The structures of glycolipids isolated from the highly thermophilic bacterium *Thermus thermophilus* Samu-SA1

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Thermophiles constitute a class of microorganisms able to grow at extremely elevated temperatures. Some of these species are classified as Gram-negative bacteria, because of the presence of an outer membrane in the cell envelope, which is located on the top of a thick murein layer. Unlike typical Gram-negative bacteria, the outer membranes of *Thermus* species are not composed of lipopolysaccharides but of peculiar glycolipids (GL), whose structures seem to be strictly involved in the adaptation to high temperatures. In this work, the complete structures of the major GL components from the cell envelope of the thermophilic bacterium *Thermus thermophilus* Samu-SA1 are presented. Protocols conventionally adopted for Gram-negative bacteria were used, and, for the first time, GL from *Thermus* were analyzed in their native form. Two GL and one phosphoglycolipid (PGL) were detected and characterized. The two GL, analyzed by nuclear magnetic resonance (NMR) spectroscopy, and electrospray ionization Fourier transform mass spectrometry (ESI FT-ICR) mass spectrometry, possessed the same tetrasaccharide structure linked to a glycerol unit or, alternatively, to a long-chain diol. Moreover, a PGL from *Thermus* was characterized for the first time, in which N-glyceroyl-heptadecanamine was present. These molecules are chemically related to other GL from thermophilic bacteria, in which they play a crucial role in the adaptation of cell membranes to heat.

**Key words:** ESI FT-MS/glycolipid/long-chain diol/NMR/*Thermus thermophilus*

Introduction

Bacteria belonging to the genus *Thermus* constitute a class of extremophile microorganisms with an optimum growth temperature between 70 and 75°C. It is generally believed that a crucial role in the thermal stability that these bacteria are endowed with is provided by the particular structure of their cell envelope. Actually, *Thermus* bacteria possess a thick murein layer (Quintela et al., 1995, 1999) like Gram-positive bacteria, bearing at its outside an outer membrane, as in Gram-negative bacteria, and the molecular arrangement of both appears to be central in the adaptation process to high temperatures (Brock, 1978). In particular, several kinds of polar glycolipids (GL) have been isolated from the membranes of *Thermus* (Pask-Hughes and Shaw, 1982; Wait et al., 1997; Lu et al., 2004) and *Meiothermus* (Yang et al., 2004) bacteria, which share some common structural features, mainly concerning kind and sequence of monosaccharides present, and the nature of the lipid component. The microorganism investigated in this study, *Thermus thermophilus* Samu-SA1, is a thermophilic bacterium first isolated in the shallow marine hot springs on Mount Grillo, in Italy (Romano et al., 2004). Applying the protocol conventionally adopted for isolation of lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria, we recovered a lipid component that was further purified to yield a fraction composed of two GL (GL1 and GL2) and one phosphoglycolipid (PGL), which were fully characterized by Gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy, and ESI Fourier transform mass spectrometry (FT-MS). This is the first time that a structure of a ‘PGL’ from *Thermus* is presented, and, interestingly, it is closely related to those of analogous molecules isolated from *Deinococcus* (Huang and Anderson, 1989, 1992), a genus highly resistant to environmental hazards, with whom *Thermus* is phylogenetically related (Hensel et al., 1986). This fact suggests that these molecules play a crucial role in the adaptation to heat.

**Results**

**Cell culture, extraction, and chemical characterization of GL**

Cells of *T. thermophilus* Samu-SA1 were grown at 75°C using the standard TH medium and harvested, in the stationary growth phase, by centrifugation. Cell yield was ∼3.5 g/L wet weight (0.9 g/L dry weight).

Dried cells were washed with 1% aqueous phenol to remove exocellular polysaccharides and subsequently extracted utilizing the hot phenol/water procedure (Westphal and Jann, 1965), optimized for Gram-negative bacteria. By chemical analyses, we detected a lipid-containing fraction in the phenol extract, which was first purified by enzymatic digestion with RNase, DNase, and Proteinase K. Analysis by thin-layer chromatography (TLC) evidenced the presence...
Glycolipids from Thermus thermophilus

of two fractions that were isolated by silica-gel, and the major fraction underwent complete chemical analysis.

