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Characterization of Glycosphingolipid Mixtures with Up to Ten Sugars by Gas Chromatography and Gas Chromatography–Mass Spectrometry as Permethylated Oligosaccharides and Ceramides Released by Ceramide Glycanase†

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ABSTRACT: A novel, effective method for structural characterization of glycosphingolipids has been devised. It employs ceramide glycanase to release intact oligosaccharides followed by analysis using high-mass gas chromatography–mass spectrometry. The oligosaccharides and ceramides released by the glycanase were permethylated and analyzed. The capillary gas chromatography gave excellent resolution and separated, for example, two isomeric 10-sugar oligosaccharides with a molecular mass of 2150 daltons differing only by a Gal1-3GlcNAc and a Gal1-4GlcNAc linkage. The oligosaccharides released from sialic acid containing glycosphingolipids (gangliosides) were also analyzed for monosialo compounds. This analytical approach is simple, is quick, and can readily allow quantitation of individual glycosphingolipids.

Glycosphingolipids (GSLs)[1] are a very heterogeneous group of substances that are localized in the outer leaflet of the plasma membrane. In this leaflet they constitute the major type of lipids in the brush border membrane of epithelial cells (Simons & van Meer, 1988; Hansson and Simons, unpublished results). No unifying function of the glycan part of the GSLs has yet been described, but they can specify several of the blood group antigens (e.g., ABO, Lewis, P, and Ii), as well as being expressed in a tumor-associated way (Hakomori, 1984). Also, many bacteria and bacterial toxins can use the carbohydrate moiety of GSLs for specific adhesion to their target cells (Mirelman, 1986).

The GSLs vary among animal species, individuals, and strains as well as among the different organs and cells within one species (Hansson, 1988). Today more than 200 different GSLs have been described. With this great variety of GSLs very high demands are put on the analytical methods for their characterization. Gas chromatography (GC) has been adapted for the analysis of oligosaccharides having up to 10 sugar residues. The high resolution of capillary GC together with the structural information obtained by mass spectrometry (MS) is shown to be very useful in the characterization of GSLs after their cleavage with the recently discovered ceramide glycanase (Li et al., 1986; Ito & Yamagata, 1986). The approach taken allows a quick and simple characterization of the oligosaccharide portion, the ceramide portion, and the nondegraded monoglycosylceramides.

Materials and Methods

Materials. The following GSLs were used in this study: total neutral GSL from white rat small intestine, both epithelial cells and nonepithelial residue (Breimer et al., 1982a); total neutral GSL from mouse small intestine (Breimer et al., 1981a); lactosylceramide, fucosyllactosylceramide, globotriaosylceramide, isoglobotriaosylceramide, globotetraosyl-

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[1] Abbreviations: GSL, glycosphingolipid; GC, gas chromatography; MS, mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine.

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ceramide, isoglobotetraosylceramide, and blood group H pentaglycosylceramide (type 1) (Breimer et al., 1982a); a mixture of two blood group H decaglycosylceramides (Breimer et al., 1982b); blood group A tetra-, hexa(type 1)-, and hexa(type 2)glycosylceramide (Breimer et al., 1982c; Smith et al., 1975); fractions D, E, and F of Ångström et al. (1982a); the penta- and hexaglycosylceramide of Ångström et al. (1982b); blood group B gangliotetraosylceramide (Hansson et al., 1987); ganglioside GM_3 (Breimer et al., 1981b); GM_1 from human brain; ganglioside B GM_1 (Bouhours et al., 1987); milk oligosaccharide LNT, LND, and LSTa from human milk (Kobata 1972). Ceramide glycanase was purified from the leach *Macrobdella decora* as described (Li et al., 1986). C-18 Sep-Pak was obtained from Waters, Milford, MA, and sodium cholate from Sigma, St. Louis, MO. HPTLC Si-60 thin-layer plates and the solvents were obtained from Merck, Darmstadt, FRG, except for the iodomethane, which was from Fluka, Buchs, Switzerland. AG 3-X4A and AG 50W-X8 were obtained from Bio-Rad, Richmond, VA.

Ceramide Glycanase Treatment. The GSLs (50 μg to 1.5 mg) were mixed with sodium cholate (0.15–1.5 mg) and dissolved in 0.1 M NaAc buffer, pH 5.0 (0.2–1.8 mL), 0.2–30 unit of ceramide glycanase was added, and the mixture was incubated at 37 °C for 24 h. The reaction was stopped by the addition of chloroform/methanol to obtain the proportions 8:4:3 (by volume) of chloroform/methanol/water. The lower phase containing the undegraded monoglycosylceramides and the released ceramides was removed to another tube and the solvent evaporated. The upper phase, containing the released oligosaccharides, was evaporated, dissolved in 10% water in methanol (1 mL), and passed over a C-18 Sep-Pak column conditioned with 20 mL of chloroform/methanol, 2:1 (by volume), 20 mL of methanol, and 30 mL of water. The oligosaccharides were eluted with 12 mL of water and lyophilized. The completeness of the degradation was tested with thin-layer chromatography using 65:25:4 chloroform/methanol/water (by volume) as solvent and the anisaldehyde reagent for detection.

