C_{15}, C_{20}, and C_{25} isoprenoid homologues in glycerol diether phospholipids of methanogenic archaebacteria

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Abstract The glycerol diether phospholipids of 25 monocultures of methanogenic bacteria were isolated and degraded with hydriodic acid. The resulting alkyl iodides were converted to acetate esters and alcohols which were examined using capillary gas–liquid chromatography. The presence of C_{20} phytanol was observed in accordance with previous studies. Soft fragmentation by chemical ionization mass spectrometry combined with selected ion monitoring enabled the detection, for the first time, of C_{15} and C_{25} isoprenologues as components of the diether phospholipids in several strains. —**Mancuso, C. A., G. Odham, G. Westerdahl, J. N. Reeve, and D. C. White.** C_{15}, C_{20}, and C_{25} isoprenoid homologues in glycerol diether phospholipids of methanogenic archaebacteria. *J. Lipid Res.* 1985. **26:** 1120–1125.

Supplementary key words isoprenologues • ether lipids • methanogens

Fatty acid components of eubacterial membranes have been employed to characterize and discriminate between different groups of microbes (1, 2). Additionally, variations in nutritional (3), chemical (4), and physical (5) parameters have been shown to cause modifications in fatty acid length (6), presence of branching (7), and degree of saturation (4, 8).

Archaebacteria, unlike eubacteria, do not possess the ester-linked phospholipid fatty acids. Instead, two isoprenoid nonpolar groups are bound to the glycerol phosphate backbone in ether linkages (9). These are sometimes found with phospholipids consisting of two diether molecules linked in a head-to-head configuration to form a tetraether (10).

Previous studies have shown that extremely halophilic archaebacteria possess only diethers (11, 12) with side groups of 20 carbon, isoprenoid branched chains. Recently, De Rosa and coworkers (13) examined the lipids of a haloalkaliphile and found that the membranes were composed mostly of diethers containing both C_{25} and C_{20} side chains. The authors suggested the presence of a "zip" type membrane where asymmetric diether lipids opposed each other to create an interlocking bilayer.

In thermoacidophiles, tetraethers are generally the only ether lipids found in membranes (14, 15). The nonpolar side chains of these lipids are characteristically 40 carbons in length. Cyclization, which occurs in the form of five membered rings, has been shown to increase with elevated growth temperature (16, 17). Ring formation has been proposed to control membrane width and density. It has also been suggested that cyclization provides greater rigidity and stability at higher temperatures (10).

Methanogens possess both diethers and tetraethers (18). The ratio of these two lipid types seems to vary from species to species, however the side chain length has been shown to be consistently 20 and 40 carbon atoms in diethers and tetraethers, respectively.

This study was undertaken in an effort to determine possible differences in the alkyl side chains of the diether lipids that could be used as signatures of specific methanogenic bacteria.

MATERIALS AND METHODS

Microorganisms

Strains of methanogenic bacteria studied were acquired as lyophilized cultures from sources and habitats of isolation as listed in **Table 1**.

Abbreviations: HFBA, heptafluorobutyric acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; EI, electron impact; PCI, positive ion chemical ionization; MS, mass spectrometry; SIM, selective ion monitoring; NCI, negative ion chemical ionization.

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TABLE 1. Methanogenic bacterial strains examined for side chains from diether phospholipids

