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Isolation and identification of novel sulfated and nonsulfated oligosialyl glycosphingolipids from sea urchin sperm*

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Novel sulfated and nonsulfated oligosialylglycosphingolipids were isolated from sperm of the sea urchin, *Hemicentrotus pulcherrimus*, and their structures were established as follows:

$$\pm HSO_3 \rightarrow 8Neu5Ac\alpha2 \rightarrow (8Neu5Ac\alpha2 \rightarrow)_n \rightarrow 6Glc\beta1 \rightarrow 1'Cer, \text{ where } n = 0, 1, 2, \text{ and } 3.$$

This provides the first evidence for the natural occurrence of a tetrasialic acid structure in glycosphingolipids. The finding of sulfated oligosialyl chains is especially noteworthy in that the sulfate group exclusively resides on the C-8 of the nonreducing terminal residues of oligo/polysialyl chains and that sulfation appears to be a termination signal for elongation of oligosialyl chains. Sulfation at the nonreducing terminal Neu5Ac residues of oligosialyl chains was also found to facilitate the formation of an inter-residue lactone between the carboxyl group at the nonreducing terminal sulfated Neu5Ac and the hydroxyl group at C-9 of the penultimate Neu5Ac residue. The long chain base was 4-hydroxysphinganine (t18:0) and the major fatty acid species were identified as C20:1, C21:1, and C22:1.

Keywords: oligosialoglycosphingolipid, sulfated sialic acid, sea urchin sperm

Abbreviations: C:M, chloroform:methanol; FAB-MS, fast atom bombardment mass spectrometry; GLC, gas-liquid chromatography; GSL, glycosphingolipid; HPTLC, high performance thin-layer chromatography; Neu5Ac8HSO_3 or $HSO_3 \rightarrow$ Neu5Ac, 5-*N*-acetyl-8-*O*-sulforylneuraminic acid; Neu5Gc9HSO_3, 5-*N*-glycolyl-9-*O*-sulforylneuraminic acid; NMR, nuclear magnetic resonance; PB, sodium phosphate buffer, pH 7.2; Sia, sialic acid; $HSO_3 \rightarrow$ Sia, sulfated sialic acid; polySia, polysialic acid; TFA, trifluoroacetic acid.

Introduction

Interestingly, while sialic acid-rich glycoconjugates had been known for several decades to be constituents of the jelly coat of sea urchin eggs, no structural characterization had been made until we were successful in demonstrating the occurrence of a novel type of polysialic acid (polySia)[1] chain [1]. Our interest in searching for sialoglycoconjugates in sea urchin sperm stemmed from previous discoveries of polySia chains and KDN as unusual components of the glycoproteins and glycosphingolipids from the gamete cells of rainbow trout [2–6]. A few sialic

*Since the dividing line between oligo- and polysialic acids is not rigidly defined, we propose here that those containing more than penta-sialic acid chains can be referred to as the polysialic acid group. The rationale for this is that $\alpha2 \rightarrow$ 8-linked pentasialic acid is the minimum chain length to exhibit the conformational property of polysialic acid [cf Michon, F, Brisson, J-R, Jennings, H (1987) *Biochemistry* **26**: 8399–405] and to act as a substrate for the endosialidase, Endo-N [Hallenbeck, PC, Vimr, ER, Yu, F, Bassler, B, Troy, FA (1987) *J Biol Chem* **262**: 3553–61].

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acid-containing glycosphingolipids (sialyl GSL) have been reported to occur in sea urchin sperm [7–9]. The objective of the present study was the isolation and characterization of minor sialyl GSLs present in sperm. To secure sufficient material for structural analysis 1000 male sea urchins were collected to search for such sialyl GSLs.

We report the isolation and structural elucidation of both sulfated and nonsulfated sialyl GSLs from sea urchin sperm and their unique structural features are discussed in the context of the facile formation of inter-residue lactone formation.

The finding of the presence of sulfated Neu5Ac as the nonreducing terminal residue of oligoSia chains supports our contention that incorporation of sulfated Sia or KDN residues into oligo/polySia chains would terminate elongation of oligo/polysialylation as previously revealed in polysialoglycoproteins [3, 10, 11].

Materials and methods

Sea urchin sperm

Sea urchin, *Hemicentrotus pulcherrimus*, in the breeding season, were collected at the sea around Tsushima, and sperm were collected by intracoelomic introduction of 0.5 M KCl, and stored at −30 °C until use.

Preparation of sialyl GSLs

During all isolation procedures the pH was never below 7. The acetone powder was prepared from a total of 200 g sea urchin sperm, and extracted with 400 ml of chloroform:methanol (C:M) (2:1 by vol) and then with 400 ml of C:M (1:2 by vol) at room temperature for 2 h [4]. The combined extract was concentrated to dryness by a rotary evaporator at 37 °C and subjected to the Folch partitioning procedure [12]. In the following partitioning procedures, 5–10 mM sodium phosphate buffer, pH 7.2 (PB) was used instead of water. The dried extract was dissolved in 200 ml of C:M (2:1 by vol) and partitioned by addition of 200 ml of 0.88% KCl-PB. The organic lower phase was washed once with C:M:0.88% KCl-PB (3:48:47 by vol) and then with C:M:PB (3:48:47 by vol). The combined aqueous phase was concentrated, dialysed against distilled water at 4 °C for 4 days and lyophilized. The glycolipid fraction was dissolved in 10 ml of C:M:water (30:60:8 by vol) and applied to a DEAE-Toyopearl 650S column (1.7 x 31 cm). After washing with three column-volumes of the same solvent to remove neutral glycolipids, the column was eluted with a linear gradient of 1 l of 0–0.1 M ammonium acetate and 5 ml fractions were collected. The elution profile was monitored by the HPTLC as described below. Each fraction was desalted by Sephadex G-50 column chromatography (1.3 x 32 cm; eluted with water) and analysed.

The Hp-s1 (for the abbreviation and the origin see Results and Fig. 1) was further subjected to DEAE-Sephadex A-25 chromatography (1.0 x 11.5 cm; equilibrated with C:M:water (30:60:8 by vol)) to remove contaminated neutral materials. The column was eluted first with C:M:water (30:60:8 by vol) and then with a linear gradient of 400 ml of 0–0.2 M ammonium acetate in the same solution. Fractions of 1.25 ml were collected and fractions 115–159 were pooled.

HPTLC analysis

Analytical HPTLC was performed on silica gel 60 plates (No. 5641; Merck, Germany), using the following solvent systems: solvent A, chloroform:methanol:0.2% $CaCl_2$ (55:45:10 by vol); solvent B, 1-propanol:25% aqueous ammonia:water (6:1:2.5 by vol). The sialyl GSL bands were visualized by spraying the resorcinol reagent [13] and heating at 80 °C for 30 min [6], or by spraying 10% sulfuric acid-ethanol followed by heating at 120 °C for 30 min [4].

Sialidase digestion

About 1 μg each of sialyl GSL fractions Hp-s1, -s2, -s4, and -s6 (see Fig. 1) were digested at 37 °C for 1 to 24 h with 12.5 mU of *Arthrobacter ureafaciens* sialidase (Nacalai Co., Japan) in 20 μl of 20 mM sodium acetate buffer, pH 5.5. The digests were lyophilized, dissolved in C:M (1:2 by vol), and analysed by HPTLC as described above.

