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Note

Identification of a new ganglioside from the starfish *Asterias rubens* *

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Gangliosides are sialic acid-containing glycosphingolipids that are present on vertebrate cell membranes, where they are responsible for a variety of biological functions[1]. They have also been discovered in a number of Echinodermata with often unusual sialic acids and carbohydrate structures[2–15].

Since the starfish *Asterias rubens* has preponderantly *N*-glycoloyl-8-*O*-methylneuraminic acid (Neu5Gc8Me) as sialic acid[16], it was interesting to study the biosynthesis of this sugar, leading to the discovery of a sialate 8-*O*-methyltransferase[17] and a *N*-acetylneuraminic acid monooxygenase[18] in this starfish species. In addition, we were interested in the type of glycoconjugates to which these sialic acids are linked. Therefore, we isolated and characterized gangliosides from *Asterias rubens* to verify the already published structure[2] and look for the occurrence of additional gangliosides whose structures have not yet been reported.

EXPERIMENTAL

Materials. – Starfish were from the Baltic Sea and obtained from the Institut für Meereskunde, University of Kiel. The animals (312 g) were homogenized for 5 min with a Waring Blendor, and total glycolipids were extracted by ultrasonication for 30 min at room temperature with 2:1, followed by 1:2 $CHCl_3$–MeOH (2 × 300 mL, each). The extracts were filtered, the residue extracted with 15:30:8 $CHCl_3$–MeOH–H_2O (2 × 500 mL), and filtered. The combined filtrates were concentrated by rotary evaporation, dialyzed against water (3 × 5 L, 16 h, 4°) and lyophilized.

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This crude glycolipid material was resuspended in 2:1 $CHCl_3$–MeOH (20 mL) and the soluble part (1.1 g, corresponding to 4 mg of sialic acid) separated for further analysis. It was applied to a 56 × 2.4 cm column of DEAE–Sephadex A-25, and the neutral glycolipids were eluted with 2:1 $CHCl_3$–MeOH (1 L), followed by MeOH (1 L). Gangliosides were obtained by elution with a linear gradient (0–1.0 M, 500 mL, each) of ammonium acetate in MeOH. Fractions (10 mL) were collected and tested for the presence of sialic acids by spotting on silica gel TLC plates, followed by spraying with the orcinol–Fe^{3+}–HCl spray reagent[19]. Sialic acid-containing fractions were pooled, concentrated, dialyzed as described above, and lyophilized. Individual gangliosides were isolated by column (50 × 2.5 cm) chromatography on silica gel using 60:40:9 $CHCl_3$–MeOH–H_2O containing 0.02% $CaCl_2 \cdot 2H_2O$ and 10:67:23 MeCN–2-propanol–50 mM KCl as solvent systems. After pooling and concentration of the fractions with individual compounds, the purified gangliosides were desalted over a column (30 × 1.1 cm) with Sephadex LH-20 with 1:1 $CHCl_3$–MeOH as solvent.

Methods. – TLC analysis of gangliosides was carried out on 0.2 mm silica gel on plastic sheets or on silica gel HPTLC plates (both from Merck, Darmstadt, Germany) using the solvent systems described above for column chromatography on silica gel and, in addition, 60:40:9 $CHCl_3$–MeOH–2.5 M NH_3. Sialic acid-containing compounds were detected by spraying with the orcinol–Fe^{3+}–HCl spray reagent.

Sialic acids were released from the individual gangliosides by acid hydrolysis followed by purification[19] and identification as per(trimethylsilyl)ated derivatives by GLC–MS[20]. Sugar analysis was performed as described earlier[21].

