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Archaeal Tetraether Lipids

Unique Structures and Applications

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Abstract

The extremely stable biomolecules manufactured by organisms from extreme environments are of great scientific and engineering interest in the development of robust and stable industrial biocatalysts. Identification of molecules that impart stability under extremes will also have a profound impact on our understanding of cellular survival. This review discusses isolation and characterization of archaeal tetraethers as well as target technologies for tetraether lipid application. The isolation and characterization of archaeal tetraether lipids has led to some interesting applications improving on ester lipid technologies. Potential applications include novel lubricants, gene-delivery systems, monolayer lipid matrices for sensor devices, and protein stabilization. Following this review, patent abstracts and additional literature pertaining to the isolation, characterization, and application of archaeal membrane lipids are listed.

Index Entries: Tetraether; archaea; liposomes; ether lipids; glycerol dialkyl glycerol tetraether; glycerol dialkyl nonitol tetraether; extremophiles; archaeosome; proteoliposome; Langmuir-Blodgett films.

Introduction

The third domain of life, now commonly referred to as the Archaea, is a surprising collection of biochemical motifs, some resembling those of prokaryotes and eukaryotes and others resembling neither. The identification of unique archaeal motifs and functions has revolutionized scientific views of life's adaptability. Members of the Archaea proliferate in the extreme habitats of hypersaline and alkaline lakes, hot acid springs, and deep-sea hydrothermal vents, as well as relatively ordinary environments

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such as anoxic lake sediments and the guts of ruminants. Even the "mesophilic" (low-temperature- and low-pressure-adapted) methane-generating archaea are extreme in their requirement of severely anoxic conditions. Thus, despite the identification of hyperthermophilic bacteria, the term *extremophile* is often applied to the Archaea as a whole.

Most commercial applications of biomolecules from extremophiles have been based on niche technologic requirements for extreme catalytic conditions. Additional development has been driven by the desire for more robust catalysts with extended processing capabilities and longer shelf lives. As a result, extremophilic enzymes have made an impact in several industries through their application in food processing, detergent formulations, and as catalysts for nucleic acid modification and synthesis *(1–4)*. Still other opportunities for novel technology development have been founded on the expanded characterization of unique biochemical structure and function among the extremophilic bacteria and archaea.

The major fraction of cell membrane lipids in the Archaea are unique among all known living organisms in the absence of ester-linked glycerol fatty acid lipids, a structural motif that once appeared to be ubiquitous to life on earth. Instead, archaeal membrane lipid profiles comprise mainly C_{20}–C_{40} isoprenoid-subunit backbones, linked by ether bonds to glycerol and/or nonitol bridge group(s). The bridge group(s) is either unsubstituted or substituted with one of a wide variety of polar or nonpolar head groups. The unusual membrane composition in archaeal species has resulted in intense study into contributions of ether lipids to extremophily. In addition to evaluating the physiologic significance of ether lipids, effort has been expended to characterize the impact of unusual lipids in stabilizing engineered systems.

Membrane Lipids

Before the elucidation of archaeal membrane composition, the bacterial and eukaryal lipid motif was generally accepted as the "universal template" for biologic membranes. Using monopolar amphiphilic lipids, bacterial and eukaryal cells construct their membranes in a bilayer arrangement. In this context, monopolar and bipolar refer not to charge, but to whether the lipid has one head group or two (one at each end of the hydrophobic backbones). The nonarchaeal monopolar amphiphilic lipids consist of a glycerol moiety covalently bound by ester linkages with an *sn*-1,2 stereochemistry to two minimally branched, or linear, hydrophobic fatty acid chains of various length and degree of saturation *(5)*. Typical chain lengths are C_{14}–C_{24} with C_{16} and C_{18} being the most common. The lipids line up in a tail-opposed orientation to form a closed vesicle of hydrophilic surface and hydrophobic interstitial space. The energy barrier that this hydrophobic core imposes on the diffusion of polar species is responsible for the very low-solute permeabilities seen in biologic membranes.

While the head group functionalities are mostly reminiscent of the ester lipids', the archaea employ a very different backbone arrangement. Instead of mostly unbranched fatty acid lipid cores, the archaeal lipid core is, without exception, based on a highly branched chain isoprene molecule called phytanyl. These novel lipid chains are attached to a glycerol, or other polyol bridge group, via ether functionalities, with a heretofore unencountered *sn*-2,3 stereochemistry. Ethers possess greater chemical stability than esters under harsh environmental conditions. Not only are the fully saturated phytanyl lipid cores much stiffer than fatty acid lipids, but also the branched chains are thought to pack much tighter than the straight-chain lipids of the nonarchaea *(6)*. This packing contributes to the much lower basal ion permeabilities of the extreme thermo(acido)philes *(7–9)*.

Adaptations to Extreme Conditions

The cellular survival response known as homeoviscous adaptation changes the lipid profile of bacterial and eukaryal membranes, both in functionality of the lipid backbone and in the ratios of the nonpolar to polar lipids. For example, as a bacterial cell's environment heats up, the cell will respond by decreasing the degree of unsaturation of its lipid backbone chains, as well as increasing the relative amounts of apolar constituents, such as sterols. These changes serve to modulate the membrane fluidity that is directly related to the permeation of solutes *(6,10)*.