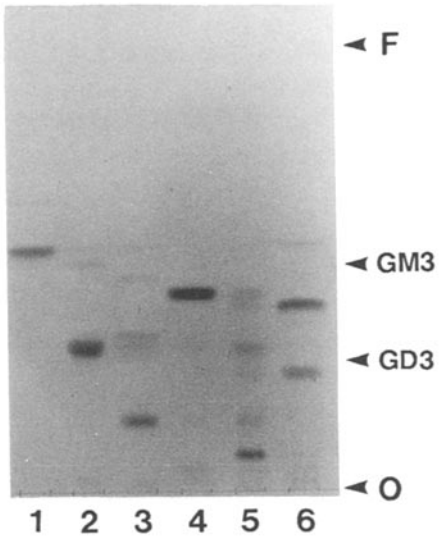

Figure 1. HPTLC of Hp-s1 to Hp-s6. Each fraction containing 1 μg as Neu5Ac was loaded on the plate, developed in Solvent A, and visualized by the resorcinol method. Lane 1, Hp-s1; lane 2, Hp-s2; lane 3, Hp-s3; lane 4, Hp-s4; lane 5, Hp-s5; lane 6, Hp-s6. The positions where GM3 and GD3 migrated are also shown on the side. O, origin; F, front.

Carbohydrate composition analysis

The monosaccharide composition of each sialyl GSL fraction was determined by GLC as previously described [14]. *N*-Acylneuraminic acid was identified by the mild methanolysis/GLC method [15].

Fatty acid analysis and identification of sphingosine base

Fatty acid composition was determined by the GLC method [4]. Sialyl GSLs (about 10 μg) were methanolyzed in 0.45 N methanolic HCl at 100 °C for 3 h. The fatty acid methyl esters formed under these conditions were extracted with hexane and analysed by GLC on a 2% OV-101 Gas Chrom Q glass column (3 mm x 2 m) with a temperature gradient from 170 to 230 °C at 2.5 °C per min using a Shimadzu GC-14A gas chromatograph.

Sulfate ion analysis

Sulfate ion was identified and quantitated by HPLC analysis of acid hydrolysate of a given sample. A sample (5–10 nmol) was hydrolysed in 6 N HCl at 110 °C for 24 h and applied to a TSK-gel IC-Anion PW column, which was pre-equilibrated and eluted with 0.5 mM sodium phthalate in solution containing 0.036% boric acid, 0.05% sodium tetraborate, 0.2% (w/v) sodium gluconate, 12% acetonitrile, and 3% (v/v) 1-butanol at the flow rate of 1.2 ml min^{-1}. Elution was monitored and quantitated by measuring the absorbance at 265 nm using sodium sulfate solution as standard.

Methylation analysis

Permethylation of intact sialyl GSLs was carried out according to the method of Anumula and Tayler [16]. Dry samples (20 μg each) were dissolved in dimethylsulfoxide (0.2 ml) by sonication for 2 min and allowed to stand at room temperature for 30 min. Then 0.2 ml of NaOH-dimethylsulfoxide and 0.1 ml of methyliodide were added, followed by sonication for 7 min. The reaction mixture was then diluted with 2 ml of distilled water and the permethylated oligosaccharides were extracted by adding the same volume of chloroform. The upper aqueous phase was discarded. The organic phase was washed twice with the same volume of water, and dried at 40 °C under nitrogen gas stream. The permethylated samples were hydrolysed in an N_2 atmosphere at 100 °C for 16 h in a solution of 0.5 ml of formic acid, 0.1 ml of water, and 0.05 ml of trifluoroacetic acid. After evaporation, 0.5 ml of freshly prepared 5% pyridine in 50% aqueous acetonitrile were added and evaporated to dryness. Partially methylated monosaccharides were reduced with 0.2 ml of 5 mg ml^{-1} $NaBH_4$ in 30% methanol containing 0.03 N NaOH at 37 °C for 4 h with occasional mixing. After neutralization with 20 μl of glacial acetic acid, the samples were dried under reduced pressure. Boric acid was removed by evaporating five times with 1 ml of 0.1% methanolic HCl. Partially methylated alditols were acetylated for 4 h in 0.15 ml of acetonitrile containing 5 mg ml^{-1} of 4-*N*,*N*′-dimethylaminopyridine, 0.05 ml of pyridine and 0.15 ml of acetic anhydride at room temperature. After the reaction was stopped by adding 2 ml of water, alditols were extracted with an equal volume of chloroform, washed three times with water, dried, and analysed by GLC using a capillary column (CBJ15, 0.32 mm x 30 m; Shimadzu) with a temperature gradient of from 180 to 260 °C at 2 °C per min on a Shimadzu GC-14A gas chromatograph [6].

Linkage analysis of oligosialic acids was carried out by the method of Inoue *et al.* [17]. The methylated samples were methanolyzed in 0.5 N methanolic HCl at 80 °C for 16 h. After evaporation, the residue was acetylated with 0.3 ml of pyridine:acetic anhydride (1:1 by vol) at 80 °C for 30 min, and dried under a nitrogen gas stream. The acetylated samples were subjected to five repeats of addition of 1 ml of toluene followed by drying under a nitrogen gas stream, and analysed by GLC using a capillary column as described above with a temperature gradient from 190 to 260 °C at 4 °C per min.

^{1}H-NMR spectroscopy

Sialyl GSL samples were separately treated twice with 99.8% D_2O and dissolved in dimethyl-sulfoxide-d_6:D_2O (98:2 by vol). One dimensional spectra were measured at 37 °C at 400 MHz with a JEOL JNM-GX400 spectrometer. Two dimensional total correlated spectroscopy (TOCSY) spectra were measured at 500 MHz on a Bruker AMX-500 spectrometer. The MLEV-17 mixing sequence of 100 ms was used. Chemical shifts were referenced to internally added tetramethylsilane and expressed in ppm.

FAB-MS

FAB-MS were measured with a VG Analytical ZAB-2SE 2FPD mass spectrometer fitted with a caesium ion gun operated at 30 and 20 kV in positive and negative modes, respectively. Data acquisition and processing were performed using the VG Analytical Opus soft ware. Samples were dissolved in 5% aqueous acetic acid (native) or methanol (derivatives). For FAB-MS, 10–20 μg samples were permethylated, or deuteroacetylated as previously described [6, 18].

Lactonization experiment

Lactonization was carried out according to the method of Endo *et al.* [19]. About 5 μg each of sialyl GSLs were dissolved in glacial acetic acid and left at 25 °C for 1 to 24 h. The reaction mixtures were neutralized with 10 N NaOH and dialysed against water at 4 °C overnight. After lyophilization, samples were dissolved in C:M (1:2 by vol) and analysed by HPTLC as described above. Delactonization was performed by treatment with 50 μl of 0.5 N NaOH for 10 min.

Results

ISOLATION AND PURIFICATION OF SIALYL GSLS FROM SEA URCHIN SPERM

The crude glycolipid fraction (8.6 mg as Neu5Ac) was obtained from sperm (200 g) from 1000 sea urchins by the procedures described under Materials and methods, and was then subjected to DEAE-Toyopearl 650S chromatography. The chromatographic profile was monitored by HPTLC followed by visualization of sialyl GSLs with the resorcinol reagent. Six fractions containing sialic acid were separated and labelled Hp-s1, eluting at 0.019–0.021 M ammonium acetate (fractions 36–43); Hp-s2, at 0.027–0.039 M (fractions 53–75); Hp-s3, at 0.048–0.054 M (fractions 93–104); Hp-s4, at 0.055–0.063 M (fractions 107–122); Hp-s5, at 0.067–0.074 M (fractions 129–143); Hp-s6, at 0.077–0.092 M (fractions 149–177). These fractions were examined for purity by HPTLC (Fig. 1).

