# Purification of glycerol dialkyl nonitol tetraether from *Sulfolobus acidocaldarius*

Shi-Lung Lo,* Charles E. Montague,* and E. L. Chang[1,†]

*Geo-Centers,* Newton Upper Falls, MA 02164, and Bio/Molecular Engineering Branch,† Code 6190, Naval Research Laboratory, Washington, DC 20375-5000*

**Summary** A modified procedure for extraction and purification of hydrolyzed archaebacterial lipids is described. Lipids were extracted from *Sulfolobus acidocaldarius* using a Soxhlet extraction procedure followed by trichloroacetic acid solvent–extraction of the residue. The yield of total extractable material by this protocol was 14% which, after a two-phase wash, yielded 10% lipid. Modifications to the published steps for purifying the subsequently hydrolyzed lipids were developed to purify glycerol dialkyl nonitol tetraether (GDNT). The nearly colorless final macrocyclic product was characterized by TLC, IR, NMR, and mass spectrometry.—**Lo, S-L., C. E. Montague, and E. L. Chang.** Purification of glycerol dialkyl nonitol tetraether from *Sulfolobus acidocaldarius*. *J. Lipid Res.* 1989. **30:** 944–949.

**Supplementary key words** archaebacteria • thermoacidophiles • *Sulfolobus acidocaldarius* • archaebacterial lipids • tetraether lipids

The membranes of thermoacidophilic archaebacteria are stable under growth conditions that can be as acidic as pH 1 and as high in temperature as 85°C (1, 2). The major lipid of the membranes is an unusual bipolar, macrocyclic, tetraether lipid with glyco- and glycophospho-polar headgroups (2). Only two hydrolyzed lipid classes differentiated by their polar headgroups are obtained from the total lipid extract: glycerol dialkyl glycerol tetraether (GDGT) and glycerol dialkyl nonitol tetraether (GDNT) (**Fig. 1**). It is thought that the high stability of the native archaebacterial membranes can be attributed to the unique chemical structure of these lipids (3).

The ruggedness of the archaebacterial membrane has attracted researchers to characterize the physical properties of the constituent lipids (4–7). In particular, the hydrolyzed archaebacterial tetraether lipids are attractive because their relative simplicity facilitates the study of certain questions concerning structure/property relationships for lipid membranes. The previously published protocols for obtaining GDNT and GDGT (8, 9), however, yielded a brown final product (S. L. Lo, C. E. Montague, and E. L. Chang, unpublished results, and personal communications, T. Langworthy) which almost assuredly implies contamination. We have developed a modified procedure which removes the contaminants to produce a

Abbreviations: GDGT, glycerol dialkyl glycerol tetraether; GDNT, glycerol dialkyl nonitol tetraether; TLC, thin-layer chromatography

[1]To whom correspondence should be addressed.

