fatty acids are palmitic (45.8%) and stearic (29.4%) acids; smaller amounts of myristic (9.1%), pentadecanoic (8.3%), and heptadecanoic (7.4%) acids are also present. According to TLC, the sphingosine base of the gangliosides is sphingenin. Unfortunately, we were unable to determine the composition of the sphingosine bases due to the deficiency of the material.

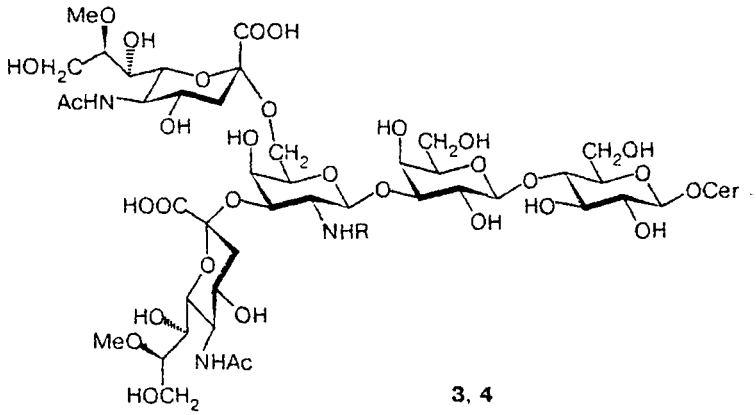
The minor sialo-containing compound isolated from the fraction of polar lipids from *E. echinosoma* upon elution from the DEAE-cellulose as monosialoganglioside was additionally purified by PTLC and obtained in a very small amount (0.7 μmol). This product was also found to contain glucose, galactose, galactosamine, and the terminal 8-*O*-methyl-*N*-acetylneuraminic acid. Partial acid hydrolysis of this compound yields mostly trihexosylceramide, and methanolysis affords sphingosine bases and methyl esters of fatty acids. The composition of the fatty acids is similar to that of the acids found in the disialogangliosides. Apparently, this minor component is a monosialoganglioside, whose structure is closely related to that of the major disialogangliosides.

The second minor sialo-containing compound obtained upon elution with a more concentrated salt solution from DEAE-cellulose contains no fatty acids and, hence, it is not a glycolipid. Its structure was not studied.

Thus, it was shown that the major components of the gangliosides from the *E. echinosoma* starfish liver are two disialogangliosides (**3**, **4**) containing 8-*O*-methyl-*N*-acetylneuraminic acid; both residues of this acid are attached to one galactosamine residue at positions 3 and 6. The oligosaccharide chains of gangliosides differ only in the *N*-acyl substituent in galactosamine, which is represented by the *N*-acetyl group in one case and by the *N*-formyl group in the other case.

To the best of our knowledge, this is the first example when an *N*-formyl derivative of an amino sugar was found in gangliosides.

When comparing the structures of the major gangliosides in the liver of the starfishes *E. echinosoma* and *E. retifera*, one can see that in both cases, they are



3, 4

R = Ac (**3**), HCO (**4**); Cer — ceramide

disialogangliosides of the ganglio series incorporating *N*-acetylneuraminic acid and the trisaccharide β-galactosaminyl-(1→3)-β-galactosyl-(1→4)-glucose as the backbone of the oligosaccharide chain, and one sialic acid residue glycosylates the galactosamine at position 3. However, substantial differences in both the oligosaccharide and the lipid moiety can also be found. Whereas in the ganglioside from *E. retifera*, the second sialic acid residue is attached to the first one by a 2→9 bond and the oligosaccharide chain is linear, the sphingosine base is represented by phytosphingosine, and about 20% of the fatty acids are α-hydroxy acids, in the gangliosides from *E. echinosoma*, the second sialic acid residue is attached to galactosamine at position 6 giving a branched structure (as in the gangliosides of starfish of the *Asterias* genus), a ganglioside with the *N*-formyl galactosamine residue is present, *N*-acetylneuraminic acid occurs as the 8-*O*-methyl derivative, the sphingosine base is represented by sphingenin, and no α-hydroxy acids were found among the fatty acids. A disialoganglioside, whose oligosaccharide chain is similar to that of the ganglioside from *E. retifera*, might be present among the minor gangliosides from *E. echinosoma*; however, its content is very low.

Thus, the main gangliosides from starfishes, even those taxonomically most closely related, can differ substantially in structure. Now it is not clear whether the structural difference between the gangliosides from one genus of starfish found here is a typical feature or an exception. To clarify this point, further studies are needed.

