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Analysis of glycosylated cardiolipins from thermophilic bacteria using GC–MS and LC–ESI–MS/MS methods

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ABSTRACT

Unusual glucose-substituted cardiolipins (Glc_x-CLs) in three genera of thermophilic bacteria, having more than one glycosidically linked glucose to the hydroxyl of the central glycerol of Glc_x-CLs were identified for the first time in thermophilic bacteria of the genera *Geobacillus, Meiothermus,* and *Thermus*. The number of glucoses reached up to five units. The structure of glycosidically linked oligosaccharides was determined based on shotgun analysis MS (electrospray high-resolution tandem mass spectrometry), partially methylated alditol acetates were identified by GC–MS, both electron ionization (EI) and positive chemical ionization (PCI), hydrophilic interaction liquid chromatography (HILIC) separation and identification of CLs glycosides by high resolution MS–ESI, and digestion by specific glycosidases.

1. Introduction

Microorganisms that live in a location that differs considerably from the usual environment are typically classified as extremophilic microorganisms. One of the most frequently researched and described environments is the high-temperature environment. Typically, microorganisms are divided into psychrophiles (<20 °C), mesophiles (20–45 °C), thermophiles (45–80 °C) and hyperthermophiles (>80 °C) [1]. Various glyco- or phosphoglycolipids have been isolated and identified from many thermophilic bacteria, e.g., the genera *Geobacillus*, *Thermus, Meiothermus,* and many others [2].

Cardiolipin (CL) is a trivial but commonly used name for a lipid whose systematic name is 1,3-bis(*sn*-3'-phosphatidyl)-*sn*-glycerol. This lipid contains four acyl residues of fatty acids and three chiral centers and can have up to two negative charges. Cardiolipin is present in Archaea (but it contains alkyls instead of acyls), in prokaryotes (e.g., in the plasma membrane) and in eukaryotes. Four acyls cause a high distribution of molecular species; of course, not all are biosynthesized and possibly detected. Another complication of CL analysis, now almost exclusively solved with MS, are structural analogues. In both Gram-positive and Gram-negative bacteria, e.g., D-glucopyranosylcardiolipin, D-alanylcardiolipin, L-lysylcardiolipin, etc., were identified [3–5].

Glycosylated cardiolipin (Hex-CL) was first detected in several strains of *Streptococcus* and identified as glucopyranosylcardiolipin at approximately 18% lipid phosphorus [3]. It has also been identified as a minor component (3.7% of membrane lipids) of *Vagococcus fluviatilis* [4]. In addition to this CL derivative, other lipids, e.g., L-lysylphosphatidylglycerol, bis(acylglycero)phosphate, α-D-glucopyranosylcardiolipin, and D-alanylbis(acylglycero)phosphate were identified [5].

Positive and negative mode fast atom bombardment mass spectrometry (FAB–MS) was used to identify substituted CLs such as α-D-glucopyranosyl-, D-alanyl- and L-lysyl-CL [5]. In the case of Glc-CL, molecular species containing even fatty acids were identified, mainly hexadecanoic (16:0), hexadecenoic (16:1), octadecanoic (18:0), and octadecenoic (18:1) acids.

High performance thin layer chromatography (HPTLC) was used to isolate the fraction containing Glc–CL from a thermophilic bacterium *Geobacillus stearothermophilus* [6]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF–MS) was also used to determine the structure of Glc–CL. Furthermore, several dozen molecular species were identified by MALDI–TOF–MS, where the majority of acyls were iso-pentadecanoic (i-15:0) and anteiso-heptadecanoic (ai-17:0) acids. A basic paper describing the mapping and sequencing of *G. stearothermophilus* cardiolipins by positive and negative ion nano-electrospray ionization quadrupole time of flight electrospray ionization (ESI–QTOF–MS) and tandem mass spectrometry

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(MS/MS) was published [7].

In the analysis of complex mixtures of cardiolipins, mass spectrometry was mainly used. Some examples have already been mentioned above, especially those related to glycosyl derivatives of CL. However, based on data from the literature (e.g., Peter-Katalinic et al., Schaffer et al., Beckedorf et al.,) [5–7] and our previously published articles Vitova et al., [8] and Rezanka et al., [9] thermophilic bacteria of the genera *Geobacillus, Meiothermus* and *Thermus* were cultured at various temperatures (55–70 °C). Lipidomic shotgun analysis was performed by high-resolution ESI tandem MS (HR–ESI/MS/MS) using a precursor ion (PIS) and neutral loss ion scans (NLS) to detect the presence of oligosaccharide glycosides of cardiolipins. The structure of these oligosaccharides was also elucidated on the basis of analysis of partially methylated alditol acetates (PMAAs) and cleavage by specific glycosidases. In addition, the use of hydrophilic interaction liquid chromatography (HILIC) connected to electrospray high-resolution tandem mass spectrometry confirmed the presence of unusual glucose substituted cardiolipins (Glcx–CL) in eight strains of thermophilic bacteria.

2. Materials and methods

2.1. Standards and Chemicals

α-Glucosidase from *Bacillus stearothermophilus* (lyophilized powder, ≥50 units/mg protein EC 3.2.1.20, α-1,4-glucosidase), β-glucosidase from almonds (lyophilized powder, ≥4 units/mg EC 3.2.1.74, β-1,4-glucosidase), pullulanase microbial (EC 3.2.1.41, i.e., α-1,6-glucanohydrolase), dextranase from *Penicillium* sp. (lyophilized powder, 400–800 units/mg protein, EC 3.2.1.11, α-1,6-glucanohydrolase), and 1′,3′-bis [1,2-dipalmitoyl-*sn*-glycero-3-phospho]-glycerol ($(16:0)_4$-CL) were purchased from Sigma-Aldrich (now Merck, Prague, Czech Republic). All other chemicals mentioned below, e.g., solvents, hexamethyldisilazane, trimethylchlorosilane, etc., were also obtained from Sigma-Aldrich.

2.2. Cultivation

The cultivation of these strains was described in previously published articles [10–12]. Briefly, the *Thermus* and *Meiothermus* genera were grown in medium B39: yeast extract 4 g/L, tryptone 8 g/L, NaCl 2 g/L, pH 7.5. Genus *Geobacillus* was cultivated in medium B10: peptone 5 g/L, meat extract 3 g/L, $MnSO_4 \times H_2O$ 0.005 g/L, pH was 7.0 and 5.5, respectively. Batch culture was carried out under aerobic conditions in 500-mL Erlenmeyer flasks filled with 200 mL of medium. The Erlenmeyer flasks were placed on a temperature-controlled shaking device (HT Inflors AG CH-4103, Hungary). The incubation continued for 48 h, 200 rpm and at different temperatures; see Table S1 (Supplements).

