Biochem. J. (1990) 266, 785–791 (Printed in Great Britain)

Calditol tetraether lipids of the archaebacterium *Sulfolobus solfataricus*

Biosynthetic studies

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Lipids from the archaebacterium *Sulfolobus solfataricus* are based on 72-membered macrocyclic tetraethers made up from two C_{40} diol units differently cyclized and either two glycerol moieties or one glycerol moiety and a unique branched-chain nonitol named calditol (glycerodialkylnonitol tetraethers, GDNTs). To elucidate the biosynthesis of calditol and related tetraethers, labelled precursors, [U-^{14}C,1(3)-^{3}H]glycerol, [U-^{14}C,2-^{3}H]glycerol, D-[1-^{14}C,6-^{3}H]glucose, D-[6-^{14}C,1-^{3}H]glucose, D-[1-^{14}C,2-^{3}H]glucose, D-[1-^{14}C,6-^{3}H]-fructose and D-[1-^{14}C]galactose, were fed to *S. solfataricus*. Without regard to stereochemistry or phosphorylation, incorporation experiments provided evidence that the biosynthesis of calditol occurs via an aldolic condensation between dihydroxyacetone and fructose, through a 2-oxo derivative of calditol as an intermediate. The latter is in turn reduced and then alkylated to yield the GDNTs. The biogenetic origins of both glycerol and C_{40} isoprenoid moieties of GDNTs are also discussed.

INTRODUCTION

The membrane of extreme thermoacidophilic archaebacteria of the genus *Sulfolobus* is based on two types of macrocyclic tetraethers, having pairs of bifunctional C_{40} isoprenoid chains, with a 16,16′-biphytanyl skeleton, that differ by as much as four cyclopentane rings [1,2]. In the tetraethers a (Fig. 1), named glycerodialkylglycerol tetraethers (GDGTs), the hydrophilic portions are two glycerol units with unusual 2,3-di-*O*-*sn* configuration, whereas in the tetraethers b (Fig. 1), named glycerodialkylnonitol tetraethers (GDNTs), a unique branched-chain nonitol, named calditol, substitutes for one of the glycerols.

A series of biogenetic studies clarified the biosynthesis of the GDGTs [3–7].

The aim of the present study was to examine the biosynthesis of GDNT lipids, which can be considered as specific taxonomic markers of the order Sulfolobales [2].

Plausibly the calditol skeleton can be formed by a variety of aldol- or acetoin-type condensations between a triose and a hexose precursor, followed by reduction. Moreover, two hypotheses for the assembly of GDNTs can be formulated: (*a*) calditol synthesis occurs before the tetraether assembly; (*b*) calditol is synthesized after the macrocyclic tetraether precursor is assembled.

In order to distinguish among different biosynthetic hypotheses formulated for the synthesis of calditol and related tetraethers, precursors appropriately labelled with ^{14}C and ^{3}H were fed to *S. solfataricus*.

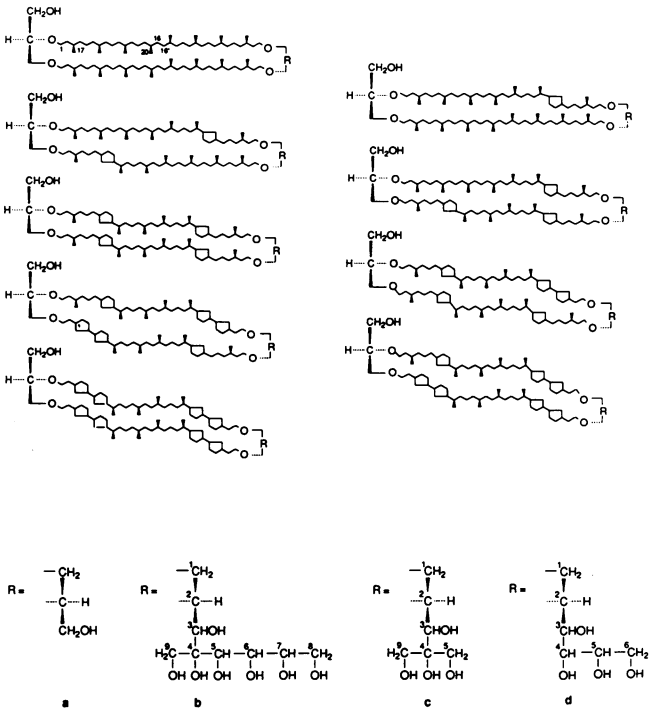

Fig. 1. Structures of (a) GDGTs and (b) GDNTs basic components of complex lipids of *S. solfataricus* (c and d are structures of $NaIO_4/NaBH_4$ degradation products of GDNTs)

Abbreviations used: GDNTs, glycerodialkylnonitol tetraethers; GDGTs, glycerodialkylglycerol tetraethers.

