〈Original Article〉

# Mild acid hydrolysis of sphingolipids yields lysosphingolipids: a matrix-assisted laser desorption and ionization time-of-flight mass spectrometry study

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**Summary** The mechanisms underlying the generation of lysosphingolipids remain unclear. The present study investigated whether sphingolipids can be de-*N*-acylated under relatively mild acidic conditions to produce lysosphingolipids. Sphingolipids (ceramide, sphingomyelin, cerebroside, lactosylceramide, trihexosylceramide, globoside and Forssman glycolipid) were dissolved in chloroform/methanol (2:1 v/v) and then treated with 0.5N HCl at room temperature. The hydrolysis products were analyzed using thin layer chromatography and matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry. We found that for all sphingolipid species tested, the relatively mild acid treatment resulted in the production of a de-*N*-acylated sphingolipid. A time-course study showed that approximately 15% of the sphingomyelin sample was hydrolyzed in the first 24 h of treatment, and that hydrolysis continued at a slower rate for 3 weeks thereafter. It appeared that sphingolipid deacylation was induced by cyclic elimination of a fatty acid due to *N*-acyl to *O*-acyl conversion. The present findings indicate that lysosphingolipids can be generated from sphingolipids under relatively mild acidic conditions.

**Key words:** Sphingomyelin, Lysosphingomyelin, Ceramide, Glycosphingolipid, Mass analysis

## 1. Introduction

Sphingolipids are important constituents of cell membranes, plasma lipoproteins, rafts and caveoli[1-6]. Sphingolipids are synthesized in cytoplasm and are hydrolyzed in lysosomes[7]. Sphingolipid metabolites have been implicated as modulators of membrane signal transduction systems[8, 9], and accumulate in

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lysosomal enzyme-deficient diseases such as sphingolipidosis[10-12]. Recently, small amounts of lysosphingolipids have been detected in plasma lipoproteins[13, 14]. However, the mechanism of generation of these lysosphingolipids and their role in plasma and cells remains unclear.

The sphingolipid amide linkage between the fatty acid and the sphingosine base is extremely stable, and as such, analysis of those long chain bases and the fatty acid composition requires strongly acidic or alkaline hydrolysis at high temperature. In general, sphingolipid hydrolysis for the preparation of lysosphingolipid is performed under strongly alkaline conditions for several hours at high temperature[15, 16]. The alternate approach of acid hydrolysis is assumed to cause an *N* -> *O* shift of the *N*-acyl chain and the formation of a cyclic intermediate such as an oxazolidine ring, after which various by-products of the long chain base are generated after deacylation. However, Van Veldhoven et al.[17] recently reported that relatively mild acid treatment (0.5N HCl for 3 hours at room temperature) of the *N*-[$^{14}$C] acetyl-sphingenine of ceramide resulted in the formation of three by-products, 1-*O*-acetyl-sphingenine, 3-*O*-acetyl-sphingenine and sphingenine, all of which were ninhydrin-positive.

In vivo, ceramide is hydrolyzed by ceramidase into fatty acids and long chain bases. However, there are no reports of enzyme hydrolysis of this sphingolipid into fatty acids and lysosphingolipid in humans. Very small amounts of lysosphingolipid are detected in humans[18], and may be associated with a predisposition to genetic sphingolipidosis diseases. Lysosphingolipid generation from accumulated sphingolipid due to enzyme deficiency may be the result of *N*-acyl conversion into *O*-acyl, which is hydrolyzed by lipid esterase in lysosomes.

Abbreviations

Chloroform/methanol; C/M, matrix-assisted laser desorption and ionization time-of-flight; MALDI-TOF, 2,5-dihydroxybenzoic acid; 2,5-DHB, thin layer chromatography; TLC, cerebroside; CMH, lactosylceramide; CDH, trihexosyl ceramide; CTH, *m/z*: mass/charge, LCB: long chain base, FA: fatty acid

Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry is useful for sphingolipid characterization[19, 20]. In addition, the products of sphingosine bases following acid hydrolysis of sphingomyelin, cerebroside or psychosine have be measured using gas-liquid chromatography, thin layer chromatography (TLC), mass spectrometry and NMR-spectrometry[21].

The present study investigated whether treatment of a variety of sphingolipids under relatively mild acidic conditions can result in the generation of lysosphingolipids due to de-*N*-acylation. MALDI-TOF mass spectrometry and TLC were used to analyze the hydrolysis.

