First Isolation and Structure Elucidation of GDNT-β-Glu – Tetraether Lipid Fragment from Archaeal *Sulfolobus* Strains

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Due to their special chemical structure, tetraether lipids (TEL) represent essential elements of archaeal membranes, providing these organisms with extraordinary properties. Here we describe the characterization of a newly isolated structural element of the main lipids. The TEL fragment GDNT-β-Glu was isolated from *Sulfolobus metallicus* and characterized in terms of its chemical structure by NMR- and MS-investigations. The obtained data are dissimilar to analogically derived established structures – in essence, the binding relationships in the polar head group are re-determined and verified. With this work, we provide an important contribution to the structure elucidation of intact TEL also contained in other *Sulfolobus* strains such as *Solfulobus acidocaldarius* and *Sulfolobus solfataricus.*

1. Introduction

The ability to form self-assembled systems is an intrinsic part of the origin of life. A well-known example in 'ordinary' cells is the formation of lipid bilayer by phospholipids. Beyond these lipids found in eukaryotes and prokaryotes,[1] tetraether lipids (TEL) as membrane lipids of archaea have shown extraordinary behavior and enable life under extreme environmental conditions. Instead of spherical structures such as liposomes, artificial biomimetic TEL preferentially form square lamellae[2] or fibers and ribbons.[3] Both the molecular structure and derived archaeosomes are characterized by an enormous stability even within the human organism.[4] Also, the incorporation of single artificial TEL in liposomes of conventional phospholipids showed an overall stabilizing effect.[5] However, the self-assembly properties of naturally occurring TEL are still insufficiently investigated due to an inadequate state-of-the-art isolation and purification to gain useful quantities and a proper purity. Therefore, to our knowledge, most studies concerning archaeal-derived TEL are performed with lipid extracts containing a variety of other compounds. For further understanding of the self-assembly behavior of TEL, it is important that the chemical structure is clearly elucidated which can only be achieved by analyzing pure TEL.

Excellent work on structure elucidation of the basic building blocks glycerol dibiphytanyl glycerol tetraethers (GDGT) and glycerol dibiphytanyl nonitol tetraethers (GDNT) was already done[6,7] and their chemical structures can be seen in Figure 1 (structures **1** and **2**, correspondingly). By using nuclear magnetic resonance (NMR) experiments, it was found that their structure is mainly formed by a larger closed cyclic backbone of biphytanyl groups linked by ether groups to either two glycerol (GDGT) or one glycerol and one calditol (GDNT) unit. This cyclic backbone, only connected by covalent bonding with mainly sp^3-hybridised carbon atoms, forces the whole molecule into a rod-like appearance. In combination with different end groups connected to the hydroxylic groups of the glycerol units, the molecules develop a lipid character where they prefer to form lamellar, columnar and various cubic phases.[8] Both main building blocks, GDGT and GDNT, can be obtained and analyzed relatively easily by hydrolysis of higher functionalized TEL and can be isolated without further side groups. It can be assumed that those building blocks are early steps in the biosynthesis of higher functionalized TEL.

Actual lipids are distinguished by the polar head groups at the corresponding backbones. An example of such a bipolar lipid (or also bolalipid) is the main polar lipid from *Sulfolobus acidocaldarius* which can be seen in Figure 1 (structure **4**).[9,10] It is characterized by a phosphate-bound *myo*-inositol and a β-D-glucose bound to the calditol of the GDNT unit.[11]

The backbones of the TEL carry a different number of cyclopentane units and therefore differ in their degree of cyclization. In contrast to the depicted structures in Figure 1, TEL derivatives usually do not possess a constant degree of cyclization, but rather are mixtures of compounds of different degrees of cyclization. The number of rings can range from zero to four per biphytanyl chain so that the full system can contain

