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New blocking method for the hydroxyl group on carbohydrate Determination of the O-acylated position of the modified glycolipid

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Abstract

To determine *O*-esterified positions, a rapid and complete acetalization to prepare an intermediate was established using ethyl vinyl ether as a new reagent. The new method was applied to *O*-esterified glycolipids followed by GC–MS analysis of the monosaccharide derivatives after methylation and methanolysis, revealing the derivatives with correctly substituted positions. This method was superior in terms of its shorter reaction time and complete acetalization, particularly of the N-glycolyl hydroxyl residue, to previously reported methods using methyl vinyl ether.

Keywords: Derivatization, GC; Ethyl vinyl ether; Lipids; Glycosphingolipids; Gangliosides

1. Introduction

Some modifications of the alkali-labile group have been found to occur on sugar and lipid moieties on glycosphingolipid (GSL), in accordance with the tumorigenicity of cells, such as 9-*O*-acetyl (Ac) GD3 expressed in melanoma cells [1–3] and *O*-Ac GM3 containing 3-*O*-Ac ceramide in rat glioma tissue [4]. The former ganglioside and other modified gangliosides were isolated in bovine buttermilk [5–7]. In normal tissue, and cells, O-acylated galactosyl ceramide (GalCer), including 2-*O*-, 3-*O*- and 6-*O*-fatty acylated derivatives, was isolated from normal human [8,9], bovine [10], pig [11], whale [12] and fish [13] brains. Furthermore, equine erythrocytes were found to have *O*-Ac GM3 containing 4-*O*-Ac-N-glycolyl neuraminic acid (NeuGc) (4-*O*-Ac GM3) [14,15], 9-*O*-Ac-NeuGc (9-*O*-Ac GM3) and 6-*O*-Ac galactose (abbreviated to 6′-*O*-Ac) (6′-*O*-Ac GM3) [16], 4,9-di-*O*-Ac-NeuGc (4,9-Ac GM3) and a 4,6′-di-*O*-Ac derivative (4,6′-Ac GM3) [17]. These acylated positions on modified glycolipids were effectively assigned by proton NMR spectroscopy. In fact, the *O*-acetylated position(s) on the gangliosides has been determined by spectroscopy, in which a proton(s) attached to a carbon with an esterified hydroxyl group characteristically resonates downfield [3–7,15–18]. NMR spectroscopy has, however, not yet been applied to the determination of O-acylated positions of the *O*-fatty acylated glycosphingolipids (GSLs). Alternatively, these modified sites were determined by GC–MS of a volatilized monosaccharide released from a suitable derivatized glycolipid. Direct methylation of the esterified GSLs

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using methylsulfinyl carbanion [19] followed by solvolysis, reduction and acetylation to derivatize to methylated alditol acetates is, however, not applicable for the determination of the *O*-esterified position, since the *O*-ester group could be partially or fully replaced with methyl residue when using carbanion [15]. After that, the *O*-esterified position would not be distinguishable from the positions with other hydroxyl groups in the intact form. To determine the *O*-esterified position, therefore, free hydroxyl groups need first to be derivatized with a carbanion-resistant group, with retention of the *O*-ester group through the derivatization reaction. For this purpose, alkyl vinyl ethers, such as methyl vinyl ether and pyranyl ether, have been employed as blocking reagents for acetalization of hydroxyl group(s) on the sugar moiety [9,12,14,15]. Methyl vinyl ether, however, having a low boiling point (5°C/1 atm), requires a low temperature for acetalization, causing slow processing of the reaction (1 atm=101 325 Pa). Additionally, since pyranyl ether has a steric bulk, the acetalization reaction could be slow and result in incomplete blocking. On the other hand, with regard to the relatively higher boiling point (36°C/atom) and lower steric bulk, ethyl vinyl ether (EVE) was predicted to be preferable for a rapid and complete acetalization reaction, but has not yet been employed for this purpose. In this paper, therefore, the use of EVE for acetalization of O-acylated glycolipids was examined for the first time, to produce an intermediate through determination of the acylated site on the sugar moiety because of its advantageous properties.