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MATERIALS AND METHODS

Radiochemicals

[U-^{14}C]glycerol (119 mCi/mmol), [1(3)-^{3}H]glycerol (0.5 Ci/mmol), [2-^{3}H]glycerol (0.5 Ci/mmol), D-[1-^{14}C]fructose (60 mCi/mmol), D-[6-^{3}H]fructose (1.6 Ci/mmol), D-[1-^{14}C]glucose (61.1 mCi/mmol), D-[6-^{14}C]glucose (59.3 mCi/mmol), D-[6-^{3}H]glucose (12.8 Ci/mmol), D-[1-^{3}H]glucose (9.1 Ci/mmol), D-[2-^{3}H]glucose (13.5 Ci/mmol) and D-[1-^{14}C]galactose (60 mCi/mmol) were purchased from Amersham International.

Doubly labelled precursors [U-^{14}C,1(3)-^{3}H]glycerol, [U-^{14}C,2-^{3}H]glycerol, D-[1-^{14}C,6-^{3}H]glucose, D-[6-^{14}C,1-^{3}H]glucose, D-[1-^{14}C,2-^{3}H]glucose and D-[1-^{14}C,6-^{3}H]-fructose were prepared by mixing the corresponding singly labelled compounds to obtain the appropriate $^{3}H/^{14}C$ ratio.

Microorganism and culture conditions

In the experiments using the labelled precursors, *Sulfolobus solfataricus*, strain MT4 (A.T.C.C. 49155), isolated from an acid hot spring in Agnano, Naples, Italy [8], was grown at 87 °C in a 2.5-litre fermentor (Chemap, Mannerdorf, Switzerland) with low mechanical agitation and aeration flux of 30 ml/min per litre of broth. The culture medium contained per litre: 2 g of yeast extract, 3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4,7H_2O$ and 0.25 g of $CaCl_2$; the pH was adjusted to 3.5 with 0.1 M-H_2SO_4, and the culture vessel was inoculated by adding a 12 h broth culture (0.1 litre/litre of medium). The labelled precursor was added to the culture (0.3 litre) at the beginning of the exponential phase. The evolved $^{14}CO_2$ was recovered as $Ba^{14}CO_3$, which was centrifuged, washed with decarbonated water to neutrality and dried over P_2O_5 under vacuum at 90 °C. Cells were harvested in the late stationary growth phase of incubation (48 h) by centrifugation at 33000 *g*. The labelled cells (yield 0.3 g of wet cells) mixed with 120 g of unlabelled wet cells, grown under the same conditions, were washed with 0.1 M-NaCl and freeze-dried.

Extraction and methanolysis of lipids

The dried cells were extracted continuously (with a Soxhlet apparatus) for 12 h with chloroform/methanol (1:1, v/v). The total lipid extract (about 3 g) was treated with 50 ml of methanolic anhydrous 1 M-HCl for 6 h under reflux; then the methanolysis mixture was dried *in vacuo*.

Purification of tetraethers

The purification of the methanolysis mixture was performed on a silica-gel column, elution being with chloroform/diethyl ether (9:1, v/v), which elutes GDGTs (300–400 mg) and then with chloroform/methanol (9:1, v/v), which elutes GDNTs (700–800 mg). To obtain a constant specific radioactivity the tetraether samples were acetylated, purified and methanolysed to obtain the parent tetraethers.

Acetylation reaction

Tetraethers (300–700 mg) were fully acetylated with 50 ml of acetic anhydride/pyridine (9:1, v/v), with refluxing for 6 h. The purification of acetylated products was performed on a silica-gel column, which was eluted with n-hexane/diethyl ether (4:1, v/v) for the GDGT diacetate, and with chloroform/diethyl ether (19:1, v/v) for the GDNT octa-acetate. The GDGT diacetate had on t.l.c. an R_F of 0.3 in n-hexane/diethyl ether (4:1, v/v), whereas GDNT octa-acetate had an R_F of 0.8 in chloroform/diethyl ether (9:1, v/v). The yield of the acetylation step was about 95%.

Methanolysis of acetylated tetraethers

Fully acetylated tetraethers were methanolysed with 50 ml of anhydrous 1 M-HCl for 6 h under reflux; the methanolysis mixtures were then dried *in vacuo*. Tetraethers were purified chromatographically on a silica-gel column as previously described. Both species of compounds, GDGT and GDNT, were pure by t.l.c. analysis; solvent system chloroform/diethyl ether (19:1, v/v) for GDGTs; chloroform/methanol (9:1, v/v) for GDNT. The yield of the methanolysis step was about 90%.