## 2. Experimental

### 2.1. Chemicals

Methanol, chloroform, hydrochloric acid, aqueous ammonia and primuline reagent were obtained from Wako Chemical Co., (Osaka, Japan), and 2,5-dihydroxybenzoic acid (2,5-DHB) was purchased from SIGMA Chemical Co. (St. Louis, MO, USA). TLC plates with silica gel 60Å (100 x100 mm, layer thickness; 250 $\mu$ m), were obtained from Whatman (Maidstone, England).

### 2.2. Materials

The following purified sphingolipids were used: ceramide, sphingomyelin, cerebroside (CMH), lactosylceramide (CDH), trihexosyl ceramide (CTH), globoside and Forssman glycolipid[21]. Purified sphingenine, various lysosphingolipids, psychosin and sphingosylphosphorylcholine were used as reference standards.

### 2.3. Treatment with aqueous methanolic HCl

Purified sphingolipid (approximately 1 mg) was dissolved in 100 $\mu$ l chloroform/ methanol (C/M, 2:1 v/v) solution containing 0.5N HCl. Following the reaction, the mixture was then dried under $N_2$ gas. Samples were re-dissolved in 2,5-DHB solution (10 mg/ml in C/M, 1:1 v/v) as a matrix, and analyzed using MALDI-TOF mass spectrometry.

2.4. Mass spectrometry

Mass spectrometer measurements were performed as previously described using a MALDI-TOF mass spectrometer (Voyager Elite XL, PerSeptive Biosystems, Framingham, MA)[19]. Extracted lipids were mixed with an equal quantity of 2,5-DHB, and 1 μl of that mixture was applied to the spectrometer metal sample plate. The sample was allowed to dry, after which routine MALDI-TOF mass spectrometry analysis in positive ion mode was performed.

2.5. Thin layer chromatography

Samples (1 μl of 1 mg/ml in C/M, 2:1 v/v) were applied to TLC silica gel plates and the plates were placed in a chamber with a solvent mixture of chloroform, methanol and aqueous ammonia (65: 35: 8, v/v/v). Lipids were visualized by spraying the plate with a solution of primuline reagent and then exposing the plate to UV light (366 nm).

## 3. Results

3.1. Degradation of sphingomyelin

Sphingomyelin was treated with 0.02, 0.1 or 0.5N HCl for 3 weeks at room temperature, and then analyzed using TLC. Untreated sphingomyelin (1 mg/ml in C/M, 2:1 v/v) appeared as two adjacent spots following TLC (Fig. 1). Treatment with 0.2 and 0.5N HCl resulted in the generation of lysosphingomyelin, whereas treatment with 0.02 or 0.1N HCl did not.

MALDI-TOF mass spectrometry analysis of untreated sphingomyelin revealed a number of ion peaks (Fig. 2-A). The peaks at *m/z* 669.8, 703.6, 725.6, 781.8, 815.7 and 837.7 represented various sphingomyelin molecular species containing different fatty acids. An ion peak at *m/z* 413.4 represented the sodium ion of 2,5-DHB ($[M+Na]^+$). Treatment of sphingomyelin with 0.5N HCl for 3 weeks at room temperature resulted in a significant reduction in the intensity of the peaks between *m/z* 669.8 and 837.7, and the appearance of new peaks at *m/z* 465.6 and 487.6 (Fig. 2-B). The latter peaks were identified as protonated and sodium-positive sphingosylphosphorylcholins ($[M+H]^+$ and $[M+Na]^+$), respectively (Table 1).

A time-course study was performed to examine

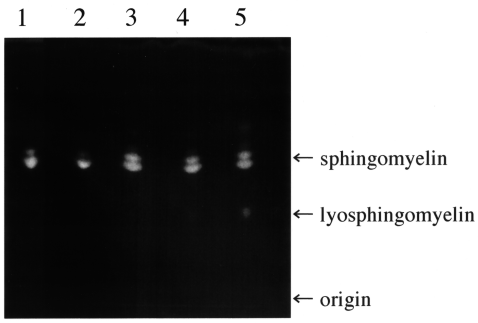


Fig. 1 The generation of lysosphingomyelin following relatively mild acid hydrolysis of sphingomyelin. Untreated sphingomyelin or sphingomyelin treated for 3 weeks with various concentrations of HCl was subjected to TLC analysis as described in the Methods (developing solvent: chloroform, methanol and aqueous ammonia at 65:35:8 v/v/v ratio). Lane 1; untreated sphingomyelin, Lanes 2-5; sphingomyelin treated with 0.02, 0.1, 0.2 and 0.5N HCl, respectively.