Modulation of membrane permeability is critical to the maintenance of osmotic pressure within the cell as well as to the control of motility, nutrient uptake, and energy generation through transmembrane electrochemical gradients. Although bacteria and eukarya can alter the degree of saturation and other lipid parameters, there is still a narrow envelope of temperatures and pHs that will support their survival. Outside of this envelope, the permeability of the membranes derived from fatty acid lipids will no longer be low enough to support the energy-generating transmembrane proton gradient (pmf). Two genera of hyperthermophilic bacteria appear to defy this model. These hyperthermophiles are obligately neutrophilic. Because the permeability of bacterial membranes to H^+ is too high at these extreme temperatures, these hyperthermophiles utilize sodium ions as the energetic coupling agent. Owing to its larger size, Na^+ is less permeable than H^+. Thus, a sodium electrochemical potential, sodium motive force (smf), for the generation of energy enables hyperthermophilic marine bacteria to survive *(7,11)*. Use of sodium as the energetic couple is common for marine organisms, but this strategy seems to aid in the survival of bacteria at extremely high temperatures.

Despite the evolution of bacterial strategies to circumvent membrane permeability issues, the fact that fatty ester lipid molecules themselves are likely to break down at extremes of pH and temperature enforces the upper limit of their thermal and acid tolerance. Thus, the relatively unstable nature of ester linkages imposes constraints on the extremophilic potential of bacteria.

Archaeal tetraethers have higher intrinsic stability in the membrane environment. To adapt further to extreme conditions, archaeal species alter lipid profiles in response to environmental changes by introducing multiple cyclopentane functionalities into the lipid core. These cyclopentane functionalities serve to stiffen further the membrane, raising the temperature at which the lipids pass from glassy to the physiologically necessary liquid crystalline state *(12–15)*. Extremophiles may also alter the relative distributions of backbones and head groups in the membrane to optimize membrane fluidity at the given environmental conditions. As a result of intrinsic stability and homeoviscous adaptation, these ether lipid membranes have been shown to have much lower basal hydrogen ion permeability rates than fatty ester lipid membranes.

Archaeal Lipid Profiles

While archaeal lipid-core profiles are not as diverse as those found in bacteria and eukarya, they are quite remarkable in their own right. In all species thus far discovered, this backbone is based on permutations of saturated isoprenoidic-alcohol chains condensed with the vicinal hydroxyls of a glycerol or complex polyol (e.g., nonitol), thus forming the ether linkage *(16–20)*. Individual chain lengths vary discretely within a highly specific range of values (C_{15}, C_{20}, C_{25}, or C_{40}). Of the diethers, the phytanyl chain (C_{20}) is the most commonly encountered archaeal lipid backbone. While branched isoprenoids, such as phytanyl, can also be found throughout nonarchaeal species (in molecules such as carotenoids, vitamin A, and retinal), archaeal organisms are the only ones to employ this as a constituent functional group in their membrane lipids *(21)*. Generally, these lipids come in two classes: the monopolar and the bipolar molecules.

Figure 1 depicts common lipid arrangements for archaeal species. Schematically, the monopolar ether lipids can be similar to bacterial and eukaryal lipids in their construction. These ether lipids consist of a derivatized amino-, glyco-, phospho-, or sulfohydrophilic head group linked to the hydrophobic backbone via a glycerol residue *(19)*. Some of the monopolar lipids consist of macrocycles in which both isoprenoidic tails are covalently linked to the same head group (Fig. 1C). Bipolar lipids, on the other hand, are an utterly unique class of membrane lipids occurring only in archaeal species. For example, instead of only one hydrophilic head group bonded to two hydrophobic chains, these lipids have two head groups, one ether linked to each end of the C_{40} chains. Thus, they resemble two monopolar lipids whose tails have been covalently bound (Fig. 1A,D). The structure of these lipids could theoretically allow the formation of monolayer membranes or a bilayer in which the lipid is bent into a U shape *(13)* with both tethered polar heads on the same side of the membrane. However, all available data point to an ubiquitously occurring monolayer membrane of roughly double the thickness of a traditional bilayer membrane, when the bipolar lipids are present *(16,22,23)*. While some bacteria

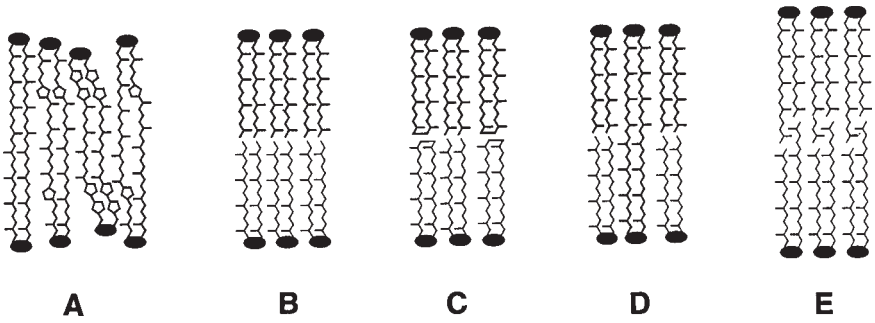

Fig. 1. Examples of backbones found in Archaeal phenotypes: **(A)** tetraethers of the extreme thermo(acido)philes *(16)*, **(B)** diethers of the halophiles *(16)*, **(C)** diethers and macrocyclic diethers of hyperthermophilic methanogens *(17)*, **(D)** di- and tetraethers of most methanogens *(20)*, **(E)** sesterterpanyl isoprenoids found predominantly in extreme haloalkaliphiles *(20)*.