Derivatization. Permethylation of the nonreduced oligosaccharides was done with solid NaOH in dimethyl sulfoxide and iodomethane as described (Larson et al., 1987). The oligosaccharides were reduced with 1 M $NaBH_4$ overnight, evaporated five times with methanol and a drop of acetic acid, and passed over AG 3-X4 (OH^- form) and AG 50W-X4 (H^+ form) ion-exchange resins. The obtained alditols were permethylated as above, but the amount of iodomethane was increased to be equal to the volume of dimethyl sulfoxide. This type of permethylation could also be done directly after $NaBH_4$ reduction without ion-exchange chromatography. Details of the permethylation are given elsewhere (Karlsson and Hansson, unpublished results).

Gas Chromatography and Gas Chromatography–Mass Spectrometry. Fused silica columns (2, 5, or 10 m × 0.25 mm i.d., HT-polyimide coated on the outside, Chrompack, Middelburg, The Netherlands) coated with 0.03–0.05 μm of cross-linked PS 264 (Fluka, Buchs, Switzerland) were used. GC was performed on a Carlo Erba 5160 or a Hewlett-Packard 5890A gas chromatograph with flame ionization detectors and hydrogen as the carrier gas. The samples were dissolved in ethyl acetate, and 1 μL was injected on-column. Details of the different experiments are given in the figure legends. GC–MS was performed on a Carlo Erba 4160 gas chromatograph directly interfaced to a VG ZAB-HF mass spectrometer equipped with a VG 11-250 data system. Fused silica columns (5 or 10 m × 0.25 mm i.d.) coated with 0.03 or 0.05 μm of cross-linked PS 264 were used. Helium was used as the carrier gas. Conditions for MS: electron energy, 70 eV; trap current, 500 μA, acceleration voltage, 8 kV; mass range scanned, m/z 1600–160; scan speed, 2 s/decade; total cycle time, 3.5 s; resolution, 1400.

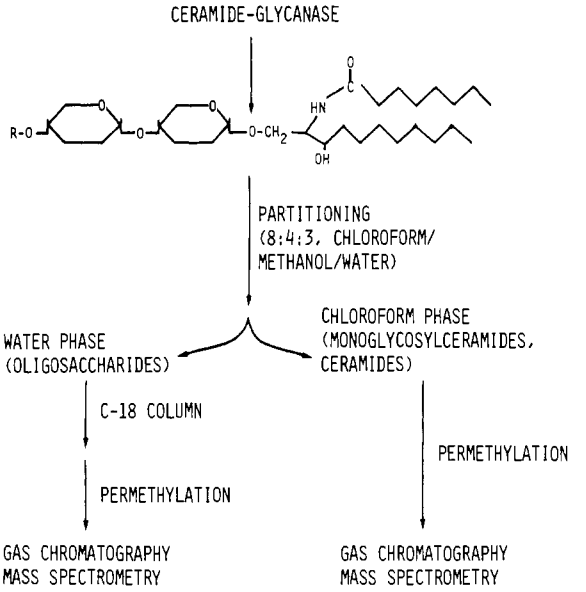

FIGURE 1: Schematic diagram showing the procedure for characterization of GSLs.

Results

The combination of ceramide glycanase treatment of GSLs with the use of thin-film capillary columns for GC at high temperatures for the characterization of GSLs is simple and involves only a few steps as shown in Figure 1.

Oligosaccharides. Table I shows the retention times relative to that of lactose of permethylated oligosaccharides analyzed by GC. The gas chromatogram of the permethylated oligosaccharides obtained from the mixture of neutral GSLs derived from the total small intestine of one strain of rat is shown in Figure 2 together with a thin-layer chromatogram of the original GSL mixture. The different oligosaccharides are separated into their β- and α-anomers with about 2:1 ratio when analyzed on a 10-m column with 0.05-μm film thickness and a temperature program at a rate of 10 °C/min. The blood group H trisaccharide (no. 3) shows only one peak. A heptasaccharide with a mass of 1516 daltons (no. 22 in Figure 2 and Table I) is the peak with highest mass identified in this chromatogram. However, when a shorter column (2 m) with a film thickness of 0.03 μm was used together with a more rapid temperature program (30 °C/min) even the largest oligosaccharides of this fraction were chromatographed (right insert of Figure 2). Due to loss of resolution with a shorter column, the oligosaccharides are not separated into their α- and β-anomers, and the two peaks shown for the decasaccharide (M_r 2150.1) are due to two isomers that differ by a type 1 sequence (Galβ1–3GlcNAc) of the 6-linked branch in component 24 and a type 2 sequence (Galβ1–4GlcNAc) in component 23 (Table I; Breimer et al., 1982b).

