Long-Chain Diols: A New Class of Membrane Lipids from a Thermophilic Bacterium

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Glycerol-derived membrane lipids are essentially absent in the thermophilic bacterium *Thermomicrobium roseum*. A series of straight chain and internally methyl-branched 1,2-diols of carbon numbers C_{18} to C_{23} were found to replace glycerolipids in this bacterium. Fatty acids were present but were ester-linked to the diols or amide-linked to polar heads groups and not to glycerol. This thermophile has evolved the integration of diols as a novel approach for the construction of its cytoplasmic membrane.

GLYCEROLIPIDS, COMPRISED OF fatty acids, aldehydes, or alcohols linked to glycerol, are universal constituents and the essence of biological membranes (*1–3*). Lipids containing fatty acid esters are characteristic of eubacterial and eukaryotic membrane lipids. A number of unusual lipids containing isopranoid glycerol ethers constitute the membranes of archaebacteria and serve as a chemotaxonomic marker for these organisms (*3*). An additional characteristic of archaebacteria is the absence of a typical cell-wall structure (*4*). *Thermomicrobium roseum* is a Gram-negative, pleomorphic rod lacking a typical peptidoglycan cell wall, although it is sensitive to penicillin (*5, 6*). It is an aerobic, heterotrophic bacterium that grows optimally at 70° to 75°C and *p*H 8.5. Since the unusual cell wall suggested that this might be an archaebacterium, we undertook an investigation of the lipids of this organism.

Cells were grown aerobically at 75°C until the late exponential phase, freeze-dried, and extracted by the method of Bligh and Dyer (*7*). Total lipids, about 3 percent of the dry weight of the cell, were fractionated on a column of silicic acid with $CHCl_3$ to remove the nonpolar neutral lipids (22.6 percent) and then with CH_3OH to elute the combined polar glycolipids and phospholipids (77.1 percent) (*8, 9*).

Polar lipids were hydrolyzed in anhydrous 1*M* CH_3OH-HCl to cleave the polar head groups (*9*), and the apolar residues were extracted by the addition of equal volumes of $CHCl_3$ and H_2O. The $CHCl_3$-soluble products were chromatographed on thin-layer chromatography (TLC) plates made with silica gel H and were developed in a mixture of *n*-hexane, diethyl ether, and acetic acid (70:30:1 by volume; solvent A). Two components were detected with I_2 vapors or after the plates were charred with 50 percent H_2SO_4 in CH_3OH. One component (28 percent) migrated with fatty acid methyl esters (R_F 0.91), and the other (72 percent) migrated in the region of 1-*O*-alkylglycerol monoethers (R_F 0.20) (*9*). The latter component tested positive for vicinal –OH groups when sprayed with $NaIO_4$-Schiff reagent and tested negative for free amine when sprayed with ninhydrin (*8, 9*), reactions displayed by glycerol monoethers. However, the infrared (IR) spectrum (*9*) of this component, measured from a thin film between NaCl crystals, showed only absorption bands of –OH groups (3400 cm^{-1}), secondary –OH groups (1075 cm^{-1}), and primary –OH groups (1045 cm^{-1}). No C–O–C ether band was present nor were any amines or double bonds detected, which eliminated the possibility that the component contained glycerol ethers or long-chain bases. The spectrum was, however, nearly identical to that of *iso*-branched, long-chain 1,2-diols prepared by saponification of wool wax (lanolin) (*10*).