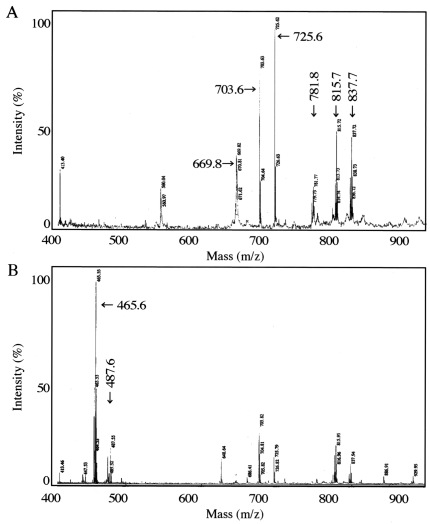


Fig. 2 The effect of mild acid hydrolysis on sphingomyelin MALDI-TOF mass spectra. Sphingomyelin was treated without (A) or with (B) 0.5N HCl for 3 weeks at room temperature, and then analyzed using positive ion mode MALDI-TOF mass spectrometry.

Table 1 Identification of MALDI-TOF mass spectrometry molecular ion peaks formed following treatment of sphin--golipids with 0.5N HCl at room temperature.
m/z: mass/charge, LCB: long chain base = sphingosine bases, FA: fatty acid, M: identified compound (sphingolipids).

| m/z mol.ion | LCB | FA | M |
|---|---|---|---|
| Sphingomyelin and lyso-sphingomyelin | | | |
| 466[M+H]$^+$ | d18:1 | - | Lyso-SM |
| 488[M+Na]$^+$ | d18:1 | - | Lyso-SM |
| 670[M+Na]$^+$ | d18:1 | C12 | SM |
| 704[M+H]$^+$ | d18:1 | C16 | SM |
| 726[M+Na]$^+$ | d18:1 | C16 | SM |
| 782[M+Na]$^+$ | d18:1 | C20 | SM |
| 816[M+H]$^+$ | d18:1 | C24 | SM |
| 838[M+Na]$^+$ | d18:1 | C24 | SM |
| Ceramide (N-acyl sphingosine) and sphingosine | | | |
| 264[M-2H$_2$O+H]$^+$ | d18:1 | - | Sphingosine |
| 282[M-H$_2$O+H]$^+$ | d18:1 | - | Sphingosine |
| 300[M+H]$^+$ | d18:1 | - | Sphingosine |
| 509[M-OH]$^+$ | d18:1 | C14h | Ceramide |
| 521[M-OH]$^+$ | d18:1 | C16 | Ceramide |
| 537[M-OH]$^+$ | d18:1 | C16h | Ceramide |
| 561[M+Na]$^+$ | d18:1 | C16 | Ceramide |
| 565[M-OH]$^+$ | d18:1 | C18h | Ceramide |
| 593[M-OH]$^+$ | d18:1 | C20h | Ceramide |
| 621[M-OH]$^+$ | d18:1 | C22h | Ceramide |
| 649[M-OH]$^+$ | d18:1 | C24h | Ceramide |
| Cerebrosides (CMH) and psychosines(Lyso-CMH) | | | |
| 463[M+H]$^+$ | d18:1 | - | Lyso-CMH |
| 485[M+Na]$^+$ | d18:1 | - | Lyso-CMH |
| 739[M+Na]$^+$ | d18:1 | C16h | CMH |
| 751[M+Na]$^+$ | d18:1 | C18 | CMH |
| 767[M+Na]$^+$ | d18:1 | C18h | CMH |
| 793[M+Na]$^+$ | d18:1 | C20:1h | CMH |
| 807[M+Na]$^+$ | d18:1 | C22 | CMH |
| 833[M+Na]$^+$ | d18:1 | C24:1 | CMH |
| 835[M+Na]$^+$ | d18:1 | C24 | CMH |
| 849[M+Na]$^+$ | d18:1 | C24:1h | CMH |
| 851[M+Na]$^+$ | d18:1 | C24h | CMH |
| 863[M+Na]$^+$ | d18:1 | C26 | CMH |