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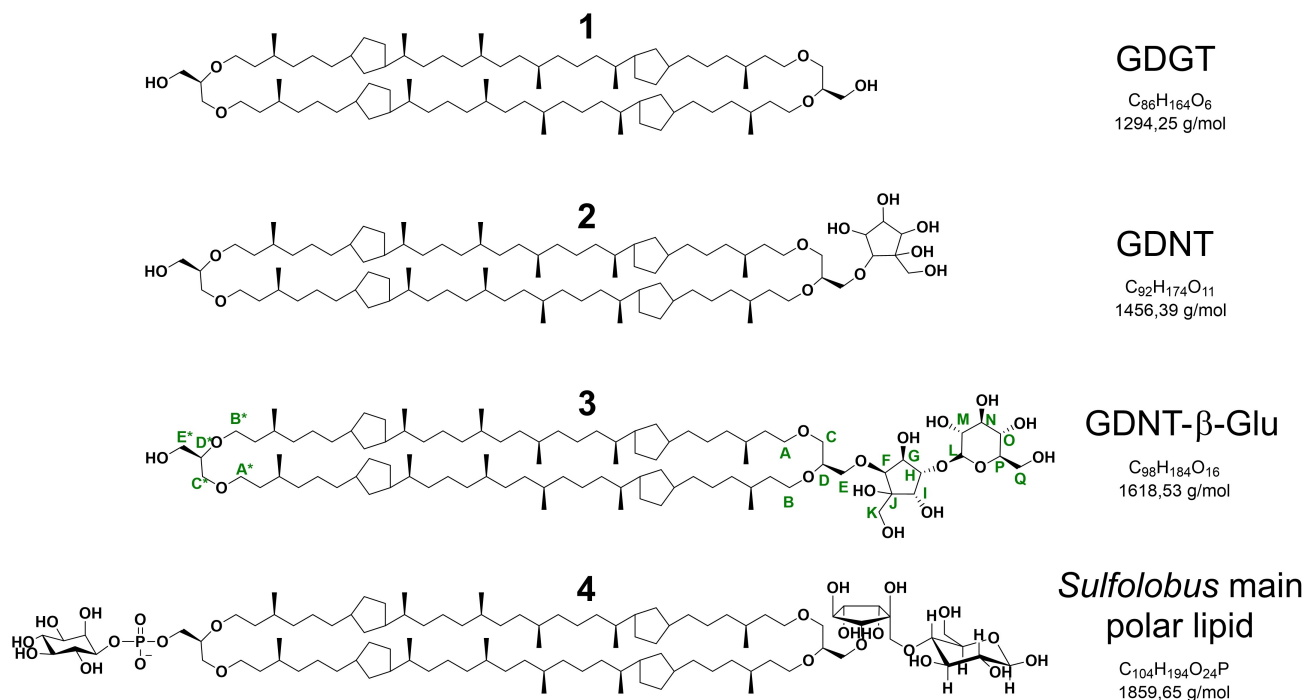European Chemical
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Figure 1. Overview of some selected TEL derivatives. **1**: Glycerol dibiphytanyl glycerol tetraether (GDGT) and **2**: glycerol dibiphytanyl nonitol tetraether (GDNT) are basic building blocks and can be obtained by hydrolysis of complex TEL; **3**: GDNT-β-Glu (with labeled carbon atoms for reproduction of the NMR elucidation) is a TEL of the main lipid of Sulfolobus strains and was newly isolated and characterized in detail in this work; **4**: Sulfolobus main polar lipid (S-MPL) is a published example of an intact main lipid.[9] All TEL and fragments are shown as their four-cyclopentane-units representatives.

up to eight cyclopentane units.[12] Additionally, different positions and other particularities (e.g. incorporation of a cyclohexane moiety in crenarchaeol derivatives)[13] can result in complex mixtures. The proportion of cyclopentane units is strongly dependent on species and cultivation conditions, for example growth state[14] and temperature.[15] In general, higher cultivation temperatures lead to an increase in the degree of cyclization – this is intended to maintain the physical properties required for the biological function of the archaeal membranes. The enzymatic formation of cyclopentane units out of the isoprenoid skeleton allows a denser packing of the lipid molecules and, as a consequence, causes an increase in the phase transition temperatures.[15] This mechanism allows archaea to survive even at higher temperatures.

The focus of this work is the isolation and characterization of a higher functionalized TEL of the main lipid from different Sulfolobus strains which is called GDNT-β-Glu (Figure 1, structure **3**). It can be classified between the basic building blocks GDGT/GDNT and, for example, the higher functionalized S-MPL (Figure 1, structure **4**). In the literature, analogously derived structures of GDNT with a hexose bound, in undefined fashion, to the backbone, have been established as GDNT-Hex.[9,16] In contrast to this molecule, we could exactly determine the binding situation of the hexose for GDNT-β-Glu. It not only allows the investigation for the targeted design of new tailor-made TEL, but the knowledge of the exact chemical structure also provides important insights into the biosynthesis of these molecules as well as aiding the investigation and understanding of its self-assembling behavior.