2. Experimental

2.1. Materials

EVE and *p*-toluenesulfonic acid (TSA) were purchased from Wako (Tokyo). Sephadex, LH-20 and DEAE–Sephadex A-25 and thin-layer chromatography (TLC) plates (silica gel 60) were from Pharmacia-LKB (Uppsala, Sweden) and Merck (Germany), respectively. Beaded silica (Iatrobeads, 8060) was obtained from Iatron (Tokyo). Other reagents were of analytical grade.

2.2. Isolation of GSLs

All solvent mixture ratios are expressed by volume. 6-*O*-Fatty acyl GalCer was isolated from porcine brain as reported by Kishimoto et al. [11]. Briefly, the glycolipids were extracted from an acetone powder (104 g dry mass), which was obtained from whole equine brain (457 g wet mass), with chloroform–methanol–water (CMW; 4:8:3, v/v/v). The total neutral glycolipid fraction obtained after passage of the extracts through a DEAE–Sephadex, A-25 (acetate form) column with CMW (40:60:10, v/v/v) was chromatographed on an Iatrobeads column by stepwise elution with CMW to isolate O-acylated GalCers. From the neutral lipid fraction, 6-*O*-acyl GalCer was obtained, with a yield of 8 mg, and the structure was identified by NMR spectroscopy. The purified 6-*O*-fatty acyl GalCer was chromatographed on a TLC plate, developed with CMW (80:20:2, v/v/v) and visualized by staining with orcinol–sulfuric acid reagent under heating conditions. 4-*O*-Ac- [15] and 9/6′-*O*-Ac GM3 [16] were purified from equine erythrocytes as reported previously. The latter lipid was a mixture composed of two gangliosides, 9-*O*-AcNeuGc-Gal-Glc-Cer and NeuGc-(6-*O*-Ac)Gal-Glc-Cer (see Fig. 1), and they were not separable on chromatography [16].

2.3. Acetalization, methylation and methanolysis of O-acyl GSLs

The purified and well-dried 6-*O*-fatty acyl GalCer from porcine brain and 4-*O*- and 9/6′-*O*-Ac GM3s from equine erythrocytes, approximately 1 to 5 mg, were separately dissolved in 0.9 ml of dimethyl sulfoxide (DMSO) and 0.5 ml of EVE. The acetalization reaction was started by adding 0.1 ml of a solution containing 10 mg of TSA in 1 ml of DMSO, with stirring, at room temperature. The progress of the reaction was monitored by TLC analysis, developed with CMW (90:10:0.5, v/v/v) for acyl GalCer and 60:35:8 (v/v/v) for Ac GM3, followed by visualization by staining with orcinol–sulfuric acid reagent under heating conditions. After the reaction was completed (within 1 h), the mixture was neutralized with pyridine and applied to an LH-20 column (40×1 cm) with chloroform, to remove

