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Analysis of Permethylated Glycosphingolipids by Desorption Chemical Ionization/Triple-Quadrupole Tandem Mass Spectrometry

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Permethylated glycosphingolipids have been studied by using desorption chemical ionization/triple-quadrupole tandem mass spectrometry. Collisional-induced dissociation of protonated molecular ions mainly produced the sugar sequence ions. Long-chain base as well as fatty acid daughter ions were observed for ceramides with a saturated long-chain base whereas only long-chain base daughter ions were detected if there was a double bond at the C(4)–C(5) position of the long-chain base. These daughter ions provide useful information for the structure of the ceramides. The absence of fatty acid daughter ions for ceramides with an unsaturation site at the C(4)–C(5) position was postulated with a rearrangement reaction involving the transfer of a hydrogen atom from the C(5) of the long-chain base to the nitrogen atom of the acylamide (fatty acid) residue.

INTRODUCTION

Glycosphingolipids (GSLs) are cell membrane components. The growing evidence that GSLs are involved in many important biological functions has stimulated the search for new analytical techniques for their structural analysis. There are three different molecular moieties in GSLs: a long-chain base, a fatty acid, and a carbohydrate residue. The hydrophobic moiety, which is a ceramide, consists of the long-chain base substituted at the amino group by a fatty acid. The carbohydrate moiety is linked at the primary hydroxy group of the long-chain base.

Mass spectrometry, a highly sensitive technique, has been a key tool for the structural elucidation of GSLs.[1–16] Classical electron impact (EI) ionization has been successfully applied to permethyl and carboxyl reduced permethyl GSLs.[1,2] The EI mass spectra of these compounds provide information on both sugar sequence and ceramide composition. Although the EI approach is quite sensitive in comparison to conventional chemical methods, the molecular ions are often of low abundance and 8 μg or more of samples are still needed to observe structurally diagnostic fragment ions.[2] Since its development in the early 1980s, fast atom bombardment (FAB) mass spectrometry has quickly become the method of choice for the mass spectral analysis of polar, non-volatile compounds such as proteins, oligosaccharides and glycoconjugates. FAB, both in the positive and in the negative ion mode, has been successfully applied to the analysis of derivatized and underivatized GSLs.[6–10] Another soft ionization technique, desorption chemical ionization (DCI), has also demonstrated its potential in the analysis of oligosaccharide and GSLs,[4,17] but the success of FAB mass spectrometry has to some extent overshadowed this technique. This is unfortunate in that DCI mass spectra often provide structural information complementary to FAB data, and unlike FAB mass spectra they are free of matrix ions.

When soft ionization techniques such as FAB, DCI, field desorption, thermospray, electrospray, etc., is chosen as the ionization method, it is not uncommon to see that the molecular ion is the base peak in the mass spectrum and the structurally informative fragment ions are either absent or present in very low abundance. In addition, the mass spectra often have high chemical background if matrix, e.g. glycerol in FAB, is used in the mass spectrometric analysis. Tandem mass spectrometry (MS/MS), especially when coupled with collisional-induced dissociation (CID), has been proved to be able to enhance the fragmentation efficiencies, increase the fragmentation pathways, and also alleviate the problem of chemical noise. Therefore it is not surprising that there are many reports on the use of MS/MS in conjunction with soft ionization techniques in the structural analysis of biomolecules.[18,19] Recently, methods based on the combination of FAB with linked scan or with four-sector MS/MS for the analysis of GSLs have been reported.[11–15] Low-energy CID has been successfully applied to the analysis of oligosaccharide;[20–22] however, only one reported, to our knowledge, is related to the structural analysis of GSLs.[16] Considering the need for unit resolution in both MS-1 and MS-2[15] in the analysis of GSLs and the great analytical potential of DCI, in this paper we explored the utility of using DCI along with triple-quadrupole MS/MS in the structural analysis of permethylated glycosphingolipids.