2.3. Isolation of glycocardiolipins

Total lipids were extracted using the method of Bligh and Dyer [13] and the aqueous phase was again extracted according to Markham and Jaworski [14] using a mixture of the lower phase of isopropanol/hexane/water (55:20:25 v/v/v). Combined lipid extracts were applied to the Sep-Pak 35 cc Cartridge Vac (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and the cartridge was eluted with 40 mL of isopropanol/hexane/water (55:20:25 v/v/v) [15]. The eluate was reduced in volume and further analyzed.

2.4. Analysis of glycocardiolipins by shotgun mass spectrometry

An LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray interface (HESI) was operated in negative ionization mode. The range of MS scans was performed in the Fourier transform (FT) cell and recorded within a window between 200 and 2500 Da. The mass resolution was set to 105,000, and the ion spray voltage was set at − 2.7 kV. The following parameters for the ionization mode were used: sheath gas flow, 20 arbitrary units (AU); auxiliary gas flow, 8 AU; ion source temperature, 285 °C; capillary temperature, 250 °C; capillary voltage, 45 V; and tube lens voltage, 170 V. Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 33% was used for the fragmentation of the parent ions. Flow injection analysis was used for sample introduction into the heated ESI–MS (H–ESI–MS) ion source. Acetonitrile was used at a flow rate of 125 μL/min. The differences between the measured and calculated values for all compounds analyzed, in negative ESI, do not exceed 0.3 ppm (0.0003 Da).

2.5. Identification of carbohydrates from glycocardiolipins

2.5.1. Methanolysis

The mixture of 2 mL of anhydrous methanol and 280 μL of acetyl chloride was mixed. After 5 min, the reagent was added dropwise to the total $(Hex)_x$-CLs and heated for 16 h (overnight) at 80 °C in tubes sealed with Teflon-lined septa in the screw caps. Methanolic HCl was evaporated under a stream of nitrogen at 40 °C. The evaporation step was repeated twice more each time after adding 250 μL of MeOH.

2.5.2. Silylation

A mixture containing 100 μL hexamethyldisilazane and 50 μL trimethylchlorosilane was added to the methylglycoside mixture in 1 mL of pyridine. The mixture was shaken vigorously for about 1 min and then allowed to stand for 10 min. The reagents were removed in a stream of nitrogen at room temperature, 100 μL of hexane was added to the mixture, and the trimethylsilyl-methyl glycosides were identified by GC–MS.

2.5.3. Gas chromatography-mass spectrometry

The methyl glycosides of TMS were analyzed using a high performance 7890 A gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with fused silica capillary column (SPB®−5 Capillary GC Column, L × I.D. 30 m × 0.25 mm × 0.25 μm, bonded; poly(5% diphenyl/95% dimethyl siloxane phase)) coupled to an Agilent 7000 MS/MS (Agilent) with chemical ionization source (CI). The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 5 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. Positive chemical ionization mass spectra (PCI; full scan mode) were recorded as normal mode, CI reagent ammonia at 1.3 mL/min [16]. All spectra were scanned within the range of m/z 50–600.

2.6. Separation of glycocardiolipins by HILIC

Semi-preparative HPLC equipment consisted of a 1090 Win system, a PV5 ternary pump (400 bar pressure), an automatic injector (HP 1090 series, Hewlett Packard, USA), and HILIC HPLC ZIC®-HILIC 250 × 10 mm × 5 μm, 200 Å column. An elution with a flow rate of 3.7 mL/min was performed and a linear gradient was performed from the mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, *v/v/v*) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, *v/v/v*) for 60 min. The column temperature was 35 °C and the re-equilibration period between runs was 30 min. The efficiency of the column was approximately 11,000 plates/25 cm. $(16:0)_4$-CL was used as an external standard. The eluent from the HPLC column was split so that 10% of the flow was introduced to ESI–MS, see above, and 90% of the flow containing fractions of lipids were collected manually. The glyco–CL fractions were further evaporated and redissolved for identification by enzyme hydrolysis and analysis of partially methylated alditol acetates [17].

2.7. Preparation and analysis of partially methylated alditol acetates (PMAAs)

2.7.1. Micromethylation procedure

The 10 μg of each of the fractions (peaks **B**–**N** not **J**, from HILIC) was methylated in a small glass tube placed in Teflon-lined screw-cap tubes with dimethyl sulfoxide (200 μL), lithium hydride (20 μL) and methyl iodide (40 μL) and the mixture was sonicated for 60 min [18,19] (Fig. 1). Methylation was stopped by adding water (0.4 mL) containing thiosulfate, and permethylated compounds were extracted with chloroform (3 × 0.4 mL). The methylated $(Glc)_x$–CLs were dried (anhydrous sodium sulfate) and filtered.

2.7.2. Hydrolysis

The organic layer was evaporated to dryness and the sample was heated with 2 mL of 3 M HCl at 100 °C for 1 h.

2.7.3. Reduction

The sample was evaporated to dryness and 2 mL of a solution of $NaBD_4$ (5 mg/mL) in a mixture of 30% methanol and 70% NaOH (0.03 M) was added. After 4 h at 50 °C, 2 mL of glacial acetic acid was added and the sample was dried by evaporation in vacuo at 50 °C. The residue was dissolved in 1 mL of methanolic HCl (0.1%) and evaporated to dryness.

2.7.4. Acetylation

To achieve peracetylation, the residue was solved in 3 mL of acetic anhydride–acetonitrile–pyridine (3:3:1, v/v/v) and heated for 2 h at 80 °C. A 2 mL volume of distilled water and 2 mL of chloroform were added, the sample was mixed with the vortex, and the aqueous layer was removed. The chloroform solution was evaporated to dryness under reduced pressure at 50 °C and the residue was dissolved in 2 mL of 2-propanol [19] and analyzed by GC–MS.