| m/z mol.ion | LCB | FA | M |
|---|---|---|---|
| Lactosylceramide (CDH) and lyso-CDH | | | |
| 624[M+H]$^+$ | d18:1 | - | Lyso-CDH |
| 646[M+Na]$^+$ | d18:1 | - | Lyso-CDH |
| 885[M+Na]$^+$ | d18:1 | C16 | CDH |
| 901[M+Na]$^+$ | d18:1 | C16h | CDH |
| 969[M+Na]$^+$ | d18:1 | C22 | CDH |
| 997[M+Na]$^+$ | d18:1 | C24 | CDH |
| 1011[M+Na]$^+$ | d18:1 | C25 | CDH |
| Trihexosyl ceramide (CTH) and lyso-CTH | | | |
| 787[M+H]$^+$ | d18:1 | - | Lyso-CTH |
| 809[M+Na]$^+$ | d18:1 | - | Lyso-CTH |
| 1046[M+Na]$^+$ | d18:1 | C16 | CTH |
| 1131[M+Na]$^+$ | d18:1 | C22 | CTH |
| 1159[M+Na]$^+$ | d18:1 | C24 | CTH |
| 1175[M+Na]$^+$ | d18:1 | C24h | CTH |
| Globoside and lyso-Globoside | | | |
| 990[M+H]$^+$ | d18:1 | - | Lyso-Globoside |
| 1012[M+Na]$^+$ | d18:1 | - | Lyso-Globoside |
| 1334[M+Na]$^+$ | d18:1 | C22 | Globoside |
| 1362[M+Na]$^+$ | d18:1 | C24 | Globoside |
| 1378[M+Na]$^+$ | d18:1 | C24h | Globoside |
| Forssman and lyso- Forssman | | | |
| 1215[M+Na]$^+$ | d18:1 | - | Lyso-Forssman |
| 1469[M+Na]$^+$ | d18:1 | C16h | Forssman |
| 1483[M+Na]$^+$ | d18:1 | C17h | Forssman |
| 1537[M+Na]$^+$ | d18:1 | C22 | Forssman |
| 1565[M+Na]$^+$ | d18:1 | C24 | Forssman |
| 1579[M+Na]$^+$ | d18:1 | C25 | Forssman |

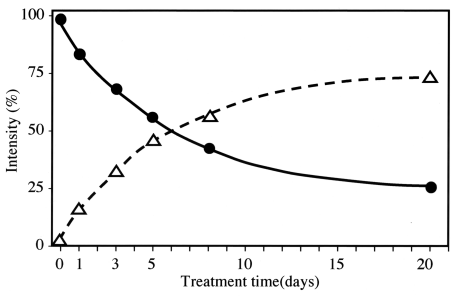


Fig. 3 Time course of sphingomyelin hydrolysis. Pure sphingomyelin (1 mg/ml in C/M, 2:1 v/v) was treated with 0.5N HCl at room temperature. Samples were taken at the times indicated and analyzed in positive ion mode using MALDI-TOF MS. ●: Sphingomyelin (total height of peaks at *m/z* 703 and 725), △: lysosphin gomyelin (total height of peaks at *m/z* 466 and 488).

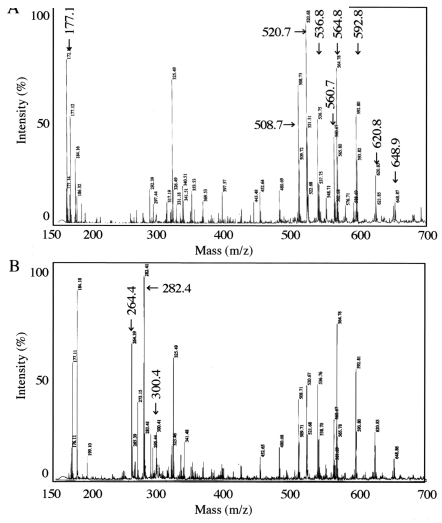


Fig. 4 Mild acidic hydrolysis of ceramide. Ceramide (Cer) was treated without (A) or with (B) 0.5N HCl at room temperature for 1 week, and the products analyzed in positive ion mode using MALDI-TOF mass spectrometry.

the rate of lysosphingomyelin production during the 3-week treatment. We found that approximately 15% of the sphingomyelin sample was hydrolyzed in the first 24 h, after which degradation occurred at a slower rate over the 3-week period (Fig. 3).