Permethylation of gangliosides was done according to Ciucanu and Kerek[22]. Partially methylated alditol acetates were prepared from the permethylated gangliosides by use of standard conditions for hydrolysis[23] and $NaBD_4$ for reduction. GLC–MS was performed on a Hewlett–Packard HSD 5970 instrument by use of a 15-m WCOT column, filled with SE 54 (Hewlett–Packard, Waldbronn, Germany), in a linear temperature program from 100 to 300° with an increase of 10°/min[23]. Partially methylated alditol acetates were identified by selected ion monitoring (SIM) as described before[24]. FAB-mass spectra of permethylated gangliosides were acquired as previously described[25,26] using a VG ZAB HF instrument (VG Analytical, Manchester, UK) at 7 kV acceleration voltage and thioglycerol as matrix. No background substraction was performed. The parts of the FAB-mass spectra showing only chemical noise or poly(ethylene) glycol repeat ions (44 amu apart) were omitted in the Figures.

RESULTS AND DISCUSSION

The extraction of total starfish *Asterias rubens* by different mixtures of chloroform–methanol–water yielded, besides of the desired lipids, also pigments and sialic acid-containing material that did not migrate in the different TLC systems

used for ganglioside analysis and was therefore, assumed to be derived from glycoprotein.

Separation of the ganglioside fraction by ammonium acetate gradient elution from Sephadex A-25 revealed the presence of two major compounds in a ratio of ~ 1 : 10, the minor one corresponding to a monosialoganglioside and the major one being eluted as a disialoganglioside. Both compounds were purified to homogeneity by column chromatography on sillica gel, as estimated with TLC.

The nature of the sialic acid residues in both gangliosides was determined, after acid hydrolysis, by GLC–MS of the per(trimethylsilyl)ated derivative, indicating Neu5Gc8Me as the only sialic acid in both compounds on the basis of the well-established fragmentation scheme[20]. Sugar analysis indicated the additional presented of glucose, galactose, and galactosamine in an equimolar ratio.

The carbohydrate structure of the gangliosides was elucidated by positive- and negative-ion FAB-MS. In the positive-ion spectrum of the permethylated monosialoganglioside (Fig. 1, Scheme 1), the presence of a terminal Neu5Gc group was shown by ions at m/z 406 and 374, the latter resulting from loss of methanol. The carbohydrate sequence was deduced from the ions at m/z 651 → 228 and 855, derived from the terminal disaccharide, Neu5Gc → $HexNAc^+$, and trisaccharide unit, Neu5Gc → HexNAc → Hex^+, respectively. Daughter ions at m/z 1067 and 1051 may stem from the tetrasaccharide sequence, [Neu5Gc → HexNAc → Hex_2 → OH] + Na^+ after elimination of methanol and a glycosidic oxygen atom.

Neu5Gc-(2-	→ 3)-GalNAc-(1-	→ 3)-Gal-(1-	→ 4)-Glc-	→ Cer
m/z 406	m/z 651	m/z 855		m/z 664
↓	↓			
m/z 374	m/z 228			
			$M + Na^+ = 1762$	

Scheme 1. Fragmentation pattern for the major ceramide component of the permethylated monosialoganglioside **1**.

The ion occurring in low intensity at m/z 1154 can be explained by a cleavage in the ceramide portion to yield, Neu5Gc → HexNAc → Hex → Hex → O → CH_2 → $CH(NH_2)$ → CH_2^+. The heterogeneity of the ceramide part was shown by a series of Cer^+ ions at m/z 664, 720 and 734, representing C_{18} phytosphingosine linked to a C_{22}, C_{26}, or C_{27} saturated fatty acid, respectively. The corresponding molecular ions MNa^+ were found at m/z 1762, 1818, and 1832, accompanied by some peaks of undermethylated products 14 amu apart. Alternatively, the Cer^+ ions could be assigned to C_{18} sphingosine (m/z 720, 734) or sphinganine (m/z 708), linked to C_{24} and C_{25} dihydroxy fatty acids. The absence of the characteristic[26] sphingosine ion $[M - acyl + 2H]^+$ favors the first proposal. The origin of the ion at m/z 893 remains unclear. The negative-ion FAB-mass spectra were of very low intensity and did not allow definitive assignments.