and eukarya employ ether linkages in their membrane lipids, they do not employ the novel isoprenoid backbone motifs *(24,25)* and thus do not form monolayer membranes.

Gambacorta et al. *(26)* and Gulik et al. *(27)* have provided good reviews of the diversity of head groups among archaeal ether lipids. For the most part, archaeal head groups comprise typical building blocks (e.g., phosphates, inositol, sugar residues, and derivatized amino groups). When rare or unusual head group functionalities occur, these structures are distributed among species within the same Archaeal genera. This division, in a manner similar to that of the lipid cores, closely follows phenotypic lines *(28–33)*.

The presence of amino head groups is a unique taxonomic marker of the methanogenic Archaea (e.g., phosphoethanolamine, phosphoserine, and glycophosphoethanolamines). Further, dimethyl and trimethyl aminopentanetetrol and glucosaminylinositol are specific to methanogens. Inositol is a common methanogenic head-group constituent thought to be shared with only a few species of thermophiles from the Kingdom Euryarchaeaota. Phosphoglycoderivatives are also mainly methanogenic in origin but have been reported in a halophile (halococci) as well as in the aforementioned Euryarchaeotic species. A notable exception to the methogenic phenotype is *Methanopyrus*, a species devoid of amino head groups, bearing mainly glyco- and phosphoderivative head groups.

The halophilic species are comprised of derivatized phospho-lipids, the main difference lying in the functional group attached to the primary phosphate. Glycerol (PG), glycerol-phosphate (PGP), glycerol-sulfate (PGS), and a methylated version of glycerol phosphate (PGP-Me) have been identified in a variety of species. In the neutrophilic halophiles, glycosyl and sulfate-glycosyl derivatives predominate, differentiated mainly by the structure, number, and interglycosidic linkages of the sugars, and the positions of the sulfate group.

In the Kingdom Crenarchaeota, there are mainly galactose and/or glucose and phosphomyoinositol head groups. Subdifferentiation arises from the stereochemistry of the glycosidic bond, and the location of the interglycosidic linkages. Thermophilic sulfur-dependent species contain mostly complex tetraether-based lipids containing phosphoglycolipids with a (poly)saccharide as one head group (e.g., β-D-galactopyranosyl-β-D-glucopyranose), and a derivatized phosphate group (e.g., phosphomyoinositol) at the other end of the molecule.

Isolation of Tetraether Lipids

Techniques for isolating tetraether lipids *(21,22,34–37)* are based on the lipid isolation protocols of Bligh and Dyer *(38)*. The Bligh & Dyer (B&D) process is the industry standard for isolation of the membrane lipids, regardless of their source *(39)*. This protocol is used to isolate the total lipid content of biologic materials by contacting a wet-cell slurry with a 2:1 (v/v) chloroform:methanol solution. For the isolation of archaeal tetraethers, researchers have used the original B&D protocol *(40)* or increased the polarity of the B&D solvent by increasing the methanol content *(14,15, 22,27,41–45)*.

Aside from solvent polarity, further distinction among purification techniques of various tetraether lipid research groups may be seen in the mode of extraction. The French and Italian groups *(14,15,27,41,42,46)* run overnight batch extractions at room temperature or below (cold extractions) on lyophilized samples. Lo et al. *(34)*, a Dutch group *(43–45)*, and Sprott and colleagues *(47,48)* extract samples using either Soxhlet extraction or a unique diethyl ether/acetone extraction. Soxhlet extraction has an advantage over the stirred-batch system, in that it uses considerably less solvent to extract the same mass of cellular material. Still other permutations of the B&D method for tetraether lipid extraction involve the addition of either an aqueous 2 *M* HCl or 5% trichloroacetic acid solution to the chloroform:methanol solvent. The acid extraction is reported to result in a sixfold increase in the recovery of lipids *(37)*. The added benefit of this acid treatment is the selection of the ether-linked archaeal lipids in the extract, as ester lipids readily hydrolyze under acidic conditions. However, the increases in yield may vary with each archaeon and with the degree of coextractions of archaeal protein *(21)*.

The nature of downstream processing of the total lipid extract (TLE) is dependent on the intended end use of the ether lipids. When lipids are used to classify an organism taxonomically, the backbones of the total lipids are of greatest importance. Thus, a TLE is generated and exposed to a strong acid and/or base to remove the headgroups hydrolytically. The lipid backbones are then separated using a combination of column chromatography and high-performance liquid chromatography before they are characterized. Without the head groups, the lipid backbones are separated based on their relative polarity, which means that they are fractionated

based on the bridge molecule (glycerol-glycerol or glycerol-nonitol) and the number of pentacycles in the backbones *(27)*.