When the plate was visualized by heating after spraying with 10% sulfuric acid-ethanol, the same chromatographic pattern as Fig. 1 was obtained except for Hp-s1, where a few new resorcinol-negative bands appeared (data not shown). The Hp-s1 fraction was further purified to homogeneity by chromatography on a column of DEAE-Sephadex A-25 column. Each of the fractions Hp-s1 to Hp-s5 consisted of one major component together with a few minor ones, and thus was considered to be homogeneous enough for chemical characterization. Hp-s2 was the most abundant among *H. pulcherrimus* sperm sialyl GSLs.

Two major components were present in Hp-s6 (Fig. 1). However, there was only a single band on HPTLC when eluants of DEAE-Toyopearl chromatography were examined (data not shown). The slowly migrating component showed a R_f value identical to that of the original one, and the fast moving material presumably appeared by lactonization during the procedures of desalting and concentration of fractions after DEAE-Toyopearl chromatography (see below).

STRUCTURAL ELUCIDATION OF HP-S1 THROUGH HP-S6

(a) Hp-s1

One residue each of Glc and Neu5Ac was found (Table 1), and methylation analysis revealed the presence of 1 mol each of 6-*O*-substituted Glc and terminal Neu5Ac, thus indicating that Hp-s1 had a disaccharide sequence of Neu5Ac2 → 6Glc. Digestion of Hp-s1 with sialidase in the presence of 0.1% sodium cholate readily gave Neu5Ac and Glc → Cer as examined by HPTLC. Release of Neu5Ac with a concomitant decrease of Hp-s1 was shown in Fig. 2. The presence in Hp-s1 of a Neu5Ac residue which resisted the action of sialidase was noted and this residue could only be partly released provided the Hp-s1 was incubated with the sialidase at 37 °C for 6 h in the absence of sodium cholate (data not shown). HPTLC analysis of acid hydrolysate (0.1 N TFA, 70 °C, 30 min) showed the liberation of Neu5Ac (Fig. 3) and the formation of Glc → Cer as visualized by 10% sulfuric acid-ethanol method (data not shown).

Fatty acid analysis on GLC showed that C20:1, C21:1, and C22:1 were predominant and present in 30 mol%, 20 mol% and 30 mol% of total fatty acid and that any of other fatty acid components detected (C16:0, C16:1, C18:0, C18:1, C19:1, and C20:0) was less than 5 mol%.

The FAB-MS experiments (Fig. 4a, Table 2) showed that Hp-s1 was a disaccharide, Neu5Ac → Hex, linked to a mixture of ceramides consisting of 4-hydroxysphinganine (t18:0) and each of three major fatty acid species (C20:1, C21:1, and C22:1) and two minor ones (C18:1 and C19:1) in good accord with the data described above. Susceptibility to the sialidase showed that the anomeric configuration of the Neu5Ac residue was α, and the coupling constant, $J_{1,2} = 8.4$ Hz (δ

Table 1. Yield and chemical composition of sialoglycosphingolipids from sea urchin sperm.

Fraction	*Yield*[a] *(mg)*	*Glc*	*Neu5Ac*	SO_4^{2-}
Hp-s1	0.54	1.0[b]	0.9	ND[c]
Hp-s2	4.8	1.0	2.1	ND
Hp-s3	0.39	1.0	2.4	ND
Hp-s4	0.69	1.0	1.2	0.90
Hp-s5	0.021	1.0	2.3	ND
Hp-s6	1.5	1.0	1.9	1.0

[a]Yield is expressed as the amount of sialic acid in each fraction as determined by the resorcinol method [13].
[b]Value are molar ratios relative to Glc set to 1.0 mol.
[c]ND, trace or not detected.

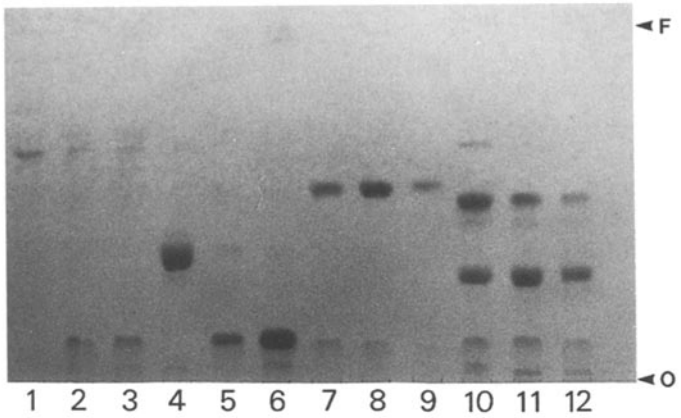

Figure 2. HPTLC analysis of sialidase digests of Hp-s1, Hp-s2, Hp-s4, and Hp-s6. Each of these fractions was incubated in 25 mM sodium acetate buffer, pH 5.5, containing 0.1% sodium cholate at 37 °C for 1 h with (lanes 2, 5, 8, and 11) and for 2 h with (lanes 3, 6, 9, and 12) or for 2 h without (lanes 1, 4, 7, and 10) *A. ureafaciens* sialidase. Lanes 1, 2, and 3, Hp-s1; lanes 4, 5, and 6, Hp-s2; lanes 7, 8, and 9, Hp-s4; lanes 10, 11, and 12, Hp-s6. O, origin; F, front.

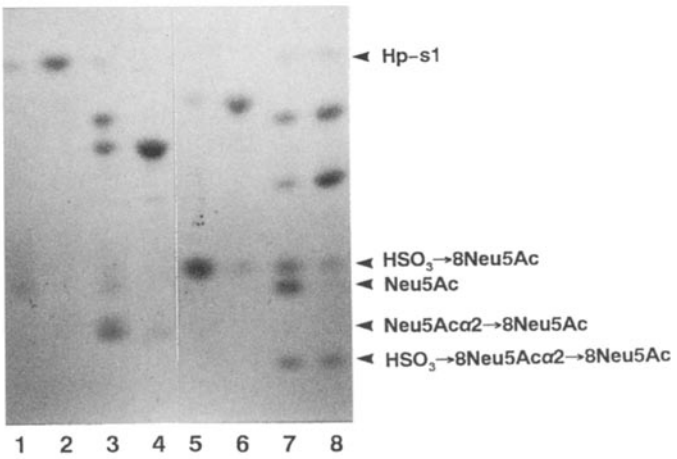

Figure 3. HPTLC of acid hydrolysates of sialyl GSLs. Hp-s1 (lanes 1 and 2), Hp-s2 (lanes 3 and 4), Hp-s4 (lanes 5 and 6), and Hp-s6 (lanes 7 and 8) were incubated with 0.1 N TFA at 70 °C for 0 min (lanes 2, 4, 6, and 8) and 30 min (lanes 1, 3, 5, and 7), and spotted on a 0.2 mm thick silica gel TLC plastic plate (20 cm long). The plate was developed in solvent B for 12 h and visualized by the resorcinol reagent. The region of 4–14 cm from the origin in the TLC plate is shown. The positions of Neu5Ac, Neu5Acα2 → 8Neu5Ac, Neu5Ac8HSO_3, Neu5Ac8HSO_3α2 → 8Neu5Ac, and Hp-s1 are indicated. Two major bands observed in lane 8 are corresponding to the fast- and slow-moving components of Hp-s6 and the former is a lactone form of the latter (see also Fig. 7).