Fatty acid analysis revealed the presence of iso- and anteiisobranched C15:0 and C17:0 and of minor amounts of iso- and anteiisobranched C14:0, C16:0, and C18:0. GC-MS analyses of the acetylated O-methyl glycosides showed the presence of glucose (Glc), galactose (Gal), and 2-deoxy-2-amino-glucose (GlcN), present in non-stoichiometric amounts. Three additional constituents, degraded by strong hydrochloric methanol treatment, could be detected after milder methanolysis and acetylation. These were identified, on the basis of the ion fragmentation in the MS spectra, as glycerol (Gro), glycerol-phosphate (GroP), and GlcNAcyl, in which the acyl moiety was C17:0. Three late-eluting components were also found, which were identified by EI-MS and CI-MS analyses after either acetylation or trimethylsililation, as octadecane-1,2-diol (OD), heptadecanoyl-amine (HA), and its N-glyceryl derivative. Long-chain alkylglycerols were previously found as components of polar GL from other Thermus species (Wait et al., 1997), where they are thought to replace acyl-glycerol in the GL structure, whereas the occurrence of glycric acid and long-chain alkylamine was a new finding. Detection of glycosylation sites and ring size of the monosaccharides was achieved by methylation analysis, identifying 2-substituted-Glc, 2-substituted-Gal, 6-substituted-GlcN and terminal GlcN, all in pyranose form, and terminal galactofuranose (Galf).

NMR and ESI FT-MS analysis of the GL extract

The poor solubility of GL in many common solvent systems is one of the major obstacles in their structure determination. This problem is typically overcome by introducing chemical modifications, usually peracetylation, to improve the solubility and to allow the execution of experiments in solution, in particular, the recording of NMR spectra. In this case, optimal solubility for the sample was found in CHCl3 : CH3OH (1:2, by volume), and this allowed the recording of a full set of 1D and 2D-NMR experiments on the native GL.

ESI FT-MS, as well as NMR analyses, demonstrated the existence of a mixture. In particular, the charge-deconvoluted ESI FT-MS mass spectrum obtained in the positive-ion mode (Figure 1) revealed at least three different species with monoisotopic masses of 1,176.857, 1,409.979, and 1,467.977 u, each one accompanied by a panel of correlated ions originating from different acyl chain length (Δm = 14 u) and from sodium attachment (Δm = 22 u).

In 1D and 2D NMR spectra, partially overlapping signals of diverse GL species were visible. In particular, in the region between 5.200 and 4.600 p.p.m. of the 1H-NMR spectrum signals for at least six anomeric protons (A–F in order of decreasing chemical shift) were visible (Figure 2). Moreover, in the same region of the spectrum, signals were present for at least four acylated carbinolic protons (G–L). In the aliphatic region between 2.500 and 0.500 p.p.m., signals were identified deriving from protons belonging to the amino-methylene group and to particularly deshielded aliphatic methylene groups (Tables I and II). By double quantum-filtered correlation spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY) spectra, the full assignment of proton resonances of the components of the mixture was possible. Subsequently, 13C chemical shifts were assigned from observed correlations in the 1H,13C-heteronuclear single quantum coherence (HSQC) spectrum (Figure 2 and Table I). For the monosaccharides, the 1JCH anomeric coupling constant values derived from the DQF-COSY spectrum established for each residue anomic and relative configurations. All residues, except residue A, were pyranoses, as proven by the observed carbon chemical shift values and by the intra-residual long-range correlations between C-1/H-5 and H-1/C-5 that appeared for each residue in the 1H,13C-HMBC spectrum. The 1H signal at 5.028 p.p.m. (H-1A) correlated in the TOCSY spectrum to proton signals at 4.079, 4.015, 3.943, and 3.725 p.p.m. All of these signals showed, in the 1H,13C-HSQC spectrum, correlations with down-field shifted carbon signals, up to 84.4 p.p.m., suggesting the occurrence of a furanose ring, as confirmed by the intra-residual H-1/C-4 and C-1/H-4 correlations in the 1H,13C-HMBC spectrum. The 1JCH anomeric coupling constant values were also observable in the 1H,13C-HMBC spectrum, because the pulse sequence used to carry out this experiment contained a low-pass
filter, set to a value of 145 Hz. In this way, the rising of \( {^1J_{C,H}} \) couplings for ring C-H could be selectively avoided, whereas it was still possible to recover the \( {^1J_{C,H}} \) for anomeric protons and carbons (Bubb, 2003). The \( {^1J_{C,H}} \) coupling constant value of 174.0 Hz for spin system A together with the \( {^{13}C} \) chemical shift of the anomeric carbon signal and the intra-residual nuclear Overhauser effect (NOE) correlations observed was diagnostic for the \( \alpha \)-anomeric configuration for A, thus identified as terminal \( \alpha \)-Gal. The \( {^1J_{C,H}} \) anomeric coupling constant values of the other spin systems were similarly obtained. For spin system B (5.019 p.p.m.), \( \alpha \)-galacto configuration was identified, on the basis of the low \( {^3J_{H,H}} \) values for H-1/H-2, H-3/H-4, and H-4/H-5 (3.4, 3.6, and <1 Hz, respectively). Typical down-field shift because of glycosylation was observed for the C-2 resonance, proving the substitution at O-2 of this residue. Thus, this residue was a 2-substituted \( \alpha \)-galactopyranose (2-\( \alpha \)-Gal).