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EXPERIMENTAL

Materials and methods

Glucocerebrosides from human (Gaucher's) spleen, type 1 and typ 2 galactocerebrosides from bovine brain, gangliosides GM1, *N*-stearoyllactocerebrosides, *N*-palmitoyl-, *N*-stearoyl-, and *N*-lignoceroyl-dihydrolactocerebrosides were all purchased from Sigma Inc., St Louis, Missouri. Methane was obtained from Matheson Co., New Jersey. Carbon dioxide, nitrogen, ammonia and argon were purchased from San-Fu Co., Hsinchu, Taiwan. Samples were permethylated as described by Ciucanu and Kerek,[23] and adapted for glycolipids by Larson *et al.*[24] The double bond in the long-chain base of GM1 was reduced with N_2D_4.[25] One milligram of permethylated GM1 was dissolved in a solution of four drops N_2H_4 in 1 ml D_2O; the solution was heated to 60 °C for four days.

Mass spectrometry

DCI/MS and DCI/MS/MS experiments were performed on a Finnigan TSQ-46C triple-quadrupole mass spectrometer (Finnigan MAT, California). Approximately 1–2 μg of sample was air-dried on a platinum or rhenium emitter; the emitter was heated by a separated power supply at a heating rate of 10 mA s^{-1} or 20 mA s^{-1} until the maximum current of 1.3 Å was reached. Argon was used as the collision gas throughout the study.

RESULTS AND DISCUSSION

The CID mass spectrum of the protonated molecular ion (*m/z* 990) from the permethylated *N*-palmitoyldihydrolactocerebroside is shown in Fig. 1. Except for the $MH^+ - CH_3OH$ ion, all other ions were sugar sequence related Y and Z ions;[15] these ions were formed by cleavage on either side of the oxygen atom of the glycosidic bonds (Fig. 1). It has been reported that glycosidic bonds are easier to cleave than peptide bonds;[26] in fact, only half of the maximum 30 eV collision energy was needed to break the glycosidic bonds as shown in Fig. 1. This readily obtained CID mass spectrum pointed to the possibility of observing glycosidic cleavage daughter ions with precursor ions of higher masses. The study was then extended to a larger GSL: GM1. Since the molecular ion of permethylated GM1 is beyond the mass range of our instrument, an α-cleavage (cleavage α to the nitrogen with elimination of long-

