Structure of gangliosides from gonads of the starfish *Evasterias retifera*

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Mono- and disialogangliosides were isolated from gonads of the starfish *Evasterias retifera*. Their structures were elucidated using chemical methods, GC-MS analysis, and enzymatic hydrolysis with neuraminidase. The monosialoganglioside has the structure 8-*O*-Me-Neu5Gc-α2→3-GalNAc-β1→3-Gal-β1→4-Glc-β1→1-Cer, while the disialoganglioside contains an additional Neu5Ac residue which glycosylates GalNAc in position 6. The lipid moieties of both gangliosides contain phytosphingosine (mainly $C_{18:0}$) and two types of fatty acids, unsubstituted (mainly $C_{16:0}$ and $C_{18:0}$) and α-hydroxy acids (mainly α-hydroxy-$C_{16:0}$).

Key words: gangliosides from gonads, starfish, *Evasterias retifera,* 8-*O*-methyl-*N*-glycolylneuraminic acid, *N*-acetylneuraminic acid.

Previously,[1,2] it was shown that the structural pattern of carbohydrate chains in gangliosides of sea urchins is common to this class of echinoderms. These chains contain sialic acids and glucose as the Sia-α2→6-Glc group. The carbohydrate chains of gangliosides of starfishes are more complicated and diverse and can differ appreciably in structure not only for representatives of different orders of Asteroidea[1—6] but also for representatives of the same order,[1—3] the same family,[1—3,7—11] and even the same genus.[12] It was of interest to find out whether the structures of gangliosides from different tissues of animals of the same species differ from one another. Tissue specificity of the set of gangliosides has been found for mammals.[13] To the best of author´s knowledge, only one such study concerning the echinoderms has been published, demonstrating that gangliosides of the body walls and hepatopancreas of the starfish *Patiria pectinifera* are characterized by a common structural pattern of the oligosaccharide chains (the same type of sialic acid and their internal position in the oligosaccharide chain and the presence of terminal arabinofuranose); however, they differ in essential structural details (the number and the ratio of monosaccharides).[14]

We continued the comparative investigation into the structures of gangliosides isolated from different tissues of one starfish species. Previously,[15,16] we demonstrated that the major ganglioside from the hepatopancreas of *Evasterias retifera* is the disialoganglioside Neu5Ac-α2→ →9-Neu5Ac-α2→3-GalNAc-β1→3-Gal-β1→ →4-Glc-β1→1-Cer, whose lipid moiety contains both normal and iso-branched phytosphingosines and unsubstituted and α-hydroxy fatty acids, the latter accounting for ~80% of the fatty acids. In this communication, we consider the structures of gangliosides from gonads of the starfish *E. retifera*.

Polar lipids obtained from the total lipid extract of the gonads of *E. retifera* as described previously[17] contained, according to TLC data, two sialoglycolipids which were separated by ion exchange chromatography on DEAE-cellulose. The less polar sialo-containing lipid eluted from the column as monosialogangliosides, while the more polar one was eluted in the disialoganglioside zone. Since the ganglioside fractions isolated by ion exchange chromatography contained not only gangliosides but also small amounts of other polar glycolipids and asterosaponins, we purified sialolipids by preparative TLC on silica gel. The structures of the isolated compounds were determined by partial and total acid hydrolysis, methylation analysis, periodate oxidation, methanolysis, oxidation with chromic anhydride, and enzymatic hydrolysis with neuraminidase.

Partial acid hydrolysis of gangliosides gave sialic acids with TLC mobilities equal to that of *N*-acetylneuraminic acid and asialoglycolipids whose TLC mobilities were the same in both cases and did not differ from the mobility of trihexosylceramide GalNAc-β1→3-Gal-β1→4-Glc-β1→ →1-Cer, which we have isolated previously by partial acid hydrolysis of the ganglioside from the hepatopancreas of this starfish species. A small amount of the product of more extensive hydrolysis, *viz.*, dihexosylceramide containing glucose and galactose, was also detected. Total acid hydrolysis of the gangliosides from gonads affords glucose, galactose, and galactosamine in the ratio 0.90 : 0.85 : 0.87 per mole of sialic acid for the less polar ganglioside and 0.46 : 0.45 : 0.43 for the more polar ganglioside. Thus, glycolipids isolated from the gonads of *E. retifera* were actually mono- and disialogangliosides

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with the same composition and the same ratio of C_6-monosaccharides.