Name	Habitat/Primary Source	Obtained from
Methanobacterium thermoautotrophicum str. Hverigerdi	hot spring,/K. Hanselman	J. P. Kaiser/Switz.
Methanobacterium thermoautotrophicum RC	river sed./R. Corder	J. Reeve/Ohio
Methanobacterium thermoautotrophicum ΔH	sewage sludge/J. G. Zeikus	J. G. Zeikus/Wisc.
Methanobacterium thermoautotrophicum ΔH	freshwater sed./R. S. Wolfe	J. Reeve/Ohio
Methanobacterium MMY	marine sed./L. Hook	J. Reeve/Ohio
Methanobacterium Ohio River isolate	river sed./P. Hamilton	J. Reeve/Ohio
Methanobacterium sp. BR-10	river sed./L. Hook	J. Reeve/Ohio
Methanobacterium GC-3A	oil well/L. Hook	J. Reeve/Ohio
Methanobacterium sp. Tenn. River isolate	river sed./P. Hamilton	J. Reeve/Ohio
Methanobacterium formicum MF1	freshwater sed./J. Robinson	J. Reeve/Ohio
Methanobrevibacter smithii	digester/M. P. Bryant	J. Reeve/Ohio
Methanobrevibacter JP2A	digester/L. Hook	J. Reeve/Ohio
Methanobrevibacter JP3	digester/L. Hook	J. Reeve/Ohio
Methanococcus maripalidus	salt marsh/W. J. Jones	J. Reeve/Ohio
Methanococcus vannielii	San. Fran. Bay/R. S. Wolfe	J. Reeve/Ohio
Methanococcus deltae ΔRC	Miss. R. Delta/L. Hook	J. Reeve/Ohio
Methanococcus voltae PSv	marine sed./R. S. Wolfe	J. Reeve/Ohio
Methanococcus voltae	marine sed./P. H. Smith	J. M. Henson/Fla.
Methanogenium marisnigri	Black Sea sed./R. S. Wolfe	J. Reeve/Ohio
Methanospirillum hungatei JF	fresh water sed./M. P. Bryant	J. Reeve/Ohio
Methanosarcina barkerii 227	digester/K. Jarrell	J. Reeve/Ohio
Methanosarcina MeS	marine mud flat/L. Hook	J. Reeve/Ohio
Methanosarcina barkerii MS	digester/M. P. Bryant	J. Reeve/Ohio
Maine coccus MMC	marine sed./L. Hook	J. Reeve/Ohio
Cuyahoga coccus PC[2]	river sed./R. Corder	J. Reeve/Ohio

Extraction

Lipids were extracted from approximately 50 mg of lyophilized cells of each strain by a modification of the Bligh and Dyer method (19). Cells were sonicated in test tubes with Teflon-lined screw-caps with 10 ml of methanol for 30 min. Five ml of chloroform and 4 ml of 50 mM phosphate buffer (pH 7.4) were added, and samples were extracted at room temperature for 18 hr. Tubes were centrifuged and the supernatant was transferred to a 50-ml separatory funnel. Sufficient chloroform and buffer were added for a final ratio for chloroform–methanol–buffer of 2:2:1.8 (v/v/v). After separation of the two phases, the lower chloroform layer was collected through a Whatman 2V filter into a screw-cap test tube and dried under a dry stream of nitrogen at less than 40°C.

Fractionation and hydrolysis of phospholipids

Total lipids were fractionated on a 1-g column of Unisil (Clarkson Chemical Co., Inc., Williamsport, PA) by an elution sequence with 10 ml each of chloroform, acetone, and methanol (20). The phospholipid fractions, recovered in methanol, were dried in a stream of nitrogen.

The phospholipids were then hydrolyzed by adding 1 ml of chloroform–methanol–6 N HCl 10:1:1 (v/v/v) and by heating to 100°C for 2 hr. Glycerol diethers created by the acidic hydrolysis of the phospholipids were extracted by the addition of 1 ml of hexane and 1 ml of water. Extraction was repeated twice and the pooled organic fractions were dried under a stream of nitrogen.

Thin-layer chromatography

Thin-layer chromatography (TLC) was used to isolate the glycerol diethers. The plates of silica gel K6, size: 20 cm × 20 cm × 250 μm (Whatman Chemical Separations, Inc., Clifton, NJ) were precleaned in hexane–diethyl ether–acetic acid 70:30:1 (v/v/v). 1,2-Di-O-hexadecyl-rac-glycerol (Sigma Chemical Co., St. Louis, MO) and authentic glycerol diether prepared from *M. thermoautotrophicum* strain Hverigerdi were applied to end lanes as reference compounds. After development, the end lanes were sprayed with Rhodamine 6G (0.01% w/v) and exposed to UV light for visualization. Glycerol diethers were found to have an R_f of 0.3 in this system. Bands 2-cm wide were scraped. The silica gel was collected in Pasteur pipettes plugged with glass wool and was eluted with 5 ml of chloroform–methanol 1:2 (v/v). The samples were dried under a stream of nitrogen.