2. Results and Discussion

2.1. GDNT-β-Glu from Sulfolobus metallicus

First, the results of extensive studies on the chemical structure of GDNT-β-Glu from Sulfolobus metallicus are shown and discussed. In principle, the TEL was isolated and purified for structure elucidation by different NMR investigations. In order to allow a stepwise analysis of the individual components of GDNT-β-Glu, the basic compounds **1** and **2** were also isolated and investigated as a reference. Since it was found that more complex compounds have only a moderate solubility in deuterated solvents, especially over a longer period, all compounds in this work were investigated in THF-d_8 for a proper comparison to each other. However, this solvent and the fact that proton spectra of TEL show large overlaps of the signals of different moieties within the molecules make it not always possible to get enough clear information from 1D NMR (^{1}H and ^{13}C) experiments for structure elucidation. Therefore, structure-relevant information of polar head groups were obtained by various 2D NMR experiments such as Heteronuclear Single Quantum Coherence (HSQC), HSQC-Total Correlation Spectroscopy (HSQC-TOCSY), Heteronuclear Multiple Bond Correlation (HMBC), Heteronuclear 2 Bond Correlation (H2BC), Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) and Rotating-Frame Nuclear Overhauser Effect Spectroscopy (ROESY). A short description of the utility of these NMR techniques, as well as a detailed look on the other experimental techniques, calculations, biotechnological production of TEL,

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TEL isolation and all NMR-spectra can be found in the Supporting Information.

The three different compounds **1**, **2** and **3** differ in their structure mainly by the number of cyclic polyol units bound to one of the functional groups of the glycerol units (zero,[7e] one or two units). The additional carbon atoms of these units can be found in their corresponding ^{13}C NMR spectra displayed in Figure 2. Only the chemical shift region of about 60–95 ppm undergoes a modulation – essentially, the incorporation of the polyols leads to additional occurrence of further signals and some additional shifts. Chemical shifts in the coloured area of 60–65 ppm are characteristic for methylene groups bound to primary hydroxyl functions. This is interesting because this function is often blocked by a connection to another polyol, such as it is the case in, for example, compound **4**. This means
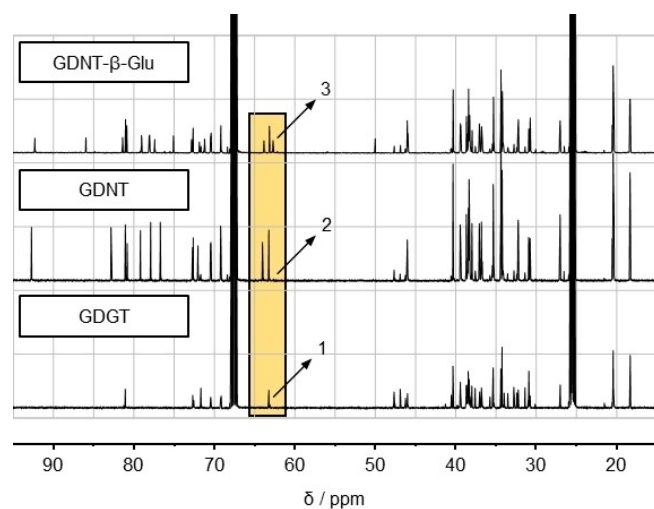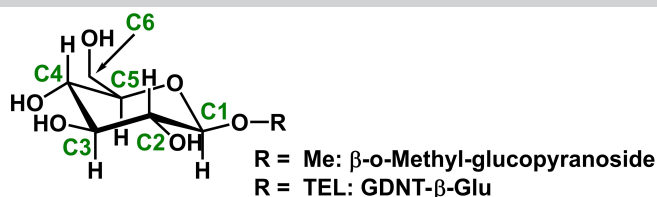
for the investigated compound **3**, the additional hexose unit is not connected over the methylene unit, but has to be connected over a different hydroxyl unit of the polyol.

