Hematoside with 8-*O*-methyl-*N*-glycolylneuraminic acid from the starfish *Linckia laevigata*

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A ganglioside was isolated from the starfish *Linckia laevigata*, and its structure was elucidated by chemical methods, mass spectrometry, and enzymatic hydrolysis with neuraminidase. The ganglioside is a hematoside containing 8-*O*-methyl-*N*-glycolylneuraminic acid, 8-O-Me-Neu5Gc-α2→3-Gal-β1→4-Glc-β1→1-Cer. The lipid part of the ganglioside consists of unsubstituted fatty acids (the major component is palmitic acid) and $C_{18:1}$-*iso*-sphingenine.

Key words: ganglioside, hematoside, starfish, *Linckia laevigata*, 8-*O*-methyl-*N*-glycolylneuraminic acid.

It was shown previously that the carbohydrate chains of gangliosides from starfish are structurally diverse and, unlike gangliosides from sea urchins, have no common structural type characteristic of this class of echinoderms.[1—3] Even for starfish species closely related taxonomically (representatives of one family) the carbohydrate chains in gangliosides can differ in the sugar composition and the site of attachment of sialic acids.[4—10] The most appreciable difference, however, has been found for gangliosides from starfish belonging to different orders. In our opinion, the most pronounced difference is the position of sialic acids in the carbohydrate chain. Whereas in the gangliosides of the order *Forcipulata* starfishes, as in the gangliosides of vertebrates, these acids either occupy a terminal position or are incorporated into oligosialosyl chains,[4—10] in the gangliosides from starfishes of the orders *Phanerozonia* and *Spinulosa* studied by now, sialic acids are located inside carbohydrate chains and glycosylated at position 4 [11—19] or even occur at a branching point.[15] In the gangliosides from *Spinulosa* starfishes, the terminal position is occupied by arabinose[11—15] or galactofuranose,[16,17] while in the gangliosides from *Phanerozonia* starfishes, it is occupied by galactopyranose.[18,19]

Since this specific structural feature of the oligosaccharide chains of gangliosides has been found so far only in two species of acerate and two species of *Phanerozonia* starfishes, we proceeded with a study whose aim was to find out whether the nonterminal position of sialic acids is peculiar to gangliosides from starfishes of these orders. In this work, we present data on the structure of the ganglioside from the starfish *Linckia laevigata* of the *Phanerozonia* order.

The sample of polar lipids, prepared from the total lipid extract of the internal organs of starfishes as described previously,[20] included, according to TLC, three sialo-containing compounds, one major compound and two minor ones, together with neutral glycolipids and phospholipids. Gangliosides were isolated from the mixture by ion exchange chromatography on DEAE-cellulose. The major ganglioside was eluted from the column as a monosialoganglioside, while minor compounds were recovered as more polar lipids. The ganglioside samples were additionally purified by PTLC. This gave 2.65 μmol of the major ganglioside and 0.24 and 0.56 μmol of the minor gangliosides (in the order of elution from the column; calculated based on sialic acid).

The structure of the major ganglioside was determined by chemical methods and by mass spectrometry. The data of total acid hydrolysis imply that the ganglioside contains glucose and galactose in 1 : 1 ratio. Mild acid hydrolysis results in the elimination of sialic acid, whose quantity is equal to that of each neutral monosaccharide and whose TLC mobility is nearly equal to that of *N*-acetylneuraminic acid, and also affords a neutral glycolipid, whose TLC mobility corresponds to that of dihexosylceramide. The same dihexosylceramide is formed on treatment of the ganglioside with the neuraminidase from *Vibrio cholerae*; thus, the ketoside bond in the sialic acid has the α-configuration. The fact that both monosaccharides were destroyed on oxidation of the acetylated derivative of dihexosylceramide with chromic anhydride[21] indicated the β-configuration of their glycoside bonds.