Residues C and D (anomeric proton shifts at 4.956 and 4.900 p.p.m., respectively) were both identified as \( \alpha \)-gluco configured residues on the basis of the large \( {^3J_{H,H}} \) values except for \( {^3J_{1,2}} \) (3.6 Hz). Gluco configuration was also confirmed by a 2D rotating frame Overhauser enhancement spectroscopy (ROESY) spectrum, in which dipolar correlations between H-2/H-4 and H-3/H-5 of both residues were observed. The finding that these two residues shared the same pattern of resonances except for H-1 supported the idea that they represented the same monosaccharide, in slightly different chemical and magnetic environments. Down-field shift because of glycosylation was observed for C-2 (80.2 p.p.m.), allowing the identification of a 2-substituted \( \alpha \)-glucopyranose (2-\( \alpha \)-Glc).

For residue E, \( {^3J_{H,H}} \) values and intra-residual NOE connectivity revealed the \( \alpha \)-gluco configuration. Moreover, the C-2 chemical shift value at 54.1 p.p.m., in the typical region of nitrogen-bearing carbons, implied the occurrence of 2-amino-2-deoxy-glucose. A typical proton resonance down-field shift because of acetylation was observable for H-2 (3.843 p.p.m.). Actually, this signal correlated in the \( {^1H, {^{13}C}} \)HMBC spectrum to a carbon at 173.4 p.p.m., which correlated to the methyl signal of the acetyl group at 1.998 p.p.m. Thus, this residue was identified as terminal 2-acetamido-2-deoxy-\( \alpha \)-glucopyranose (t-\( \alpha \)-GlcNAc).

Residue F (H-1 at 4.629 p.p.m.) was identified as GlcN on the basis of the C-2 resonance at 57.1 p.p.m. and of the high \( {^3J_{H,H}} \) values, whereas the diagnostic NOE correlations observed between H-1, H-3, and H-5 and \( {^1J_{C,H}} \) anomeric coupling constant value (165 Hz) unambiguously proved the \( \beta \)-gluco configuration. Substitution occurred at O-6, as testified by the glycosylation shift for C-6 (67.1 p.p.m.). Also, an acylation shift was observed for H-2 (3.553 p.p.m.). In the HMBC spectrum, H-2 correlated to a carboxyl signal at 176.9 p.p.m., which correlated to a proton at 2.218 p.p.m., was identified as H\(_g\) to the carboxyl group of a fatty acid alkyl chain. This information proved the existence of the amide linkage with a fatty acid, namely, on the basis of previous chemical analysis, with C\(_{17}\). Thus, residue F was identified as 6-substituted 2-acylamido-\( \beta \)-glucopyranose (6-\( \beta \)-GlcNAcyl).
Of the anomeric region of the $^1$H-NMR spectrum, signals at 5.169, 5.176, 4.950, and 4.661 p.p.m. were correlated in the $^1$H,$^1$C-HSQC spectrum with carbon resonances at 70.4, 70.2, 73.2, and 76.4 p.p.m. These signals allowed the identification of four spin systems, designed G, L, H, and I respectively. In particular, the signal at 5.169 p.p.m. (H-2\text{G}) showed correlations in the DQF-COSY with two diastero-topic methylene groups, at 4.140/4.381 (H-3\text{a}/H-3\text{b}\text{G}) and 3.631/3.710 (H-1\text{a}/H-1\text{b}\text{G}) p.p.m. On the basis of the chemical shifts for protons and carbon signals (Table I) and of the observed long-range correlations with carboxyl group resonances at 174.4 and 174.5 p.p.m., it was possible to identify residue G as a Gro moiety acylated at O-2 and O-3.