Ether cleavage and derivatization

Alkyl iodides and alkyl acetates were prepared according to procedures fully detailed elsewhere (9, 21). The diether lipids were degraded by digestion with 55% hydriodic acid (Mallinckrodt, Inc., Paris, KY) heated to 100°C for 18 hr. The resulting alkyl iodides were extracted into hexane and washed with 10% (w/v) Na_2CO_3 and then 50% (w/v) $Na_2S_2O_3$. The alkyl acetate was formed by mixing the alkyl iodide with silver acetate and acetic acid and heating at 100°C for 18 hr. After adding water, the acetate esters were extracted into hexane and washed with

10% (w/v) $Na_2S_2O_3$.

Alcohols were produced from the acetates by alkaline methanolysis with 0.5 M KOH in methanol heated to 100°C for 3 hr. The alcohols were extracted into hexane, dried under a stream of nitrogen, and then derivatized with heptafluorobutyric acid (HFBA) according to the procedures described by Hogge and Olson (22).

Gas–liquid chromatography

GLC was performed using a Varian 2100 gas chromatograph equipped with a flame ionization detector (FID) and a Hewlett Packard 3390 integrator. Alkyl acetates were injected at 80°C in the splitless mode (vent time = 0.5 min) onto a 25-m fused silica capillary column coated with SE-54 (Alltech Assoc., Inc., Deerfield, IL). After 2 min the oven temperature was increased linearly to 270°C at a rate of 6°C min^{-1}. The injector and detector temperatures were maintained at 300°C. Hydrogen was used as a carrier gas (linear velocity = 55 cm sec^{-1}).

Gas–liquid chromatography–mass spectrometry

Samples were analyzed using a Ribermag R10-10C quadrapole gas chromatograph-mass spectrometer data acquisition system equipped with a Carlo Erba model 4160 GC. Separations were performed on a column similar to the one described above. Helium was used as the carrier gas (linear velocity = 40 cm sec^{-1}). Samples (1–2 μl) were injected in the splitless mode (vent time = 0.5 min) at an injector temperature of 230°C and an oven temperature of 180°C. Immediately after injection, the oven temperature was increased linearly to 270°C at a rate of 6°C min^{-1}.

The electron energy used for electron impact (EI) GLC-MS was 70 eV and for chemical ionization (CI) GLC-MS was 94 eV. The temperature of the ion source for EI/GLC-MS was 140°C.

Positive ion chemical ionization (PCI) MS was performed on the acetate esters using ammonia (99.96%) at an ion source temperature of 110°C and a pressure of 0.06

