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Structural Elucidation of Diglycosyl Diacylglycerol and Monoglycosyl Diacylglycerol from *Streptococcus pneumoniae* by Multiple-Stage Linear Ion-Trap Mass Spectrometry with Electrospray Ionization

Raju Venkata Veera Tatituri², Michael B. Brenner², John Turk¹, and Fong-Fu Hsu^{1,*}

¹Mass Spectrometry Resource, Division of Endocrinology, Diabetes, Metabolism, and Lipid research, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110

²Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, Harvard Medical School, 1 Jimmy Fund Way, Boston, Massachusetts 02115

Abstract

The cell wall of the pathogenic bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) contains glucopyranosyl diacylglycerol (GlcDAG) and galactoglucopyranosyldiacylglycerol (GalGlcDAG). The specific GlcDAG consisting of vaccenic acid substituent at sn-2 was recently identified as another glycolipid antigen family recognized by invariant natural killer T cells (NKT cells). Here, we describe a linear ion-trap (LIT) multiple-stage (MSⁿ) mass spectrometric approach towards structural analysis of GalGlcDAG and GlcDAG. Structural information derived from MSⁿ (n = 2,3) on the [M + Li]⁺ adduct ions desorbed by electrospray ionization (ESI) affords identification of the fatty acid substituents, assignment of the fatty acyl groups on the glycerol backbone, as well as the location of double bond along the fatty acyl chain. The identification of the fatty acyl groups and determination of their regio-specificity were confirmed by MSⁿ (n = 2,3) on the [M + NH₄]⁺ ions. We establish the structures of GalGlcDAG and GlcDAG isolated from *S. pneumoniae*, in which the major species consists of a 16:1- or 18:1-fatty acid substituent mainly at sn-2, and the double bond of the fatty acid is located at ω-7 (n-7). More than one isomers were found for each mass in the family. This mass spectrometric approach provides a simple method to achieve structure identification of this important lipid family that would be very difficult to define using the traditional method.

Keywords

Diglycosyl Diacylglycerol; Monoglycosyl Diacylglycerol; *Streptococcus pneumoniae*; Glycolipids; Ion-trap mass spectrometry; ESI; Lithium adduct ion

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is gram-positive, alpha-hemolytic, bile-soluble aerotolerant anaerobe and a member of the genus Streptococcus.^[1] It is a leading cause of pneumonia in all ages. Diseases caused by *S. pneumoniae* constitute a major global public health problem. In 2000, about 14.5 million episodes of serious pneumococcal disease were

*To whom the correspondence should be addressed: Dr. Fong-Fu Hsu, Box 8127, Washington University School of Medicine, 660 S Euclid, St. Louis, MO 63110. Tel: 314-362-0056, fhsu@im.wustl.edu.

estimated to occur, resulting in about 826 000 deaths (www.who.int/nuvi/pneumococcus/en/).

Diglycosyl diacylglycerols (DGDG) are ubiquitous membranous components of gram-positive bacteria.^[2, 3] Depending on the nature of the disaccharide glycosidically bound to the 3-position of a sn-1,2-diglyceride, several structural types have been characterized. For example, the DGDG found in *S. pneumoniae* is 1,2-diacyl-3-*O*-[α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-galactopyranosyl]-sn-glycerol (or 3-[*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl]-sn-1,2-diglyceride (GalGlcDAG)) (See Scheme 1 for structure).^[4, 5] In addition to GalGlcDAG, monoglucosyldiacylglycerol (MGlcDAG) and monoacyl-3-*O*-[α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-galactopyranosyl]-sn-glycerol (GalGlcMAG) were also found.

Glycolipids are important antigens for microbes to be recognized by T cell receptor (TCR). For example, DGDG and MGDG from *S. pneumoniae* were recently reported to be recognized by invariant natural killer T cells (iNKT cells). The glycolipid response depends on the presence of vaccenic acid at sn-2 of the glycerol backbone.^[6] These findings underscore the importance of the specificity of the fatty acid substituent for recognition by the invariant T cell antigen, and consistent with the earlier reports that the regio-specificity of the fatty acid substituents in the glycerolipids is one of the essential factors for endogenous and microbial lipid antigen recognition by CD1 molecules to be presented to iNKT cells.^[7–9] DGLcDAG in *Enterococcus Faecalis* is a glycolipid and lipoteichoic acid precursor involving in biofilm accumulation, adherence to host cells, and virulence *in vivo*.^[10] α -Galactosyl diacylglycerols from *B. burgdorferi*, the causative agent of Lyme disease, directly stimulated iNKT cells through TCR engagement rather than iNKT cell activation through antigen-presenting cell stimulation.^[11] Therefore, development of a simple method for characterization of DGDG and MGDG in particular, the determination of the regio-specificity and the location of double bond of the unsaturated fatty acid substituent is essential for advancing the biological study related to these lipids.