The same mixture of released oligosaccharides has been analyzed after reduction and permethylation by GC/MS as shown in Figure 3. Two examples of mass spectra are given, at the top isoglobotetraol and at the bottom blood group H type 1 pentaol (LNF I). The isoglobotetraol is characterized by the terminal HexNAc- at m/z 260, terminal HexNAc-Hex- at m/z 464, terminal HexNAc-Hex-Hex- at m/z 668, and reduced Hex at m/z 235. The H-type pentaol has an internal

Table I: Relative Retention Times of Permethylated Oligosaccharides Released from Glycosphingolipids

saccharide	trivial name[a]	retention time relative to that of lactose[b]	no. in Figure 2	relative amount in GSL mixture from rat (Figure 2) (globotriaose, 100)
non sialic acid containing				
Glc	glucose	not released	1	
Galβ1-4Glc	lactose	1.0	2	23
Fucα1-2Galβ1-4Glc	fucosyllactose	1.46	3	2.8
Galα1-4Galβ1-4Glc	globotriaose	1.54	4	100
Galα1-3Galβ1-4Glc	isoglobotriaose	1.61	5	27
Galα1-3Galα1-4Galβ1-4Glc		1.97	6	25
GalNAcβ1-3Galα1-4Galβ1-4Glc	globotetraose	2.08	7	9
Galβ1-3GalNAcβ1-4Galβ1-4Glc	gangliotetraose	2.15	8	4.1
GalNAcβ1-3Galα1-3Galβ1-4Glc	isoglobotetraose	2.20	9	40
Galβ1-3GlcNAcβ1-3Galβ1-4Glc	lactotetraose	2.24		
Fucα1-2Galα1-3Galα1-4Galβ1-4Glc		2.33	10	<1
Galα1-3Galα1-3Galα1-4Galβ1-4Glc		2.35	11	1.8
Fucα1-2Galβ1-3GalNAcβ1-4Galβ1-4Glc		2.37		
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	(H-5-1)	2.44	12	28
GalNAcβ1-3Galα1-3Galα1-4Galβ1-4Glc		2.48	13	<9
Hex-HexNAc-Hex-Hex-Hex		2.52	14	1.1
GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc	Forssman antigen	2.59		
Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-4Galβ1-4Glc		2.60	15	2.9
Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glc		2.68	16	1.3
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	(Le^b-6-1)	2.71		
Hex-Hex-Hex-Hex-Hex-Hex		2.75	17	1.4
GalNAcβ1-3Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glc		2.79	18	1.6
GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glc	(A-6-2)	2.81		
GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc	(A-6-1)	2.83		
HexNAc-Hex-Hex-Hex-Hex-Hex		2.86	19	1.0
Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Galα1-3Galβ1-4Glc		2.94		
Fuc-Hex-Hex-Hex-Hex-Hex-Hex		2.95	20	<1
Galα1-3Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glc		2.96	21	<1
GalNAcβ1-3Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glc		3.07	22	3.2
Fucα1-2Galβ1-4GlcNAcβ1-6 Galβ1-3GlcNAcβ1-3Galβ1-4Glc Fucα1-2Galβ1-3GlcNAcβ1-3	(H-10-1,1,2)	3.67	23	nd[c]
Fucα1-2Galβ1-3GlcNAcβ1-6 Galβ1-3GlcNAcβ1-3Galβ1-4Glc Fucα1-2Galβ1-3GlcNAcβ1-3	(H-10-1,1,1)	3.69	24	nd[c]
sialic acid containing				
NeuAcα2-3Galβ1-4Glc	sialyllactose	1.93		
Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc	GM_1 oligosaccharide	2.61		
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	LSTa	2.75		
Galα1-3(Fucα1-2)Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc	B-GM_1 oligosaccharide	2.98		

[a] Designations in parentheses mean blood group reactivity-number of sugar residues-type of core sequence. [b] Calculated on the β-peak. [c] Not determined.

HexNAc which gives prominent ions at both sides of this residue, i.e., m/z 638 and 684. The terminal Fuc-Hex sequence is shown at m/z 189, 393, and 361 (393 - 32) and by the peak at m/z 949 for the loss of 145 from the molecular ion (Karlsson et al., 1987). The mass spectra of the larger oligosaccharides lacking HexNAc show little information, and the identities of these peaks have been shown by comparing mass spectra and retention times with those of the oligosaccharides of pure GSLs. It has not been possible to record any mass spectra of the 10-sugar oligosaccharides due to GC–MS interface problems, and the identities of the two peaks (no. 23 and 24) are based on their relative abundance.