### 3.2. Degradation of ceramide

We examined the effect of mild acid hydrolysis on

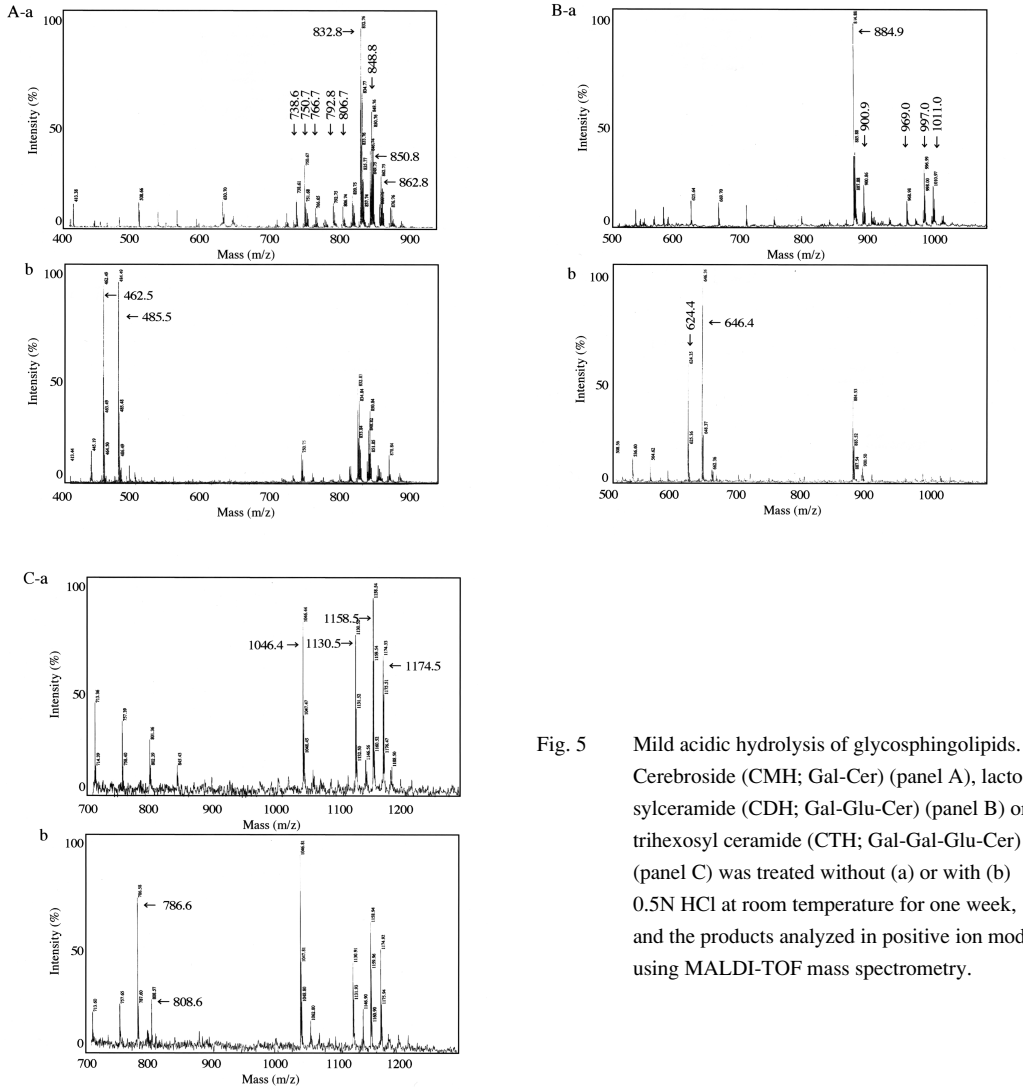


Fig. 5 Mild acidic hydrolysis of glycosphingolipids. Cerebroside (CMH; Gal-Cer) (panel A), lactosylceramide (CDH; Gal-Glu-Cer) (panel B) or trihexosyl ceramide (CTH; Gal-Gal-Glu-Cer) (panel C) was treated without (a) or with (b) 0.5N HCl at room temperature for one week, and the products analyzed in positive ion mode using MALDI-TOF mass spectrometry.