In previous work in the field of structure elucidation of TELs originating from *Sulfolobus* strains, it was shown that the sugar end sequence bound to the end groups of the GDNT skeleton is always a glucose unit.[11] This can also be confirmed in the case of GDNT-β-Glu. Table 1 shows data of the ^{13}C NMR experiments of the bound hexose unit of GDNT-β-Glu (Figure S18) and a reference spectrum of commercially available β-*o*-Methyl-glucopyranoside (Figure S33), as well as H2BC- (Figure S23) and HSQC-TOCSY-derived signals (Figure S26) of GDNT-β-Glu recorded in THF-d$_8$. The chemical shifts in the ^{13}C spectrum and specific signals from HSQC (Figures S19/20) and H2BC experiments show that the hexose in GDNT-β-Glu obtained from *Sulfolobus metallicus* must be a β-glucose. The coupling of *H1* at 7.6 Hz from the HSQC can be clearly assigned to a β-pyranose (the designations H1…H6, H6' and C1…C6 correspond to the usual ones used for sugars; C1 denotes the anomeric carbon atom). Furthermore, ROESY reveals that there is a spatial interaction between *H1*, *H3* and *H5* – so these should be located on one side in the case of β-glucose, which is therefore a strong indicator of this sugar.

To find the correct carbon atom which connects the calditol unit to the β-glucose unit, data of the HMBC of GDNT-β-Glu (Figures S21/22) are used and reveal an interaction of the anomeric *C1* to a proton with a chemical shift of 4.00 ppm which belongs to the spin system of the cyclic polyol of the calditol function. It is correlated to the carbon atom labeled with **H** in structure **3** (Figure 1). Further interactions between atoms of the two spin systems which could indicate a connection of the hexose cannot be observed. It is also possible to detect two free primary hydroxyl functions, one belonging to the calditol function and the other one corresponding to the β-glucose unit. This is another proof that the binding of the glucose must be assigned to another hydroxyl function.

Figure 2. Stacked ^{13}C NMR spectra in THF-d$_8$ of GDGT **1**, GDNT **2** and GDNT-β-Glu **3** (derived from *Sulfolobus metallicus*) in the range from 15 to 95 ppm. The stepwise increase in signals from carbon atoms bound to free primary hydroxyl groups (1 to 3) is obvious.

Table 1. Chemical shifts from the ^{13}C NMR, H2BC and HSQC-TOCSY experiments of GDNT-β-Glu (Figures S18, S23, S26) and the ^{13}C NMR experiment of beta-*o*-methyl-glucopyranoside (Figure S33) for comparison.

R = Me: β-*o*-Methyl-glucopyranoside
R = TEL: GDNT-β-Glu

Carbon no.	Hexose in GDNT-β-Glu			β-o-Methyl-glucopyranoside
	^{13}C Chemical Shift [ppm]	Signals derived from HSQC-TOCSY [ppm]	H2BC-Signals [ppm]	^{13}C Chemical Shift [ppm]
C1	104.8	4.46 (JH–H = 7.6 Hz)	3.22 (H2) 3.26 (H5)	105.7
C2	75.1	3.22	3.32 (H3) 4.46 (H1)	75.2
C3	78.1	3.32	3.22 (H2) 3.32 (H4)	78.4
C4	71.3	3.32	3.26 (H5) 3.33 (H3)	71.8
C5	78.1	3.26	3.32 (H4) 3.64 (H6) 3.77 (H6')	77.9
C6	62.7	3.77 3.64	3.26 (H5)	63.3

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In the case of the cyclic polyol of the GDNT backbone, distinct chemical shifts in the HMBC can be assigned to the atoms at the following positions (see proposed chemical structure in Figure 1): **F** – due to the mutual coupling with atoms at positions **C**, **D**, and **E**; **J** due to the unambiguous signal of a quaternary carbon atom in the [13]C Attached Proton Test (APT) spectrum, and **K** due to the already described nature of the attached primary hydroxyl group. The assignment of the further signals is done accordingly to the assignment of the glucose characterization – the signals of the described spin system are known from a HSQC-TOCSY experiment. By TOCSY and H2BC experiments, the corresponding chemical shifts in the respective ^{1}H and ^{13}C NMR spectra can also be attributed to the positions **G**, **H** and **I**. With regard to the stereochemistry, it can be seen from the ROESY experiment (Figure S25), that on one hand the protons at positions **F** and **G**, and, on the other hand, those at **H**, **I** and **L** are spatially close to each other. Based on the data described so far, it can be shown that the head group structure of GDNT-β-Glu isolated from *Sulfolobus metallicus* is definitely the one shown in structure **3** (Figure 1).
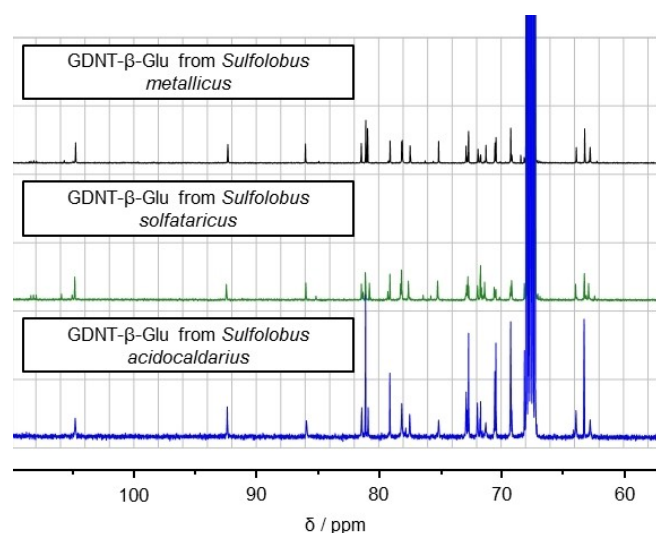

