

Resemblance of Carbocycle Formation from Carbohydrates between Archaea and Eucarya/Eubacteria. Biosynthesis of Calditol, the Characteristic Lipid-Content Molecule in *Sulfolobus acidocaldarius*

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We investigated the biosynthesis of the unique structure of calditol, a part of the characteristic lipid molecules in the major part of thermophilic archaea, *Sulfolobus* sp. Deuterium-labeling experiments with deuterated glucose substrates, were carried out. The results clarified that calditol was biosynthesized from glucose with C–C bond formation between C-1 and C-5, and no loss of deuterium at C-6 of glucose was observed. Two reaction mechanisms were possible when these experimental findings, and conventional reports were considered. Among these, the course that assumes C-4 oxidation as the starting point would be preferable from the resemblance to *myo*-inositol biosynthesis. Furthermore, the high deuterium incorporation at C-1 of calditol from C-1 of glucose suggests that activation, such as oxidation at C-1, is not involved in the ether C–O bonding.

Archaea are clearly distinguished from eucarya and eubacteria by their 16S ribosomal RNA sequences, and constitute the third domain in the phylogenetic tree.[1] Among them, thermophilic archaea live under harsh conditions for normal living organisms, such as at high temperatures (above 100 °C) and low pH (1 to 3). The resemblance of their living environment to primitive Earth conditions has been pointed out. Furthermore, some investigators regard the archaea as the oldest among living organisms based on their molecular biological characteristics. In particular, their membrane lipid patterns are characteristic and quite different from those of eucarya and eubacteria. The existence of an unusual ether lipid in archaea is one of the clearest distinctions between archaea and other organisms, and the lipids of archaea characteristically consist of saturated isoprenoids bonded with glycerol by an ether-linkage.[2] The stereochemistry of the ether linkage from glycerol is *sn*-2,3-di-O-alkylated, which is antipodal to other organisms, which are *sn*-1,2-di-O-acylated. We have been interested in the biochemical features of these archaeal lipids; recently, the characteristic biosynthetic pathway of isoprenoidal lipids in halophilic archaea was reported.[3] Furthermore, thermophilic archaea have di-bisphytanylated ethers as a membrane lipid component. Furthermore, these macrocyclic tetraether-containing lipid molecules form a double-face monolayer membrane. These peculiar lipid molecules form remarkably stable liposomes and lipid films compared with other "ordinary" lipid molecules from eucarya and eubacteria, while other archaea, such as halophilic archaea, have di-phytanyl ethers that form the usual double-layer membrane as the lipid component.[4] The diglycerol tetraether structure **1** has been termed diglycerocardarcheol or GDGT (glyceroldialkylglycerol tetraether). The structure of GDGT was finally confirmed by a synthetic approach.[5] The total synthesis of **1** and the preparation and observation of the properties of liposomes from derivatives of **1** were carried out recently.[6]

Furthermore, the existence of a 5-membered ring part, such as **2** in the saturated isoprenoid portion at the lipid-core of archaea, has been reported. Archaea change the number of 5-membered rings and the ratio of lipids including the 5-membered ring with the environmental growth temperature, and alter the properties of the cell membrane to adapt themselves to a high-temperature environment.[7] Recently, it has been discovered that there are archaea around 1000 m from the marine outer layer. The oceanic archaea (pelagic euryarchaeota and pelagic crenarchaeota) have a 6-membered ring structure in a saturated isoprenoid chain at the lipid core at the same body in a lipid that contains a 5-membered ring. In addition, the temperature and a change in the ratio of lipids including the 5-membered ring portion were observed, as mentioned above.[8]

Among the thermophilic archaea, the genus *Sulfolobus* (belonging to the order *Sulfolobales*), which account for one of the main parts of the thermophilic archaea, have a characteristic lipid termed carditoglycerocaldarcheol, or GDCT (glyceroldialkyl calditol tetraether) **3**, as shown in Fig. 1.

The structure of **3** had been believed to be the ether linkage of polyol to glycerol (nonitol structure **4**), as in the structure determination of De Rosa et al. in 1977.[9] Molecule **4** had been termed dialkylnonitol tetraether (GDNT), and the structure had been accepted for a long time. Nicolaus et al. reported a proposal for the biosynthesis of nonitol structure **4** from their ^{14}C- and ^{3}H-incorporation experiments.[10] The starting material was glucose (or fructose) and dihydroxyacetone; an aldol-type C–C bond formation of the two molecules was proposed.

