Synthesis of a glioma-related ganglioside, *O*-Ac GM3 having 3-*O*-Ac ceramide and its substrate property toward hydrolases

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Abstract An *O*-acetyl group was selectively introduced into the ceramide moiety at the C-3-*O* on ganglioside GM3 containing *N*-acetyl neuraminic acid, the product of which has been previously found in rat glioma tissue as a glioma-associated ganglioside. The introduction of the acetyl residue involved a two-step process involving per *O*-acetylation of GM3 and saponification with a mild alkaline solution in a bilayer system constituted of water and water-immiscible organic solvent. Of the several solvents studied, 2-pentanol and diethyl ether gave the highest yields (68% and 62%, respectively). The chemical structure of the synthesized 3-*O*-acetyl GM3 was confirmed by proton nuclear magnetic resonance spectroscopy and fast atom bombardment–mass spectrometry, as well as by comparing the mobilities on thin-layer chromatography of its exoglycosidase-digested products with those of the synthesized, authentic 3-*O*-acetyl-lactosylceramide and -ceramide. Furthermore, the substrate specificities of both 3-*O*-acetyl GM3 and 3-*O*-acetyl sphingomyelin toward exo- and endo-hydrolases were examined, revealing that they were hardly cleaved by the endoglycoceramidase and sphingolipid *N*-deacylase for the 3-*O*-acetyl GM3 and by sphingomyelinase for 3-*O*-acetyl sphingomyelin. Thus, the enzymes were found to recognize a free C-3 hydroxyl group on ceramide.—**Tsuchihashi, K., T. Daino, T. Akino, and S. Gasa.** Synthesis of a glioma-related ganglioside, *O*-Ac GM3 having 3-*O*-Ac ceramide and its substrate property toward hydrolases. *J. Lipid Res.* 1996. **37:** 2136–2144.

Supplementary key words *O*-acetyl GM3 • *O*-acetylation • NMR • ganglioside • *O*-acetyl ceramide

O-acetylated gangliosides occur in a wide variety of species with tissue specificity, as well as in accordance to tumorigenicity of epithelial cells. For example, GM3 containing 4-*O*-acetyl (Ac) *N*-glycolylneuraminic acid (NeuGc) (1, 2), 9-*O*-Ac NeuGc and 6-*O*-Ac galactose (3) has been found in equine erythrocytes; GD3 with 9-*O*-Ac NeuAc (4, 5) and with 7-*O*-Ac- and 7,9-*di*-*O*-Ac NeuAc (5) in buttermilk; GD3 with 9-*O*-Ac NeuAc in melanoma (6, 7); GT2 (8) and GT3 (9) with 9-*O*-Ac NeuAc in codfish brain. All these gangliosides were *O*-acetylated derivatives at their sugar moiety. Recently, we isolated and characterized another novel *O*-Ac ganglioside, *O*-Ac GM3 containing NeuAc (GM3 (Ac)) having 3-*O*-Ac sphingosine from transplanted rat glioma tissue (10). In addition, the immunological property of the modified GM3 (Ac) at the lipid moiety was significantly different from unacetylated GM3 (Ac) toward monoclonal antibody (M2590) in terms of the liposome-lysis system containing complement (10). An elevation of the immunological activity was observed on the liposome with phospholipids containing a mixture of GM3 (Ac) and 3-*O*-Ac GM3 (Ac) rather than on that containing GM3 (Ac), suggesting that recognition of the sugar moiety by the antibody might be ascribed to a modification of the lipid moiety like *O*-acetylation. Further, to investigate the properties of the 3-*O*-Ac GM3, selective chemical introduction of the Ac residue to the sphingosine on GM3 (Ac) was, therefore, required.

With respect to the chemical *O*-acetylation of monosaccharides and gangliosides, Haverkamp et al. (11) have reported the synthesis of 9-*O*-Ac and 4,9-*di*-*O*-Ac NeuAc methyl ester using *N*-Ac imidazolide under basic conditions, and the reaction was further developed by Ritter et al. (12) to synthesize *O*-acetylated GD3; however, the acetylation of GD3 did not bring about lipid acetylation. Another *O*-acetylation reaction of sialic acid was presented by Ogura et al. (13) using trimethyl orthoacetate under acidic conditions, giving the 4-*O*-Ac derivative, but was not applicable for lipid-acetylation. The establishment of the lipid acetylation of the glycolipid described herein is the first to be reported.

Abbreviations: Cer, ceramide; GM3 (Gc), GM3 containing *N*-glycolylneuraminic acid (NeuGc) (II^3NeuGc α LacCer); GM3 (Ac), GM3 containing *N*-acetylneuraminic acid (NeuAc); TLC, thin-layer chromatography; NMR, nuclear magnetic resonance spectroscopy; FAB–MS, fast atom bombardment–mass spectrometry; CMW, chloroform–methanol–water.