The identified resonances for the acyl moieties are summarized in Table II. Full attribution was impossible because of the merging of methylene signals of the long fatty acid chains into one broad signal at 1.242 p.p.m.

Residue H was identified as the expected OD, on the basis of the observed scalar correlations (DQF-COSY) of the signal at 4.950 p.p.m. (H-2\text{H}), with one hydroxymethylene group (3.523/3.580 p.p.m., H-1\text{a}/H-1\text{b}\text{H}) and a methylene group in the aliphatic region (1.532 p.p.m.). Chemical shifts for the other protons and carbons of the alkyl chain were only partially distinguishable (Table I). In this case, acylation occurred at O-2, as proven by the long-range correlation with the carboxyl at 177.1 p.p.m.

Residue I was identified as the N-glyceroil-HA unit. The signal at 4.661 p.p.m. (H-2\text{I}) showed a correlation with a methylene signal at 3.830 p.p.m., which gave a scalar correlation with a carboxyl carbon at 170.1 p.p.m. (C-1\text{I}, $^1$H,$^1$C-HMBC). The same carbon signal also showed a correlation to the methylene signal at 3.152/3.237 p.p.m., which was identified as H-1\text{I} and, thus, as the aminomethylene position, on the basis of the carbon chemical shift value (39.7 p.p.m.).
A second Gro unit was identified (L), starting from the resonances of H-2 at 5.176 p.p.m., from which two correlations with hydroxymethylene groups, resonating at 4.084/4.312 (H-3/a/H-3/bL) and 3.983 p.p.m. (H-1/aL), could be identified. Correlations were observed for H-2 and H-3 with carbonyl groups (172.5 and 173.1 p.p.m.), suggesting acylation at O-2 and O-3, whereas the observed down-field displacement of the H-1 resonance, compared with the analogous position of residue G, implied phosphorylation at this site, in consistency with chemical analyses.

Connectivity between the identified spin systems were established on the basis of the inter-residual dipolar correlations detected in the 2D ROESY spectrum and scalar long-range correlations observed in the $^1$H,$^{13}$C-HMBC spectrum. In particular, proton H-1A gave a strong NOE connectivity with H-2B and a scalar correlation with the carbon resonance at 76.7 p.p.m., suggesting the attachment at O-2 of the α-Galp residue. This was linked at O-6 of residue F, namely the β-GlcNAcyl residue, as confirmed by the occurrence of the dipolar correlation H-1B/H-6F and of the long-range correlation between H-1B and C-6B. A cross peak appeared in the ROESY spectrum between H-1F and a proton at 3.472 p.p.m., identified as H-2 of residues C and D. The information deriving from both ROESY and $^1$H,$^{13}$C-HMBC spectra showed that residue C was linked to O-1 of residue G. In fact, a long-range correlation existed between H-1C and carbon at 66.6 p.p.m. (C-1G). Residue D appeared to be linked at O-1 of residue H, namely the OD.

These data can be summarized in the following structure:

$$\alpha$$-Gal/(1-2)$$\alpha$$-Gal-(1-6)$$\beta$$-GlcNAcyl-(1-2)$$\alpha$$-Glc-(1-1)-R

A B F C/D

with R = Gro (G) or OD (H), thus, the tetrasaccharide backbone is linked either to the glycerol unit G or to the OD H.

