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Structure Elucidation of Glycosphingolipids and Gangliosides Using High-Performance Tandem Mass Spectrometry†

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ABSTRACT: Glycosphingolipids and gangliosides have been investigated by using fast atom bombardment high-performance tandem mass spectrometry (FABMS/MS). Homologous compounds have been investigated in order to ascertain the fragmentation. Collision-induced dissociation spectra of the molecular species in the positive ion mode mainly afford information on the ceramide constitution (aglycon as a whole, *N*-acyl residue, and long-chain base), whereas negative ion spectra show fragments informative of the sugar sequence and the degree of branching of the carbohydrate. In the case of gangliosides carrying a complex oligosaccharide moiety, collision spectra of fragment ions have been performed in order to gain additional structural data. The advantage of tandem mass spectrometry over conventional fast atom bombardment mass spectrometry (FABMS) consists in the fact that collision spectra of the individual components from mixtures, as usually encountered with these kinds of samples, can be recorded. Furthermore, the exclusion of most of the interfering signals from the matrix allows the identification of pertinent fragments at low mass.

The growing interest in glycosphingolipids (GSLs)[1] and gangliosides, involved in many vital functions on cell membranes, such as cell surface antigens, cell–cell recognition sites, specific receptor for signal molecules, and signal transductor through the membrane (Hakomori, 1981; Ledeen & Yu, 1982; Karlsson, 1986), has stimulated the investigation of new techniques for their structural analysis. Mass spectrometry, a highly sensitive method, has been a key tool for the determination of of the primary structure of derivatives of these compounds (Sweeley & Nunez, 1985). Soft ionization techniques, such as field desorption (FD) (Costello et al., 1980; Kushi & Handa, 1982) and more recently fast atom bombardment (FAB) (Arita et al., 1983a,b; Hemling et al., 1983; Sonnino et al., 1986; Egge & Peter-Katalinic, 1987), have now made possible the analysis of underivatized glycosphingolipids. The latter method yields pertinent fragment ions as well as molecular weight information, but the identification of these signals, especially at low mass, is often hindered by the matrix background. On the other hand, heterogeneity in the ceramide as well as in the oligosaccharide moiety reflects the complexity of these structures and can complicate the interpretation of spectra.

Tandem mass spectrometry (MS/MS) (McLafferty, 1983) overcomes some of these limitations and thus increases the structural information. The recent availability of commercial high-performance tandem mass spectrometers has added a new dimension in the analysis of biomolecules (Biemann, 1986). In MS/MS, the ion associated with the molecular weight ($[M + H]^+$, $[M - H]^-$) or a fragment ion is selected in the first mass spectrometer MS-1 at a resolution of 1 mass unit. These ions collide with an inert gas such as helium in a collision cell located in the field-free region between MS-1 and MS-2. Finally, the product ions are analyzed, also at unit resolution, in the second mass spectrometer MS-2. The major advantage of this two-step process is that mixtures can be analyzed in a manner that yields structural information related solely to the ions selected by MS-1. This technique has already been used successfully for the analysis of polypeptides (Biemann, 1986; Biemann et al., 1986) and the determination of the structure of small peptides (Johnson & Biemann, 1987). The present work describes the application of this approach for the characterization of glycosphingolipids and gangliosides.

Experimental Procedures

Materials. Semisynthetic *N*-palmitoyl- (**1**), *N*-stearoyl- (**2**), and *N*-lignoceroyl- (**3**) dihydrogalactocerebrosides, *N*-palmitoyl- (**4**), *N*-stearoyl- (**5**), and *N*-lignoceroyl- (**6**) dihydrolactocerebrosides, and *N*-palmitoyl- (**7**), *N*-stearoyl- (**8**), and *N*-oleoyl- (**9**) galactocerebrosides were obtained from Sigma Chemical Co., St. Louis, MO (see Chart I). Glycosphingolipid asialo-GM_1 (**10**) and gangliosides GM_1 (**11**) and GD_{1a} (**12**) were obtained from Supleco Inc., Bellefonte, PA. Compound GD_{1b} (**13**) was supplied by Bachem Inc., Torrance, CA. Samples were dissolved in DMSO (5 $\mu g/\mu L$), and this solution

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[1] Abbreviations: FABMS, fast atom bombardment mass spectrometry; FABMS/MS, fast atom bombardment tandem mass spectrometry; CID, collision-induced dissociation; FD, field desorption; $[M + H]^+$, protonated molecular ion; $[M - H]^-$, deprotonated molecular ion; E, electric sector; B, magnetic field; MS-1, first of two high-resolution mass spectrometers in tandem; MS-2, second of two high-resolution mass spectrometers in tandem; GSL, glycosphingolipid; LCB, long-chain base; HPLC, high-performance liquid chromatography; *m/z*, mass to charge ratio; Hex, hexosyl or hexose; Sia, sialyl or sialic acid; Cer, ceramide.

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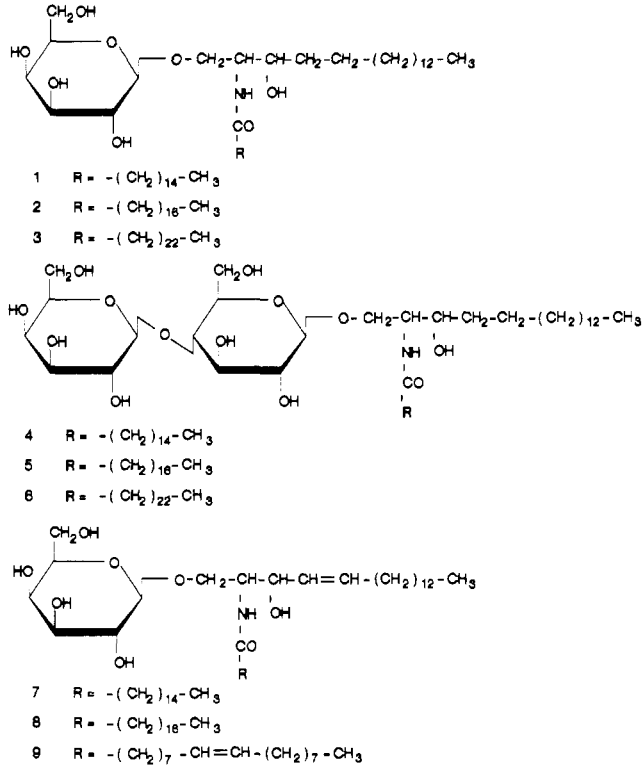
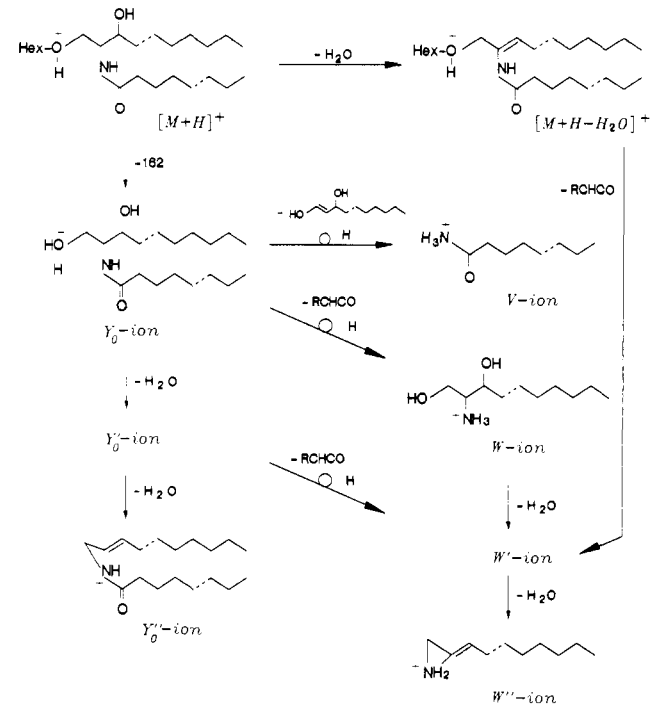
Chart I



Scheme I: Fragmentation Pathways of the Ceramide Moiety from Hexocerebrosides in the Positive Ion Mode[a]



[a] Dotted lines represent structural variations.

was mixed 1:1 with the matrix: glycerol (for the positive ion mode) or triethanolamine (for the negative ion mode). Spectra were recorded by using 0.3 μL of the resulting solution (ca. 1 μg of sample).