To determine the sequence of monosaccharides in the oligosaccharide chains of gangliosides, the positions of substitution, the type of *N*-acyl substituents in amino sugars, and the presence of *O*-methyl groups in sialic acids, we used methylation and trideteriomethylation. Dihexosylceramides formed in a small amount upon partial acid hydrolysis of gangliosides were isolated by preparative TLC and methylated. Analysis of partially methylated hexitol acetates derived from methylated dihexosylceramides showed that the galactopyranose residue occupies a terminal position, while the glucopyranose residue is substituted in position 4*, *i.e.*, glucose is located at the "reducing" terminus of carbohydrate chains of gangliosides.

The mono- and disialogangliosides were trideuteriomethylated and the resulting derivatives were isolated by column chromatography on Sephadex LH-20. For analysis of sialic acid, 1/4 of the volume of solutions of trideuteriomethylated gangliosides was concentrated and subjected to methanolysis. The resulting trideuteriomethylated derivatives of the methyl esters of sialic acid methyl ketosides were analyzed using GLC and GLC-MS. Analysis of the methanolysis products obtained from the monosialoganglioside demonstrated the presence of one compound whose mass spectrum corresponded to the methyl ester of pertrideuteriomethylated 8-*O*-methyl-*N*-glycolylneuraminic acid methyl ketoside. Thus, the sialic acid of the monosialoganglioside is represented by 8-*O*-methyl-*N*-glycolylneuraminic acid located at the end of the oligosaccharide chain. Analysis of the methanolysate of pertrideuteriomethylated disialoganglioside revealed two compounds present in approximately equal amounts. One of them with a smaller retention time was identified as methyl ester of pertrideuteriomethylated *N*-acetylneuraminic acid methyl ketoside, while the other did not differ in the retention time and the mass spectrum from the component of the methanolysate of trideuteriomethylated monosialoganglioside. Hence, the disialoganglioside contains *N*-acetylneuraminic and 8-*O*-methyl-*N*-glycolylneuraminic acids, which occupy terminal positions in the oligosaccharide chain, *i.e.*, this chain is branched.

The remaining solutions of trideuteriomethylated gangliosides were concentrated and subjected to acetolysis followed by hydrolysis, reduction of the resulting sugar derivatives to alditols, acetylation, and GC-MS analysis of partially trideuteriomethylated alditol acetates. The analysis showed that the glucose residue in the monosialoganglioside is substituted in position 4, the galactose residue is substituted in position 3, and the galactosamine residue is acetylated at the amino group and is substituted in position 3. In the disialoganglioside, the glucose residue is also substituted in position 4, the galactose residue, in position 3, and the *N*-acetylgalactosamine residue is substituted in positions 3 and 6. Thus, the basic oligosaccharide chains of the gangliosides from the gonads of *E. retifera* are represented by the trisaccharide GalNAc-1→3-Gal-1→4-Glc, which is sialylated in position 3 of the amino sugar with 8-*O*-methyl-*N*-glycolylneuraminic acid in the case of the monosialoganglioside and in positions 3 and 6 with 8-*O*-methyl-*N*-glycolylneuraminic and *N*-acetylneuraminic acids in the case of the disialoganglioside.

The configurations of the glycosidic bonds of glucose, galactose, and *N*-acetylgalactosamine were determined using chromic anhydride oxidation of the acetylated derivatives of trihexosylceramides obtained upon partial acid hydrolysis of the gangliosides.[18] The fact that in both cases all sugars were destroyed upon this treatment indicated the β-configuration of their glycosidic bonds. The configurations of the bonds of sialic acids were determined by enzymatic hydrolysis. Upon treatment of the gangliosides with neuraminidase from *Vibrio cholerae*, sialic acids were split off; hence, they are linked by α-ketosidic bonds.

The lipid part of the gangliosides was analyzed using methanolysis and periodate oxidation. The methanolysis products were found to contain higher fatty acid methyl esters and sphingosine bases. Analysis of the fatty acid methyl esters by TLC showed that the gangliosides contain unsubstituted and α-hydroxy acids, the latter being present in larger amounts. The methyl esters were separated into unsubstituted and α-hydroxy acid derivatives by preparative TLC, and the components were analyzed by GLC and GC-MS; the methyl esters of hydroxy acids being acetylated beforehand. The results showed similar compositions of the fatty acids of the mono- and disialogangliosides. In both cases, α-hydroxypalmitic acid accounts for more than half of the α-hydroxy acids. Of the unsubstituted acids, palmitic and stearic acids are the major components, myristic acid consitutes about 10%, and C_{15}-, C_{17}-, C_{20}-, C_{22}-, and C_{24}-acids are present in smaller amounts (Table 1).