The GSLs of this mixture from rat small intestine have earlier been studied in detail (Ångström et al., 1982a,b; Breimer et al., 1982a–c), and almost all components shown before have now been identified together with three additional compounds. These are component 14, which could be isoglobotetraose (or globotetraose) extended by a Gal, component 17 that is a hexahexose, and component 19 that is a hexasaccharide with terminal HexNAc. In a previous analysis the oligosaccharides from GSL fraction F of Table I in Ångström et al. (1982a), those corresponding to no. 16 and 17, were found in a ratio of 1:0.7 (data not shown). According to the data from the methods used at that time, only the major hexaglycosylceramide with the structure Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glcβ1-Cer was deduced. The present results of GC and GC–MS show that there are two isomers that may differ in the sequence of Galα1-3 and Galα1-4 residues. Components 18 and 19, which both have terminal HexNAc, are probably related in a similar way as the oligohexahexoses.

Gangliosides are also cleaved by the ceramide glycanase, and Table I shows the structure and relative retention times of four sialic acid containing oligosaccharides. Figure 4 shows a mass spectrum from a GC–MS analysis of the permethylated nonreduced oligosaccharide from ganglioside GM_1. Ions specific for terminal sugars are found at m/z 219 (Hex) and 376 (NeuAc) and for terminal disaccharide at m/z 464. The

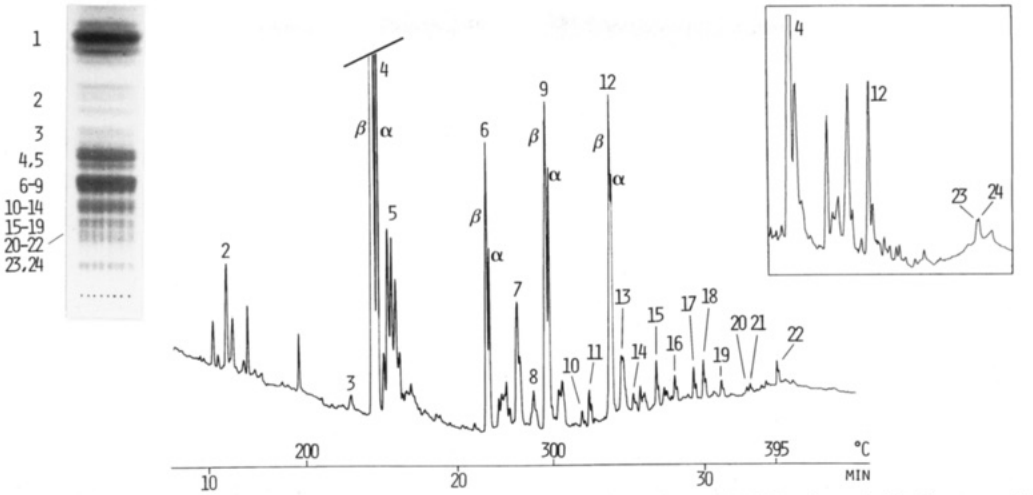

FIGURE 2: Gas chromatogram of permethylated oligosaccharides from the mixture of neutral GSLs from (white) rat small intestine (both epithelial and nonepithelial part) released by ceramide glycanase. The inserted gas chromatogram to the right is of the same sample. The thin-layer chromatogram to the left shows the original GSL mixture. The numbers on the gas chromatogram refer to the permethylated oligosaccharides of Table I, the numbers in the thin-layer chromatogram to the corresponding GSLs, and the β and α designations to the anomeric forms. The fused silica column was 10 m, film thickness 0.05 μm, carrier gas H_2 with a pressure of 1.1 bar, detector temperature 395 °C, initial temperature 70 °C for 1 min, programmed by 10 °C/min to 395 °C with a final time of 10 min. A Carlo-Erba 5160 chromatograph was used. The inserted chromatogram to the right shows the results from a 2-m column: film thickness, 0.03 μm; carrier gas, H_2 with a pressure of 0.45 bar; detector temperature, 400 °C, initial temperature, 70 °C for 1 min, programmed by 30 °C/min to 400 °C with a final time of 1 min. A Hewlett-Packard 5890A chromatograph was used. Conditions for the thin-layer chromatography were HPTLC silica gel plates, chloroform/methanol/water (68:35:8, by volume) as solvent, and detection by the anisaldehyde reagent.

ion at m/z 1030 contains the sialic acid and three sugar residues of the core saccharide. Loss of 59 ($COOCH_3$), typical for sialic acid containing oligosaccharides, gives a fragment ion at m/z 1206. The mass spectra for the other analyzed sialic acid containing oligosaccharides showed similar fragmentation patterns.

Monoglycosylceramides and Ceramides. The chloroform phase after the ceramide glycanase treatment was directly permethylated and analyzed by GC and GC–MS. Figure 5 shows the gas chromatogram of these components from the same experiment as in Figure 2, i.e., neutral GSLs from the small intestine of the rat. To identify the different peaks, we have used GC–MS as shown in Figure 6. The first part of the chromatogram is made up of the ceramides and the second of the monoglycosylceramides, in this case only glucosylceramides (Ångström et al., 1982a). The mass spectrum of the ceramide with hydroxysphinganine (phytosphingosine) and nonhydroxylated 24:0 fatty acid has an immonium ion as the base peak at m/z 438 (M − 285). Other dominant ions are at m/z 482 (M − 241) and 450 (482 − 32).