High-Performance Liquid Chromatography. Reversed-phase HPLC was carried out with Waters Associates Model 510 pumps, a Model 680 solvent programmer, a U6K injector, and a Waters 490 multiwavelength detector, monitoring at 206 nm (unless specified differently). A Waters μBondapak C_{18} column was used. Glycosphingolipids carrying only one sugar unit could be separated by reversed-phase HPLC using methanol as eluent at a flow rate of 1.2 mL/min (detection at 210 nm). More polar compounds (two sugar units or more) required the addition of 5–10% water to the mobile phase (isocratic conditions). Gangliosides were chromatographed under isocratic conditions with the acetonitrile/5 mM ammonium acetate buffer (pH 6.8) solvent system at a flow rate of 1.2 mL/min. The following compositions were used: 70:30 for GM_1 and 60:40 for GD_{1a} and GD_{1b}.

Fast Atom Bombardment Mass Spectrometry. FABMS of the glycosphingolipids and gangliosides was carried out on the first (MS-1) of the two mass spectrometers of a tandem high-resolution mass spectrometer (JEOL HX110/HX110) at 10-kV accelerating voltage and 1:1500 resolution, with 100-Hz filtering. For calibration $(CsI)_nCs^+$ and $(CsI)_nI^-$ cluster ions were used for the positive and negative ion modes, respectively. Single scans were acquired by scanning the magnet from m/z 200 to m/z 2000 in about 2.0 min. The JEOL FAB gun was operated at 6 kV with xenon as the FAB gas. Spectra shown are profile data of single scans and were recorded with a JEOL DA5000 data system.

Tandem Mass Spectrometry. FABMS/MS was carried out by using all four sectors of the JEOL HX110/HX110 spectrometer, an instrument of $E_1B_1E_2B_2$ configuration. Collision-induced fragmentation took place in the third field-free region, thus operating both MS-1 (E_1B_1) and MS-2 (E_2B_2) as double-focusing instruments. Helium was used as the collision gas at a pressure sufficient to reduce the precursor ion signal by 50%. The FABMS/MS spectra (linked scans of MS-2 at constant B/E ratio) were recorded at 30-Hz filtering, and the scan rate was the same as used for FABMS. MS-2 was usually operated at a resolution of 1:1000. MS-2 was calibrated with a mixture of CsI, NaI, KI, and LiCl in the positive ion mode and a solution of CsI in glycerol in the negative ion mode (Sato et al., 1987). FABMS/MS spectra shown are profile data of two or three accumulated scans.

Results and Discussion

Study of Glycosphingolipids in the Positive Ion Mode. The spectra of glycolipids carrying homologous fatty acid chains were investigated in order to ascertain the fragmentation.

Dihydroglycosphingolipids. Figure 1A shows the collision-induced decomposition (CID) MS/MS spectrum of the FAB-generated protonated molecular ion (m/z 702.6) of *N*-palmitoyldihydrogalactocerebroside (**1**). The most abundant fragment at m/z 540 results from the loss of the hexosyl unit, to yield the protonated ceramide moiety. This ion $(Y_0)^2$ can undergo subsequent fragmentation as summarized in Scheme I. The different pathways, which afford fragments that contain either the long-chain base (LCB) or the acyl residue, allow an easy characterization of the aglycon subunits. The ceramide can lose one molecule of water to yield the ion at m/z 522 (Y'_0) and a second to produce the ion at m/z 504 (Y''_0).

[2] Symbols different from those previously described (Hemling et al., 1984; Ohashi, 1987) are used in the present work for representing the various fragmentations because the fragmentation in CID spectra of glycosphingolipids is more complex than that observed in normal FABMS. V and W represent positive fragment ions, and S and T represent negative ions. A, B, and C and X, Y, and Z are used to designate ions resulting from cleavages within the carbohydrate portion in either mode. The former represent fragments containing the terminal sugar moiety (linear sugar moiety), whereas the latter refer to ions still containing the aglycon. Superscripts indicate the bond cleavages within the sugar rings. Subscripts indicate position relative to the termini analogous to the system in use for FABMS of peptides. A detailed description of this nomenclature will be published elsewhere.

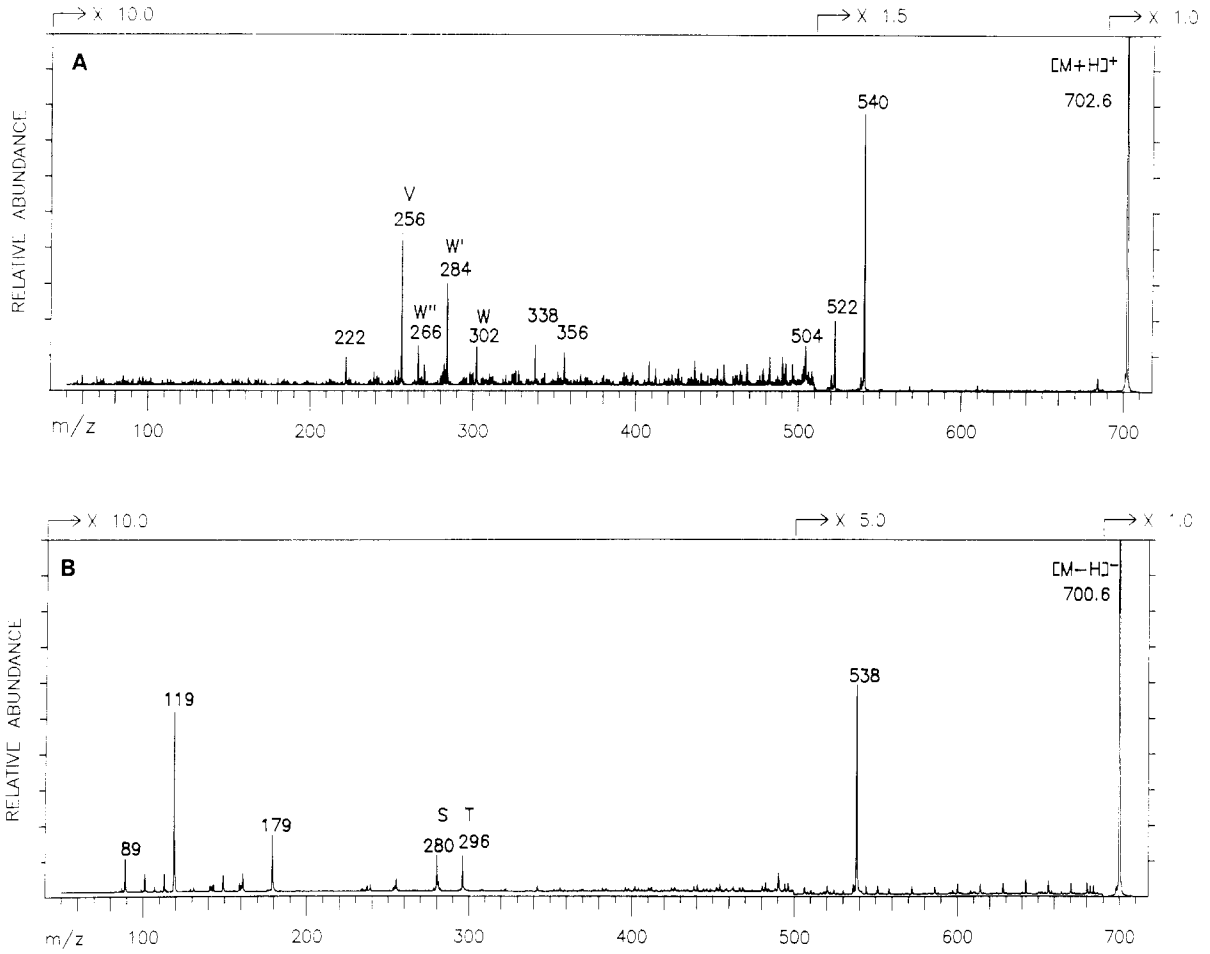


FIGURE 1: FABMS/MS spectra of *N*-palmitoyldihydrogalactocerebroside (**1**): (A) CID of $[M + H]^+$ m/z 702.6; (B) CID of $[M - H]^-$ m/z 700.6.