However, the structure of **3** (**4**) was proposed to be cyclic polyol by Langworthy et al. in 1974, although the structure was not determined.[11] Furthermore, synthetic studies of the nonitol structure revealed doubts as to the structural determination of the first nonitol structure.[12] In addition, two independent structural determinations of calditol structure indicated a mis-

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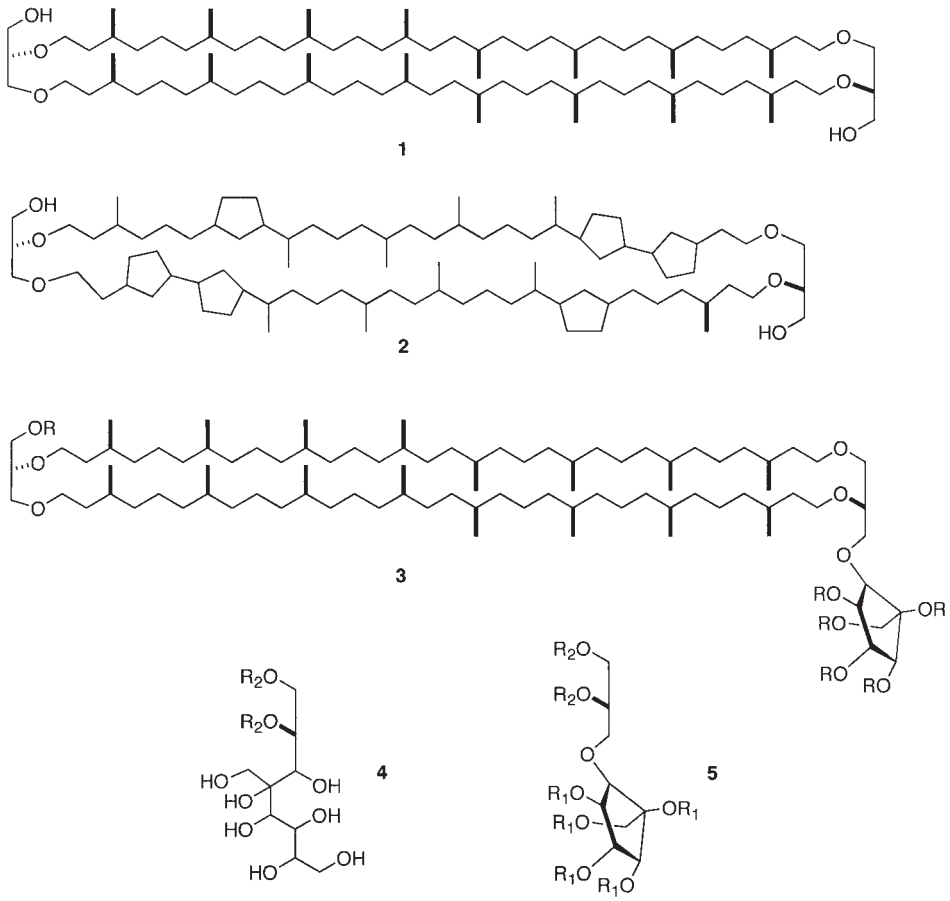

Fig. 1. Structure of the lipid core and related compounds in *Sulfolobus*.

take in the open-chain structure of calditol. Sugai et al. (using *Sulfolobus acidocaldarius*)[13] and Arigoni et al. (using *Sulfolobus solfataricus*) showed the 5-membered-ring structure of calditol **5**. The synthesis of the new calditol structure by Siney et al. revealed the correct structure and the stereochemistry of the hydroxy groups in **5**, as shown in Fig. 1.[15]

Compared with isoprenoidal chains and glycerol, ether-bond formation of glycerol and calditol is in a more hindered position three-dimensionally. Needless to say, an ether bond is more stable for an acid than ester and glycoside bonding. It seems that the existence of calditol is the one of the reasons why *Sulfolobus* is suitable for growth under low pH and high-temperature conditions. It is interesting to observe the timing and mechanism of carbocycle formation and ether bond formation in the stages of calditol biosynthesis. In addition, it is also intriguing what kind of enzyme is involved in the ether bond formation of calditol and glycerol.

A rereading of the report on biosynthetic studies of the old structure of calditol showed that the carbocyclic structure was formed from glucose, although the structure was not correct.[10] So far, the formation of many carbocyclic compounds from carbohydrates has been proved and the mechanistic implications of the biosynthesis of each compound have been discussed. In the biosynthesis of five-membered ring carbocycles, Rinehart et al. pointed out that pactamycin produced by *Streptomyces pactum* was biosynthesized from ring closure at C-1 and C-5 of glucose.[16] Parry et al. showed the precise mechanism of the formation of the 5-membered ring portion of aristeromycin and neplanocin A.[17,18] They proved that a C–C bond was formed between the C-2 and C-6 carbons of glucose, and proposed the formation of a fructose-like intermediate.