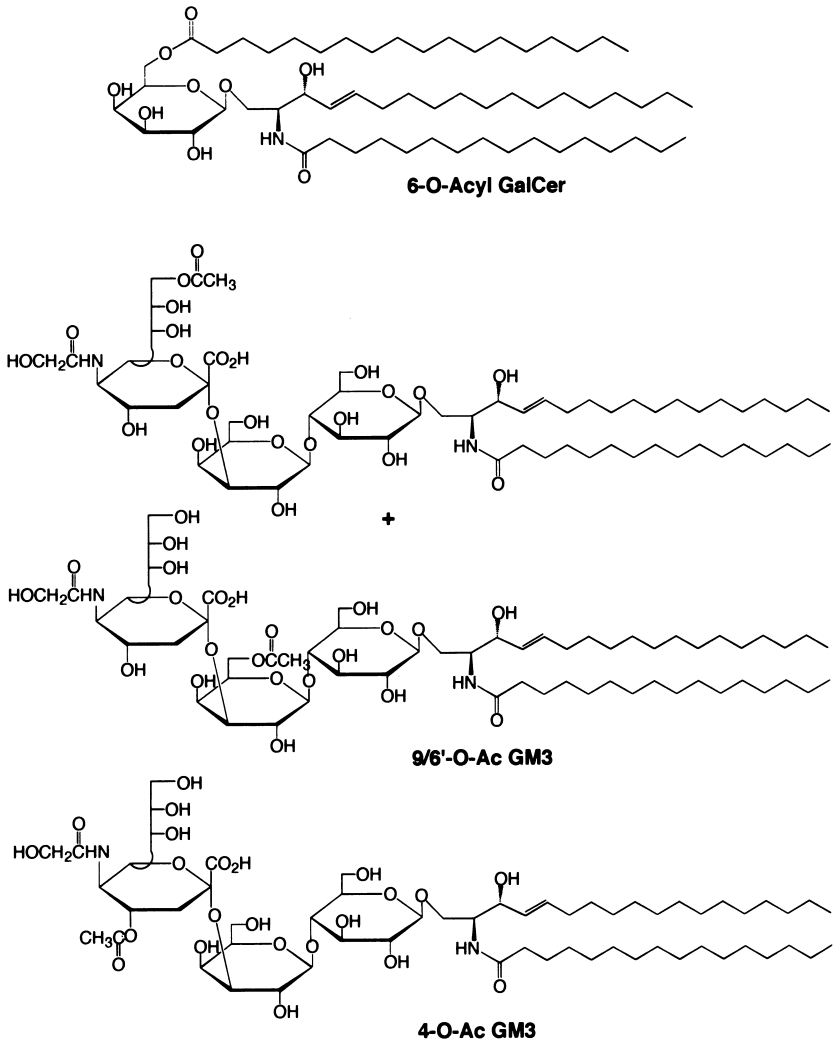

Fig. 1. *O*-Esterified GSLs employed. 9/6′-*O*-Ac GM3 is a mixture of 9-*O*-Ac GM3 (9-*O*-AcNeuGc-Gal-Glc-Cer) and 6′-*O*-Ac GM3 (NeuGc-(6-*O*-Ac)Gal-Glc-Cer).

DMSO and salts. The sugar-positive fractions excluded from the column were combined and the solvents were evaporated in vacuo to dryness. The per-acetalized material was next methylated without further purification, because of instability, with 0.2 ml of 4 *M* methylsulfinyl carbanion and 0.5 ml of methyl iodide in 1 ml of DMSO [19] for 16 h, followed by removal of the carbanion and DMSO by LH-20 column chromatography, as described above, after evaporation of methyl iodide. The per-acetalized and methylated glycolipid was methanolyzed with 1 ml of 1 *M* HCl in methanol at 80°C for 16 h for acyl GalCer and with 0.3 *M* HCl in methanol at 80°C for 3 h for Ac GM3, and the methanolysates were washed with 1 ml of *n*-hexane three times, to remove fatty acid methyl ester, followed by evaporation of the solvents to dryness in vacuo.

2.4. GC–MS

The methanolysates obtained above were derived to the trimethylsilyl (TMS) derivative with 50 ml of a mixture of *N*,*O*-bis TMS dimethylformamide and TMS–Cl (8:2, v/v) by heating at 60°C for 20 min.

An aliquot of the solution was subjected to GC–MS on a JEOL JMS-HX 100 using a capillary column (25 m×0.25 cm) that was coated with 1% OV-1 at the NMR and MS Laboratory of the Faculty of Agriculture in Hokkaido University, at a programmed temperature increase from 160 to 280°C at 5°C/min, a pressure of $1 \cdot 10^{-6}$ Torr, a source temperature of 250°C and an acceleration voltage of 70 eV, as reported previously [16,17] (1 Torr= 133.322 Pa). GC–MS chromatograms were obtained with selected ion monitoring using m/z 409 and 654, due to the $[M^{+}-Me]$ for 1,6-di-*O*-Me-2,3,4-tri-*O*-TMS galactoside and 4-*O*-Me or 9-*O*-Me neuraminoside Me ester derivative, respectively.