The intense signal at m/z 256 represents the protonated palmitamide (V fragment). An alternative fragmentation yields the ions at m/z 302 (W ion), 284 (W′ ion) and 266 (W″ ion), thus characterizing the long-chain base as dihydrosphingosine. The peak at m/z 222 very likely represents the protonated (iminoethyl)galactoside.

The collision spectra of *N*-stearoyldihydrogalactocerebroside (**2**) and *N*-lignoceroyldihydrogalactocerebroside (**3**) showed a very similar pattern with a shift of 28 and 112 daltons, respectively, of the fragments containing the acyl residue. In the spectrum of compound **2**, the acylamide fragment was observed at m/z 284, but in this case there was overlap with the isobaric long-chain base ion. The ceramide ions were shifted to m/z 568 and 550. In a similar way, all fragments of homologue **3** still containing the lignoceroyl group were shifted by 112 daltons, and thus the V ion was clearly observed at m/z 368.

The study was then extended to compounds carrying a disaccharide moiety; Figure 2A shows the tandem mass spectrum of *N*-palmitoyldihydrolactocerebroside (**4**; $[M + H]^+$ precursor ion at m/z 864.7). The main fragmentation pathway involves the elimination of the sugar moiety, to yield the protonated aglycon at m/z 540 and its dehydration product at m/z 522, the cleavage of the interglycosidic bond being comparatively only a minor process (ion at m/z 702). The fragments characteristic of the ceramide subunit are identical with those previously observed in the monoglycosylated analogue **1**. Additionally, two small signals at m/z 163 and 145, resulting from the sugar moiety, are observed in this case. Here again, the two homologues, the *N*-stearoyl (**5**) and *N*-lignoceroyl (**6**) derivatives, were investigated in order to ascertain the fragmentation of the ceramide moiety. As expected, the ions carrying the acyl residue showed a shift of 28 and 112 daltons, respectively, while the signals attributed to the long-chain base remained unchanged.

Finally, two series of homologous fragments, originated from the protonated aglycon and its dehydrated form, are observed. These correspond to the elimination of alkane [$H(CH_2)_nH$, n = 1–12, in the case of palmitoyl derivatives]. Similar fragmentations along hydrocarbon chains remote from the charge site have previously been reported for alkali metal cationized fatty acids and have been used for the determination of double bond location (Adams & Gross, 1987).

Cerebrosides. Glycosphingolipids containing an unsaturated long-chain base are much more common. Three homologous cerebrosides with various fatty acid chains have been investigated, namely, *N*-palmitoyl- (**7**), *N*-stearoyl- (**8**), and *N*-oleoyl- (**9**) galactocerebrosides. The presence of a double bond at C(4)–C(5) greatly affects the fragmentation pattern. The CID spectrum of the protonated molecular ion of compound **7** (precursor ion at m/z 700.6) is shown in Figure 3 as an example. The pathway yielding a characteristic ion of the acyl group (V ions), is mostly suppressed in favor of the pathway producing W fragments (in analogy with Scheme I). In this case, the W ion at m/z 300 is not observed, but its dehydration products (m/z 282 and 264) show strong signals, that at m/z 264 being the most abundant fragment. The dominant elimination of water from the molecular species and the dehydration of the aglycon also contrast with the saturated compounds.

Glycosphingolipids that contain a saturated base appear to decompose via the the route $[M + H]^+$ to Y_0 to Y'_0 to Y''_0

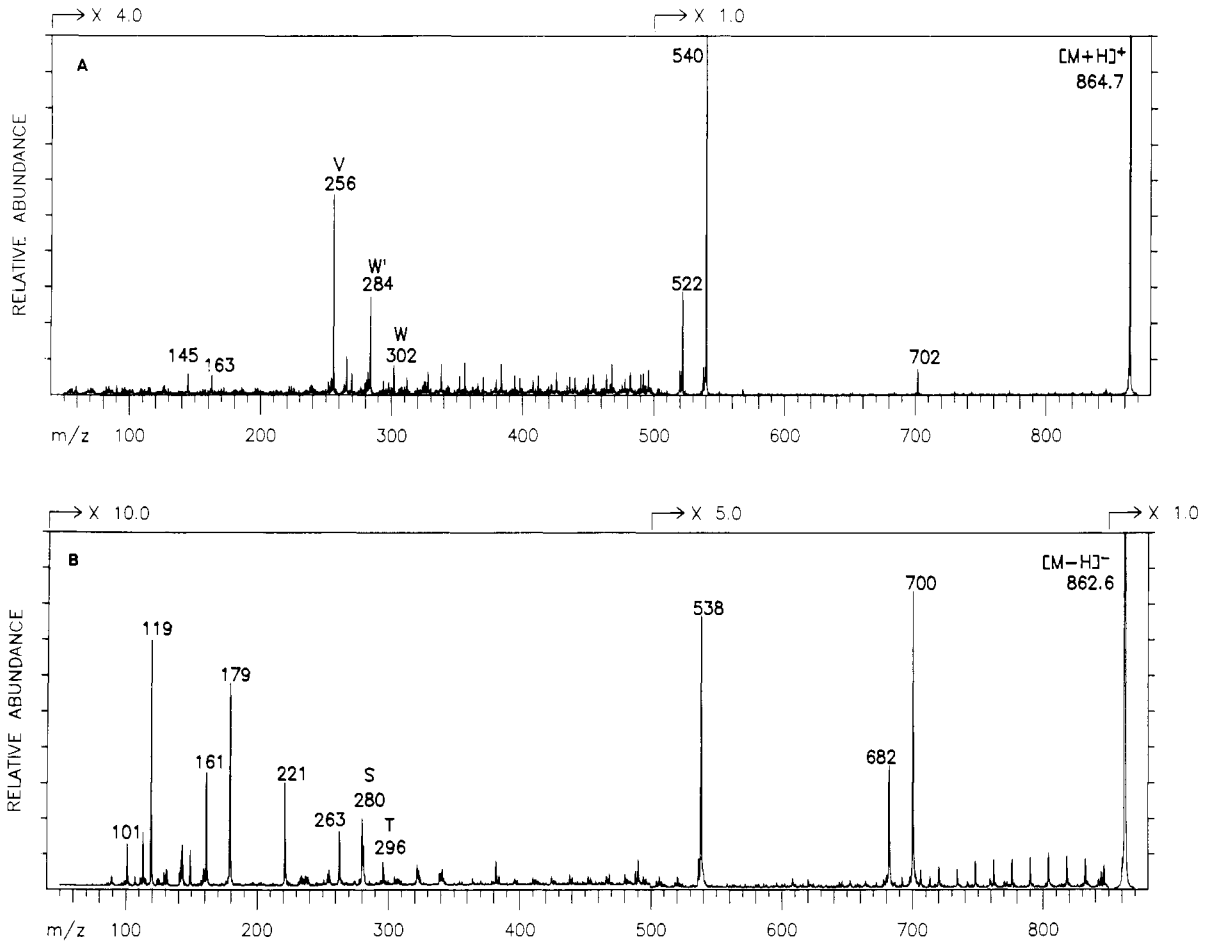


FIGURE 2: FABMS/MS spectra of *N*-palmitoyldihydrolactocerebroside (**4**): (A) CID of $[M + H]^+$ m/z 864.7; (B) CID of $[M - H]^-$ m/z 862.6.

(low abundance). Dehydration from $[M + H]^+$ is only a very minor process. From Y_0, either hydroxy group may be lost in the first dehydration step to form Y'_0 and the second lost to form Y''_0. GSLs with a double bond proximate to the original hydroxy group very easily lose water from $[M + H]^+$ to form the conjugated diene $[(M + H) - H_2O]^+$ and then lose hexose (162 daltons) to form the Y'_0 ion. Because Ohashi et al. (1987) did not observe m/z 538 in their two-sector constant B/E linked scan mass spectrum of **7**, they concluded that the hexose was lost in a single 180-dalton step from $[M + H]^+$. On the basis of our results with this and related compounds reported here, we would rather propose that the decomposition occurs as indicated in Scheme I, an interpretation that will also accommodate their constant B/E and B^2/E scan data.