2.7.5. GC–MS of PMAAs

GC–MS of the PMAA mixtures was performed on a 7890 A gas chromatograph. Splitless injection was performed at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d. × 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, then increased at 20 °C/min

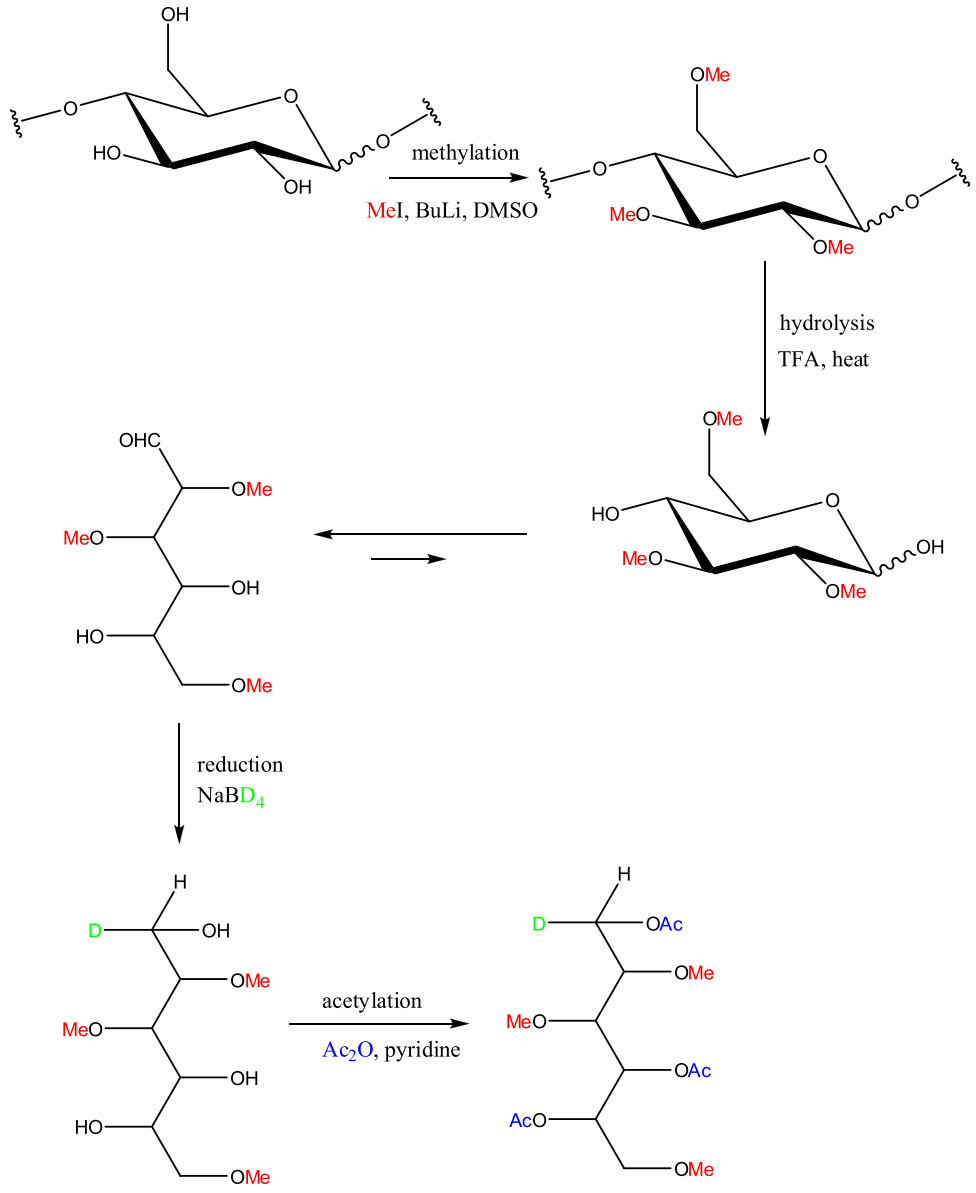

Fig. 1. Preparation of PMAAs which are prepared by a series of reactions, i.e. methylation, hydrolysis, reduction, and acetylation.

to 160 °C and at 2 °C/min to 240 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range of *m/z* 50–500. The structures of PMAAs were confirmed by comparison of retention times and fragmentation patterns, see the paper Sims et al. [18]. Their molar ratios were estimated from the peak areas and response factors [20].

2.8. Enzymatic hydrolysis by glucosidases

α-Glucosidase from *Bacillus* (EC 3.2.1.20, α-1,4-glucosidase), β-glucosidase from almonds (EC 3.2.1.74, β-1,4-glucosidase), pullulanase microbial (EC 3.2.1.41, α-1,6-glucanohydrolase), and dextranase from *Penicillium* sp. (EC 3.2.1.11, α-1,6-glucanohydrolase) from Sigma-Aldrich were used. Samples of glycosyl-CLs (10–15 μg, i.e., fraction **B**–**N** non **J**, from HILIC) were suspended in 0.1 mL of 0.1 M citrate buffer (pH 4.5) for α-glucosidase and β-glucosidase, 0.1 M Tris buffer (pH 6.5) for pullulanase and dextranase, each containing 0.1 mg of sodium taurodeoxycholate. The reaction was carried out with ~0.5 units of enzymes at 37 °C for 24 h. The incubation was stopped by adding 0.5 mL of a mixture of chloroform-methanol (2:1, v/v) and the derivatives of CLs, after extraction into the lower layer, were dried in a nitrogen stream and then analyzed by shotgun HR–ESI–MS.

3. Results and discussion

3.1. Cultivation and isolation of glycosylated cardiolipins

The genera *Geobacillus*, *Meiothermus*, and *Thermus* were cultivated as previously described [10–12]. After cultivation, the biomass was concentrated by centrifugation and lyophilized. However, due to the different relative hydrophobicities of lipid molecules, it is impossible to extract all lipid classes from biomass [21]. The chloroform/methanol mixture (extraction according to Bligh and Dyer) has already been published several times [14,22,23] that it is not suitable for very polar lipids such as mono- to oligo-glycosides of lipids. Therefore, after extraction according to Bligh and Dyer, a mixture of lower phase of isopropanol/hexane/water (55:20:25 v/v/v) was used for further extraction of polar lipids [14,23,24]. Total lipid extracts were applied to the Sep-Pak Cartridge (aminopropylsilica phase) and lipids were subsequently eluted, see Experimental. After concentration, total glyco-CLs were separated and identified by shotgun lipidomic analysis and HILIC/ESI–MS/MS.

3.2. Identification of $(Hex)_x$–CLs by shotgun MS

Total $(Hex)_x$–CLs were analyzed by high-resolution mass spectrometry (HR–MS) negative ESI on an Orbitrap instrument, see Materials and Methods. Structures of $(Hex)_x$–CLs were deduced based on previously published descriptions of negative mass spectra [5,7–9,25–28]. The ions