3. Results

3.1. Acetalization, methylation and methanolysis of O-acyl GSLs

The chemical structures of the *O*-acyl- and *O*-Ac-GSLs employed herein are illustrated in Fig. 1. The per-acetalization product with EVE of 6-*O*-acyl GalCer migrated to the front on the TLC, together with a polymerized product of EVE, indicating that it is less polar than the original GSL, as demonstrated in Fig. 2. This was similar to the reaction with methyl vinyl ether [14,15]. The acetalization reaction of all the O-acylated GSLs used herein (Fig. 2) was completed within 1 h at room temperature, and further reaction gave only an increased amount of the above polymerized product (asterisked band in Fig. 2B). The amount of TSA used as a catalyst was optimal in the reaction at 1–5 mg of glycolipid per 1 mg of the acid. The acetalized product was very labile in acidic conditions, such as silica gel column chromatography using an aqueous solvent mixture, through which the product was converted to several degraded spots in TLC analysis (data not shown). The acetalization product was next methylated with methyl iodide using methylsulfinyl carbanion, by which *O*-Ac and *O*-acyl groups in the intact GSLs were replaced with *O*-methyl groups. Finally, the acetalized and methylated GSL was methanolyzed with methanolic HCl, resulting in removal of the acetal groups to give partially methylated methyl glycoside. The derivatization reaction described above is summarized in Fig. 3.

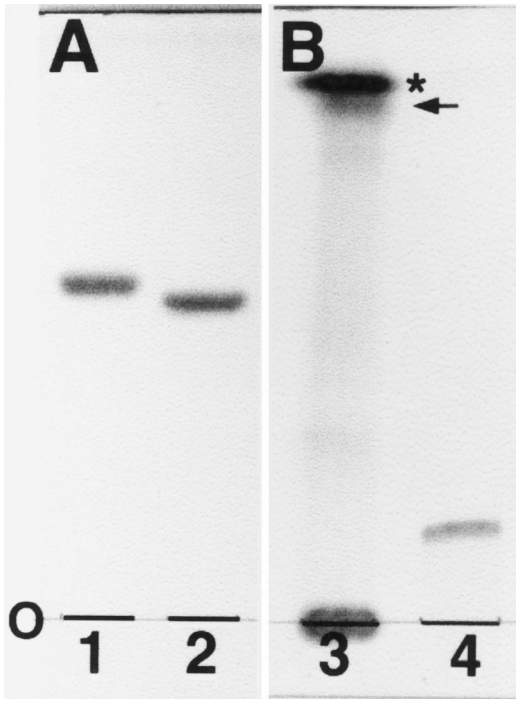

Fig. 2. TLC of *O*-esterified GSLs. 4-*O*-Ac GM3 (lane 1), 9/6′-*O*-Ac GM3 (2), 6-*O*-Acyl GalCer (3) and the acetalization mixture of 6-*O*-acyl GalCer (4) were chromatographed on TLC, developed with CMW (65:25:4, v/v/v) for panel A and CMW (80:20:2, v/v/v) for panel B, and all were stained using orcinol–sulfuric acid reagent. The asterisked band is not sugar-positive, whereas the band indicated by an arrow is sugar-positive.

3.2. GC–MS

Prior to GC–MS analysis, the sugar moiety of 6-*O*-acyl GalCer was confirmed to be Gal by GLC analysis of the TMS derivative from methanolysates of the acyl GalCer without acetalization, and by TLC analysis after saponification (0.2 *M* sodium methoxide in methanol for 2 h at room temperature) of the acyl GalCers, giving a mobility identical to that of authentic GalCer. Similarly, the O-acylated sugars in 4-*O*-Ac GM3 and 9/6′-*O*-Ac-GM3 employed herein were already assigned as GM3, having 4-*O*-Ac N-glycolyl neuraminic acid (4-*O*-Ac NeuGc) [15] in the former and 9-*O*-Ac NeuGc or 6-*O*-Ac Gal [16],