A few ESI tandem mass spectrometric approaches toward identification of DGDG molecules have been reported.^[12–14] Among them, Guella et al employed MS² on the [M + Na]⁺ adduct ions with an ion-trap instrument for assignments of the fatty acid moieties on the glycerol backbone of galactolipids. The determination is based on the findings that loss of the fatty acid substituent at sn-1 is more facile than that at sn-1.^[14] This discrimination in the losses of the fatty acid substituents dependent on their position on the glycerol backbone has been reported in our earlier structural characterization of glycerophospholipids and triacylglycerols (TAG) that were desorbed as [M + Li]⁺ adduct ions and sequentially subjected to low energy CID tandem mass spectrometry.^[15, 16] However, none of aforementioned approaches affords location of the position of double bonds along the fatty acyl chain. Kunjo et al applied the traditional GC/MS method to determine double bond position of the monounsaturated fatty acid in DGDG.^[11] This approach is laborious, requiring first hydrolysis of the glycolipid and isolation of the fatty acids, followed by derivatization steps to the final methyl ester of dimethyl disulfide derivative before GC/MS analysis. Herein, we described a simple LIT MSⁿ method to achieve structural analysis of DGDG and MGDG as the lithiated adduct ions, leading to identification of the fatty acid substituents, their regio-specificity, and the location of double bond of the unsaturated fatty acid moieties.

Materials and methods

Strain and Culture conditions

S. pneumoniae, strain URF918,^[17, 18] provided by K. Kawakami was cultured in THY (Difco) or BHI (Oxoid) medium at 37°C until mid- to late-log phase. Bacterial samples for lipid analyses were harvested by centrifugation at 3000 RPM in 50 ml falcon tubes. These pellets were washed×3 in LPS-free PBS (Gibco) and heat-inactivated at 65°C for 45 min. These heat inactivated bacterial samples were lyophilized for 24–48 h. before lipid extractions

Lipid extraction and TLC purification

Crude lipids were extracted as previously described.^[19, 20] Lyophilized bacteria (8 g) were treated with methanolic saline and petroleum ether and the resulting upper phase comprising apolar lipids was removed and stored. The lower phase was again treated two to three times with petroleum ether and all the upper petroleum ether phases were removed. The leftover biomass and the lower phase were treated with various combinations of chloroform, methanol and saline solutions in the following step-wise fashion: Step-1, 260 ml of CHCl₃, CH₃OH, 0.3% NaCl (9:10:3, v/v/v) was added and the solution was stirred for 4 h. and this mixture was allowed to settle and was filtered. Step-2, the filter cake was re-extracted twice with 85 ml of CHCl₃, CH₃OH, 0.3% NaCl (5:10:4, v/v/v). Step-3, CHCl₃ (145 ml) and 0.3% NaCl (145 ml) were added to the combined filtrates which were achieved in step 1 and 2. This mixture was stirred for 1 h. and allowed to settle down to form a biphasic, and the lower layer containing the polar lipids was recovered and dried under reduced pressure. The recovered crude polar lipid fractions containing glycolipids were loaded on a 10 × 10 cm plastic-backed silica gel 60 F254 TLC plates (Merck scientific), first eluting with CHCl₃:CH₃OH:H₂O (60:30:6, v/v/v) in one direction, followed with CHCl₃:CH₃COOH:CH₃OH:H₂O (40:25:3:6, v/v/v/v) in the second direction. The TLC plates were then sprayed with 0.01% 1,6-diphenyl-1,3,5-hexatriene dissolved in petroleum ether/acetone (9:1, v/v), and lipids were visualized and marked under UV light (See supplementary material Figure S1, panel a and b). The lipid spots corresponding to MGDG and DGDG were separately scraped from the plates and the lipids were extracted using CHCl₃/CH₃OH (2:1, v/v). The amount ratio of MGDG to DGDG from the preparation was measured and is close to 1/2.5 (See supplementary material Figure S1, Panel c).

