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Polar lipids of a non-alkaliphilic extremely halophilic archaebacterium strain 172: a novel bis-sulfated glycolipid ☆

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Abstract

Extremely halophilic archaebacteria which require high salt concentrations for growth and survival contain glycerol diether analogues of phospholipids and sulfated glycolipids as major membrane polar lipids. A non-alkaliphilic, non-pigmented rod-shaped extreme halophile, isolated from sea sand in Japan and designated 'strain 172', was found to contain two phospholipids, phosphatidylglycerol (PG) and phosphatidylglyceromethylphosphate (PGP-Me), derived from both C_{20}-C_{20}- and C_{20}-C_{25}-glycerol diethers, and a novel major glycolipid (designated SGL-X). This glycolipid has been identified as a bis-sulfated diglycosyl C_{20}-C_{20}- or C_{20}-C_{25}-glycerol diether, on the basis of its TLC mobility, positive-staining behavior with sugar and sulfate-staining reagents, its mole ratio sulfate/glycolipid = 2.2, and by spectrometric analysis (IR and FAB-MS) of the intact and the desulfated SGL-X. The sugars were identified as mannose and glucose, after acid hydrolysis of SGL-X, by paper chromatography of the free sugars and GC-MS of the derivatized sugars (alditol acetates). Permethylation analysis and ^{1}H- and ^{13}C-NMR analysis established the position and configuration of the sugar linkages and the positions of the sulfate groups. The final structure of SGL-X (now designated S_2-DGD-1) is proposed to be: 2,3-diphytanyl- or phytanyl-sesterterpenyl-1-[2,6-$(HSO_3)_2$-α-Man*p*-1 → 2-Glc*p*]-*sn*-glycerol. This lipid is the first bis-sulfated glycolipid to be reported in extremely halophilic archaebacteria, and is the first in the biosphere that possesses two sulfate groups attached to the same monosaccaride.

Key words: Diphytanylglycerol diether analogue; C_{20}-C_{25}-glycerol diether analogue; Phosphatidylglyceromethylphosphate; Glycolipid bis-sulfate; Spectroscopy; FAB-MS

Abbreviations: CI-MS, chemical ionization mass spectrometry; DGD, diglycosylglyceroldiether; ECNSS-M, ethyleneglycol succinate polyester-cyanoethyl silicone polymer; FAB-MS, fast atom bombardment spectrometry; IR, infrared spectroscopy; MGD, monoglycosyldiether; NMR, nuclear magnetic resonance spectroscopy; NPGS, neopentylglycol succinate; PG, phosphatidylglycerol (diether analogue); PGP-Me, phosphatidylglycerophosphate methyl ester (diether analogue); PGS, phosphatidylglycerosulfate (diether analogue); S-DGD, monosulfated diglycosylglyceroldiether; S-DGD-1, monosulfated diglycosylglyceroldiether from *Hf. mediterranei* (6-HSO_3-Man*p*-α1 → 2-Glc*p*-α1 → 1-glyceroldiether); SGL-X or S_2-DGD-1, bis-sulfated diglycosylglyceroldiether from strain 172; SIMS, secondary ion mass spectrometry; S-TGD-1, sulfated TGD-1 (3-HSO_3-Gal*p*-β1 → 6-Man*p*-α1 → 2-Glc*p*-α1 → 1-glyceroldiether); S-TeGD, sulfated tetraglycosylglyceroldiether (3-HSO_3-Gal*p*-β1–6-Man*p*-(α1–3-Gal*f*)-α1–2-Glc*p*-α1 → 1-glyceroldiether); TGD-1, triglycosylglyceroldiether (Gal*p*-β1–6-Man*p*-α1 → 2-Glc*p*-α1 → 1-glyceroldietherl); TGD-2, triglycosylglyceroldiether [Glc*p*-β1 → 6-Man*p*-α1 → 2-Glc*p*-α1 → 1-glyceroldiether); TMS, trimethylsilyl.

☆ This paper is dedicated to the memory of our co-author, Dr. Toshiko Matsubara, who passed away on December 21, 1992.

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1. Introduction

Red pigmented extremely halophilic archaebacteria of the genera *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium* and *Natronococcus* contain polar lipids which are derived exclusively from a glycerol diether lipid core, 2,3-di-*O*-phytanyl-*sn*-glycerol [1,2] (C_{20}-C_{20}-diether). Strains belonging to these genera contain two main phospholipids characteristic of extreme halophiles and haloalkaliphiles [1,3]: glyceroldiether analogues of phosphatidylglycerolmethylphosphate (PGP-Me) [4] and phosphatidylglycerol (PG); and genus-specific glycolipids and/or sulfated glycolipids [1,3,5]. The structures of seven genus-specific glycolipids have so far been elucidated [1,3,5]. However, strains of the genera *Natronobacterium* and *Natronococcus* (red alkaliphilic extreme halophiles growing optimally at pH 9–10 [6]) possess two other core diether lipids, 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol(C_{20}-C_{25}-diether) [7,8] and 2,3-di-*O*-sesterterpanyl-*sn*-glycerol (C_{25}-C_{25}-diether) [9], in addition to the C_{20}-C_{20}-diether core lipid. Subsequent analyses by Tindall et al. [10] revealed that several strains of *Halococcus morrhuae* (halophilic cocci) also contain the C_{20}-C_{25} diether core lipid, and this has also been found for *Halococcus saccharolyticus* and other halococci from salt flats in Spain [11], and in *Halobacterium*, *Haloferax*, and *Natronobacterium* strains from hypersaline environments in India [12].

Onishi et al. [13] have isolated a non-alkaliphilic, non-pigmented, rod-shaped extreme halophile (strain 172) from sea sand in Japan, and found that this strain had the C_{20}-C_{25}-diether core lipid as well as the C_{20}-C_{20}-diether core [14]. These authors also observed the presence of an unidentified glycolipid (SGL-X; designated S-DGD-2 [1]) with low TLC mobilities [14].

In the studies reported here, we have examined the polar lipids of strain 172 in detail and have determined the complete chemical structure of the isolated unknown glycolipid. This glycolipid turns out to be a bis-sulfated diglycosyl archaeol, a structure not previously encountered in halophilic archaebacteria.

2. Materials and methods

2.1. Organism and culture conditions

An unidentified extreme halophile, designated strain 172 [13,14], was grown aerobically at 38°C for 3 days in 1 l of the Sehgal and Gibbons complex medium [3], containing 4 M NaCl, in a 3 l Erlenmeyer flask. The cells were harvested by centrifugation in the late exponential growth phase and washed with a 4.3 M NaCl solution containing 0.1 M $MgCl_2$. *Halobacterium cutirubrum* (NRC 34001) and *Haloferax mediterranei* (strain R4, ATCC 33500) were also cultured in the same way [3,4].