Furthermore, Sakuda et al. recently studied the cyclopentane ring formation of allosamizoline, a part of a *Streptomyces* metabolite (a chitinase inhibitor), allosamizine.[19] They observed C–C bond formation of the C-1 and C-5 carbons of glucose, retention of the C-3 and C-4 hydrogens, and stereospecific loss of pro-*R* hydrogen during the ring closure. These results suggested the involvement of NAD(H)-dependent oxidoreduction for the activation of aldol condensation-type ring closure of glucose. This NAD(H)-dependent catalytic oxidoreduction was also observed in the 6-membered ring formation from carbohydrates, like *myo*-inositol-1-phosphate,[20] dehydroquinate,[21] and 2-deoxy-*scyllo*-inosose (the key intermediate of deoxystreptamine-containing antibiotics).[22] Further mechanistic implications were discussed. In addition, trehazoline, a potent trehalase inhibitor isolated from *Micromonospora*, had a substituted cyclopentane structure with the same functional group as calditol (hydroxymethyl substituent and quaternary center at the substituent). C–C bond formation at C-1 and C-5 of glucose was also observed.[23]

A reinvestigation of the biosynthesis of the 5-membered ring in calditol was reported very recently by Gambacorta et al.[24] They proposed that the mechanism for the carbocycle formation of this part was just like the *myo*-inositol ring closure from the incubation experiment of C-3 and C-4 deuterated glucose. However, several ambiguities have remained. The ring closure

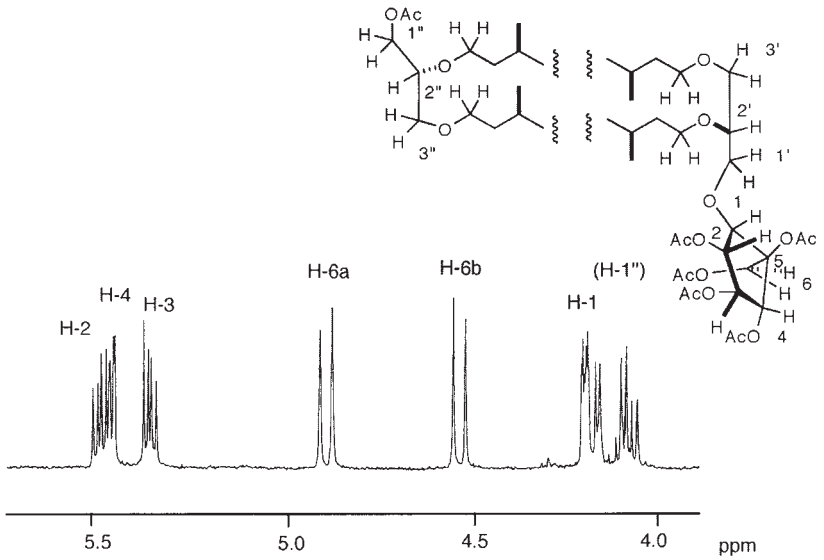

Fig. 2. Partial ^{1}H NMR and assiginments of each hydrogen of GDCT (acetate derivative).

Table 1. Assignments of the Hydrogens of GDCT at the Calditol-Portion

Hydrogen	Chemical shift	Coupling constants/Hz
H-1	4.20	2.4 (H-4, "W-type coupling") and 4.6 (H-2)
H-2	5.47	4.6 (H-1) and 8.5 (H-3)
H-3	5.35	8.5 (H-2) and 4.0 (H-4)
H-4	5.43	2.4 (H-1, "W-type coupling") and 4.0 (H-3)
H-6a	4.52	13.4
H-6b	4.89	13.4
(H-1″)	4.10 and 4.19	5.7 and 11.0

W-type coupling was observed at H-1 and H-4 of calditol.

mechanism and the C–O bond formation between the cyclopentane ring and glycerol should be examined by precise observations of the labeling experiments. Thus, we investigated the precise mechanistic implications for the formation of the unique structure of calditol using deuterium labeling, and tried to predict the "timing" of the ether-bond formation in the calditol structure. Fortunately, the labeling pattern and the degree of deuterium incorporation can be clearly observed by a ^{1}H NMR measurement with GDCT acetate.

Assignment of Ring Protons of GDCT Acetate at ^{1}H NMR

At first, deuterium incorporation was evaluated with underivatizated GDCT, but it was difficult to compare the precise incorporation of deuterium with no doubtful standard about the integral value of hydrogens at the ring proton in the glucose and the product. Furthermore, the signal separation of each ring proton was not sufficient for estimating the deuterium incorporation. Thus, GCDT was converted to its acetate according to the results of Gambacorta et al.,[24] and, the assignment of each proton was carried out. GCDT acetate was prepared by pyridine–acetic anhydride–catalytic 4-dimethylaminopyridine and purified by silica gel. Acetyl-CH_3 proton, the non-deuterated proton standard, could be introduced by the procedure. Furthermore, all of the signals of the protons on the cyclopentane-ring portion of calditol were separated by the acetylation shift. The C-1 proton of calditol was ether-bonded and not shifted, and could be distinguished from other protons. From decoupling experiment and H–H COSY measurements, all of the protons on the cyclopentane ring could be assigned as shown in Fig. 2 and Table 1. Unfortunately, the C-1 proton of calditol and the acetylated hydroxymethyl CH_2 proton on the other side of the tetraether lipid overlapped, but would be distinguishable in the labeling experiment by a slight difference in the chemical shift. Furthermore, labeling with ^{2}H on the proton on the cyclopentane-portion would cause a change in the proton–proton coupling.