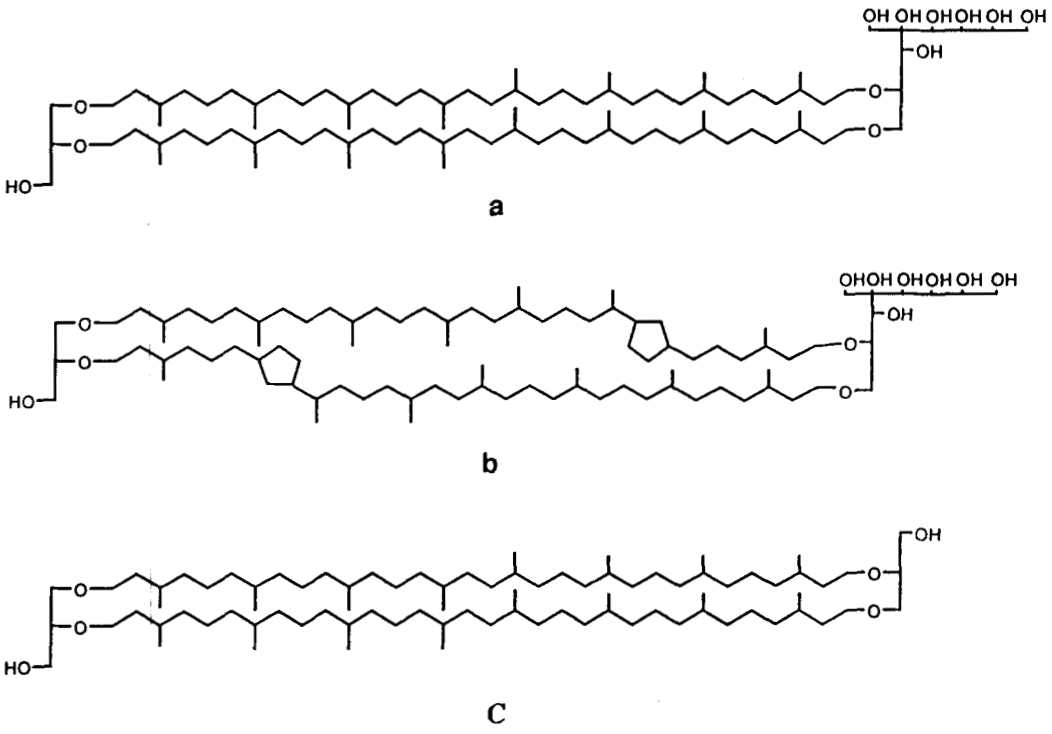


**Fig. 1.** Chemical structure of (a) glycerol dialkyl nonitol tetraether (GDNT) and (b) GDNT with two cyclopetanes; *S. acidocaldarius* lipids can have 0 to 4 rings per alkyl chain; (c) glycerol dialkyl glycerol tetraether (GDGT) possesses only one hydroxyl per glycerol.

higher purity lipid. This will allow the use of spectroscopic techniques such as Raman spectroscopy, which is highly sensitive to fluorescent contaminants, for the study of the lipids in aqueous media. In this report, we detail a modified protocol developed that not only produces a cleaner lipid but also increases the total yield of crude lipids extracted from the organism.

## MATERIALS AND METHODS

### Growth of Cells.

*Sulfolobus acidocaldarius* (ATCC strain 33909, Rockville, MD) was grown at 65–67°C in 20-L glass carbuoys containing 15 L of ATCC medium 1256 supplemented with 1 g/l glucose (10). Air was bubbled through the aqueous medium at 1 l/min and growth was monitored by absorbance at 420 and 540 nm. Cells were harvested by centrifugation at the onset of stationary phase, lyophilized, and stored at −20°C until used. Average yield was 300–400 mg dry cells/l culture.

### Extraction of lipids.

Lipids were extracted for 12 h by the Soxhlet extraction method (8), with 300 ml of chloroform–methanol 1:1 for each 1.5 g lyophilized cells. The residue from the initial extraction then underwent a second extraction with 140 ml of chloroform–methanol–5% trichloroacetic acid 1:2:0.8 at room temperature for 2 h (11). After centrifugation, the pellet was washed twice with 32 ml of chloroform–methanol–water 1:2:0.8. The supernatants from the acid extraction and the two washes were pooled and chloroform and water were added to obtain a ratio of chloroform–methanol–water 8:4:3. The upper phase was extracted with an equal volume of chloroform (system I) and the lower phase was washed with an equal volume of chloroform–methanol–water 3:48:47 (sytem II). The lower phase from system II was then washed with the upper phase of system I. The lower phase from this mixture was then pooled with the lower phase of system I.

### Thin-layer chromatography

Thin-layer chromatography was carried out on LK5F and PLK5F plates (Whatman, Clifton, NJ) using the following solvents: A, chloroform–methanol 9:1; B, diethyl ether–methanol 6:4. Lipids were detected by exposure to iodine vapor or charring with phosphomolybdic acid (12).

