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Preparation of Various Lysogangliosides Including Lyso-Fucosyl GM1 and Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometric Analysis

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Our rapid method of microwave-mediated saponification for preparing lysoglycosphingolipids from their parent glycosphingolipids was also able to prepare lysogangliosides or modified lysogangliosides, which were identified by delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometric (DE MALDI-TOF MS) analysis. When GM3, GM2, and GM1 isolated from adult human brain gangliosides were subjected to the saponification, GM3 was found to give rise to only lyso-GM3 containing de-*N*-acetylneuraminic acid (de-*N*-acetyl lyso-GM3), whereas the GM2 produced both lyso-GM2 and the de-*N*-acetyl compound, and GM1 also gave both lyso-GM1 and the de-*N*-acetyl compound. In the saponification of GM1 and GDla, isolated from rat brain gangliosides, GM1 similarly produced both lyso-GM1 and the de-*N*-acetyl compound, but GDla was found to give rise to both dehydrated de-*N*-monoacetyl and dehydrated de-*N*-diacetyl lyso-GDla. However, the saponification of the GM1 fraction isolated from porcine brain gangliosides gave rise not only to both lyso-GM1 and the de-*N*-acetyl compound, but also unexpectedly to both lyso-fucosyl GM1 and its de-*N*-acetyl compound. The untreated GM1 fraction was examined by TLC and DE MALDI-TOF mass spectrometry, and proved to contain fucosyl-GM1. The DE MALDI-TOF MS analysis of the prepared lyso-gangliosides showed that their long chain bases consisted of d18:1 and d20:1 sphingosines in various ratios reflecting those of the different mammalian brain gangliosides.

Key words: delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry, fucosyl-GM1 ganglioside, ganglioside, lysoganglioside, sphingosine.

We introduced a method for the preparation of lysohematoside from equine erythrocyte hematoside (*N*-glycolyl GM3) by means of an alkaline reaction for the first time in 1970 (*1*). Subsequently, other groups applied, and in some cases improved, the procedure for the preparation of more complex deacylated gangliosides (*2, 3*). The alkaline conditions used gave rise to products lacking not only the fatty acyl group, but also the acetyl group of *N*-acetylneuraminic acid (*2*) or of both *N*-acetylneuraminic acid and *N*-acetylgalactosamine (*4*). The preparation of lysogangliosides lacking only the fatty acyl group was achieved by Neuenhofer *et al.* (*2*) and later improved by Gasa *et al.* (*3*) using different blocking agents for amino residues. Sonnino *et al.* (*5*) reported a simple procedure for preparing lyso-GM1 that lacks the fatty acyl group, but not the acetamide groups of galactosamine and sialic acid units, by using a one-pot reaction starting from GM1 ganglioside. We (*6, 7*) have recently developed a new, rapid method for preparing various kinds of lysoglycosphingolipids which can be precisely identified by a new technology, DE MALDI-TOF MS. In this study, this rapid method was also applied to various gangliosides to prepare their lysogangliosides. It was clarified by DE MALDI-TOF MS that the microwave-mediated saponification for 2 min of gangliosides gives rise to products lacking not only the fatty acyl group, but also the acetamide group of the sialic acid unit. Also, the DE MALDI-TOF mass spectrometric analysis of prepared lysogangliosides led to the finding that fucosyl-GM1 containing *N*-acetylsialic acid is present in the porcine brain. These experimental results are presented in detail in this paper.

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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.

MATERIALS AND METHODS

Materials—GM3, GM2, and GM1 isolated from human brain gangliosides, GM1 and GDla from rat brain and the GM1 fraction from porcine brain (*8*) were available for this experiment. Lysohematoside containing de-*N*-glycolylneuraminic acid prepared from equine erythrocyte hematoside by our orthodox method (*1, 9*) was used as a control. α-Cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (2,5-DHB), used as the matrices for DE MALDI-TOF mass spectrometry, were purchased from

Sigma Chemical (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid were from Nacalai Tesque (Kyoto).

Rapid Method of Microwave-Mediated Saponification of Various Gangliosides for Preparation of Their Lysogangliosides—Approximately 1 mg of a sample of gangliosides 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 maximum power of the microwave oven (500 W, Toshiba model ER-V 11) for 2 min as already reported by us (*6, 7*).