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MATERIALS AND METHODS

Chemicals

DEAE-Sephadex, A-25 and Sephadex LH-20 were obtained from Pharmacia-LKB (Uppsala, Sweden); silica beads (Iatrobeads) from Iatron Laboratories (Tokyo); silica gel thin-layer chromatography (TLC) plate (silica gel 60) and [$^2H_6$]dimethylsulfoxide (Me_2SO-d_6) were from Merck (Germany). Exo- and endo-glycosidases were obtained as follows: neuraminidase (*Arthrobacter ureafaciens*) and β-galactosidase (jack bean) from Seikagaku Kogyo Corp. (Tokyo); β-glucosidase (sweet almonds) from Boehringer-Mannheim (Germany); endoglycoceramidase (*Rhodococcus sp.*) from Takara Inc., (Tokyo). The endoglycoceramidase as well as sphingolipid *N*-deacylase were kindly donated by Dr. Makoto Ito, Faculty of Agriculture of Kyushu University. Phospholipase C (*Clostridium welchii*), sphingomyelinase (*Staphylococcus aureus*), and sphingomyelin (bovine brain) were from Sigma (St. Louis, MO). Ganglioside GM3 (Gc) was previously prepared from equine erythrocyte membranes (2, 3). Other standard glycolipids were prepared in this laboratory, and all other reagents were of analytical grade.

Per *O*-acetylation of GM3 (Ac)

The ratio of solvent mixture is expressed by volume. To obtain a large amount of GM3 (Ac), the *N*-Gc residue at the sialic acid moiety of GM3 (Gc) from equine erythrocytes (2, 3), was replaced with an *N*-Ac group through the de-*N*-glycolyl GM3 derivative, which was derived with mild alcoholic alkaline solution under heating according to the method of Taketomi and Kawamura (14) with a yield of 90%. The *N*-acetylation of the de-*N*-glycolyl GM3 after purification by silica-gel column chromatography as described below was performed with acetic anhydride (20 μl per 10 mg of de-*N*-glycolyl GM3) in a bilayer solution (1 ml per 10 mg of the lipid) composed of aqueous 0.3 M sodium bicarbonate and diethyl ether with a ratio of 3:2, to quantitatively give GM3 (Ac). The purity of the converted GM3 (Ac) was determined by TLC analysis and proton nuclear magnetic resonance spectroscopy (NMR) to be over 99%.

The GM3 (Ac) was first per *O*-acetylated as follows: 50 mg of well-dried GM3 (Ac) was dissolved in 3 ml of dried pyridine (distilled over potassium hydroxide) followed by addition of 1 ml of acetic anhydride, and the mixture was stirred for 16 h at room temperature. After the GM3 (Ac) was completely converted to faster-migrating compound(s) on TLC, developed with chloroform–methanol–water (CMW) 60:35:8 and stained by orcinol-sulfuric acid reagent, 5 ml of methanol was added to the reaction mixture followed by evaporation of the solvents to dryness. The crude *O*-acetylated products were subjected to limited alkaline hydrolysis as described below without further purification.

Limited alkaline hydrolysis of per *O*-acetylated GM3

The per *O*-acetylated GM3 (Ac) (10 or 20 mg) obtained as above was suspended in a mixture consisting of 0.57 ml of water and 0.33 ml of water-immiscible organic solvent, followed by sonication and vigorous stirring to make emulsion at 25°C in a water bath. To the mixture, 0.1 ml of aqueous 1 N NaOH was added under vigorous stirring. The resultant reaction mixture consisted of 10 or 20 mg of GM3 in 1.0 ml of the solvent mixture of water and organic solvent with a ratio of 2:1 and a final concentration of 0.1 N NaOH. An aliquot (50 μl) of the reaction mixture was neutralized with 5 μl of 1 N HCl at the indicated reaction time followed by dilution with 0.2 ml of CM, 1:4. An aliquot (5 μl) of the diluted mixture was chromatographed on a TLC plate and stained as described above, and resultant spots on the stained plate were scanned using a chromatoscanner (CS-910, Shimadzu) equipped with a data processor (Chromatopac C-R6A, Shimadzu) to assess the generation of 3-*O*-Ac GM3 (the structure of which was determined as described below). As the water-immiscible organic solvents, alcohols such as 1-butanol, 2-butanol, 1-pentanol, 2-pentanol, and 3-pentanol, and ethers such as diethyl ether, *di-n*-butyl ether and *di-n*-hexyl ether were examined.

To prepare a large amount of 3-*O*-Ac GM3 (Ac), 50 mg of per *O*-acetylated GM3 (Ac) was hydrolyzed with 0.1 N NaOH in a final concentration in 2.5 ml of water–2-pentanol 2:1 at 25°C for 70 min. After neutralization of the mixture with 0.25 ml of 1 N HCl, the mixture was concentrated and applied to an LH-20 (Pharmacia-LKB, Sweden) column (1 × 50 cm) with CMW (60:30:4.5) to remove salts. The sugar-positive fractions excluded from the column were combined, evaporated for the solvents, and applied on a silica gel (Iatrobeads, Iatron, Tokyo) column (1 × 30 cm) with CMW (90:10:0.1). The ratio of the CMW mixture for elution was changed stepwise from 90:10:0.5 to 85:15:1.5, 80:20:2, 75:25:2.5 and 70:30:3 (200 ml each). The 3-*O*-Ac GM3 (Ac) and starting GM3 (Ac) were eluted from the column with CMW (80:20:2) and CMW (75:25:2.5) to (70:30:3), to give 30 mg and 7 mg of pure materials, respectively.