Polar fractions of the TLE, the polar lipid extract (PLE) *(22,41)*, or even subfractions of the PLE *(15,43)* are of interest in nontaxonomic applications. The processes by which PLE is isolated from a TLE are varied. For instance, after obtaining the TLE, a PLE may be generated by extracting the nonpolar lipids from the TLE by a two-phase extraction of *n*-hexane added to the TLE *(14,27)*. This hexane-washed PLE can then be further separated into constituent fractions using thin-layer chromatography (TLC) or silica gel column chromatography by using ever more polar solvents to elute the constituent lipid species *(15,27,39,41)*.

To generate preparative samples of the tetraether lipids, methods more amenable to scale-up may be beneficial. Lo and Chang *(22)* use reverse-phase extraction on a C_{18} column, eluted with three decreasingly polar solvents (1:1 methanol:chloroform, 0.8:2:0.8 chloroform:methanol:water, 65:25:4 chloroform:methanol:water), followed by preparative TLC to isolate the wide PLE band from the intermediately polar C_{18} lipid fraction. Elferink et al. *(43)* have followed a similar protocol, choosing to stop the purification at the C_{18} extraction step. The TLE is separated into fractions of differing polarity on a silica gel column. Again, the lipids separate according to their relative polarities, but unlike the backbones, the intact lipids are fractionated primarily by head group *(15,20)*, resulting in nonhomogeneous lipid solutions, rich in several types of complex lipids *(15,27)*.

The salient feature common to all tetraether isolation protocols is that the solvents used are nonpolar enough to take advantage of the large apolar regions characteristic of lipids, while polar enough to be miscible with the water in a wet-cell pellet or bound in dried cell samples. To elute the TLE fractions from a silica gel column, a variety of solvent mixtures may be employed *(15,27,39,41)*.

Applications of Tetraether Lipids

Archaeal Lipid Backbones: A Taxonomic Tool

Unlike the vast majority of bacterial and eukaryal membrane lipids, archaeal lipid backbones are extremely useful as taxonomic markers *(49)*. When classifying archaeal species based on their lipid content (as opposed to using rRNA or other genetic methods), the taxonomic positions typically divide archaeal species along phenotypic lines. Consequently, great attention has been paid to isolating and characterizing the lipid cores of archaea.

The lipids of the thermoacidophilic genus *Sulfolobus* are the best-studied representatives of the thermophiles *(50–52)*. The Sulfolobales also have the widest lipid spectrum in the archaea at the level of polar head structures and of isoprenoid chain variety *(19)*. The vast majority of *Sulfolobus* membrane lipids are the glycerol dialkyl glycerol tetraethers (GDGTs) and glycerol dialkyl nonitol tetraethers (GDNTs). GDNTs are unique to the Sulfolobales. As with all organisms, lipid content and lipid profiles are

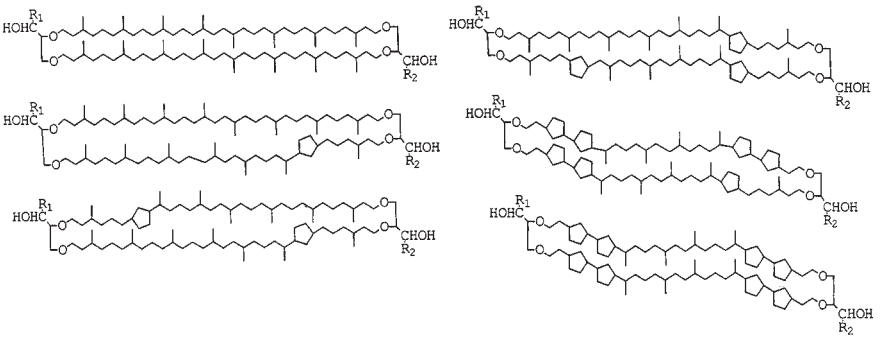

Fig. 2. Lipid backbones of thermo(acido)philes depicting varying degree of pentacyclization. For GDGT, R_2 = H; for GDNT, R_2 = $CH_2OHCOH(CHOH)_3CH_2OH$.

affected by the conditions under which the cells are grown *(36,40)*. Within *Sulfolobus* species, diether:tetraether ratios vary from 0 to 0.1, while GDNT:GDGT ratios vary from 1.3 to 5 *(19)*. Altered distribution and character of tetraether lipids have also been reported for hyperthermophilic methanogens *(47)*. Thus, the degree of pentacyclization in the tetraethers (Fig. 2) varies with growth conditions (e.g., temperature, anaerobiosis, and hetero- or autotrophy) as well as with genus and species *(19,36,40,47)*.