The signal at m/z 222 is again observed as well as a new fragment at m/z 332, which can probably be attributed to an ion formed by the cleavage of the Y''_0 bond β to the conjugated diene. In this case, the losses of alkane from the aglycon (or its dehydrated form) are of minor importance. Fragments are observed at m/z 518, 502, and 488, corresponding to elimination of H_2, water, and methanol, respectively, from the Y_0 ion. The spectra of the homologous compounds **8** and **9** showed a very similar pattern, fragments W′ and W″ remaining unshifted. The "new" fragment (m/z 332 for **7**), on the contrary, underwent the expected displacement to m/z 360 and 358 for **8** and **9**, respectively.

As demonstrated by these examples, CID MS/MS of the protonated molecular ion of *N*-acyldihydrohexocerebrosides affords pertinent information on the ceramide and its constituents, the long-chain base and the acyl residue. This fact is of immediate interest, since samples from biological sources often contain mixtures resulting from heterogeneity in the lipophilic part. The method is very useful for specifying the location of the double bond, which could occur either in the long-chain base or in the fatty acid. Such a distinction would not be possible by conventional mass spectrometry. This approach has been applied to the structure determination of glycosphingolipids from parasitic helminths (tapeworms), in which the MS/MS data made evident the presence of isomeric compounds that contained phytosphingosine or sphingosine and nonhydroxylated or hydroxylated *N*-fatty acids (Singh et al., 1987).

Study of Glycosphingolipids in the Negative Ion Mode. In order to gain complementary information regarding the sugar moiety, data not found in the positive ion collision spectra, a simultaneous investigation of the CID spectra in the negative ion mode was carried out.

Dihydroglycosphingolipids. The spectrum obtained for the dihydroglycosphingolipid **1** (precursor ion m/z 700.6) is shown in Figure 1B. In this mode, information on both the sugar moiety and the ceramide can be gained. The deprotonated aglycon ion can be identified at m/z 538. Subsequent fragmentation has yielded the two ions observed at m/z 280 and 296. These fragments (S and T) can also be observed in the conventional negative FAB mass spectrum (as long as the sample concentration is relatively high, so that the matrix background is not too important) as reported previously by Hemling et al. (1986). The structure of these ions, which can be formulated as the cleavage of the C(2)–C(3) bond of the long-chain base, is shown in Scheme II. Since the S and T fragments contain the acyl residue, they are very informative

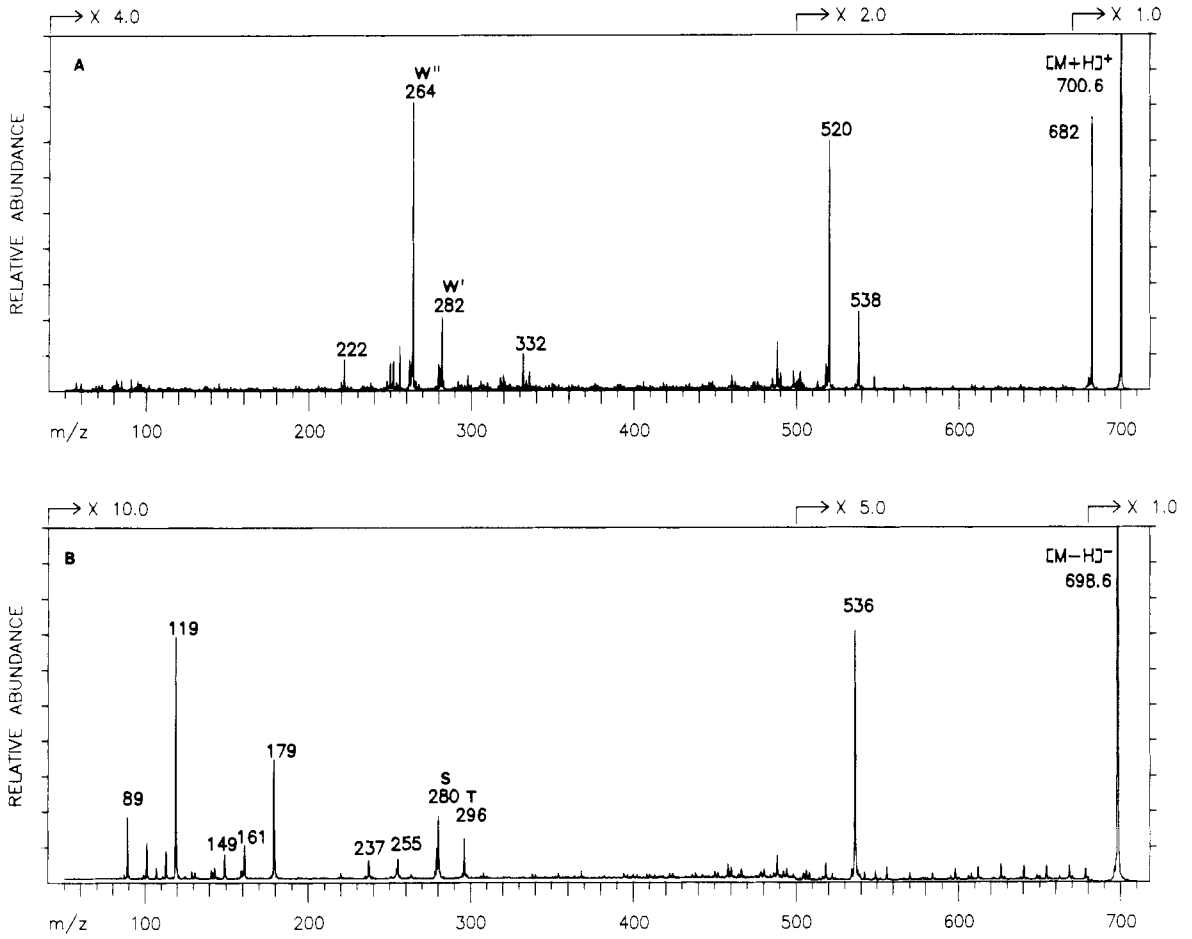
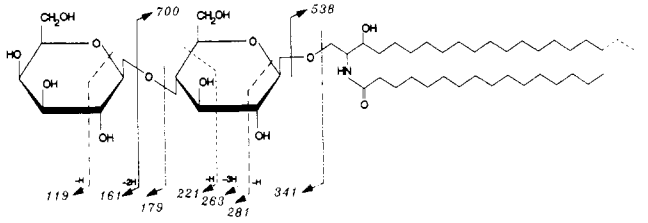


FIGURE 3: FABMS/MS spectra of *N*-palmitoylgalactocerebroside (**7**): (A) CID of $[M + H]^+$ m/z 700.6; (B) CID of $[M - H]^-$ m/z 698.6.

Chart II: Fragmentation of the Lactosyl Moiety of Lactocerebrosides (Negative Ion Mode)



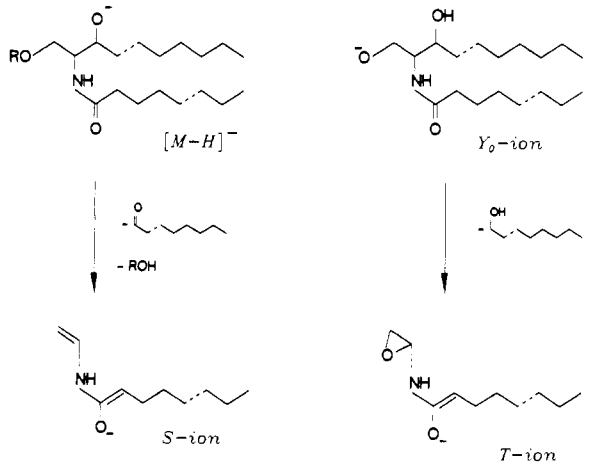
regarding the constitution of the ceramide. It should be noted that these fragments are not observed in the positive ion mode and thus provide an independent set of data.