Preparation of authentic 3-*O*-Ac-LacCer and -Cer

The *O*-Ac residue was inserted at C-3-*O* of Cer on LacCer in same manner as described above but with a half alkaline concentration, to give authentic 3-*O*-Ac LacCer with a yield of 50% when diethyl ether was used as the organic solvent. The structure of the 3-*O*-Ac LacCer was characterized by fast atom bombardment-mass spectrometry (FAB–MS) and NMR. Two major pseudomolecular ions, [*O*-Ac LacCer-H]$^-$ at *m/z* 1014 (24:0 as a fatty acyl) and 1012 (24:1), as well as fragment ions at 852 (24:0) and 850 (24:1) due to [*O*-Ac GlcCer-H]$^-$, and 690 (24:0) and 688 (24:1) due to [*O*-Ac Cer-H]$^-$ were observed in the FAB–MS spectrum, and *O*-Ac methyl protons and C-3 methine proton on sphingenine resonated at δ1.93 and 5.17 ppm, respectively, in the NMR spectrum. Authentic 3-*O*-Ac glucosylceramide (3-*O*-Ac GlcCer) was, however, not obtained from the bilayer saponification; therefore, the 3-*O*-Ac GlcCer could not be used as a reference. Reference

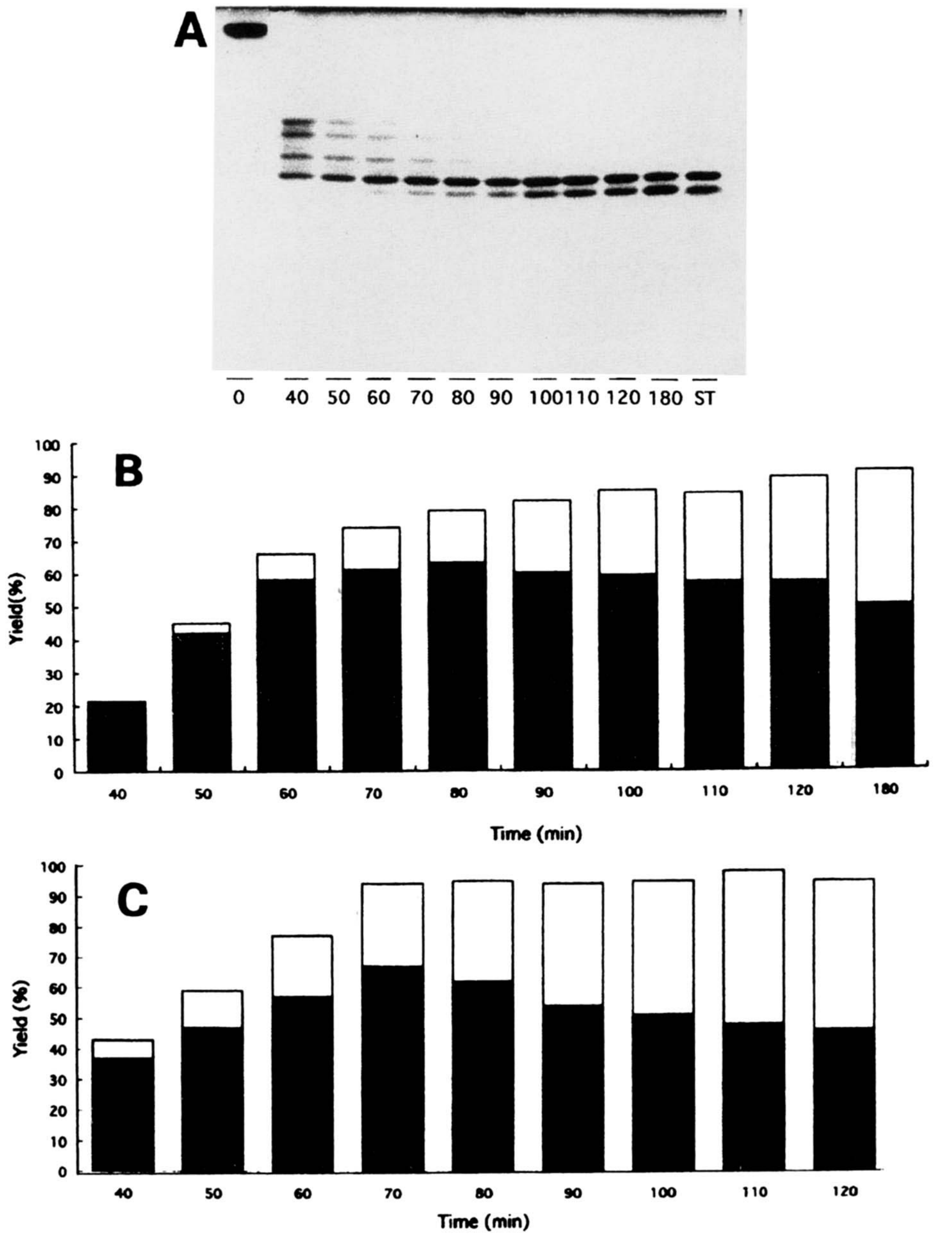

Fig. 1. TLC patterns of products obtained through limited alkaline hydrolysis of per *O*-Ac GM3 as a function of the time course. Panel A shows the product on TLC at the indicated reaction times in the bilayer hydrolysis using 0.1 N NaOH and diethyl ether; lane 0, starting per *O*-Ac GM3 (Ac), 40–180, respective reaction times; ST, authentic 3-*O*-Ac GM3 (Ac) from rat glioma (10) revealed in the upper band and unacetylated GM3 (Ac) in the lower. The plate was developed with chloroform–methanol–water 60:35:8, and stained by an orcinol–sulfuric acid reagent. Panels B and C demonstrate quantification of the yields of 3-*O*-Ac GM3 (Ac) (dark bar) and unacetylated GM3 (Ac) (white) at the indicated reaction times in the bilayer reaction using diethylether and 2-pentanol, respectively.

