J. Biochem. 120, 573-579 (1996)

Rapid Method of Preparation of Lysoglycosphingolipids and Their Confirmation by Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

Tamotsu Taketomi,[1] Atsushi Hara, Kei-ichi Uemura, and Eiko Sugiyama

Department of Biochemistry, Division of Neuro-Aging, Research Center on Aging and Adaptation, Shinshu University School of Medicine, Matsumoto, Nagano 390

Received for publication, April 17, 1996

We have recently developed a rapid method for the preparation of lysoglycosphingolipids on a small scale, in high yield. This procedure of microwave-mediated saponification of about 1 mg of glycosphingolipid with 0.5 ml of 0.1 M NaOH in methanol for two minutes can be easily repeated if larger amounts of lyso-compounds are needed. We have also found that the new methodology of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry in the reflector mode is extremely effective for the confirmation of different lysoglycosphingolipids together with their long chain base components. The combined method of preparation and confirmation of lysoglycosphingolipids is also important for the identification of long chain bases of various sphingolipids, because the usual analytical method of long-chain bases of sphingolipids depends on acidic methanolysis, which results in the formation of by-products such as *O*-methylsphingosines and *threo*-sphingosines.

Key words: glycosphingolipids, lysoglycosphingolipids, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, sphingolipids, sphingosines.

Various procedures have been used for the preparation of lysoglycosphingolipids from their parent glycosphingolipids (*1-7*). In general, these procedures are tedious, time-consuming, and offer low yields, but they are still useful for the preparation of lysoglycosphingolipids on a large scale. Recently we have found that microwave-mediated saponification of glycosphingolipids (about 1 mg) with 0.5 ml of 0.1 M NaOH in methanol is effective for the preparation of the lyso-compounds in high yields within a few minutes. This rapid preparation method can be easily and quickly repeated if larger amounts of lysosphingolipids are required. Each lysoglycosphingolipid thus prepared can be confirmed by chemical analysis as well as FAB mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy as described elsewhere (*3, 5-9*), but in this paper the new methodology of delayed ion extraction (DE) in matrix-assisted laser desorption ionization (MALDI) mass spectrometry using time-of-flight (TOF) analyzer with reflector detector is introduced for the first time for the confirmation of lysoglycosphingolipids. MALDI-TOF mass spectrometry has recently been shown to be a viable technique for analysis of glycolipids and gangliosides, since the problem of poor resolution in the analyzers has been overcome by the use of a magnetic sector mass spectrometer (*10*). The DE MALDI-TOF mass spectrometer we used has excellent resolution and allows accurate mass measurement. Various lysoglycosphingolipids, together with their long-chain base components, have been confirmed accurately and rapidly by this analytical method. Since the lysosphingolipids are of interest from the viewpoints of biological activities and metabolism (*11-17*), these new methodologies for the preparation and confirmation of lysoglycosphingolipids are important for further studies of lysoglycosphingolipids, as well as glycosphingolipids.

MATERIALS AND METHODS

Materials—Globo-series glycosphingolipids (Table I) were available for this experiment, that is, glucosylceramide prepared from spleen tissue of a patient with Gauchers disease (*18*), lactosylceramide, globotriaosylceramide, and globotetraosylceramide prepared from porcine erythrocytes (*19*) and globopentaosylceramide (Forssman) prepared from caprine erythrocytes (*19*). Various lysoglycosphingolipids prepared by the orthodox method as described by us (*3*) were available as control samples. α-Cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrices for MALDI-TOF mass spectrometry were purchased from Sigma Chemical (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid were from Nacalai Tesque (Kyoto).

Microwave-mediated saponification of glycosphingolipids for rapid preparation of lysoglycosphingolipids—About 1 mg of each glycosphingolipid was dissolved in 0.5 ml of 0.1 M NaOH in methanol in a Teflon-lined screw-capped Pyrex glass vial (10 ml) and exposed to the

[1] To whom correspondence should be addressed. Phone: +81-263-35-4600 (Ext. 5345), Fax: +81-263-33-5718, E-mail: agingol@gipac.shinshu-u.ac.jp

Abbreviations: α-CHCA, α-cyano-4-hydroxycinnamic acid; 2,5-DHB, 2,5-dihydroxybenzoic acid; DE MALDI-TOF MS, delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry; FAB, fast atom bombardment; TLC, thin laser chromatography.