The sphingosine bases isolated from the methanolysates of the mono- and di-sialogangliosides produced identical TLC patterns and contained each two ninhydrin-positive compounds, one with the mobility of phytosphingosine and the other with a mobility similar to that of sphingenine. These compounds were oxidized with HIO_4 in order to obtain higher aldehydes, reduced with KBH_4 to give alcohols, and analyzed by TLC. Higher alcohols were found only in the samples obtained from sphingosine bases with the mobility of phytosphingosine. Then all samples were acetylated and analyzed by GLC

* The presence of glucose in the furanose form substituted in position 5 can be excluded, as the glucosidic bond in the glycolipid is stable under conditions of partial acid hydrolysis.

Table 1. Composition of higher fatty acids of mono- (I) and disialogangliosides (II) from gonads of the starfish *Evasterias retifera*

Acid	Unsubstituted acids[a] (%)		α-Hydroxy acids[b] (%)	
	I	II	I	II
$C_{14:0}$	8.9	9.1	9.4	11.1
$C_{15:0}$	5.3	4.6	15.6	17.7
$C_{16:0}$	45.9	54.4	55.6	59.2
$C_{17:0}$	4.7	1.8	7.5	5.0
$C_{18:0}$	23.0	18.3	11.9	7.0
$C_{20:0}$	3.6	3.3	—	—
$C_{22:0}$	5.3	4.2	—	—
$C_{24:0}$	3.3	4.3	—	—

[a] Of the unsubstituted acids.
[b] Of the α-hydroxy acids.

and GC-MS. This analysis also showed that alcohol acetates are present only in the samples obtained from compounds with the mobility of phytosphingosine. Apparently, the ninhydrin-positive compound with the sphingenine mobility is the methyl ether of phytosphingosine, which could be produced during methanolysis, as in the case of sphingenine.[19] This compound contains no vicinal hydroxy groups; therefore, it is stable against periodate oxidation. The sets of alcohols obtained from phytosphingosines of the mono- and di-sialogangliosides are virtually identical, although the contents of alcohols in the mixture are somewhat different (Table 2). The $C_{15:0}$-alcohol is the major component, which accounts for about one-third of the mixture; C_{13}-, C_{14}-, and C_{16}-alcohols are present in substantial amounts. Compounds with longer aliphatic chains (up to C_{25}) are found only in minor amounts. Thus, C_{18}-phytosphingosine is the major sphingosine base of the mono- and di-sialogangliosides, and C_{16}-, C_{17}-, and C_{19}-phytosphingosines are present in somewhat lesser amounts. It is noteworthy that the chromatograms obtained in the GC-MS analysis of alcohol acetates showed the presence of several peaks with similar retention times and identical molecular weights. Since higher fatty acid methyl esters and acetates of higher alcohols with branched aliphatic chains have somewhat smaller retention times in GLC than normal compounds, we concluded that virtually all major phytosphingosines we found represent a set of normal- and branched-chain compounds.

Table 2. Composition of phytosphingosines of gangliosides from gonads of the starfish *Evasterias retifera*

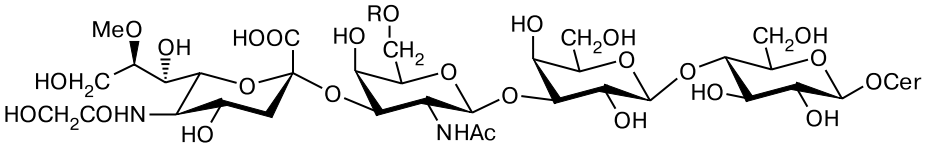
Alcohols	Corresponding phytosphingosines	Content of phytosphingosines in gangliosides (%)	
		Monosialo-ganglioside	Disialo-ganglioside
$C_{13:0}$	$C_{16:0}$	8.7	13.0
$C_{14:0}$	$C_{17:0}$	17.0	20.7
$C_{15:1}$	$C_{18:1}$	2.2	—
$C_{15:0}$	$C_{18:0}$	34.6	32.0
$C_{16:0}$	$C_{19:0}$	13.3	16.9
$C_{17:0}$	$C_{20:0}$	5.7	6.2
$C_{18:0}$	$C_{21:0}$	13.4	5.6
$C_{19:0}$	$C_{22:0}$	0.6	1.4
$C_{20:0}$	$C_{23:0}$	1.7	—
$C_{21:0}$	$C_{24:0}$	—	1.2
$C_{22:0}$	$C_{25:0}$	2.8	—
$C_{23:0}$	$C_{26:0}$	—	2.6