TABLE 1. Yield of 3-*O*-Ac GM3 (Ac) in the limited saponification of per *O*-Ac GM3 in the various solvents

Solvents	GM3 Concentration *mg/ml*	Yield %
Ethers		
Diethyl	10	62
Di-isopropyl	10	57
Di-*n*-butyl	10	53
Alcohols		
1-Butanol	10	30
2-Butanol	10	36
1-Pentanol	10	38
1-Pentanol	20	49
2-Pentanol	10	50
2-Pentanol	20	68
3-Pentanol	10	56

3-*O*-Ac Cer was prepared by incubation of *O*-acetylated sphingomyelin with phospholipase C. Briefly, 14 mg of sphingomyelin was acetylated with 0.5 ml of acetic anhydride in 1 ml of pyridine for 16 h at room temperature. After decomposition of the acetic anhydride with methanol, the solvent was evaporated in vacuo and the residue was dried in a desiccator under reduced pressure. The purity of the acetylated sphingomyelin was estimated by TLC analysis to be over 99%, and the structure was confirmed by the NMR spectrum, in which signals at δ1.96 ppm due to *O*-Ac methyl protons and at 5.22 ppm due to the C-3 methine proton were observed, indicating it to be 3-*O*-Ac derivative. The 3-*O*-Ac sphingomyelin was dispersed in 2 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 30 mM $CaCl_2$ by sonication for 1 min, and then 2 mg (38 U) of phospholipase C was added to the mixture. A small amount (1 ml) of diethyl ether was overlaid slowly onto the reaction mixture followed by incubation for 16 h at 37°C, maintaining the two phases of the mixture under gentle stirring. The lipid product was extracted with 1 ml of diethyl ether from the reaction mixture three times followed by evaporation of the solvent, and further purified by silica gel column (1 × 10 cm) chromatography using chloroform, giving 9.8 mg of 3-*O*-Ac Cer. The structure of the authentic 3-*O*-Ac Cer was confirmed with FAB–MS and by the NMR spectra, which showed pseudomolecular ions at *m/z* 690 (24:0) and 688 (24:1) due to $[O\text{-Ac Cer-H}]^-$ in the MS spectrum, and δ1.95 and 5.21 ppm due to 3-*O*-Ac methyl protons and C-3 methine proton, respectively.

Simultaneously, *O*-Ac sphingomyelin prepared as described above was incubated with sphingomyelinase similar to the phospholipase reaction as above without $CaCl_2$, followed by quantification of the product on a TLC plate as described above, except for staining the plate with 70% sulfuric acid for the generated Cer.

Glycosidase digestion of 3-*O*-Ac GM3

The 3-*O*-Ac GM3 was sequentially digested with exoglycosidases to confirm the position of the Ac residue located on the Cer moiety. One mg of the 3-*O*-Ac GM3 was dispersed in 1 ml of 50 mM acetate buffer, pH 5.0, by sonication followed by incubation with neuraminidase (10 U) for 16 h at 37°C. The reaction mixture was heated at 95°C for 3 min, centrifuged, and the resultant supernatant was applied on an LH-20 column (1 × 30 cm) with CMW, 30:60:10 to remove salts. The digested products were further chromatographed on a DEAE-Sephadex column (1 × 3 cm) with CMW, 30:60:10, to isolate glycolipid unbound to the column, giving 0.7 mg of the unbound lipid. The product (0.7 mg) was next incubated with β-galactosidase as above except for 0.3 U of enzyme. After by desalting on an LH-20 column as above, the product was isolated by silica gel column (1 × 5 cm) chromatography with CM (9:1), to obtain 0.4 mg of the product. Finally, the product (400 μg) from β-galactosidase digestion was incubated with β-glucosidase (1 U) and purified in a manner similar to the β-galactosidase reaction, giving 19 μg of the product. The amounts of the products from these enzymatic digestions were determined by chromatoscanner as described above. The mobilities of these sequential enzymatic products from 3-*O*-Ac GM3 (Ac) were compared with those of authentic 3-*O*-Ac-LacCer and -Cer on TLC developed with CMW, 60:35:8 for the glycolipids and 90:10:0.1 for the Cer, and stained by orcinol–sulfuric acid for the former and 70% sulfuric acid for the latter.

On the other hand, the 3-*O*-Ac GM3 (65 μg) was incubated with endoglycoceramidase (0.1 mU) in 50 μl of 50 mM acetate buffer, pH 6.0, containing taurodeoxy-

TABLE 2. Cleavages of 3-*O*-Ac GM3 (Ac) and 3-*O*-Ac sphingomyelin with hydrolases

Hydrolases[a]	Substrates	Product	Yield %
Exoglycosidases			
Neuraminidase	3-*O*-Ac GM3	3-*O*-Ac LacCer	90
β-Galactosidase	3-*O*-Ac LacCer	3-*O*-Ac GlcCer	70
β-Glucosidase	3-*O*-Ac GlcCer	3-*O*-Ac Cer	6
Endoglycoceramidase	3-*O*-Ac GM3	3-*O*-Ac Cer	0
N-Deacylase	3-*O*-Ac GM3	3-*O*-Ac lysoGM3	0
Phospholipase C	3-*O*-Ac SM[b]	3-*O*-Ac Cer	89
Sphingomyelinase	3-*O*-Ac SM	3-*O*-Ac Cer	0

[a] Incubation was performed for 16 h and detailed reaction was as described in the text.