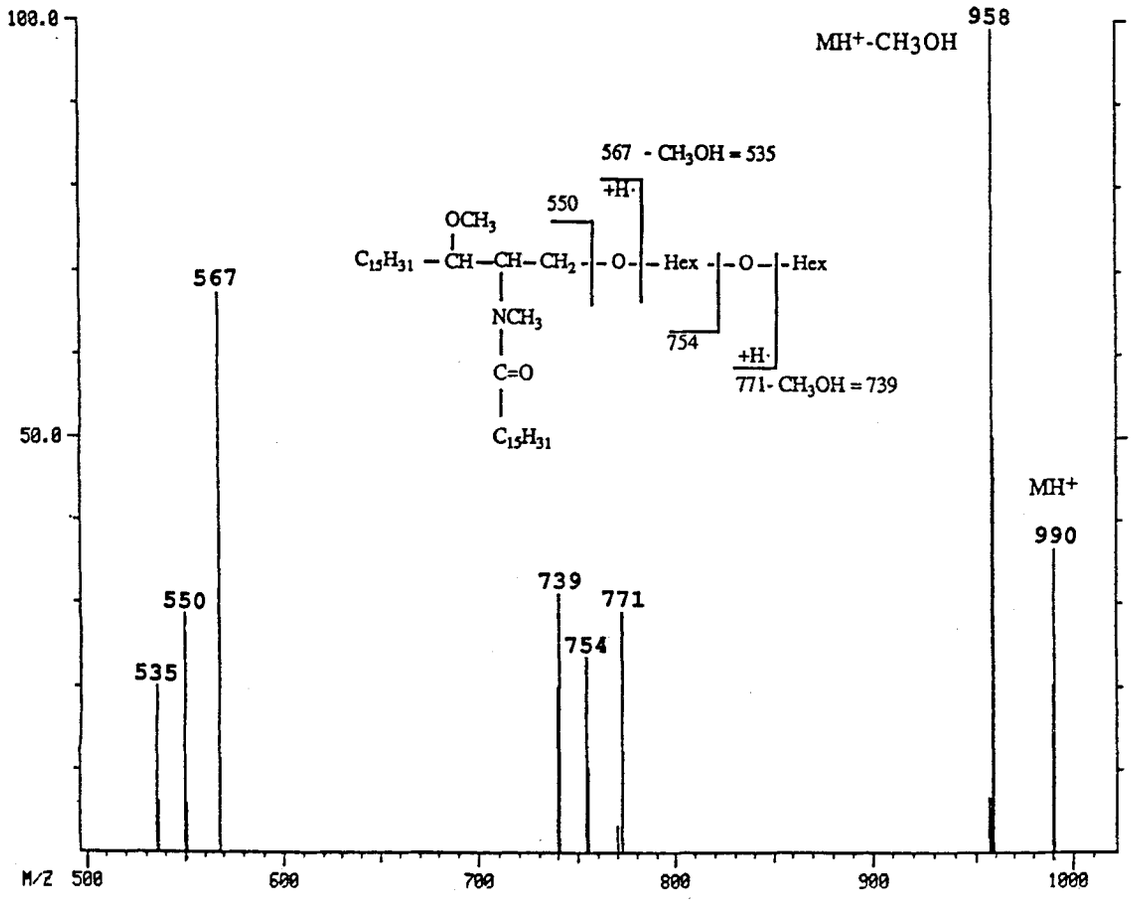


Figure 1. CID mass spectrum of protonated molecule of permethylated *N*-palmitoyldihydrolactocerebroside.