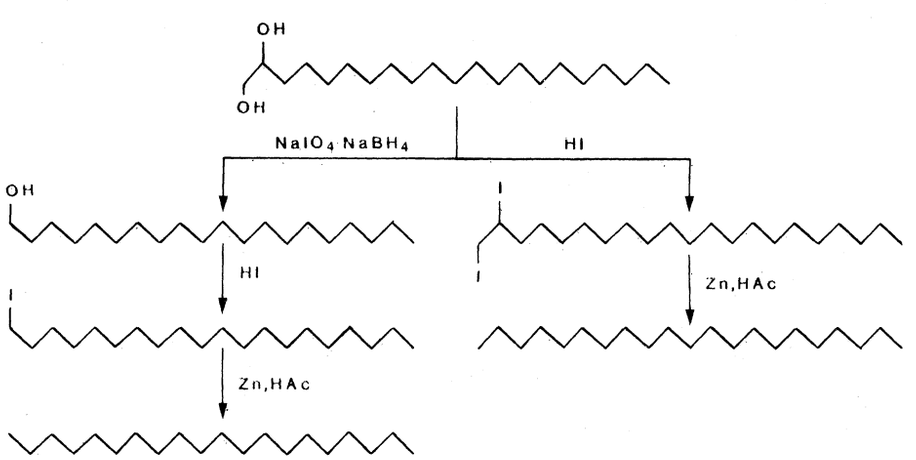

Fig. 1. Degradation scheme of the 1,2-diols of *T. roseum* represented as the *n*-C_{21} diol, 1,2-eicosanediol. Oxidation with $NaIO_4$ resulted in the loss of one carbon atom from the alkyl chain; HAc, acetic acid.

To establish chain length and the position of the vicinal –OH groups on the hydrocarbon chain, we cleaved the diols with $NaIO_4$ and reduced them to the monoalcohols with $NaBH_4$ (*11*). These diols and monoalcohols were also converted to their respective alkyl iodides and then reduced with zinc in acetic acid to their respective alkane derivatives (*8*) (Fig. 1).

Intact diols, derived monoalcohols, and both of the respective alkane derivatives were identified by gas chromatography (GC) and GC–mass spectrometry (GC-MS) as described (*9*). Analysis by GC of the original diols as the acetate or trifluoroacetic anhydride derivatives revealed at least 11 components, none of which eluted with a series of glycerol monoethers (*9*) or the *iso*-branched diols generated from lanolin. Gas chromatography of alkanes derived from the original diols showed the same relative peaks, including peaks corresponding to *n*-alkanes of carbon number 19, 20, 21, 22, and 23 and to branched-chain alkanes. None of the branched alkanes eluted with *iso*- or *anteiso*-branched standards. Alkanes from the monoalcohol derivatives showed the same GC pattern, but all the compounds were one carbon atom shorter than those from the original diols. The loss of one carbon after oxidation with $NaIO_4$ established the position of the vicinal –OH groups on positions C-1 and C-2 of the hydrocarbon chain.

Analysis by GC-MS confirmed the straight chain structures and established the positions of the methyl branches. The mass spectra of the diols showed no molecular ion but a dominant peak at $M - 31$ and a series of olefin peaks common to alcohols. Structures were established by a comparison of the mass spectra from the alkanes derived from the original diols and the alkanes derived from the monoalcohols (Fig. 2). The distribution of the diols is shown in Table 1.

The structures of the fatty acids were similarly identified by GC-MS analysis of derivative alkanes. The esters were convert-

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ed to alcohols with $NaBH_4$; the alcohols were converted to the iodides with HI; and the iodides were reduced to the alkanes with $LiAlD_4$. The fatty acids consisted mainly of the *n*-C_{18} (16 percent) and internally branched 12-methyl-C_{18} (68 percent) components. The remainder included fatty acids with the following carbon numbers: *n*-C_{16}, *n*-C_{17}, *n*-C_{19}, *n*-C_{20}, 10-methyl-C_{16}, 10-methyl-C_{17}, 12-methyl-C_{17}, 7,11-dimethyl-C_{17}, 12-methyl-C_{19}, and 14-methyl-C_{20}. To determine if the fatty acids arose from typical ester-linked acylglycerols, we performed an assay for glycerol with the CH_3OH-H_2O phase after acid methanolysis of the total polar lipids. No free glycerol could be detected enzymatically (*12*) or by TLC (*8*).