maximum power of the microwave oven (500 W, Toshiba model ER-V-11) for 2 min. The volume of alkaline solution must not exceed 0.5 ml because excess reagent and rapid heating may lead to explosion of the tubes as suggested by Khan and Williams (*20*). After saponification, the sample was cooled to room temperature and acidified with one drop of 6 N HCl together with one drop of distilled water. The reaction mixture was vigorously shaken with 1 ml of hexane using a vortex mixer and separated into an upper phase of hexane and a lower phase of aqueous methanol by centrifuging. The upper phase was removed carefully with a micropippet, and the remaining lower phase was again treated twice with 1 ml of hexane to extract fatty acids thoroughly. The hexane extracts were combined and subjected to GLC analysis after methanolysis with 3% anhydrous HCl in methanol according to Khan and Williams (*20*), though these data are not shown here. The lower phase of aqueous methanol was routinely applied to a Sep-Pak C-18 cartridge to remove salt. Each lysoglycosphingolipid thus eluted with chloroform/methanol/water (60 : 30 : 4.5, by volume) was not only detected by TLC (*3*), but also confirmed by DE MALDI-TOF mass spectrometry in the reflector mode.

DE MALDI-TOF Mass Spectrometry—Chloroform/methanol (1 : 1, v/v) solution (1.5 μl) containing about 10–100 pmol of lysoglycosphingolipid and 1.5 μl of matrix solution (10 mg of α-CHCA in 1 ml of a 1 : 1 mixture of acetonitrile/water containing 0.1% of trifluoroacetic acid, or 10 mg of 2,5-DHB in 1 ml of a 9 : 1 mixture of water/ethanol) in a microcentrifuge tube were shaken vigorously by a vortex mixer and centrifuged by a microcentrifuge (Chibitan, Japan Millipore, Tokyo) for about 1 min. One microliter of the supernatant of the mixture was loaded on a sample plate that has 100 sample positions. The plate was

TABLE I. **Chemical structures of globo-series glycosphingolipids and chemical formula and monoisotopic molecular weights of their lyso-compounds.**

Globo-series	Chemical structure	Lyso-compound (d18:1 sphingosine)	
		Chemical formula	Monoisotopic M.W.
Glucosylceramide	Glcβ1-1′Cer	$C_{24}H_{47}NO_7$	461.60
Lactosylceramide	Galβ1-4Glcβ1-1′Cer	$C_{30}H_{57}NO_{12}$	623.72
Globotriaosylceramide	Galα1-4Galβ1-4Glcβ1-1′Cer	$C_{36}H_{67}NO_{17}$	785.84
Globotetraosylceramide	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer	$C_{44}H_{80}N_2O_{22}$	989.00
Globopentaosylceramide (Forssman)	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer	$C_{52}H_{93}N_3O_{27}$	1,192.17

M.W., molecular weight; Glc, glucose; Cer, ceramide; Gal, galactose; GalNAc, *N*-acetylgalactosamine.