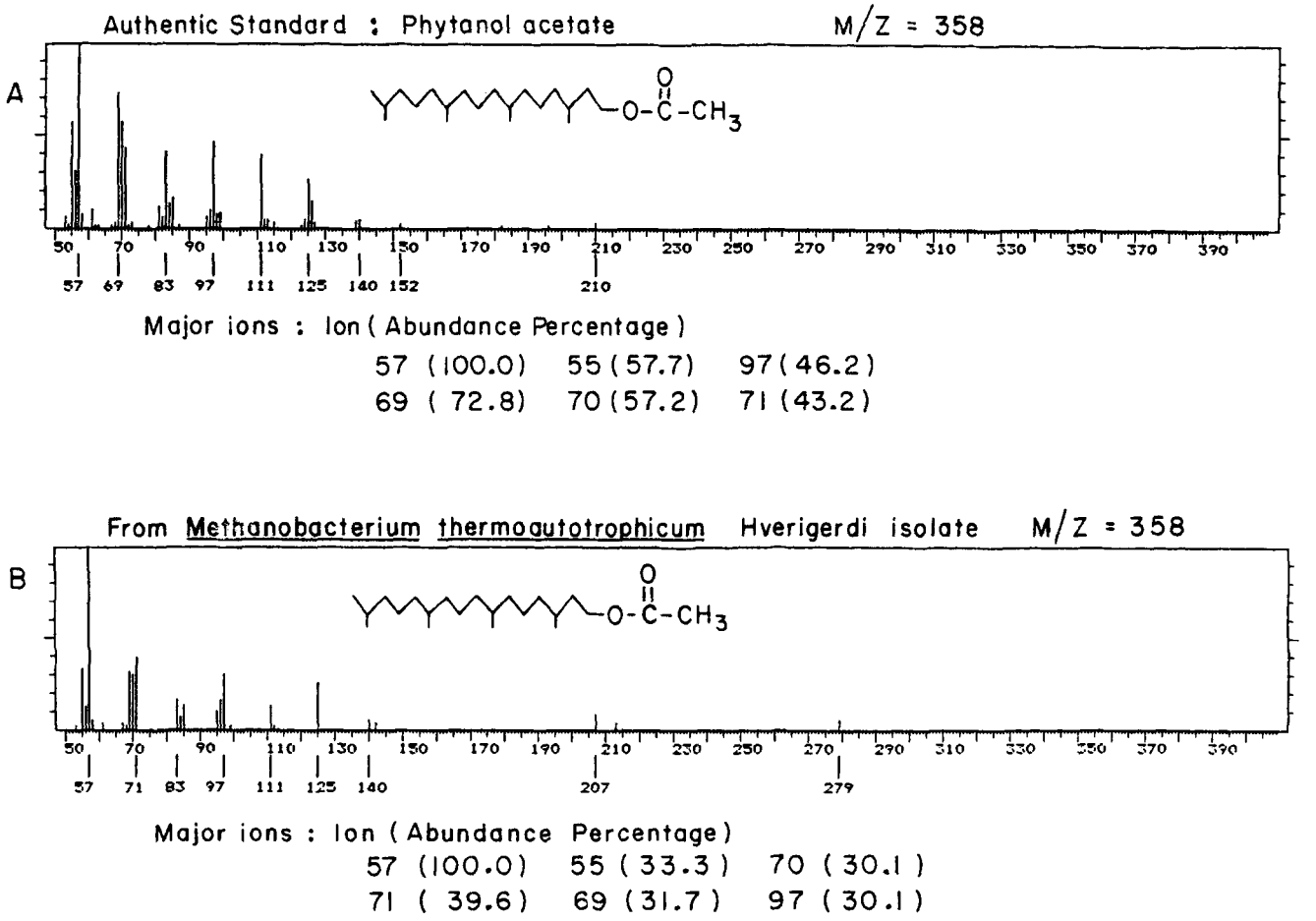

Fig. 1. Electron impact (70 eV) mass spectra of (A) 20 ng of acetate esters from authentic phytanol standard and (B) 10 ng of a prominent component derived from phospholipid diethers of *M. thermoautotrophicum* strain Hverigerdi.

Torr. Methane (99.995%) at a pressure of 0.06 Torr and with an ion source temperature of 180°C served as a reagent gas in the negative ion chemical ionization (NCI) for the analysis of the HFBA derivatized alcohols.

Authentic reference compound

Phytol was partially purified by liquid chromatography on alumina. It was provided in this form by Dr. Liljenberg, from the Department of Plant Pathology at the University of Gothenberg, Sweden. Phytol was hydrogenated after the addition of methanol and platinum oxide catalyst, for 3 hr with mechanical agitation in a Parr Hydrogenator (270 kPa, 20°C). The resulting phytanol was acetylated and purified further by TLC.