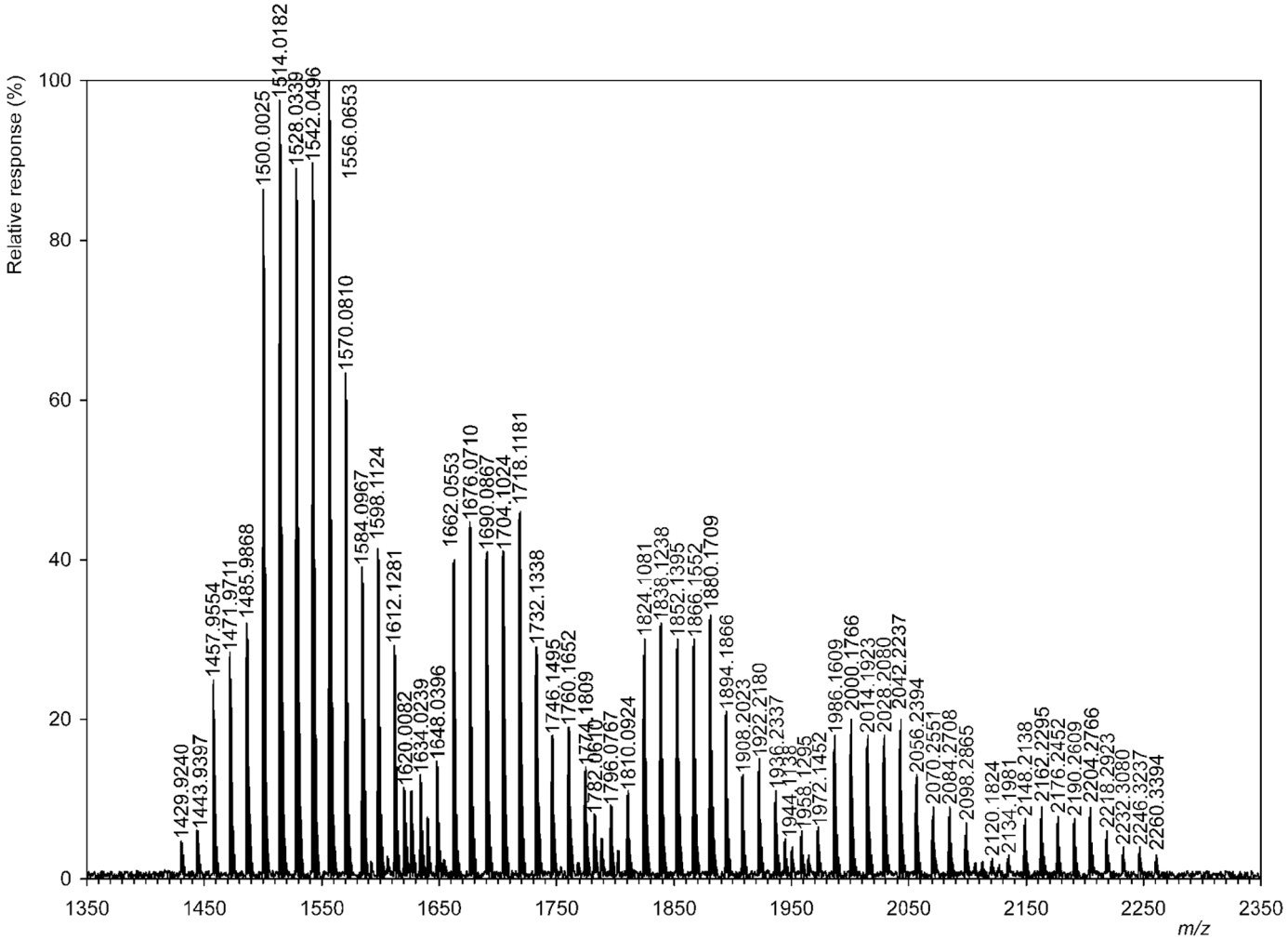

Fig. 2. High-resolution mass spectrum using the neutral loss scan method (neutral fragment at *m/z* 162.0528) by negative ESI.

in the MS differed by ~162 Da which was the neutral loss glucose fragment. The assumed structure of the neutral fragment with mass 162.0528 is shown in Fig. S1 (Supplements). Already in 2002, Beckedorf et al. [7] wrote in their article "*An additional interesting feature of the parent ion scan was the detection of fragment ions due to the neutral loss of a hexose moiety*". The mass spectrum of $(Hex)_x$–CLs using the neutral loss scan method (neutral fragment at *m/z* 162.0528) of the strain *Meiothermus ruber* strain (CCM 4212) cultured at 65 °C is shown in Fig. 2. Characteristic mass differences such as 14, 28 or 162 are shown in Table S2. The *m/z* values and their relative intensity are listed in Supplements (Table S3), where the content of molecular species of glucosylated cardiolipins from eight thermophilic bacteria is listed. Unfortunately, the overall mass spectrum was very complicated by the presence of heavier isotopes, especially ^{13}C and ^{2}H (D). It is obvious that the abundance of $[M\text{-}H+1]^-$ ion, mainly produced by the ^{13}C isotope, is always greater than the abundance of $[M\text{-}H]^-$ ion, which applies to ions with *m/z* values higher than about 1500 Da. As an example, molecular species $(Hex)_5$–67:0-CL can be mentioned, where the ratio $[M\text{-}H]^-$ versus $[M\text{-}H+1]^-$ is 88:100 (calculated 87.2:100) at *m/z* 2204.2763.

As an example of cleavage in tandem MS, the molecular species at *m/z* 2204.2763, i.e., $(Hex)_5$-67:0-CL, is given, see Fig. 3 and fragmentation pathways (Fig. 4). The predicted structure of the ion is shown in Fig. S1. Since the structures and cleavage in tandem MS have already been described several times, see above, the results are presented only briefly. The ion $[M\text{-}H]^-$ found at *m/z* 2204.2763 (calculated 2204.2760) gradually loses hexose (Hex) to form an aglycone ion at *m/z* 1394.0121. The characterization of $(Hex)_5$-67:0-CL consisting of two different diacylglycerol moieties is illustrated by the product-ion spectrum at *m/z* 2204.2763. The spectrum contains sets of ions at *m/z* 605.4190 (**a** ion), at *m/z* 661.4452 (**a** + **56**), at *m/z* 741.4115 (**a** + **136**) and at *m/z* 731.5598 (**b**), at *m/z* 661.4452 (**b** + **56**), at *m/z* 867.5524 (**b** + **136**), nomenclature according to Hsu et al. [27]. In the spectrum, there is an ion of *m/z* 731.5598, for which the structure was determined as 19:0/19:0-PA. This ion is formed by the loss of a neutral particle with a mass of 135.9925, structure, see Fig. S1. In addition to the ion at *m/z* 605.4190, the spectrum is also dominated by the ion at *m/z* 363.1943, resulting from the loss of the 15:0 acid in *sn*-2, and a less prominent ion at *m/z* 377.2099 resulting from the loss of the 14:0 acid in *sn*-1, showing a structure of 14:0/15:0-PA. Similarly, the "right" part of the CL was identified, where the dominant ions, see Fig. S1, indicate the presence of **b**-type ions. Based on the above facts, it can be stated that the ion at *m/z* 2204.2763 represents the majority component 14:0/15:0–19:0/19:0–CL aglycone.

Both the content of CLs in bacteria and their identification are described quite frequently [29,30], so in the next part the main attention was paid to the carbohydrate parts of glycosides. First, the mixture of total $(Hex)_x$–CLs was hydrolyzed and the resulting monosaccharides were identified in the form of derivatives by GC–MS.