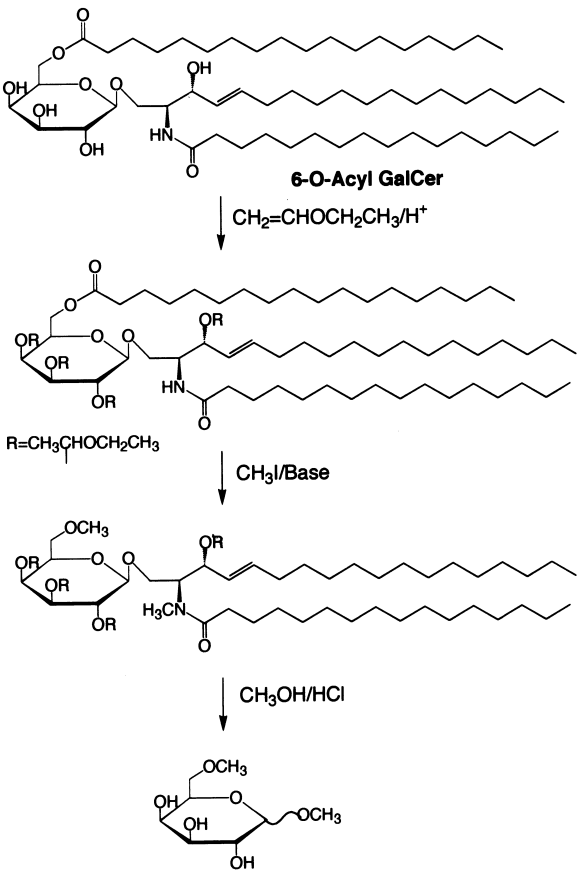

Fig. 3. Schematic diagram of the derivatization of monosaccharide from 6-*O*-acyl GalCer.

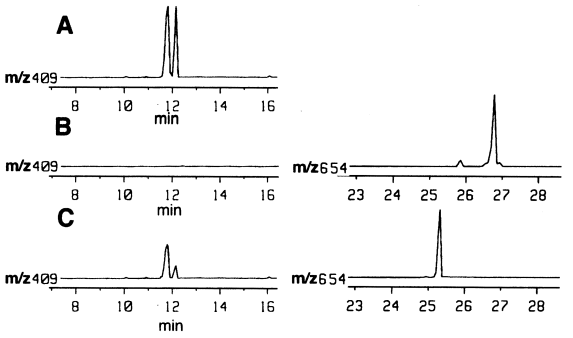

Fig. 4. GC–MS chromatogram of the derivatized glycosides from *O*-esterified GSLs. The chromatograms were performed using the selected ions, m/z 409 and 654, due to $[M^+-Me]$ of methylated galactoside (M_r=424), and neuraminoside (M_r=669), respectively. Panels A, B and C indicate chromatograms of the derivatives from 6-*O*-acyl GalCer, 4-*O*-Ac GM3 and 9/6′-*O*-Ac GM3, respectively.

which we were unable to separate by chromatography, in the latter.

The methylated position of the *O*-methylated methyl glycosides obtained through acetalization and methylation followed by methanolysis was analyzed by GC–MS as a TMS derivative. The GC–MS chromatograms were first monitored using selected ions due to $[M^+-Me]$ at m/z 409 and 654 for the 1,6-di-*O*-Me galactoside and 4-*O*-Me or 9-*O*-Me neuraminoside derivative, respectively. As shown in Fig. 4, 6-*O*-acyl GalCer afforded two peaks, at 11.8 and 12.1 min of the retention time monitored with m/z 409, and the peaks were observed after similar derivatization of 9/6′-*O*-Ac GM3. These two peaks presented similar MS spectra (see below), suggesting that they were anomeric isomers. In contrast, neuraminoside derivatives with different retention times were detected at 26.8 and 25.3 min from 4-*O*-Ac GM3 and 9/6′-*O*-Ac GM3, respectively (Fig. 4). No peak was detected in the derivatives from these GM3s when monitored with m/z 596, the ions of which should be observed if Gc-*O* was not acetalized following methylation (data not shown).