2.2. Extraction and purification of polar lipids

Total lipids were extracted from the washed cells by the method of Bligh and Dyer [15], as modified for extreme halophiles [16]. The total lipids were fractionated by chromatography on silicic acid columns eluted with chloroform (neutral lipid fraction), acetone (glycolipid fraction), and methanol (phospholipid fraction) [16]; final isolation and purification of individual polar lipids was by preparative thin-layer chromatography (TLC) in the solvent system B (given below) [11,16]. Phospholipids (PGP-Me and PG) and the glycolipid sulfate (SGL-X) were converted to their ammonium salt forms as described elsewhere [11,16]. The standard 6-HSO_3-Man*p*-α1 → 2-Glc*p*-α-1 → 1-glyceroldiether (S-DGD-1) was isolated from *Haloferax mediterranei* as described elsewhere [11].

2.3. Thin-layer chromatography

Analytical TLC of polar lipids was performed on silica-gel G plates (Brinkmann SIL G-25 or Baker Si 250, 0.25 mm layers) in the following solvent systems: A, chloroform/methanol/acetic acid/water (85:22.5:10:4, v/v; double development); B, chloroform/methanol/acetic acid/water (90:15:54:6, v/v); C, chloroform/methanol/water (65:25:4, v/v); permethylated lipids were run in solvents: D, chloroform/methanol (95:5, v/v), or E, petroleum ether/ethyl ether (5:20, v/v); glyceroldiethers were run in solvent F, petroleum ether/ethyl ether/glacial acetic acid (70:30:1, v/v). Preparative chromatography was carried out in the same way using either preparative plates (0.75–1.0 mm thick layers) or analytical plates (0.25 mm layers).

Lipids were detected with the following spray reagents [16]: (1) ammonium molybdate/sulfuric acid for phospholipids; (2) 0.5% orcinol/sulfuric acid, or 0.5% α-naphthol/sulfuric acid for glycolipids; azure A/sulfuric acid for sulfatides including sulfoglycolipids [17,18]; and (3) sulfuric acid/ethanol (1:1, v/v) followed by charring at 150°C for detection of all lipids.

2.4. Quantitative analysis

Phosphorus was determined by the modified method of Bartlett [16] and total hexose was estimated by a modified phenol-sulfuric acid method using a mixture of glucose, galactose and mannose (1:1:1, w/w) as a standard [16]. Quantitative analysis of total sulfate in the glycolipid sulfate (SGL-X) was performed colorimetrically [17,18] or by densitometry at 620 nm on TLC plates with the azure A method [18,19]. Absorbance of

the color on TLC plates was linear with the amount of standard galactosylceramide sulfate in the range 0.5 to 4.0 nmol.

2.5. Chemical procedures

Lipids were subjected to acid methanolysis in methanolic 2.5% HCl (0.69 N) under reflux at 80–90°C for 2 h in a screw-cap (Teflon-lined) tube [16,20]. After addition of 10% water, the mixture was extracted with petroleum ether (b.p. 40–60°C) or hexane, and the extracts were subjected to chemical ionization mass spectrometry (CI-MS) to identify the diether core lipids. Sugar methyl glycosides in the methanol-water phase were converted to trimethylsilyl (TMS) ethers with pyridine/hexamethylene-disilazane/trimethylchlorosilane (10:2:1, v/v) at 60°C for 5 min, and analyzed by gas chromatography [21].

Time-course studies of partial methanolysis of SGL-X were done in chloroform/methanol/12 M HCl (1:1:0.02, v/v; final HCl concentration, 0.12 N) at 25°C or in 0.052 M methanolic-HCl at 23°C. At intervals of 30 min samples were taken and checked by TLC in solvent A or B for partial hydrolysis products. Hydrolysis of SGL-X into two partially degraded glycolipid products (monodesulfated SGL-X (S-DGD) and bis-desulfated SGL-X (DGD)) was observed on TLC after 1 h, and after 24–30 h, a third product appeared (monoglycosylarchaeol (MGD)). For isolation of these products on a preparative scale, SGL-X was hydrolysed in 0.0068 M methanolic-HCl overnight at room temperature, in 0.025 M methanolic-HCl overnight at room temperature, or in 0.1 M methanolic-HCl for 5 h at 100°C to obtain S-DGD, DGD or MGD, respectively. These products were isolated in pure form by preparative TLC of the partial hydrolysates in solvent C or by chromatography on an Iatrobeads column [22], using an eluting gradient solvent system of chloroform/methanol/water from 75:35:1.5 (v/v) to 50:50:5 (v/v).

Permethylation of the native and the partially degraded glycolipids was performed as described by Kushwaha et al. [20] or Hakomori [23]. Intact permethylated glycolipids, after final purification by preparative TLC in solvent D or E, were first analyzed by FAB-MS, then methanolysed in 0.7 M methanolic HCl as described above to form the partially methylated sugar methylglycosides. The latter were analyzed as such [21], or after conversion to partially methylated alditol acetates, by GC and/or GC-MS [23].

2.6. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

GC and GC-MS analyses of TMS-methylglycosides were performed on a 2 m glass column packed with 3% SE-30 at 170°C [21]. Partially methylated methylglycosides and partially methylated alditol acetates were analyzed on a 3 m column of 3% NPGS at 160°C and a 1 m column of 3% ECNSS-M at 160°C, respectively [20,23]. The GC-mass spectra were measured at 70 eV.

2.7. Fast-atom bombardment mass spectrometry (FAB-MS)

FAB-MS analyses of the intact or permethylated glycolipids were carried out using a JEOL JMS HX-100 double focus mass spectrometer equipped with a FAB xenon gun operated at an accelerating potential of 6 kV. Samples dissolved in chloroform/methanol (2:1, v/v) were mixed with matrix: triethanolamine alone for negative ion FAB-MS, and a mixture of triethanolamine, tetramethylurea and NaI for positive ion FAB-MS. The mass spectrometer was operated at 5 kV accelerating voltage and a post accelerating type detector was used for detection of positive and negative ions. The conversion dinode was given a potential of −15 kV or +15 kV for positive and negative FAB, respectively. Spectra were acquired with a JEOL DA 5000 data system. Some negative SIMS spectra were obtained with a Kratos Concept IIH mass spectrometer fitted with a cesium gun ion source operated at an accelerating voltage of −6kV with a potential of +4kV applied to the gun to form the Cs^+ beam. Samples dissolved in ethylene dichloride were evaporated onto 2–3 μl of glycerol containing 1–2% of thioglycerol as carrier. Spectra were acquired with a SUN data system.

Chemical ionization mass spectrometry (CI-MS) was performed on a VG 7070E (Vacuum Generators) mass spectrometer using a 6 kV chemical ionization accelerating voltage and diethyl ether as the chemical ionization element. Spectra were acquired with a DEC, PDP8 data system.

2.8. Spectroscopic analyses (IR, ^{1}H- and ^{13}C-NMR)

IR spectra were taken on approx. 200 nmol of SGL-X or S-DGD-1 from *Hf. mediterranei*, mixed with KBr, using an A-302 infrared spectrophotometer (Japan Spectroscopic, Tokyo).