Deuterium-Labeling Experiment

Deuterium-labeling experiments with each of [1-^{2}H]-, [2-^{2}H]-, [6,6-$^{2}H_2$]- and (6*S*)-[6-^{2}H]-D-glucoses were carried out to investigate the cyclization mechanism and verify the proposal of Gambacorta et al.[24] Usually, deuterium incorporation experiments can only be observed at ^{2}H NMR because the deuterium incorporation is so small. The natural abundance of deuterium content (0.02%) allows the observation of labeled compounds selectively with ^{2}H NMR. However, several experiments to observe the deuterium contents using ^{1}H NMR have been reported for feeding experiments of lipid biosynthesis of archaea with deuterated starting materials.[24,25] As was the case for experiments of Gambacorta et al., the incorporation of deuterium was observed qualitatively. Their experiments were as follows: (1) fully deuterated glucose + non-labeled glucose (1:1) were administered with virtually no loss of deuterium at C-1, C-2, C-3, C-4, and C-6 of glucose; (2) [3-^{2}H]glucose

was administered and deuterium was observed at C-3 of calditol with no rearrangement or transformation; (3) [4-^{2}H]glucose was administered and deuterium was observed at the C-4 of calditol also without rearrangement. Therefore, they proposed a *myo*-inositol-like cyclization mechanism and that involvement of oxidoreduction of C-4 was the predominant pathway of the cyclization.

Our results are shown in Fig. 3 by ^{1}H NMR charts; the incorporation percentages that were obtained by the integration of ^{1}H NMR are summarized in Table 2. At first, [6,6-$^2H_2$]glucose was administered and the incorporation pattern was observed. Deuterium was clearly incorporated at the C-6 methylene position (spectrum A compared with spectrum E) with the same degree of incorporation as that shown in Fig. 3 (about 50% incorporation of deuterium). This result suggested that the C-6 hydrogen of glucose did not participate during the ring-formation reaction due to the deuterium labeling of [6,6-$^2H_2$]glucose being taken in by the high efficiency and the same degree of incorporation to the C-6 hydrogen of calditol. However, if a non-stereospecific reaction occurred on the C-6 hydrogen (for example, non-enzymatic enolization at C-5 and C-6), the degree of C-6 hydrogen labeling was equally observed at each methylene hydrogen in calditol. A compound stereoselectively deuterated at one of the prochiral hydrogens at C-6 of glucose was prepared, and the fate of the labeling was observed. (6*S*)-[6-^{2}H]glucose was synthesized according to the method of Ohrui et al.,[26] and an incorporation experiment was performed. The calditol provided by a labeling experiment of (6*S*)-[6-^{2}H]-