Additionally, fragments from the sugar moiety are observed at m/z 179, 161, 149, 119, 101, and 89. The ion at m/z 179 corresponds to the deprotonated galactose unit. All other fragments involve a cleavage of the sugar ring, as rationalized in Chart II, for the spectrum shown in Figure 2B. In contrast to the positive ion CID spectrum (in which the sugar moiety is eliminated prior to the alkane losses), alkanes $[H(CH_2)_nH$, n = 1–12] are directly eliminated from the molecular species $[M - H]^-$. Such eliminations have been reported for negative ion CID spectra of fatty acids and used for the characterization of branched hydrocarbon chains (Tomer et al., 1986).

In the CID MS/MS spectrum of compound **2** ($[M - H]^-$ precursor ion at m/z 728.6), the ceramide and S and T ions were shifted to m/z 566, 324, and 308, respectively. In a similar way, the corresponding fragments of glycolipid **3** [$(M - H]^-$ precursor m/z 812.6) were observed at m/z 650, 392, and 408.

The negative ion collision spectrum of glycosphingolipid **4** ($[M - H]^-$ precursor m/z 862.6) (Figure 2B) shows abundant

Scheme II: Fragmentation Pathways of the Ceramide Moiety of GSLs in the Negative Ion Mode[a]



[a] Dotted lines represent structural variations.

product ions in the upper part of the spectrum (m/z 700 and 538) that arose by cleavage of the glycosidic bonds. These ions clearly indicate the sugar sequence. The two peaks at m/z 280 and 296 correspond to ions S and T and allow the identification of the fatty acid part of the aglycon. All other ions represent fragments from the disaccharide unit. Besides ions characterizing the terminal moiety already encountered (m/z 179, 161, 149, 119, and 101), additional signals at m/z 221, 263, and 281 resulting from the fragmentation of the second sugar unit are observed.

Cerebrosides. Negative ion CID spectra of the unsaturated species showed a pattern very similar to that of the corre-

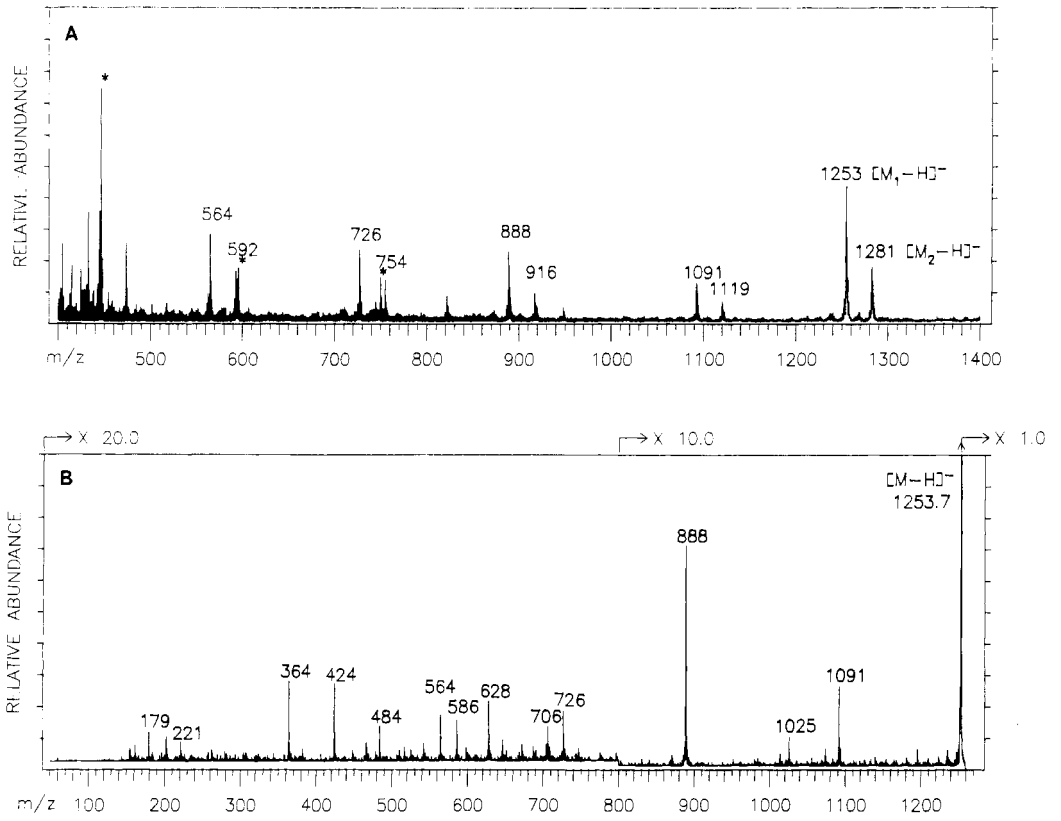


FIGURE 4: (A) Negative ion FABMS spectrum of glycosphingolipid asialo-GM_1 (*represents triethanolamine signals); (B) FABMS/MS spectrum of $[M_1 - H]^-$ m/z 1253.7.

sponding dihydro homologues. The fragments were identical with those observed for the glycosphingolipids containing saturated bases, except for those containing the long-chain base, which were shifted to lower mass by 2 daltons (see Figure 3B). Here again, a series of homologous ions corresponding to the alkane elimination from the molecular ion is observed.

More Complex Glycosphingolipids. The glycosphingolipids encountered in biological systems often contain larger oligosaccharide moieties (up to eight sugar units or even more) and are frequently end capped with sialic acid (*N*-acetylneuraminic acid) and are then called gangliosides. As the FAB sensitivity for underivatized gangliosides in the positive ion mode is more adversely affected by the presence of minor contaminants than is the negative ion sensitivity and thus may not be sufficient for carrying out tandem mass spectrometric experiments, investigation of these compounds was performed exclusively in the negative ion mode. Since sialic acid units can be selectively released, by chemical (mild hydrolysis) or sometime enzymatic (*N*-acetylneuraminidase) degradation, to produce the asialo glycoconjugate, the tetraglycoside asialo-GM_1, which results from the degradation of gangliosides of the series GM_1, GD_1, or GT_1, was also included in this study.

The normal negative ion mass spectrum of asialo-GM_1 (Figure 4A) shows two homologous molecular ions at m/z 1253 ($[M_1 - H]^-$) and 1281 ($[M_2 - H]^-$) due to the presence of both sphingosine (C_{18}) and eicosasphingosine (C_{20}) as long-chain bases. Fragments of the former species can be observed at m/z 1091 (Y_3), 888 (Y_2), 726 (Y_1), and 564 (Y_0), allowing a quick identification of the linear sugar sequence. The differences between the fragments of 162, 203, 162, and 162 again indicate the following sequence: hexose (terminal unit), *N*-acetylhexosamine, hexose, hexose, the last ion of this series (m/z 564) representing the deprotonated aglycon. The other signals result from the presence of the homologous compound (28 daltons heavier). However, no fragments allowing the characterization of the ceramide units (the variation being possible either in the long-chain base or in the fatty acid) can be observed at lower masses because of the interference from matrix-related ions. Fragments representing the sugar moiety alone also cannot be distinguished.

Some of this information can be gained from a CID MS/MS mass spectrum of the deprotonated molecular species, shown in Figure 4B. In this case, the precursor ion at m/z 1253.7 was selected for the collision spectrum, and the resulting product ions are summarized in Chart III. The most abundant product ion at m/z 888 (Y_2) results from the preferential cleavage of the interglycosidic bond involving the 2-deoxy-2-acetamido sugar unit, fragmentation of the other acetal bonds being observed at m/z 1091 (Y_3, loss of the terminal hexose), m/z 726 (Y_1, loss of the trisaccharide moiety), and m/z 564 (Y_0, deprotonated ceramide).