### Isolation and purification of GDNT

The lipid extracts were first treated with 1 N methanolic HCl at 75°C for 18 h in a tightly capped flask, followed by adjusting the mixture to a 50% methanol–water solution with water and to pH 14 with 8 N KOH. After hydrolyzing again at 80°C for 1 h, the mixture was brought to

pH3 with HCl and the hydrolyzed products were recovered by adding chloroform and methanol to obtain a solution of chloroform–methanol–water 8:4:3. The lower phase was washed once with an equal volume of chloroform–methanol–water 3:48:47.

After drying the chloroform layer, the residue was suspended in chloroform and transferred to a silica gel column (Merck Kiesel gel, 6 mg lipids per gram of silica gel). The column was eluted with 5 bed-volumes each of the following solvents: chloroform, chloroform–diethyl ether 8:2, and chloroform–methanol 8:2.

The GDNT-containing fraction, chloroform–methanol 8:2, was purified by preparative TLC on PLK5F plate (100 mg lipid per plate) with solvent B. The lipid bands on the plate were located with iodine vapor. A wide band corresponding to GDNT, with $R_f$ = 0.45, was scraped and eluted with chloroform–methanol–water 65:25:4. Methanol and water were added to the solvent mixture to adjust the chloroform–methanol–water ratio to 8:4:3. The lower phase, containing the GDNT, was further purified by preparative TLC on a PLK5F plate with solvent A ($R_f$ = 0.54). The final TLC purification was achieved with solvent B using a prewashed LK5F plate (5 mg lipids per plate). The $R_f$ of the purified GDNT was 0.38.

GDNT obtained from the above procedure was dissolved in a minimum amount of solvent A. The solution was diluted with chloroform to 10 volumes and filtered through a 0.45 $\mu$m Millex-HV filter (Millipore, Bedford, MA). The filtrate was dried and redissolved in warm acetone (40°C). Any insoluble solid was filtered by Millex-HV filter. The acetone filtrate was stored at + 4°C for 18 h then at – 10°C for 30 min to precipitate the GDNT, which was collected by centrifugation.

### Infrared spectroscopy

Infrared spectra were recorded from dried films on KBr windows using Perkin-Elmer Model 1800 infrared spectrophotometer. GDNT was transferred to the windows in chloroform and allowed to air dry.

### Nuclear magnetic resonance

NMR spectra were acquired with a Bruker MSL 300 Fourier Transform NMR Spectrometer system that includes a 7.0 Tesla wide-bore (89 mm) magnet and an Aspect 3000 computer system. Measurements were carried out at 22 ± 1°C. The samples were spun at 15 Hz in 5.0 mm NMR tubes. Samples were dissolved in $CDCl_3$. Tetramethylsilane (1%) was used as an internal reference and assigned a value of 0.0. The $^1H$ NMR spectra were acquired at 300.130 MHz. Free-induction decays were acquired with 5.0 $\mu$sec $^1H$ pulses and a 2.0 sec pulse delay.

### Mass spectrometry

Chemical-ionization mass spectrometry was performed on a Finnigan TSQ-70 triple quadrupole mass spectrometer using ammonia or isobutane as reagent gas.

### UV/vis spectrometry

UV/visible spectrometry of GDNT was performed on a Perkin-Elmer Lamda 4c Spectrometer. Pathlength was 1 cm.

## RESULTS AND DISCUSSION

The recovery of total material from the initial Soxhlet extraction step was 9.5% of dry weight, which was nominally better than the 8% literature value (13). The additional acid extraction of lipids from the residue was originally developed by Nishihara and Koga (11) for methanogens. Our application of this step to the Soxhlet extraction residue from *S. acidocaldarius*, a thermoacidophile, yielded another 4.5% lipids for a total material yield of 14%. However, the initial Soxhlet yield included some possibly proteinaceous material which partitions into the interphase region of a chloroform–methanol–water 8:4:3 two-phase system. A two-phase wash of the Soxhlet extraction decreased the final lipid yield for the Soxhlet ex-