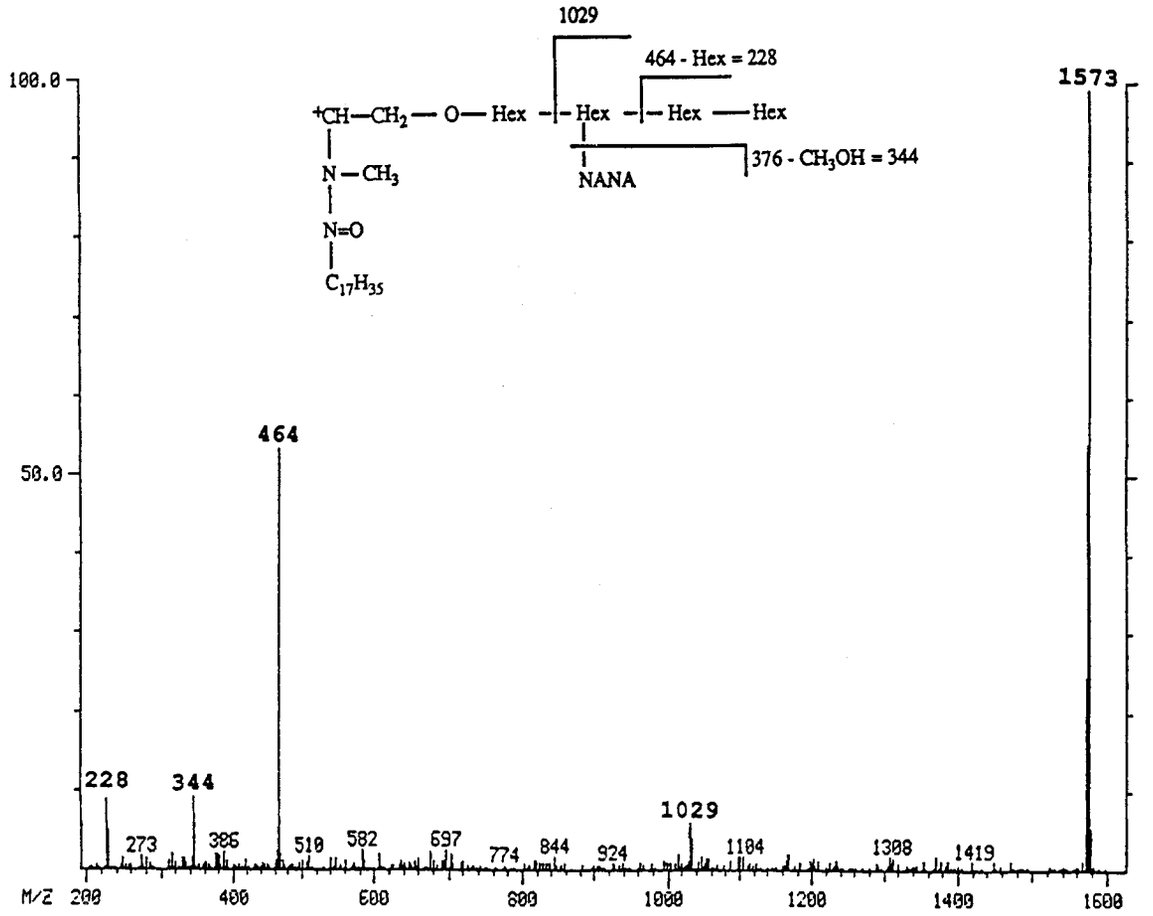


Figure 2. CID mass spectrum of the *m/z* 1573 precursor on from permethylated GM1.

chain base) ion,[27] with *m/z* value of 1573, was studied by low-energy CID. Figure 2 shows the daughter ion mass spectrum of the *m/z* 1573 precursor ion. The ion at *m/z* 344 corresponds to the loss of a methanol molecule from the terminal *N*-acetyl neuraminic acid residue. The presence of a second terminal sequence, hexose-*N*-acetyl hexose, is confirmed by the *m/z* 464 ion. The ion at *m/z* 1029 is important in that it indicates the branching point where the sialic acid is attached. The above data provide clear information about the glycan structure of GM1. Since this CID mass spectrum (Fig. 2) was obtained with 20 eV collision energy, it is very likely that sugar sequence information can be obtained with GSLs larger than GM1 if the full collision energy (30 eV) is used.

Ceramide ions were observed in the methane DCI mass spectra of permethylated *N*-alkyldihydrolactocerebrosides. These ceramide ions were subjected to MS/MS analysis and the CID mass spectra of these ions are presented in Fig. 3. Two types of daughter ions, one originating from the long-chain base and the other from the fatty acid, were observed (Fig. 3); these daughter ions provided very useful information about the structure of the ceramide.

When the popular methane or ammonia was used as the DCI reagent gas, ceramide ion was one of the major fragment ions in the DCI mass spectra, but the total ion current was mainly carried by the protonated molecular ion and not by the ceramide ion. In order to increase parent ion intensity to facilitate the collision experiment, EI and chemical ionization with a variety of reagent gases were studied for the absolute abundance of the ceramide ions. The results showed that DCI with charge-exchange reagent gases such as carbon dioxide or nitrogen produced the best results; the ceramide ions were often the base peaks in the mass spectra and their absolute abundances were much higher than under the methane and ammonia conditions.