After the microwave-mediated saponification, the reaction mixture was cooled to room temperature and acidified with one drop of 6 N HCl together with one drop of distilled water. The solution 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 lower phase was routinely applied to a Sep-Pak C-18 cartridge to remove salt.

The desalting was performed with 10 ml each of methanol-water (3:7, v/v) and water, and the lysogangliosides were eluted with 2 ml of ethanol and 10 ml of chloroform-methanol-water (60:30:4.5, by volume) (*10*).

DE MALDI-TOF Mass Spectrometric Analysis of Lysogangliosides and Gangliosides—As described elsewhere (*6, 7*), lysogangliosides or gangliosides were subjected to DE MALDI-TOF mass spectrometric analysis. Chloroform/methanol (1:1, v/v) solution (1.5 μl) containing about 10-100 pmol of the sample 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% 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 on a vortex mixer and centrifuged on a microcentrifuge (Japan Millipore, Tokyo) for about 1 min. One microliter of the supernatant was loaded on a sample plate with 100 sample positions. The plate was loaded into a Voyager™ Elite XL (6.6 m flight length) Biospectrometry™ Workstation (PerSeptive Biosystems, Framingham, MA, USA) and the mass spectrum of the sample was acquired by the software of the instrument, with an N_2 laser (337 nm) in the reflector mode, in the negative ion mode. The resolution of the ion peak was determined by the resolution calculator from the GRAMS/386 software supplied with the instrument. The ion peaks were calibrated with α-CACH dimer ($[M+H]^+$, 379.093) and angiotensin I ($[M+H]^+$, 1,296.685) in the calibration file of the software. The mass spectra were smoothed by 5-point Savitsky-Golay smoothing.

Thin-Layer Chromatography of Gangliosides—Samples were loaded on HPTLC plates, which were developed with the solvent system, chloroform/methanol/0.2% $CaCl_2$ aqueous solution (60 : 35 : 8, by volume). Gangliosides were visualized with a diphenylamine reagent at 110°C for 20 min.

RESULTS

Lyso-Compounds Prepared from Human Brain GM3, GM2, and GM1 Gangliosides—First, for comparison, the de-*N*-glycolyl lysohematoside (monoisotopic M.W.= 872.48 as d18:1 sphingosine) prepared from equine erythrocyte hematoside by our orthodox method (*1*) was subjected to DE MALDI-TOF mass spectrometry in the reflector mode, in the negative ion mode. As shown in Fig. 1, the negative ion peak of $[M-H]^-$ at *m/z* 871.63 in the mass spectrum confirmed the sample as the de-*N*-glycolyl lysohematoside containing a major d18:1 sphingosine. The de-*N*-glycolyl lysohematoside prepared from the same hematoside by our new rapid method of microwave-mediated saponification (*6, 7*) gave the same mass spectrum(data not shown). The new rapid method was then applied to human brain GM3 ganglioside to prepare the lyso-compound, which was also subjected to the mass spectrometric analysis.

The mass spectrum showed two negative ion peaks at *m/z* 871.78 and at *m/z* 899,79 (Fig. 2), indicating that the de-*N*-acetyl lyso-GM3 (monoisotopic M.W.=872.48 as d18:1, 900.51 as d20:1) contained a higher ratio of d18:1/d20:1. In this case, it was hard to prepare the lyso-GM3 lacking only the fatty acid moiety.

Secondly, the lyso-GM2 fraction similarly prepared from GM2 ganglioside by the rapid method showed four major negative ion peaks at *m/z* 1,075.29, at *m/z* 1,103.35, at *m/z* 1,117.32 and at *m/z* 1,145.39 (Fig. 3), indicating that de-*N*-acetyl lyso-GM2 (monoisotopic M.W.=1,075.56 as d18:1, 1,103.59 as d20:1) and lyso-GM2 (monoisotopic M.W.=1,117.56 as d18:1, 1,145.59 as d20:1) containing a slightly lower ratio of d18:1/d20:1 had been obtained, and that the microwave-mediated saponification of GM2 split