While the lipids of most thermophiles are based solely on the tetraethers, three extremely thermophilic archaea have been shown to contain only the monopolar diphytanyl-glycerol-diether. Cyclized tetraethers have also been found in the mesophilic methanogen *Methanosarcina*. These discoveries make sweeping statements about the connection of membrane thermal stability with pentacyclized tetraether lipid content difficult to defend.

Other Applications of Ether Lipids

Tetraether lipids have been evaluated for their use in the lubrication of engines. This research has resulted in a patent for novel boundary lubricants *(53)*. The patent claims to provide a boundary lubrication composition with a friction coefficient of <0.1. The thermal stability of the ether lipids from extremophilic archaea is a key feature in the generation of a lubricant that does not require preheating or a carrier lubricant. The ether bonds convey chemical resistance to peroxidation, which is a problem for conventional lubricants. Furthermore, the patent purports that the bipolarity of the tetraether will enhance surface contact and packing of the lipid-lubricants. Consequently, a solution of archaeal or modified archaeal tetraether lipids could be used to lubricate engine parts. A typical lubricant formulation may include variously derivatized GDGTs and GDNTs, with and without internal pentacycles. The lipids may also be modified to include halogenations along the isoprenoid backbone for added stability *(53)*.

Applications of Tetraether Liposomes and Lipid Films

A body of academic and industrial research has been conducted to understand better the thermodynamic and kinetic driving forces for membrane behavior. A primary goal of these efforts has been to elucidate how the membranes of living organisms work. More specifically, since the structure and morphology of liposomes can be made to resemble cell membranes, vesicle-cell membrane fusion, membrane fission, and membrane phase thermodynamics and thermotropism can be evaluated *(54–56)*. Based on the behavior of liposomes and of proteins reconstituted into the lipid environment, several biomedical and bioengineering applications have been developed *(16,57–60)*. Such applications include the transfer of materials to cells in vitro and in vivo, for genetic manipulation, immunomodulation, or chemical modification.

Outside of their more obvious application in the medical and biological sciences, archaeal lipid models are of interest specifically in their applicability to the production of novel heterogeneous catalysts, bioelectronics, and nanotechnology *(16,60)*. Interest in these areas is stimulated primarily because the tetraether lipids self-assemble into ordered structures whose structural and functional parameters change as a function of temperature and solvent characteristics.

Preparation and Processing of Liposomes

Liposomes form spontaneously when dried amphiphiles are rehydrated by an aqueous medium, forming a population of vesicle sizes from hundreds of nanometers to tens of microns. Spontaneous liposome formation has been reported for purified archaeal tetraethers *(34,41,61)*. However, it is clear that not all fractions of a PLE will form liposomes outside of mixtures with other lipids *(41)*. For example, nonamphiphilic lipids, such as quinones, will not form liposomes independently. Elferink et al. *(61)* evaluated liposome formation from each of three reverse-phase extracted ether lipid fractions of increasing polarity. This work demonstrated that only the intermediately polar fraction was capable of forming liposomes in the absence of exogenously added *Escherichia coli*–derived phosphatidylcholine. Similarly, Lo and Chang *(22)* demonstrated that their more highly purified PLE, based on the intermediate fraction from the C_{18} column, also autonomously generated liposomes. However, contrary to Elferink et al. *(43)*, Lo et al. *(34)* claimed that the only way to obtain PLE capable of forming liposomes was through their more extensive lipid purification protocol.

The desired end use of the liposome has implications for methodologies for the preparation of liposomes. For archaeal tetraethers, vesicles have been produced through a variety of traditional physical techniques including the hand-shaken method, bath or probe sonication, and solvent extrusion *(55,62)*. The protocol used for production most strongly affects the size and lamellarity of liposomes. In addition to these physical parameters, many applications of liposomes involve the entrapment of aqueous sub-

strates within the vesicle. As a result, methods have been developed in order to maximize substrate loading.

Drug Delivery

Liposomes have practical applications in the medical field as immunocompatible vehicles for the introduction and controlled release of drugs and for vaccine delivery *(58,63–65)*. Efforts toward utilizing liposomes as vehicles for the transfer of genetic information in molecular biology applications have also been pursued. Regarding the introduction of vaccines, and of particular note in the area of archaeal lipid technology, is the recent discovery that tetraether containing archaeosomes (liposomes made from archaeal lipids) can stimulate the immune system *(40)*. Specifically, the mouse humoral immune response to the proteins bovine serum albumin and cholera toxin B, when delivered by archaeosome, is much superior to the response generated when the antigens are encapsulated by liposomes of ester lipids, or not encapsulated at all.

Gene Delivery

Genetic research in archaea is hampered by the paucity of mechanisms for cellular uptake of DNA *(66–69)*. Many archaeal species are not naturally able to take up external DNA. Furthermore, the extreme environmental conditions for cellular function are often too harsh for the introduction of unprotected DNA into the medium. One way to overcome these obstacles is to deliver the gene(s) to the cells mechanically. As such, a solution of the DNA to be introduced is entrapped in a liposome and mixed with the culture to be transformed such that the vesicles fuse with the cell membranes, delivering their contents directly to the cytoplasm. Liposome-mediated transfer of plasmid DNA into archaea has been successfully applied for synthetic lipid systems with low-temperature methanogenic archaea *(66)*. Membrane fusion studies with archaeal tetraethers *(61)* provide a basis for the feasibility of archaeosome-mediated transformation. Because direct uptake of DNA by hyperthermo(acido)philes may prove impractical owing to the acidity and temperature of the cultures, this technology may become useful for hyperthermo(acido)philic archaea as techniques for cellular mutation and selection of transformants are further developed.