BCl_3 degradation of GDNTs

GDNTs (250 mg) were treated with BCl_3 (5 ml, in 5 ml of chloroform, at 18 °C for 12 h). The reaction mixture was evaporated under N_2 and chromatographed on silica-gel column. Chloroform eluted C_{40} dichlorides, chloroform/methanol (7:3, v/v) eluted glycerol, and acetone/methanol/water (3:1:1, by vol.) eluted calditol, with the relative molar proportions (C_{40} dichlorides/glycerol/calditol) 2:1:1; t.l.c. was developed with chloroform/methanol (7:3, v/v) for glycerol, n-hexane for C_{40} dichlorides and chloroform/methanol (3:2, v/v) for calditol. The yield of the BCl_3 degradation step was 85%.

$NaIO_4/NaBH_4$ degradation of GDNTs

GDNTs (200 mg) dissolved in chloroform/methanol (1:1, v/v; 20 ml) were treated twice for 48 h with aq. 8% $NaIO_4$ (5 ml) with stirring. Excess reagent was destroyed with glycerol, and the oxidation products were recovered by extraction with chloroform. The extract, dried under N_2, was dissolved in chloroform/methanol (3:7, v/v; 5 ml) and treated with excess $NaBH_4$ for 3 h with stirring. Excess borohydride was destroyed with 6 M-HCl and reduction products (180 mg), extracted with chloroform, were resolved by column chromatography: chloroform/diethyl ether (9:1, v/v) eluted a GDGT mixture not chromatographically distinguishable from the GDGTs a (Fig. 1; 21 mg), chloroform/methanol (49:1, v/v) eluted tetraethers c (Fig. 1; 75 mg) and chloroform/methanol (24:1, v/v) eluted tetraethers d (Fig. 1; 84 mg). The products were acetylated as described above and purified to constant specific radioactivity on a silica-gel column eluted with n-hexane/diethyl ether (4:1, v/v) for acetylated GDGTs and with chloroform/diethyl ether (19:1, v/v) for fully acetylated tetraethers c and d (Fig. 1). On t.l.c. with chloroform/methanol (19:1, v/v), fully acetylated tetraethers c and d (Fig. 1) showed R_F values of 0.50 and 0.45 respectively.

Chromatographic procedures

Columns containing Merck Kieselgel 70-230 mesh silica gel were used; t.l.c. was performed on 0.25 mm-thick silica-gel plates (F254 Merck), activated by heating at 100 °C for 2 h. Complex lipids, tetraethers and C_{40} dichlorides were detected either by exposure to I_2 vapour or by spraying with $Ce(SO_4)_2$. Specific reagents included the Dittmer & Lester [9] reagent for phospholipids, periodate/Schiff [10] for *vic*-glycols and diphenylamine [11] for glycolipids.

Radioactivity measurements

The labelled samples, weighed on a Cahn electrobalance, were dissolved in 5 ml of Bray's [12] solution and the radioactivity was measured with a Prias liquid-scintillation counter (Packard, model Tri-Carb PLD), equipped with an automatic system for the selective evaluation of the absolute radioactivity from two different radionuclides.

In the case of $Ba^{14}CO_3$ samples, 0.1 g of Cab-O-Sil thixotropic gel were added to the scintillation vial. In order to measure the radioactivity incorporated into whole cells and in the medium, 1 ml of culture was rapidly filtered on a Millipore filtration apparatus. The filter was then washed twice with 2 ml of water, dried and counted for radioactivity in the scintillation solution in the presence of Cab-O-Sil (0.1 g). The filtrate (0.25 ml) was counted for radioactivity in 5 ml of Bray's [12] solution.

RESULTS

In seven independent experiments, growing cultures of *Sulfolobus solfataricus* were incubated with D-[1-^{14}C,6-^{3}H]glucose, D-[6-^{14}C,1-^{3}H]glucose, D-[1-^{14}C,2-^{3}H]glucose, D-[1-^{14}C,6-^{3}H]fructose, D-[1-^{14}C]galactose, [U-^{14}C,1(3)-^{3}H]glycerol and [U-^{14}C,2-^{3}H]glycerol. The lipids were extracted from the harvested cells and methanolysed to obtain GDGTs a (Fig. 1) and GDNTs b (Fig. 1).

The radioactivity detected in whole cells as well as that in both total lipids and GDGTs is reported in Table 1. The results of experiments using different precursors were comparable, since the relevant cell yields were found to be equivalent.

The radioactivity of whole cells in the experiments when using glycerol and galactose as precursors was lower than that observed when using labelled glucose and fructose.