Mass spectrometry

Low-energy CID MSⁿ experiments and high resolution (R=100,000 at *m/z* 400) mass measurements on the [M + Li]⁺ ions of MGDG and DGDG and their subsequent MSⁿ fragment ions were performed on a Thermo LTQ Orbitrap Velos (San Jose, CA) mass spectrometer with Xcalibur operating system. MGDG and DGDG from *Streptococcus pneumoniae* were dissolved in chloroform/methanol (1/4), and CH₃CO₂Li (2 μm/μL) was added to achieve a final concentration of 1 mM before infusion (2 μL/min) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and the temperature of heated capillary was 300 °C. The automatic gain control of the ion trap was set to 5 × 10⁴, with a maximum injection time of 200 ms. Helium was used as the buffer and collision gas at a pressure of 1×10⁻³ mbar (0.75 mTorr). The MSⁿ experiments were carried out with an optimized relative collision energy ranging from 30–40% and with an activation *q* value at 0.25, and the activation time at 10 ms to leave a minimal residual abundance of precursor ion (around 20%). The isolation window for the precursor ions were set at 1 Da. Mass spectra were accumulated in the profile mode, typically for 3–10 min for MSⁿ-spectra (n=2,3,4). The mass resolution of the instrument was tuned to 0.6 Da at half peak height for LIT.

Nomenclature

All diglycosyl diacylglycerols (DGDG) including those found in *S. pneumoniae* contain a dihexose residue attached to C-3 of 1,2-diacylglycerol. The distinction among DGDG structures varied by the glycosides (i.e., glucose, galactose, or mannose) and their linkage is beyond the scope of this study. To simplify data interpretation, the molecule such as 1-hexadecanoyl-2-octadecenoyl-3-*O*-[α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-galactopyranosyl]-*sn*-glycerol that consists of C16:0 and C18:1-fatty acid substituents at *sn*-1 and *sn*-2, respectively, is designated as 16:0/18:1-DGDG to reflect that the compound contains a dihexosyl group without specifying the sugar species and the linkage. The corresponding monoglucosyldiacylglycerol (MGlcDAG) with the same fatty acid substituents is designated as 16:0/18:1-MGDG.

Results and Discussion

Upon subjected to ESI, both DGDG and MGDG isolated from *S. pneumoniae* formed abundant $[M + \text{Alk}]^+$ (Alk = Li, Na, NH_4) ions in the positive-ion mode in the presence of alkaline metal ions (Figure 1); as well as $[M + X]^-$ ($X = \text{CH}_3\text{CO}_2$, HCO_2 , Cl) ions in the negative-ion mode (see supplementary material Figure S2 for the ESI mass spectra of the corresponding $[M + \text{NH}_4]^+$ (Panel a) and $[M + \text{CH}_3\text{CO}_2]^-$ (Panel b) ions of TDM). The profiles of these mass spectra are nearly identical, indicating the utility of these adduct ions in profiling this lipid family. Elemental compositions deduced from high-resolution mass spectrometric analysis showed the presence of homologous ion series with 1 or 2 double bonds, and a minor ion series possessing an additional oxygen, probably at the fatty acid substituents (Table 1). The structural characterization of this glycerolipid family with an extra oxygen will be reported separately. The multiple-stage LIT mass spectrometric approaches toward identification of the major DGDG and MGDG separated from *S. pneumoniae* are described below.