1.14 ppm), observed for the H-1 proton of Glc indicated that the Glc was β-linked. The structure of Hp-s1 was thus established as Neu5Acα2 → 6Glcβ1 → Cer.

(b) Hp-s2

Hp-s2 contained one Glc and two Neu5Ac residues (Table 1). Methylation analysis showed the presence of 6-*O*-substituted Glc and 1 mol each of unsubstituted Neu5Ac and 8-*O*-substituted Neu5Ac. As shown in Fig. 2, sialidase digestion in the presence of 0.1% sodium cholate resulted in complete removal of Neu5Ac residues to yield Glc → Cer. Acid hydrolysis in 0.1 N TFA at 70 °C for 30 min gave rise to Neu5Ac and Neu5Ac dimer as revealed by HPTLC (Fig. 3). Neu5Ac dimer was detected as a major product formed upon mild acid hydrolysis of Hp-s2 with 25 mM sodium acetate (pH 5.5) at 65 °C for 16 h. These results suggested that Neu5Acα2 → 8Neu5Acα2 → was linked to 6-*O*-position of the Glc residue in Hp-s2. Anomeric configuration of Glc was β on the basis of its coupling constant value in ^{1}H-NMR spectroscopy (δ 4.12 ppm, $J_{1,2}$ = 7.3 Hz).

The same fatty acid species as found for Hp-s1 were also detected for Hp-s2 on GLC. This was confirmed by FAB-MS measurements which showed that t18:0 was amidated with fatty acids of mainly C20:1, C21:1, and C22:1 (Fig. 4b, Table 2).

(c) Hp-s4

Glc, Neu5Ac, and sulfate groups were present in a molar proportion of 1:1:1 (Table 1). Methylation analysis showed the presence of 6-*O*-substituted Glc and only 8-*O*-substituted Neu5Ac but no unsubstituted Neu5Ac. Acid hydrolysis with 0.1 N TFA at 70 °C for 30 min yielded Glc → Cer but not Cer when examined by HPTLC by visualizing with 10% sulfuric acid-ethanol (data not shown). These results indicated that the linkage of Hp-s4 is HSO_3 → 8Neu5Ac2 → 6Glc1 → Cer. HPTLC of the acid hydrolysate, when visualized by resorcinol reagent, showed a new band migrating differently from Neu5Ac (Fig. 3). The results of sugar composition and sulfate analysis showed that this component was a sulfated Neu5Ac. Anomeric configurations of Glc and Neu5Ac residues were determined as β and α, respectively, based on ^{1}H-NMR data (H-1 of Glc, δ 4.10 ppm, $J_{1,2}$ = 8.4 Hz; H-3_{eq} of Neu5Ac, δ 2.48 ppm; H-3_{ax} of Neu5Ac, δ 1.45 ppm). These results were consistent with the structure, Neu5Ac8HSO_3α2 → 6Glcβ → Cer.

FAB-MS data supported these results and showed that Hp-s4 contained the same ceramide structure as Hp-s1 (Fig. 4c, Table 2).

The failure of *A. ureafaciens* sialidase to cleave Neu5Ac from Hp-s4 is attributed to sulfation at the 8-*O*-position of the nonreducing terminal Neu5Ac residue (Fig. 2).

(d) Hp-s6

Hp-s6 contained one Glc and two Neu5Ac residues (Table 1). Sulfate was also present in a 1:1 molar ratio relative to

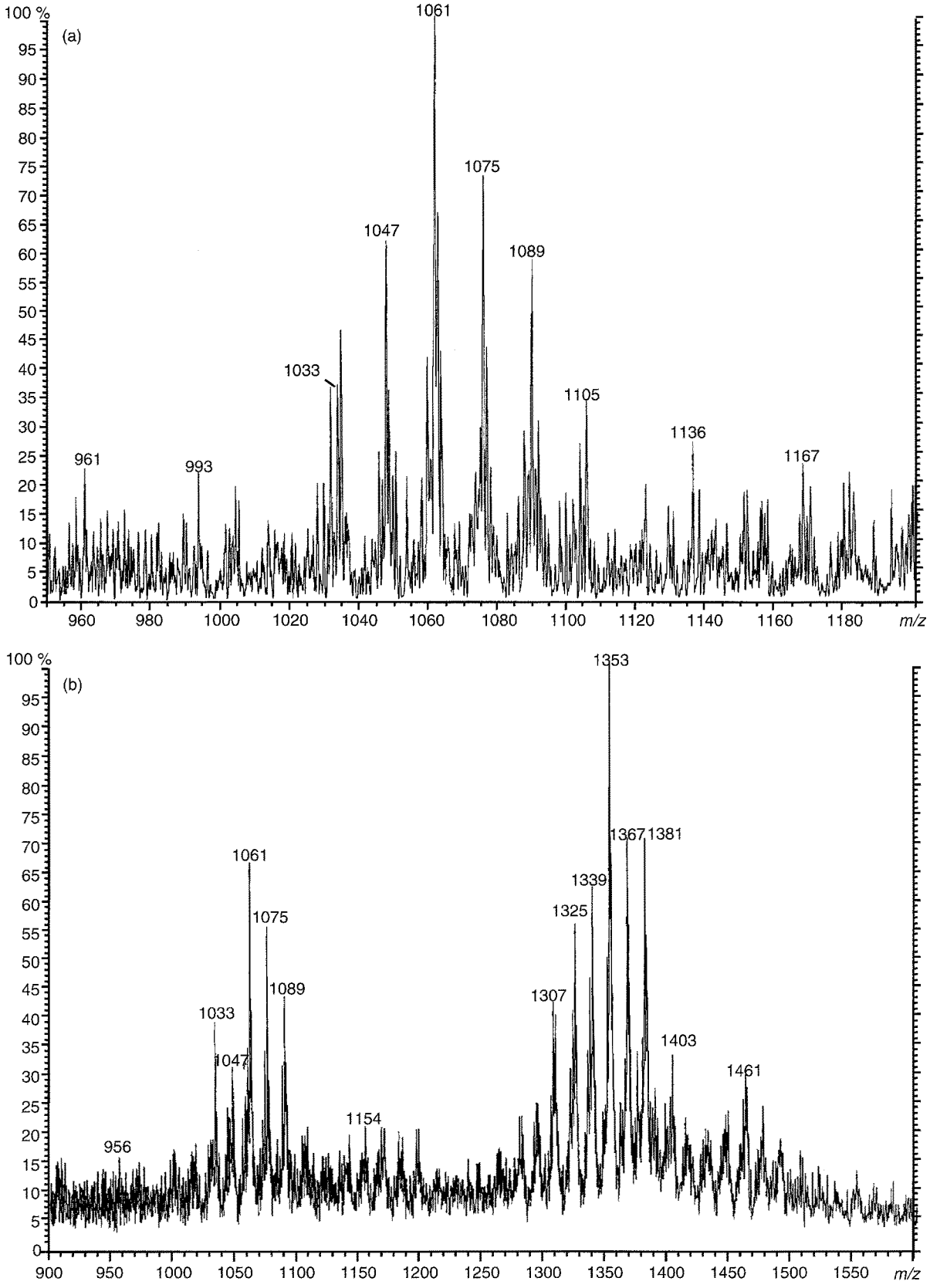

Figure 4. Molecular ion regions of negative FAB spectra of underivatized (a) Hp-s1; (b) Hp-s2; (c) Hp-s4; (d) Hp-s6. Major signals are assigned in Table 2. In some spectra ions 2 mass units below major signals are quite prominent. These most likely correspond to minor components which have an additional double bond in the acyl moiety or the sphingosine.