The recovery of ^{3}H and ^{14}C radioactivity in the cells in the experiments with glucose differently labelled was dependent on the label position in the glucose skeleton, indicating the presence in *S. solfataricus* of glucose metabolism characterized by cell excretion of catabolites containing C-1 of the glucose. The lower value of ^{3}H cell radioactivity in the experiments with D-[1-^{14}C,2-^{3}H]glucose, when compared with the corresponding value with D-[1-^{14}C,6-^{3}H]glucose, indicated a glucose$\rightleftharpoons$fructose interconversion that specifically causes a loss of ^{3}H from the C-2 position. The metabolic equivalence of glucose and fructose in *S. solfataricus* is confirmed by the similar values of the ^{3}H and ^{14}C radioactivity recovered in the cells with D-[1-^{14}C,6-^{3}H]glucose and D-[1-^{14}C,6-^{3}H]fructose. In these experiments the ^{3}H/^{14}C ratio in the medium, after cell removal, increased about 9–10-fold with respect to the initial ^{3}H/^{14}C value of the labelled precursors. This is indicative of metabolic processes with a selective loss of $^{14}CO_2$. Conversely no significant changes of ^{3}H/^{14}C ratio in the growth medium were observed with doubly labelled glycerols as precursors. A high $^{14}CO_2$ production was also observed when galactose was used as a precursor, only 15% of ^{14}C radioactivity being recovered in the growth medium in this experiment (results not shown).

The cell radioactivity associated with total lipids (Table 1) in the experiments with differently labelled glycerols was 2–6-fold higher than that recovered with differently labelled hexoses, owing to the different metabolic pathways involving these precursors. The ^{3}H/^{14}C ratio of GDGTs (Table 1) in the experiments with [1-^{14}C,6-^{3}H]glucose, [6-^{14}C,1-^{3}H]glucose and [1-^{14}C,6-^{3}H]fructose was indicative of the selective recovery of the radioactivity localized on C-6 of the sugar.

Data on radioactivity distribution in GDGTs, when using labelled glycerols, have been previously discussed [5]. With these precursors a high incorporation of radioactivity occurs in the glycerol moieties without changes in ^{3}H/^{14}C ratio. Conversely the isotopic ratio of C_{40} isoprenoid chains appears to be strictly dependent on the labelling pattern of the glycerol precursor [5].

GDNTs generally show a higher specific radioactivity than do GDGTs (Table 2). Some information on the radioactivity localization on GDNTs was obtained by the analysis of BCl_3 and $NaIO_4/NaBH_4$ degradation products of tetraethers (see the Materials and methods section). Results for BCl_3 degradation showed a high incorporation of radioactivity into the calditol moiety. In particular, with [1-^{14}C,6-^{3}H]glucose, [6-^{14}C,1-^{3}H]glucose and [1-^{14}C,6-^{3}H]fructose, the ^{3}H/^{14}C ratio in the calditol moiety was almost the same as the one in the supplied precursor. These data indicated that both hexoses are direct precursors of the calditol; furthermore, they are intactly incorporated into the carbon skeleton of the polyol and are metabolically equivalent. Information about the biogenetic origin of C-4–C-9 of the calditol can be obtained from the results of the experiment with [1-^{14}C,2-^{3}H]glucose. In this case the ^{3}H/^{14}C ratio in the calditol is 50% lower than that of the precursor. Moreover, the ^{3}H label is specifically located on C-9 of the calditol (which has its origin in C-1 of the hexose precursor), as shown by the loss of ^{3}H radioactivity in the tetraethers d (Table 2; Fig. 1). Therefore the 50% loss and C-1 $\rightarrow$ C-2 shift of ^{3}H label can be regarded as a consequence of a glucose$\rightleftharpoons$fructose isomerization process, characterized by exchange or transfer of the proton on C-2 of the glucose. With differently labelled glycerols the ^{3}H/^{14}C ratio of the calditol depends on the position of ^{3}H in the precursor; in fact with [U-^{14}C,1(3)-^{3}H]glycerol about 70% of the ^{3}H is retained, whereas ^{3}H is almost completely lost when [U-^{14}C,2-^{3}H]glycerol is used. The labelling pattern of glycerol and C_{40} dichlorides, obtained from BCl_3 degradation of GDNTs, strictly reflects the situation occurring in the corresponding GDGTs [5].

The $NaIO_4$ degradation data (Table 2) allowed us to clarify the pattern of radioactivity distribution on the calditol skeleton. With C-1/C-6 differently labelled glucose and fructose the radioactivity is specifically localized at C-8 and C-9 of the calditol, corresponding to C-6 and C-1 of the labelled precursor. In fact in the tetraethers c (Table 2), which originate from the removal of C-6–C-8 of the calditol skeleton, only the radioactivity localized at C-6 of the sugar precursor is lost. Conversely, in tetraethers a and d (Table 2), in which C-4–C-9 and C-7–C-9 of the calditol moiety are removed respectively, the radioactivity localized on both C-6 and C-1 of the precursor is lost. When differently labelled glycerols were used as precursors, tetraethers a, c, and d (Table 2) showed the same molar radioactivity as the intact GDNTs. This evidence, together with data on the calditol radioactivity obtained with BCl_3 degradation of GDNTs, indicates a specific localization of the labelled glycerol on C-1–C-3 of the calditol skeleton.