[b] Sphingomyelin.

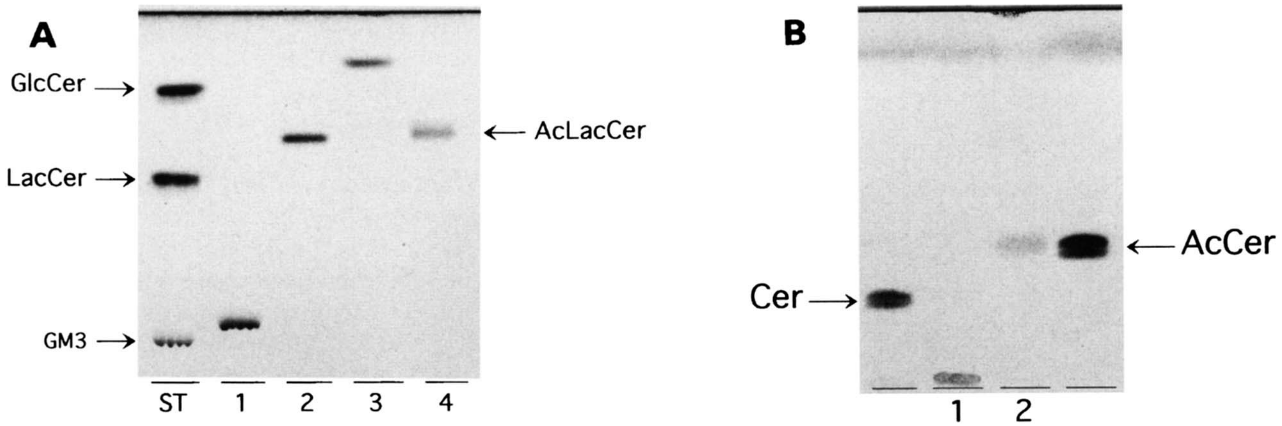

Fig. 2. TLC of glycosidase-digested products from 3-*O*-Ac GM3. In panel A, lane ST shows a mixture of standard GM3 (Ac), LacCer and GlcCer; 1, 3-*O*-Ac GM3 (Ac) obtained from limited saponification; 2, the product from 3-*O*-Ac GM3 (Ac) with neuraminidase digestion; 3, the product from the glycolipid of lane 2 with β-galactosidase digestion; 4, authentic 3-*O*-Ac LacCer. The plate was developed with chloroform–methanol–water 65:25:4. In panel B, lanes 1 and 2 indicate the glycolipid in lane 3 of the above panel A and the product from the glycolipid with β-glucosidase digestion, respectively. The plate was developed with chloroform–methanol–water 90:10:0.5 and stained by aqueous 70% sulfuric acid.

cholate (0.5 mg/ml), or the 3-*O*-Ac-GM3 (13 µg) or -sphingomyelin (10 µg) with *N*-deacylase (2 mU) in 20 µl of 20 mM acetate buffer, pH 5.0, containing 0.8% Triton X-100 for 16 h at 37°C, and the product in aliquots of the reaction mixture was separately quantified on TLC plates as described above.

NMR and mass spectrometry

The one-dimensional proton NMR spectrum of purified glycolipid (0.5 to 2 mg) was measured in a solution of $Me_2SO\text{-}d_6/D_2O$ (98:2) at 90°C by 400 MHz, in an NMR–MS Laboratory in the Faculty of Agriculture, Hokkaido University, as reported earlier (10). The spectrum of the negative FAB–MS of the sphingolipids was obtained with JEOL HX-500 in the above NMR–MS laboratory, using triethanolamine as a matrix as reported previously (10).

RESULTS

Per *O*-acetylation of GM3

The per *O*-acetylation of GM3 (Ac) was carried out with acetic anhydride and pyridine (15), and complete *O*-acetylation at the C-3-*O* on Cer moiety was estimated with the NMR spectrum (data not shown). As the starting material for the next limited hydrolysis, a peracetylated derivative of de-*N*-glycolyl GM3, which was derived from GM3 (Gc) with mild alkaline solvolysis, was also used without further purification; however, slight amounts of byproducts were generated, causing failure of the yield of 3-*O*-Ac GM3 (Ac).