RESULTS

Acetate esters

The lower limit of detection for authentic phytanol (retention time = 25.75 min) when analyzed by GLC(FID) was approximately 5 ng. Using GLC-MS in the PCI mode, an m/z at 358.3 $[M + 18]^+$ was verified and the limit of detection was determined to be 40 pg at a signal to noise ratio of 2 to 1. The EI/GLC-MS analysis of this molecule produced a characteristic fragmentation pattern. The major ions are listed in **Fig. 1** in descending order of abundance.

In most bacterial samples, one prominent peak was detected. The GLC retention time and EI/GLC-MS fragmentation of this component isolated from *M. thermoautotrophicum* strain Hverigerdi was nearly identical to that of the standard (Fig. 1). When analyzed by GLC-MS with PCI mode this component, like the standard, showed an m/z at 358.3 (**Fig. 2**).

Apart from the major component, two additional peaks with GLC retention times of 0.75 and 1.34 relative to phytanol acetate (1.0) were found for the *M. thermoautotrophicum* RC sample. Using GLC/MS with PCI in selective ion monitoring (SIM) mode, ions at 288.3 and 428.4 were detected (Fig. 2). These compounds were tentatively assigned as the acetate esters of 15 and 25 carbon isoprenoid-branched molecules, homologues of the 20 carbon isoprenoid molecule, phytanol.

Alcohols

GLC-MS in the NCI mode was employed to detect the HFBA-derivatized phytanol standard. The sensitivity of this technique was increased eightfold compared to the PCI method which employed NH_3 as the reagent gas. A lower limit of detection of 5 pg with a signal to noise ratio of 2 to 1 was obtained. The remaining samples, found by GLC to contain phytanol acetate in reduced amounts relative to *M. thermoautotrophicum* RC sample, were thus converted to alcohols for further analysis.

Ions having m/z values of 404.4, 474.4, and 544.5 which corresponded, respectively, to the $[M-HF]^-$ ion of the C_{15}, C_{20}, and C_{25} carbon containing molecules of the isoprenoid type were detected when the HFBA derivatives of *Methanobacterium* MMY were analyzed by GLC-MS using NCI (reagent gas, CH_4) operating in the SIM mode.

The HFBA-derivatized alcohols of several other samples were analyzed with NCI/GLC-MS. *Methanosarcina barkerii* MS, *Methanobacterium* sp. (Ohio River isolate), *M. thermoautotrophicum*ΔH (Wisconsin isolate), and Cuyahoga coccus PC[2] were found to contain the C_{15} isoprenoid side