In the experiment with [1-^{14}C]galactose, the molar radioactivity of GDNTs is 8.8×10^4 d.p.m./mmol. This value, about 20-fold lower than that reported in Table 2

Table 1. Incorporation of labelled precursors into whole cells and in lipids of *S. solfataricus*

Precursor			Cells			Total lipids			GDGTs		
	Specific radioactivity (mCi/mmol)			10^{-6} × Total radioactivity (d.p.m.) (% of recovered radioactivity)‡			10^{-6} × Total radioactivity (d.p.m.) (% of recovered radioactivity)§		10^{-3} × (d.p.m./mmol) radioactivity		
Compound (μmol of labelled precursor; $^3H/^{14}C$)	^3H	^{14}C	Yield* (mg)	^3H	^{14}C	Yield† (g)	^3H	^{14}C	^3H	^{14}C	$^3H/^{14}C$
[1-^{14}C,6-^{3}H]Glucose (0.82; 2)	125	63	78	48.2 (21.8)	16.1 (14.6)	3.2	5.2 (10.8)	2.2 (13.7)	1150	139	8.3
[6-^{14}C,1-^{3}H]Glucose (0.85; 2)	119	60	89	33.7 (15.3)	24.3 (22.1)	3.0	4.4 (13.1)	2.8 (11.5)	201	1080	0.2
[1-^{14}C,2-^{3}H]Glucose (0.82; 2)	125	63	76	15.9 (7.2)	16.6 (15.1)	3.4	2.1 (13.2)	2.1 (12.7)	31	93	0.3
[1-^{14}C,6-^{3}H]Fructose (0.89; 2)	112	56	83	51.8 (23.2)	17.0 (15.5)	3.1	4.9 (9.5)	2.1 (12.4)	1635	147	11.1
[1-^{14}C]Galactose (0.83)	–	60	81	–	2.7 (2.5)	2.8	–	0.3 (11.1)	–	34	–
[U-^{14}C,1(3)-^{3}H]Glycerol (2.88; 3)	207	69	84	51.5 (3.9)	23.2 (5.3)	3.0	23.5 (46.0)	12.4 (53.5)	15400	5880	2.6
[U-^{14}C,2-^{3}H]Glycerol (2.88; 3)	207	69	80	21.0 (1.6)	25.4 (5.8)	3.1	8.5 (40.5)	12.7 (50.0)	14600	6000	2.4

* The yields refer to the amount of freeze-dried cells recovered from 330 ml in incubation experiments with labelled precursors.
† The yields refer to the amount of lipids extracted from labelled freeze-dried cells, diluted about 400-fold with unlabelled freeze-dried cells.
‡ Percentage of radioactivity recovered in the whole cells; '100' refers to the total radioactivity added to the growing cultures.
§ Percentage of radioactivity recovered in the crude lipid fraction; '100' refers to the total radioactivity recovered in the whole cells.

Table 2. Distribution of radioactivity into GDNTs

		$10^{-3} \times {}^{14}C$ or ^{3}H Radioactivity (d.p.m./mol)						
			BCl_3 degradation			$NaIO_4$ degradation		
Precursor ($^{3}H/^{14}C$)	Radionuclide	GDNTs*	Calditol†	Glycerol	C_{40} dichlorides	Tetraethers c†	Tetraethers d†	Tetraethers a*‡
[1-^{14}C,6-^{3}H]Glucose (2)	^{14}C	1644	1464	1	71	1610	137	148
	^{3}H	5040	3135	18	859	1851	1081	1840
	$^{3}H/^{14}C$	3.0	2.1	18.0	12.1	1.2	13.1	12.4
[6-^{14}C,1-^{3}H]Glucose (2)	^{14}C	2620	1542	7	501	1038	997	1020
	^{3}H	3090	2950	1	90	3001	185	191
	$^{3}H/^{14}C$	1.2	1.9	0.1	0.2	2.9	0.2	0.2
[1-^{14}C,2-^{3}H]Glucose (2)	^{14}C	1409	1303	1	60	1391	131	127
	^{3}H	1351	1401	1	15	1300	90	36
	$^{3}H/^{14}C$	1.0	1.1	1	0.3	0.9	0.7	0.3
[1-^{14}C,6-^{3}H]Fructose (2)	^{14}C	1701	1519	1	74	1730	164	156
	^{3}H	4620	2950	16	830	1741	1750	1701
	$^{3}H/^{14}C$	2.7	1.9	16.0	11.2	1.0	10.6	10.9
[U-^{14}C,1(3)-^{3}H]Glycerol (3)	^{14}C	10600	7340	2100	630	10700	10100	11000
	^{3}H	25000	15488	6200	870	24800	24800	25100
	$^{3}H/^{14}C$	2.4	2.1	3.0	1.4	2.3	2.4	2.3
[U-^{14}C,2-^{3}H]Glycerol (3)	^{14}C	11200	7438	2300	650	11200	11350	11300
	^{3}H	7440	240	6900	95	7490	7510	7550
	$^{3}H/^{14}C$	0.7	0.1	3.0	0.2	0.7	0.7	0.7