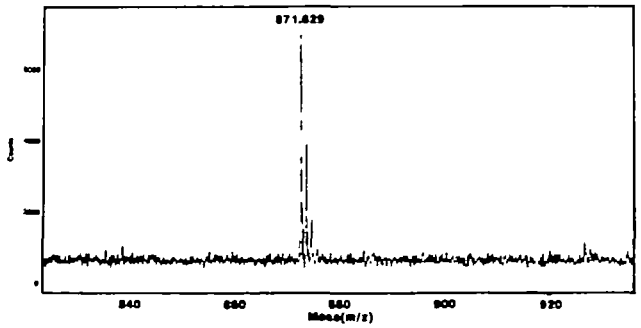


Fig. 1. **DE MALDI-TOF mass spectrum of de-*N*-glycolyl lysohematoside (NeuNH$_2\alpha$2-3Galβ1-4Glcβ1-1′sphingosine) prepared from equine erythrocyte hematoside in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Accelerating voltage: 20,000, grid voltage: 74.0% of the accelerating voltage, guide wire voltage: 0.05% of the accelerating voltage, delay: 100 ns, laser step: 2,000, scan average: 128.

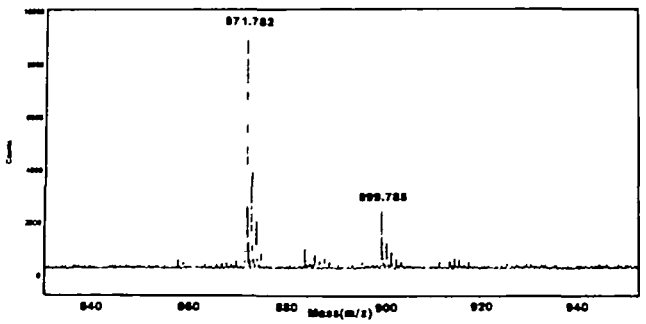


Fig. 2. **DE MALDI-TOF mass spectrum of de-*N*-acetyl lyso-GM3 (NeuNH$_2\alpha$2-3Galβ1-4Glcβ1-1′sphingosine) prepared from human brain GM3 ganglioside in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Conditions were the same as in Fig. 1, except for the laser step: 1,630.

the *N*-fatty acyl group more easily than the acetamide groups of *N*-acetylneuraminic acid. Also, the deprotonated molecular ions of lyso-GM2 at *m/z* 1,117.32 and at *m/z* 1,145.39 suggested that the acetamide group of *N*-acetylneuraminic acid of lyso-GM2 was a little more resistant to the microwave-mediated saponification, because no lyso-GM3 except for de-*N*-acetyl lyso-GM3 was obtained, as described above. The acetamide group of *N*-acetylgalactosamine of lyso-GM2 seems to be resistant to microwave-mediated saponification, like those of lysogloboside and lyso-Forssman, as already reported by us (*6*).

Thirdly, the lyso-compound fraction prepared from GM1 ganglioside by the rapid method showed four major negative ion peaks at *m/z* 1,237.42, at *m/z* 1,265.47, at *m/z* 1,279.47, and at *m/z* 1,307.52 (Fig. 4), indicating that de-*N*-acetyl lyso-GM1 (monoisotopic M.W.=1,237.62 as d18:1, 1,265.65 as d20:1) and lyso-GM1 (monoisotopic M.W.=1,279.62 as d18:1, 1,307.65 as d20:1) containing a slightly lower ratio of d18:1/d20:1 had been formed, and that the microwave-mediated saponification of GM1 split the *N*-fatty acyl group and partially split the acetamide group of *N*-acetylneuraminic acid.

Lyso-Compounds Prepared from Rat Brain GM1 and GDla Gangliosides—With regard to the lyso-GM1 fraction prepared from rat brain ganglioside GM1, four negative ion peaks at *m/z* 1,237.24, at *m/z* 1,265.27, at *m/z* 1,279.26, and at *m/z* 1,307.29 were seen in the mass spectrum (Fig. 5), indicating that de-*N*-acetyl lyso-GM1 and lyso-GM1 contained a higher ratio of d18:1/d20:1, which was in contrast with those prepared from human brain GM1 ganglioside.