The mass spectrum of the corresponding monoglycosylceramide (Figure 6, bottom) is typical for this derivative (Karlsson et al., 1974). The base peak at m/z 187 is due to the loss of methanol from the pyran oxonium ion (m/z 219). Other major fragment ions are the ceramide ion at m/z 692, the ion at m/z 686 (M − 241), and the ion at m/z 292. The latter is an immonium ion cleaved at two bonds with the addition of hydrogen and is typical for monoglycosylceramides. The 20:0 fatty acid predominates in the cleaved GSLs, whereas the glucosylcermides have larger amounts of 16:0 and 24:0 fatty acids (Figure 5).

Discussion

Glycosphingolipids, both neutral and sialic acid containing, have been cleaved by ceramide glycanase, simply separated by partitioning into oligosaccharides and ceramides/monoglycosylceramides, permethylated, and analyzed by GC and GC–MS. The excellent resolution obtained by capillary GC allows separation of most of the 34 oligosaccharides analyzed in this paper. Oligosaccharides that differ in one linkage position, for example, type 1 chain (Galβ1–3GlcNAc) and type 2 chain (Galβ1–4GlcNAc), can be separated as shown for the oligosaccharides from two blood group A hexaglycosylceramides and the two blood group H decaglycosylceramides. Another advantage of the high resolution is the separation of the permethylated nonreduced oligosaccharides into their α- and β-anomers, which gives a simple way to distinguish oligosaccharides from impurities directly by GC. Fucosyllactose is an exception to this as it only gives one peak, a phenomenon not yet explained.

To allow the analysis of the larger oligosaccharides, shorter columns and a more rapid temperature program should be used. By this approach a fast screening of the sample is achieved with a lower resolution. These chromatograms do not show separation of the α- and β-anomers but separate isomers as exemplified by the two decasaccharides (Figure 2). The oligosaccharides of gangliosides can also be analyzed after ceramide glycanase treatment and permethylation by GC and GC–MS. The upper limit for the number of sialic acid residues has not been investigated, but it will probably not allow the analysis of gangliosides with three to four sialic acids.

GC of permethylated oligosaccharides up to pentasaccharides has been reported (Wang et al., 1983). Mass spectra of permethylated oligosaccharides, similar to the ones presented here, have been published before (Hallgren & Lundblad, 1977; Ando et al., 1977; Sweeley et al., 1980), the largest ones with five sugars only by direct-inlet MS (Sweeley et al., 1980).

Permethylation using solid NaOH gave good yields for the nonreduced oligosaccharides as is discussed elsewhere (Karlsson and Hansson, unpublished results). The permethylated nonreduced oligosaccharides are preferred for GC as it eliminates one extra step and the α- and β-anomeric peaks are diagnostic for peaks containing oligosaccharide. However, for GC–MS this derivative has its limitations as it is often impossible to distinguish the reducing sugar residue from the nonreducing ones in a mass spectrum. One way to solve this is to reduce the oligosaccharides to the corresponding alditols. Permethylation of reduced oligosaccharides with solid NaOH gives lower and variable recoveries of the different components