Methylation analysis (Table I) showed the presence of a 3-*O*-substituted GalNAc as the *N*-acetylhexosamine unit, and of a 3-*O*-substituted galactose and a 4-*O*-sub-

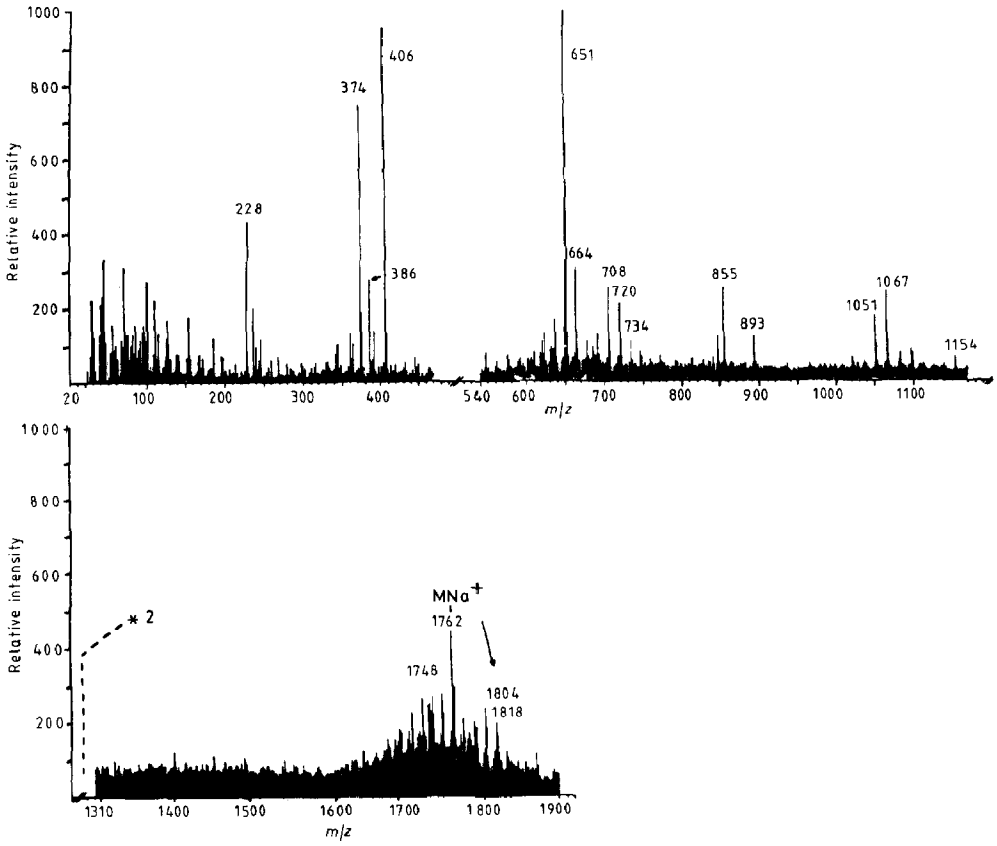


Fig. 1. Positive-ion FAB-MS of the permethylated monosialoganglioside from the starfish *Asterias rubens*.

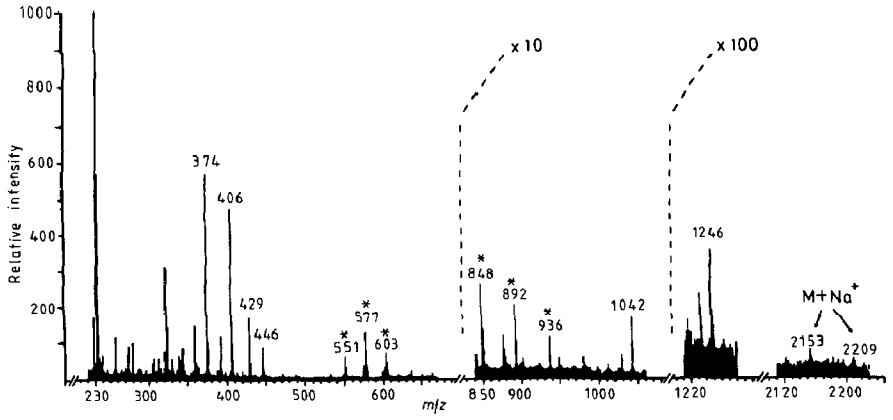


Fig. 2. Positive ion FAB-MS of the permethylated disialoganglioside from the starfish *Asterias rubens*. The ions marked with an asterisk are derived from impurities.