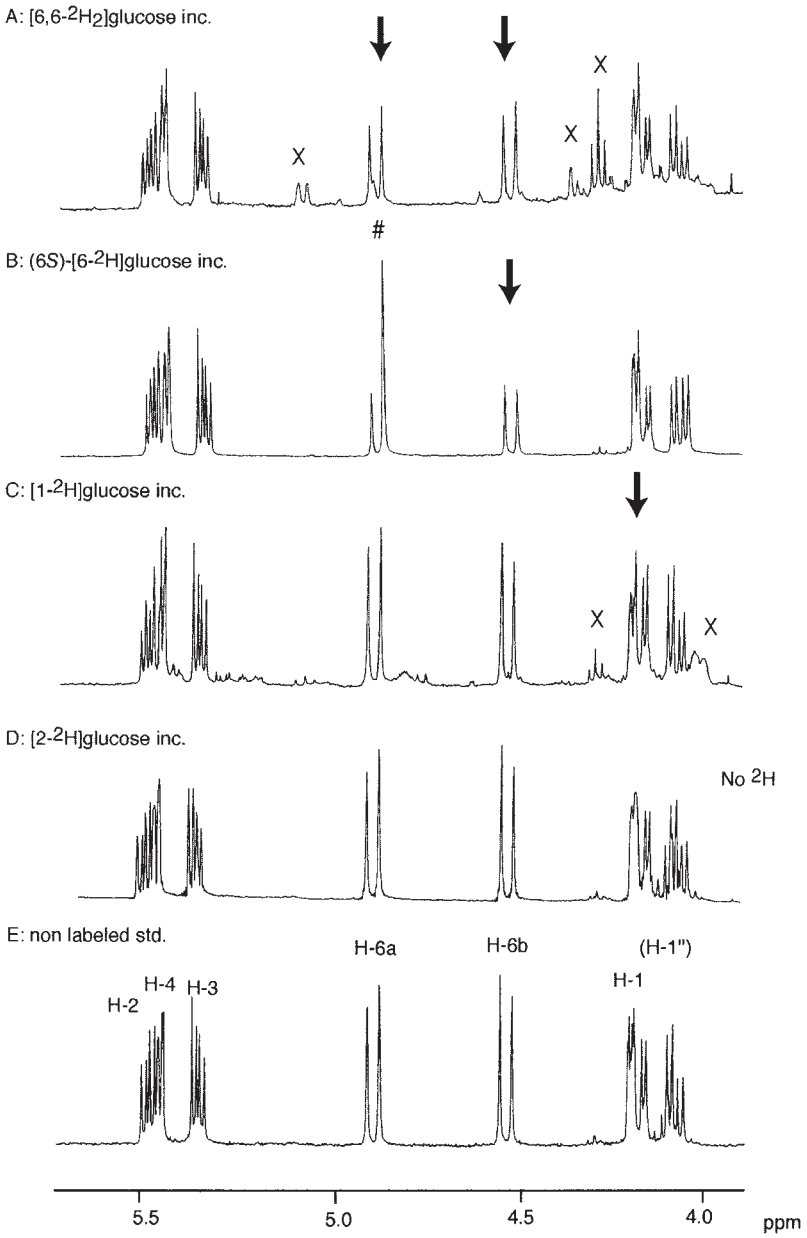

Fig. 3. Partial ^{1}H NMR of calditol in deuterium-incorporation experiments. In this figure, peaks marked by × represent signals of contaminated material. The peaks marked by # in spectrum B represents the overlap of the singlet signal derived from the deuterium-incorporated compound and the doublet signal derived from non-labeled compound.

Table 2. Integral Value and Deuterium Incorporation Value at Each Hydrogen of Calditol in Spectra A to E in Fig. 2

	H-2,3,4	H-6a	H-6b	H-1 + H-1″	Site (^{2}H %)
A: [6,6-$^2H_2$]Glucose	2.98	0.60	0.60	3.05	H-6a,b (40%)
B: (6*S*)-[6-^{2}H]Glucose	2.99	0.98	0.52	2.73	H-6b (48%), H-1″ ?
C: [1-^{2}H]Glucose	3.04	0.99	1.02	2.38	H-1 (62%)
D: [2-^{2}H]Glucose	3.01	0.99	0.98	(3.11)	No (0%)
E: Non-labeled	3.01	1.00	1.05	3.01	No (0%)

The standard for the number of the hydrogen was settled 18 at all acetyl-CH_3 introduced after the incorporation experiment. Hydrogen numbering was shown in Fig. 2 and Table 1.

glucose was deuterated with high efficiency. The degree of labeling was similar to that of the same experiment performed with [6,6-$^2H_2$]glucose. Furthermore, on the C-6 methylene hydrogen of calditol, the one hydrogen was specifically deuterium-labeled (spectrum C). From these results, it became certain that the two C-6 hydrogens did not participate in the process of this reaction.

Next, experiments with deuterium labeling of C-1 of glucose ensured that cyclization occurred between the C-1 and C-5 carbons of glucose. It is possible that the hydrogen of the C-4 of glucose becomes that of C-1 of calditol and that the ether-bond is formed between the C-1 of calditol (originating from C-4 of glucose) and glycerol in the reaction mechanism. The deuterium at C-1 of glucose was incorporated at C-1 of calditol (Fig. 3, spectrum D) with high efficiency, as shown in Table 2. These results clarified that calditol was biosynthesized from glucose with C–C bond formation between C-1 and C-5. In addition, it became clear that the C-1 hydrogen of glucose was not lost during this reaction.

Next, the fate of the C-2 hydrogen was investigated. The spectrum of a [2-^{2}H]glucose labeling experiment almost agreed with the spectrum of calditol without a label, as the deuterium of the C-2 of glucose was completely lost during the biosynthesis of calditol. Two possibilities arise from the complete disappearance of the C-2 hydrogen. The first possibility was the loss of deuterium during the equilibrium of glucose-6-phosphate and fructose-6-phosphate in an initial stage of the glycolytic system. The second was the loss of deuterium in the reaction between the C-2 and C-6 carbons of glucose, such as occurs in the case of aristeromycin reported by Parry.[18] The latter possibility was excluded by the C-6 labeling experiment mentioned above. If the C–C bond formation occurred between the C-2 and C-6 of glucose, the methylene hydrogen of the hydroxymethyl substituent of calditol should have originated from the C-1 hydrogen of glucose. The result mentioned above is a pathway occurring via the former possibility. These labeling experiments and the observations of Gambacorta et al.[24] about the behavior of the C-3 and C-4 hydrogens strongly suggest that the cyclizaton reaction was triggered by the oxidation of the C-4 of glucose, an increase in the acidity of the C-5 hydrogen, and an aldol-like condensation between C-1 and C-5 of glucose. In addition, the C-4 is reduced at a point in time before or after the ring-formation reaction (aldol reaction). Regarding the C-4 hydrogen of glucose, the relative stereochemistry compared with the other hydroxy groups is reversed in calditol compared with glucose at this point, and the existence of some oxidation–reduction is regarded as the reason for the inversion.