Limited alkaline hydrolysis of per *O*-acetylated GM3 (Ac)

The protective effects of several water-immiscible organic solvents on the Ac group at the lipid moiety against aqueous alkaline in the bilayer system were examined. Typical reactions of hydrolysis using diethyl ether and 2-pentanol are demonstrated in **Fig. 1** as a function of time. The generation of the 3-*O*-Ac GM3 (Ac) occurred at 40 min in the diethyl ether–alkaline bilayer containing 10 mg of peracetylated GM3, increased until 80 min, and then gradually decreased from 90 min to 200 min, whereas fully de-*O*-acetylated GM3 (Ac) was increased from 40 to 200 min. A pattern similar to that of the ether bilayer was obtained in the 2-pentanol-alkaline bilayer containing 20 mg of the starting material except for the time at 70 min giving maximum yield of the 3-*O*-Ac GM3. For the solvents used herein, the reaction times giving the highest yields of the 3-*O*-Ac GM3 were observed at 70 to 80 min from the start, and the yields obtained among different solvents are summarized in **Table 1.** Of the solvents used in the reaction, 2-pentanol containing 20 mg of per Ac GM3 per ml and diethyl ether containing 10 mg per ml gave a desirable product with higher yields of 68 and 62%, respectively. As 2-pentanol containing 10 mg of the lipid per ml gave a lesser yield of 50% and diethyl ether containing 20 mg per ml afforded a much lesser yield under 10%, the starting GM3 concentration in the bilayer system might affect the generation of the 3-*O*-Ac GM3 (Ac). The chemical structure, especially the position of the Ac residue of synthesized 3-*O*-Ac GM3 (Ac), was characterized with NMR and FAB–MS spectra as well as comparing its digested glycolipids with authentic 3-*O*-Ac LacCer or 3-*O*-Ac Cer as described below.

Preparation of authentic 3-*O*-Ac-LacCer and -Cer

The limited saponification of per *O*-Ac LacCer under the bilayer reaction using diethyl ether gave *O*-acetylated LacCer at the Cer moiety with a yield of 50%, whereas per *O*-Ac GlcCer did not convert to 3-*O*-Ac GlcCer in this system. A high yield of authentic 3-*O*-Ac Cer was obtained from digestion of Ac sphingomyelin with phospholipase C. The position of the newly inserted *O*-Ac residue of the authentic 3-*O*-Ac LacCer and that of Cer were confirmed by NMR and FAB–MS analyses (data not shown). These authentic lipids were used to identify sequentially digested products from 3-*O*-Ac GM3 (Ac) using exoglycosidases by TLC analysis. The reaction of *O*-Ac sphingomyelin with sphingomyelinase did not yield 3-*O*-Ac Cer. The yield of 3-*O*-Ac Cer from Ac sphingomyelin after incubation with phospholipase C and sphingomyelinase is summarized in **Table 2,** together with those products from 3-*O*-Ac glycolipids obtained through exo- and endo-glycosidase digestions.

Glycosidase digestion of 3-*O*-Ac GM3 (Ac)

A digested product from 3-*O*-Ac GM3 (Ac) with neuraminidase gave a less polar glycolipid on the TLC plate, the mobility of which was identical to authentic 3-*O*-Ac LacCer as shown in **Fig. 2.** Likewise, the glycolipid due to the 3-*O*-Ac LacCer was converted to an even less polar glycolipid, probably 3-*O*-Ac GlcCer, by the sequential digestion with β-galactosidase. The glycolipid obtained after β-galactosidase digestion was a lipid with mobility similar to standard 3-*O*-Ac Cer with β-glucosidase. These conversions of the mobility identical to those of standard substances on the TLC plate through the sequential digestion indicated that the *O*-Ac residue localized at the Cer moiety on the original *O*-Ac GM3. The structures of these digested products from 3-*O*-Ac GM3 (Ac) were further confirmed with respective FAB–MS spectra (data not shown). The products of both neuraminidase and β-galactosidase digestions of the 3-*O*-Ac glycolipid substrates were obtained with high yields, whereas that in β-glucosidase reaction was less than 10%.

The digestion of 3-*O*-Ac GM3 with endoglycoceramidase gave no 3-*O*-Ac Cer (see Table 2), whereas unacetylated GM3 is reported to be degraded quantitatively under the same conditions used herein (16). Similarly, the 3-*O*-Ac GM3, as well as 3-*O*-Ac sphingomyelin, were markedly insensitive to *N*-deacylase digestion, though positive control was not determined because of the limited amount of the enzyme. The amount of the