3.3. Structure of carbohydrates

The glycosyl composition was determined by combined gas chromatography–mass spectrometry (GC–MS) of the trimethylsilyl (TMS) methyl glycosides produced from the total $(Hex)_x$–CL by acid

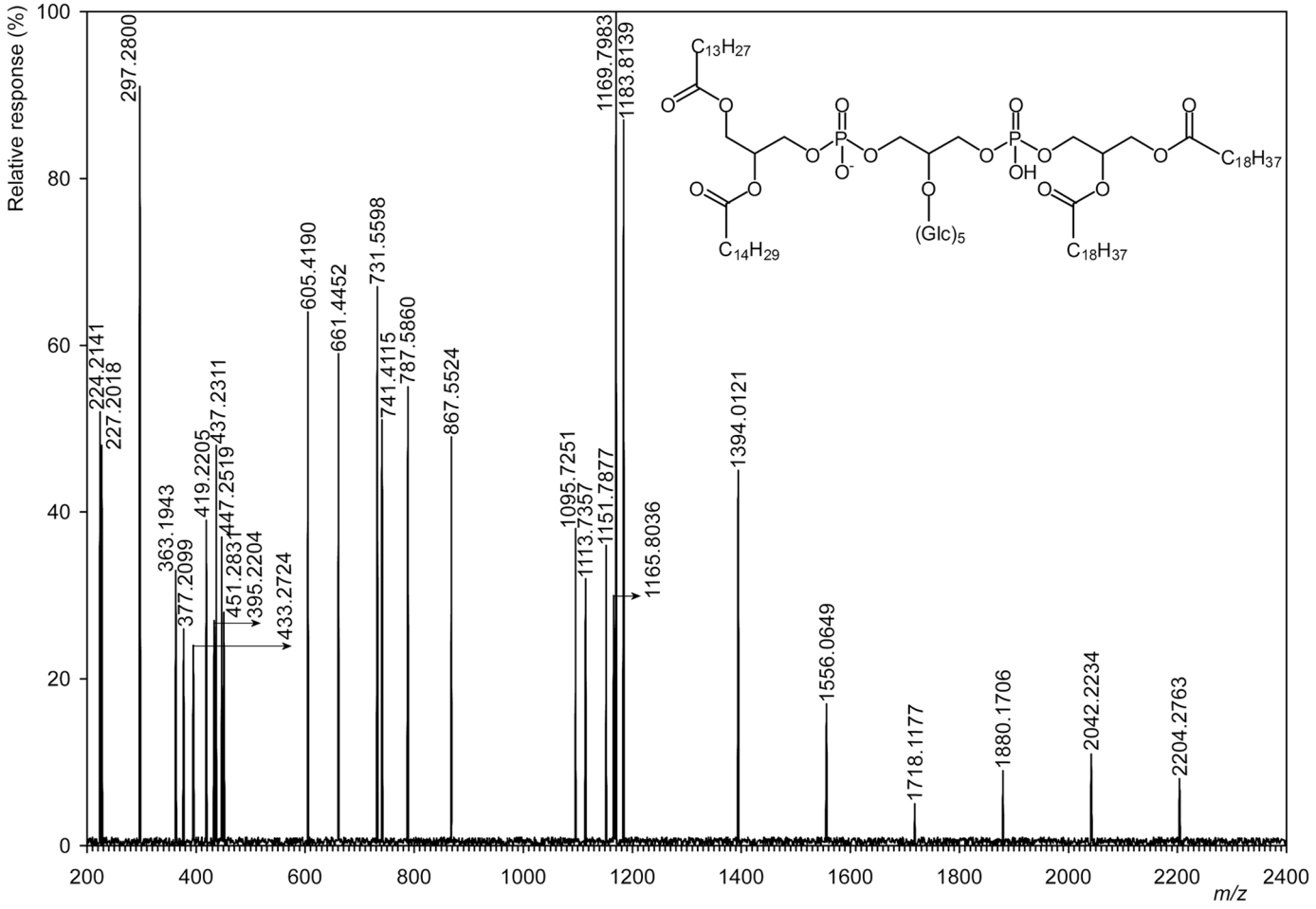

Fig. 3. High-resolution tandem mass spectrum of the molecular species at *m/z* 2204.2763, i.e., $(Glc)_5$–67:0-CL, i.e. ($(Glc)_5$–14:0/15:0–19:0/19:0–CL) by negative ESI.