Figure 3. Stacked ^{13}C NMR spectra of GDNT-β-Glu from *Sulfolobus metallicus*, *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* in the polar head group region. The general position of the signals is the same for all three different strains

2.2. GDNT-β-Glu from Different Sulfolobus Strains

The extensive investigations above are based on the analysis of substances isolated from *Sulfolobus metallicus* biomass of high purity. It is also highly probable that the isolated GDNT-β-Glu is a part of other *Sulfolobus* strains such as *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*, too. In order to investigate whether the GDNT-β-Glu isolated from *Sulfolobus metallicus* can also be found in other *Sulfolobus* species, a simple comparison is done.

Figure 3 shows stacked ^{13}C NMR spectra of GDNT-β-Glu derived from the three species, clearly indicating that the chemical structure is obviously nearly identical. Table 2 lists the corresponding chemical shifts of chosen characteristic carbon atoms which can be extracted from the respective spectra, even though the GDNT-β-Glu fractions' purities were not ideal. With **E***, **K** and **Q**, carbon atoms are chosen which carry a free primary hydroxyl group. **L** is the anomeric carbon atom of the hexose. Finally, **H** and **J** are characteristic positions in the cyclic polyol of the calditol unit – **H** is bound to the hexose, and **J** is the only quaternary carbon.

It becomes clear that all signals detected for GDNT-β-Glu of *Sulfolobus metallicus* can also be found in the compounds of the other two archaea species with very little deviation. It can thus be concluded that the chemical structure of GDNT-β-Glu presented here represents an essential structural feature of all MPL of these *Sulfolobus* species.

2.3. Degree of Cyclization

As already mentioned in the introduction, TEL can carry a different amount of cyclopentane units within the biphytanyl part of the backbone. After the head group structure of GDNT-β-Glu is now elucidated and clearly detected in all three *Sulfolobus* strains, there are still pronounced differences in the degree of cyclization. Hence, we performed mass spectrometry (MS) experiments to classify the degree of cyclization.

Figure 4 shows the mass range relevant to the study of the described GDNT-β-Glu extracts. Especially in Figure 4a, the respective differences are clearly visible. While GDNT-β-Glu from *Sulfolobus metallicus* and *Sulfolobus acidocaldarius* have a similar distribution pattern in the mass spectrum, the GDNT-β-Glu from *Sulfolobus solfataricus* clearly shows a shift to lower

Table 2. Chemical shifts of selected characteristic carbons in the ^{13}C NMR spectra of the GDNT-β-Glu fractions from *Sulfolobus metallicus*, *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. The corresponding spectra can be found in the Supporting Information (Figures S18, S31, S32).

Carbon atom[a]	GDNT-β-Glu – Characteristic chemical shifts [ppm]		
	Sulfolobus metallicus	*Sulfolobus solfataricus*	*Sulfolobus acidocaldarius*
E*	63.1	63.3	63.2
F	92.3	92.5	92.4
H	86.0	85.9	85.9
J	80.9	80.8	80.9
K	63.8	63.9	63.9
L	104.8	104.8	104.8
Q	62.7	62.9	62.7

[a] The labeling refers to the chemical structure proposed in structure **3** (Figure 1).