* Radioactivity is measured on tetraethers purified to constant specific radioactivity.
† Radioactivity is measured on fully acetylated derivatives, purified to constant specific radioactivity (for structures, see Fig. 1).
‡ These GDGTs, obtained from GDNTs by the removal of C-4–C-9 of the calditol, have the same structural features as natural GDGTs and are not spectroscopically and chromatographically distinguishable from these tetraethers.

for the other hexoses, indicates that galactose is not a direct precursor of the calditol, as are glucose or fructose. In fact experiments involving the degradation of GDNTs by BCl_3 and $NaIO_4/NaBH_4$ indicated a randomization of ^{14}C radioactivity, with a preferential localization at the level of C_{40} isoprenoids (results not shown).

DISCUSSION

In a series of experiments with singly and doubly labelled precursors such as glycerol, glucose, fructose and galactose, the general features which underline the GDNTs b (Fig. 1) biosynthesis in *Sulfolobus solfataricus*, have been focused.

The unequivocal rationalization of incorporation data using labelled precursors requires the evaluation of the possible role of isotopic effects due, for example, to the ability of the enzymes involved in the utilization of precursors to discriminate against ^3H. In previous papers [5,13,14] concerning lipid biosynthesis and glucose/fructose metabolism in archaebacteria, a significant role for such an effect has been excluded.

C-1–C-3 of the calditol moiety are specifically labelled with glycerol, whereas C-4–C-9 are selectively labelled with glucose and fructose. On the other hand the calditol labelling with galactose is very low and the radioactivity is distributed throughout the whole of the GDNTs, indicating that this sugar is not a direct precursor of the polyol.

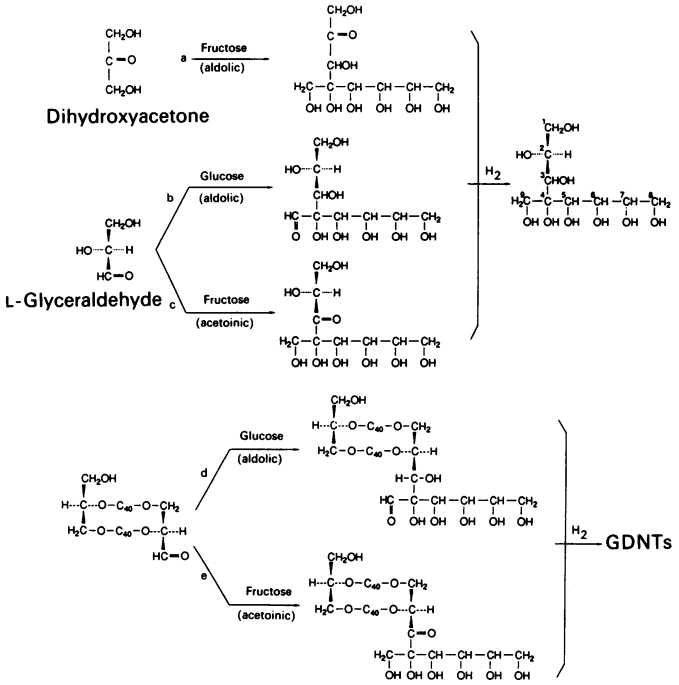
This evidence suggests that the C_9 skeleton of the calditol can be formed by a variety of aldol- or acetoin-type condensations between a triose and glucose or fructose, followed by reduction, leading to calditol, (a–c, Scheme 1), without implications as to stereochemistry, phosphorylation etc. However, a condensation between glucose or fructose and an aldehydic macrocyclic tetraether precursor, via an aldolic or acetoinic condensation process cannot be ruled out (d and e, Scheme 1).

The analysis of the labelling of GDNTs when double labelled precursors are fed to *S. solfataricus* allows us to consider only a limited number of biogenetic hypotheses.

The observation that [U-^{14}C,2-^3H]glycerol labels C-1–C-3 of the calditol, with the total loss of ^3H, favours route a (Scheme 1), based on a dihydroxyacetone intermediate, and excludes hypothetical routes d and e (Scheme 1) involving an aldehydic tetraether precursor. In fact, previous biosynthetic studies on glycerol ether lipids of the archaebacteria [5,13] showed that ether linkage formation does not involve methylene and methyne glycerol protons. Therefore the ^3H loss observed with this precursor can be regarded as specifically related to the calditol biosynthesis rather than to the ether linkage formation between polyol and isoprenoid precursors.

Confirmatory evidence on the role of dihydroxyacetone (or its activated derivative) in the calditol biosynthesis comes from the experiment with [1-^{14}C,1(3)-^3H]glycerol. This precursor is incorporated in the calditol moiety of GDNTs with a loss of 30% of ^3H, as a consequence of the condensation mechanism of the dihydroxyacetone with the hexose precursor of C-4–C-9 of the calditol.