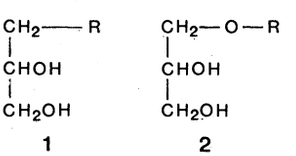
To establish the diols as the hydrophobic backbone, we separated the polar lipids into glycolipids (65 percent) and phospholipids (35 percent) by DEAE-cellulose chromatography and into individual species by TLC as described (*9, 11*). Five glycolipids and four phospholipids were detected; each yielded diols after acid methanolysis, but not all released fatty acid esters. The IR spectra of the glycolipids showed that all but one contained only amide-linked fatty acids. The one ester-containing glycolipid, which accounted for about half the polar lipids, was permethylated (*13*) before and after mild saponification (*11*) to cleave the fatty acid esters. After acid methanolysis, the IR spectrum of the diols from the intact glycolipid showed both primary and secondary –OH bands whereas the diols of the saponified glycolipid showed only primary –OH and a methoxy (1115 cm^{-1}) band. This indicates that the polar head group of the major complex lipid is linked to the terminal –OH of the diols and the fatty acids are ester-linked to the secondary –OH.

The neutral lipids were examined by TLC in solvent A. Free diols and a component migrating in the region of diacylglycerols (R_F 0.55) were present. The latter component contained about equal amounts of diols and fatty acids, and its IR spectrum showed an ester band (1730 cm^{-1}) but only a primary –OH band (1045 cm^{-1}), indicative of 2-acyldiols. This also indicates that the terminal –OH of the diols can provide the attachment site for polar head groups, as the terminal –OH of glycerolipids does in other organisms (*3*). Whether polar head groups are linked to the terminal –OH in all of its polar lipids remains to be established.

Although long-chain 1,2-diols are components of the external surface waxes of some plants (*14*) and the sebum of animals (*10, 15*), their occurrence as membrane constituents is unique. They could, however, be viewed as structural analogs of glycerolipids if there is a bend in their configuration at C-3 instead of a straight-chain structure (**1**). In this manner they are perhaps most analogous to the most reduced glycerolipid counterpart, 1-*O*-alkylglycerol (**2**).

Table 1. Distribution of long-chain diols that replace glycerolipids in *Thermomicrobium roseum*; Tr, trace or less than 0.5 percent.

Carbon number	Compound	Relative percentage	Structure in Fig. 1
Branched C_{18}	11-Methyl-1,2-heptadecanediol	Tr	A
n-C_{19}	1,2-Nonadecanediol	10.6	B
Branched C_{20}	13-Methyl-1,2-nonadecanediol	21.1	C
n-C_{20}	1,2-Cosanediol	6.1	D
Branched C_{21}	13-Methyl-1,2-cosanediol	2.5	E
Branched C_{21}	15-Methyl-1,2-cosanediol	0.9	F
n-C_{21}	1,2-Eicosanediol	48.5	G
Branched C_{22}	15-Methyl-1,2-eicosanediol	7.8	H
n-C_{22}	1,2-Docosanediol	1.0	I
n-C_{23}	1,2-Tricosanediol	1.1	J
n-C_{24}	1,2-Tetracosanediol	Tr	K



The exception is that at C-3 of the diols, which corresponds to C-1 of glycerolipids, there is no functional group present. Also, in this configuration, the extended length of the hydrocarbon chain becomes reduced by three carbons. For example, the chain length of the principal *n*-C_{21} diol is reduced to 18 carbons, which also corresponds to the length of the major 12-methyl-C_{18} fatty acid of *T. roseum*. The diols thus would have a glycerol-like equivalent built into their structure. When acylated with fatty acids at C-2 providing a double chain form, they would appear capable of forming a membrane bilayer in a manner analogous to glycerolipids, although these ideas must be tested experimentally. The presence of internally methyl-branched chains is unusual in thermophiles, which are normally enriched in *iso*- and *anteiso*-branched chains (*2*). The absence of isopranoid-branched glycerol ethers in this organism (*3*) indicates that *T. roseum* is not an archaebacterium but a eu-