Protein Reconstitution

To date, relatively little research has been conducted on archaeal proteoliposomes. Research on archaeosomes has been limited primarily to developments of liposome construction techniques, based mostly on pre-existing techniques developed for ester lipids *(70)*. A few reports have been published in which nonarchaeal proteins have been reconstituted into archaeal liposomes or hybrid liposomes of tetraethers and phosphatidylcholines *(43,44,71–74)*. These studies dealt with membrane-spanning

polypeptides involved in ion and electron transport systems. Cavangetto et al. *(71)* demonstrated that ionophores in PLE/phosphatidyl choline vesicles were functional in facilitating transport. Lower transport rates than in native membranes were attributed to charge interactions from head groups of the PLE and basic amino acids on the polypeptide.

In 1992, Elferink et al. *(43)* demonstrated that tetraether lipids of archaea can be used to produce a suitable matrix for the function of exogenous membrane proteins originating from lipid bilayers. The investigated membrane proteins carried out transport functions, generating transmembrane potentials. High concentrations of tetraether lipids were found to inhibit activity of proteins from fatty ester membranes under physiologic temperature and pH *(44)*. Proteins reconstituted from diether lipid bilayers were fully functional *(44)*. Further studies investigated the potential for increased thermophily induced by the tetraether monolayers *(45)*. This research showed that a lipidic environment enhances the stability of transmembrane enzymes over that in detergent systems. Respiratory enzymes fared better in lipid environments most similar to the native membrane. The work to date demonstrates an interest to study mixed lipid systems for combined effects on transport and stability.

Preparation and Processing of Lipid Films

Supported membrane systems are key features of emerging nanotechnologies. Contributing to growing interests in this field are three major objectives: (1) "biofunctionalizing" inorganic solids and polymeric materials; (2) providing natural environments for protein immobilization without denaturation; and (3) preparing ultrathin layers on conductors for biosensor design *(60,75)*. Additional applications of lipid films involve the study of cell-surface interactions related to physiologic function. As such, research to date on the subject of tetraether monolayers encompasses potential applications in bioelectronics. This area of inquiry also suggests interesting prospects for understanding archaeal physiology and for developing novel interfacial catalysts.

Monolayer lipid films, also termed *black lipid membranes*, have been prepared from archaeal tetraethers for the evaluation of lipid structure and membrane transport, as well as for potential application in nanotechnology *(76)*. Membranes have been evaluated at the air-water interface or after deposition onto solid supports. Gliozzi et al. *(23)* prepared monolayer lipid membranes above 70°C with the dispersion of GDNT in squalene using the Rudin and Mueller technique *(77)*. This method of membrane formation utilizes a wire loop to form an annulus for bilayer formation. By applying tetraether lipids with chloroform or butanol solvents, black lipid membranes could form at temperatures lower than 70°C, but not below 40°C.

Other researchers have used the method of Wilhelmy to study Langmuir monolayers of archaeal lipids *(60)*. These techniques have also been applied to form Langmuir-Blodgett (LB) films of archaeal lipids *(79–81)*. The process of forming such monolayers requires the deposition of a lipid

and/or protein monolayer on a water surface and compression in a Langmuir trough. LB films are formed on a solid substrate by contact of the substrate with the monolayer through a method of "vertical lift" *(75,78)*.

Deposition of protein films is desirable for the development of biosensors and other bioelectronic applications. Because of the instability of water-soluble proteins at the biomolecule-semiconductor interface, effective formation of densely packed molecular arrays has been demonstrated for few proteins. Lipid systems have been used to facilitate the formation of such arrays *(79)*. Such protein lipid composites may be of use in exploiting the functional principles of membrane-associated and membrane-integrated molecules *(80)*.

Bioelectronics

In 1994, De Rosa et al. *(16)* touted the potential application of archaeal lipids to the field of bioelectronics. The advantage of using ether lipids in biosensors can be attributed to the intrinsic stability of the ether molecules. However, an even more compelling attribute is the ability of archaeal tetraether lipids to form self-assembled asymmetric membranes *(23)*. The membrane structures are characterized by two external polar surfaces with different chemical, physical, and electrical properties. Further, the archaeal lipids offer novel structural opportunities in terms of protein lipid interactions *(16,79)*.

The ability to construct synthetic bipolar lipids has added to the body of knowledge about monolayer orientation and provided models for monolayer evaluation. However, both synthetic single-chain and double-chain bipolar lipids are more flexible than natural tetraether lipids. The methyl branches and pentacycles in the natural tetraethers provide a more stable lipid component of the monolayer *(81)*.