Experimental

Commercial samples of *N*-acetylneuraminic acid (Koch-Light, UK), *N*-glycolylneuraminic acid (Sigma, USA), DEAE-cellulose (DE-23, Whatman, UK), and neuraminidase from *Vibrio cholerae* (5 a.u. mg^{-1}, Serva, Germany) were used. Chloroform and methanol were distilled prior to use.

Analytical TLC and PTLC were carried out on silica gel 60 H (Merck, Germany). The following solvent systems were used: for gangliosides, $CHCl_3$—MeOH—H_2O (6 : 4 : 1) and $CHCl_3$—MeOH—2 *M* NH_4OH (60 : 35 : 8), visualization carried out by resorcinol[19] and orcinol[20] reagents; for neutral glycolipids, $CHCl_3$—MeOH—H_2O (62 : 32 : 7), visualization by orcinol reagent; for sphingosine bases, $CHCl_3$—MeOH—2 *M* NH_4OH (40 : 10 : 1), visualization by a 0.2% solution of ninhydrin in acetone; for methylated and trideuteriomethylated glycolipid derivatives, $CHCl_3$—MeOH (19 : 1), visualization by orcinol reagent; and for methyl esters of fatty acids, $CHCl_3$, visualization by a solution of bromothymol blue and H_2SO_4.

GLC analysis was performed on a Hewlett Packard 5890A instrument (USA) with an HP Ultra-1 capillary column (25 m × 0.2 mm, thickness of the phase layer 0.33 μm) at a nitrogen flow rate of 30 mL min^{-1} and in the 200—290 °C temperature range (10 °C min^{-1}). Neutral monosaccharides were analyzed as polyol acetates, sialic acid was analyzed as the trideuteriomethylated methyl ester of methyl ketoside, methylated and trideuteriomethylated derivatives of sugars were determined as acetates of the corresponding polyols, and fatty acids were converted into methyl esters.

GLC/MS analysis was carried out on an M-80A instrument (Hitachi, Japan, EI, 70 eV) at a temperature in the ionization chamber of 200 °C. Chromatographic separation was performed on a packed column (3×1000 mm) with the OV-1 stationary phase (on Gas Chrom Q, 100—200 mesh) at a column temperature of 280 °C with preheating from 240 to 280 °C at a rate of 10 °C min^{-1}, an injector temperature of 250 °C and a He flow rate of 25 mL min^{-1}.

^1H NMR spectra were recorded on a Bruker DRX-500 spectrometer for 0.1% solutions in DMSO-d_6 and a DMSO-d_6—D_2O mixture (98 : 2). The signals were assigned using ^1H—^1H COSY spectra and published data. Two-dimensional spectra were recorded using standard Bruker software.

The following analytical methods were used. Sialic acid was quantitatively determined with resorcinol reagent,[21,22] hexoses were determined as hexitol acetates by GLC (using inositol as the internal standard), and amino sugars were analyzed on a Biotronic LC2000 amino acid analyzer (Germany).

The *E. echinosoma* starfishes were gathered in Possjet Bay of the Sea of Japan in September. The lipid extract from the liver and the preparation of polar lipids were prepared by a previously described procedure.[11] From 1 L of liver homoge-

nate in MeOH (the tissue : MeOH ratio was 1 : 3 (v/v)), 0.674 g of polar lipids was isolated.

Column chromatography of polar lipids on DEAE-cellulose (AcO^- form) was carried out as described previously.[7,23] The minor ganglioside was eluted with a 0.025 *M* solution of $AcONH_4$ in MeOH, and the major one was eluted with a 0.1 *M* solution of $AcONH_4$. Fractions of the same composition containing resorcinol-positive compounds were combined and concentrated, and the residue was dissolved in water, dialyzed against distilled water, and lyophilized. The minor ganglioside was purified by PTLC, and the substance was eluted from silica gel with a $CHCl_3$—MeOH—H_2O (50 : 50 : 7) mixture. The major ganglioside was dissolved in a $CHCl_3$—MeOH mixture (2 : 1) and re-chromatographed on a column with DEAE-cellulose. A specimen of the major ganglioside, containing 16 μmoles of sialic acid, and a specimen of the minor ganglioside, containing 0.7 μmol of sialic acid, were isolated from 0.674 g of polar lipids.

Analyses were carried out using solutions of the gangliosides in a $CHCl_3$—MeOH—H_2O mixture (60 : 30 : 4.5) containing ~1 μmol mL^{-1} of sialic acid. Aliquots of the solutions were concentrated in test tubes at a reduced pressure and a water bath temperature not exceeding 37 °C.