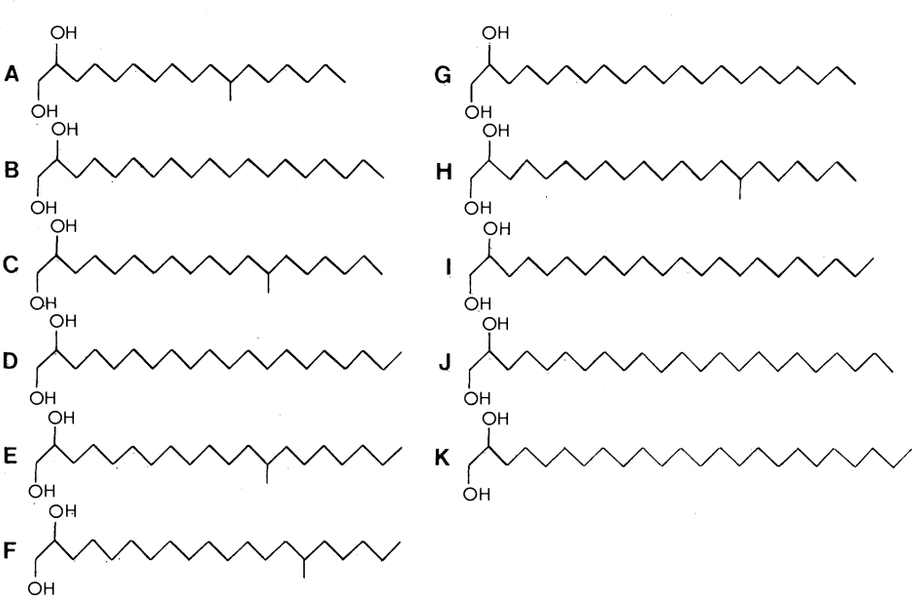


Fig. 2. Structures of the long-chain 1,2-diols of *T. roseum*: (A) branched C_{18}; (B) *n*-C_{19}; (C) branched C_{20}; (D) *n*-C_{20}; (E) branched C_{21}; (F) branched C_{21}; (G) *n*-C_{21}; (H) branched C_{22}; (I) *n*-C_{22}; (J) *n*-C_{23}; and (K) *n*-C_{24}. For GC analysis, we used a column (1.83 m by 4 mm) of 5 percent SP2100 isothermally at 235°C (for diols) and 180°C (for monoalcohols and alkanes) or a 30-m DB5 bonded-phase fused-silica capillary column programmed from 50° to 300°C. Mass spectra were recorded at 70 eV on a GC-MS system (Extranuclear Simulscan) interfaced with a GC (Carlo Erba). Comparison of the mass spectra of alkanes from diols and monoalcohols afforded identification (*17, 18*).

bacterium, which correlates with recent 16S ribosomal RNA analysis (*16*). The diols also reveal a new molecular marker for assessing phylogenetic and biogeochemical relationships of prokaryotes.

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18. For example, the alkane derivative, derived from the branched C_{22} diol, showed a molecular ion at a mass-to-charge ratio (*m/e*) = 310. The enhanced olefin peaks at *m/e* = 112 and 224 identified the compound as 7-methyleicosane. A 7,14-dimethylcosane could generate a similar spectrum, but the alkane from the corresponding monoalcohol showed enhanced peaks only at *m/e* = 112 and 210, not at *m/e* = 224 and 98, as would be expected. The *m/e* = 210 fragment confirmed also the position of the –OH groups in the diol. The observed shift of 14 amu in the spectrum of the alkane from the monoalcohol indicated that the –OH-bearing carbon was lost from the long end of the branch. Thus, 15-methyl-1,2-eicosanediol was the structure of the original diol.