Structural characterization of DGDG and MGDG as $[M + \text{Li}]^+$ ions

We recently applied LIT multiple-stage mass spectrometry for near-complete structural characterization of glycerophospholipid (GPL), cardiolipin and triacylglycerol (TAG) as lithiated ions.^[21–23] The identification of the fatty acyl substituents and assignment of their position on the glycerol backbone is based on the findings that the loss of the fatty acid group at the *sn*-1/*sn*-3 (*sn*-3 fatty acid only seen for TAG) is more facile than that at *sn*-2, when subjected to CID and analyzed with a tandem quadrupole or ion-trap instrument.^[16, 21, 22] As seen in Figure 2a, the MS^2 spectrum of the $[M + \text{Li}]^+$ ion at m/z 925 contained the ions at m/z 763, arising from neutral loss of a hexose residue, and ions at m/z 669 and 643 arising from loss of 16:0- and 18:1-fatty acid substituents, respectively. The ion at m/z 669 is more abundant than the ion of m/z 643, indicating that the 16:0- and 18:1-fatty acid moieties are located at *sn*-1 and *sn*-2, respectively,^[16] and the presence of major 16:0/18:1-DGDG isomer. The spectrum (Figure 2a) also contained the ions at m/z 671 and 641, arising from losses of 16:1-, and 18:0-fatty acid residues, respectively. The ion at m/z 641 is more abundant than the ion at m/z 671, suggesting the presence of the minor 18:0/16:1-DGDG isomer. The ion at m/z 763 is equivalent to the $[M + \text{Li}]^+$ ion of the corresponding MGDG and yielded a MS^3 spectrum ($925 \rightarrow 763$; Figure 2b) identical to the MS^2 spectrum of the MGDG ion of m/z 763 (Scheme 1). The spectrum (Figure 2b) contained ions at m/z 507 and 481, arising from losses of 16:0-, 18:1-fatty acids, respectively, along with the ion-pair of m/z 509 and 479, arising from further losses of 16:1- and 18:0-fatty acid substituents, respectively. The former ion pairs (i.e., ions at m/z 507 and 479) are respectively more abundant than the latter ion pairs, consistent with the presence of 16:0/18:1-DGDG and the minor 18:0/16:1-DGDG isomers.

The assignments of the above structures were further confirmed by MS⁴ on the ion of m/z 583 arising from loss of the sugar (galactosylglucose) residue. As shown in Figure 2c, the MS⁴ spectrum of the ion of m/z 583 ($925 \rightarrow 763 \rightarrow 583$) contained ions at m/z 303 arising from loss of 18:1-fatty acid as α,β -unsaturated fatty acid (loss as 18:2-fatty acid) (Scheme 1, route **a**), along with ions at m/z 345 and 327, arising from losses of 16:0-fatty acid as ketene and as acid, respectively. The results indicated that the 18:1-fatty acid resides at sn-2; while the 16:0-fatty acid is located at sn-1,^[22] consistent with the presence of 16:0/18:1-DGDG. The spectrum also contained the ion at m/z 329 ($583 - 254$), arising from loss of the 16:0-fatty acid as an α,β -unsaturated fatty acid (loss as Δ^2 16:1-fatty acid), indicating the presence of 16:0-fatty acid substituent at sn-2; together with the ions at m/z 319 and 301, arising from loss of 18:1-fatty acid at sn-1 as ketene and as free fatty acid, respectively. The results show the presence of the 18:1/16:0-DGDG isomer. By contrast, this latter structure cannot be extracted from the MS² spectrum of the m/z 925 ion (Figure 2a), because fragment ions arising from acid losses observed for 16:0/18:1-DGDG and 18:1/16:0-DGDG isomers are identical and are indistinguishable by mass spectrometry. The presence of 16:0/18:1-DGDG and 18:1/16:0-DGDG isomers is consistent with the notion that the differences in the abundances of the ions of m/z 641 and 671 is not as drastic as that seen for the analogous ions of m/z 669 and 671 for the $[M + Li]^+$ ion of 18:0/18:1-DGDG at m/z 953, which represents mainly a single isomer (see Figure 3a). The spectrum (Figure 2c) also contained the ion at m/z 331 corresponding to loss of 16:1-fatty acid as an α,β -unsaturated fatty acid (loss as 16:2-fatty acid), along with the ions of 307 and 299 arising from loss of 18:0-fatty acid at sn-1, consistent with the assignment of the minor 18:0/16:1-DGDG isomer as seen earlier.