ceramides. MALDI-TOF mass spectrometry analysis of untreated ceramides with various fatty acids revealed ion peaks at *m/z* 508.7, 520.7, 536.8, 560.7, 564.8, 592.8, 620.8 and 648.9 (Fig. 4-A). The ion peak at *m/z* 177.1 represented the sodium ion of 2,5-DHB ($[M+Na]^+$). Following treatment with 0.5N HCl for one week at room temperature, additional ion peaks were detected at *m/z* 300.4, 282.4 and 264.4 (Fig. 4-B). Those peaks corresponded to protonated sphingosine ($[M+H]^+$), dehydroxy sphingosine ($[M-OH]^+$), and two protonated molecules of dehydrated sphingosine ($[M-2H_2O+H]^+$) (Table1). In addition, there was a decrease in the intensity of peaks in the region from *m/z* 508.8 to 648.9 (i.e., ceramide).

### 3.3. Degradation of glycosphingolipids

The effect of a 1-week treatment with 0.5N HCl at room temperature on three glycosphingolipids was investigated. MALDI-TOF mass spectrometry analysis of untreated cerebroside (CMH) revealed mass ion peaks at *m/z* 738.6, 750.7, 766.7, 792.8, 806.7, 832.8, 834.8, 848.8, 850.8 and 862.8 (Fig. 5-A-a). After treatment, two new ion peaks were detected at *m/z* 462.5 and 484.5 (Fig. 5-A-b), which were identified as individual molecular species of protonated and sodium-positive lysocerebroside (Table 1).

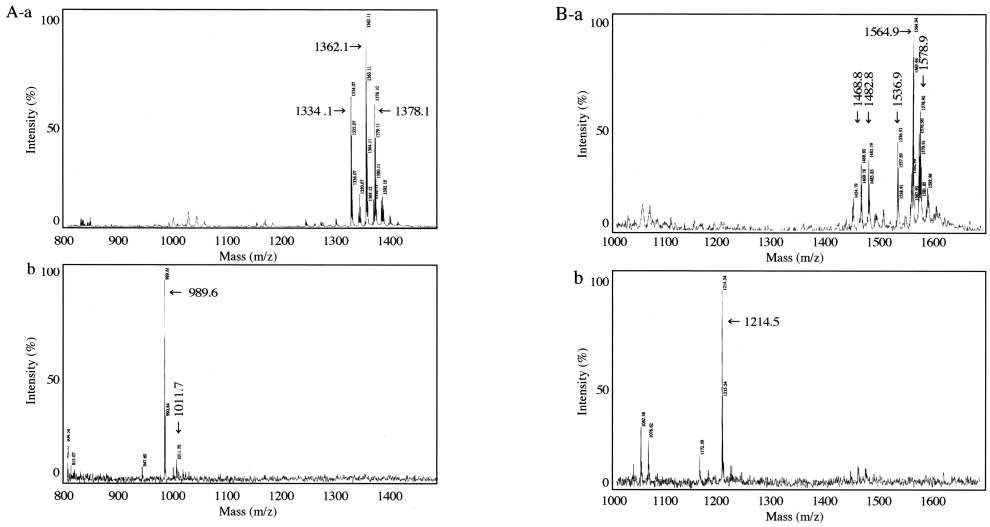


Fig. 6 Mild acidic hydrolysis of globosides with N-acetyl glucosamine.
Globoside (GalNac-Gal-Gal-Glu-Cer) (panel A) or Forssman glycolipid (GalNac-GalNac-Gal-Gal-Glu-Cer) (panel B) was treated without (a) or with (b) 0.5N HCl at room temperature for one month, and the products analyzed in positive ion mode using MALDI-TOF mass spectrometry.

Untreated lactosylceramide (CDH) showed MALDI-TOF mass ion peaks at *m/z* 884.9, 900.9, 969.0, 997.0 and 1011.0 (Fig. 5-B-a). After treatment, two new ion peaks were detected at *m/z* 624.4 and 646.4 (Fig. 5-B-b), and these were identified as protonated and sodium-positive lysolactosylceramide (Table 1).

Untreated globotriaosylceramide (CTH) produced MALDI-TOF mass ion peaks at *m/z* 1046.4, 1130.5, 1158.5 and 1174.5 (Fig. 5-C-a). Following treatment, two new ion peaks were detected at *m/z* 786.6 and 808.6 (Fig. 5-C-b), and these were identified as protonated and sodium-positive lysoglobotriaosylceramide (Table 1).