Next, the lyso-GDla fraction prepared from GDla ganglioside by the rapid method showed four major negative ion peaks at *m/z* 1,468.43, at *m/z* 1,496.5, at *m/z* 1,510.45, and at *m/z* 1,538.55 (Fig. 6), suggesting that the saponification had produced dehydrated de-*N*-diacetyl-lyso-GDla (monoisotopic M.W.=1,469.70 as d18:1, 1,497.73 as d20:1) and dehydrated de-*N*-monoacetyl lyso-GDla (monoisotopic M.W.=1,511.70 as d18:1, 1,539.73 as d20:1), because the mass spectrometric analysis of GDla showed only the deprotonated molecular ions (data not shown). The modified lyso-GDla contained a higher ratio of d18:1/d20:1, which was similar to the sphingosine ratio of

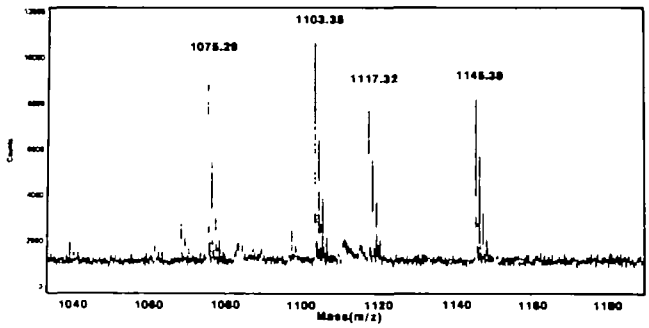
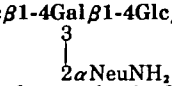


Fig. 3. **DE MALDI-TOF mass spectrum of de-*N*-acetyl lyso-GM2 (GalNAcβ1-4Galβ1-4Glcβ1-1′sphingosine) and lyso-GM2**



prepared from human brain GM2 in the reflector mode, in the negative ion mode with α-CHCA as the matrix. Conditions were the same as in Fig. 1, except for the laser step: 1,700.

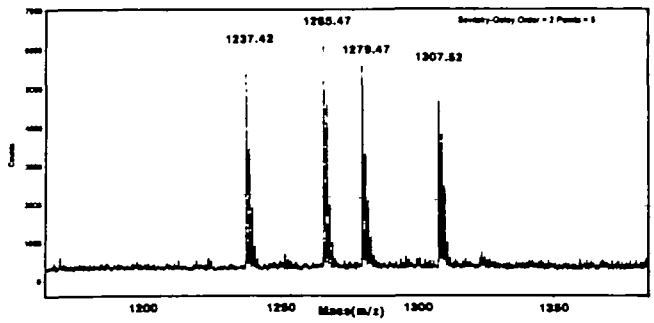
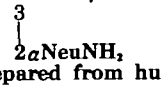


Fig. 4. **DE MALDI-TOF mass spectrum of de-*N*-acetyl lyso-GM1 (Galβ1-3GalNAcβ1-4Galβ1-4 Glcβ1-1′sphingosine) and**



lyso-GM1 prepared from human brain GM1 in the reflector mode, in the negative ion mode with α-CHCA as the matrix. Conditions were the same as in Fig. 1 except for the laser step: 1,600.

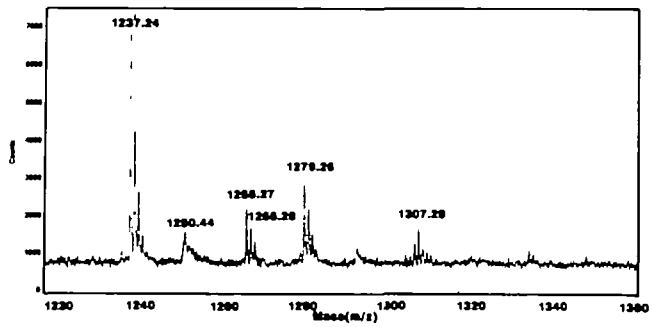


Fig. 5. **DE MALDI-TOF mass spectrum of de-*N*-acetyl lyso-GM1 and lyso-GM1 prepared from rat brain GM1 in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Conditions were the same as in Fig. 1 except for the laser step: 1,950.