^{1}H- and ^{13}C-NMR-spectra of the native glycolipid (SGL-X), the S-DGD-1 from *Hf. mediterranei* and the desulfated glycolipid (DGD), after deuterium exchange, were taken in $[^2H]Me_2SO/[^2H]_2O$ (98:2, v/v; final glycolipid concentration, ca. 20 mM) on a JEOL GX-400 spectrometer at 400 MHz and 100 MHz, respectively. Double-quantum-filtered COSY (DQF-COSY) and ^{1}H-^{13}C heteronuclear COSY (^{1}H-^{13}C COSY) spectra were measured in the phase-sensitive mode. ^{13}C-NMR spectra were obtained under continuous proton broad-band decoupling conditions. Further experimental details are given in the figure legends.

3. Results

3.1. Lipid composition

Growth of strain 172 under the conditions described above yielded 3.0 g of wet cell pellet, or 320 mg of cell protein per liter of culture. Total lipid content was about 8.5% on a cell protein basis. Analytical TLC in solvent A of the total lipids showed the presence of two major phospholipids (PGP-Me and PG), one major glycolipid (S-GLX) and one minor glycolipid (GLY) (Fig. 1A). The TLC mobilities in solvent A of the two phospholipids (R_f 0.68 and 0.92, respectively) corresponded closely with those of the diether analogues of phosphatidylglyceromethylphosphate (PGP-Me) [4] and phosphatidylglycerol (PG) (reference phospholipids from *Hb. cutirubrum* [1,3,4]). The main glycolipid (S-GLX) gave a positive test both with the sugar stain (orcinol or α-naphthol reagent) and the sulfate stain (azure A reagent); it had a mobility on TLC in solvent A (R_f 0.18) between that of the sulfated triglycosylglyceroldiether (S-TGD-1; R_f 0.29) and the sulfated tetraglycosylglyceroldiether (S-TeGD; R_f 0.12) (Fig. 1A and B), and much lower than that of the S-DGD-1 from *Hf. mediterranei* [3,20]. The major glycolipid component thus appeared to be a highly polar sulfated glycolipid with unusual structure and was designated SGL-X. The minor glycolipid (GLY, R_f 0.63) was not further studied because of insufficient material.

PGP-Me, PG, and SGL-X were purified by preparative TLC and subjected to methanolysis as described in Section 2. The petroleum ether-soluble (hydrophobic) methanolysis products of each polar lipid were identified as glyceroldiethers by TLC in solvent F [11]. CI-MS spectra of these products showed two molecular ion peaks ($[M + H]^+$) at m/z 653 (major) and 723 (minor), corresponding to C_{20}-C_{20}- (M = 652) and C_{20}-C_{25}-(M = 722) diethers, respectively, consistent with the difference of 70 mass units (C_5 fragment) in the structures of these diethers. Thus, each of the major polar lipid components were derived from both C_{20}-C_{20}- and C_{20}-C_{25}-lipid cores, in different ratios, in contrast to the lipids of *Hb. cutirubrum* which contained only the C_{20}-C_{20}-diether.

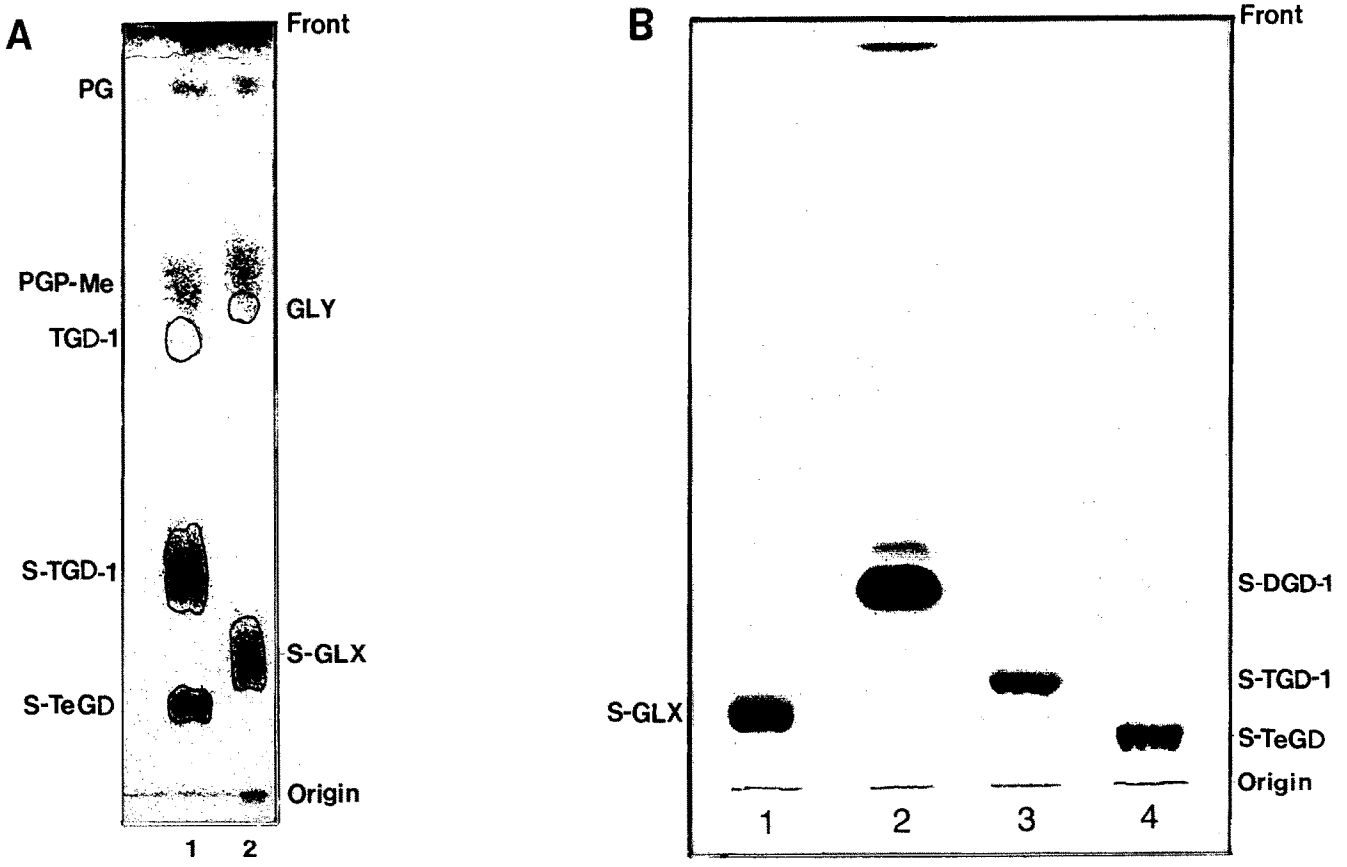

Fig. 1. TLC plate of: A, total polar lipids of 1, *Hb. cutirubrum* and 2, strain 172; and B, isolated glycolipids: 1, SGL-X from strain 172; 2, S-DGD-1 from *Hf. mediterranei*; 3, S-TGD-1 and 4, S-TeGD from *Hb. cutirubrum*. Solvent A, chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, v/v, double development). The chromatograms were stained with α-naphthol/sulfuric acid (circled spots are sugar positive) and charred. PG, phosphatidylglycerol; PGP-Me, phosphatidylglyceromethylphosphate; TGD-1, triglycosyl diether; S-TGD-1, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether; S-GLX, major glycolipid; GLY, minor glycolipid.