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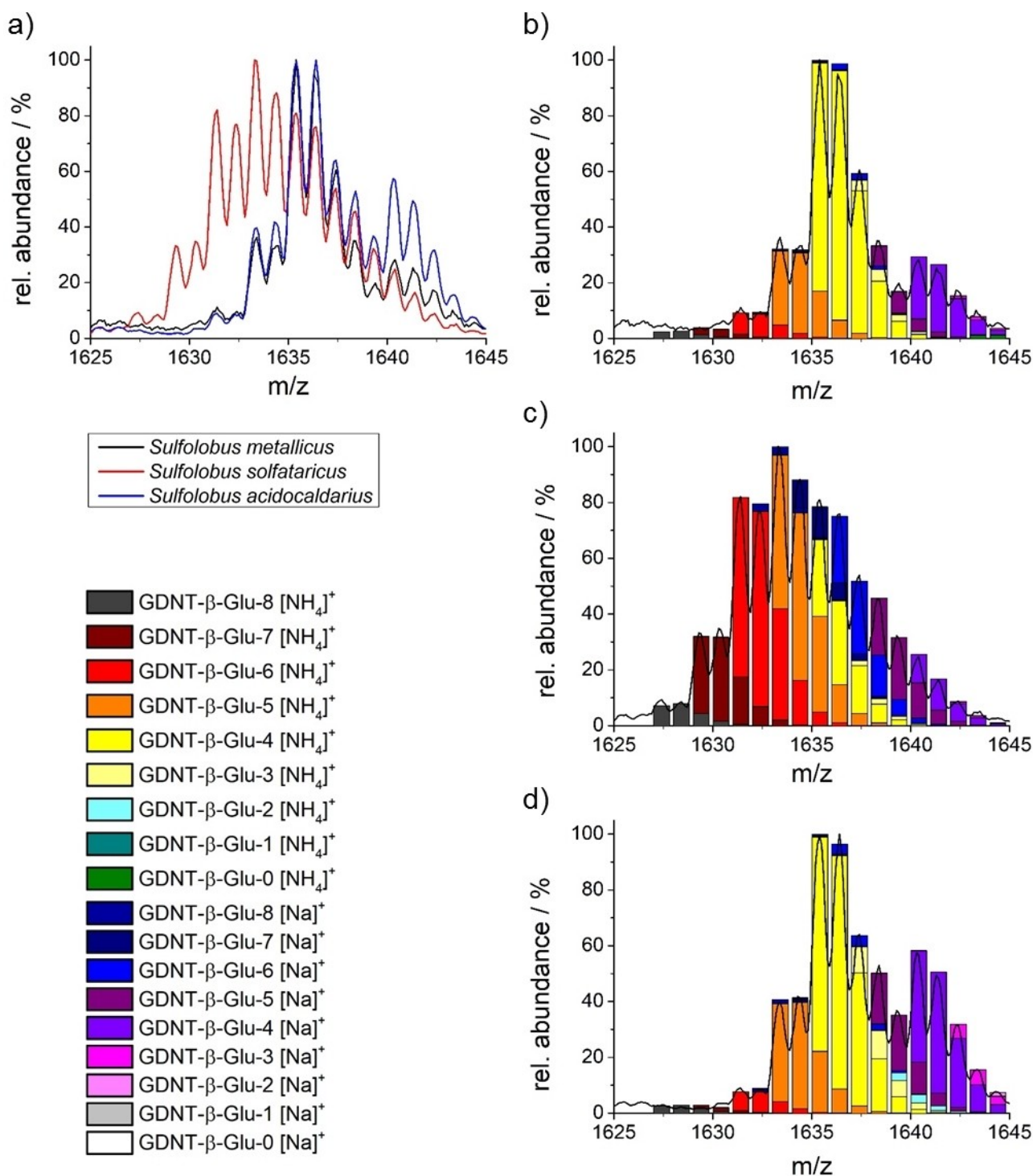European Chemical
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Figure 4. Mass-spectrometric analysis of the GDNT-β-Glu fractions. a) Representation of the experimental data of all three derivatives. Mass spectra of GDNT-β-Glu obtained from b) *Sulfolobus metallicus*, c) *Sulfolobus solfataricus* and d) *Sulfolobus acidocaldarius* including the distribution of the cyclization degree.

m/z values. Although these spectra are not directly suitable to find the distribution of the degree of cyclisation, it becomes apparent that all three GDNT-β-Glu derivatives differ in the distribution of their degree of cyclization (Figure 4b–d).