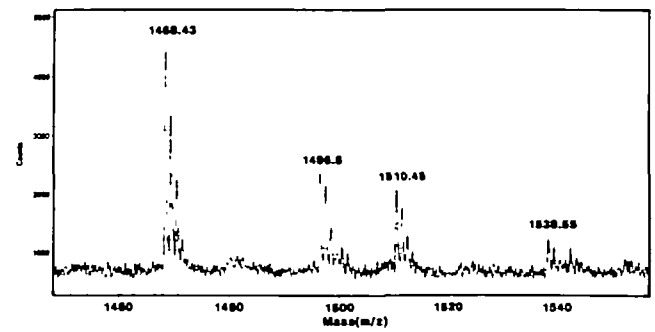
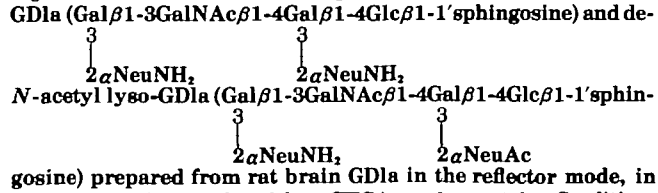


Fig. 6. **DE MALDI-TOF mass spectrum of de-*N*-diacetyl lyso-GDla (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′sphingosine) and de-**



***N*-acetyl lyso-GDla (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′sphingosine) prepared from rat brain GDla in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Conditions were the same as in Fig. 5.

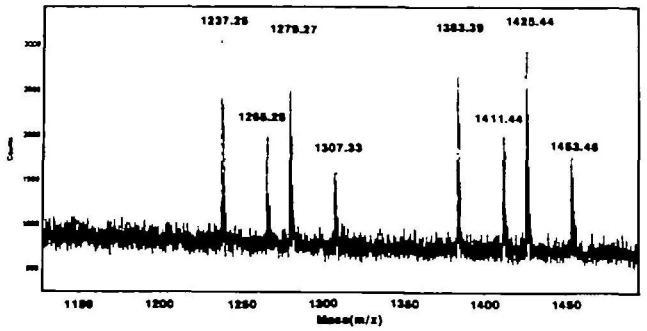


Fig. 7. **DE MALDI-TOF mass spectrum of de-*N*-acetyl lyso-GM1, lyso-GM1, de-*N*-acetyl lyso-fucosyl GM1 (Galβ1-3Gal-**
2
|
1α**Fuc**
NAcβ1-4Galβ1-4Glcβ1-1′sphingosine) and lyso-fucosyl GM1
3
|
2α**NeuNH$_2$**
prepared from porcine brain GM1 fraction in the reflector mode, in the negative ion mode with α-CHCA as the matrix. Conditions were the same as in Fig. 5 except for the laser step: 2,180.

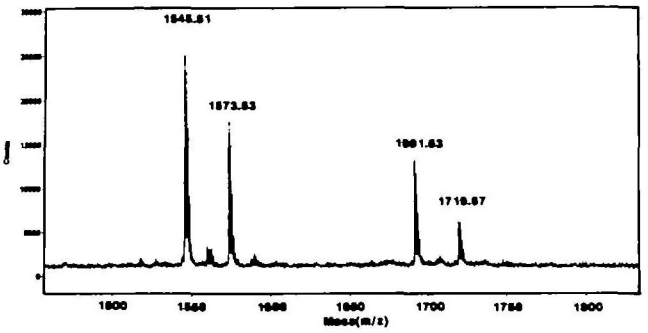


Fig. 8. **DE MALDI-TOF mass spectrum of porcine brain GM1 fraction (containing fucosyl GM1) in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Conditions were the same as in Fig. 7 except for the laser step: 1,960.

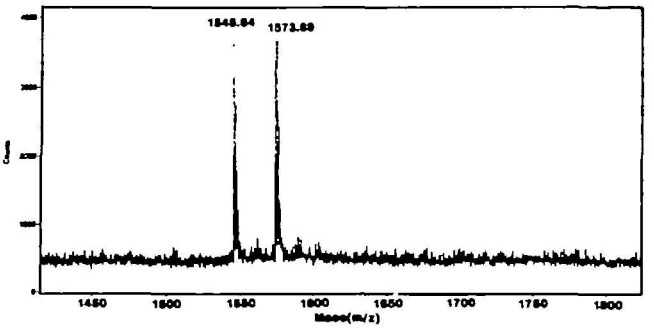


Fig. 9. **DE MALDI-TOF mass spectrum of human brain GM1 fraction in the reflector mode, in the negative ion mode with α-CHCA as the matrix.** Conditions are the same as in Fig. 8 except for the laser step: 2,000.