The glycoside derivatives from 6-*O*-acyl GalCer were determined for both of the above peaks to be 1,6-di-*O*-Me-2,3,4-tri-*O*-TMS galactoside from the mass spectrum. Typically, m/z 409, 393 and 377, due to $[M^+-Me]$, $[M^+-MeO]$ and $[M^+-Me-MeOH]$, indicated a mono-*O*-methylated structure, except for a glycosidic methyl residue, as shown in Fig. 5A. The ion at m/z 305, due to $[TMSOCH_2CH(OTMS)COTMS]^+$ provided a non-methylated structure at C-2, -3 and 4-*O* in the whole molecule, and m/z 290, due to $[M^+-TMSO-CH_2OMe]$ indicated the presence of a methylated hydroxymethylene group. The *O*-methylated positions on the galactoside, except for the anomeric position, indicated conclusively an acylated site at C-6-*O* in the intact 6-*O*-acyl GalCer. Similarly, the mass spectrum (Fig. 5B) of the Me neuraminoside derivative (retention time, 26.8 min) released from acetalized and methylated 4-*O*-Ac GM3 by methanolysis gave ions at m/z 654 and 610, due to $[M^+-Me]$ and $[M^+-CO_2Me$ (''d'' in the figure)], respectively. These indicated the presence of mono-*O*-methylated NeuGc. The ions at m/z 566, 464 and