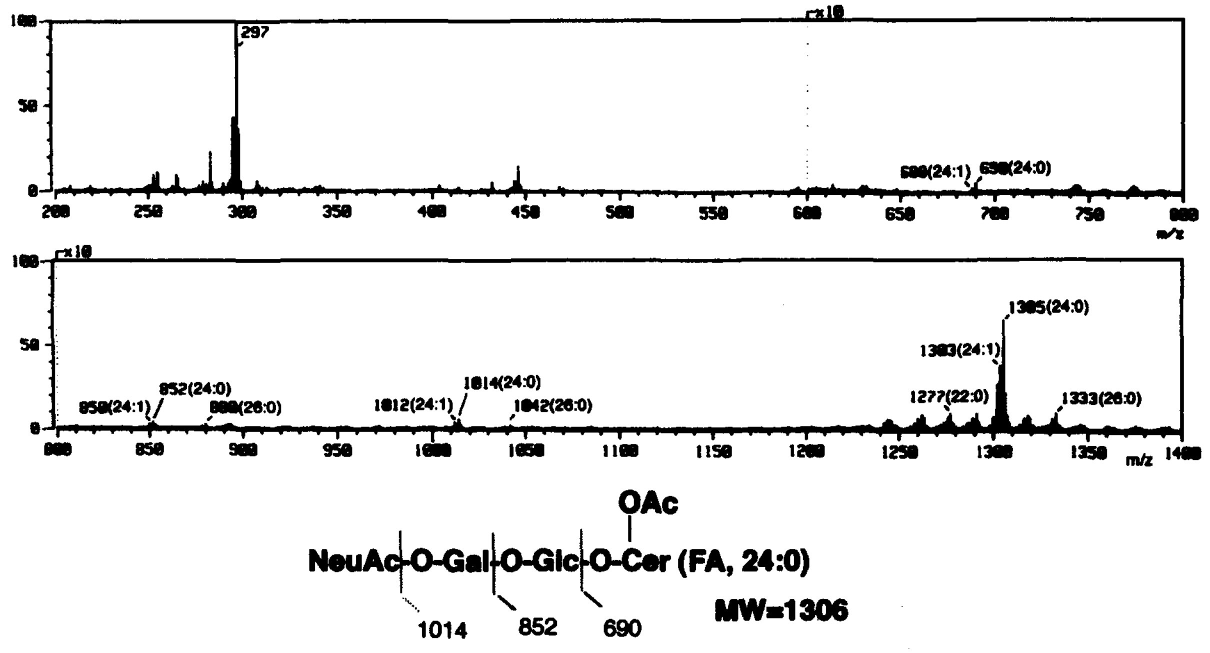

Fig. 3. Negative FAB–MS spectrum of 3-*O*-Ac GM3. The numbers in parentheses at the fragments indicate the carbon number and the unsaturated grade of the fatty acid.

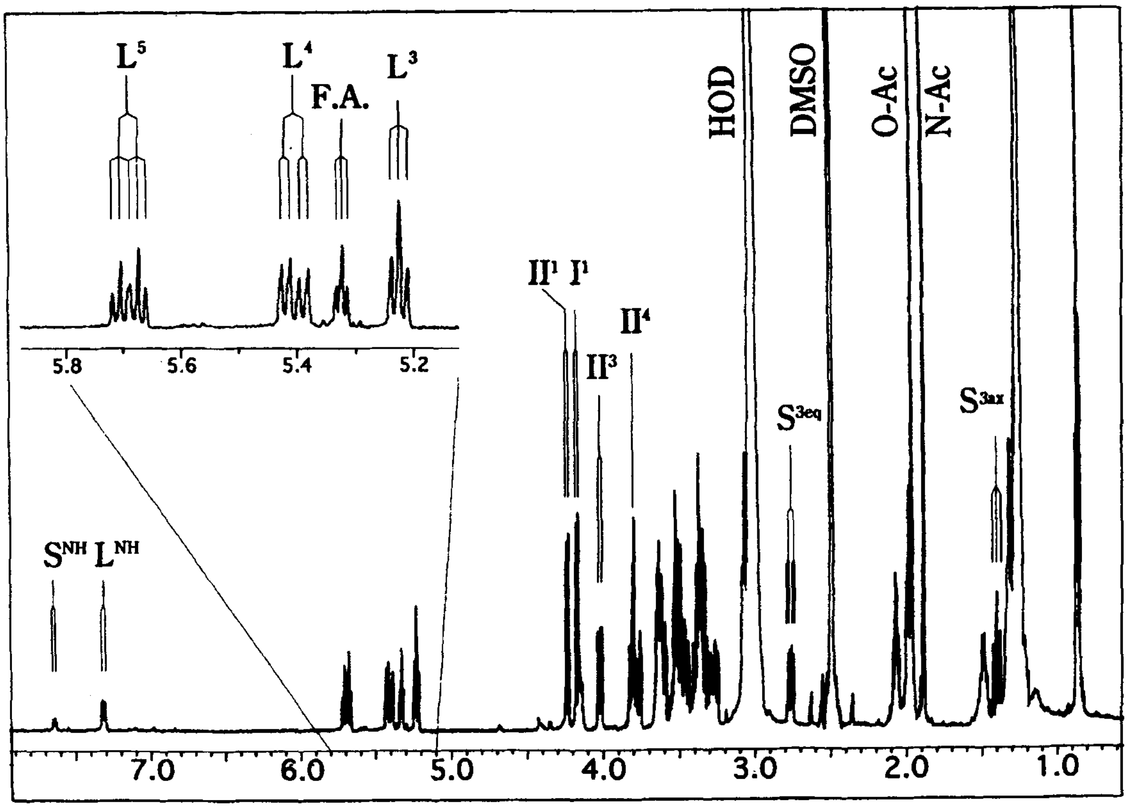

Fig. 4. Proton NMR spectrum of 3-*O*-Ac GM3. In the spectrum, S shows the proton on sialic acid, L, sphingenine, I^1, H-1 on glucose; II^1, H-1 on galactose; F.A., fatty acid.

enzyme used herein is also reported to cleave unacetylated GM3 with a yield of 45% and sphingomyelin with 28% (17).