Total acid hydrolysis of gangliosides (0.2—0.5 μmol of sialic acid) was performed with 2 *M* aqueous HCl (1 mL) at 100 °C for 4 h; the hydrolyzate was treated as described previously.[7] The resulting polyol acetates were analyzed by GLC. To analyze amino sugars, gangliosides (0.07 μmol of sialic acid) were hydrolyzed with 4 *M* aqueous HCl (1 mL) at 100 °C for 20 h, and the hydrolyzate was concentrated and analyzed on an amino acid analyzer.

Partial acid hydrolysis of gangliosides (0.2—1.5 μmol of sialic acid) was carried out with 0.05 *M* H_2SO_4 (4 mL) at 80 °C for 2 h. The reaction mixture was dialyzed for 24 h against distilled water. The solution inside the tube was lyophilized and analyzed by TLC, and neutral glycolipids were isolated by PTLC; their monosaccharide composition was determined after total acid hydrolysis. The outer aqueous solution was concentrated to 5—7 mL and sialic acids were isolated on a column with Dowex 2×8 (AcO^- form), as described previously.[21] The fraction of neutral sugars and an aliquot portion of the fraction of sialic acids were concentrated and the residue was subjected to total acid hydrolysis, treated under the conditions for the preparation of polyol acetates, and analyzed by GLC. No polyol acetates of neutral sugars were detected.

Methylation of dihexosylceramide, obtained upon partial acid hydrolysis of the major ganglioside, and trideuteriomethylation of the gangliosides (4 μmol of sialic acid) were carried out in DMSO (1 mL) in the presence of NaOH powder (60 mg) at ~20 °C for 1 h.[24] Chloroform (7 mL) and water (3 mL) were added to the reaction mixture; the organic layer was separated, washed with water (3×3 mL), and concentrated. The residue was dissolved in a $CHCl_3$—MeOH mixture (1 : 1, 0.5 mL), applied onto a column with Sephadex LH-20 (1.2×20 cm) washed with the same mixed solvent, and eluted with this solvent; 2-mL fractions were collected and analyzed by TLC. The fractions containing glycolipid derivatives were combined, concentrated, and dissolved in 2 mL of $CHCl_3$. An aliquot portion of the solution (0.5 mL) was concentrated and subjected to methanolysis with 0.5 *M* HCl in MeOH (1 mL) at 80 °C for 16 h. The methyl esters of fatty acids were extracted with hexane (3×1 mL) and analyzed by TLC and GLC. The methanolic solution was concentrated and sialic acid derivatives were analyzed by GLC and GLC/MS. The remaining 1.5 mL of the chloroform solution was concentrated, and the residue was subjected successively to acetolysis, hydrolysis, KBH_4 reduction, and acetylation, as described previously.[25] The partially methylated and trideuteriomethylated polyol acetates thus obtained were analyzed by GLC and GLC/MS.

Total acid methanolysis of the gangliosides (0.2—1.0 μmol of sialic acid) was carried out with 1 *M* HCl in MeOH (1 mL) at 80 °C for 16 h. An equal volume of water was added to the reaction mixture, and the methyl esters of fatty acids were extracted with hexane (3×2 mL) and analyzed by TLC and GLC. The remaining solution in aqueous methanol was made alkaline with 4 *M* KOH in 90% MeOH to pH 10, sphingosine bases were extracted with ether (3×3 mL), and the extract was washed with water to neutral reaction and analyzed by TLC.

Oxidation of trihexosylceramide with CrO_3 was carried out by a known procedure.[13] The reaction products were subjected to acetolysis, hydrolysis, subsequent reduction with KBH_4, and acetylation, and the resulting derivatives were analyzed by GLC.

Enzymatic hydrolysis of the disialogangliosides (0.5 μmol of sialic acid) by neuraminidase were carried out in a 0.05 *M* sodium acetate buffer solution, pH 5.5, as described previously.[26] Several drops of toluene were added to the reaction mixture, the mixture was dialyzed against distilled water, the diffusate was concentrated to a small volume, and sialic acids were isolated on a column with Dowex 2×8 (AcO^- form).[21] The solution of sialic acids was applied onto a column with IR-120 cation-exchanger (H^+ form) and eluted with water, the eluate was concentrated, and the sialic acids were analyzed by TLC. The content of the dialysis bag was concentrated and neutral glycolipids were analyzed by TLC.

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