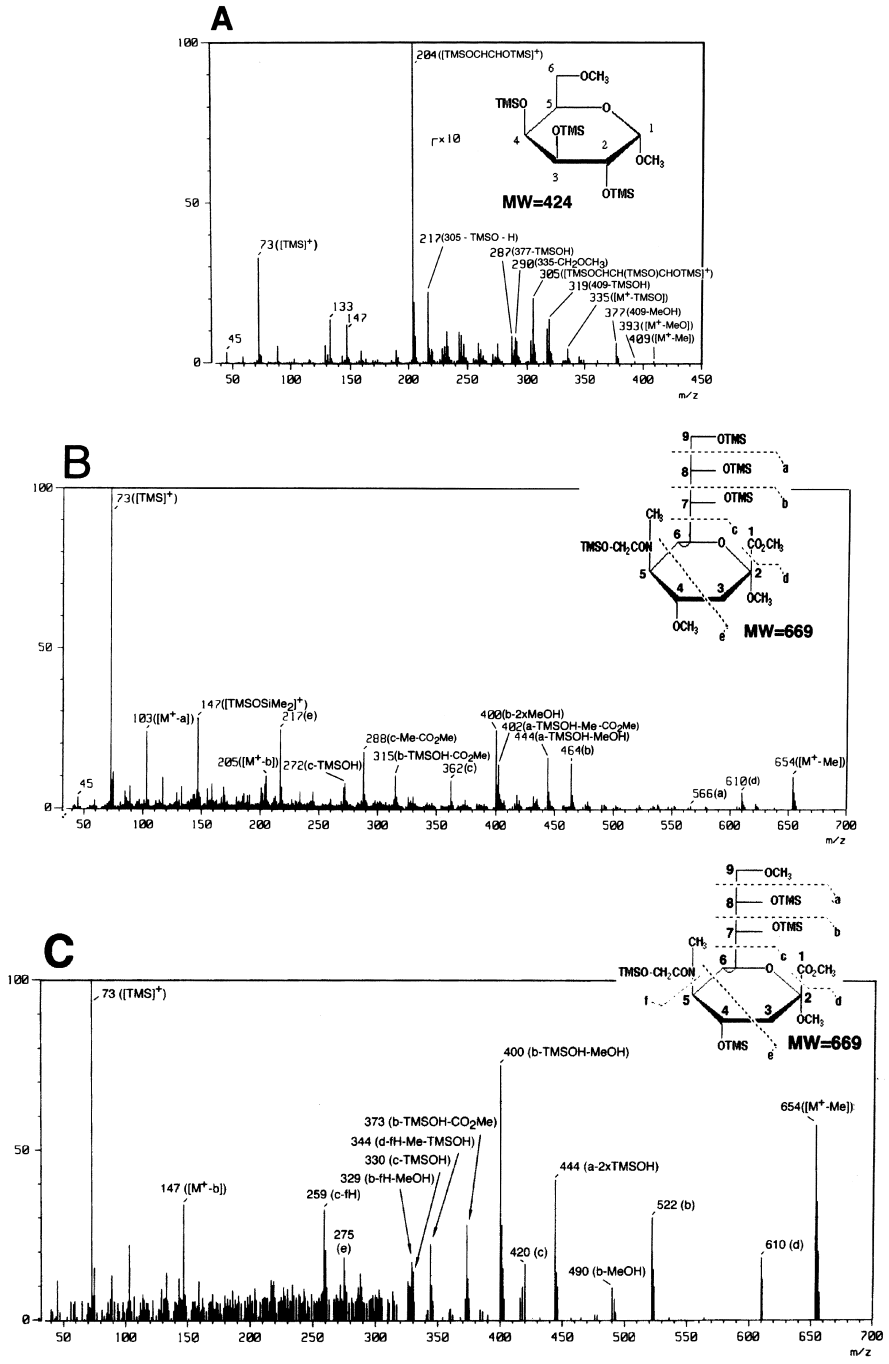

Fig. 5. Mass spectra of partially methylated glycosides derived from *O*-esterified GSLs. *O*-Esterified GSL was acetalized with EVE, methylated, methanolyzed, and derivatized as a TMS compound, which was followed by analysis by GC–MS. Panels A, B and C indicate mass spectra of partially methylated glycosides derived from both 6-*O*-acyl GalCer and 9/6′-*O*-Ac GM3, 4-*O*-Ac GM3 and 9/6′-*O*-Ac GM3, respectively. MW=Molecular mass.

362 represented a non-methylated structure at C-7, 8 and 9-*O* of NeuGc, while *m/z* 217 and 201, due to $[M^+-TMSOGc-N(Me)CHCHOMe]$ (and/or $[TMSOCHCH(OTMS)CH]^+$) and [*m/z* 362 (c), TMSOGc–NHMe], respectively, suggested a C-4-*O*Me structure. Both indicated a methylated structure at C-4-*O* of the NeuGc. The observations on these ions indicated the structure of 2,4-di-*O*-Me *N*-Me *N*-Gc (TMS)-7,8,9-tri-*O*-TMS neuraminoside Me ester, being identical to the previously reported spectrum [15]. The result indicated an acetylated structure at C-4-*O* of the NeuGc moiety of intact 4-*O*-Ac GM3. On the other hand, acetalized and methylated 9/6′-*O*-Ac GM3 gave two partially methylated glycoside derivatives after methanolysis, one was 1,6-di-*O*-Me-2,3,4-tri-*O*-TMS galactoside, as described above, and the other was a 9-*O*-Me derivative of NeuGc (retention time, 25.3 min in Fig. 4C). The structure of 9-*O*-Me NeuGc was confirmed from ions at *m/z* 654 and 610, due to $[M^+-Me]$ and $[M^+-CO_2Me]$, which indicated a mono-*O*-methylated structure, except for a glycosidic Me group. The ions at *m/z* 522 (b) and 420 (c) suggested non-*O*-methylated structures at C-4-*O*, Gc-*O* and C-7-*O*. Similarly, the ions at *m/z* 344, 329, 275 and 259, due to reduced ions with $TMSOCH_2CONH(CH_3)$ (''f'' in the figure plus H), indicate non-*O*-methylated structures at C-4-*O* and Gc-*O*. The presence of the ion at *m/z* 444, due to $[a-2TMSOH]^+$ demonstrated a methylation site at C-9-*O*, not C-8-*O*, since an ion at *m/z* 502, due to $[a-TMSOH-MeOH]^+$, the fragment of which should have appeared if C-8-*O* was methylated, was not observed in the spectrum of the *O*-Me NeuGc derivative. Comparing the ion at *m/z* 444 that is commonly observed between 4-*O*-Me NeuGc and 9-*O*-Me NeuGc with their structures, the ion in the former might have been due to a decrease of MeOH at C-4-*O*Me and an adjacent hydrogen from [a, TMSOH] and the ion in the latter might be suitable for removal of TMSOH at C-4-OTMS and an adjacent hydrogen from [a, TMSOH]. The ion at *m/z* 147, due to $[MeOCH_2CHOTMS]^+$ and/or $[TMSOSiMe_2]^+$, was not useful for assigning the methylated position at C-9-*O* of the 9-*O*-Me NeuGc derivative, since it was also observed in 4-*O*-Me NeuGc (Fig. 5B). The spectrum of 9-*O*-Me NeuGc was identical to that previously reported, which was derived after acetalization using methyl vinyl ether [16]. The assignment of the neuraminoside derivatives as well as the galactoside derivative indicated the presence of mixtures of acetylated structures at C-9-*O* of the NeuGc and at C-6-*O* of the Gal of intact 9/6′-*O*-Ac GM3.