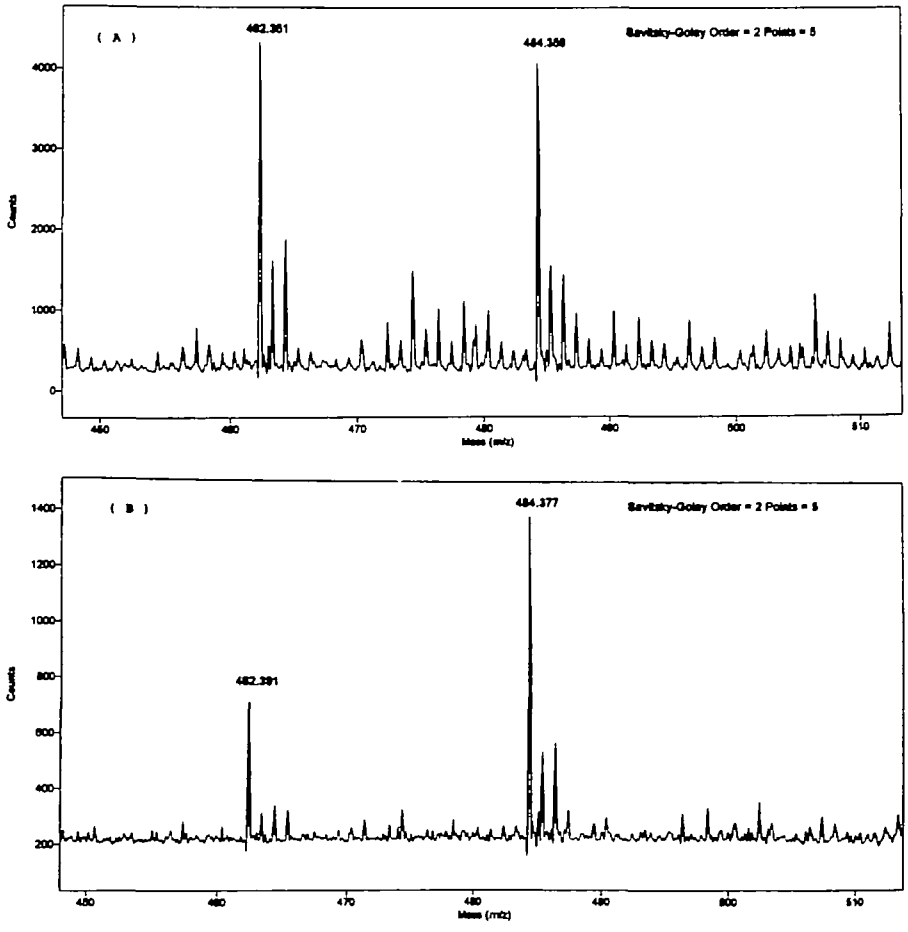

Fig. 1. **(A) High-resolution DE MALDI-TOF mass spectrum of lysoglucosylceramide in the reflector mode with α-CHCA as the matrix.** Accelerating voltage: 20,000, grid voltage: 74.0% of the accelerating voltage, guide wire voltage: 0.05% of the accelerating voltage, delay: 100 ns, laser step: 1,610, scan average: 128. **(B) High-resolution DE MALDI-TOF mass spectrum of lysoglucosylceramide in the reflector mode with 2,5-DBA as the matrix.** Conditions are the same as in (A) except for the laser step: 1,900.

loaded into a Voyager™ Elite XL (6.6 m flight length) Biospectrometry™ Workstation (PerSeptive Biosystems, Framigham, MA, USA) and the mass spectrum of the sample was acquired by the software of the instrument, with an N_2 laser (337 nm; 3-nanosecond-wide pulses at up to 20 pulses per second). The resolution of the ion peak is $M/\Delta M$ (peak centroid mass/right mass−left mass, see Fig. 2) determined by the resolution calculator from the GRAMS/386 software supplied with the instrument. The ion peaks were calibrated with potassium+1 (mass, 39.00), α-CHCA+1 (mass, 190.05), and angiotensin I (mass, 1,297.50) in the calibration file of the software and 5-point Savitsky-Golay smoothing was applied to the mass spectra as shown in the figures.

RESULTS

Lysoglucosylceramide—As expected, the lysoglucosylceramide which was prepared by the rapid method described above and detected by TLC (data not shown) gave a positive ion mass spectrum showing $(M+H)^+$ at m/z 462.36 and 462.39 and $(M+Na)^+$ at m/z 484.36 and 484.38 with α-CHCA and 2,5-DHB, respectively, as shown in Fig. 1, A and B. These ion peaks are shown at high resolution. For example, as shown in Fig. 2, A and B, the resolution of the $(M+H)^+$ ion peaks was calculated to be 3,302 and 2,884 with α-CHCA and 2,5-DHB, respectively. The protonated molecule of lysoglucosylceramide showed that the long-chain base is almost entirely d18:1 sphingosine (sphingenine). As showm in Fig. 1, A and B, the lysoglucosylceramide gave stronger signals with α-CHCA as the matrix than with 2,5-DHB. The latter matrix showed a tendency for $(M+Na)^+$ to dominate rather than $(M+H)^+$. As it was suggested that α-CHCA is better than 2,5-DHB as a matrix for lysoglycosphingolipids in TOF-mass spectra, the data obtained with 2,5-DHB as the matrix are not given below.

Lysolactosylceramide—The positive ion mass spectrum of lysolactosylceramide showed $(M+H)^+$ at m/z 624.29 and $(M+Na)^+$ at m/z 646.26 with α-CHCA as a matrix (Fig. 3A). These ion peaks, also seen at high resolution (data not shown), suggested that long-chain base of lysolactosylceramide is mainly d18:1 sphingosine. However, in addition to these ion peaks, the ion peak at m/z 638.30 was assumed to be due to lysolactosylceramide containing d19:1 sphingosine, though it is not clear why the corresponding $(M+Na)^+$ at m/z 660.30 was not found. Moreover, other smaller ion peaks near m/z 462 and 476 may be due to contaminating lysomonohexosylceramides, suggesting monohexosylceramide contamination in the lactosylceramide sample before the saponification. At any rate, it was found that the rapid method of microwave-mediated saponification of lactosylceramide is capable of producing the lyso-compounds.