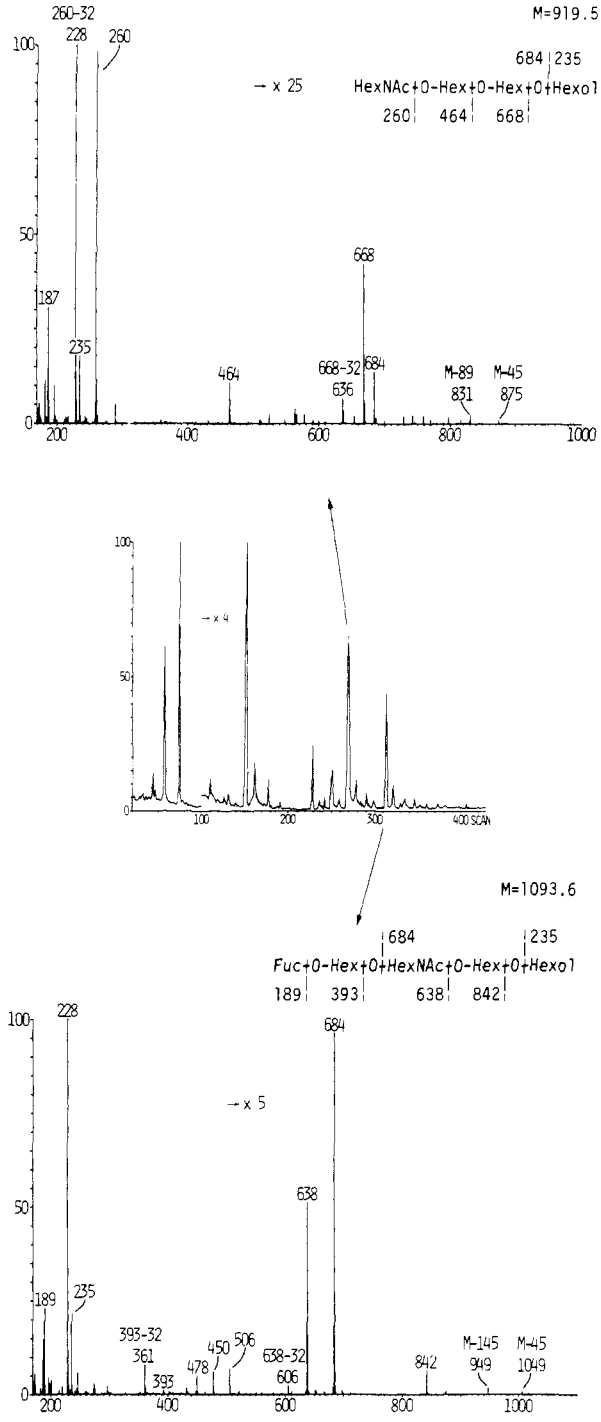

FIGURE 3: GC–MS of reduced and permethylated oligosaccharides from the mixture of neutral GSLs of (white) rat small intestine (both epithelial and nonepithelial part) released by ceramide glycanase. The total ion chromatogram is shown in the middle with arrows pointing from the peaks to the mass spectra at the top (scan 269) and bottom (scan 313). The fused silica column was 10 m: film thickness, 0.03 μm; carrier gas, He with a pressure of 0.2 bar; initial temperature, 88 °C for 1 min, programmed by 10 °C/min to 390 °C with a final time of 3 min. The GC–MS interface was kept at 400 °C and the ion source at 380 °C.

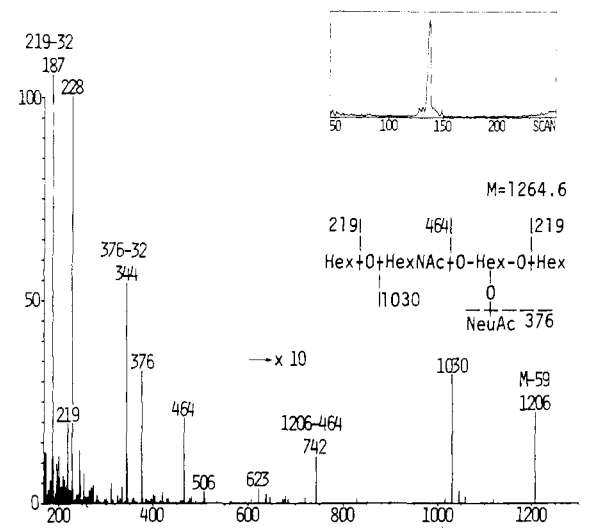

FIGURE 4: Mass spectrum obtained by GC–MS of the oligosaccharide released from the ganglioside GM_1 by ceramide glycanase. The mass spectrum of scan 138 is shown together with an interpretation formula. Inserted in the upper right-hand corner is the total ion chromatogram. The chromatography column was 10 m: film thickness, 0.05 μm; carrier gas, He with a pressure of 0.2 bar and a linear gas velocity of 90 cm/s; initial temperature, 200 °C for 1 min, programmed by 10 °C/min to 395 °C. The GC–MS interface was kept at 360 °C and the ion source at 340 °C.

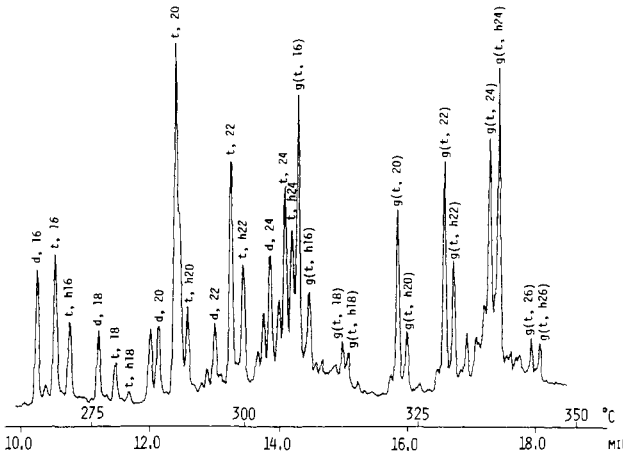

FIGURE 5: GC of permethylated ceramides and monoglycosylceramides recovered in the chloroform phase after ceramide glycanase treatment of the mixture of neutral GSLs from (white) rat small intestine (both epithelial and nonepithelial part). The structures in the different peaks were interpreted from mass spectrometry and are marked at the top of the individual peaks. The first part of the chromatogram represents the ceramides and the second part the glucosylceramides, abbreviated g. d means d18:1, dihydroxylated long-chain base (sphingosine); t means t18:0, trihydroxylated long-chain base (hydroxysphinganine, phytosphingosine); the digits are the number of carbons in the fatty acid, and h means hydroxylated fatty acid. The fused silica column was 10 m: film thickness, 0.05 μm; carrier gas, H_2 with a pressure of 0.7 bar; detector temperature, 390 °C; initial temperature, 70 °C for 1 min, programmed by 10 °C/min to 390 °C. A Hewlett-Packard 5890A chromatograph was used.

probably due to partial degradation. As for example, the amount of tetrahexaose (no. 6 in Figure 2) is larger than the corresponding amount of tetrahexol in the total ion curve of the alditols (Figure 3). The yields of alditols can be increased by using larger amounts of iodomethane in the permethylation.