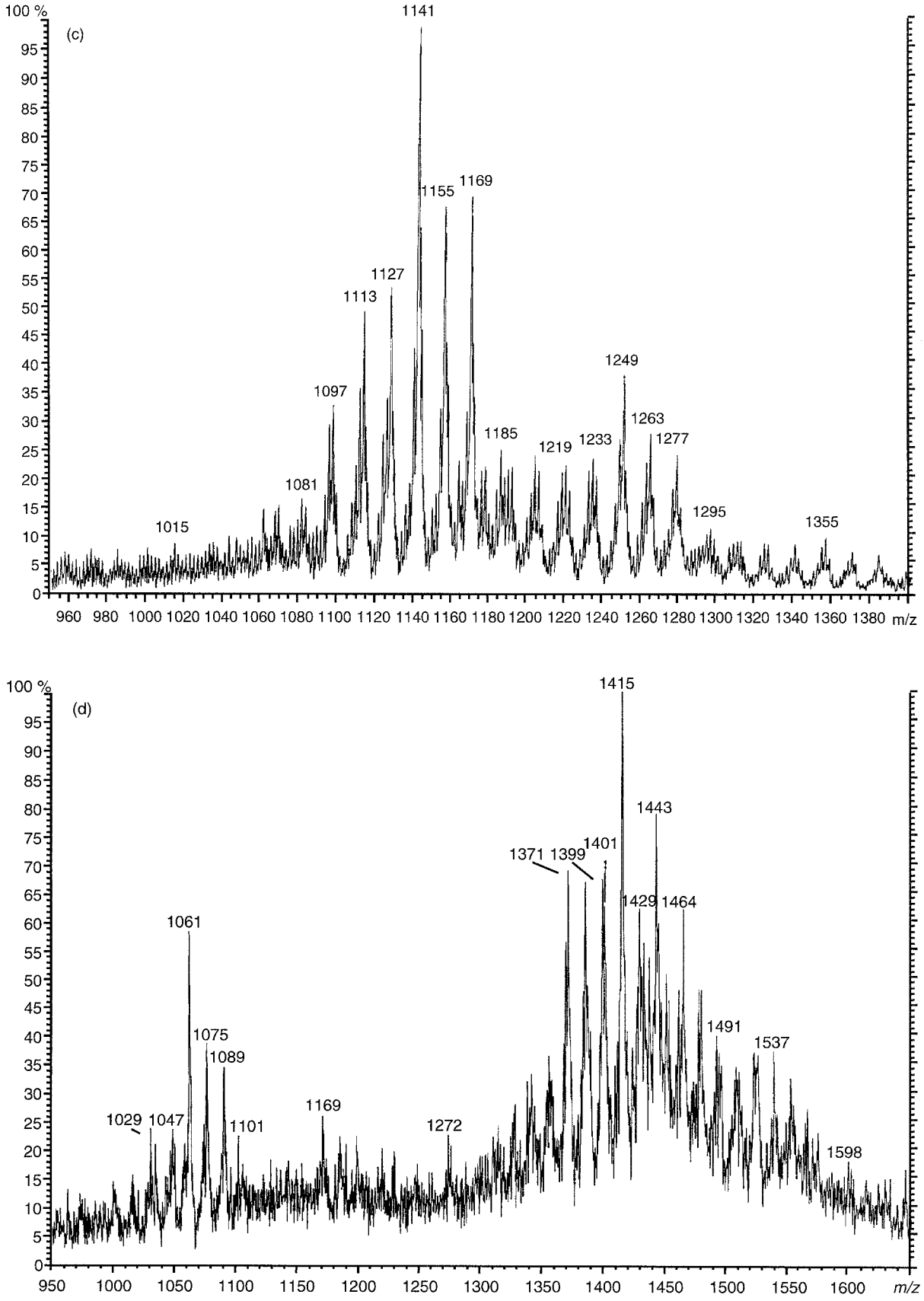

Figure 4. c and d.

Table 2. Summary of the FAB-MS data on sea urchin sialyl GSLs.

	m/z *for [M–H]⁻ (underivatives)*	m/z *for [M + H]⁻ and key fragment ions (permethylated)*	m/z *for [M–H]⁻ (permethylated)*	m/z *for [M–H]⁻ (deutoacetylated)*	*Assignment*
Hp-s1	1033, 1047, 1061, 1075, 1089	1203, 1217, 1231, 1245, 1259	ND	1439, 1453, 1467, 1481, 1495	$Neu5Ac_1Hex_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
		606, 620, 634, 648, 662			Ceramide, (C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
		376			$Neu5Ac^+$
		939			$(M–N–acyl)^+$
HP-s2	1325, 1339, 1353, 1367, 1381	1564, 1578, 1592, 1606, 1620	ND		$Neu5Ac_2Hex_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
				1802, 1816, 1830, 1844, 1858	$Neu5Ac_2Hex_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0, lactonized
		606, 620, 634, 648, 662			Ceramide, (C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
	1033, 1047, 1061, 1075, 1089				$(M–NeuAc–H)^-$
		376			$Neu5Ac^+$
		737			$Neu5Ac_2^+$
Hp-s4	1113, 1127, 1141, 1155, 1169		1267, 1281, 1295, 1309, 1323	1473, 1487, 1501, 1515, 1529	$Neu5Ac_1Hex_1/sulfate_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
				1455, 1469, 1483, 1498, 1512	$(M–H–18)^-$
		1211, 1225, 1239, 1253, 1267			desulfated $(M + Na)^+$
		606, 620, 634, 648, 662			Ceramide, (C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
		362			$Neu5Ac(OH)^+$
Hp-s6			1629, 1643, 1657, 1671, 1685		$Neu5Ac_2Hex_1/sulfate_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
	1387, 1401, 1415, 1429, 1443			1836, 1850, 1864, 1878, 1892	$Neu5Ac_2Hex_1/sulfate_1$/(C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0, lactonized
	1033, 1047, 1061, 1075, 1089				(M(lactone)-sulfated Neu5Ac (lactone))⁻
		606, 620, 634, 648, 662			Ceramide, (C18:1, C19:1, C20:1, C21:1, C22:1)/t18:0
		723			$Neu5Ac_2(OH)_1^+$
		362			$Neu5Ac(OH)_1^+$

Glc. Hp-s6 was a single component on HPTLC when fractions obtained by the DEAE-Toyopearl chromatography were monitored before pooling. An additional faster moving band was detected on HPTLC after desalting followed by concentrating to dryness. These two materials with different mobilities were separately purified by preparative TLC. The fast moving material was not distinguished in the carbohydrate composition from the slow moving material, and the former was converted to the latter on treatment with 0.1 M NaOH at 37 °C for 5 min. These results suggested that the fast moving material was a lactone form of the slow moving one. Hp-s6 was shown to undergo facile lactonization under the conditions usually employed for purification.