Additional fragments originating from the oligosaccharide portion are observed. Some of them result from the cleavage of the interglycosidic bond on either side of the oxygen atom, to yield either B_i or C_i ions (the former being formally dehydration products of the latter). Thus signals at m/z 179 (C_1), 706 (C_4), 161 (B_1), and 364 (B_2) involve such cleavages. Another set of signals, already encountered in the simpler glycosphingolipids, results from the cleavage of the sugar rings (A ions). In the present case, where all interglycosidic linkages are either (1→3) or (1→4), ions ($^{2,4}A_i$ ions) are observed 42 daltons higher than the corresponding C_{i-1} fragments, a shift that represents the additional C(3)H=C(4)H—O unit of the adjacent sugar moiety. These quite abundant signals are present at m/z 221 ($^{2,4}A_2$), 424 ($^{2,4}A_3$), and 586 ($^{2,4}A_4$), respectively. One other type of cleavage involves the elimination of the C(1)–C(2) unit ($^{0,2}A_i$ ions). Such ions are observed at m/z 484 ($^{0,2}A_3$) and 646 ($^{0,2}A_4$). A further fragmentation involving the sugar unit attached to the ceramide is observed at m/z 628 ($^{2,5}A_4$).

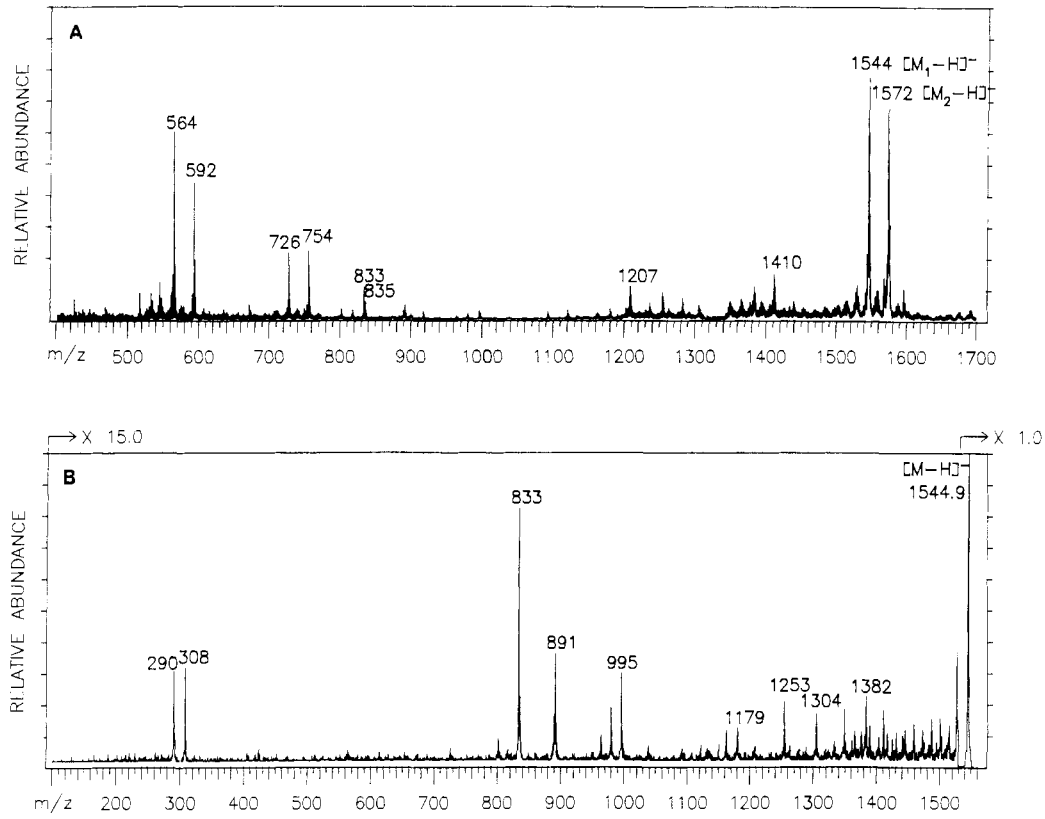
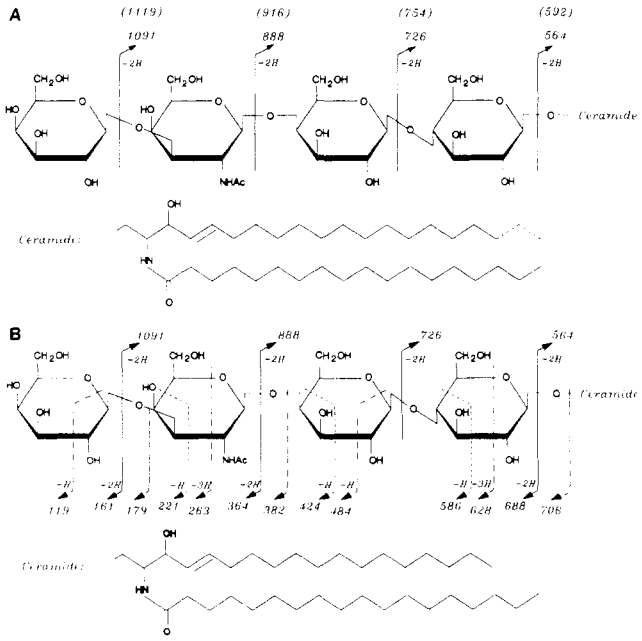


FIGURE 5: (A) Negative ion FABMS spectrum of ganglioside GM_1; (B) FABMS/MS spectrum of $[M_1 - H]^-$ m/z 1544.9.

Chart III: Fragmentation of the Glycosphingolipid Asialo-GM_1[a]



[a] (A) Negative ion FABMS (dotted lines represent the heterogeneity of the long-chain base, and the corresponding fragments are given in parentheses); (B) FABMS/MS of $[M_1 - H]^-$ [(—) represents cleavage of the glycosidic bond without retention of the oxygen atom, (-·-) represents cleavage of the glycosidic bond with retention of the oxygen atoms, and (---) represents the sugar ring cleavages].

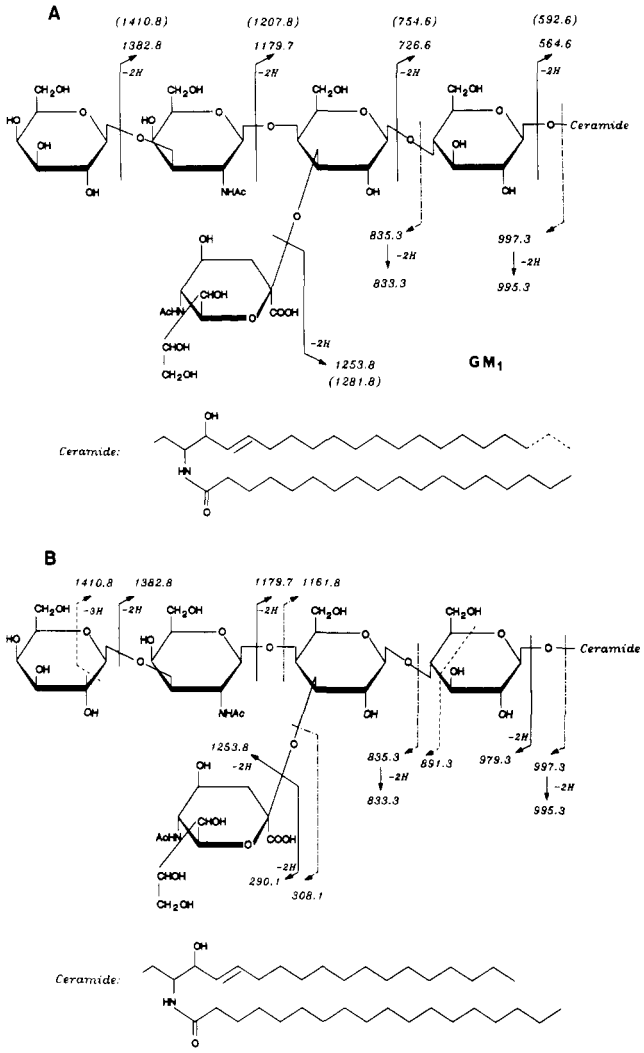
A similar collision spectrum was observed for the homologous precursor ion at m/z 1281.7. All sugar fragments remained unshifted, whereas the Y_i-type ions appeared 28 daltons higher. We speculate that other substitution patterns [i.e., (1→2) or (1→6)] would probably not yield these ions.