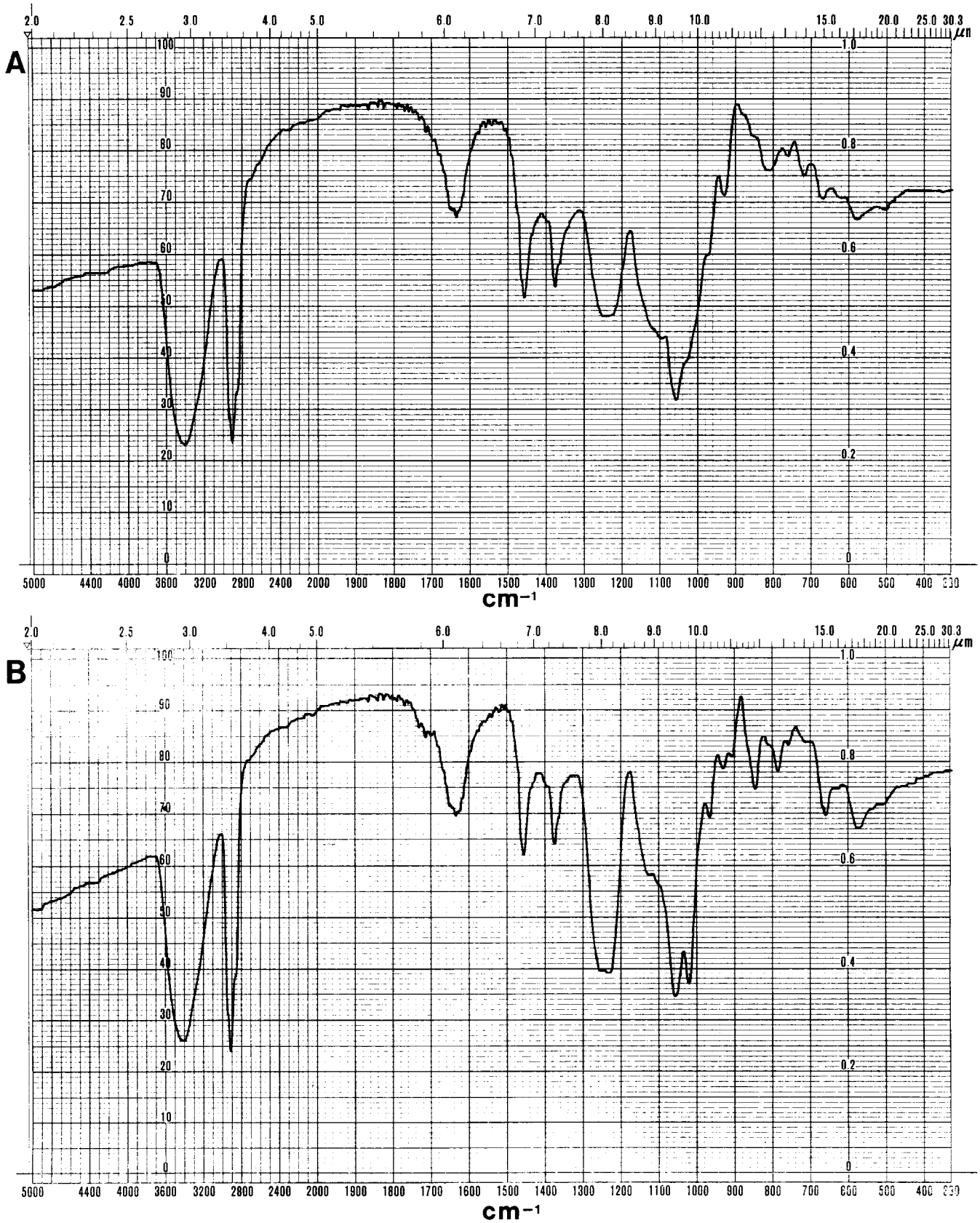

Fig. 2. Infrared spectra in KBr of: A, standard S-DGD-1 (ammonium salt) of *Hf. mediterranei*; and B, SGL-X (ammonium salt).

3.2. Structure determination of SGL-X

The purified SGL-X (Fig. 1B) had a sulfate content of 2.27 mol sulfate/mol glycolipid, as compared to a value of 0.93 mol sulfate/mol glycolipid for the S-DGD-1 of *Hf. mediterranei*. Hexose analysis by GC and GC-MS showed the presence of glucose and mannose in 1:1 mol ratio. Thus SGL-X appeared to be a bis-sulfated diglycosyl glyceroldiether (previously designated S-DGD-2 [1], but now designated as S_2-DGD-1).

The IR spectrum of SGL-X (Fig. 2B) resembled that of S-DGD-1 (Fig. 2A) except for the following diagnostic differences: (1) the S = O (asymmetrical stretch) absorption intensity at 1230–1260 cm^{-1} is about twice that of S-DGD-1, indicating the presence of at least two sulfate groups in S-GLX (see Ref. [24]); (2) the C-**O-S** absorption at 843 cm^{-1}, indicative of a secondary sulfate ester group at an axial position in the hexopyranose ring [25,26] (Fig. 2B), compared to the absence of this band in the spectrum of the standard S-DGD-1 (6-HSO_3-Man*p*-α1 → 2-Glc*p*-α1 → 1-glyceroldiether); the latter shows instead a band at 810 cm^{-1} for the primary sulfate at the equatorial C-6 of mannose [25,26] (Fig. 2A); absorption attributable to a sulfate group at the equatorial C-6 of a hexopyranose may also be present in the spectrum of S-GLX, but at a lower frequency (790 cm^{-1}) perhaps due to interaction of the two sulfate groups [25]; and (3) the presence of C-**O-S** bands for both primary and secondary sulfates at 1020 and 960 cm^{-1}, respectively [16,27] in SGL-X