FAB–MS study

The main pseudo molecular ion, $[M-H]^-$ of the 3-*O*-Ac GM3 at *m/z* 1305 was due to a GM3 molecule (M_r = 1264) including an additional Ac residue, composed of sphingenine as a long chain base, 24:0 as a fatty acyl and an *N*-Ac neuraminic acid moiety, as demonstrated in **Fig. 3.** The minor molecular ions at *m/z* 1333, 1303, and 1277 were consequently assigned to *O*-Ac GM3 with 26:0, 24:1, and 22:0 as the fatty acyls. These fatty acid components of the starting equine GM3 (Gc) were mostly similar to those of our previous report (2). The fragment ions due to *O*-Ac-*di*-hexosylCer, -mono-hexosylCer, and -Cer were also observed with the same major fatty acyl species as those of the 3-*O*-Ac GM3. The observation of these fragments indicated the presence of an Ac group on Cer of the GM3 molecule, and the Ac group was probably attached to the hydroxyl oxygen at C-3 of sphingenine, as the Cer moiety of the starting GM3 (Gc) has no hydroxyl group except for sphingenine. Thus the observation of the MS spectrum of the saponification product from per *O*-Ac GM3 (Ac), similar to that of rat glioma 3-*O*-Ac GM3 (10), led to identification of the structure as rat glioma 3-*O*-Ac GM3 (Ac).

NMR study

The NMR spectrum of the 3-*O*-Ac GM3 (Ac) demonstrated two singlet signals at δ1.96 and 1.88 due to *O*-Ac- and *N*-Ac-methyl protons, respectively, as shown in **Fig. 4.** In addition, a characteristic downfield shift of a pseudo-triplet signal at δ5.22 (L^3 in the figure) suitable for the C-3 proton on sphingenine was also observed in the spectrum. The chemical shifts and the coupling constants of these signals, as well as other protons located on sugar and lipid moieties, were completely identical to those of glioma 3-*O*-Ac GM3 (10), indicating the GM3 obtained from limited saponification of per *O*-Ac GM3 to be GM3 having 3-*O*-Ac sphingenine.

DISCUSSION

The principle of the selective lipid acetylation described herein is due to protection of the *O*-Ac residue bound to the Cer moiety on the GM3 molecule against exposure to alkaline solution using a water-immiscible or hardly soluble organic solvent. The organic solvents giving higher yields of the desired product such as diethyl ether and 2-pentanol appear to have slight solubility with water, as *di*-alkyl ethers with longer carbon chains such as *di*-*N*-butyl ether and *di*-*N*-hexyl ether (Table 1) or hydrocarbons such as *N*-hexane and benzene (data not shown), the solubilities of which were inferior to those of the above two solvents, gave poor or no yields. This bilayer system could be applicable for other distinctive modifications of lipid and sugar moieties of glycolipids. In fact, *N*-acylation of lysoglycolipid occurred quantitatively in the bilayer system using acylchloride to obtain a glycolipid having homogeneous fatty acid (S. Gasa, unpublished result). The direct *O*-acetylation of GM3 sphingosine was examined in the bilayer system using *N*-Ac imidazolide (11) and trimethyl orthoacetate (13), as these Ac donors were soluble in an organic solvent rather than water and the lipid acetylation might occur in the organic layer, resulting in no acetylation at sphingosine but slight acetylation at the sugar moiety.

The position of an alkali-labile group like the *O*-Ac residue bound to glycolipid, in particular, localized in the lipid moiety, was effectively analyzed by NMR, the spectrum of which demonstrated a significant downfield shift of the proton attached to the carbon bearing the *O*-Ac group. The FAB–MS spectrum also indicated the location of the Ac residue in the sugar or lipid moiety level, but not the carbon number class. Furthermore, gas chromatography–MS analysis of methanolysates and hydrolysates from the *O*-Ac glycolipid with degradation methods using acidic and alkaline conditions was inadequate for determination of the modification of the lipid moiety, because of removal of the modifier before analysis.

With respect to an interactive activity of the sphingolipids having 3-*O*-acetylated sphingosine, 3-*O*-Ac GM3 was hardly degraded by endoglycoceramidase, which cleaved the linkage between the oligosaccharide and Cer of glycosphingolipid (16). Likewise, 3-*O*-Ac GM3 and Ac sphingomyelin were insensitive toward *N*-deacylase, catalyzing cleavage of *N*-acyl linkage of Cer in glyco- or phosphosphingolipid (17). On the other hand, the 3-*O*-Ac-GM3 (Ac) and -LacCer were cleaved by neuraminidase and β-galactosidase, respectively, similarly to the reactions of corresponding unacetylated glycolipids. These results suggest that both endoglycoceramidase and *N*-deacylase recognize the Cer moiety in the glycolipid molecule, particularly the C-3 hydroxyl group, through degradation. The failure of the degradation may be ascribed to a steric hindrance of the 3-*O*-Ac residue against the enzymes and/or to loss of a crucial hydrogen bond involving the C-3 hydroxyl group due to the acetylation. The lower reactivity of 3-*O*-Ac LacCer toward β-glucosidase could also be responsible for a similar hindrance and/or loss of a hydrogen bond. Furthermore, Ac sphingomyelin was degraded by phospholipase C to give 3-*O*-Ac Cer, whereas sphingomyelinase did not afford the product. These different reactivities to Ac sphingomyelin could also be interpreted via different recognition sites of the substrate with the enzymes; phospholipase C from *Cl. welchii* recognizes a phosphorylcholine group (18), while sphingomyelinase recognizes the Cer moiety rather than phosphorylcholine (19). Based on the insensitivity of sphingomyelinase, the biological activity of the 3-*O*-Ac sphingomyelin might be useful as, for example, an anti-apoptotic substance in programmed cell death.■

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