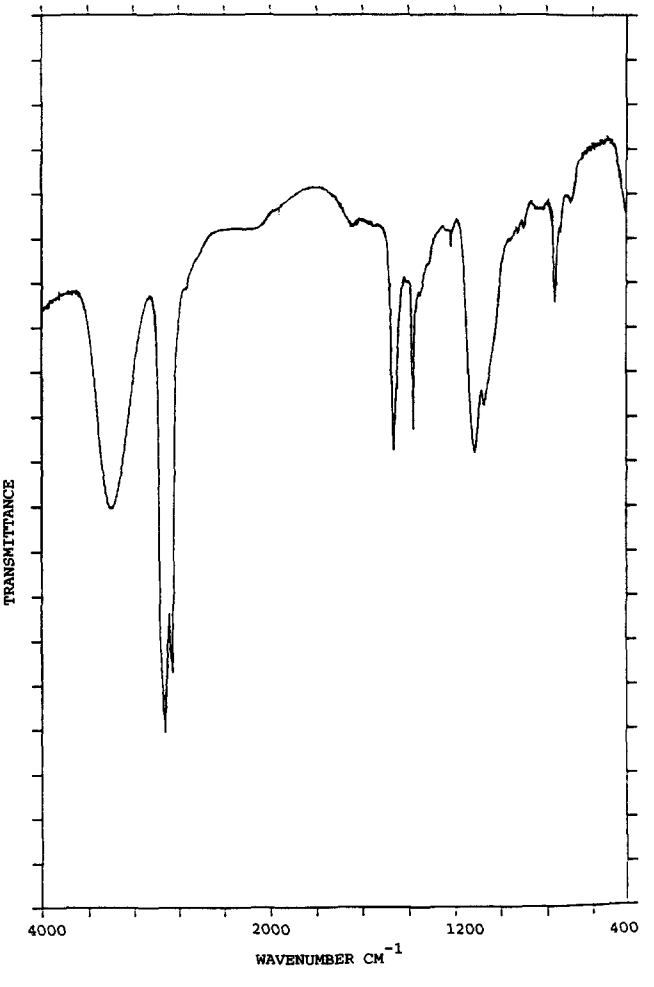


**Fig. 2.** FTIR spectrum of GDNT film.

traction by about 50%, resulting in a net yield of 5% for Soxhlet extraction. The upper phase was re-extracted by a second two-phase wash. The lower phase of this second wash was found to contain negligible amounts of lipids. Thus reported yields on *S. acidocaldarius* by Soxhlet extraction alone should be treated with caution (e.g., ref. 13), as the final amount of usable lipids may be much less. The acid extraction procedure does not suffer this ambiguity as a two-phase wash is standard to the protocol. Therefore, for practical purposes, we define our yield to be the amount of lipids remaining after a two-phase extraction. Thus our net yield from a two-phase extraction of the Soxhlet material (5%) plus the lipids obtained from the acid extraction of the Soxhlet residue (4.5%) is 9.5%. To compare this net yield with published values of 8% for Soxhlet extraction alone (8, 13), the published yields must be reduced by 50% (see above).

An alternative procedure would be to extract the dried cells in acidic solvent directly. This procedure yielded 9.1% lipids. Further extraction of the residue by Soxhlet extraction resulted in an additional 1.4% lipid. Thus, the total yield of lipid, taken to be the material after a two-phase wash, is around 10% for either method.

Both acid methanolysis and base hydrolysis of the total lipids were performed in order to assure complete cleavage of the phosphodiester and sugar linkages. In the TLC purification step, the hydrolyzed lipids were overloaded to improve the separation of the colored material from GDNT. Most of the colored material was removed by repeated preparative TLC on PLK5F plates. Some colored material that was not resolvable from GDNT on PLK5F plates was removed on 250 $\mu$m LK5F plates. Final purification was achieved by acetone precipitation. The purified GDNT has the same $R_f$ values in solvents A and B as the GDNT provided by Dr. Langworthy. Average yield was 1-1.5% of dry cell mass.