To identify the sialic acid and the positions of linkages between the monosaccharides, the ganglioside was subjected to trideuteriomethylation. The sialic acid derivatives obtained after methanolysis of the trideuteriomethylated ganglioside were analyzed by GLC/MS. The analysis showed the presence of two compounds, the major and the minor components, in ~10 : 1 ratio. The former compound was the methyl

Translated from *Izvestiya Akademii Nauk. Seriya Khimicheskaya*, No. 1, pp. 162—165, January, 2000.

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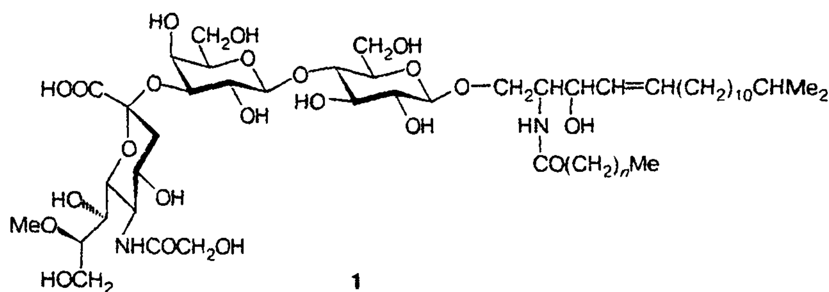

n = 12, 14, 15, 16, 20

ester of 4,7,9-tri-*O*-trideuteriomethyl-8-*O*-methyl-5-*N*-trideuteriomethyl-*N*-(*O*-trideuteriomethylglycolyl)-neuraminic acid methyl ketoside, and the latter was the methyl ester of 4,7,8,9-tetra-*O*-trideuteriomethyl-5-*N*-trideuteriomethyl-*N*-(*O*-trideuteriomethylglycolyl)-neuraminic acid methyl ketoside.[4] Thus, the ganglioside incorporates *N*-glycolylneuraminic acid, mainly as an 8-*O*-methyl derivative, which occupies the terminal position in the chain. To identify the positions of linkages in the glucose and galactose residues, the trideuteriomethylated derivative of the ganglioside was subjected to acetolysis followed by hydrolysis, reduction of the resulting sugar derivatives into polyols, acetylation, and GLC/MS analysis of the partially trideuteriomethylated polyol acetates. It was found that the glucose residue is substituted at position 4, and the galactose residue is substituted at position 3. Both monosaccharides exist in the pyranose form, as indicated by the fact that their glycoside bonds are stable under conditions of partial acid hydrolysis.

The sequence of neutral monosaccharides in the dihexosylceramide resulting from partial acid hydrolysis of the ganglioside was determined by methylation followed by analysis of the partially methylated polyol acetates. The analysis showed that glucose is substituted at position 4 and, hence, it is located in the beginning of the chain, and galactose is at the terminal position. Thus, the oligosaccharide chain of the major ganglioside has the structure of (8-*O*-methyl-*N*-glycolylneuraminyl)-$\alpha 2 \rightarrow 3$-galactosyl-$\beta 1 \rightarrow 4$-glucoside.

The lipid fragment of the ganglioside was analyzed using methanolysis. As shown by TLC, the methanolysis products contained the methyl esters of unsubstituted fatty acids and sphingosines. The composition of the acids was determined by GLC. Palmitic acid accounted for 51% of the acid mixture. In addition, stearic (19.1%), myristic (13.1%), margaric (3.7%), and behenic (4.8%) acids were found.

To determine the composition of the sphingosine bases, they were subjected to periodate oxidation, the fatty aldehydes thus formed were reduced by KBH_4, and the products were acetylated and analyzed by GLC and GLC/MS. Analysis showed the presence of one major compound, whose mass spectrum corresponded to the acetate of a monounsaturated hexadecyl alcohol having a branching at the end. Hence, the major sphingosine base of the ganglioside is $C_{18:1}$-*iso*-sphingenine.

Thus, the data obtained allow the conclusion that the major ganglioside from the starfish *L. laevigata* has the structure **1**.