Under the conditions used in this study the ceramide glycanase does not cleave monoglycosylceramides (Li et al., 1986). Extended specificity studies have also shown that it also does not cleave lactosylceramide with GalNAc added α1–3 and Fuc added α1–2, i.e., the smallest blood group A GSL identified (Breimer et al., 1982c).

The ceramides and monoglycosylceramides partitioned to the chloroform phase can also be analyzed on the same column after permethylation. The ceramides separate from the monoglycosylcermides, and analysis by GC–MS identifies the different components by their mass spectra. Ceramides have earlier been analyzed by GC and GC–MS as their trimethylsilyl derivatives (Samuelsson & Samuelsson, 1969), but

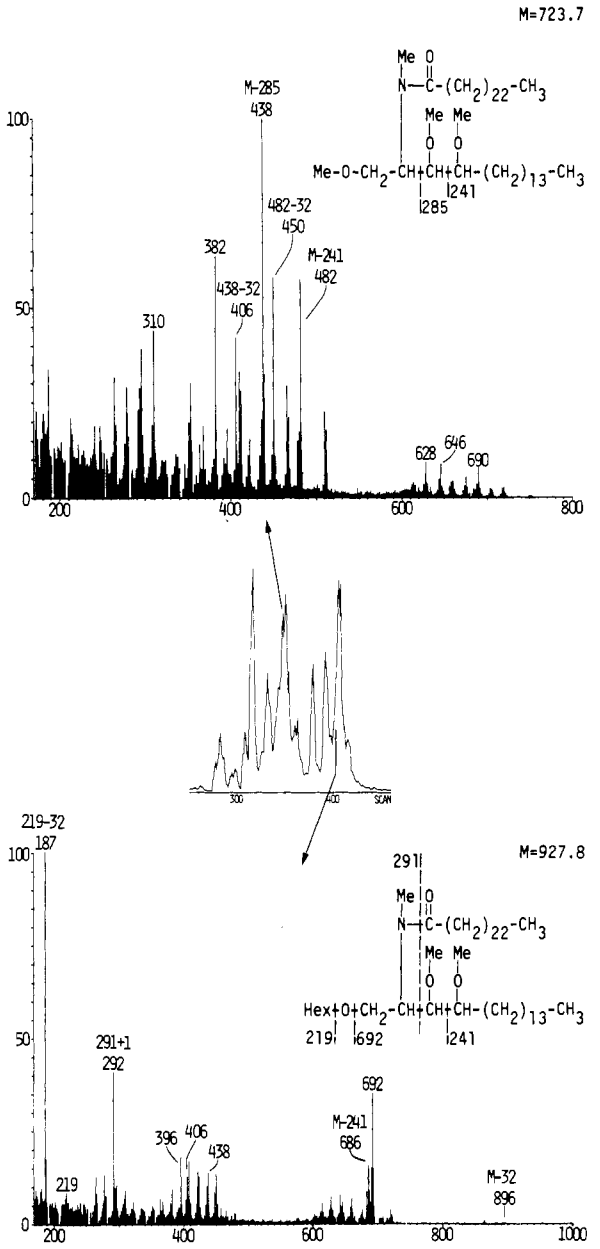

FIGURE 6: GC–MS of the same sample as analyzed in Figure 5. The total ion chromatogram is shown in the middle with arrows pointing from the peaks to the spectrum of one ceramide species at the top (scan 348) and to the spectrum of the corresponding glucosylceramide at the bottom (scan 404). The conditions for GC–MS are as described in Figure 3.

the permethylated derivative has not been used. Mass spectra after direct-inlet MS of permethylated monoglycosylceramides have been described before (Karlsson et al., 1974); however, GC analysis of intact permethylated glycolipids has never been reported.

The fatty acid patterns are different in the ceramides and glucosylceramides, which is in accordance with earlier results obtained from the intact GSLs (Breimer et al., 1982a,d). The ceramides come from all the GSLs with two or more sugar residues, and the major component is the hydroxylated 20:0 fatty acid, which is abundant in the predominant globotriaosylceramide. The long-chain base is both sphingosine and hydroxysphinganine (phytosphingosine), a reflection of the epithelial and nonepithelial origins of the GSLs of the small intestine. The major drawback of the approach taken is that the information on specific association of oligosaccharides with ceramides is lost. An example is that all GSLs with four or more sugars and terminal GalNAc from the nonepithelial part of the small intestine have nonhydroxylated 18:0 as a dominating fatty acid (Breimer et al., 1982a).