Mass spectral analysis showed peaks at 1460, 1456, and 1452 a.m.u., corresponding to $[M-H_2O]^+$, where M is the GDNT molecule with degrees of cyclization from 1 to 5 (major peak for cyclization = 3). Diagnostic fragments missing a glycerol backbone ($C_3H_6O_3$), [M-90], or missing an alditol ($C_6H_{13}O_6$), [M-181], were also seen. No peak around 1480 for the intact GDNT was found. The absence of this parent peak has been reported previously (8).

Infrared spectroscopy of the GDNT in chloroform (**Fig. 2**) showed a broad band centered at 3400 $cm^{-1}$ for OH stretch, two strong peaks at ~2920 and 2850 $cm^{-1}$ due to CH stretch modes, a peak at 1460 $cm^{-1}$ from $CH_2$ and at 1375 $cm^{-1}$ from $CH_3$ bending modes. In addition,

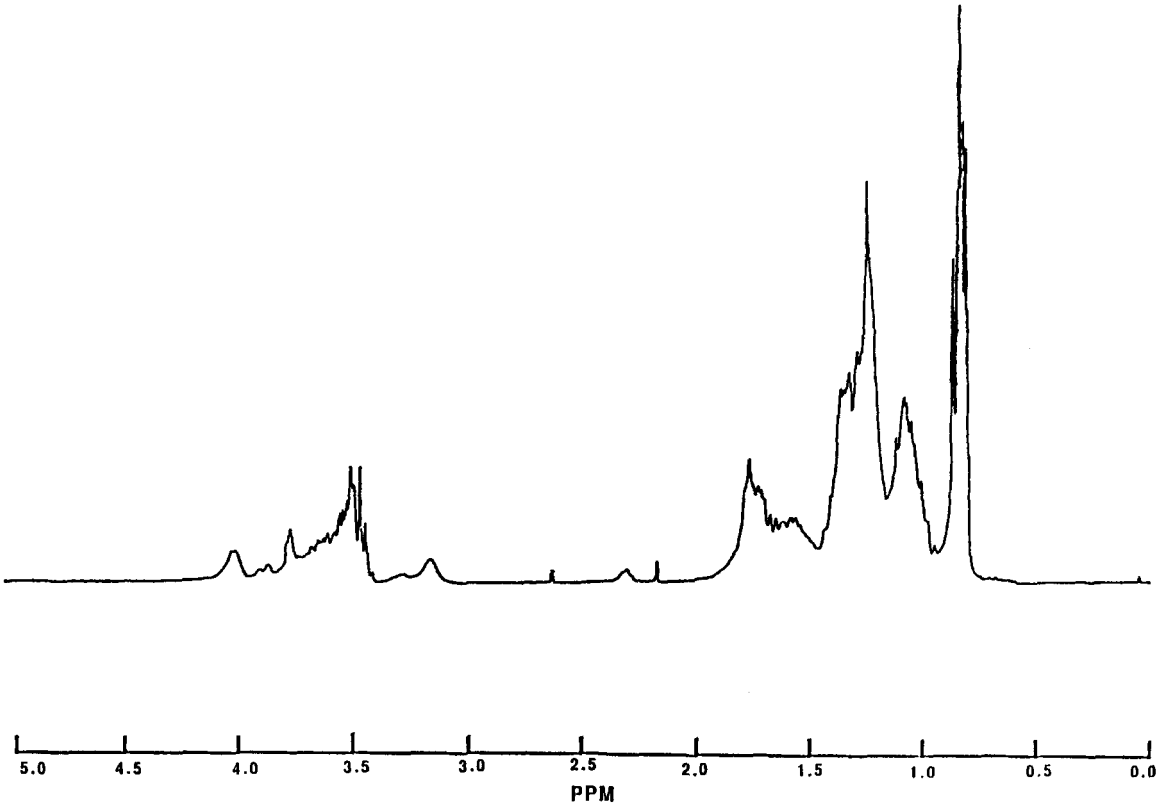


**Fig. 3.** NMR spectrum of GDNT.

a strong band was found at ~1110 $cm^{-1}$ for the ether stretching vibrations and a peak at 1070 $cm^{-1}$ is most likely due to primary hydroxy stretch. The ratio of the 3400 $cm^{-1}$ to 2920 $cm^{-1}$ peaks exhibited by the spectrum is consistent with the high amount of hydroxyls in GDNT. The existence of ether bonds is confirmed by the 1100 $cm^{-1}$ peak, while the high content of methyl groups is indicated by the shoulders at ~2960, 2870, and 1450 $cm^{-1}$.