Discussion

The mechanism of the cyclization reaction of calditol was reconsidered using the results of these experiments. A study of the reaction mechanisms of the process that produces five-membered and six-membered ring compounds and consideration of the results of Gambacorta et al. indicate several kinds of mechanisms. The possible mechanisms are shown in Fig. 4. From conventional studies of the conversion of carbohydrates to carbocycles, the first step in all the reaction mechanisms is the same oxidation of one hydroxy group in the carbohydrate, and one hydroxy group is activated on the occasion of the cyclization. When the degree of oxidation of a starting material for calditol is considered, the oxidation state is equal for the starting material and the product. Therefore, it becomes necessary for a reduction reaction to be caused after the activation by oxidation. The possible mechanisms are illustrated in Fig. 4 from conventional reports when the oxidation reaction of each carbon of glucose is used as the starting point. It seems that the C-2 carbon of glucose can be regarded the same as in the biosynthesis of aristeromycin for the oxidized system.[18] However, since in this case it is bonding of the C-2 and C-6 of glucose, it seems to be different from the system of calditol having bonding of the C-1 and C-5 of glucose. The oxidation of C-3 is thought to be about the same as in the reaction of dehydroquinate synthase where the oxidized system is a key reaction in the biosynthesis of an aromatic amino acid.[21] As for this system, a study of the enzyme itself, and clarification of the reaction mechanism for an enzyme involved in the carbocyclic ring formation reaction have proceeded. After activation by the oxidation of a carbon in the β-position from the oxidized hydroxy group, an enolate-like intermediate product causes β-elimination, nucleophilic attack on the carbonyl carbon occurs, and the hydroxy group first oxidized is reduced in the reactive site with the same cofactor molecule. In a total sequence of the enzyme reaction, the cofactor is used with a catalytic amount ("catalytic oxidation–reduction"). As for the formation mechanism of this compound, 2-deoxy-*scyllo*-inosose synthase, the key intermediate of the carbocyclic compound 2-deoxystreptamine (a part of the major group of aminoglycoside antibiotics, such as neomycin), has a similar mechanism.[22] However, the C-4 hydrogen of glucose should be completely lost when the reaction proceeds with a similar mechanism to dehydroquinate synthase compared with calditol, and this is excluded by the result showing a retention of the C-4 hydrogen in the report by Gambacorta et al.[24] In addition, after cyclization, the introduction of a hydroxy group to the C-5 of calditol is necessary. This