These results suggest that under mild acidic conditions, a gradual deacylation of neutral glycosphingolipids occurred irrespective of the number of hexose chains in the glycosidic linkage with the ceramide moiety.

### 3.4 Degradation of globosides with *N*-acetyl glucosamine

We investigated the effect of treatment with 0.5N HCl at room temperature on globoside, which contains one *N*-acetyl glucosamine molecule, and on Forssman glycolipid, which contains two *N*-acetyl glucosamine molecules.

MALDI-TOF analysis of untreated globoside revealed ion peaks at *m/z* 1334.1, 1362.1 and 1378.1 (Fig. 6-A-a), and these were identified as individual molecular species of sodium-positive globoside (Table 1). After treatment with 0.5N HCl for approximately one month, those peaks could no longer be detected, and two new ion peaks were detected at *m/z* 989.6 and 1011.7 (Fig. 6-A-b), which were identified as protonated and sodium-positive lysoglobotriaosylceramide (Table 1).

Untreated Forssman glycolipid MALDI-TOF mass ion peaks were detected at *m/z* 1468.8, 1482.8, 1536.9, 1564.9, and 1578.9 (Fig. 6-B-a). Treatment with 0.5N HCl for approximately one month resulted in the disappearance of those peaks, and the appearance of a new ion peak at *m/z* 1214.5 (Fig. 6-B-b), which was identified as an individual molecular species of the sodium-positive lyso-Forssman glycolipid (Table 1).

These data suggest that mild acidic treatment resulted in deacylation of *N*-acetylglucosamine-

containing glycosphingolipids without any hydrolysis of the glycosidic linkage.

## 4. Discussion

The present study using MALDI-TOF mass spectrometry found that deacylation of ceramide and sphingomyelin can occur under relatively mild acidic conditions. Indeed, this deacylation occurred in a range of glycosphingolipids with differing numbers of hexose chains. In addition, the deacylation was observed in glycosphingolipids with either one or two *N*-acetyl glucosamine molecules, and occurred without any hydrolysis of the glycosidic linkage. The findings indicate that relatively mild acidic treatment of sphingolipids can result in the generation of lysosphingolipids.

The current data indicate that sphingolipid deacylation under relatively mild acidic conditions may be induced by *N*-acyl conversion to *O*-acyl (*N* -> *O*-acyl migration) through cyclic elimination of fatty acid. In general, *N* -> *O*-acyl migration reactions occur under strongly acidic or high temperature conditions (e.g., *N*-acetyl-ephedorine, cyclosporine or cyclic and linear peptides)[22, 23]. It was previously reported that the potential of the *N* -> *O*-acyl migration reaction depends on a cyclic elimination of a fatty acid via a pyrolytic reaction and allylic rearrangement, as suggested by Weiss[24] in studies on the preparation of lysohematoside from hematoside of equine erythrocytes[16]. Recently, Van Overloop et al. reported that a very small amount of long chain bases was produced from *N*-[$^{14}$C]acetyl-sphingenine hydrolyzed in 0.5N HCl for 3 hours at room temperature[17]. The by-products of *N*-[$^{14}$C]acetyl-sphingenine deacetylation were identified as 1-*O*-acetyl-sphingenine, 3-*O*-acetyl-sphingenine and sphingenine based on their ninhydrin-positive reaction.

MALDI-TOF mass spectrometry in the reflector mode was found to be extremely effective in the analysis of the various lysosphingolipids together with their long chain base components. An advantage of this technique is that intramolecular decomposition is not required. The lysosphingolipid mass spectra provided the precise mass of the molecular-related ions, and simultaneously indicated the constituents of the long chain base[18].

To date, few studies have examined sphingolipid hydrolysis under relatively mild acidic conditions. The present study suggests that *N*-acyl to *O*-acyl conversion of sphingolipids is possible under such conditions. These findings that lysosphingolipids can be created under such conditions, and without a by-product, are likely to provide an important approach for future structural, functional and metabolic studies of lysosphingolipids.

## 5. Conclusion

In the present study, relatively mild acidic treatment of a range of sphingolipids resulted in the production of lysosphingolipids, and no by-products. This deacylation occurred for all sphingolipids species tested, and therefore appeared to be independent of the number or type sugar moiety.

## Acknowledgments

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