The proton NMR spectrum of the pure compound (**Fig. 3**) showed signals at $\delta$ 0.7-0.9 ($-CH_3$), $\delta$ 0.9-1.5 ($-CH_2$ and -CH), $\delta$ 1.6-2.0 (cyclopentanyl protons), and $\delta$ 3.2-4.0 ($CH_2$-O-, CH-O-, and -OH). The peaks corresponded well to the general structure of GDNT with cyclopentane rings. Quantitative interpretation of the overlapping peaks, especially in the high-field region, was difficult; but the results were consistent with a GDNT molecule with multiple rings. Thus, the NMR, combined with the IR and the mass spectral data, provided conclusive support that the final product was GDNT, containing from one to five cyclopentane rings per molecule.

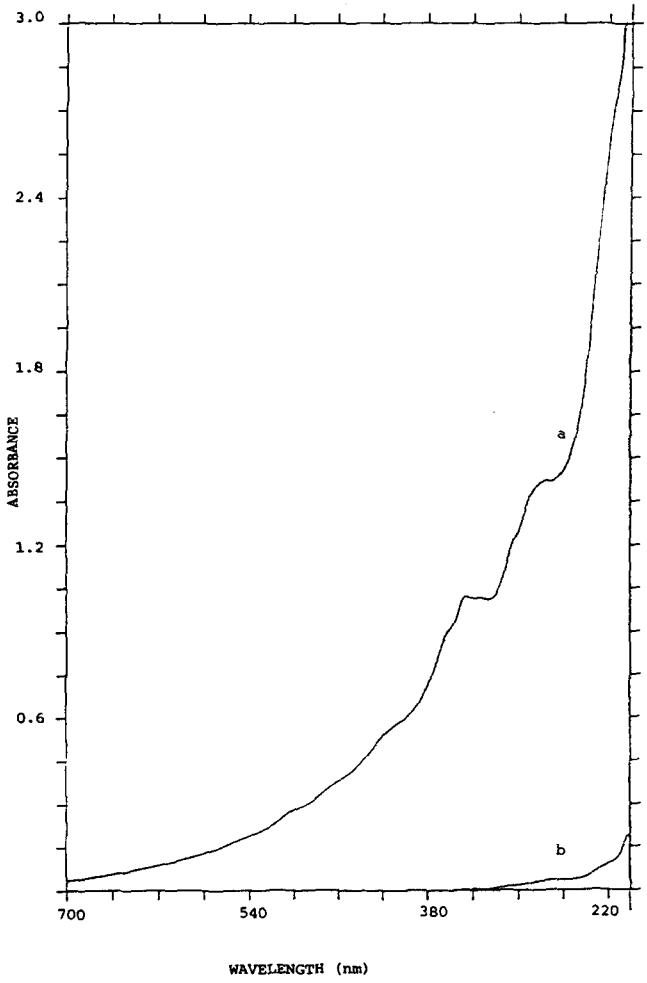


**Fig. 4.** UV/vis spectra of GDNT (1 mg/ml in isopropanol-hexane 1:1); upper curve for GDNT purified by published method (9), lower curve by present method.

The UV/visible spectra (**Fig. 4**) demonstrates that the total absorption of the purified GDNT decreased by over an order-of-magnitude over lipid obtained by published procedure (9).