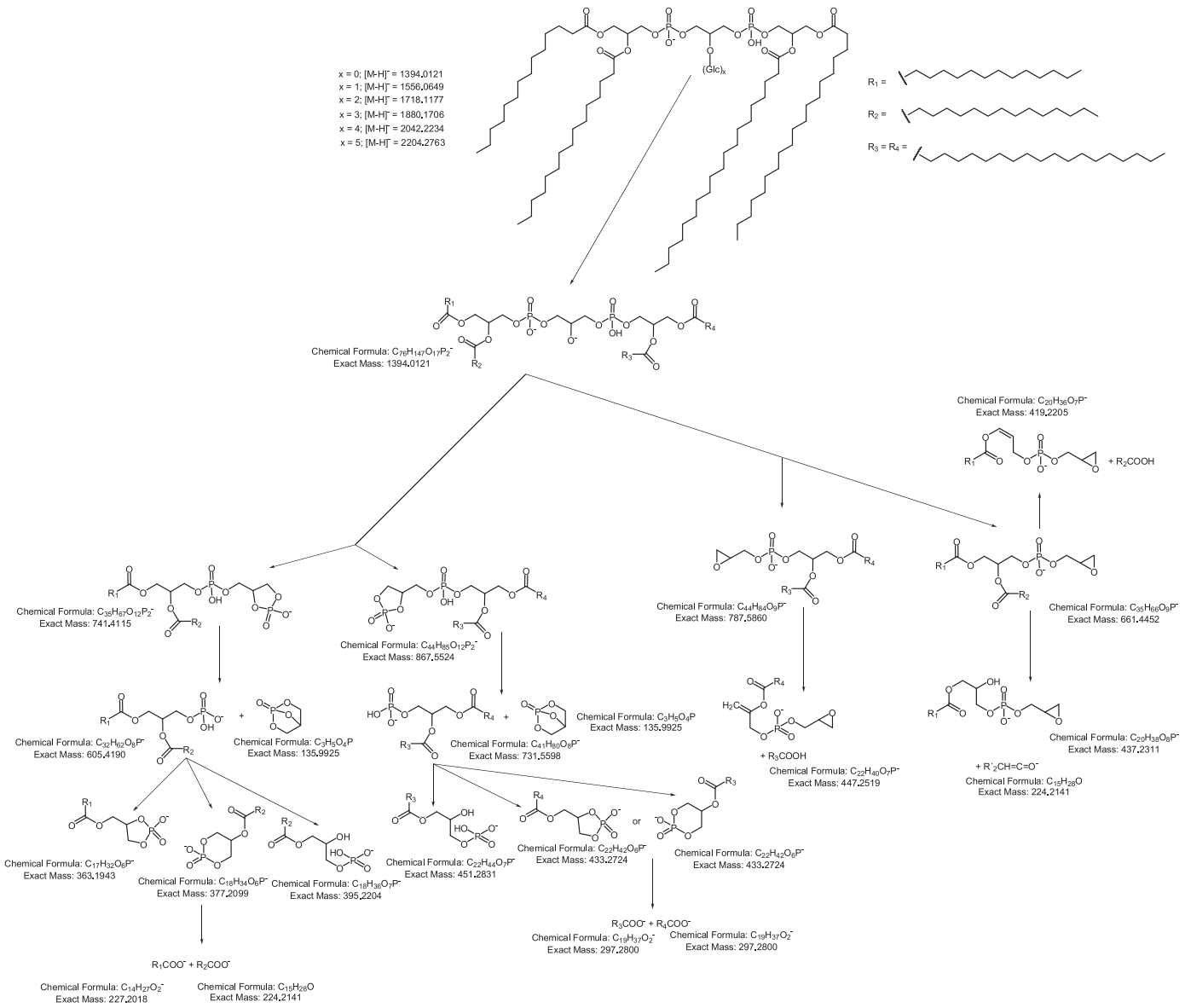

Fig. 4. Probable major fragmentation pathways of $(Glc)_5$-67:0-CL using high-resolution tandem mass spectrum by negative ESI.

methanolysis and silylation [20]. Multiple peaks derived from each monosaccharide can produce a characteristic pattern that further aids in identification by retention time. Two major peaks were identified whose retention times corresponded to standards of TMS α-methylglucoside and TMS β-methylglucoside were identified. The standards of TMS methylglycosides were prepared from commercially obtained methyl α-D-glucopyranoside and methyl β-D-glucopyranoside. In the area of molecular ions of positive chemical ionization mass spectrometry (PCI–MS), there were abundant ions of the type $[M+H]^+$ and the ammonium adduct ion $[M+NH_4]^+$. Other ions were formed by the gradual loss of neutral molecules, predominantly methanol and trimethylsilanol; see below. The ions at *m/z* 207 and at *m/z* 217, whose structure is similar to that of electron impact MS, were also found [31]. The base peak of both TMS methyl glycosides identified by PCI–MS was an ion of type $[M+H\text{-}CH_3OH\text{-}TMSiOH]^+$ at *m/z* 361. Further abundant ions were $[M+NH_4]^+$ at *m/z* 500, $[M+NH_4\text{-}CH_3OH]^+$ at *m/z* 468, $[M+NH_4\text{-}CH_3OH\text{-}TMSiOH]^+$ at *m/z* 378, $[M+NH_4\text{-}CH_3OH\text{-}2\times TMSiOH]^+$ at *m/z* 288, $[M+NH_4\text{-}CH_3OH\text{-}3\times TMSiOH]^+$ at *m/z* 198, etc., see also the paper Bleton et al. [31] GC–MS/PCI allowed confirming that the only one saccharide present in the substituted molecule $(Hex)_x$–CLs was glucose (Glc). To further elucidate and confirm the structure of $(Glc)_x$–CLs, total glucosylated CLs were separated by HILIC.

3.4. HILIC

Shotgun analysis confirmed that in very polar lipids, which were obtained after prepurification by Sep-Pak cartridge, molecular species containing up to five Glc units are present. Unfortunately, shotgun analysis does not allow linear and branched glycosidically bound glucosyl derivatives to be distinguished from each other. Therefore, total $(Glc)_x$–CLs were separated by HILIC. Fig. 5 shows the HILIC separation of the total $(Glc)_x$–CLs, identified as the neutral loss scan (NLS) of the 162.0528 Da. A total of 13 peaks were separated (Fig. 5). The structure of the peak marked as J in Fig. 5 could not be determined. Twelve peaks were identified as mono- to penta- glucosyl derivatives of CLs. The structure of these glycosides was determined based on tandem MS analysis of PMAAs and cleavage by glucosidases (Table S4). Separation of glycosides according to the number of glucoses was on the baseline, but separation of linear and branched chain oligosaccharides was not so successful.