As was the case with Hp-s4, methylation analysis showed the presence of 6-*O*-substituted Glc and only 8-*O*-substituted Neu5Ac but not unsubstituted Neu5Ac. Mild acid hydrolysis of Hp-s6 with 0.1 N TFA at 80 °C for 30 min, but not sialidase (Fig. 2), liberated the sulfated Neu5Ac, unsubstituted Neu5Ac, and monosulfated Neu5Ac dimer leaving the rest of the molecule (Glc → Cer; results not shown) intact when HPTLC of the hydrolysate was visualized by the resorcinol reagent (Fig. 3). The data are consistent with the structure, Neu5Ac8HSO$_3$2 → 8Neu5Ac2 → 6Glc → Cer, in which a lactone can readily be formed. ^{1}H-NMR spectral data showed that Glc was in the β configuration (H-1 of Glc, δ 4.08 ppm, $J_{1,2}$ = 7.7 Hz). The anomeric configuration of the Neu5Ac residues was concluded to be α by the previously established criteria on the basis of H-3 proton chemical shift values {(H-3$_{eq}$, H-3$_{ax}$) of each Neu5Ac residue, (2.26 ppm, 1.45 ppm) or (2.42 ppm, 1.35 ppm),

these signals were not assignable unambiguously; [20]}. Hp-s6 was quantitatively transformed to a lactone form during preparation of the sample for ^{1}H NMR measurement, when monitored by HPTLC analysis of the solution in an NMR tube (data not shown).

FAB-MS data showed that Hp-s6 also shared the same ceramide structure with other sialyl GSL components studied here (Fig. 4d; Table 2).

(e) Hp-s3 and Hp-s5

These fractions were minor components of sperm sialyl GSLs and yields of Hp-s3 and Hp-s5 were: 4.9% and 0.27%, respectively, of the total sialic acid-containing glycosphingolipids based on the content of Neu5Ac. Hp-s3 and Hp-s5 gave a major band at $R_f = 0.18$ and 0.092, respectively, on HPTLC (Fig. 1). Insufficient material made an exact determination of their structure impossible. However, the migratory behaviour on TLC gave an indication of possible structures: logarithmic plots of R_f values against ammonium acetate concentrations at which they eluted on DEAE-Toyopearl chromatography for each sialyl GSL including Hp-s3 and Hp-s5 are shown in Fig. 5. A homologous series of sialyl GSLs having a common core glycosylceramide but differing in chain length of oligoSia is anticipated to give a unique straight line [21]. As shown in Fig. 5, a straight line was constructed, suggesting the presence of a common core glycosylceramide structure in these four sialyl GSLs. Thus, the result suggests the major components in Hp-s3 and Hp-s5 have the structure $(Neu5Ac)_3 \rightarrow Glc \rightarrow Cer$ and $(Neu5Ac)_4 \rightarrow Glc \rightarrow Cer$, respectively.

Supporting evidence was obtained from FAB-MS experiments and from HPTLC analysis of the products formed from Hp-s3 by acid hydrolysis under mild

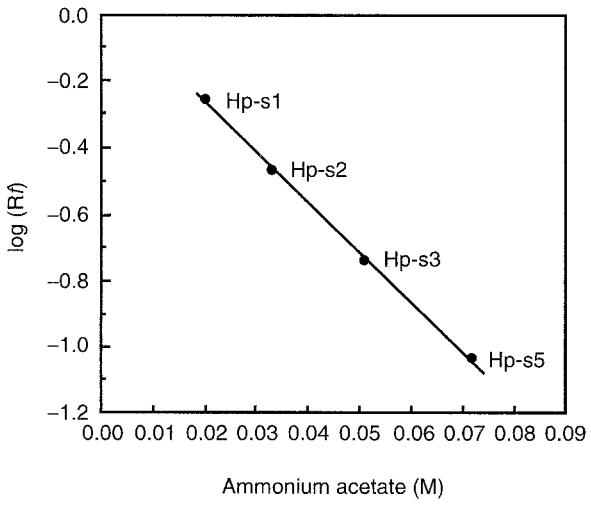

Figure 5. Plot of log R_f of Hp-s1, Hp-s2, and the major component of Hp-s3 and Hp-s5 against ammonium acetate concentrations where eluting on DEAE-Toyopearl 650S chromatography.

conditions (in 0.1 N TFA at 70 °C for 20 min). Two spots were observed when the TLC chromatogram was sprayed with resorcinol reagent. A TLC comparison between these two oligoSia and authentic oligoNeu5Ac allowed the identification of the fast- and slow-running materials as $Neu5Ac\alpha2 \rightarrow 8Neu5Ac$ and $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 8Neu5Ac$, respectively (not shown). On positive FAB-MS, A-type fragment ions, Neu5Ac (*m/z* 376), $(Neu5Ac)_2$ (*m/z* 737), and $(Neu5Ac)_3$ (*m/z* 1099), were observed for the permethylated Hp-s3. Among clusters of weak molecular ions, the major peaks were observed at *m/z* 1925, 1939, 1953, 1967, and 1981 as predicted (data not shown). On positive FAB-MS of the deuterioacetylated derivative of Hp-s3, prominent molecular ion peaks were observed at *m/z* 2167, 2181, 2195, 2209, and 2223, corresponding to lactonized $(Neu5Ac)_3(Hex)_1Cer$ with fatty acid and sphingosine content identical to those observed for other sperm sialyl GSL fractions (Fig. 6a; note that the derivatization procedure catalyses lactone formation). The presence of a major A-type ion at *m/z* 1135 [lactonized $(Neu5Ac)_3^+$] supported this composition. The TLC and FAB-MS data indicated that structure of a major component of Hp-s3 was $Neu5Ac2 \rightarrow 8Neu5Ac2 \rightarrow 8Neu5Ac2 \rightarrow Glc \rightarrow Cer$.

Similar FAB-MS experiments of deuterioacetylated Hp-s5 gave a series of molecular ions corresponding to a Neu5Ac increment above Hp-s3 (major signals at *m/z* 2559, 2573, 2587; Fig. 6b), consistent with the presence of $(Neu5Ac)_4(Hex)_1Cer$. The A-type ion of *m/z* 1499 [lactonized $(Neu5Ac)_4^+$] supported this composition. The negative FAB-MS of permethylated Hp-s5 showed the presence of small amounts of sulfated sialyl GSLs such as Hp-s4 and Hp-s6 (data not shown). Although lack of material prevented further characterization, the results were in good agreement with the TLC data (Fig. 1) showing the presence of minor components corresponding to these sulfated sialyl GSLs. On TLC analysis of the mild acid hydrolysate of Hp-s5 using a sodium acetate buffer, pH 5.5, at room temperature for several hours, bands migrating at the rates identical with those for $Neu5Ac \rightarrow Neu5Ac \rightarrow Neu5Ac \rightarrow Glc \rightarrow Cer$ and $Neu5Ac \rightarrow Neu5Ac \rightarrow Glc \rightarrow Cer$ appeared. These results, together with those shown in Fig. 5, strongly indicated the presence of $Neu5Ac \rightarrow Neu5Ac \rightarrow Neu5Ac \rightarrow Neu5Ac \rightarrow Glc \rightarrow Cer$.