Electrophysical characteristics of tetraether monolayers are important to bioelectronic applications. Tetraether monolayer membranes are intrinsically good insulators, but the electrical conductance can be increased by the addition of ionophores *(49)*. Application of an external electric field to polar ether lipids produces unequal potential profiles across the monolayer, resulting in an internal potential difference within the membrane *(23)*. The membrane thus displays asymmetrical conductance. Formation of electrically oriented monolayers is possible only when the lipid system is warmed (above 60°C) to increase sufficiently the flexibility of the lipids that would otherwise be in the frozen state *(23)*.

Insertion of ionophores into a membrane alters its intrinsic electrical properties by allowing free diffusion of ions through the membrane. Films with a uniformly oriented distribution of the mesophilic, K^+-selective ionophore valinomycin were obtained. Nicolini *(79)* found that these films were stable at high temperatures (100°C) and in aqueous solutions, thus, demonstrating that ionophores of nonarchaeal origin may be utilized to modify thermophilic archaeal lipid vesicles.

Conflicting data exist on the structure of the tetraether lipid monolayer films at the air-water interface. Some researchers have determined through surface pressure isotherms area/lipid values that suggest an upright arrangement of lipids. Other investigators have determined a folded (U-shaped) arrangement of the lipid molecules at the interface. Nicolini *(79)* reported the fabrication of LB thin films of nine different archaeal lipids. The electron micrographs and electron diffraction patterns of these films revealed a U-shaped configuration for the lipids *(79)*. Dote et al. *(82)* evaluated GDNT and GDGT monolayers at the air-water interface. Surface pressure/area and surface potential/area isotherms were consistent with single polar head groups at the lipid-water interface. No evidence supported the lipids having both polar heads at the lipid-water interface. GDNT films were more sensitive to subphase pH and ionic strength than GDGT films. Thus, they inferred that the nonitol head groups of GDNT lipids orient at the lipid-water interface.

Recent work by Bakowsky et al. *(81)* sought to clarify the structure of the monomolecular film at the air-water interface using film balance, ellipsometry, X-ray, and atomic force microscopy experiments. This research team proposed a time-dependent model for the organization of tetraether lipids in monolayer films, which shows mixed lipid arrangements. Atomic force microscopy revealed elevated domains, which represent upright lipid populations surrounded by "horseshoe-forming" lipid molecules. The organization of lipids at the air-water interface strongly depends on spreading time and surface pressure. The upright standing orientation appears to be a metastable state encountered at high surface pressure.

The state of the lipids within the monolayer may be influenced by interactions with proteins. Schuster et al. *(80)* studied the interactions of GDNT monolayers with cell-surface proteins. S-layer proteins of *Bacillus coagulans* E38-66 were recrystallized on the surface of preformed GDNT monolayers. After formation of GDNT monolayers with vertical arrangements as determined by surface pressure measurements in the Langmuir trough, an S-layer solution was injected into the subphase. Recrystallization occurred at the GDNT-water interface. The electrophysical features of this biomimetic membrane were evaluated in comparison with plain GDNT films, demonstrating that the S-layer-supported monolayer displayed a lower conductance than the plain GDNT monolayer.

Conclusion

The unique characteristics of archaeal tetraether membrane lipids have spawned several novel applications of these molecules in the chemical industry. Intrinsic thermostability, resistance to chemical modification, and asymmetric self-assembly are special characteristics that are attractive for a variety of applications. As these lipid systems continue to be characterized in liposomes and on monolayer surfaces, still more biofunctionalized

materials may be designed. In addition, the elucidation of molecular interactions that contribute to extremophily in natural systems will enable the further utilization of archaeal tetraethers in bioengineered systems.

Patents and Literature

Introduction

The objective of this section is to summarize and cite recent developments in industrial and academic research as represented within the scope of current patents and literature and to highlight emerging, biotechnologic research areas. The subject of this section is the development and application of technologies using archaeal tetraether lipids.

Patents

This section covers patents concerning the use of archaeal tetraether lipids from January 1970 to October 2000. This time period was considered in order that the total history of the isolation and application of archaeal ether lipids could be highlighted. The major search headings were archaea, archaeabacteria, ether lipid, and tetraether. Many of the abstracts were edited for clarity. The following patents are listed: European Patent Organization (EP), Japanese Patent Organization (JP), World Intellectual Property Organization (WO), and Deutsches Patent und Markenamt (DE). Copies of US patents can be obtained from the Office of Public Records, Document Services Division, Crystal Gateway 4, Suite 300, Jefferson Davis Highway, Arlington, VA 22202.

Chang, E. L.
New Class of Lubricants Derived from Archaebacterial Lipids
5,098,588, March 24, 1992
Assignee: The United States of America as represented by the Secretary of the Navy (Washington DC)

A new class of lubricants based on the general features of archaebacterial lipids but not limited to lipids solely extracted from archaebacteria is described. These lubricant/additive molecules have the following features: bipolarity, ether bonds, and branched biphytanyl chains. They can be double or single chained. This new class has the potential to be highly thermally and chemically stable because of these features.