Lysoglobotriaosylceramide—As shown in Fig. 3B, the positive ion mass spectrum of lysoglobotriaosylceramide gave $(M+H)^+$ at m/z 786.41 and $(M+Na)^+$ at m/z 808.38 with α-CHCA as a matrix. These ion peaks, also seen at high resolution (data not shown), suggested that the long-

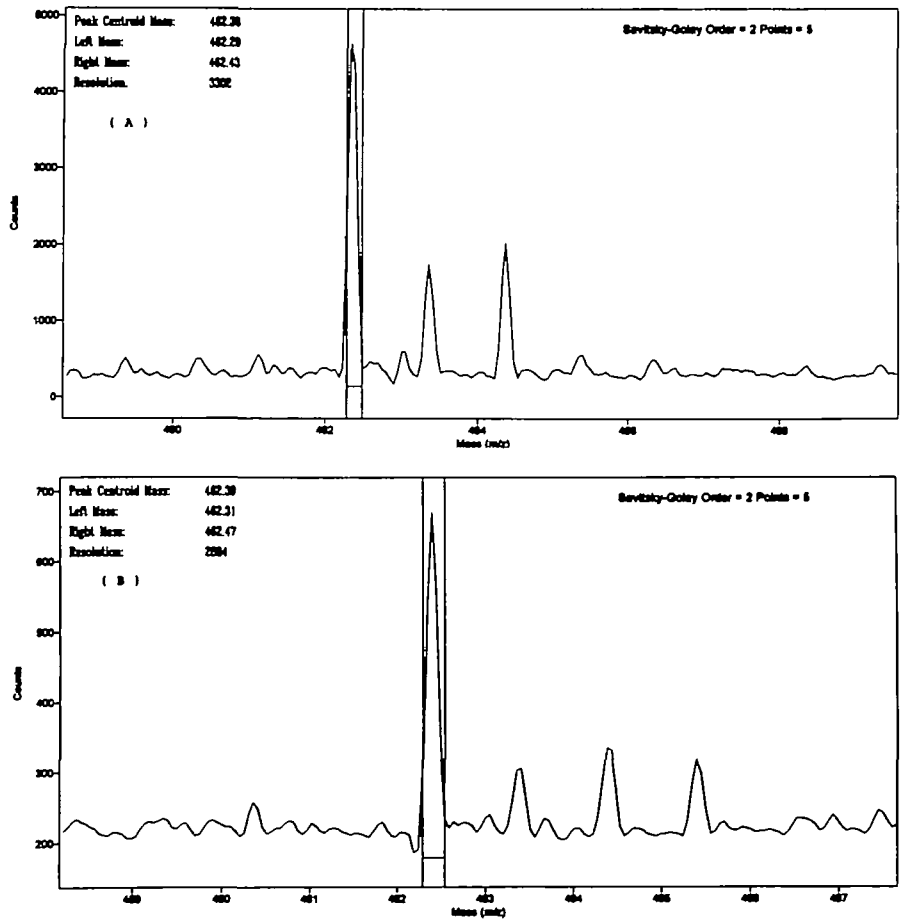

Fig. 2. **(A) Calculated resolution of $(M+N)^+$ at m/z 462.36 in the spectrum in Fig. 1A. (B) Calculated resolution of $(M+N)^+$ at m/z 462.39 in the spectrum in Fig. 1B.**