The above structural assignments were also readily extractable from the MSⁿ on the $[M + NH_4]^+$ ions.^[18] As seen in Figure 2d, the MS² spectrum of the $[M + NH_4]^+$ ions of m/z 936, the ion analogous to the $[M + Li]^+$ ion of m/z 925, is dominated by the ions at m/z 595 arising from the combined losses of the sugar moiety (324 Da) and NH_3 , together with the ion at m/z 577 arising from further loss of H_2O . The spectrum also contained the ions at m/z 339 (loss of 16:0-fatty acid) and 313 (loss of 18:1-fatty acid) that identify the 16:0- and 18:1-fatty acid substituents. Further dissociation of the ion of m/z 577 ($936 \rightarrow 577$; Figure 2e) gave rise to ions at m/z 265 and 239, representing the 18:1- and 16:0-acylium ions, respectively. The former ion is more abundant than the latter, indicating that the 18:1-fatty acyl substituent is located at sn-2, while the 16:0-fatty acid is located at sn-1.^[22, 24] The spectrum also contained the minor ions at m/z 237 and 267, representing the 16:1- and 18:0-acylium ions respectively. The ion at m/z 237 is more abundant than the ion at m/z 267, consistent with the presence of the minor 18:0/16:1-DGDG isomer. The differential formation of the feature ions resulting from the preferential losses of the fatty acid substituents as seen in the MSⁿ spectra of $[M + Li]^+$ and $[M + NH_4]^+$ readily permit confident assignment of the fatty acid substituents and determination of their position on the glycerol backbone.

In Figure 2c, the ions at m/z 513 and 447 (inset) were observed. These ions arose from β -cleavage with γ -H rearrangement (Scheme 1, route **b**) and have been reported in our previously studies.^[21, 22, 25] The observation of these two ions signifies that the double bond of the 18:1-fatty acid is located at C-11; and the ion of m/z 925 represents mainly a 16:0/ Δ^{11} 18:1-DGDG.

Similarly, the MS² spectrum of the $[M + Li]^+$ ion at m/z 953 (Figure 3a), contained the ion at m/z 669 and 671, arising from losses of 18:0-, and 18:1-fatty acid substituents, respectively. The former ion is more abundant than the latter, indicating the presence of 18:0/18:1-DGDG. The ion at m/z 791 arose from loss of a hexose residue and gave rise to ions at m/z 507 and 509 by losses of 18:0- and 18:1-fatty acid substituents (Figure 3b). The

ion at m/z 507 is more abundant than the ion at m/z 509, consistent with that the 18:0-, 18:1-fatty acids reside at sn-1 and sn-2, respectively. The ion of m/z 611 is analogous to the ion of m/z 583 as seen in Figure 2b and represents a lithiated ion of the dehydrated 18:0/18:1-diacyl glycerol. The MS⁴ spectrum of the ion of m/z 611 ($953 \rightarrow 791 \rightarrow 611$; Figure 3c) contained the ions at m/z 541 and 485 (see inset) arising from the similar β -cleavage with γ -H rearrangement fragmentation. These two ions are 28 Da higher than the analogous ions of m/z 513 and 447 as seen in Figure 2c, suggesting that the double bond of the 18:1-fatty acid chain resides at C-11, in agreement with the location of double bond assigned for the 18:1-fatty acid substituent in 16:0/ Δ^{11} 18:1-DGDG. The spectrum (Figure 3c) is dominated by the ion of m/z 331 arising from loss 18:1-fatty acid as α,β -unsaturated fatty acid (loss as $\Delta^2,^{11}$ 18:2-fatty acid). The result further supports that the 18:1-fatty acid resides at sn-2.

The $[M + Li]^+$ ion of m/z 897 gave rise to major ions at m/z 641 and 643, arising from losses of 16:0-, 16:1-fatty acid substituents, respectively, together with ion at m/z 735, arising from loss of a hexose residue (Figure 3d). The ion at m/z 641 is more abundant than the ion of m/z 643, indicating the presence of 16:0/16:1-DGDG. The regio-specificity of the fatty acid substituents and the location of double bond of the 16:1-fatty acid, again, were further recognized from the MS⁴ spectrum of the ion of m/z 555 ($897 \rightarrow 735 \rightarrow 555$; Figure 3e), representing a lithiated ion of dehydrated 16:0/16:1-diacyl glycerol arising from elimination of sugar. The spectrum contained the ions at m/z 485 and 429, arising from the similar fragmentation process involving β -cleavage with γ -H rearrangement. These two ions signify that the double bond of the 16:1-fatty acid substituent is located at C-9; and the ion of m/z 897 mainly represents a 16:0/ Δ^9 16:1-DGDG. The spectrum also contained the prominent ion of m/z 303 arising from loss of 16:1-fatty acid as α,β -unsaturated fatty acid (loss as $\Delta^{2,9}$ 16:2-fatty acid), confirming that the 16:1-fatty acid is indeed located at sn-2. The minor ion pairs at m/z 613/671 arising from losses of 18:0/14:1-fatty acid substituents, and at m/z 615/669 arising from losses of 18:1/14:0-fatty acid substituent were also observed, indicating the presence of the minor 18:0/14:1-DGDG and 18:1/14:0-DGDG isomers. The presence of these latter two isomers is consistent with the observation of the ion at m/z 331, and 329 arising from losses of 14:2- and 14:1-fatty acids, respectively (loss as α,β -unsaturated fatty acid) in Figure 3e, reassuring that the 14:1- and 14:0-fatty acids are situated at sn-2.