GSLs containing an unsaturated long-chain base are much more common. When the ceramide ion with *m/z* 576 from the permethylated *N*-stearoyllactocerebroside was analysed by DCI/MS/MS, only long-chain base daughter ions were observed (Fig. 4). The absence of the acylamide ion (fatty acid related ion) indicated the significant influence of a double bond at C(4)–C(5) of the long-chain base. A charged site rearrangement reaction involving the transfer of a hydrogen atom from the C(5) of the long-chain base to the nitrogen atom of the amide is postulated (Scheme 1). To support this assumption, the double bond in the long-chain base of the *N*-stearoyllactocerebroside was reduced with deuterium and then analysed by DCI/MS/MS. Besides the long-chain base ions (*m/z* 314, 282), the acylamide (fatty acid) ions were observed, as expected, at *m/z* 298 and 299 with similar intensity. The *m/z* 298 ion was believed to be formed by transferring a hydrogen atom from the C(5) (or less likely C(4)) of the long-chain base to the nitrogen atom, whereas the *m/z* 299 ion was formed by

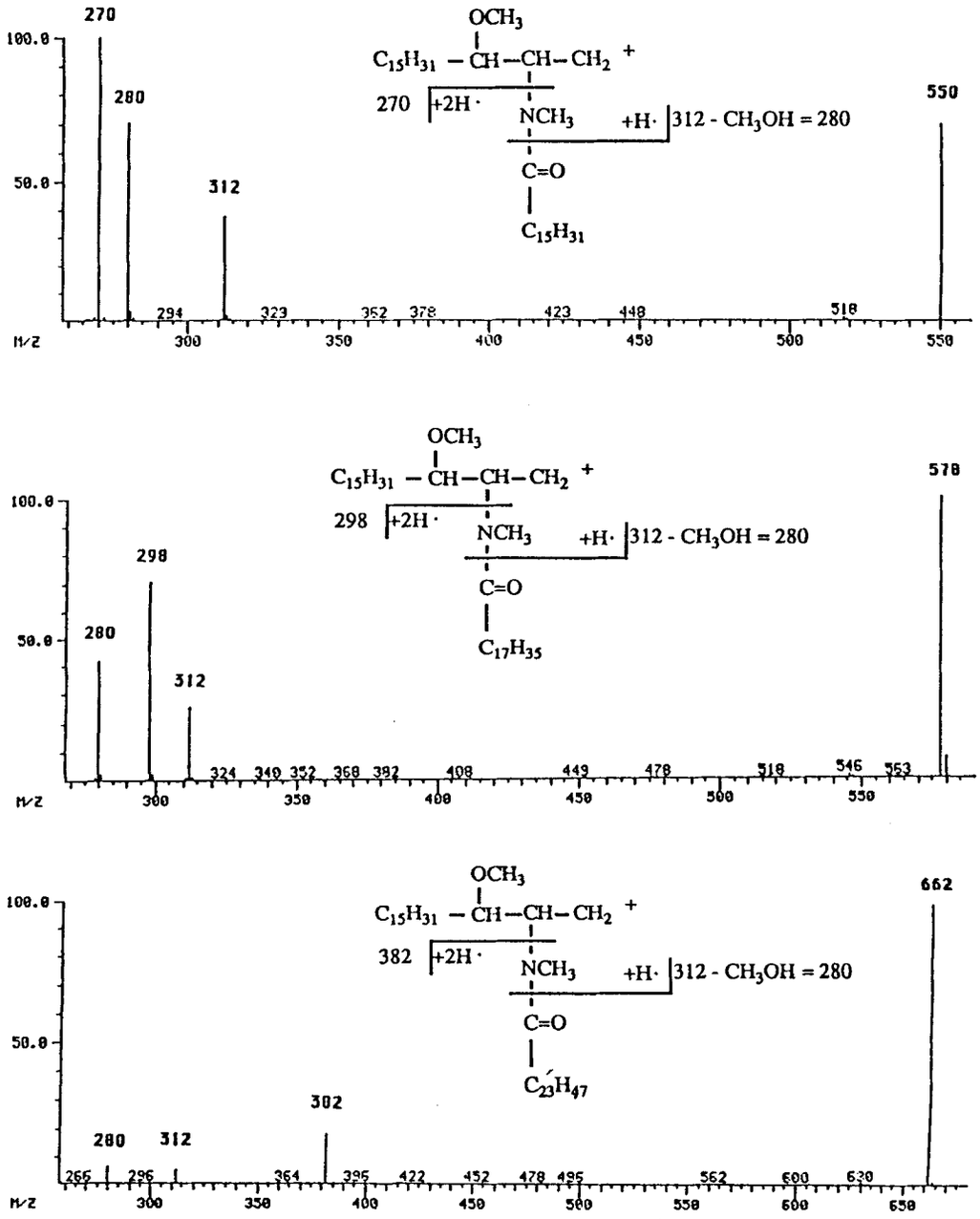


Figure 3. CID mass spectra of the ceramide ions from permethylated (a) *N*-palmitoyldihydrolactocerebroside, (b) *N*-stearoyldihydrolactocerebroside, and (c) *N*-lignoceroyldihydrolactocerebroside.

transferring a deuterium atom from the C(5) of the D-labelled long-chain base to the nitrogen atom.

It is generally believed that the higher the mass of the precursor ion, the higher the collision energy needed to produce the daughter ion. Ceramide ions are significantly smaller than the protonated molecular ions; it is, however, interesting to see that higher collision energies were needed to break the ceramide ions. In addition, it was noticed that ceramides with a saturated long-chain base took significantly higher collision to produce the daughter ions even though they are only two mass units larger than their unsaturated analogues. These experiments are consistent with a well-known but often neglected fact that in addition to the size of the parent ion, the degree of fragmentation in the low-energy CID experiment is also closely related to the structure of the precursor ions.

For ceramides with a saturated long-chain base, besides the effect on the fragmentation pattern and collision energy, a dramatic source temperature effect was observed. For example, in the CID analysis of the ceramide precursor ion (*m/z* 578) from *N*-stearoyldihydrolactocerebroside, the daughter ion abundance increased, on average, more than five times when the source temperature was increased from 100 °C to 190 °C (the maximum source temperature). This temperature effect is even more critical for precursor ions of higher mass. In fact, only at a source temperature of 150 °C or higher did the ceramide ion from the *N*-lignoceroyldihydrolactocerebroside (*m/z* 662) fragment