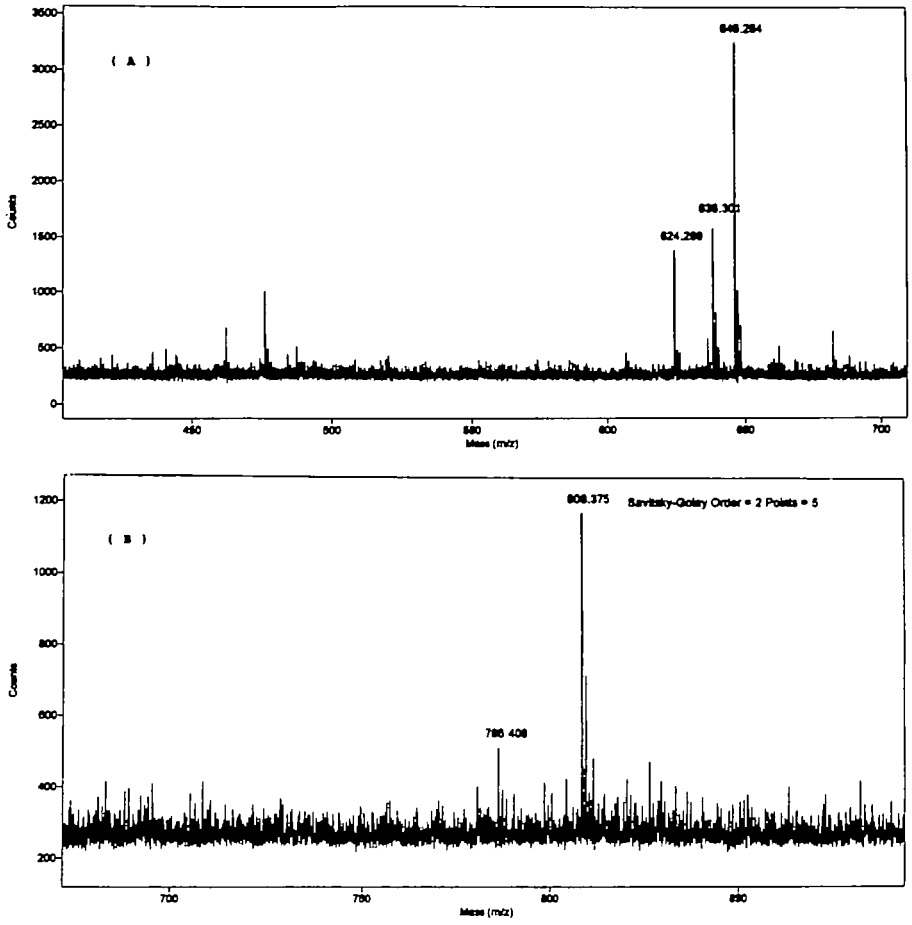

Fig. 3. **(A) High-resolution DE MALDI-TOF mass spectrum of lysolactosylceramide in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A except for the laser step: 1,600. **(B) High-resolution DE MALDI-TOF mass spectrum of lysoglobotriaosylceramide in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A.

chain base of the lysoglobotriaosylceramide is almost entirely d18:1 sphingosine.

Lysoglobotetraosylceramide—As shown in Fig. 4A, the positive ion mass spectrum of deacetylated lysoglobotetraosylceramide and lysoglobotetraosylceramide showed $(M-N\text{-acetyl}+Na)^+$ at m/z 969.01 and $(M+Na)^+$ at m/z 1,010.98, indicating the presence of d18:1 sphingosine, whereas Fig. 4B shows the positive ion mass spectrum of deacetylated lysoglobotetraosylceramide prepared by our orthodox method (*3*), in which $(M-N\text{-acetyl}+Na)^+$ ion peak at m/z 968.92 is dominant. These findings in Fig. 4, A and B, suggested that the microwave-mediated saponification with 0.5 M NaOH in methanol split the *N*-fatty acyl group of the ceramide moiety more easily than the acetamide group of *N*-acetylgalactosamine in the globotetraosylceramide. On the other hand, the orthodox method for the preparation of lysoglycosphingolipids completely split the *N*-fatty acyl group of ceramide as well as the acetamide group of *N*-acetylgalactosamine.

Lysoglobopentaosylceramide (lyso-Forssman)—The positive ion mass spectrum of partially deacetylated lyso-Forssman and lyso-Forssman prepared by the rapid method using microwave-mediated saponification of Forssman glycolipid showed $(M-N\text{-acetyl}+Na)^+$ at m/z 1,172.19 and $(M+Na)^+$ at m/z 1,214.18 in Fig. 5A, indicating the presence of d18:1 sphingosine. Figure 5B shows that the positive ion mass spectrum of completely deacetylated lyso-Forssman prepared by the orthodox method gave $(M-2\times N\text{-acetyl}+H)^+$ at m/z 1,107.93 and $(M-2\times N\text{-acetyl}+Na)^+$ at m/z 1,129.98. These findings in Fig. 6, A and B, also suggested that the rapid method of microwave-mediated saponification split the *N*-fatty acyl group of ceramide more easily than the acetamide groups of the two molecules of *N*-acetylgalactosamine in Forssman glycolipid.

DISCUSSION

We (*1, 3, 8, 9, 11*) have already reported an orthodox method for the preparation of various lysoglycosphingolipids, as well as their physicochemical and physiological properties. The lysoglycosphingolipids are important starting materials for the preparation of artificial antigens, such as psychosine-protein antigens and other glycosphingosyl-protein conjugates (*21*). Many reports (*13-16*) have appeared on the bioactivities of lysosphingolipids. Also, various lysosphingolipids have been reported to be present in abnormal tissues of patients with various sphingolipidoses (*12, 22-24*), though the mechanism of their accumulation remains to be resolved. Nevertheless, the occurrence of lysosphingolipids in various tissues should be more carefully reinvestigated, because even if they are present, the amounts are so small that it is hard to detect and analyze them. Also, the study of lysosphingolipids involves two difficult problems; restricted availability of standard samples of various lysosphingolipids, and precise identification. Our rapid method for the preparation of lysoglycosphingolipids on a small scale is extremely reproducible