In order to confirm that phytosphingosines are the only sphingosine bases in the gangliosides from the *E. retifera* gonads, we performed periodate oxidation of a mixture of the gangliosides. The resulting higher aldehydes were extracted with hexane, reduced to form alcohols, acetylated, and analyzed as described above. The aqueous solution of gangliosides following oxidation was treated with KBH_4, subjected to dialysis, concentrated to a small volume, freeze-dried, and then subjected to methanolysis. No sphingosine bases were found in the methanolysis products; hence, phytosphingosines are actually the only sphingosine bases present in gangliosides.

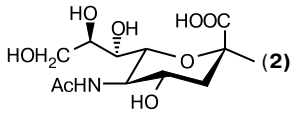
Thus, from the gonads of the starfish *E. retifera*, we isolated mono- and disialogangliosides having the same trisaccharide as the base of the olisaccharide chain, GalNAc-β1→3-Gal-β1→4-Glc, where the terminal amino sugar residue in the monosialoganglioside (**1**) is sialylated at the O(3) atom with 8-*O*-methyl-*N*-glycolylneuraminic acid, while that in in disialoganglioside (**2**) is sialylated at the O(3) and O(6) atoms with 8-*O*-methyl-*N*-glycolylneuraminic and *N*-acetylneuraminic acids (Scheme 1). The fact that the structures of the oligosaccharide chains in the asialo derivatives of gangliosides are identical and their lipid moieties have similar compositions suggests that, as in mammal gangliosides, the disialoganglioside is formed in the biosynthesis from monosialoganglioside through the addition of one more sialic acid residue. In conformity with this assumption, the 8-*O*-Me-Neu5Gc residue in the disialoganglioside from *E. retifera* should be located in position 3, while the Neu5Ac residue should be in position 6 of *N*-acetylgalactosamine.

This study showed that gangliosides isolated from the gonads of the starfish *E. retifera* and the gangliosides from hepatopancreas of this starfish species studied previously[15,16] have much in common as regards the structures of both carbohydrate and lipid moieties. They contain the same trisaccharide fragment linked to the lipid moiety, the sphingosine bases are represented by phytosphingosines, some of which are compounds with a branched aliphatic chain; fatty acids are characterized by a high content of α-hydroxy acids with α-hydroxypalmitic acid

Scheme 1



1, **2**

Cer — ceramide; R = H (**1**),  (**2**)

as the major component, while among unsubstituted acids, more than half of the acids falls to palmitic and stearic acids. However, substantial differences between the structures of the gangliosides from hepatopancreas and gonads can also be seen. This refers to the composition of the gangliosides and also to the composition and location of sialic acids. In hepatopancreas gangliosides, disialoganglioside is the major component, while the content of the monosialoganglioside does not exceed 5%. The sialic acid of the disialoganglioside is represented by *N*-acetylneuraminic acid in which both residues are linked to each other by a 2—9 bond and the disialyl fragment is attached to position 3 of *N*-acetylgalactosamine to form a linear oligosaccharide chain.[15,16] The gangliosides from gonads contain approximately equal amounts of mono- and disialogangliosides, the disialoganglioside contains 8-*O*-methyl-*N*-glycolylneuraminic and *N*-acetylneuraminic acids attached to *N*-acetylgalactosamine in positions 3 and 6 giving rise to a branched oligosaccharide chain. Analogous branched chains have been found previously in gangliosides from the starfishes of the genus *Asterias*[7—9,16] and the starfish *E. echinosoma*.[12] The results of the present study, together with the data on gangliosides from the hepatopancreas and the body walls of the starfish *Patiria pectinifera*,[14,20,21] show that different tissues of starfishes, like those of mammals, can have a peculiar set of gangliosides specific for the separate tissue.

Experimental

Materials and methods. The commercial reagents used in the study included *N*-acetylneuraminic acid (Koch-Light, UK), *N*-glycolylneuraminic acid (Sigma, USA), DEAE-cellulose DE-23 (Whatman, UK), *Vibrio cholerae* neuraminidase (5 U mg^{-1}, Serva, Germany), and yeast phytosphingosine (Serva, Germany).