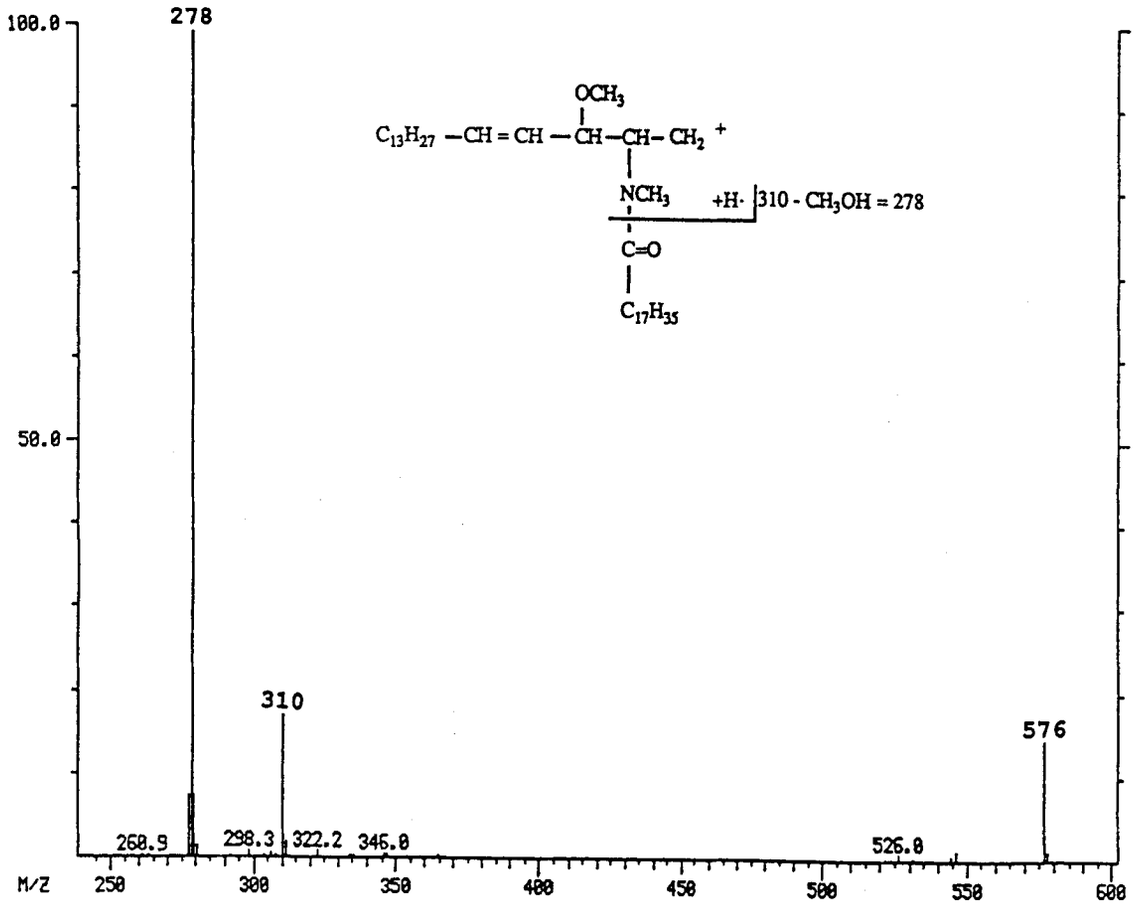


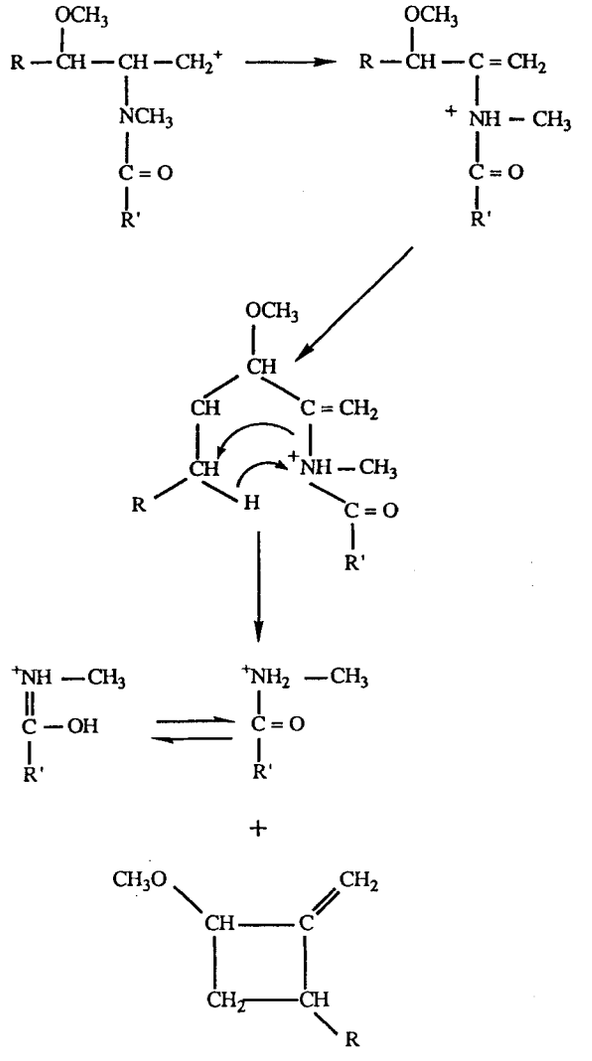
Figure 4. CID mass spectrum of the ceramide ion (*m/z* 576) from permethylated *N*-stearoyllactocerebroside.

significantly. It is noteworthy that source temperature has very little, if any, effect on ceramides with a double bond in the C(4)–C(5) position of the long-chain base.