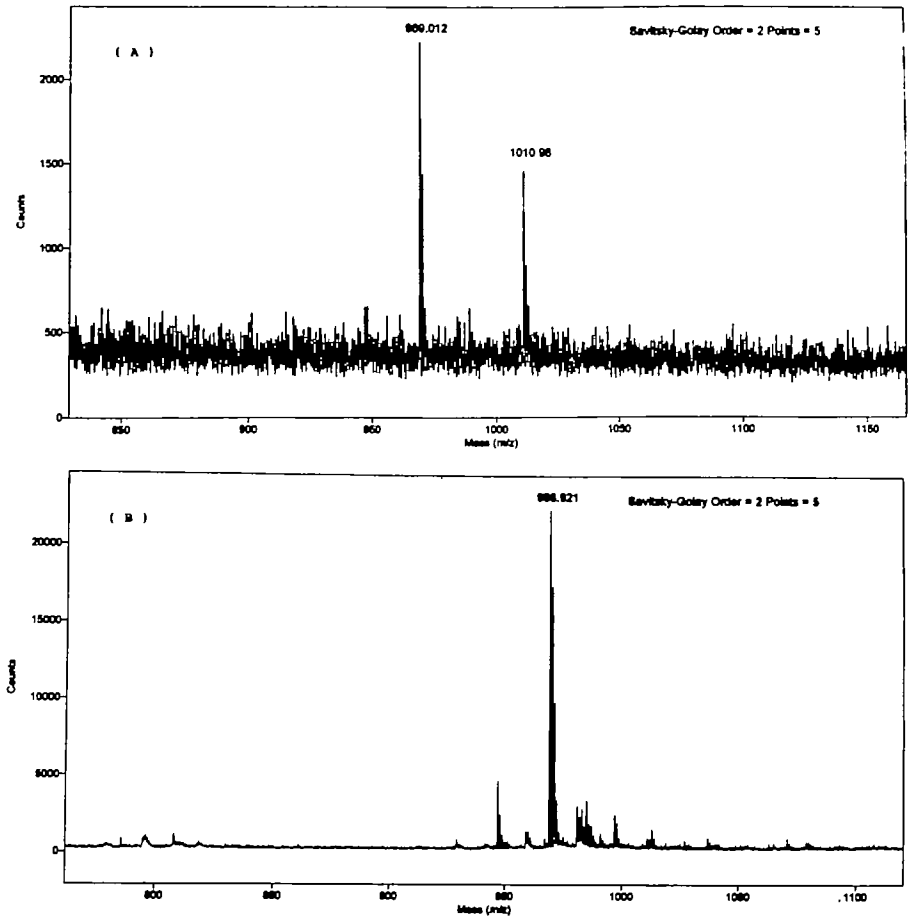

Fig. 4. **(A) High-resolution DE MALDI-TOF mass spectrum of lysoglobotetraosylceramide prepared by the new method in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A except for the laser step: 1,690. **(B) High-resolution DE MALDI-TOF mass spectrum of lysoglobotetraosylceramide prepared by the orthodox method in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A except for the laser step: 1,600.

and offers high yield as compared with the tedious and time-consuming orthodox method. Although various experimental conditions of microwave-mediated saponification have been examined, those described in "MATERIALS AND METHODS" seem to be the best we have so far found. It is interest that the microwave-mediated saponification with 0.5 M NaOH in methanol more easily hydrolyzes the *N*-fatty acyl group of the ceramide moiety than the acetamide group of *N*-acetylgalactosamine in both globotetraosylceramide and Forssman glycolipid as compared with the orthodox method of preparation of lysosphingolipids (*3*). These findings are reminiscent of the report by Neuenhofer *et al.* (*5*) that when a solution of the appropriate ganglioside (20 μmol) in 1 M methanolic potassium hydroxide (10 ml) was stirred under argon at 102°C in a screw-capped vial for various periods of time (18–27 h), the fatty acyl and acetyl group of the sialic acid residues were completely removed, but the acetamide group of the *N*-acetylgalactosamine moiety of the ganglioside was hardly (~10%) hydrolyzed. They were inclined to explain this in terms of shielding of this acetamide group by the negatively charged carboxyl group of the sialic acid residue. However, since the globotetraosylceramide and Forssman glycolipid have no sialic acid, their explanation is not applicable to the globo-series glycosphingolipids in this experiment.

DE MALDI-TOF mass spectrometry in the reflector mode is a highly sensitive, high-resolution, accurate method of mass measurement not only for lysosphingolipids, but also sphingolipids (data not shown). Thus, the mass spectra of lysoglycosphingolipids gave the precise mass of the molecule-related ions, indicating the constituents of long chain bases at the same time, but the appearance of $(M+Na)^+$ could not be avoided despite desalting by Sep-Pak C-18. For TOF mass spectra, the lysoglycosphingolipids were examined in α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid as the matrix. However, since it was suggested that α-CHCA is better than 2,5-DHB as the matrix for the lysoglycosphingolipids, the data with 2,5-DHB are not shown except in the case of lysoglucosylceramide. Owing to the acid lability of the glycosidic linkages, splitting of the glycosphingolipid amide bonds should be performed under alkaline conditions. Thus, our rapid methods for the preparation of lysoglycosphingolipids by microwave-mediated saponification and for sensitive and accurate confirmation of them by DE MALDI-TOF mass spectrometry are also very useful for the determination of natural D-*erythro*-sphingosine components of glycosphingolipids; the usual method for the determination of sphingosine components of sphingolipids involves acidic methanolysis, which leads to the formation of by-products such as *O*-methyl ethers of sphingosine and *threo*-sphingosine (*25, 26*). These new methods can be applied not only to globo-series glycosphingolipids, but also to gala-series, ganglio-series, and other sphingolipids, as will be described elsewhere.