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Crystal Structures at Megabar Pressures Determined by Use of the Cornell Synchrotron Source

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X-ray diffraction studies have been carried out on alkali halide samples 10 micrometers in diameter (volume 10^{-9} cubic centimeter) subjected to megabar pressures in the diamond anvil cell. Energy-dispersive techniques and a synchrotron source were used. These measurements can be used to detect crystallographic phase transitions. Cesium iodide was subjected to pressures of 95 gigapascals (fractional volume of 46 percent) and rubidium iodide to pressures of 89 gigapascals (fractional volume of 39 percent). Cesium iodide showed a transformation from the cubic B2 phase (cesium chloride structure) to a tetragonal phase and then to an orthorhombic phase, which was stable to 95 gigapascals. Rubidium iodide showed only a transition from the low-pressure cubic B1 phase (sodium chloride structure) to the B2 phase, which was stable up to 89 gigapascals.

X-RAY DIFFRACTION STUDIES ON MATERIALS subjected to high pressures provide direct information about pressure-induced crystallographic phase transitions and allow measurement of pressure-volume relations (equation of state). Diamond-window, high-pressure cells have been used for x-ray diffraction studies on a variety of materials because diamonds are transparent to x-rays above 10 keV. X-ray diffraction studies in the megabar pressure range have been carried out with conventional x-ray sources (*1, 2*). In these studies, film techniques were used to record diffraction patterns; x-ray collimators of 50 to 150 μm are typically used, and the data collection time is 100 to 400 hours for each pressure.

Ultrahigh pressures have been generated in the diamond anvil cell, and recently static pressures of 2.8 Mbar have been measured (*3, 4*) on stainless steel and ruby crystal composites. Large pressure gradients are present across the sample region between the two single-crystal diamonds, and in some situations (*3, 4*) gradients of 3 GPa/μm have been measured. These pressure gradients limit the maximum pressures attainable in the diamond anvil cells and also affect the quality of the x-ray diffraction data obtained. They also give rise to diffraction line-broadening in x-ray experiments, and the effect is pronounced in compressible materials. Pressure gradients can be reduced and the state of stress can be made relatively hydrostatic if a soft pressure-transmitting medium such as a rare gas solid is used. However, using such a medium somewhat reduces the maximum attainable pressures. It is important to develop experimental techniques that can be used to measure physical properties on small areas (10 μm diameter or 75 μm^2) so that the state of stress is homogeneous in the sample region.

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The energy-dispersive diffraction technique coupled with a high-brilliance synchrotron source can be used to perform very rapid diffraction studies of samples in the diamond anvil cell (*5*). The Cornell High Energy Synchrotron Source (CHESS) is particularly suitable for this type of work because at normal operating conditions (beam energy 5 GeV; beam current 30 mA) it is an intense x-ray source in the spectral region from 10 to 50 keV (*6*). We have modified the experimental technique of Baublitz *et al.* (*7*) and have used an energy-dispersive technique at CHESS. The essential improvement is in the collimator system, where a stainless steel tube with a lead pinhole is located close to the table of the diamond (about 15 mm from the sample) and is adjusted with motor-driven motion in the two directions perpendicular to the beam. The pinhole is driven by a computer with a video display of the collimator position and can be moved with a precision of 0.2 μm. The adjustment and alignment procedures allow rapid optimization of the sample signal. Using this system, we have been able to record diffraction patterns with a pinhole 10 μm in diameter (sample volume less than 10^{-9} cubic centimeter). This volume is 0.05 to 0.1 of that used in earlier x-ray experiments. With the new collimator system, it is also possible to measure the pressure gradients across the diamond anvil face by x-ray techniques.

The alkali halides have been the subject of extensive study under high pressure because of the possibility of observing transitions from insulator to metal in some of these compounds with the present megabar capability of the diamond anvil cell. Cesium iodide (CsI) and rubidium iodide (RbI), which have band gaps of 6.4 and 6.1 eV, respectively, at ambient pressure, are highly