Experiments with [1-^{14}C,6-^3H]glucose, [6-^{14}C,1-^3H]glucose, and [1-^{14}C,6-^3H]fructose showed that both sugars build up C-4–C-9 of the calditol, since both the



Scheme 1. Biogenetic hypotheses for the assembly of the calditol skeleton of GDNTs, basic components of complex lipids of *S. solfataricus*

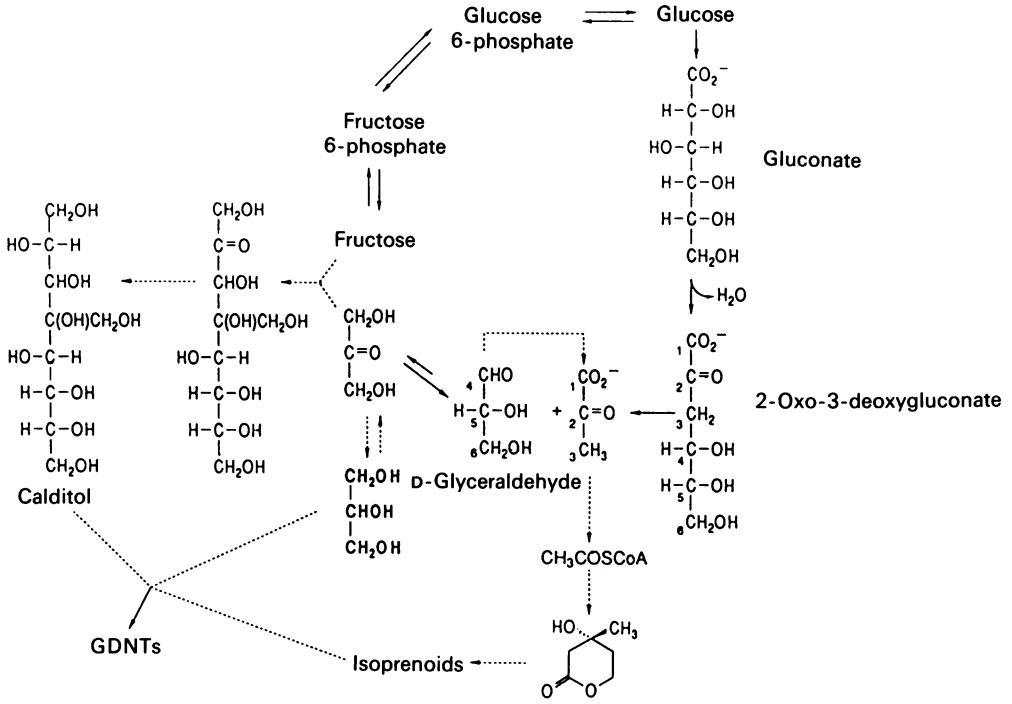
Details of C_{40} isoprenoid chains are given in Fig. 1.

effective incorporation of precursor radioactivity and full retention of the original $^3H/^{14}C$ ratio were observed. This metabolic equivalence of glucose and fructose is consistent with previous observations on the glucose pathway in *S. solfataricus* [14].

As shown in route a (Scheme 1), without implications for activation, fructose more than glucose was an effective precursor of calditol synthesis. This occurs through an aldolic condensation process between fructose and dihydroxyacetone, followed by a reduction of the C-2 oxo group.

The role of the fructose in calditol biosynthesis is indirectly confirmed by the experiment with [1-^{14}C,2-^3H]glucose, indicating the occurrence of a glucose⇌fructose isomerization in the utilization of the precursor in the calditol skeleton. In fact with [1-^{14}C,2-^3H]glucose, only 50% of the ^3H was recovered in the calditol, but the radioactivity was specifically located on C-9 of the polyol, corresponding to the C-1 of the glucose, which was not originally ^3H-labelled. This $1 \rightarrow 2$ shift on the [1-^{14}C,2-^3H]glucose precursor can be rationalized in terms of a glucose⇌fructose isomerization that probably, in *S. solfataricus*, involves an equivalent amount of both transfer of proton and exchange, as reported in literature for some other isomerases [15].

The main features of the biosynthesis of GDNTs in *S. solfataricus* are summarized in Scheme 2. Regarding the glucose metabolism in this micro-organism [14], the poor labelling at C-1–C-3 of the calditol with 6-^{14}C- and 6-^3H-labelled glucose and fructose indicates that D-glyceraldehyde (Scheme 2) is not a direct precursor of the calditol and that triose isomerase does not play a significant role in the biosynthesis of C-1–C-3 of the calditol. It is worth noting in this respect that C-2 of the calditol in GDNTs shows the same unusual stereochemistry observed in the chiral centre of the glycerol



Scheme 2. Biosynthetic pathway of glycerodialkylcalditol tetraethers, basic components of membrane lipids of *S. solfataricus*

moiety of all archaebacterial lipids [2,6], that is, opposite to that of D-glyceraldehyde.