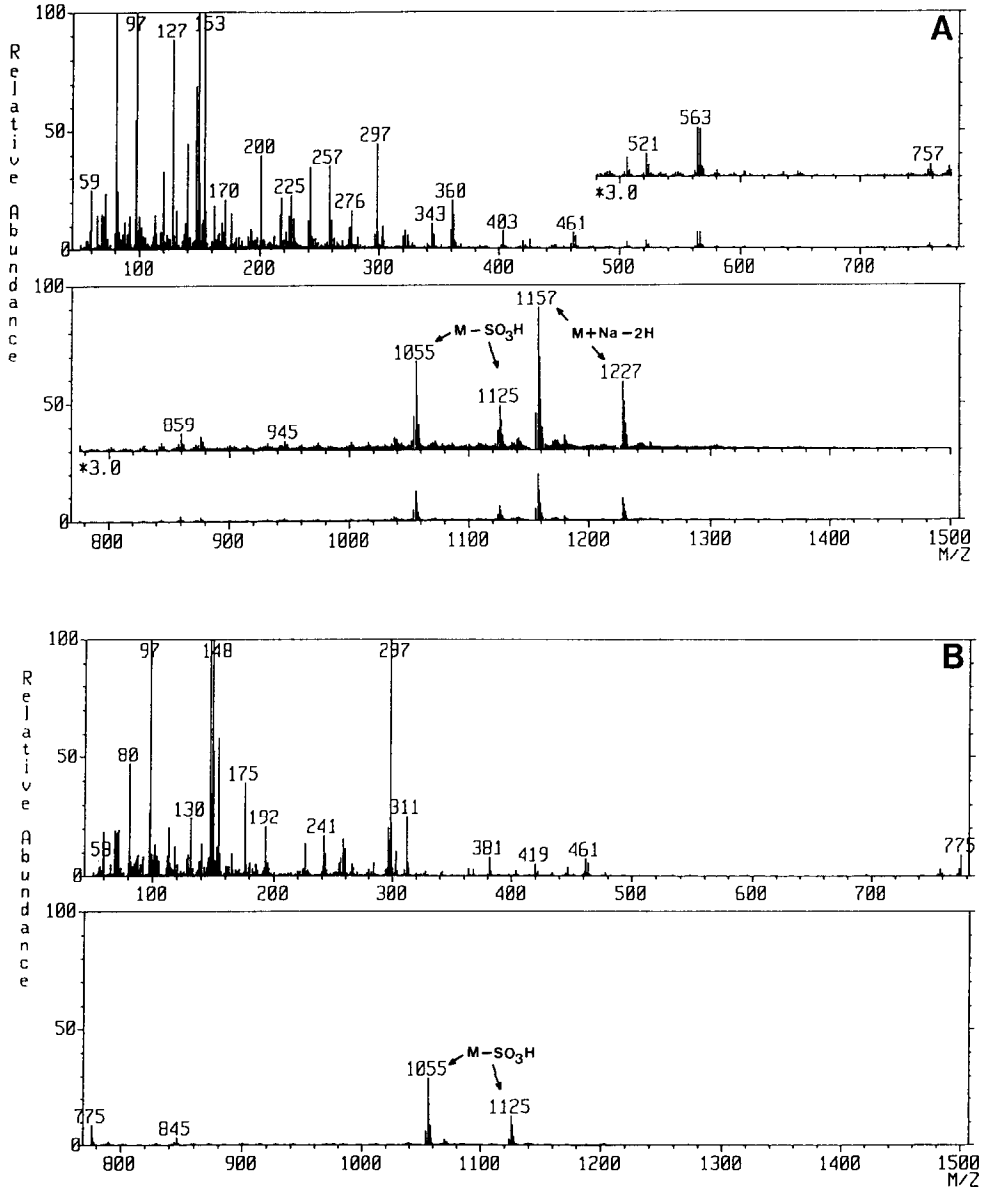

Fig. 3. Negative FAB-MS spectra of: A, intact SGL-X (NH_4 salt); B, monodesulfated SGL-X (S-DGD); C, desulfated SGL-X (DGD); D, MGD; and E, permethylated SGL-X.

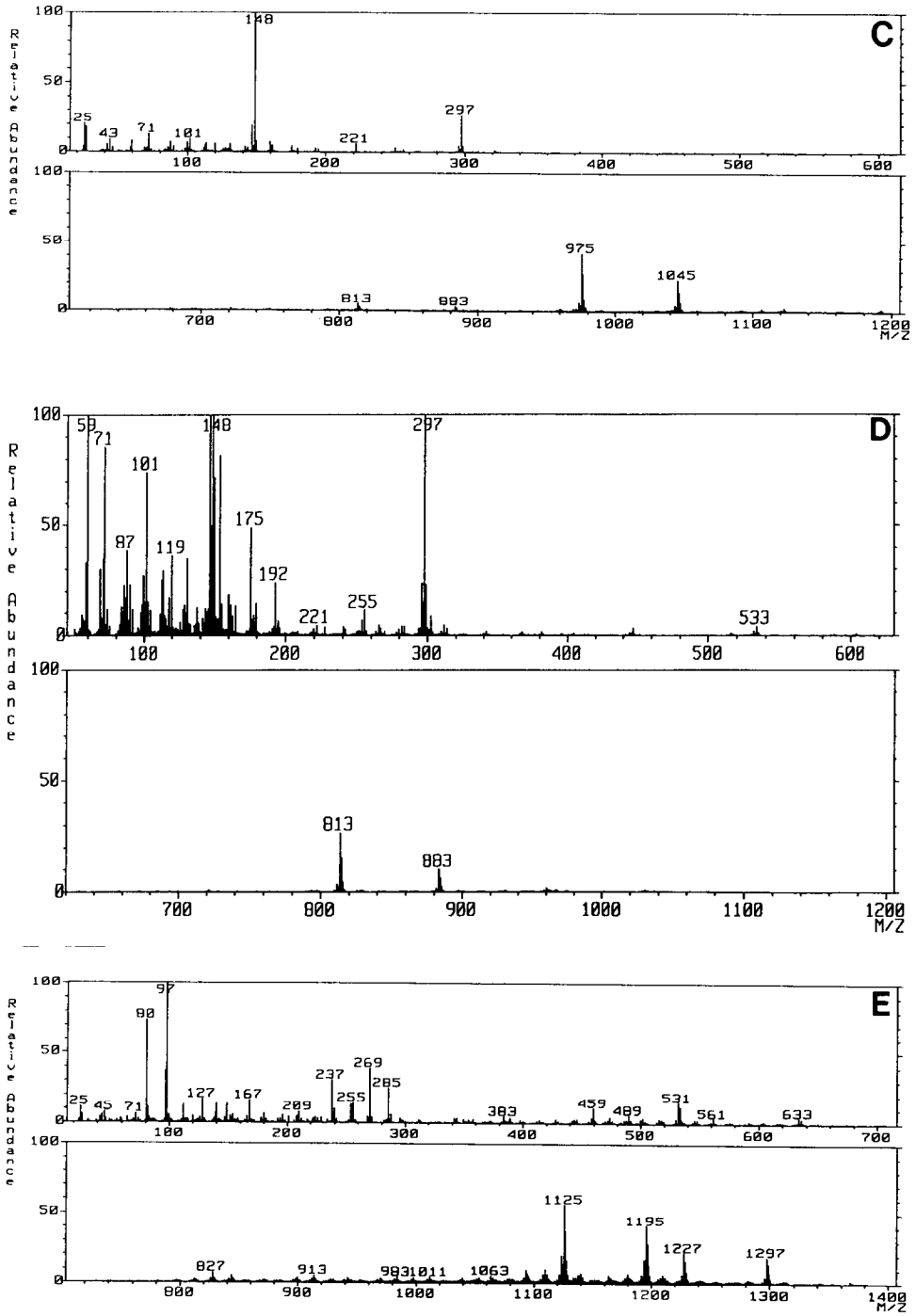

Fig. 3 (continued).

but only the primary sulfate band at 1020 cm^{-1}(sh) was present in the standard S-DGD-1 (Fig. 2A and B). Both S-GLX and S-DGD-1 showed strong S = O (symmetrical stretch) absorption (+alcoholic C-O absorption) at 1060 cm^{-1} [16].