The minor gangliosides also contain glucose, galactose, and 8-*O*-methyl-*N*-glycolylneuraminic acid in 1 : 1 : 1 ratio; they also form dihexosylceramide on partial acid hydrolysis and contain sphingenine as the sphingosine base; however, both materials also contain amino acids. The sphingenine : amino acid ratio in the sample prepared by elution with a 0.1 *M* solution of $AcONH_4$ amounts to 1 : 10, while that in the sample resulting from elution with a 0.25 *M* solution of $AcONH_4$ is 1 : 25. Apparently, these materials are complexes of the major ganglioside with a protein or peptides; previously we had found such complexes in some species of Echinodermata.[20,22] They were not further studied.

Thus, a ganglioside of the hematoside type, which is characteristic of the erythrocytes of vertebrates, was isolated from the *Phanerozonia* starfish *L. laevigata*. It differs from the other known hematosides by the nature of the sialic acid; a hematoside with 8-*O*-methyl-*N*-glycolylneuraminic acid was found for the first time. Unlike the gangliosides from the two other species of starfishes of this order studied previously, namely, *Luidia quinaria bispinosa*[18] and *Leptichaster anomalus*,[19] in which the sialic acid is *N*-acetylneuraminic acid or its 8-*O*-methyl derivative located in a penultimate position in the carbohydrate chain and glycosylated at position 4 by galactose, in the ganglioside from *L. laevigata*, as in the gangliosides from vertebrates, the sialic acid is located at the terminal position. Apparently, the penultimate position of the sialic acid is not a characteristic feature of the carbohydrate chain of gangliosides from starfishes of this order, or, perhaps, this species is an exception. The final solution of this problem requires further studies.

Experimental

Commercial preparations of *N*-acetylneuraminic acid (Koch-Light, UK), *N*-glycolylneuraminic acid (Sigma, USA), neuraminidase from *Vibrio cholerae* (5 a.u. per mL, Serva, Ger-

many), and DE-23 DEAE-cellulose (Whatman, UK) were used. Chloroform and methanol were distilled prior to use.

Analytical TLC and PTLC were carried out on 60 H silica gel (Merck, Germany). The solvent systems used were the same as those in the previous study.[23]

GLC and GLC/MS analyses were carried out using the same instruments and the same conditions as in the previous paper.[23]

The following analytical methods were used. Sialic acids were quantitatively determined using resorcinol reagent[24,25]; hexoses were determined as hexitol acetates by GLC (using inositol as the internal standard); sphingosine bases were quantified by colorimetry in the presence of methyl orange using the procedure reported previously[26]; the calibrating curve was constructed against phrenosin; and amino acids were analyzed on a Biotronic LC 2000 amino acid analyzer (Germany).

The *L. laevigata* starfishes were gathered in Nha Trang Bay at the Vietnam coast. The internal organs were dehydrated by acetone, dried in air, homogenized in MeOH, and extracted with 2 : 1 and 1 : 1 $CHCl_3$—MeOH mixtures, as described previously.[20] The extracts were dialyzed; the aqueous phase formed in the dialysis bag was concentrated *in vacuo* and lyophilized. From 50 g of dry tissue, 0.56 g of polar lipids was obtained.

Column chromatography of polar lipids on DEAE-cellulose (AcO^- form) was carried out as described previously.[7,27] The major ganglioside was eluted with a 0.025 *M* solution of $AcONH_4$ in MeOH; the minor sialo-containing compounds were eluted with 0.1 *M* and 0.25 *M* solutions of $AcONH_4$. All compounds were additionally purified by PTLC: the gangliosides were eluted from silica gel with a $CHCl_3$—MeOH—H_2O mixture (50 : 50 : 7). A sample of the major ganglioside, containing 2.65 µmol of sialic acid, and samples of the two minor gangliosides, containing 0.24 and 0.56 µmol of sialic acid, were obtained from 0.56 g of polar lipids.