The advantage with the present approach is that it allows a good separation of different components found in natural GSL mixtures. An example of this is the two hexahexosylceramides (oligosaccharides 16 and 17 of Figure 2) and the two hexasaccharides with terminal HexNAc (no. 18 and 19) that it was not possible to distinguish earlier (Ångström et al., 1982b). The characterization of the GSLs of the rat intestine (Ångström et al., 1982a,b; Breimer et al., 1982a–c) should have been much easier if the present approach had been available. The relative amounts of the individual GSLs in the original mixture, or even absolute amounts if internal standards are added, can easily be obtained by GC of the nonreduced oligosaccharides. The amounts given in Table I for neutral GSLs of rat small intestine are in general agreement with the results published earlier (Breimer et al., 1982a), with the exception that the amount now given for isoglobotriaosylceramide is larger and that for globotetraosylceramide is smaller. However, the amounts given earlier were primarily based on gravimetry of purified components, and the results presented in this paper are probably more accurate.

Nilsson and Zopf (1983) have published a procedure for the analysis of GSLs by GC–MS after trifluoroacetolysis. Only GSLs with ceramides having a double bond (sphingosine) are cleaved; the method is more difficult and takes longer time. Although the *N*-(trifluoroacetyl) derivatives are more volatile, a maximum of six sugar residues was reached. With the columns now used permethylated decasaccharides were chromatographed, but the range could probably be increased by exchanging the *N*-acetyl with *N*-(trifluoroacetyl) groups.

The analysis of the ceramide and oligosaccharide parts of GSL mixtures after ceramide glycanase treatment and permethylation using high-mass GC and GC–MS will provide a rapid and simple method for the separation and characterization of GSLs. The excellent chromatographic resolution obtained by capillary GC and the availability of gas chromatographs in most laboratories working on GSLs will make the introduction of this analytical approach easy. Also, the information obtained by comparing retention times with those of known standards when no GC–MS equipment is available will be very useful.

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Fluorescent Molecular Rotors: A New Class of Probes for Tubulin Structure and Assembly†

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ABSTRACT: 9-(Dicyanovinyl)julolidine (DCVJ) is a fluorescent dye whose intramolecular rotational relaxation is solvent dependent. Since its quantum yield increases with decreasing free volume, this molecule has been very useful in monitoring synthetic polymer reactions and measuring local microviscosity changes in phospholipid bilayers [Loutfy, R. O. (1986) *Pure Appl. Chem. 58*, 1239-1248; Kung, C. E., & Reed, J. K. (1986) *Biochemistry 25*, 6114-6121]. We have used DCVJ to follow the polymerization of tubulin, a protein that can assemble into a variety of polymorphic microstructures. DCVJ binding to free tubulin is accompanied by an increase in quantum yield, indicating that DCVJ has become partially immobilized. At 4 °C, DCVJ binds to a single population of high-affinity hydrophobic sites (K_d = 1.12 ± 0.26 μM) with a stoichiometry that is protein concentration dependent. n, the number of moles of DCVJ bound per mole of $\alpha\beta$ dimer, approaches 1 at concentrations ≤0.5 mg/mL but decreases to a lower limit of ≈0.3 at concentrations ≥2.0 mg/mL. The quantum yield also increases with increasing protein concentration. This trend is unaltered by the presence of microtubule-associated proteins. These results are analyzed in terms of a concentration-dependent oligomerization of tubulin at 4 °C. When tubulin is polymerized at 37 °C to microtubules or to sheets in the presence of Zn^{2+}, the fluorescence intensity of DCVJ increases although the magnitude of this increase differs significantly. We are able to use the distinct fluorescent and binding characteristics of the bound dye to distinguish between these two polymorphs on a molecular level.

Microtubules are cylindrically ordered polymers that play a key role in the cytoskeletal architecture of all eukaryotic cells, providing form, shape, and mobility. They participate in a wide range of dynamic cellular functions including exocytosis, intracellular transport, and mitotic spindle formation to name but a few [for reviews, see Dustin (1984) and Roberts and Hyams (1979)].

The predominant protein of microtubules, comprising approximately 75-85% of the total mass, is tubulin, a heterodimer composed of two nonidentical α and β subunits, each of approximately 55 000 daltons. In addition, a number of nontubulin proteins, termed microtubule-associated proteins (MAPs),[1] are isolated with tubulin during purification and appear to play a crucial role in in vivo microtubule formation (Borisy et al., 1975).

The assembly of tubulin dimers to form complex oligomeric structures has been the subject of considerable research in a

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[1] Abbreviations: MAPs, microtubule-associated proteins; MTP, microtubule protein(s); DCVJ, 9-(dicyanovinyl)julolidine; PC-tubulin, phosphocellulose-purified tubulin; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DPPC, dipalmitoylphosphatidylcholine; GTP, guanosine 5′-triphosphate; SDS, sodium dodecyl sulfate.

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