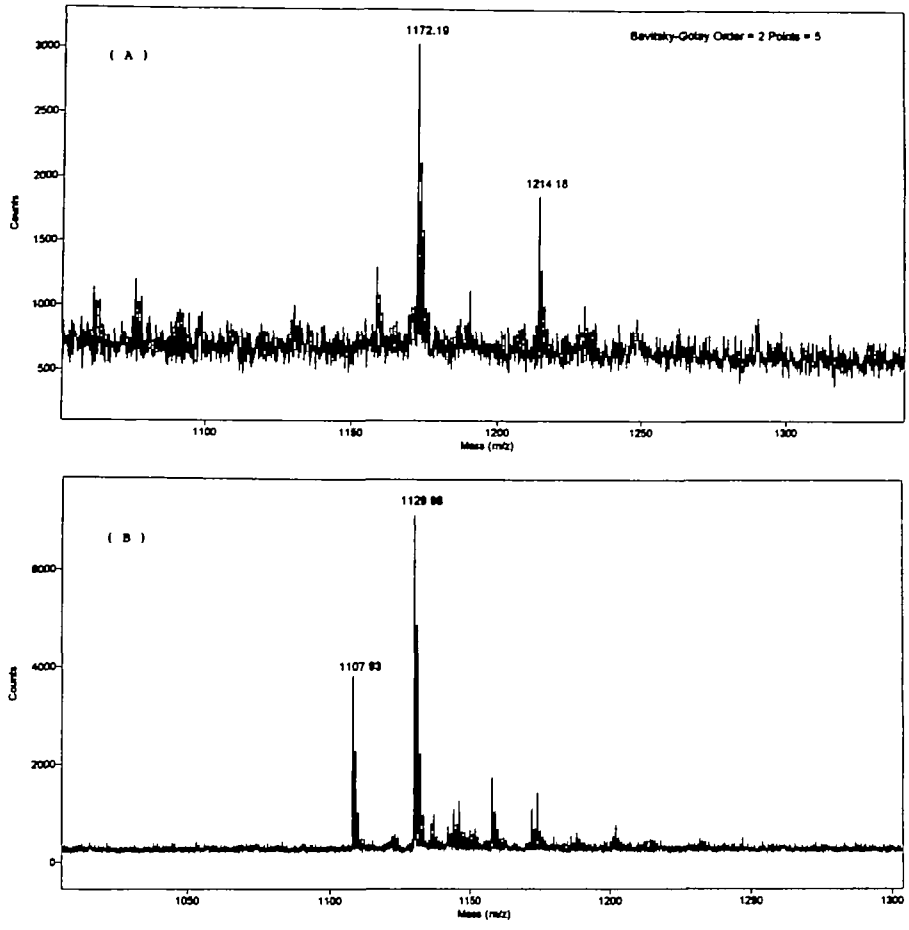

Fig. 5. **(A) High-resolution DE MALDI-TOF mass spectrum of lyso-Forssman glycolipid (lysoglobopentaosylceramide) prepared by the new method in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A except for the laser step: 1,700. **(B) High-resolution DE MALDI-TOF mass spectrum of lyso-Forssman glycolipid prepared by the orthodox method in the reflector mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 1A except for the laser step: 1,650.