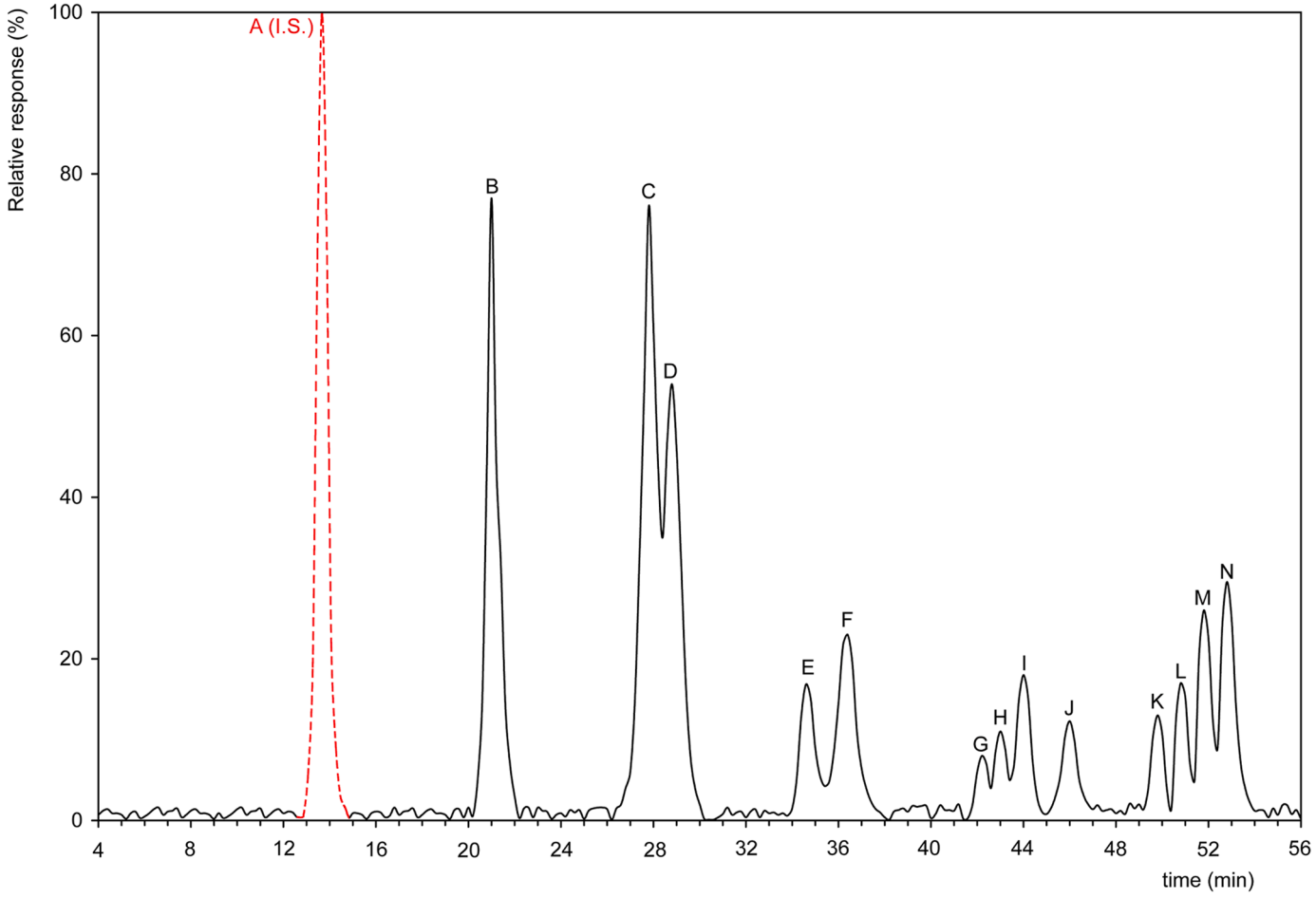

Fig. 5. Hydrophilic interaction liquid chromatography – HILIC/ESI–MS separation of the total $(Glc)_x$–CLs, identified as the neutral loss scan (NLS) of the 162.0528 Da by high-resolution negative ESI. A=internal standard $((16:0)_4$-CL, letters B, C, D, etc., see Table S4.

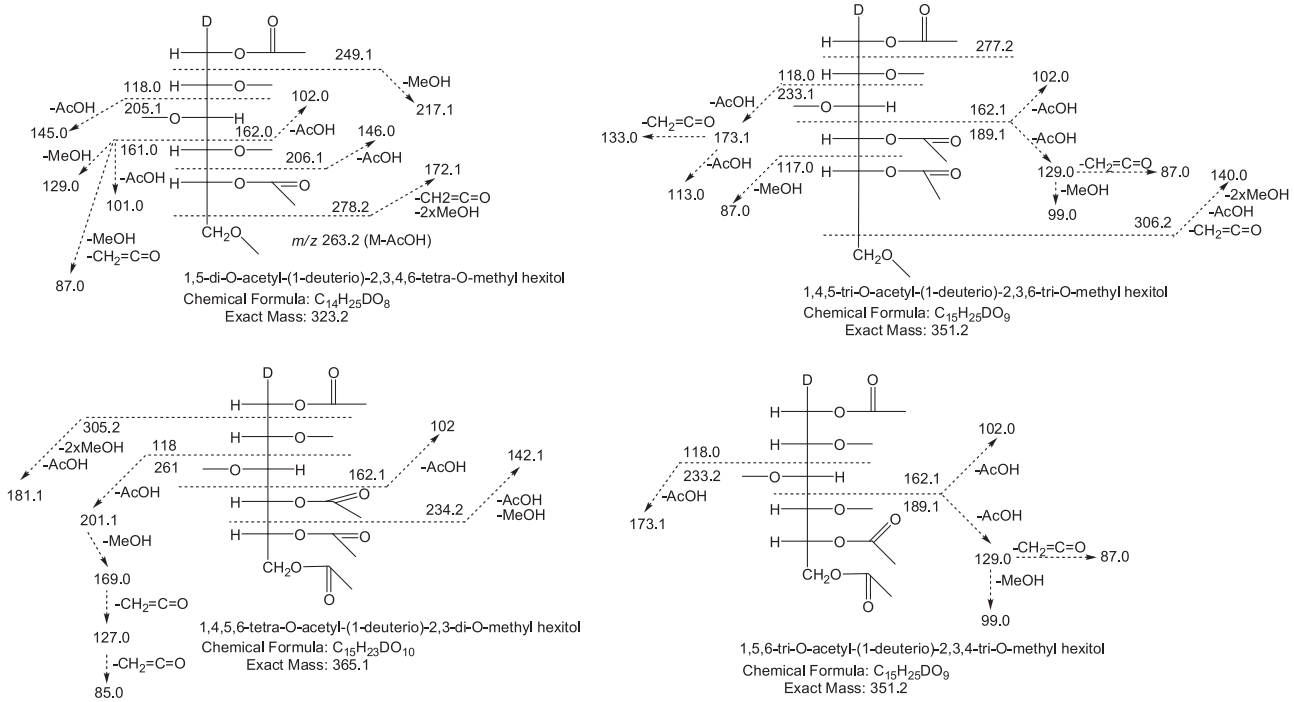

Fig. 6. Structure of PMAAs; description of fragmentation of partially methylated alditol acetates identified by GC–MS (electron impact ionization).

3.5. Structure of glycosidically bound oligosaccharides to cardiolipins

Individual peaks marked as **B**–**N**, see Fig. 5 were manually collected, and preparation of PMAAs was performed according to the methods described by Blakeney and Stone [32] (Fig. 6). The methylated CLs were hydrolyzed at 120 °C with 2 M trifluoroacetic acid for 90 min; the products were reduced with $NaBD_4$, and subsequently acetylated. The identification of PMAAs was based on GC–EI–MS (Table 1) and GC–PCI–MS.

The separation of all four PMAAs was performed on a polar capillary column, the tRs were sufficiently different, and the peaks were separated on the base line. Table 1 shows both the retention times (tR) and the majority of the ions from all four PMAAs. Furthermore, Table 2 contains the molar ratio of individual PMAAs. The full structures of the glycosides are shown in Table S4. However, it should be noted that, for example, peak B, i.e., mono–Glc–CLs, contains 21 molecular species that differ in the length of acyls bound to glycerol (Table S3). The mass spectra of the four PMAAs were very similar, if not identical, to the published mass spectra [33,34]. In any case, no molecular ion was identified, only fragment ions were found were found; see Fig. 6.