Solutions of the gangliosides in a $CHCl_3$—MeOH—H_2O mixture (60 : 30 : 4.5) containing ~1 µmol mL^{-1} of sialic acid were used for analysis. Aliquot portions 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 the glycolipids (0.1—0.3 µmol of sialic acid) was carried out by 2 *M* aqueous HCl (1 mL) at 100 °C for 6 h. The hydrolyzate was washed with $CHCl_3$ (1 mL) to remove fatty acids, and the aqueous layer was neutralized by AV 17×8 resin (HCO_3^- form), treated with KBH_4 (10 mg) at 20°C for 12 h, applied onto a column with IR-120 cation exchanger (H^+ form), and eluted with water. The eluate was concentrated with the addition of MeOH at the end of the process, the residue was treated with a Ac_2O—Py mixture (1 : 1, 0.5 mL) at 20 °C for 16 h, and the resulting hexitol acetates were analyzed by GLC. To analyze amino acids, the gangliosides (0.05 µmol of sialic acid) were hydrolyzed with 4 *M* HCl (0.5 mL) at 100 °C for 20 h.

Partial acid hydrolysis of the gangliosides (0.1—0.5 µmol of sialic acid) was performed by 0.05 *M* H_2SO_4 (4 mL, 80 °C, 2 h). The reaction mixture was dialyzed for 1 day against distilled water, the outer aqueous solution was concentrated *in vacuo* to 5—7 mL, and sialic acids were isolated on a column with Dowex 2×8, as described previously.[24] The solution inside the dialysis bag was lyophilized and analyzed by TLC, and neutral glycolipid was isolated by PTLC.

Total acid methanolysis of the ganglioside (0.04—0.40 µmol of sialic acid) was performed by 1 *M* HCl in MeOH (1 mL) at 80 °C for 18 h. The methyl esters of fatty acids were extracted with hexane (3×1 mL) and analyzed by TLC and GLC. The remaining methanolic solution was made alkaline by adding 4 *M* KOH in 90% MeOH to pH 10, sphingosine bases were extracted with ether (3×3 mL), and the ethereal solution was washed with water to neutral reaction and analyzed by TLC.

The periodate oxidation of the resulting sphingenine was carried out in a MeOH—H_2O mixture, as described previously.[30] The aliphatic aldehydes thus formed were reduced with KBH_4 (15 mg) and acetylated with Ac_2O in Py (1 : 1, 0.5 mL) at 20 °C for 16 h, the reaction mixture was co-evaporated with toluene, and alcohol acetates were analyzed by the GLC and GLC/MS methods.

The methylation and trideuteriomethylation of the glycolipids (0.1—1.0 µmol of sialic acid) were carried out as described previously.[28] Chloroform (7 mL) and water (3 mL) were added to the reaction mixture, the mixture was stirred (without shaking), and the organic layer was separated, washed with water (3×3 mL), and evaporated to dryness. The residue was dissolved in 0.5 mL of a $CHCl_3$—MeOH mixture (1 : 1), applied onto a column with Sephadex LH-20 (2×20 cm), and eluted with the same mixed solvent; 2-mL fractions were collected and analyzed by TLC. Fractions containing glycolipid derivatives were combined and concentrated and the residue was dissolved in 2 mL of $CHCl_3$. Some 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 methanolyzate was washed with hexane (3×1 mL) and concentrated and sialic acid derivatives were analyzed by the GLC/MS method. The remaining solution was concentrated and subjected to acetolysis followed by hydrolysis, reduction of sugar derivatives with KBH_4, and acetylation, as described previously.[29] The acetates of partially methylated or trideuteriomethylated derivatives of polyols were analyzed by GLC.

Oxidation of the previously acetylated dihexosylceramide with chromic anhydride was carried out by a known procedure[21]: monosaccharides were analyzed by GLC as polyol acetates.

Enzymatic hydrolysis of the ganglioside (0.3 µmol of sialic acid) by neuraminidase (50 µL) was carried out[31] in 0.05 *M* sodium acetate buffer, pH 5.5. Several drops of toluene were added to the reaction mixture, which was then dialyzed against distilled water, and the diffusate and the content of the dialysis bag were concentrated separately and analyzed by TLC.

The author is grateful to V. S. Levin for gathering and classification of starfishes and V. L. Sadovskaya for performing GLC/MS analysis.

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Received June 30, 1999;
in revised form September 16, 1999