REFERENCES

1. Taketomi, T. and Yamakawa, T. (1963) Immunochemical studies of lipids I. Preparation and immunological properties of synthetic psychosine-protein antigens. *J. Biochem.* **54**, 444-451
2. Radin, N.S. (1974) Preparation of psychosine (1-*O*-hexosylsphingosine) from cerebrosides. *Lipids* **9**, 358-360
3. Taketomi, T., Kawamura, N., Hara, A., and Murakami, S. (1976) Comparative studies on chemical hemolytic and diffusion-in-gel precipitation properties of various lysosphingolipids. *Biochim. Biophys. Acta* **424**, 106-113
4. Suzuki, Y., Hirabayashi, Y., and Matsumoto, M. (1984) Hydrazinolysis of glycosphingolipids. A new method for preparation of *N*-deacylated (Lyso) glycosphingolipids. *J. Biochem.* **95**, 1219-1222
5. Neuenhofer, S., Schwarzmann, G., Egge, H., and Sandhoff, K. (1985) Synthesis of lysogangliosides. *Biochemistry* **5**, 525-532
6. Gasa, S., Kamio, K., and Makita, A. (1992) Improved preparation method for lysogangliosides. *J. Lipid Res.* **33**, 1079-1084
7. Sonnino, S., Acquotti, D., Kirschner, G., Uguaglianza, A., Zecca, L., Rubino, F., and Tettamanti, G. (1992) Preparation of lyso-GM1 (II^{3}Neu5AcGgOse4-long chain base) by a one-pot reaction. *J. Lipid Res.* **33**, 1221-1226
8. Hara, A. and Taketomi, T. (1986) Chemical properties and stereoisomerism of heterogeneous long chain bases in lysosphingolipids by positive ion fast atom bombardment mass spectrometry and carbon-13 NMR spectroscopy. *J. Biochem.* **100**, 415-423
9. Taketomi, T., Hara, A., Kutsukake, Y., and Sugiyama, E. (1990) New approach for characterization of lysosulfatide by TLC, fast atom bombardment mass spectrometry and NMR spectroscopy. *J. Biochem.* **107**, 680-684
10. Harvey, D.J. (1995) Matrix-assisted laser desorption/ionization mass spectrometry of sphingo- and glycosphingolipids. *J. Mass Spectrom.* **30**, 1311-1324
11. Taketomi, T. and Nishimura, K. (1964) Physiological activity of psychosine. *Jpn. J. Exp. Med.* **34**, 255-265
12. Nilsson, O. and Svennerholm, L. (1982) Accumulation of glucosylceramide and glucosylsphingosine (psychosine) in cerebrum and cerebellum in infantile and juvenile Gaucher disease. *J. Neurochem.* **39**, 709-718
13. Igisu, H. and Suzuki, K. (1984) Progressive accumulation of toxic metabolite in a genetic leukodystrophy. *Science* **224**, 753-755
14. Strassberg, P. (1986) Cerebroside and psychosine disrupt mitochondrial functions. *Biochem. Cell Biol.* **64**, 485-489
15. Igisu, H. and Nakamura, M. (1986) Inhibition of cytochrome c oxidase by psychosine (galactosylsphingosine). *Biochem. Biophys. Res. Commun.* **137**, 323-327
16. Hannun, Y.A. and Bell, R.M. (1987) Lysosphingolipids inhibit protein kinase C: Implication for the sphingolipidoses. *Science* **235**, 670-674
17. Hakomori, S. and Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling. *J. Biochem.* **118**, 1091-1103
18. Kubota, M. (1972) Studies on neural and visceral glycolipids in a case of infantile form of Gaucher's disease. *Jpn. J. Exp. Med.* **42**, 513-526
19. Taketomi, T. and Kawamura, N. (1972) Chemical and immunological characterization of caprine erythrocyte glycolipids in comparison with porcine erythrocyte glycolipids. *J. Biochem.* **72**, 799-806
20. Khan, M.U. and Williams, J.P. (1993) Microwave-mediated methanolysis of lipids and activation of thin-layer chromatographic plates. *Lipids* **28**, 953-955

21. Taketomi, T. (1969) Immunochemical studies of lipids III. Precipitation of sera of various animals with synthetic glycosphingosyl-protein conjugates. *J. Biochem.* **65**, 239-246
22. Neuenhofer, S., Conzelmann, E., Schwarzmann, G., Egge, H., and Sandhoff, K. (1986) Occurrence of lysoganglioside Lyso-GM2 (II^3-Neu5Ac-gangliotriaosylsphingosine) in GM2 gangliosidosis brain. *Biol. Chem. Hoppe-Seyler* **367**, 241-244
23. Nilsson, O., Mansson, J.-E., Hakansson, G., and Svennerholm, L. (1982) The occurrence of psychosine and other glycolipids in spleen and liver from the three major types of Gaucher's disease. *Biochim. Biophys. Acta* **712**, 453-463
24. Kobayashi, T., Goto, I., Okada, S., Orii, T., Ohno, K., and Nakano, T. (1992) Accumulation of lysosphingolipids in tissues from patients with GM1 and GM2 gangliosidoses. *J. Neurochem.* **59**, 1452-1458
25. Taketomi, T. and Kawamura, N. (1972) Degradation of sphingosine bases during acid hydrolysis of sphingomyelin, cerebroside or psychosine. *J. Biochem.* **72**, 189-193
26. Kisic, A., Tsuda, M., Kulmacz, R.J., Wilson, W.K., and Schroefer, G.J., Jr. (1995) Sphingolipid bases. A revisitation of the *O*-methyl derivatives of sphingosine. Isolation and characterrization of diacetate derivatives, with revised ^{13}C nuclear magnetic assignments for D-*erythro*-sphingosine. *J. Lipid Res.* **36**, 787-803