LACTONE FORMATION OF SULFATED AND NONSULFATED OLIGOSIALYL GSLS

As described above, Hp-s6 ($= Neu5Ac8HSO_3\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow Cer$) was found to readily form a lactone during preparation procedures after DEAE-Toyopearl chromatography while sialyl GSLs characterized in this study were not lactonized. In view of the fact that neither the nonsulfated counterpart (Hp-s2 or $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow Cer$), nor the

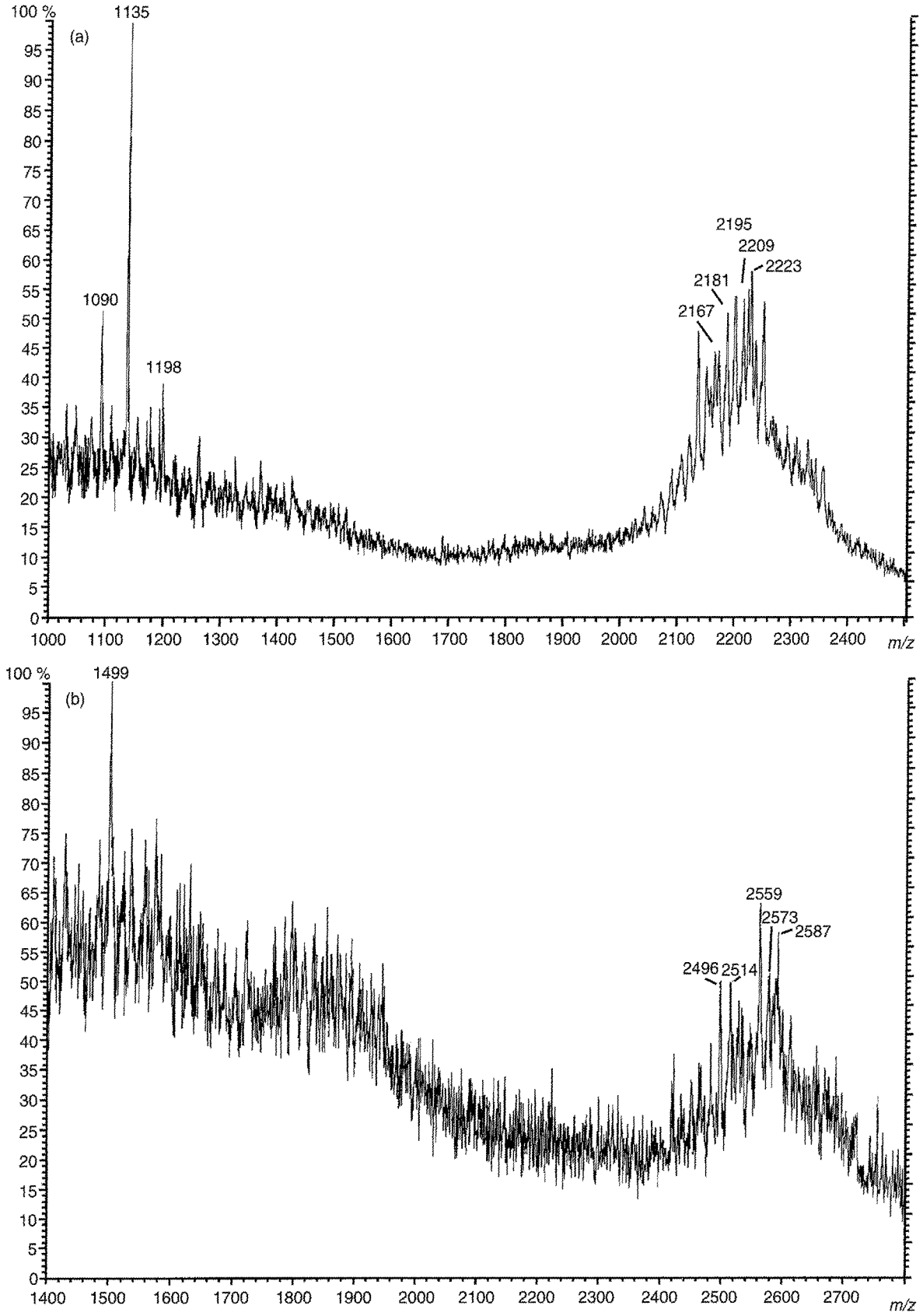

Figure 6. Molecular ion and high mass fragment ion regions of the positive FAB spectra of deuterioacetylated derivatives of (a) Hp-s3 and (b) Hp-s5. The data are consistent with fully lactonized Neu5Ac residues. Lactonization is known to be catalysed by the derivatizing reagents. In addition to the signals assigned in the text there are associated signals corresponding to sodium adducts and to under-deuteroacetylated components.

sulfated monosialyl GSL (Hp-s4 or Neu5Ac8H-$SO_3\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer) underwent similar facile lactone formation, sulfation at the nonreducing terminal Neu5Ac residue of disialyl Glc $\rightarrow$ Cer is apparently important for lactonization to occur.

More than 60% of Hp-s6 was converted to a lactone form on treatment with glacial acetic acid at 25 °C for 2-4 h, when monitored by HPTLC, while only a small proportion of Hp-s2 appeared to undergo lactonization upon similar treatment. It should be also noted that no lactone formation was observed with GD3 even after 4 h incubation under the same conditions. Under mild alkaline conditions the lactone ring was readily saponified (Fig. 7).

Lactonization of Hp-s2 and Hp-s6 also occurred on drying in vacuo at room temperature during preparation of the sample solutions for ^{1}H-NMR measurements. The lyophilized samples were dissolved in 2% D_2O/DMSO-d_6 for ^{1}H-NMR measurements. In the ^{1}H-NMR spectrum of Hp-s2 (Fig. 8b), the geminal H-9 signals of the two Neu5Ac residues resonate at δ 4.83 ppm and 4.56 ppm, which are down-field shifted as compared to the corresponding protons of Hp-s4 (Fig. 8a; δ for H-9a and H-9b <4 ppm). The chemical shifts of a pair of H-9 protons of Hp-s6 (Fig. 8c) were 4.84 and 4.48 ppm, being almost identical with those observed for Hp-s2 (Fig. 8b). The signals for H-9's of the 8-*O*-sulfated Neu5Ac residue in Hp-s4 appeared at the region higher than 4.23 ppm (Fig. 8a). Therefore, the signals appearing at 4.84 ppm and 4.4–4.6 ppm in Hp-s2 and Hp-s6 are assigned to H-9's protons of the internal Neu5Ac residues. We have no direct evidence for the structure of a lactone. However, the possibility of attack of the carboxyl group of the nonreducing terminal Neu5Ac residue at hydroxyl group at C-9 of the penultimate Neu5Ac residue may be suggested for intramolecular esterification.

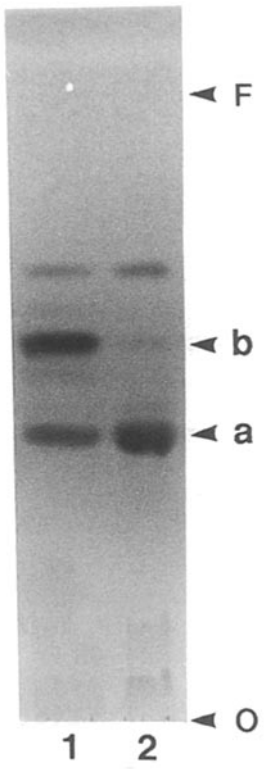

Figure 7. Mild alkali treatment of Hp-s6, under which conditions only lactone ring would open immediately. Hp-s6 was treated with 0.1 N NaOH at 25 °C for 3 min, and analysed by HPTLC. The HPTLC plate was developed in solvent A and visualized with the resorcinol reagent. The fast-moving band (marked by b) was converted to the slow-moving one (marked by a). O, origin; F, front. Lane 1, before treatment; lane 2, after treatment.