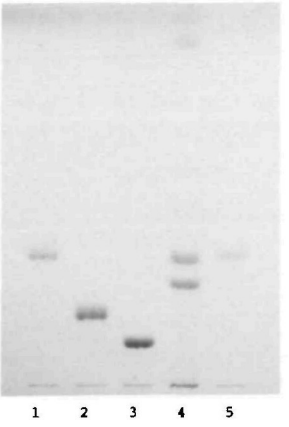


Fig. 10. **HPTLC of porcine and human brain GM1 fractions.** The plate was developed in chloroform-methanol-0.2% $CaCl_2$ 60:35:8 and gangliosides were detected with diphenylamine reagent spray. Lane 1: authentic GM1; lane 2: authentic GDla; lane 3: authentic GDlb; lane 4: porcine brain GM1 fraction (containing fucosyl GM1); lane 5: human brain GM1 fraction.

the previous lyso-GM1 fraction.

Lyso-Compounds Prepared from Porcine Brain GM1 Ganglioside—For the lyso-GM1 fraction prepared from porcine brain GM1 ganglioside, in addition to four major ion peaks of de-*N*-acetyl lyso-GM1 and lyso-GM1 containing both d18:1 and d20:1 sphingosines as described above, four major ion peaks at *m/z* 1,383.39, at *m/z* 1,411.44, at *m/z* 1,425.44, and at *m/z* 1,453.46 were newly found in the mass spectrum (Fig. 7). Judging from the mass numbers, they corresponded to molecule-related ions of de-*N*-acetyl lyso-fucosyl GM1 (monoisotopic M.W.=1,383.67 as d18:1, 1,411.70 as d20:1) and lyso-fucosyl GM1 (monoisotopic M.W.=1,425.67 as d18:1, 1,453.70 as d20:1), which showed a higher ratio of d18:1/d20:1, like the above de-*N*-acetyl lyso-GM1 or lyso-GM1. As the porcine brain GM1 ganglioside fraction was assumed to contain fucosyl-GM1, the GM1 fraction was analyzed by DE MALDI-TOF mass spectrometry and TLC. As shown in Fig. 8, the two major deprotonated ion peaks at *m/z* 1,545.51 and at *m/z* 1,573.53 corresponded in molecular weight to deprotonated GM1 containing both d18:1 and d20:1 sphingosines, as well as stearic acid, whereas the other two major ion peaks at *m/z* 1,691.63 and at *m/z* 1,719.67 corresponded to the molecular weights of deprotonated fucosyl-GM1 containing both d18:1 and d20:1, as well as stearic acid. The ceramide moieties of GM1 and fucosyl GM1 were considered to be very similar to each other, suggesting they are common in the synthetic pathway of gangliosides in the porcine brain.

However, fucosyl GM1 was not found in the human brain GM1 ganglioside fraction, which showed only the two major deprotonated ion peaks at *m/z* 1,545.64 and at *m/z* 1,573.69 (Fig. 9). The GM1 contained a slightly lower ratio of d18:1/d20:1, as well as stearic acid. The fucosyl GM1 in the porcine brain was also clearly detected between the GM1 and GDla spots on TLC (Fig. 10) in this study.

DISCUSSION

The preparation of lysogangliosides has been reported by several workers (*1-3, 5*), and the structures of the lysogangliosides have been confirmed by chemical analysis as well as by negative ion FAB mass spectrometry and ^{1}H NMR spectroscopy. However, further investigation are needed because the lysogangliosides are important starting mate-

TABLE I. **Chemical structures of various gangliosides and chemical formula and monoisotopic molecular weights of their lyso-compounds.**