The IR spectrum thus confirms the presence of two sulfate groups in SGL-X (S_2-DGD) and indicates that one sulfate may be located on a primary (equatorial) hydroxyl, the other on a secondary (axial) hydroxyl.

The negative FAB-MS spectrum of the intact SGL-X (S_2-DGD)(Fig. 3A) showed major parent ion peaks at m/z 1157 and 1227 ($[M + Na - 2H]^-$) corresponding to the deprotonated monosodium salts of the C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of a bis-sulfated diglycosyl glyceroldiether ($C_{55}H_{108}O_{19}S_2$, M = 1136; and $C_{60}H_{118}O_{19}S_2$, M = 1206, respectively). Less intense ion peaks at m/z 1055 and 1125 ($[M-SO_3-H]^-$) corresponded to the deprotonated free acid forms of

C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of a monosulfated diglycosyl glyceroldiether ($C_{55}H_{108}O_{16}S$, M = 1056; and $C_{60}H_{118}O_{16}S$, M = 1126, respectively). These spectra are similar to those of bis-sulfated glycolipids obtained previously by negative SIMS [28]. The presence of ion peaks corresponding to monodesulfated SGL-X ($[M-SO_3-H]^-$) was probably due to the high lability of the sulfate group at C-2 of the mannose residue, as will be discussed below. In contrast, the sulfate group at C-6 of mannose is much more stable as shown by the fact that the negative FAB-MS spectrum of the reference S-DGA-1 from *Hf. mediterranei* gave a single parent ion peak at 1055 (data not shown). The FAB-MS data are thus consistent with a bis-sulfated diglycosyl glyceroldiether structure.

In other analyses (data not shown), additional diagnostic ion peaks were observed at m/z 975 and 1045, corresponding to the C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of the desulfated SGL-X (DGD: M = 976 and 1046, respectively), and at m/z 813 and 883 corresponding to further loss of a hexose group to form the monoglycosyl glyceroldiether molecular species (MGD: M = 814 and 884, respectively).

Further evidence supporting the bis-sulfated glycolipid structure was obtained by mild acid hydrolysis of SGL-X as described in the Section 2. The first partial hydrolysis product that was formed stained positively for sugar and sulfate and had a TLC mobility (R_f, 0.35 in solvent A) corresponding to that of the standard monosulfated diglycosyl glyceroldiether (S-DGD-1) from *Hf. mediterranei* [1,20]. This observation was consistent with the loss of a labile sulfate group (SO_3^-, M = 80; $NaSO_3$, M = 103) or of a tetrose unit (M = 102) from SGL-X. The latter possibility was eliminated when no free tetrose was detected in the hydrolysate. A sulfate-free glycolipid was also formed in this hydrolysate, with TLC mobility (R_f, 0.74 in solvent A; 0.69 in solvent C), similar to that of the diglycosyl diether (DGD-1) from *Hf. mediterranei* [1,20]. This was indicative of the loss of the second sulfate group from SGL-X. After further hydrolysis (up to 5 h) in 0.1 M methanolic HCl at 100°C, a fast moving glycolipid corresponding to a monoglycosyl diether (MGD; R_f, 0.91 in solvent A; 0.88 in solvent C) was formed.

All of these products were isolated by preparative TLC and subjected to negative FAB-MS. The spectrum of the first partial hydrolysis product (S-DGD) showed two main $[M-H]^-$ ion peaks at m/z 1055 and 1125 (Fig. 3B) (calc. for the free acid form of C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of S-DGD: M = 1056 and 1126, respectively); the second product (DGD, desulfated SGL-X) showed two main $[M-H]^-$ ion peaks at m/z 975 and 1045 (calc. for C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of DGD, M = 976 and 1046, respectively) (Fig. 3C); and the third product, MGD, presumably formed by release of the terminal hexose in DGD, showed $[M-H]^-$ ions at m/z 813 and 883 (calc. for C_{20}-C_{20}- and C_{20}-C_{25}-molecular species of MGD, M = 814 and 884, respectively) (Fig. 3D).

Analysis of the hexose components of the partial hydrolysis products showed that the MGD product contained glucose as the sole sugar, while the DGD

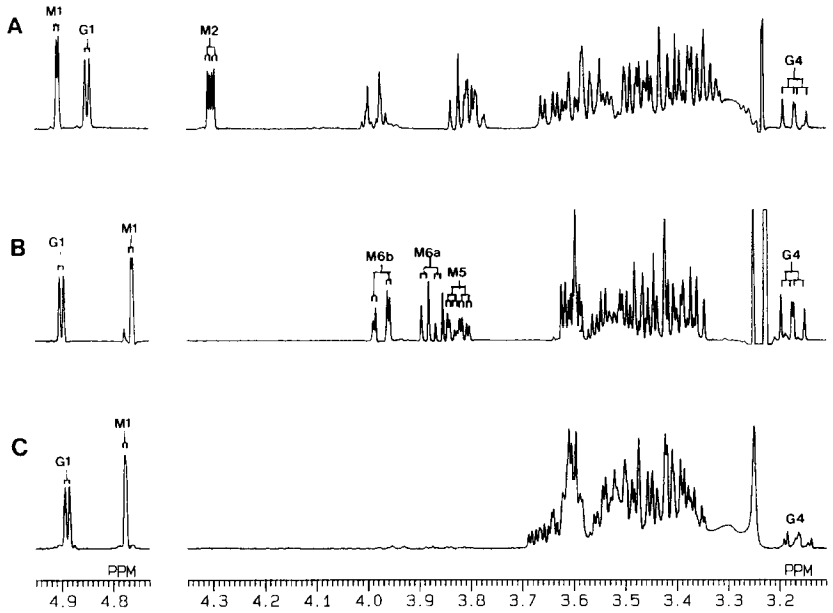

Fig. 4. ^{1}H-NMR spectra of sugar residues in: A, SGL-X (S_2-DGD); B, S-DGD-1 from *Hf. mediterannei*; C, desulfated SGL-X (DGD), in perdeuterated dimethyl sulfoxide/perdeuterated water (98:2, v/v). The operating conditions were: frequency, 400 MHz; sweep width, 4 kHz; sample points, 65 000; temperature, 55°C (328K). Signals were assigned according to the cross peaks in the phase-sensitive DQF-COSY spectrum (data not shown).

contained both glucose and mannose in equimolar proportion. Thus, the sequence of sugars in SGL-X was found to be: mannose → glucose → glyceroldiether.