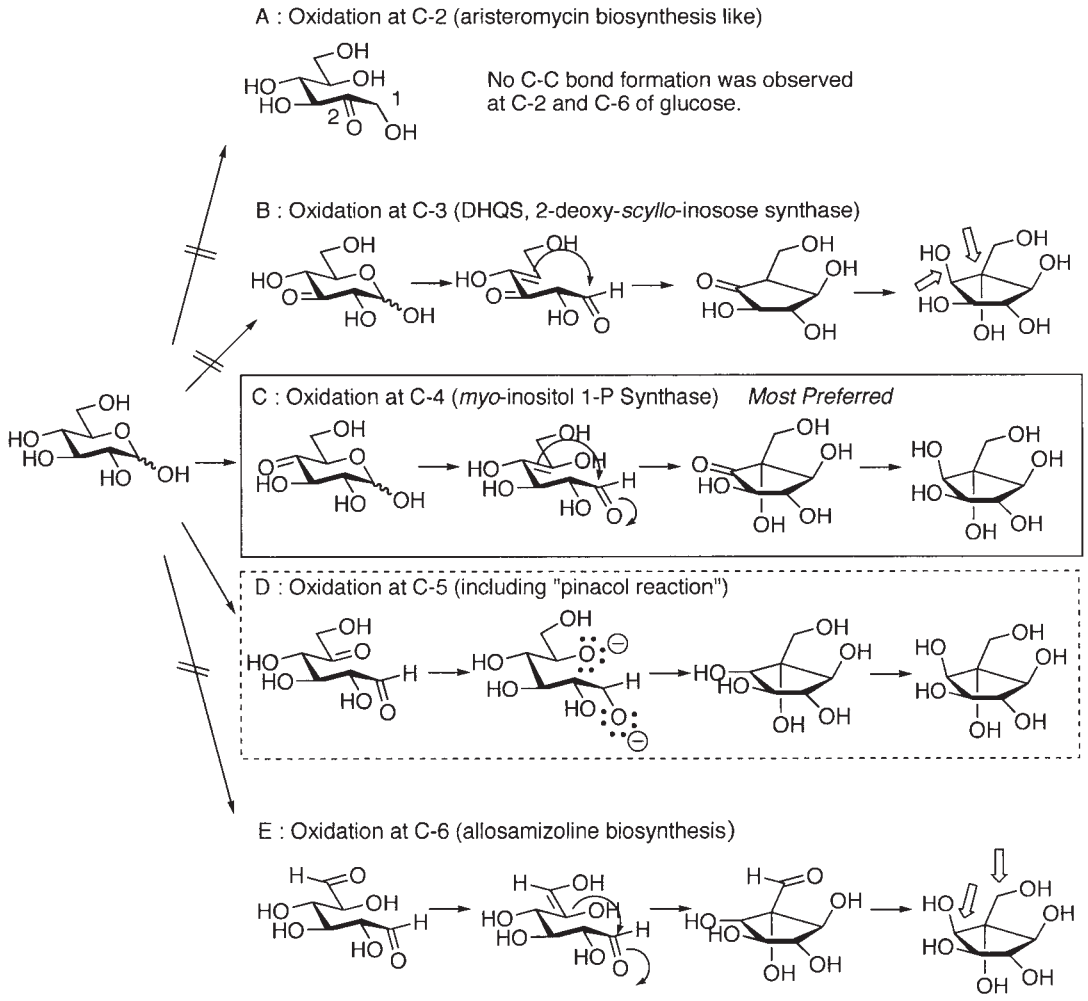

Fig. 4. Possible mechanisms of calditol formation.

step(s) is a circuitous one. Furthermore, it is thought that the mechanism that assumes oxidation of the C-6 hydroxy group as the starting point resembles the biosynthesis of the five-membered ring component in the chitinase inhibitor allosamidin, a secondary metabolite of Actinomycetes. Recently, the details of the mechanism were reported.[19] However, the pro-*S* hydrogen at C-6 of glucose was stereospecifically lost in the reaction course of allosamidin biosynthesis. However, it seems that the C-6 hydrogen does not participate in the reaction from the results of our experiments. This course is completely excluded. Furthermore, an inversion of the stereochemistry of the C-4 hydroxy group is necessary regardless of the cyclization. Again, this is a circuitous route for the metabolic pathway.

In other words, only two reaction mechanism courses are possible when the efficiency of the mechanism and the experimental findings are considered. One course resembles the biosynthesis of *myo*-inositol that assumes C-4 oxidation as the starting point. The second course is where the enzyme performs the coupling reaction of a pinacol reaction-like reductive coupling after C-5 oxidation. It will be extremely interesting if an enzyme does exist which can conduct pinacol reaction-like reductive coupling. In addition, a radical-coupling reaction was suggested at the end of C-20 at the interval where an isoprenoid chain of C-40 of archaebacteria can leave the biosynthesis.[27] However, the reaction mechanism which assumes oxidation at C-4 as the starting point can happen easily as shown in the next sentence. First, it is shown that "catalytic oxidation-reduction" happens with the C-4 of glucose by the reaction of 2-deoxy-*scyllo*-inosose synthase.[22] In addition, it is thought that the reaction mechanism resembles the one with *myo*-inositol 1-synthase well.[20] Furthermore, many phosphorylated *myo*-inositols are included in the polar group of the membrane lipids of thermophilic archaea.[2] *myo*-Inositol and calditol are the chief ingredients of the polar group of cell membranes in the archaea *Sulfolobus*. There may be some relationship between the biosynthesis of these carbocycles. Further details will become clear by the progress of reactions in cell-free systems, enzymatic studies regarding the enzymes concerned, or a hereditary study of the genes.

The existence of the characteristic L-gulose has been shown in the membrane lipids of the major lipid constituent of other thermophilic archaebacteria such as *thermoplasma*.[28] Perhaps this unique sugar should be considered to be biosynthesized from D-glucose. All of the hydroxy groups, except for one, are accompanied by an inversion of stereochemistry to achieve this change. Catalytic oxidoreduction of a certain hydroxy group the same as in the carbocyclic ring formation reaction might be pointed out.