The above assignment of the position of the double bond of the 16:1 and 18:1-fatty acid substituents at C-9, and C-11, respectively, is consistent with the earlier report that Δ^9 16:1- and Δ^{11} 18:1-Fatty acids are the major monounsaturated fatty acid found in *S. pneumoniae*.^[11, 26–28] The structural assignment, including the location of double bond of the unsaturated fatty acid was further confirmed by structural characterization applying MSⁿ on the corresponding $[M - H + 2Li]^+$ ions, which gave rise to the dilithiated ions of the monosaturated fatty acid that undergo further dissociation to yield feature ions applicable for locating the double bond position (see supplementary material Figure S3).

Structural characterization of MGDG and the fragmentation pathways supported by H-D exchange

Both DGDG and MGDG contain the glycerol backbone to which fatty acid substituents are attached to sn-1 and sn-2, and a sugar moiety is attached to sn-3. As shown earlier (Figure 1), the profiles of DGDG (Panel a) and MGDG (Panel b) isolated from *S. pneumoniae* are similar. The MS² spectrum of the MGDG ion of m/z 763 (Figure 4a) is identical to the MS³ spectrum of the ion of m/z 763 (Figure 2b) deriving from m/z 925, consistent with the notion that DGDG first eliminates a hexose residue to yield a lithiated MGDG ion, upon subjected to CID in an ion-trap. The results showed that the ion of m/z 763 represents a major 16:0/18:1-MGDG isomer together with minor 18:0/16:1-MGDG and 18:1/16:0-MGDG isomers.

The lithiated ion of m/z 763 shifted to m/z 767 after H-D exchange, consistent with the notion that MGDG contains 4 exchangeable hydrogens in the sugar moiety. The profile of the MS² spectrum of the $[M + Li]^+$ ion of d₄-MGDG ion of m/z 767 (Figure 4b) is identical to that of m/z 763 (Figure 4a). The spectrum is dominated by the ions of m/z 511 and 537, arising from losses of the 18:1- and 16:0-fatty acid substituents, together with the ions at m/z 263 and 289, representing the lithiated ions of 16:0- and 18:1-fatty acids, respectively. The results demonstrate that the loss of the fatty acid moieties does not involve the participation of the exchangeable hydrogen atoms on the sugar ring; and probably involves the α -hydrogen of the neighboring fatty acid substituents (Scheme 1, route I). The spectrum also contained the ion of m/z 602, analogous to the ion of m/z 601 (Figures 2b and 4a) and the ion of m/z 583. These two ions arise from losses of the various hexose residues and consistent with the notion that the ion of m/z 602 yielded the ion of m/z 583 by further loss of a water molecule (loss of HDO) (data not shown). The MS⁴ spectrum of the ion of m/z 583 ($767 \rightarrow 602 \rightarrow 583$; data not shown) is identical to that shown in Figure 2c. The results further support the proposed fragmentation processes in which the α -hydrogen of the fatty acid substituent at sn-2 in MGDG also participates in the loss of the glucose residue that results in the formation of the ion of m/z 583 (Scheme 1, route II), similar to that previously observed for TAG.^[22]