GC–PCI–MS analysis was also used to fully confirm the structures of the four PMAAs. In contrast to EI mass spectra, in which fragment ions are more abundant, ammonia PCI spectra of PMAAs are dominated by molecular weight-related ions, i.e., predominantly to an $[M+NH_4]^+$ ion and minor ions of the type $[M+NH_4\text{-}32]^+$ and or $[M+NH_4\text{-}60]^+$[20, 33,35]. From Fig. 5 it is clear that the peaks (**B**–**N**) after separation by HILIC yield the mixture of PMAAs. Analysis revealed that the glycosides contain a maximum of four PMAAs, i.e., of combination 1, 5-di-*O*-acetyl-(1-deuterio)− 2,3,4,6-tetra-*O*-methyl hexitol, 1,5,6-tri--*O*-acetyl-(1-deuterio)− 2,3,4-tri-*O*-methyl hexitol, 1,4,5-tri--*O*-acetyl-(1-deuterio)− 2,3,6-tri-*O*-methyl hexitol, and 1,4,5, 6-tetra-*O*-acetyl-(1-deuterio)− 2,3-di-*O*-methyl hexitol.

Enzymatic hydrolysis of the above mentioned glycosides (peaks **B**–**N**, excluding peak **J**) using four glucosidases confirmed the presence of only glucose units. Peak **B** was not hydrolyzed by any of the four glucosidases, and subsequent analysis by HR–ESI–MS would confirm the structure of this glycoside as Glc–CLs. Glycoside **C** was hydrolyzed only by α-1,6-glucosidases (i.e., both dextranase and pullulanase) to Glc and Glc–CLs.

The glycosides (peaks **D**, **F**, **I**, and **N**) were hydrolyzed by α-1,4-glucosidase to form Glc and Glc–CLs. Upon enzymatic hydrolysis with β-1,4-glucosidase and both α-1,6-glucosidase, no Glc was identified after the reaction. From these results, it can be concluded that Glc has α configuration and that the oligosaccharides are linear 1,4-glucans.

Glucose was released from glycosides **E**, **G**, **H**, **K**, **L**, and **M** by successive α-1,6-glucosidase hydrolysis, but by HR–ESI–MS it was found that glycosides with two or more Glc units were still present. Subsequent hydrolysis of α-1,4-glucosidase identified only Glc and Glc-CLs in the reaction mixture.

Enzymatic hydrolysis thus confirmed that α-D-glucose was present in the $(Hex)_x$-CLs molecules as a common saccharide. Furthermore, by combining HILIC, four hydrolases, and HR–ESI–MS, glycoside structure was determined, see Table S3. This is the first finding of new Glc-linked oligosaccharide chains in cardiolipins of thermophilic bacteria.

Therefore, the structure of $(Glc)_x$-CL was confirmed by four independent analyzes, that is, shotgun analysis (Table S3), HILIC separation based primarily on neutral loss of 162.0528 Da, enzyme cleavage using four glucosidases and GC–MS of PMAAs labeled with deuterium.

Table 1
Partially methylated alditol acetates obtained from the permethylated $(Glc)_x$-CLs.

Methylated alditols	tR	Diagnostic ions (*m/z*) (relative abundance in %)
1,5-di-O-acetyl-(1-deuterio)− 2,3,4,6-tetra-O-methyl hexitol	9.49	87 (46); 101 (43);102 (100); 118 (81); 129 (77); 145 (72); 146 (69);161 (17); 162 (14); 172 (9); 205 (13); 206 (14); 217 (5); 249 (3); 263 (12)
1,5,6-tri-O-acetyl-(1-deuterio)− 2,3,4-tri-O-methyl hexitol	15.83	87 (61); 99 (57); 102 (100);118 (92); 129 (73); 162 (6); 173 (13); 189 (5); 233 (17)
1,4,5-tri-O-acetyl-(1-deuterio)− 2,3,6-tri-O-methyl hexitol	16.81	87 (49); 99 (68); 102 (54); 113 (11); 117 (88); 118 (100); 129 (19); 131 (15); 140 (9); 162 (6); 173 (13); 189 (13); 233 (17); 277 (3)
1,4,5,6-tetra-O-acetyl-(1-deuterio)− 2,3-di-O-methyl hexitol	23.02	85 (63); 102 (39); 118 (100); 127 (42); 142 (5); 162 (6); 169 (8); 181 (16); 201 (11); 217 (3); 234 (8); 261 (10); 305 (2)

Table 2
Partially methylated alditol acetates obtained from the permethylated $(Glc)_x$-CLs (molar %).

Peak	1,5-di-O-Ac-2,3,4,6-tetra-Me-Glc[a]	1,5,6-tri-O-Ac-2,3,4-tri-Me-Glc[b]	1,4,5-tri-O-Ac-2,3,6-tri-Me-Glc[c]	1,4,5,6-tetra-O-Ac-2,3-di-Me-Glc[d]
B	1.1	-	-	-
C	1.1	0.9	-	-
D	1.0	-	0.9	-
E	1.9	-	-	0.9
F	1.1	-	1.9	-
G	2.1	-	1.0	1.0
H	2.1	-	1.0	0.9
I	0.9	-	3.1	-
K	1.9	0.9	0.9	1.1
L	2.1	-	1.9	1.1
M	2.1	-	2.0	1.1
N	1.0	-	4.3	-

[a] 1,5-di-O-Ac-2,3,4,6-Me_4-Glc is 1,5-di-O-acetyl-(1-deuterio)− 2,3,4,6-tetra-O-methyl hexitol
[b] 1,5,6-tri-O-Ac-2,3,4-Me_3-Glc is 1,5,6-tri-O-acetyl-(1-deuterio)− 2,3,4-tri-O-methyl hexitol
[c] 1,4,5-tri-O-Ac-2,3,6-Me_3-Glc is 1,4,5-tri-O-acetyl-(1-deuterio)− 2,3,6-tri-O-methyl hexitol
[d] 1,4,5,6-tetra-O-Ac-2,3-Me_2-Glc is 1,4,5,6-tetra-O-acetyl-(1-deuterio)− 2,3-di-O-methyl hexitol

4. Conclusions

The use of two-stage extraction, i.e., according to Bligh and Dyer [13] and Markham and Jaworski [14] (lower phase of isopropanol/hexane/water (55:20:25 v/v/v)) combined with several analytical techniques, such as shotgun HR–ESI–MS, GC–MS (both EI and PCI) of partially methylated alditol acetates, HILIC separation and identification of CL glycosides by high-resolution MS–ESI, and digestion of specific glycosidases made it possible to identify dozens of molecular species of cardiolipin glycosides. For the first time, it was possible to identify substituted CLs in thermophilic bacteria that have more than one glucose bound glycosidically to the central hydroxyl of cardiolipins. In total, CLs with up to five glucosyl units were identified in eight strains of thermophilic bacteria. Thus, these combinations of extraction and analysis open up the possibility of analysis and identification of highly polar lipids, which $(Glc)_x$-CLs are undoubtedly.

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CRediT authorship contribution statement

Lucie Kyselová: Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing, analysis. **Tomáš Řezanka**: Conceptualization, Funding acquisition, Supervision,

Writing – original draft, Writing – review & editing, analysis. Both authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115800.

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