Residue E (t-$$\alpha$$-GlcNAc) showed a NOE correlation with H-2I and an additional long-range correlation with C-2I that indicated the presence of the fragment α-GlcNAc-(1–2)-N-glyceryl-heptadecane-amine. These two spin systems, as well as the GroP L, did not show any dipolar or long-range correlation in NMR spectra with the structures so far identified, thus appearing as isolated fragments, likely belonging to a different molecular species within the blend.

**Purification and complete characterization of GL1, GL2, and PGL from T. thermophilus Samu-SA1**

To find out further structural details, we performed mild de-O-acylation with anhydrous hydrazine, and the product was purified on silica gel. Two fractions were obtained, composed by de-O-acylated GL (de-O-GL1 and de-O-GL2), and ESI FT-MS and 1D and 2D NMR analyses were performed on both the products. The approximate molar ratios of monosaccharides in both fractions were Gal:Glc:GlcN, 2:1:1.

NMR spectra recorded on the more abundant fraction (de-O-GL1) appeared rather simplified compared with that of the initial mixture (Figure 3). Nevertheless, the tetrasaccharide

![Fig. 3. Comparison of 1H-NMR spectra of the products obtained after purification of de-O-acylation of the glycolipid extract from Thermus thermophilus Samu-SA1. The identified spin systems are representative of the conserved structure of the tetrasaccharide moiety.](image-url)
linked to the OD already identified was clearly recognizable. This affirmation was proven by the NOE correlation in the ROESY spectrum between H-1 of the 2-α-Glc residue and H-1H and by the observation of the scalar long-range inter-residual correlation in the $^1$H,$^1^3$C-HMBC with C-1H. Final confirmation of the proposed structure was provided by ESI FT-MS of the de-O-GL1 (Figure 4). The charge-deconvoluted mass spectrum revealed an abundant peak with monoisotopic mass of 1185.760 u, which is in an excellent agreement with the calculated mass (1185.7597 u) of a molecule composed of OD-Hex$_3$-HexN-C$_{17}$:0. Comparison with the ESI-MS spectra of the mixture led to the identification of the first molecular species in the native form (GL1, Figure 5), where the molecule appeared to be O-acylated at O-2 of the OD residue by a C$_{15}$:0, as testified by the molecular mass of 1409.979 u, differing from 1185.760 u by one C$_{15}$:0 unit.

The NMR data (Figure 3) of the second isolated fraction (de-O-GL2) showed high analogy with de-O-GL1, the only structural difference being the occurrence of a Gro moiety instead of the long-chain diol. Also in this case, the tetrasaccharide structure was recognized after complete 2D NMR analysis and resulted identical to the structure already found in de-O-GL1.

ESI-MS spectrum on de-O-GL2 (Figure 4) showed an intensive molecular peak with 991.519 u, in agreement to

![Fig. 4. Charge-deconvoluted ESI FT mass spectra of the separated fractions obtained from the glycolipid extract of *Thermus thermophilus* Samu-SA1 after de-O-acylation. The major peaks correspond to the exact mass of characterized de-O-GL1 and de-O-GL2 (see Table IV).](image)

The proposed species Gro-Hex$_3$-HexN-C$_{17}$:0, with a minor peak at 829.465 u ($\Delta m = 162$ u), suggesting the presence of a minor compound lacking of a hexose residue. This minor form was not distinguishable in the NMR spectra. The two ion peaks visible in this spectrum at 1027.497 u ($\Delta m = 36$ u) and 1051.536 u ($\Delta m = 60$ u) were likely because of artifacts deriving from the purification procedure applied, since neither chemical analysis nor NMR investigation showed any species that could generate these molecular ions. Moreover, analogous mass differences are undetected in the
intact mixture spectrum. The comparison with the ESI-MS spectrum of the blend allowed to relate de-O-GL2 to the species represented by the peak with a mass of 1467.977 u, identified as the molecular ion peak for the second GL (GL2, Figure 5). In fact, the mass difference of 476 u between the two peaks was consistent with the presence of one C_{15:0} and one C_{17:0} residues, suggesting that O-2 and O-3 of the Gro unit must be esterified by these two residues. In the same way, the species with a molecular mass of 1439.949 u was in account for a minor compound acylated by two C_{15:0} units.