Conclusions

The present LIT MSⁿ mass spectrometric approaches for characterization of DGDG and MGDG as the $[M + Li]^+$ ions afford a confident and near complete structural identification. In contrast, structural identification employing MS² on the $[M + Na]^+$ ions is incomplete and confirmation of the assignment requires tedious enzymatic reaction for specific cleavage of the fatty acid moiety.^[14] In the present study, we revealed that the mass spectrum profiles of DGDG and MGDG in *S. pneumoniae* are very similar (Figure 1); and the diacyl structures between the corresponding DGDG and MGDG species are also nearly identical (Table 1). These results suggest that DGDG in *S. pneumoniae* may be synthesized from MGDG, similar to the previous findings that MGalDAG is the substrate for DGalDAG biosynthesis in plant cells.^[29, 30] and diglucosyl diglyceride is synthesized from monoglucosyl diglyceride in *Mycoplasma laidlawii*.^[31] Our preliminary data including those from high-resolution mass measurement suggested the presence of a new family of DGDG and MGDG containing an additional oxygen in the fatty acyl chain. The structural characterization of this new glycolipid family is currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The ESI mass spectra of the $[M + Li]^+$ ions of MGDG (a) and DGDG (b) isolated from *S. pneumoniae*.

Figure 2. The MS² spectrum of the $[M + Li]^+$ ion of m/z 925 (a), its MS³ spectrum of m/z 763 (925 $\rightarrow$ 763) (b), MS⁴ spectrum of m/z 583 (925 $\rightarrow$ 763 $\rightarrow$ 583) (c), and the MS² spectrum of the analogous $[M + NH_4]^+$ ion of m/z 936 (d), and its MS³ spectrum of the ion of m/z 577 (936 $\rightarrow$ 577) (e). Panel (f) shows the cleavages that lead to the denoted fragment ions. Ions marked with “o” locate the double bond of the 18:1-fatty acid substituent (Panel c, inset).

Figure 3.

The MS^2 spectrum of the $[M + Li]^+$ ion of m/z 953 (a), its MS^3 spectrum of m/z 781 (953 $\rightarrow$ 791) (b), MS^4 spectrum of m/z 611 (953 $\rightarrow$ 791 $\rightarrow$ 611) (c); and the MS^2 spectrum of the $[M + Li]^+$ ion of m/z 897 (d), and its MS^4 spectrum of m/z 555 (897 $\rightarrow$ 735 $\rightarrow$ 555) (e). Ions marked with “o” locate the double bond along the 18:1-fatty acid (c) and the 16:1-fatty acid (e) substituents, respectively.

Figure 4. The MS^2 spectra of the $[M + Li]^+$ ions of the MGDG ion of m/z 763 (a), and of the analogous d_4 -MGDG ion of m/z 767 (b) obtained by H-D exchange.

Scheme 1.

The proposed fragmentation pathways leading to structural characterization of the $[M + Li]^+$ ion of 16:0/ Δ^{11} 18:1-DGDG (*masses observed for deuterium labeled analogs)

Table 1

The elemental compositions deduced from high-resolution mass measurements and the structural assignments based on MSⁿ on the [M + Li]⁺ ions of MGDG (A) and DGDG (B) from *S. pneumoniae*