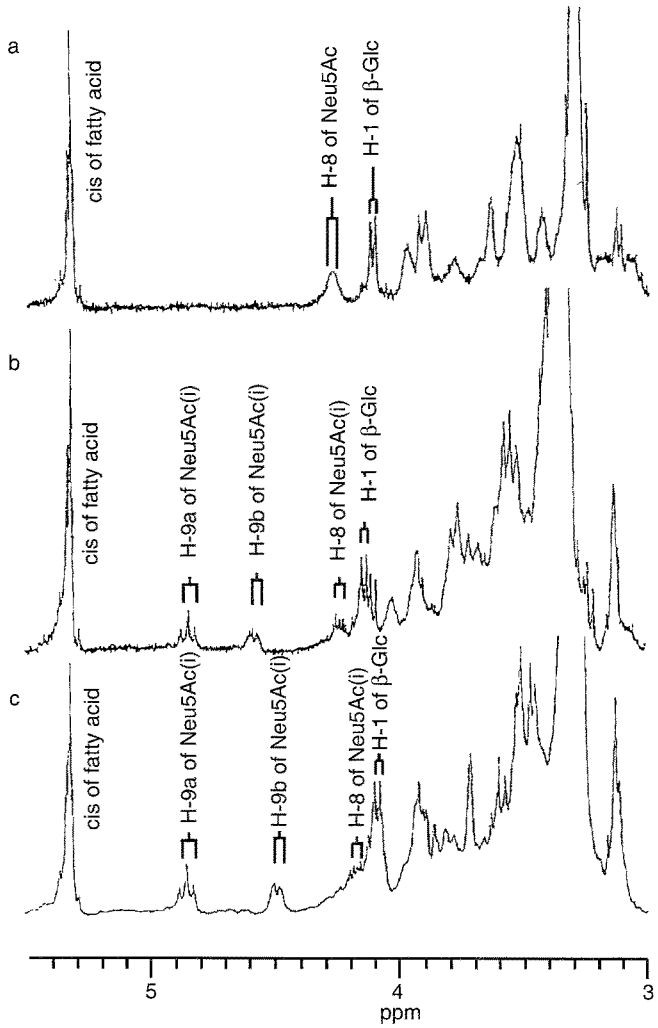

Figure 8. ^{1}H-NMR spectra (the 3.0–3.5 ppm region) of (a) Hp-s4 (Neu5Ac8$HSO_3\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer), (b) Hp-s2 (Neu5Ac$\alpha2 \rightarrow 8Neu5Ac(i)\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer), and (c) Hp-s6 (Neu5Ac8$HSO_3\alpha2 \rightarrow 8Neu5Ac(i)\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer). Neu5Ac(i) represents the penultimate Neu5Ac residue in the disialyl groups.

Discussion

Sialyl GSLs designated Hp-s1 to Hp-s6 were obtained in the range of 0.017×10^{-3} to 5.4×10^{-3}% yields based on wet weight of sea urchin sperm and it was considered to be pure enough for structural analysis by HPTLC criteria. The structures of six sialyl GSLs (Hp-s1 to Hp-s6) isolated from sea urchin sperm were elucidated by FAB-MS in combination with chemical analysis. All of these belong to the family of Glc → Cer type glycosphingolipids to which $\pm HSO_3 \rightarrow 8Neu5Ac\alpha2 \rightarrow (8Neu5Ac\alpha2 \rightarrow)_n \rightarrow$, where $n = 0$, 1, 2, and 3, are linked at *O*-6 (Fig. 9). Hp-s3 and Hp-s5 were not fully structurally analysed but it is of note that they contained the same Glc → Cer as that proposed for the core terminal part of the other sialyl GSLs (Hp-s1, Hp-s2, Hp-s4, and Hp-s6) whose structures were firmly established.

This paper provides the first evidence for the natural occurrence of $Neu5Ac8HSO_3\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer and a oligosialo GSL, $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer in biological material although $Neu5Ac8HSO_3\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer and a trisialic acid structure, $Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow$, in certain gangliosides have been identified previously [9, 22, 23]. Glycoproteins containing $Neu5Gc9HSO_3\alpha2 \rightarrow (5\text{-glycolyl-}O\text{-}Neu5Gc\alpha2 \rightarrow)_n$ structures have recently been found on the cell surface of sea urchin eggs [11]. The occurrence of the nonreducing terminal structure $HSO_3 \rightarrow$ Sia appears to represent a common terminating signal for elongation of oligo/polySia chains not only in glycoproteins but also in glycosphingolipids. Whether there is any relationship between the observed resistance of $HSO_3 \rightarrow Sia\alpha2 \rightarrow R$ ketosidic linkages to sialidases and biological phenomena is an open question. Demonstration of the occurrence of such sulfated Neu5Ac- or higher oligoNeu5Ac-containing GSLs has been made possible by the use of sensitive analytical methods, such as FAB-MS. A closer examination than heretofore may well reveal similar polySia components in glycoconjugates from other animal sources. It is noted that lower vertebrates such as the fish or amphibian contain a higher proportion of oligosialogangliosides which contain four or five Neu5Ac residues per ganglioside molecule, and a lower proportion of monosialogangliosides. It is interesting that in mammalian brain, the cerebellum also contains a relatively higher proportion of oligosialogangliosides [23, 24].

Perhaps the most interesting property of $Neu5Ac8HSO_3\alpha2 \rightarrow 8Neu5Ac\alpha2 \rightarrow 6Glc\beta1 \rightarrow$ Cer (Hp-s6) was the almost quantitative formation of the lactone which was produced under the conditions commonly used in the preparation of sialyl GSL. The explanation we have invoked to explain this anomalous result is a straightforward lactonization during the procedures of evaporation or dissolving in organic solvent although we were not able to rationalize such a property and the precise structure of the lactone(s). However, it is reasonable to state that, in aqueous solution at biological pH and temperature, $Neu5Ac8HSO_3\alpha2 \rightarrow (8Neu5Ac\alpha2 \rightarrow)_n \rightarrow$ chains, where $n = 1$, 2, --- exist as an equilibrium mixture of free oligoNeu5Ac chains and their lactone form.

Sialo- and/or sulfoglycolipids are known to occur on the sperm surface of various animals, and their possible involvement in cell-cell recognition and adhesion during spermatogenesis and fertilization was discussed in previous studies [4, 6, 25, 26]. (KDN)GM3, a major glycosphingolipid component on sperm surface of rainbow trout, has been suggested to bind specifically to a certain glycoprotein on the homologous egg surface at sperm-egg adhesion [4, 6]. Sulfated glycolipids appear to occur widely in sperm of a wide variety of animal ranging from sea urchin to mammals, and sulfogalactosyllipids are shown to be involved in sperm-egg interactions [26]. In this regard, the present finding of a series of glycosphingolipids having sulfated and/or nonsulfated sialic acid residues in sea urchin sperm provides an interesting system for future study of such unique acidic glycolipid-mediated cell-cell adhesive interaction.

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Hp-s1	Neu5Acα2→ 6Glcβ1→ 1'Cer
Hp-s2	Neu5Acα2→ 8Neu5Acα2→ 6Glcβ1→ 1'Cer
Hp-s3	Neu5Acα2→ 8Neu5Acα2→ 8Neu5Acα2→ 6Glcβ1→ 1'Cer
Hp-s4	HSO_3→ 8Neu5Acα2→ 6Glcβ1→ 1'Cer
Hp-s5	Neu5Acα2→ 8Neu5Acα2→ 8Neu5Acα2→ 8Neu5Acα2→ 6Glcβ1→ 1'Cer
Hp-s6	HSO_3→ 8Neu5Acα2→ 8Neu5Acα2→ 6Glcβ1→ 1'Cer

Figure 9. Structure of sialyl GSLs from sea urchin sperm.

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