It was not possible to find any product related to the phospholipid present in the native mixture, likely because of total degradation during de-O-acylation. Nevertheless, it was possible to compare the composition of the two GL so far identified with the results of the chemical and spectroscopical analyses performed on the native mixture (Table III), deducing the identity of the third expected molecule. This was identified as a phospholipid (PGL), containing in its structure the fragment \( \text{Gro} - \text{AN} - \text{CH}_{2}\text{CH}_{3} - \text{P} - \text{C}_{17:0} - \text{C}_{15:0} \) (PGL, Figure 5). From this mass spectrum, it was also possible to deduce that the Gro unit was esterified in the most abundant species by one C_{15:0} and one C_{17:0}. It was also possible to detect a highly heterogeneous acylation pattern, indicated by the occurrence of ions with \( \Delta m = 14 \text{ u} \), a methylene group, suggesting variability in the chain length of fatty acid residues. Moreover, the family of ions centered at 1134.846 u (\( \Delta m = 42 \text{ u} \), acetyl group) indicated the presence of a small amount of a compound, where the GlcN residue was not acetylated. The absence of dipolar correlations between the glyceramide and the acyl-glycerol units, observed in the previous NMR data, was explained with the existence of a phospho-diester bridge connecting the two fragments, as confirmed by the detection of glycerol phosphate in chemical analysis. Such a structure was earlier found in bacteria belonging to the genus Deinococcus, a genus phylogenetically related to Thermus (Huang and Anderson, 1989, 1992). Figure 5 shows the structures of GL1, GL2, and PGL.

**Table III.** Comparison between chemical compositions, obtained by GC-MS and NMR, of the native glycolipid extract and the constituent species, GL1, GL2, and PGL

<table>
<thead>
<tr>
<th>Native mixture</th>
<th>GL1</th>
<th>GL2</th>
<th>PGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-(\alpha)-Galf</td>
<td>X</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>2-(\alpha)-Gal</td>
<td>X</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>2-(\alpha)-Glc</td>
<td>X</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>t-(\alpha)-GlcNAc</td>
<td>–</td>
<td>–</td>
<td>X</td>
</tr>
<tr>
<td>6-(\beta)-GlcNAcI</td>
<td>X</td>
<td>X</td>
<td>–</td>
</tr>
<tr>
<td>Gro</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GroP</td>
<td>–</td>
<td>–</td>
<td>X</td>
</tr>
<tr>
<td>octadecane-1,2-diol</td>
<td>X</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GroAN(CH(_2)(_2)CH(_3))</td>
<td>–</td>
<td>–</td>
<td>X</td>
</tr>
</tbody>
</table>

Table IV. Measured and calculated exact molecular masses of the identified glycolipids from Thermus thermophilus Samu-SA1

<table>
<thead>
<tr>
<th>Measured mass (u)</th>
<th>Calculated mass (u)</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL1 1409.979</td>
<td>1409.9739</td>
<td>OD-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>GL2 1467.979</td>
<td>1467.9793</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>1439.944</td>
<td>1439.948</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>PGL 1176.858</td>
<td>1176.8502</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>1204.889</td>
<td>1204.8817</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>1134.846</td>
<td>1134.8399</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})-C(_{15:0})</td>
</tr>
<tr>
<td>de-O-GL1 1185.760</td>
<td>1185.7597</td>
<td>OD-Hex(<em>2)-HexN-C(</em>{17:0})</td>
</tr>
<tr>
<td>de-O-GL2 991.519</td>
<td>991.5199</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})</td>
</tr>
<tr>
<td>829.465</td>
<td>829.4671</td>
<td>Gro-Hex(<em>2)-HexN-C(</em>{17:0})</td>
</tr>
</tbody>
</table>

The measured masses were taken from the charge-deconvoluted mass spectra given in Figures 1 and 4.
Materials and Methods

Cell growth

_T. thermophilus_ Samu-SA1 (DSM 15284, ATCC BAA-951) was grown at 75°C in a 50 L fermenter (Biostat-D, Braun) with a mechanical agitation of 100 rpm and an aeration flux of 56%. Growth was followed turbidimetrically at 540 nm. The whole culture medium (TH medium) contained (g/ L) peptone (Oxoid) 8.0, yeast extract (Oxoid) 4.0, NaCl 2.0 at pH 7.0.