The labelling pattern of the glycerol moiety of GDNTs using [U-^{14}C,2-^{3}H]glycerol and [U-^{14}C,1(3)-^{3}H]glycerol as precursors is similar to that previously observed in GDGTs of *S. solfataricus* [5], with a total retention of ^{3}H from both precursors. Moreover, the poor labelling of the glycerol moiety in the experiments with 6-^{14}C- and 6-^{3}H-labelled glucose and fructose is further evidence of the very minor role of triose isomerase in the recovery of C-4–C-6 of glucose and fructose in the C-3 metabolites involved in the biosynthesis both of the calditol and the glycerol moiety of GDNTs. In this respect the *S. solfataricus* pathway differs from that observed in *Halobacterium halobium* [13]. In fact the experiments with D-[6,6-$^{2}H_2$]glucose using this halophilic archaebacterium showed an efficient incorporation of ^{2}H into the glycerol moiety of lipids.

The modified Entner–Doudoroff pathway operating in *S. solfataricus* [14] justifies the absence of the radioactivity in the isoprenoid moiety of lipids when C-1-labelled glucose or fructose were used. Conversely the efficient labelling of isoprenoidic chains with C-6-labelled glucose and fructose indicates the occurrence in *S. solfataricus* of normal glycolytic and mevalonate pathways that have D-glyceraldehyde, pyruvate and mevalonate as intermediates.

Similar results were obtained with [1-^{14}C,2-^{3}H]glycerol and [U-^{14}C,1(3)-^{3}H]glycerol as precursors. As expected, [U-^{14}C,1(3)-^{3}H]glycerol gave substantial ^{3}H retention in bisphytanyl C_{40} chains, whereas [U-^{14}C,2-^{3}H]glycerol gave a loss of ^{3}H label in these isoprenoids.

These results strongly suggest that the calditol biosynthesis in *S. solfataricus*, without implication as to stereochemistry, phosphorylation, etc., occurs via an aldolic condensation between dihydroxyacetone and fructose, followed by a reduction of the C-2 oxo group. This polyol as well as glycerol [5] are subsequently alkylated by an isoprenoid activated precursor to give isoprenoid–ether intermediate(s) of the GDNTs biosynthetic pathway.

We thank Mrs. Ida Romano for her technical assistance and Mr. Raffaele Turco for the art work.

REFERENCES

1. Langworthy, T. A. & Pond, Y. L. (1986) System. Appl. Microbiol. **7**, 253–257
2. De Rosa, M. & Gambacorta, A. (1988) Prog. Lipid Res. **27**, 153–175
3. De Rosa, M., Gambacorta, A. & Nicolaus, B. (1980) Phytochemistry **19**, 791–793
4. De Rosa, M., Esposito E., Gambacorta, A., Nicolaus, B. & Bu'Lock, J. D. (1980) Phytochemistry **19**, 827–831
5. De Rosa, M., Gambacorta, A., Nicolaus, B. & Sodano, S. (1982) Phytochemistry **21**, 595–599
6. De Rosa, M., Gambacorta, A., Nicolaus, B., Sodano, S. & Bu'Lock, J. D. (1980) Phytochemistry **19**, 833–836
7. Bu'Lock, J. D., De Rosa, M. & Gambacorta, A. (1983) in Biosynthesis of Isoprenoid Compounds (Porter, J. W. & Spurgeon, S. L., eds.), vol. 2, pp. 161–187, John Wiley and Sons, New York
8. De Rosa, M., Gambacorta, A. & Bu'Lock, J. D. (1975) J. Gen. Microbiol. **86**, 156–164
9. Dittmer, J. C. & Lester, R. J. (1964) J. Lipid Res. **5**, 126–127
10. Baddily, J., Buchanan, J. G., Hanschumacher, R. E. & Prescott, J. F. (1956) J. Chem. Soc. 2818–2823
11. Shively, J. M. & Benson, A. A. (1967) J. Bacteriol. **94**, 1679–1684
12. Bray, G. A. (1960) Anal. Biochem. **1**, 270–273
13. Kakinuma, K., Yamagishi, M., Fujimoto, Y., Ikekawa, N. & Oshima, T. (1988) J. Am. Chem. Soc. **110**, 4861–4863
14. De Rosa, M., Gambacorta, A., Nicolaus, B., Giardina, P., Poerio, E. & Buonocore, V. (1984) Biochem. J. **224**, 407–414
15. Noltman, E. A. (1972) Enzymes 3rd Ed. **6**, 272–287

Received 19 June 1989/20 September 1989; accepted 29 September 1989