Ganglioside	Chemical structure	Lyso-compound (d18:1 sphingosine)	
		Chemical formula	Monoisotopic M.W.
Hematoside (GM3)	NeuGcα2-3Galβ1-4Glcβ1-1′Cer	$C_{41}H_{74}N_2O_{21}$	930.48
GM3	NeuAcα2-3Galβ1-4Glcβ1-1′Cer	$C_{41}H_{74}N_2O_{20}$	914.48
GM2	GalNAcβ1-4Galβ1-4Glcβ1-1′Cer 3 2αNeuAc	$C_{49}H_{87}N_3O_{25}$	1,117.56
GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer 3 2αNeuAc	$C_{55}H_{97}N_3O_{30}$	1,279.62
Fucosyl-GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer 2 3 1αFuc 2αNeuAc	$C_{61}H_{107}N_3O_{34}$	1,425.67
GDla	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer 3 3 2αNeuAc 2αNeuAc	$C_{66}H_{114}N_4O_{38}$	1,570.71

M.W., Molecular weight; Glc, glucose; Gal, galactose; NeuGc, *N*-glycolylneuraminic acid; NeuAc, *N*-acetylneuraminic acid; GalNAc, *N*-acetylgalactosamine; Cer, ceramide.

rials for the preparation of derivatives used as reagents for the elucidation of physicochemical, biochemical, physiological, pharmacological, and biological features of gangliosides (*11*).

We have recently introduced a rapid method for the preparation of lysoglycosphingolipids by means of microwave-mediated saponification of gala- and globo-series glycosphingolipids (*6, 7*). We have established here that our new rapid method is also applicable to various gangliosides to prepare their lyso-compounds, although it also gives rise to the lyso-compounds lacking the acetyl group of *N*-acetylneuraminic acid. However, as already reported by Neuenhofer *et al.* (*2*), the acetamide group of the *N*-acetylgalactosamino moiety of the GM2, GM1, and GDla gangliosides was very little hydrolyzed by the rapid method.

Although we (*6, 7*) have already pointed out that the new technology, DE MALDI-TOF MS, is extremely effective for the confirmation of various lysoglycosphingolipids in the positive or negative ion mode, it has been clarified that this mass spectrometric analysis is also effective for the identification of various lysogangliosides in the negative ion mode. This analytical instrument shows sufficiently high resolution to distinguish between lysogangliosides and de-*N*-acetyl lysogangliosides containing d18:1 and d20:1 sphingosines. For the DE MALDI-TOF MS analysis of various lysogangliosides, the negative ion mode is better than the positive ion mode because the former suppresses alkali metal adduct production and salt formation (*12*). It was also suggested that α-CHCA is a better matrix than 2.5-DHB (data not shown) for lysogangliosides, as well as for lysoglycosphingolipids (*6*). DE MALDI-TOF mass spectrometric analysis of the lyso-GM1 fraction derived from porcine brain GM1 suggested the presence of lyso-fucosyl GM1 in addition, and the mass spectrometric analysis of the untreated GM1 fraction resulted in the finding of fucosyl GM1 containing both d18:1 and d20:1 sphingosines as well as stearic acid, which are characteristics of the ceramide moiety of brain gangliosides. The compositions of fatty acid and long chain base of the porcine brain fucosyl GM1 are different from those of boar testis fucosyl GM1 (*13*). Although fucosyl GM1 was found in beef brain (*14*), the ceramide moiety was not described at all. Similarly, although fucosyl GDlb [$II^3\alpha(NeuAc)_2IV^2\alpha$FucGgOse4Cer] containing a 3:2 ratio of d18:1/d20:1 as well as a large amount of stearic acid was found in pig cerebellum (*15*), the fucosyl GM1 was not described. We previously (*8*) failed to detect fucosyl GM1 in porcine white and gray matter. It seems to us now that the fucosyl GM1 may have overlapped with GM1 on TLC, and thus, was not detected. Consequently, DE MALDI-TOF MS should be utilized for the reconfirmation of some lyso-glycosphingolipids or lysogangliosides found in the brain and other tissues from patients with sphingolipidoses, because the reported evidence for them seems weak, even though some were analyzed by negative ion FAB mass spectrometry or other procedures (*16, 17*) and by HPLC (*18*). Despite the weak evidence, speculative hypotheses concerning sphingolipidoses due to lysosphingolipids and related substances inhibiting protein kinase C or concerning functions of sphingolipids and sphingolipid breakdown products in cellular regulation have been presented (*19, 20*). Although they appear to be attractive, further investigation on the lysogangliosides or lysoglycosphingolipids should be carried out with advanced technology to obtain stronger evidence.

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