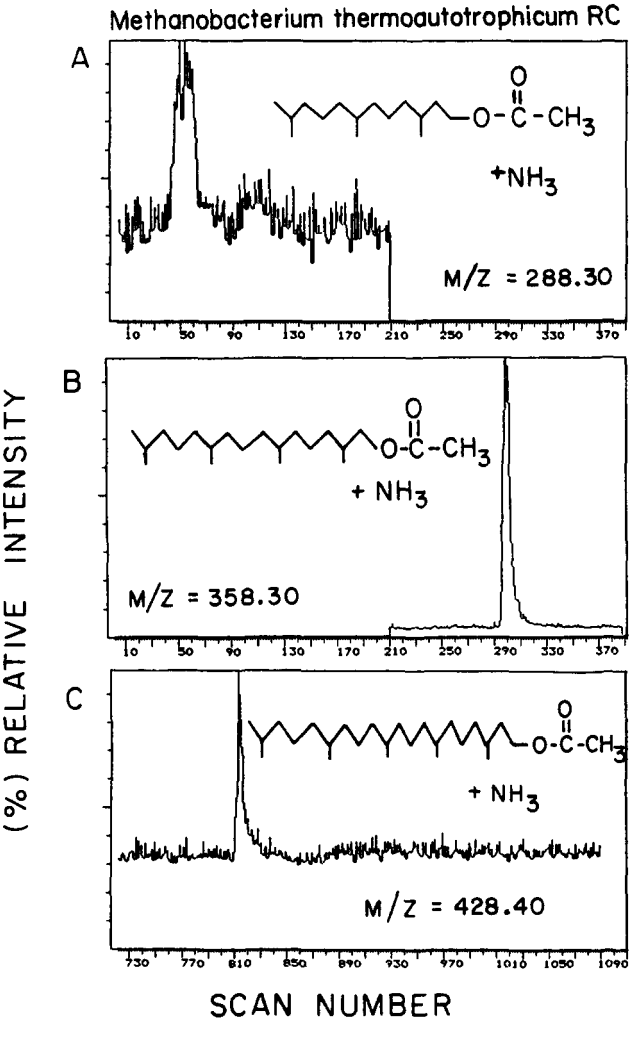

Fig. 2. Mass fragmatogram obtained using PCI/GLC-MS (reagent gas, NH_3) for the acetate esters of the diether side chains monitoring at A) 288.30, B) 358.30, and C) 428.40.

TABLE 2. GLC and GLC-MS features of derivatized diether side chains

Features	Side Chain Length		
	C_{15}	C_{20}	C_{25}
Acetate ester			
Empirical formula	$C_{17}H_{34}O_2$	$C_{22}H_{44}O_2$	$C_{27}H_{54}O_2$
Molecular weight	270	340	410
PCI/GLC-MS $[M + 18]^+$	288.3	358.4	428.4
Scan #	50	300	810
GLC retention time (min)[a]	19.55	25.75	34.56
GLC retention time (relative to C_{20})	0.75	1.00	1.34
EI/GLC-MS[b]		57 (100)[c] 71 (43) 69 (73) 210 (3) 55 (58) 152 (2) 70 (57) 182 (1) 97 (46) 196 (1)	
Alcohol			
Empirical formula	$C_{15}H_{32}O$	$C_{20}H_{42}O$	$C_{25}H_{52}O$
Molecular weight	228	298	368
NCI/GLC-MS $[M-HF]^-$[d]	404.4	474.4	544.5
Scan #	200	335	1150

[a] Temperature program: 80–270°C at 6°C/min.
[b] Phytanol acetate, reference compound.
[c] Ion (relative abundance), base peak = 100%.
[d] HFBA derivative.

chain, in addition to the C_{20} phytanyl chain. The GLC-MS data for the isoprenologues are summarized in **Table 2**.

DISCUSSION

In this study, the side chains of the diether phospholipids from 25 strains of methanogenic bacteria were chemically isolated as acetate esters and alcohols. Capillary GLC and GLC-MS using NCI and selective ion monitoring provided extreme selectivity and sensitivity (23, 24). After close examination of the side chains using these techniques, it was determined that C_{20} phytanol was the most frequently detected side chain component. This finding confirms that of previous workers (10, 18). In addition, C_{15} and C_{25} homologues were also tentatively identified for the first time in the phospholipids of methanogenic bacteria (Fig. 2).

It was hoped that strains of methanogenic bacteria could be identified from the patterns of alkyl side chains. Although two additional isoprenologues were documented in the glycerol diether lipids in some strains, the relatively simple pattern of alkyl side chains, together with the difficulty of preparing alkyl iodides in adequate yields from small samples, mitigate against this technique in routine use. No cyclic or novel alkyl side chains were detected in the diether lipids of the 25 strains of methanogenic bacteria that were examined.

It has been demonstrated that the degree of cyclization in the tetraether lipids of thermoacidophilic archaebacteria increases with elevations in growth temperatures (16, 17). A higher degree of cyclization allows for membrane fluidity at higher temperatures. Similarly, methanogens may vary the amounts of different isoprenologue side chains, such as those reported in the present study. Additionally, the relative amounts of diether and tetraether lipids in cell membranes could be manipulated to maintain adequate membrane fluidity in the range of environments in which methanogens are found. Future studies of the effects of environmental parameters on these cell membranes will give some insight into the physiology and biochemistry of methanogens and other archaebacteria.

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