Analytical and preparative TLC (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), visualization by resorcinol[22] and orcinol[23] reagents; for neutral glycolipids, $CHCl_3$—MeOH—H_2O (62 : 32 : 7), visualization by the orcinol reagent; for sphignosine bases, $CHCl_3$—MeOH—2 *M* NH_4OH (40 : 10 : 1, two runs), visualization by a 0.2% solution of ninhydrin in acetone; for methylated and trideuteriomethylated glycolipid derivatives, $CHCl_3$—MeOH (19 : 1), visualization by the orcinol reagent; for higher fatty acid methyl esters, $CHCl_3$, visualization by a solution of Bromothymol Blue and H_2SO_4; for higher alcohols and their acetates, $CHCl_3$—MeOH (24 : 1), visualization by H_2SO_4.

GLC analysis was performed on a Hewlett—Packard 5890A instrument (USA) using an Ultra-2 capillary column (25 m × 0.2 mm, 0.33-μm thick phase layer) and a nitrogen flow rate of 30 mL min^{-1} in the temperature range of 175—290 °C (10 °C min^{-1}). Neutral monosaccharides were analyzed as alditol acetates; sialic acids, as trideuteriomethylated methyl esters methyl ketosides; methylated and trideuteriomethylated sugar derivatives, as acetates of the corresponding polyols; unsaturated fatty acids, as methyl esters; α-hydroxy acids, as *O*-acetylated methyl esters; and higher alcohols, as acetates.

GC-MS analysis was carried out on a Finnigan MAT 111 instrument (Germany) coupled with a Carlo Erba Fractovap 4200 chromatograph (Italy), an HP Ultra-1 column (25 m × 0.2 mm, 0.33-μm thick phase layer) at a temperature of 150—280 °C (heating rate 5 °C min^{-1}).

The following analytical methods were used: sialic acid was quantified by the resorcinol reagent,[24,25] hexoses were determined as hexitol acetates by GLC (with inositol as the internal standard), and amino sugars were analyzed using a Biotronik LC 2000 amino acid analyzer (Germany).

Isolation of gangliosides. The starfishes *E. retifera* were collected in the Possiet Bay of the Japanese sea in September. The lipid extract of gonads and the total polar lipids were prepared by a previously described procedure.[17] From 1.2 L of a gonad homogenate (a tissue—MeOH ratio was 1 : 2 (v/v)), 1.3 g of polar lipids were isolated.

Column chromatography on DEAE-cellulose (AcO^- form) was carried out as described previously.[9,26] The less polar ganglioside was eluted with a 0.025 *M* solution of $AcONH_4$ in MeOH, while the more polar one, with a 0.1 *M* solution of $AcONH_4$ in MeOH. The fractions containing resorcinol-positive compounds were combined and concentrated, the residues were dissolved in water, dialyzed against distilled water, and freeze-dried. The resulting preparations were purified by TLC, the gangliosides being eluted from silica gel with a $CHCl_3$—MeOH—H_2O mixture (50 : 50 : 7). This gave a monosialoganglioside preparation containing ~15 μmol of sialic acid and a disialoganglioside preparation containing ~17 μmol of sialic acid.

Determination of the structure of carbohydrate chains. Total acid hydrolysis of gangliosides was carried out with 2 *M* aqueous HCl at 100 °C for 4 h and the hydrolysate was worked up as described previously.[9] For analysis of amino sugars, gangliosides were heated with 4 *M* aqueous HCl at 100 °C for 20 h, and the hydrolysates were concentrated and analyzed on the amino acid analyzer.

Partial acid hydrolysis of gangliosides was performed with 0.05 *M* H_2SO_4 at 80 °C for 2 h. The reaction mixture was dialyzed for 24 h against distilled water. The solution inside the tube was freeze-dried and analyzed by TLC. Neutral glycolipids were isolated by PTLC and analyzed after total acid hydrolysis to determine the monosaccharide composition. The outer aqueous layer was concentrated to 5—7 mL and sialic acids were isolated on a column with Dowex 2×8 (AcO^- form) as described previously.[24]