At this stage, no information regarding the ceramide constituents could be obtained. Nevertheless, fragments corresponding to the aglycon ion and its monoglycosylated conjugate are present in the normal FAB mass spectrum, and by performing CID on these fragment ions, such data may be gained. The collision spectra (not shown) of the fragments at m/z 564 and 592 (corresponding to the ceramides) as well as those at m/z 726 and 754 (monoglycosylated ceramides) all showed signals at m/z 308 and 324. These ions corresponded to the S and T fragments (both containing the *N*-acyl group) and thus clearly indicated, as they remained unshifted, that the homology is located in the long-chain base.

Gangliosides. Besides the molecular species (m/z 1572 and 1544) the most abundant ions in the negative ion FAB spectrum of the ganglioside GM_1 are those of the ceramide fragments and their monoglycosylated homologues (Figure 5A). A pair of two intense signals at m/z 835 (C_3 ion) and 833 (C_3 − 2H) corresponds to the branched terminal tetrasaccharide moiety and its dehydro form. A similar set of signals is observed at m/z 997 (C_4) and 995 (C_4 − 2H), resulting from the cleavage of the pentasaccharide moiety. Additionally, the corresponding B_4 ion is observed at m/z 979. The primary ions (containing the sialic acid unit) formed by the fission of the glycosidic bonds with retention of the glycosidic oxygen are always accompanied by another ion 2 daltons lower, formed by elimination of H_2 as previously observed by Egge et al. (1985). The CID spectra of fragments at m/z 833 and 835 (not shown) indicate that H_2 elimination, which may likely be related to the presence of the sialic acid unit, takes place on the disubstituted sugar unit. For both m/z 833 and m/z 835, the collision spectra showed signals at m/z 290 and 308, characterizing the intact *N*-acetylneuraminic acid unit. In addition, product ions corresponding to the elimination of the terminal hexose and Hex-HexNAc moieties were observed, thus demonstrating that none of these sugar units was involved in the hydrogen elimination.

In the normal negative ion spectrum weaker signals were observed at m/z 1410 (1382) and 1207 (1179), corresponding

Chart IV: Fragmentation of the Ganglioside GM_1[a]



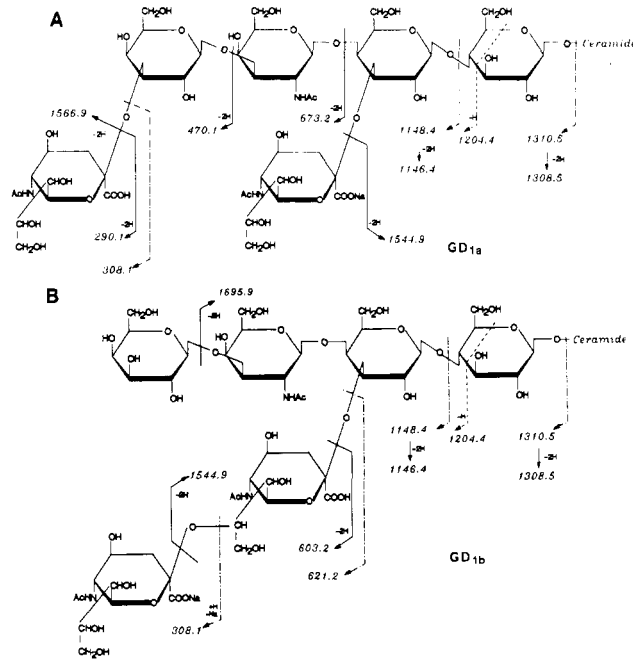
[a](A) Negative ion FABMS; (B) FABMS/MS of $[M_1 - H]^-$ (see Chart III for explanation of the fragmentations).

to the split of the terminal hexose and the hexosylacetamidohexose moieties (Y_4 and Y_3 ions), thus providing some information on the sugar sequence. However, only a partial structure could be established from such data.

In order to get a more informative pattern, the collision spectra of both molecular species were recorded. Figure 5B shows the product ion spectrum of m/z 1544.9. The most intense signals are sugar fragments containing the sialic acid unit: m/z 290, 308, 833, 979, and 995. It should be noted that the cleavage of the sugar bond (with retention of the glycosidic oxygen) is accompanied by fragments resulting from the the loss of hydrogen (H_2) or water and these fragments are abundant only in the collision spectra (see Chart IV).

Another fragmentation involving the cleavage of the sugar unit attached to the ceramide is also observed (m/z 891). In contrast to the previous observation, the C(3)–C(4) bond is broken as well as the C(5)–O bond, to yield an ion containing the C(4)–C(5)–C(6) unit. Some other signals, namely, those at m/z 1382, 1179, 1161, and 1253 are indications of the sugar sequence; they correspond to the elimination of a hexosyl, a hexosylacetamidohexosyl, and a sialyl moiety, respectively. They fit perfectly with the expected structure, assuming that single glycosidic bond cleavages occur (primary fragments). Contrary to the normal mass spectrum, no "secondary" fragmentation is observed in the collision spectrum. The upper end of the spectrum contains the peaks resulting from alkane

Chart V: Structures and Fragmentation of Isomeric $[M - 2H + Na]^-$ Gangliosides GD_{1a} (A) and GD_{1b} (B)



losses. Except m/z 1253 (formally the asialo-GM_1 ion), all fragments retain the sialic acid residue, which very likely carries the negative charge. Obviously, the presence of sialic acid unit thus drives the fragmentation in a very specific direction and produces spectra completely different from those of neutral glycosphingolipids.

As noted above, since the sialic acid moieties may be released specifically, by mild acidic hydrolysis, for example, information regarding the neutral oligosaccharide moiety may be obtained by measuring CID spectra of the resulting asialoglycosphingolipid. Further characterization of the ceramide was made possible by recording the collision spectra of fragments at m/z 564, 592, 726, and 754 (not shown). They corresponded to the spectra described above for simpler glycosphingolipids.

The two isomeric gangliosides GD_{1a} and GD_{1b} have also been investigated. In order to obtain single pure compounds, samples were purified by reversed-phase HPLC using a solvent system containing acetonitrile/ammonium acetate buffer (pH 6.8) as eluent. The FAB spectrum spectrum of the ganglioside GD_{1a} showed two molecular species, namely, the deprotonated $[M - H]^-$ ion and its sodium adduct $[M - 2H + Na]^-$ at m/z 1835 and 1857, respectively. Y_i-type ions were observed at m/z 1544, 1382, 1179, 1091, 726, and 564 (ceramide) and have been formulated in Chart V. Fragments resulting from subsequent cleavages were also observed at m/z 1091 and 888. Some sugar ions (C_i ions) could be observed at m/z 308, 470, and 673. The isomeric GD_{1b} compound showed the following fragments: m/z 1544, 1253, 726, and 564 (Y_i ions), fragments resulting from double cleavages at m/z 1091 and 888, as well as a signal at m/z 553, absent in the previous spectrum but characteristic of a disialyl unit ($599 - CO_2 - H_2$). Although this spectrum clearly indicated that two sialic acid units were bonded together, the simultaneous presence of secondary fragements did not allow the differentiation between the proposed branched structure (Chart V) and a compound with a linear oligoglycosidic portion (i.e., Sia-Sia-Hex-HexNAc-Hex-Hex-Cer).