For ceramides without double bond(s) and/or other substituted group(s) in the fatty acids, this DCI/MS/MS approach can provide clear assignments about the structure of the ceramides, although no fatty acid daughter ions will be detected if there is a C(4)–C(5) double bond in the long base. Fatty acid could still be assigned according to the mass interval between the ceramide precursor ions and the long-chain base daughter ions. For example, when the permethylated GM1 was analysed by DCI/MS, two ceramide ions (*m/z* 576, 604) were observed. When these ceramide ions were subjected to CID analysis, the *m/z* 576 ion produced the *m/z* 310, 278 ions, and the *m/z* 604 ion produced the *m/z* 338, 306 ions. Since these daughter ions were long-chain base ions, these two pairs of ions (310/338, 278/306) suggested that the 28 u difference between the *m/z* 576 and the *m/z* 604 ions resided in the long-chain base and not the fatty acid, thus the long-chain bases were assigned as sphingosine and eicosasphingosine. Based on the mass interval between the ceramide precursor ions (*m/z* 576, 604) and the long-chain base ions (*m/z* 310, 338), the fatty acid was assigned as a stearic acid residue. For those ceramides which have a double bond or a hydroxy group in the fatty acid, our investigation of type 1 and type 2 galactocerebrosides showed that this approach is able to identify the fatty acid constituents as C18-hydroxy, C24-hydroxy, C24:1, etc.; however, this method is, as might be expected for low-energy CID, not able to locate the position of the double bond in the type 2 sample as well as the hydroxy group in the type 1 sample.

When aprotic gas is used as the DCI reagent gas, an ion with elimination of the long-chain base from the molecular ion (an α cleavage ion) provides information about the chain length of the base. However, problems may arise if the sample under study is a complex mixture—a common occurrence for a biological system. Under these circumstances, the α cleavage ions could mix with Y and/or Z ions in a rather small mass range, thus making the assignment difficult. For example, in the analysis of permethylated glucocerebrosides there were many ions in the 500–700 u range, including the α cleavage ions and the ceramide ions; we therefore applied the CID method. The results showed that the *m/z* 660, 632 and 548 were the major ceramide ions; the same daughter ion mass spectrum (*m/z* 278, 310) indicates that these three ceramide ions have the same long-chain base: sphingosine. The mass intervals between the ceramide ions (*m/z* 660, 632, 548) and the sphingosine ion (*m/z* 310) correspond, respectively, to palmitic acid, behenic acid and lignoceric acid.

When protonated molecular ions and ceramide ions were studied by DCI/high-energy CID, our preliminary results, obtained with *B/E* linked scan, showed that the daughter ion mass spectra were significantly different



Scheme 1

from the low-energy CID results. For the protonated molecular ion from *N*-palmitoyldihydro- lactocerebroside, in addition to the interglycosidic cleavage ions observed in low-energy CID (Fig. 1), E ion, V ion and B ions were also detected in high-energy CID. For ceramide ions, the difference is even bigger; under high-energy CID, the ceramide ion from *N*-palmitoyldihydrolectocerebroside produced many more daughter ions than low-energy CID and the two types of daughter ions observed in the low-energy CID (Fig. 3(a)) were not detected, at least not in significant abundance, in high-energy CID.

CONCLUSION

Unit resolution for precursor ion selection and daughter ion analysis is often needed in the structural analysis of complex GSLs.[15] The combination of DCI and triple-quadrupole mass spectrometry makes for a useful tool for the structural analysis of permethylated GSLs. This is particularly true when the more expensive four-sector tandem mass spectrometer is unavailable for use. This investigation has shown that, upon low-energy CID, useful information regarding the structure of the glycan as well as the ceramide can be obtained, provided that both molecular ions and ceramide ions were chosen as the precursor ions.

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