Cells were harvested in the stationary phase of growth by continuous-flow centrifugation on a Alfa Laval Model LAB 102 B-20 centrifuge. The pellet obtained was lyophilized.

Isolation and enzymatic purification of the GL component

Dried cells (3.5 g) were washed with 140 mL 1% aqueous phenol and kept at 4°C under stirring for 16 h. After centrifugation (8000 x g, 4°C, 1 h), the supernatant was removed, and the cells were extracted thrice with 60 mL of 45% aqueous phenol at 68°C, according to the conventional hot phenol-water procedure (Westphal and Jann, 1965). The phenol phase was diluted and dialyzed against water (3.500 kDa molecular weight cut-off). After dialysis, the extract was again centrifuged (8000 x g) and lyophilized, obtaining 210 mg of dried mass. The extract was digested with DNase, Rnase, and Proteinase K, dialyzed and freeze dried (49 mg, 1.4% of dry cells).

Sugar and fatty acid analyses

Monosaccharide analyses were realized by means of GC-MS of acetylated O-methyl glycosides derivatives, obtained after methanolysis (2 M HCl/MeOH, 85°C, 24 h) and acetylation with acetic anhydride in pyridine (85°C, 30 min). The absolute configuration of the monosaccharides was obtained according to the published method (Leontein and Lönngren, 1978).

Methylation analysis was performed using the modified Hakomori procedure (Hakomori, 1964) by Ciucanu and Kerek (1984). After chloroform/water extraction, the organic phase was evaporated and hydrolyzed with 4 M trifluoroacetic acid (100°C, 3 h), carbonyl reduced with NaBD₄, acetylated with acetic anhydride : pyridine (1:1, v/v), and analyzed by GC-MS. For identification of OD, heptadecane-1-amine, and N-glyceroxy-heptadecane-1-amine, trimethylsilation was achieved treating the sample with bi(trimethylsilyl)trifluoroacetamide, 60°C, 30 min, followed by vacuum centrifugation.

Purification of crude and de-O-acylated glycolipid extract

Chromatography for purification of GL was performed on Silica gel (Merk, 230–400 mesh) eluted with CHCl₃ : MeOH (9:1 to 1:1, by volume), and the fraction collected were monitored by TLC, developed with CHCl₃ : MeOH : H₂O (65:25:4, by volume), and visualized with 0.1% Ce(SO₄)₂·4H₂O. 5% (NH₄)₆Mo₇O₂₄·4H₂O in 5.8% v/v H₂SO₄. The most abundant fraction eluted was treated with anhydrous hydrazine at 37°C for 30 min. The hydrazine was removed by evaporation under nitrogen and the product was again purified on Silica gel, eluting with CHCl₃ up to CHCl₃ : MeOH (1:1, by volume) followed by TLC performed as already described.

ESI FT-MS analysis of GL

FT-MS was performed in the negative- and positive-ion modes using an APEX II—Instrument (Bruker Daltonics, Billerica, MA) equipped with an actively shielded 7 T magnet and an (nano) ESI source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. For the negative-ion spectra samples (–10 ng µL⁻¹) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine. For the positive-ion mode 50:50:0:03 (v/v/v) mixture of 2-propanol, water, 30 mM ammonium acetate adjusted with acetic acid to pH 4.5 was used. The samples were sprayed at a flow rate of 2 µL min⁻¹. Capillary entrance voltage was set to 3.8 kV and drying gas temperature to 150°C. The spectra shown are charge-deconvoluted, using the xMASS-6.1 software, and
mass numbers given refer to the monoisotopic molecular masses.

**NMR spectroscopy**

Glycolipids from Thermus thermophilus