To make this distinction, collision mass spectrometry can be a useful tool. The product ion spectra of both species [M

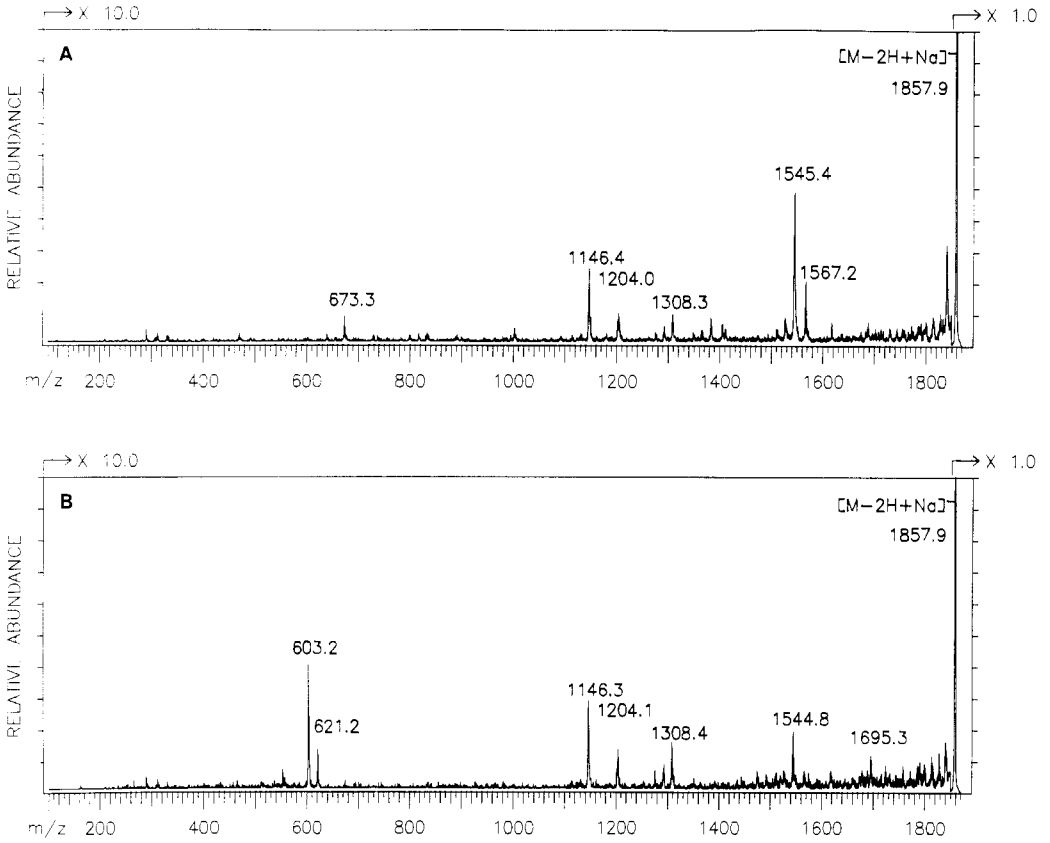


FIGURE 6: FABMS/MS spectra of isomeric gangliosides GD_{1a} (A) and GD_{1b} (B); precursor was $[M - 2H + Na]^-$ cluster m/z 1857.9. (Note that, for the m/z values in the upper part of the spectra, rounding off of the high fractional masses would yield values 1 dalton higher than the nominal mass. Calculated values are given in Chart V.)

$- H]^-$ and $[M - 2H + Na]^-$ were measured, and the latter are shown in Figure 6. For GD_{1b}, the main fragment at m/z 603 results from the dehydration of the sodium adduct of the disialyl moiety at m/z 621. The signals at m/z 1146 and 1308 correspond to the dehydropentaglycosyl unit and the dehydrohexasaccharide unit, respectively. The peak at m/z 1204 originates from the cleavage of the sugar unit linked to the ceramide, as previously observed for GM_1. Loss of the terminal sialyl unit yields the ion observed at m/z 1544.8. The ion at m/z 1696, although not very abundant, is a key fragment since it results from the elimination of a terminal hexose moiety and therefore indicates that the two sialic acid units, linked together, must be bonded to an internal sugar unit.

The spectrum of GD_{1a} is completely different from that of GD_{1b} and thus demonstrates that MS/MS spectra of sodium adducts distinguish isomers that differ in the position of sialic acid(s). The fragments containing the carbohydrate portion are observed at m/z 290, 469, 673, 1146, and 1308 and allow the determination of the complete sugar sequence. Again, a fragment involving a cleavage across the sugar moiety attached to the aglycon is present (m/z 1204).

Conclusion. This investigation of glycosphingolipids and gangliosides by high-performance tandem mass spectrometry, in both the positive and negative ion modes, has shown that much information regarding the lipophilic part as well as the glycosidic moiety can be gained and has thus demonstrated the potential of this new method for the structure elucidation of glycoconjugates using only a few micrograms of sample.

Further studies will explore the MS/MS spectra of derivatized glycosphingolipids and gangliosides in order to increase the sensitivity in the positive ion FAB mode for compounds carrying a negatively charged sugar moiety and larger oligosaccharides. At the same time, more control over fragmentation may be achieved.

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Structural Characterization of Several Galactofuranose-Containing, High-Mannose-Type Oligosaccharides Present in Glycoproteins of the Trypanosomatid *Leptomonas samueli*†

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ABSTRACT: It was reported before that cells of the trypanosomatid *Leptomonas samueli* incubated with [^{14}C]glucose synthesized dolichol-P-P-linked $Man_9GlcNAc_2$ as the main and largest derivative. It is now reported that this protozoan is deficient in dolichol-P-Glc synthesis as judged from results obtained in a cell-free assay. We have structurally characterized several endo-β-*N*-acetylglucosaminidase H sensitive oligosaccharides present in mature glycoproteins of this parasite. The compounds appeared to have the compositions $Gal_3Man_9GlcNAc_2$, $Gal_2Man_9GlcNAc_2$, $Gal_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $Gal_1Man_8GlcNAc_2$, $Man_8GlcNAc_2$, $Gal_1Man_7GlcNAc_2$, and $Man_7GlcNAc_2$. The galactose residues were in all cases in the furanose form and linked to mannoses in nonreducing ends. In the cases of $Gal_1Man_8GlcNAc_2$ and $Gal_1Man_7GlcNAc_2$, the galactose-substituted mannose units were the nonreducing residues originally present in the oligosaccharide transferred from dolichol-P-P ($Man_9GlcNAc_2$) and not the nonreducing termini generated by demannosylation of the latter oligosaccharide. Except for $Gal_3Man_9GlcNAc_2$, the other galactosylated compounds appeared to be mixtures of several isomers.

Protein N-glycosylation in most eucaryotes is initiated by the transfer of an oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a dolichol-P-P derivative to asparagine residues in incompleted or recently completed polypeptide chains. Processing of the oligosaccharide occurring as the glycoproteins migrate through the endoplasmic reticula and the Golgi apparatus leads, in mammalian cells, to the formation of high-mannose-, complex-, or hybrid-type compounds. The high-mannose compounds contain two *N*-acetylglucosamine and five to nine mannose units, and the complex oligosaccharides contain two *N*-acetylglucosamine, three mannose, and additional *N*-acetylglucosamine, galactose, sialic acid, and fucose units, whereas the hybrid compounds have both high-mannose- and complex-type characteristics (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985).

Trypanosomatids are parasitic protozoa, some of which are the causative agents of endemic diseases in developing countries (Chagas's disease, sleeping sickness, visceral leishmaniasis or kala-azar disease, etc.). Protein N-glycosylation in those microorganisms involves transfer of oligosaccharides devoid of glucose and containing six, seven, or nine mannose units depending on the species (Parodi et al., 1981, 1984a; Parodi & Quesada-Allue, 1982; Previato et al., 1986). *Crithidia fasciculata* and *Crithidia harmosa* were found to transfer $Man_7GlcNAc_2$. Processing of this oligosaccharide involved removal of a single mannose unit from either one of the two nonreducing ends and addition of one galactofuranose residue to the nonreducing mannoses originally present in the transferred oligosaccharide (Figure 1). Thus, two $Gal_1Man_6GlcNAc_2$ isomers were generated (Mendelzon & Parodi, 1986). It should be noted that only galactopyranose units have been found to be present in complex-type, protein-linked oligosaccharides of mammalian cells.

We have also detected galactofuranose residues in high-mannose-type oligosaccharides present in glycoproteins of two species of trypanosomatids transferring $Man_9GlcNAc_2$ from the dolichol-P-P derivative, *Leptomonas samueli* and *Herpetomonas samuelpessoai*. Galactose (and not mannose) residues were liberated from the oligosaccharides under relatively mild acid conditions, and in addition, galactose was converted to arabinose units when the oligosaccharides were first treated with sodium periodate under conditions in which only bonds between exocyclic diols were broken and then reduced with sodium borohydride (Mendelzon et al., 1986).

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