The nature of the molecules makes it necessary to use more energy than normal to get a signal from the ionized derivatives in the MS. This means that the fragmentation probability of the molecule gets higher. To avoid high energies, it is possible to use larger ions as carriers such as ammonium (NH_4^+, $M = 18.0$ g mol^{-1}) and sodium (Na^+, $M = 23.0$ g mol^{-1}). Therefore, TEL with different degrees of cyclization bound to these ions are mainly detected. In total, 18 different species are expected – 0 to 8 cycles per molecule in connection with two different ions – that superimpose each other in the spectrum.

Table 3. Overview of the proportions of GDNT-β-Glu molecules with different degrees of cyclization (theoretical derivation on the base of the experimental data for the least squares of errors).

Strain	Portion of degree of cyclization [%]									Na-factor
	8	7	6	5	4	3	2	1	0	
Sulfolobus metallicus	2	2	6	21	65	3	0	0	1	0.27
Sulfolobus solfataricus	4	15	35	30	15	1	0	0	0	0.37
Sulfolobus acidocaldarius	2	1	5	26	57	7	2	0	0	0.52

Based on the experimental data, the most probable cyclization distribution was calculated by the least squares method (SLA, see Supporting Information). Since sodium is a heavier ion than ammonium, the sodium adducts can be found at higher m/z-values. Because the GDNT-β-Glu extracts were isolated from different strains and have been purified in different ways, sodium adducts can occur in different ratios. This circumstance was taken into account by considering a sodium factor (Na-factor). This additional parameter was added to find the best calculated distribution for the experimental data. The factor, which gave out the best suitable distribution, as well as the numerical values of the proportions of the respective degree of cyclization are shown in Table 3. It states that GDNT-β-Glu from *Sulfolobus metallicus* and from *Sulfolobus acidocaldarius* have a very similar distribution of the cyclopentane units. In more detail, the four-ring variant shown in Figure 1 dominates with 65% in *Sulfolobus metallicus*, followed by the representative with five cyclopentane units (21%). In the case of *Sulfolobus solfataricus*, there is a shift towards a higher degree of cyclization (6 rings: 35%, 5 rings: 30%, and 7 and 4 rings: 15%). With *Sulfolobus acidocaldarius*, the four-ring variant again represents the largest fraction with 57%, followed by 26% of the five-ring variant.

However, a deeper mathematical analysis reveals that the proportion of four cyclopentane units in *Sulfolobus metallicus* is almost 10% higher than that of *Sulfolobus acidocaldarius*. In addition, there is a narrower distribution. Strong deviations are found in GDNT-β-Glu from *Sulfolobus solfataricus*. Here, mainly the five- and six-ring variants are present in almost equal proportions, followed by the four- and seven-ring TEL derivatives. This shift towards a higher degree of cyclization is expected because *Sulfolobus solfataricus* was cultivated at a higher temperature of 85°C than *Sulfolobus acidocaldarius* at 70°C. The growth conditions for *Sulfolobus metallicus* are not exactly known, but due to their reference as a by-product of an industrial process, it can be assumed that (in relation) moderate temperatures prevailed here. This would lead to the conclusion that, by controlling the growth conditions, quite specific GDNT-β-Glu derivatives can be preferentially prepared; for example, lower temperatures apparently result in a narrower distribution of the number of ring moieties.

3. Conclusion

Within the scope of the present study, the GDNT-β-Glu obtainable from archaeal *Sulfolobus* strains could be isolated in pure form and described comprehensively with respect to the chemical structure. Remarkably, the determined structure was not yet published for any *Sulfolobus* species. However, it could be demonstrated that the described GDNT-β-Glu can be isolated as a main component from *Sulfolobus metallicus*, *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. Furthermore, the nature of the polar head group was identical in all cases. Therefore, it can be assumed that the described GDNT-β-Glu represents an essential structural feature of the intact lipids of these species. From a chemical point of view, it also represents a very interesting intermediate. The actual practice, with regard to a more intense exploitation, suffers from the circumstance that TEL are very difficult to isolate and that the so-far achieved yields are extremely low. GDNT-β-Glu seems to be a very good compromise – it can be isolated in relatively high yields and contains of a sufficient number of polar functions. The in-depth structure elucidation in this work now allows the further investigation of the newly isolated TEL species for self-assembling behavior and the formation of supramolecular structures.

Conflict of Interest

The authors declare no conflict of interest.

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