3.3. Permethylation analysis

Negative FAB-MS analysis of permethylated SGL-X, after TLC purification, showed two minor $[M-H]^-$ ion peaks at m/z 1227 and 1297, corresponding to the C_{20}-C_{20}- and C_{20}-C_{25}-permethylated molecular species of SGL-X (mono Na salts), as well as two major ion peaks at m/z 1125 and 1195 (Fig. 3E). The decrease of 102 mass units between the minor ion peaks (m/z 1227 and 1297) and the major ion peaks (m/z 1125 and 1195) is consistent with the loss of the labile sulfate group (as $NaSO_3$) from the fully permethylated SGL-X, similar to the loss of the labile sulfate group observed in the spectrum of the intact SGL-X (Fig. 3A). The increase of 70 mass units between the parent ion peaks m/z 1227 and 1297 in the spectrum of permethylated SGL-X (Fig. 3E) and those of the native SGL-X (m/z 1157 and 1227, respectively) (Fig. 3A) indicated the addition of 5 methyl (ether) groups to the SGL-X molecular species, as expected for a fully *O*-methylated bis-sulfated diglycosylarchaeol. For comparison, the negative FAB-MS spectrum of permethylated standard S-DGD-1 showed a single [M-H] ion peak at m/z 1139, indicating the addition of 6 methyl groups, as expected for a fully *O*-methylated monosulfated diglycosyldiether (data not shown).

GC-MS analysis of the partially methylated alditol acetates obtained from permethylated SGL-X showed two major peaks (data not shown), with retention times and mass spectra corresponding to those of standard acetylated 3,4,6-tri-*O*-methyl glucitol (prepared from kojibiose), and to those of standard acetylated 3,4-dimethyl mannitol [20,23]. These results indicated that the sulfate groups are both attached to the terminal mannopyranosyl residue at positions 2- and 6-, and not to the internal glucopyranosyl residue, and that the mannose group is linked 1 → 2 to the glucose group. At this stage the structure of SGL-X can be formulated as:

2,6-(HSO_3)$_2$-*Manp*-1 → 2-*Glcp*-1 → 1-*glyceroldiether*

3.4. ^1H- and ^{13}C-NMR spectra

Stereochemistry of the glycosidic linkages and the location of the sulfate groups was established by proton NMR studies of SGL-X (Fig. 4A). The doublet at 4.85 ppm with a coupling constant ($J_{1,2}$) of 3.4 Hz (Table 1) can be assigned to the anomeric proton of an α-glucopyranosyl residue [5,29–31]. The presence of coupling constants $^3J_{2,3}$, $^3J_{3,4}$, and $^3J_{4,5}$ larger than 8

Table 1
^{1}H-NMR assignment of the sugar residues in SGL-X, S-DGD and DGD

Chemical Shifts (ppm)

	Glc							Man						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
SGL-X	4.849	3.387	3.171	3.395	3.395	3.482	3.598	4.907	4.303	3.647	3.349	3.790	3.817	3.987
S-DGD	4.899	3.408	3.467	3.175	3.383	3.489	3.607	4.762	3.60	3.61	3.482	3.823	3.874	3.972
DGD	4.889	3.402	3.497	3.165	3.388	3.370	3.602	4.776	3.604	3.604	3.449	3.665	3.479	3.621

Coupling constants (Hz)

	Glc							Man						
	^{3}J1,2	^{3}J2,3	^{3}J3,4	^{3}J4,5	^{3}J5,6a	^{3}J5,6b	^{2}J6a,6b	^{3}J1,2	^{3}J2,3	^{3}J3,4	^{3}J4,5	^{3}J5,6a	^{3}J5,6b	^{2}J6a,6b
SGL-X	3.4	9.8	8.7	9.8	5.1	2.3	−11.9	1.7	3.4	9.7	9.7	n.d.	n.d.	n.d.
S-DGD	3.2	10.0	8.3	10.0	5.6	2.6	−11.7	n.d	n.d.	n.d.	n.d.	5.6	1.8	−10.9
DGD	3.7	10.0	8.7	9.8	5.1	2.3	−11.9	n.d.	n.d	n.d	n.d	5.6	2.7	−11.7

Chemical shifts were determined relative to internal Me $_4$Si at 328 K.
n.d.: Coupling constants could not be determined due to strong coupling.

Table 2
^{13}C-Chemical shifts of the sugar residues in SGL-X, S-DGD and DGD

	Glc						Man					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
SGL-X	95.74	74.78	71.44	70.14	72.54	60.84	95.66	75.41	69.42	67.76	71.44	66.08
S-DGD	95.39	73.87	71.39	70.15	72.48	60.80	96.67	70.41	70.29	66.87	71.23	66.05
DGD	95.54	74.42	71.40	70.08	72.37	60.72	96.97	70.49	70.63	67.04	73.15	61.17

Chemical shifts (ppm) were determined relative to internal Me $_4$Si at 328 K.

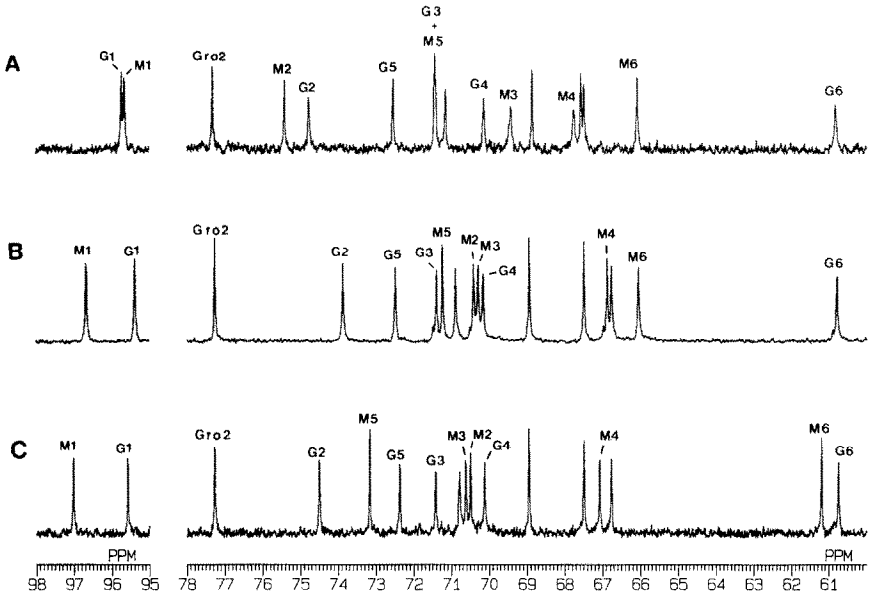

Fig. 5. ^{13}C-NMR spectra of sugar residues in: A, SGL-X (S_2-DGD-1); B, S-DGD-1 from *Hf. mediterannei*; and C, desulfated SGL-X (DGD). The spectral width was 20 kHz. Assignments were made by ^{13}C-DEPT sequence with a polarization transfer pulse of 135° and ^{1}H-^{13}C-COSY (data not shown). Identification of ^{13}C-signals in H-C COSY was based on the assignment of ^{1}H signals in Fig. 4.

confirms that the configuration is that of an α-glucopyranoside [29,30]. The doublet at 4.91 ppm with $J_{1,2}$ of 1.7 Hz and the resonance at 4.30 ppm with $J_{2,3}$ of 3.4 Hz (Table I) indicate the presence of equatorial protons at the C-1 and C-2 positions, corresponding to the configuration of an α-mannopyranoside [5,31]. For comparison, the ^{1}H-NMR spectrum (Fig. 4B) of S-DGD-1 from *Hc.saccharolyticus* [11] or *Hf. mediterranei* [20], which contain only α-glycosidic linkages (α-mannopyranosyl and α-glucopyranosyl) showed corresponding doublets centered at 4.76 and 4.90 ppm, respectively (Table 1). These results are consistent with both the mannose and the glucose glycosidic linkages in SGL-X having the α-configuration.