Thus, the mass spectra of Me glycoside or neuraminoside Me esters derived from O-acylated GSLs enabled us to employ EVE to fully mask free hydroxyls of the sugar moiety on the GSL by acetalization before methylation analysis. In addition, a partially acetalized derivative of the Gc hydroxyl residue of the NeuGc moiety, which was observed when acetalized using methyl vinyl ether [15], was not detected.

4. Discussion

The O-acylated or *O*-acetylated position(s) on the GSLs has been effectively assigned by proton NMR as described above. To chemically follow the assignment, the O-acylated site was determined by GC–MS analysis of a volatile monosaccharide derivative, which was methylated at the *O*-esterified site after blocking of free hydroxyls of the sugar moiety using alkyl vinyl ether [9,12,14,15]. Direct methylation of the *O*-esterified GSLs under basic conditions, to derivatize methylated alditol acetates, would provide no evidence for the esterified position of intact GSL, since *O*-ester groups are labile in a basic solution, even in an aprotic solvents. Similarly, direct methanolysis of the esterified GSL, to obtain the Me glycoside following GC–MS analysis, would provide only the de-esterified derivative. Therefore, acetalization to block free hydroxyls is required to derivatize the ether intermediate, which is stable in a basic solution but unstable in an acid solution, prior to methylation and methanolysis, whereby the *O*-esterified position and free hydroxyls are distinct. Alkyl vinyl ether is known as an active reagent that forms ether derivatives of sugar under acidic conditions, to which the *O*-ester group is insusceptible. Compared to the structures of the three alkyl vinyl ethers shown in Fig. 6, methyl vinyl ether has a smaller molecule. An acetalization reaction using it, however, requires a low temperature, since the compound has a low boiling point, causing slow

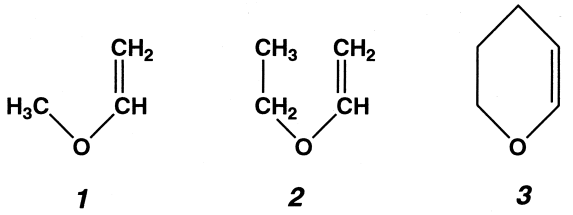

Fig. 6. Structures of alkyl vinyl ethers. Structures 1, 2 and 3 indicate methyl vinyl ether, EVE and pyranyl ether, respectively.

progress of the reaction. In fact, 4-*O*-Ac GM3 is incompletely acetalized with methyl vinyl ether, even after more than 6 h, and the Gc hydroxy group is partially retained in that duration, since the Gc hydroxy residue was hardly acetalized, as described earlier [15]. In contrast, the use of EVE caused the acetalization reaction to progress rapidly and completely, even for GM3 containing NeuGc, as described herein. EVE has a slight bulky structure compared to that of methyl vinyl ether, but not to that of pyranyl ether, and it has a higher boiling point than the former. Since methyl vinyl ether is a gas at room temperature and atmosphere, the handling of this reagent is not simple, and an acetalization reaction using this requires a temperature lower than 5°C. The higher boiling point of EVE, however, allowed us to handle it easily and the reaction could take place at room temperature. The rapid and complete acetalization, even of the Gc hydroxy residue in the NeuGc moiety of 4-*O*-Ac GM3, with EVE of *O*-acyl GSLs might, therefore, be ascribed to these advantages.

GC–MS chromatographies were performed by monitoring using respective selected ions due to $[M^+-Me]$ of the sugar derivatives (Fig. 4), since their total ion chromatograms were very complex. The complexity could be ascribed to the interference of derivatives from the sphingosine base, fatty acids and/or non-methylated sugar(s) other than the glycosidic position in the methylated monosaccharide region. The two peaks observed in the chromatogram from derivatized 6-*O*-Ac GalCer (Fig. 4) might be anomeric isomers, because of their identical MS spectra.

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