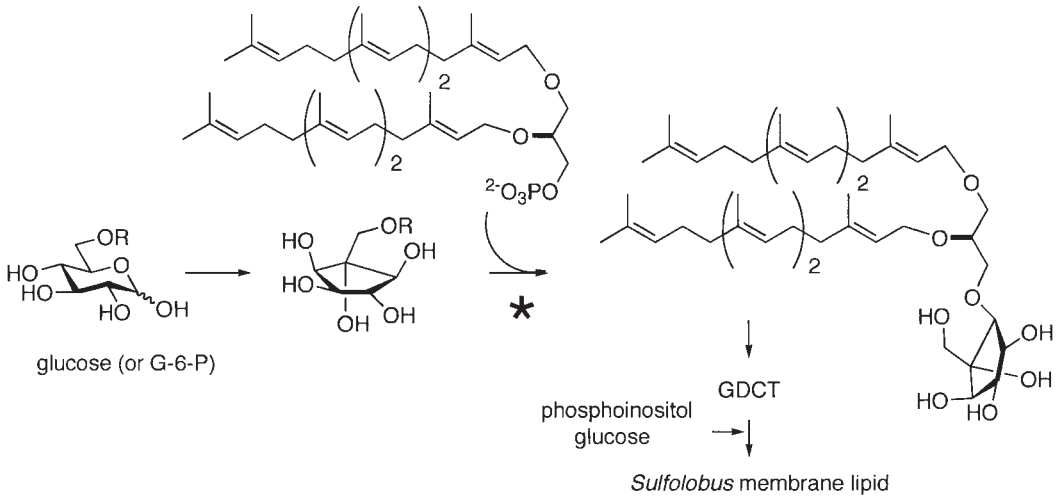

Fig. 5. GDCT and *Sulfolobus* membrane lipid biosynthesis.

On the other hand, it was an interesting result to obtain equal high rates of incorporation of deuterium at the C-1 of calditol from the C-1 of glucose with the C-6 of glucose which did not participate in oxidation–reduction in the reaction mechanism. Under the present conditions, the order of the stages of ether formation to the glycerol portion and the cyclization reaction in the biosynthetic pathway of calditol is unclear. However, this high deuterium incorporation at the C-1 of calditol suggests that the activation of oxidation is not involved on the calditol side at the stage of ether C–O bonding. Furthermore, it is also suggested that the ether formation occurs after the cyclization reaction as shown in Fig. 5. As a result, etherification to the substituent of the secondary hydroxy group of the cyclic compound in the comparatively crowded position was suggested. It will be interesting to investigate this ether formation reaction since it is not easy to accomplish chemically. Currently, experiments involving labeled compounds as an approach to this problem and the search for the intermediates of calditol biosynthesis (such as the five-membered carbocycles) are planned.

Experimental

Infrared spectra were obtained with a Perkin Elmer 1600 FT-IR spectrometer. ^{1}H NMR spectra were recorded with a JEOL EX-90 or GX-400 spectrometer. For the ^{1}H spectra, tetramethylsilane (0 ppm) was used as an internal standard in $CDCl_3$. GC–MS were recorded with a Shimadzu QP-5000 spectrometer. Chromatographic separations were carried out with Merck Kieselgel 60, 70–230 mesh columns. [1-^{2}H]-D-glucose and [2-^{2}H]-D-glucose was prepared according to methods in the literature. [6,6-$^2H_2$]-D-glucose was prepared by the method of Lemiux and Stevens,[29] and (6*S*)-[6-^{2}H]-D-glucose was prepared by the method of Ohrui.[26] The deuterium content of all the deuterated compounds was confirmed by the ^{1}H NMR peak integration of each anomer of tetraacetate, and GC–MS of the mixture of the anomer of tetraacetate and > 95% at each hydrogen. *Sulfolobus acidocaldarius* (JCM 8929) was obtained from the Japan Collection of Microorganisms (JCM, RIKEN). The cultivation and isolation of lipid diether were carried out as follows. One liter of L medium (containing $(NH_4)_2SO_4 \cdot 7H_2O$, 1.30 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; KH_2PO_4, 0.07 g, and yeast extract (Difco) 1.00 g) was incubated for each experiment, and 1.00 g of deuterium-labeled glucose was administered to the medium before autoclaving. Five mL of precultured (L medium without deuterated material) cells was inoculated into the 1 liter of L medium described above and incubated for 7 days. Cells were harvested by centrifugation (8500 g, 10 min), washed with H_2O, and dried by lyophilization to yield typically 20 mg of dry cells. Lipids were extracted and GDCT was purified according to the method of De Rosa. Lipid was extracted twice for 18 h each with refluxing $CHCl_3$:CH_3OH (2:1), and the extracted mixture was applied to acid hydrolysis (3% HCl–MeOH) for 18 h. The mixture was evaporated to dryness and the residue was chromatographed with silica gel (chloroform:ether = 9:1 to chloroform:methanol = 8:1). GDCT was obtained in the chloroform–methanol fraction with a slight impurity. The main GDGT fraction was further acetylated with acetic anhydride–pyridine (each 1 mL) and stirred for 5 h. The GDGT acetate was purified by silica-gel column chromatography (hexane:ethyl acetate = 4:1), and then analyzed by NMR. Typically, 1.5–2 mg of GDCT acetate was obtained from 1 liter of broth.

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