The position of the sulfate groups on C-2 and C-6 of the mannose residue was established by ^{1}H- and ^{13}C-NMR spectral analysis of SGL-X, S-DGD-1 from *Hf. mediterranei* [20] and desulfated SGL-X (DGD), using single pulse and phase sensitive DQF and H-C COSY (see Tables 1 and 2). The ^{13}C-NMR data (Table 2; Fig. 5A and B) showed that C-6 of the mannose residue in SGL-X and S-DGD-1 resonated at 4.9 ppm downfield from that of desulfated SGL-X (DGD), due to substitution with a sulfate group. Resonance of C-2 of mannose in SGL-X was also shifted downfield by 4.9 ppm (Table 2). No significant shift increments were observed in any of the C-resonances from the glucose residue. The ^{1}H-NMR data (Table 1; Fig. 4A and B) also showed downfield shift increments of H-2, H-6a and H-6b of mannose in SGL-X and only at H-6a and H-6b in S-DGD relative to the corresponding resonances in DGD; no significant shift increments were observed in the glucose H-resonances. Furthermore, the shift increments of H-2, H-6a and H-6b of SGL-X were comparable to those of H-3 of 3-HSO_3-Gal-ceramide [19] and H-6a and H-6b of 6-HSO_3-Gal-ceramide, respectively (N. Iida-Tanaka and I. Ishizuka, unpublished results). The NMR data thus fully support the location of the two sulfate groups in SGL-X at C-2 and C-6 of the mannose residue.

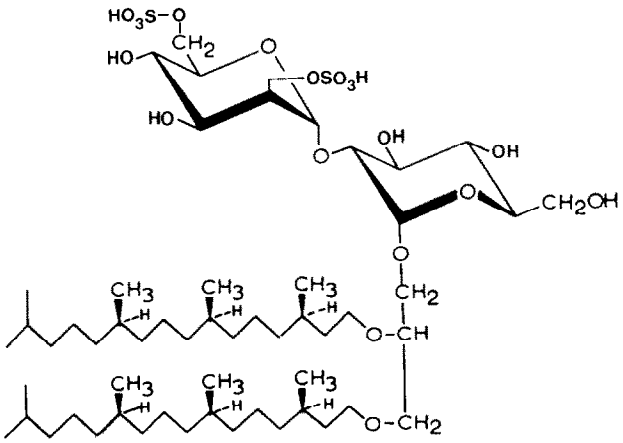

Fig. 6. Proposed structure of SGL-X (S_2-DGD-1) from halophilic archaebacterium strain 172.

On the basis of the above results the final structure of SGL-X is proposed to be: *sn*-2,3-diphytanyl- or phytanyl-sesterterpenyl-1-[2,6-$(HSO_3)_2$-α-Man*p*-1 → 2-α-Glc*p*]-glycerol (Fig. 6).

4. Discussion

Strain 172 is a long rod-shaped non-pigmented extreme halophile which has been isolated from sea sand

in Japan by Onishi and coworkers [1,13]. The content of bacterioruberins, the red pigment in extreme halophiles, was estimated to be less than 0.1% of that in *Hb. cutirubrum*. The authors considered that strain 172 might be a pigmentless spontaneous mutant of the genus *Halobacterium*. However, the polar lipid composition reported here shows that the major phospholipids are PGP-Me and PG without any PGS, and that the major glycolipid is a bis-sulfated-DGD, i.e., a sulfated derivative of the S-DGD-1 of *Hf. mediterranei* [20]. Another unusual feature of the lipids of strain 172 is the presence of both C_{20}-C_{20}- and C_{20}-C_{25}-lipid cores. The presence of both of these lipid cores have been reported previously only in *Natronobacterium*, *Natronococcus* and *Halococcus* species, although recent studies indicate the presence of both lipid cores also in some halococci [11] and in some non-alkaliphilic rod-shaped halobacteria [12,37].

Torreblanca et al. [3] proposed a reclassification of non-alkaliphilic halophilic archaebacteria based on numerical taxonomy and polar lipid composition, designating 3 genera: *Halobacterium, Haloarcula,* and *Haloferax*. All three genera contain PGP-Me and PG but only *Hb.* and *Ha.* species contain PGS as well. The glycolipid composition is more discriminating in that each of the above genera has its characteristic major glycosyldiether(s): *Halobacterium*, sulfated Gal-Man-Glc-diether (S-TGD-1) and sulfated tetraglycosyldiether (S-TeGD); *Haloarcula*, Glc-Man-Glc-diether (TGD-2); and *Haloferax*, Man-(6-sulfate)-Glc-diether (S-DGD-1) [3,5,32]. Note, however, that S-DGD-1 has also been found in *Hb. saccharovorum* [33] and in *Hc. saccharolyticus* [11].

Strain 172 would appear to have the *Haloferax* phospholipid composition and a *Haloferax*-like glycolipid (S_2-DGD-1) which has an extra sulfate group, on C-2 of the mannose residue, as compared to S-DGD-1. This would suggest that strain 172 may be a mutant of a *Haloferax* strain that acquired a sulfating enzyme specific for the 2-position of mannose. It is of interest in this connection that a related glycolipid (S-DGD-5), an isomer of S-DGD-1 in which the single sulfate is attached to C-2 of mannose, has been identified in *Hb. trapanicum* (NRC 34021, NCMIB 767) [34]. An analogue (S-DGD-3) of this sulfated glycolipid (S-DGD-5), in which the 2-sulfated mannose is linked $1 \rightarrow 4$ to glucose has been found in *Hb. sodomense* [35]. Both of these extreme halophiles should then possess the mannose-C-2 sulfating enzyme. It is possible that the mutation which formed strain 172 may have involved gene fusion of a *Haloferax* strain and a *Hb. trapanicum* strain. Comparison of the nucleotide sequences of 16S rRNA encoding genes of strain 172 and *Hb. trapanicum* (NRC 34021, NCMIB 767) suggested that the latter is not a species of the genus *Halobacterium* and that these two halophilic archaebacteria belong to different new genera [36]. A more detailed taxonomical study is required to clarify the classification of these extreme halophiles and of halophilic archaebacteria in general.

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