A (MGDG)					
Measured Mass (Da)	Theoretical Mass (Da)	Deviation (mmu)	Rel. Intensity (%)	Elemental Composition	Structures
681.5125	681.5124	0.18	3.30	C37 H70 O10 Li	16:0/12:0
705.5127	705.5124	0.30	1.13	C39 H70 O10 Li	16:0/14:2; 16:1/14:1; 18:2/12:0; 18:1/12:1
707.5285	707.5280	0.50	7.87	C39 H72 O10 Li	16:0/14:1; 18:1/12:0; 16:1/14:0; 18:0/12:1
709.5441	709.5437	0.44	6.53	C39 H74 O10 Li	12:0/18:0; 16:0/14:0
721.5077	721.5073	0.44	1.34	C39 H70 O11 Li	*
723.5232	723.5229	0.31	1.29	C39 H72 O11 Li	*
733.5443	733.5437	0.66	5.13	C41 H74 O10 Li	16:1/16:1; 18:1/14:1; 18:0/14:2; 16:0/16:2; 18:2/14:0
735.5598	735.5593	0.49	22.76	C41 H76 O10 Li	16:0/16:1; 18:0/14:1; 18:1/14:0
737.5750	737.5750	0.06	3.73	C41 H78 O10 Li	18:0/14:0; 16:0/16:0
749.5391	749.5386	0.54	1.18	C41 H74 O11 Li	*
751.5547	751.5542	0.47	1.13	C41 H76 O11 Li	*
759.5600	759.5593	0.65	2.83	C43 H76 O10 Li	18:2/16:1; 16:2/18:1
761.5755	761.5750	0.53	30.30	C43 H78 O10 Li	18:1/16:1; 18:2/16:0
763.5905	763.5906	-0.09	100.00	C43 H80 O10 Li	16:0/18:1; 18:0/16:1
775.5548	775.5542	0.58	1.61	C43 H76 O11 Li	*
777.5702	777.5699	0.32	5.77	C43 H78 O11 Li	*
779.5856	779.5855	0.07	4.45	C43 H80 O11 Li	*
787.5910	787.5906	0.37	8.80	C45 H80 O10 Li	18:2/18:1
789.6065	789.6063	0.20	54.56	C45 H82 O10 Li	18:1/18:1; 18:2/18:0
791.6214	791.6219	-0.51	93.40	C45 H84 O10 Li	18:0/18:1
803.5858	803.5855	0.24	4.00	C45 H80 O11 Li	*
805.6012	805.6012	0.07	7.75	C45 H82 O11 Li	*
807.6167	807.6168	-0.08	3.91	C45 H84 O11 Li	*
B (DGDG)					
815.5340	815.5339	0.14	1.87	C41 H76 O15 Li	16:0/10:0; 14:0/12:0; 18:0/8:0
841.5497	841.5495	0.11	3.72	C43 H78 O15 Li	16:1/12:0; 16:0/12:1

A (MGDG)						
Measured Mass (Da)	Theoretical Mass (Da)	Deviation (mmu)	Rel. Intensity (%)	Elemental Composition	Structures	
843.5651	843.5652	-0.06	15.97	C43 H80 O15 Li	16:0/12:0	
867.5653	867.5652	0.11	2.07	C45 H80 O15 Li	16:0/14:2; 16:1/14:1; 18:2/12:0; 18:1/12:1	
869.5809	869.5808	0.11	11.96	C45 H82 O15 Li	16:0/14:1; 18:1/12:0; 16:1/14:0; 18:0/12:1	
871.5964	871.5964	0.00	13.81	C45 H84 O15 Li	12:0/18:0; 16:0/14:0	
885.5759	885.5757	0.11	1.43	C45 H82 O16 Li	*	
895.5968	895.5965	0.27	5.56	C47 H84 O15 Li	16:1/16:1; 18:1/14:1; 16:0/16:2; 18:0/14:2; 18:2/14:0	
897.6121	897.6121	-0.01	30.84	C47 H86 O15 Li	16:0/16:1; 18:0/14:1; 18:1/14:0	
899.6275	899.6278	-0.25	5.83	C47 H88 O15 Li	18:0/14:0; 16:0/16:0	
911.5917	911.5914	0.33	2.59	C47 H84 O16 Li	*	
913.6073	913.6070	0.27	3.07	C47 H86 O16 Li	*	
921.6126	921.6121	0.43	2.95	C49 H86 O15 Li	18:2/16:1; 18:1/16:2	
923.6280	923.6278	0.19	25.03	C49 H88 O15 Li	18:1/16:1; 18:0/16:2	
925.6427	925.6434	-0.73	100.00	C49 H90 O15 Li	16:0/18:1; 18:0/16:1; 18:1/16:0	
937.6073	937.6070	0.21	3.22	C49 H86 O16 Li	*	
939.6227	939.6227	0.04	16.90	C49 H88 O16 Li	*	
941.6380	941.6383	-0.31	11.98	C49 H90 O16 Li	*	
943.6536	943.6540	-0.43	2.26	C49 H92 O16 Li	*	
949.6434	949.6434	-0.05	7.00	C51 H90 O15 Li	18:2/18:1	
951.6587	951.6591	-0.39	37.30	C51 H92 O15 Li	18:1/18:1; 18:0/18:2	
953.6732	953.6747	-1.56	76.56	C51 H94 O15 Li	18:0/18:1	
965.6381	965.6383	-0.28	7.60	C51 H90 O16 Li	*	
967.6534	967.6540	-0.57	16.72	C51 H92 O16 Li	*	
969.6688	969.6696	-0.79	14.92	C51 H94 O16 Li	*	

* structures not assigned