We have described here a procedure for the extraction and purification of GDNT, a hydrolyzed lipid from *S. acidocaldarius*. Whereas the published extraction procedures yielded a product that was visibly contaminated, the modified procedure presented here eliminates the contamination. The modifications to existing procedures include the addition of an acidic solvent extraction of the residue from the initial chloroform-methanol extraction, a base hydrolysis of the extracted total lipids, a multiple-step TLC purification, and a final acetone precipitation step. The final product from this process is a nearly colorless oily lipid.

The authors would like to thank Dr. Robert Kelly for supplying us with the starting cultures of *S. acidocaldarius*, Drs. Mark Ross and John Callahan for obtaining the mass spectra, and Dr. Walt Gerasimowicz for the NMR spectra. We would also like to acknowledge the kind gift of hydrolyzed lipid standards from Dr. Tom Langworthy. Part of this work was supported by ONR contract no. N0001488wx24088.

*Manuscript received 21 July 1988, in revised form 10 October 1988, and in re-revised form 6 December 1988.*

## REFERENCES

1. Brock, T. D. 1985. Life at high temperatures. *Science.* **230:** 132-138.
2. Langworthy, T. A., and J. L. Pond. 1986. Membranes and lipids of thermophiles. *In* Thermophiles: General, Molecular, and Applied Microbiology. John Wiley & Sons, Inc., New York. 107-135.
3. Langworthy, T. A. 1985. Lipids of archaebacteria. *In* The Bacteria. Vol. 8. C. R. Woese and R. S. Wolfe, editors. Academic Press, New York. 459-497.
4. Luzzati, V., and A. Gulik. 1986. Structure and polymorphism of tetraether lipids from *Sulfolobus solfataricus.* II. Conjectures regarding biological significance. *System. Appl. Microbiol.* **7:** 262-265.
5. Gliozzi, A., G. Paoli, D. Pisani, F. Gliozzi, M. DeRosa, and A. Gambacorta. 1986. Phase transition of bipolar lipids of thermophilic archaebacteria. *Biochim. Biophys. Acta.* **861:** 420-428.
6. Rolandi, R., H. Schindler, M. DeRosa,and A. Gambacorta. 1986. Monolayers of ether lipids from archaebacteria. *Eur. Biophys. J.* **14:** 19-27.
7. Bauer, S., K. Heckmann, L. Six, C. Strobl, D. Blöcher, B. Henkel, T. Garbe, and K. Ring. 1983. Hyperfiltration through cross-linked monolayers II. *Desalination* **46:** 369-378.
8. DeRosa, M., A. Gambacorta, B. Nicolaus, B. Chappe, and P. Albrecht. 1983. Isoprenoid ethers: backbone of complex lipids of the archaebacterium *Sulfolobus solfataricus. Biochim. Biophys. Acta.* **753:** 249-256.
9. DeRosa, M., S. deRosa, A. Gambacorta, and J. D. Bu'Lock. 1977. Lipid structures in the *Caldariella* group of extreme

thermoacidophile bacteria. *J. Chem. Soc. Chem. Comm.* 514–515.

10. Brock, T. D., K. M. Brock, R. T. Belly, and R. L. Weiss. 1972. *Sulfolobus*, a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch. Mikrobiol.* **84:** 54–68.
11. Nishihara, M., and Y. Koga. 1987. Extraction and composition of polar lipids from the archaebacterium, *Methanobacterium thermoautotrophicum:* effect of extraction of tetraether lipids by an acidified solvent. *J. Biochem.* **101:** 977–1005.
12. Kates, M. 1972. Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids. North-Holland Publishing, Amsterdam. 437–438.
13. DeRosa, M., A. Gambacorta, B. Nicolaus, and J. D. Bu'lock. 1980. Complex lipids of *Caldariella acidophila*, a thermoacidophile archaebacterium. *Phytochemistry.* **19:** 821–825.