The methylation of dihexosylceramides prepared by partial acid hydrolysis of gangliosides and trideuteriomethylation of gangliosides were carried out in DMSO in the presence of the NaOH powder at 20 °C for 1.5 h using a known procedure.[27] The resulting derivatives were isolated on a column with Sephadex LH-20 as described previously[12] and dissolved in 2 mL of $CHCl_3$. Aliquots of the solutions (0.5 mL) were concentrated and subjected to methanolysis with 0.5 *M* HCl in MeOH at 80 °C for 16 h, the higher fatty acid methyl esters were extracted with hexane, the methanolic solution was concentrated, and the sialic acid derivatives were analyzed using GLC and GC-MS. The remaining 1.5 mL of the chloroform solution was concentrated and the residue was subjected successively to acetolysis, hydrolysis, reduction with KBH_4, and acetylation as described before.[28] The resulting partially methylated and trideuteriomethylated alditol acetates were analyzed by GLC and GC-MS.

Oxidation of trihexosylceramides with CrO_3 was carried out as described previously.[18] The reaction products were subjected to acetolysis followed by hydrolysis, KBH_4 reduction, and acetylation. The derivatives thus obtained were analyzed by GLC.

The enzymatic hydrolysis of the gangliosides with neuraminidase was carried out in a 0.05 *M* Na-acetate buffer, pH 5.5, as described previously.[29] Several drops of toluene were added to the reaction mixture, the mixture was dialyzed against distilled water, the outer aqueous solution was concentrated to a small volume and sialic acids were isolated on a column with Dowex 2×8 anion exchange resin (AcO^- form).[24] The eluate containing sialic acids was passed through a column with Amberlite IR-120 cation exchanger (H^+ form) using water for elution. The resulting eluate was concentrated and sialic acids were analyzed by TLC. The content of the dialysis bag was concentrated and neutral glycolipids were analyzed by TLC.

Analysis of the lipid moiety of gangliosides. Acid methanolysis of gangliosides was performed by a conc. HCl—MeOH—H_2O mixture (3 : 29 : 4) for 18 h at 80 °C. Fatty acids and their methyl esters were extracted with hexane, the solvent was evaporated, and the residue was subjected to methanolysis with 0.5 *M* methanolic HCl at 80 °C for 16 h. The resulting derivatives were analyzed by TLC and separated into methyl esters of unsubstituted and α-hydroxy acids; the latter were acetylated with acetic anhydride in pyridine (1 : 1) at 20 °C for 16 h. The derivatives of fatty acids were analyzed by GLC and GLC-MS. The aqueous methanolic solution remaining after the extraction with hexane was alkalifed with 1 *M* NaOH to pH 8 and sphingosine bases were extracted with chloroform and analyzed by TLC. The ninhydrin-positive compounds were isolated by PTLC on silica gel using a $CHCl_3$—MeOH mixture (2 : 1) as the eluent. The solutions were concentrated, 5 mL of MeOH and 1 mL of 0.2 *M* $NaIO_4$ were added to the residues, and the mixtures were magnetically stirred in the dark for 5 h at 20 °C. Higher aldehydes were extracted with hexane, hexane was evaporated, and 1 mL MeOH and 1.4 mL of a freshly prepared 10% solution of $NaBH_4$ in 0.1 *M* NaOH were added to the residue. The mixture was kept at 20 °C for 16 h, 1 *M* HCl was added to pH ≅ 6, and then H_2O (1 mL), MeOH (0.6 mL), and $CHCl_3$ (3.4 mL) were added.[30] After shaking and layer separation, the chloroform layer was separated, concentrated, and analyzed by TLC. Higher alcohols were isolated by PTLC, acetylated with acetic anhydride in pyridine (1 : 1), purified by PTLC, and analyzed by GLC and GC-MS.

The periodate oxidation of the gangliosides was carried out by treatment with a 0.02 *M* solution of $NaIO_4$ at 20 °C for 20 h; several drops of a 10% solution of ethylene glycol were added and 20 min later, higher aldehydes were extracted with hexane, hexane was evaporated, MeOH and KBH_4 were added to the residue, and the mixture was kept for 18 h at 20 °C and neutralized with 2 *M* AcOH. The higher alcohols were extracted with hexane, analyzed with TLC, acetylated as described above, purified by PTLC, and analyzed by GLC and GC-MS. The aqueous solution of gangliosides, following oxidation, was treated with KBH_4 under the same conditions, neutralized with 2 *M* AcOH, dialyzed against distilled water, concentrated to a small volume, freeze-dried, and subjected to methanolysis to detect sphingosine bases as described above.

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