TABLE I

Methylation analysis of [1-^{2}H]alditols obtained from the monosialoganglioside of *Asterias rubens* in SIM

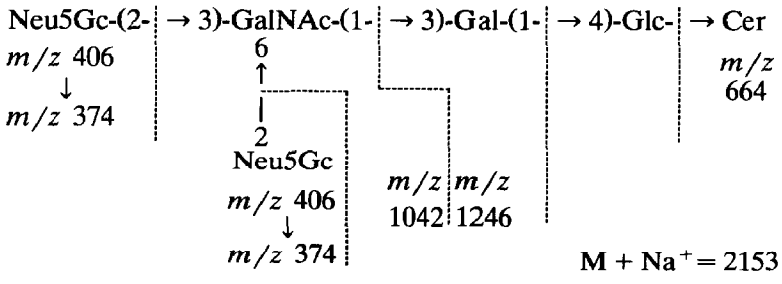
Partially methylated alditol acetate	Retention time on SE 54 (min)	Characteristic ions (m/z)
1,3,5Ac$_3$4,6Me$_2$ GalN(AcMe)	14.8	159 → 117, 161, 275, 308
1,3,5Ac$_3$2,4,6Me$_3$ Gal	10.7	118, 161, 234
1,4,5Ac$_3$2,3,6Me$_3$Glc	10.6	162, 233, 234

stituted glucose as the two hexose units, supporting structure **1** having a GalNAc residue linked (1 → 3) to the Gal residue. Thus, the carbohydrate backbone is different from the β-D-Gal*p*Nac-(1 → 4)-D-Gal*p* type of the *ganglio* series.

Neu5Gc8Me-(2 → 3)-D-Gal*p*NAc-(1 → 3)-D-Gal*p*-(1 → 4)-D-Glc*p* → Cer

1

In the permethylated disialoganglioside of *Asterias rubens*, *N*-glycoloylneuraminic acid was again found as the only sialic acid, as deduced from the ions at m/z 406 and 374 in the positive-ion FAB-MS (Fig. 2, Scheme 2). Ions identifying the carbohydrate sequence were found at m/z 1042 and 1246 stemming from the (Neu5Gc)$_2$ → HexNAc$^+$ and (Neu5Gc)$_2$ → HexNAc → Hex$^+$ structures, respectively. The absence of an ion at m/z 797 that is characteristic for a Neu5Gc → Neu5Gc-disialosyl structure suggested that both Neu5Gc groups are linked to the subterminal HexNAc unit. This substitution pattern became evident from the methylation analysis showing a 3,6-disubstituted Gal*p*NAc residue. Several molecular ions (MNa$^+$) were found, *e.g.* at m/z 2153 and 2209 with corresponding Cer$^+$ ions at m/z 664 and 720, however of low intensity and accompanied by impurities. Thus, the structure **2** of the disialoganglioside is identical with that of



Scheme 2. Fragmentation pattern for the major ceramide component of the permethylated disialoganglioside **2**.

the gangliosides isolated from *Asterias amurensis*[3] and *Asterias rubens*[2] already published

Neu5Gc8Me-(2 → 3)-D-Gal*p*NAc-(1 → 3)-D-Gal*p*-(1 → 4)-D-Glc*p* → Cer
6
↑
2
Neu5Gc8Me

2

The monosialogunglioside (structure **1**), however, has not yet been described.

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