Sprott, G. D. (Ottawa, CA); Patel, G. B. (Nepean, CA); Choquet, C. G. (Quebec, CA); Ekiel, I. (Quebec, CA)
Formation of Stable Liposomes from Lipid Extracts of Archaeobacteria (Archaeu)
5,989,587, November 23, 1999
WO9308202, April 29, 1993
EP0610289
Assignee: National Research Council of Canada (Ottawa, CA)

Novel ether lipids were obtained from methanogenic (*Methanospirillum hungatei*, *Methanococcus jannaschii*, *Methanococcus voltae*, *Methano-*

sarcina mazei, and *Methanobrevibacter smithii*) and extremely halophilic (*Halobacterium cutirubrum*) representatives of the archaebacteria. Several of the ether lipids produced by *Methanospirillum* and *Methanosarcina* genera were purified and characterized structurally for the first time. Unilamellar liposomes were prepared from emulsions of the total polar-ether lipid extracts of such bacteria by pressure extrusion through membranes of various pore sizes. Liposome populations were shown by dynamic light scattering and electron microscopy to range in size depending on the pore size of the filter, the source of the lipids, and the composition of the suspending buffer medium. In all cases, the size ranges indicated highly homogeneous preparations. Leakage of entrapped fluorescent or radioactive compounds established that the ether liposomes were stable to attack by phospholipase A(2) and B and were stable for at least 60 d to storage in an atmosphere of air. The detergent dialysis method of forming liposomes also produced unilamellar liposomes from most of the archaeobacterial total polar lipid extracts tested.

Kazuhiro, I., Hiroyoshi, M., Sadami, O., and Shuhei, K.

Method for Measuring Amount of Methane Bacterium Utilizing Acetic Acid

JP 05-068590, March 23, 1993

Assignee: TOTO Ltd.

A method to measure surely, highly sensitively, and accurately the amount of a methane bacterium utilizing acetic acid by measuring the amount of a monoalkyl glycerol contained in a specimen to be tested is described. The amount of a monoalkyl glycerol in a specimen to be tested is measured. The measurement of the monoalkyl glycerol is preferably performed by extracting an ether-bonded lipid from a test specimen containing a methane bacterium utilizing acetic acid, removing polar groups existing in the lipid from the lipid, introducing a labeled substituent in the obtained neutral lipid, and subsequently detecting the substituent of the monoalkyl glycerol among the introduced substituents in the produced derivative. Specifically, 9-anthroyl nitrile is introduced as the labeled substituent in a neutral lipid, and, subsequently, the fluorescence of the monoalkyl glycerol derivated with the anthroyl nitrile is measured.

Freisleben, H.-J. Dr (DE), Gropp F. (DE), Hartmann K. (DE), Antonopoulos E. (DE), Balakirev M. (RU), Balakireva L. (RU)

New Macrocyclic Tetraether Lipid Derivatives

DE19736592, February, 25 2000

Assignee: Syrinx Diagnostika Gmbh (DE); Freisleben Consulting Dr (DE)

Conventional liposomes that are used for transporting pharmaceutical active agents in eukaryotic cells or for lipofection can only be preserved for a limited period, are not acid stable, and require a number of set parameters in order to achieve satisfactory results. Less sensitive liposomes are therefore highly desirable. The macrocyclic tetraether lipid derivatives described in this patent and liposomes or liposome aggregates containing

these derivatives are new. The form of tetraether lipid derivatives, consisting of a 72-membered macrocyclic dibiphytanyl-diethyl-tetraether ring derivatized by two substituted carbamoyl groups, and derivatives of such modified by the formation of pentacycles in the tetraether structure, are new. Polar head groups, S1,S2 = -CONH-X1-N(R1)*n*-X2-Y; Y = NR2R3 or -N<+>R4R5R6; X1,X2 = 2-20C alkylene or alkenylene; R1–R6 = H or alkyl, alkenyl, aralkyl, or aryl (all of up to 12C); or one of R1–R6 = antibody against cell surface molecules or ligand for cell-surface receptors; *n* = 0–10. Independent claims are included for liposomes and lipid aggregates containing at least one of these novel lipids optionally together with a nucleic acid molecule. The inventive tetraether lipid derivatives are extremely stable and well suited to lipofection.

Freisleben, H.-J. Dr (DE), Bakowsky, U. (DE), Rothe, U. Dr (DE), Antonopoulos, E. (DE)

No Title Available

DE19607722, September 4, 1997

Assignee: Freisleben, H. J. Dr (DE)

The invention relates to a tetraether lipid in which one of the branch groups is a modified or an unmodified gulose radical or an oxidation product of said gulose, and the other is hydrogen, modified or unmodified phosphoglycerine, or an oxidation product thereof. It also relates to the use of a tetraether lipid according to the invention and liposomes that contain 0.1–100% by weight, in relation to the whole lipid, of the tetraether lipid according to the invention. Conventional liposomes have a low level of stability in storage and are acid labile. The use thereof for the oral administration of pharmaceuticals that should not be released until they are in the duodenum is therefore not possible. By contrast, liposomes containing the tetraether lipid according to the invention are acid stable and can therefore move easily through the stomach into the small intestine and cause resorption therein of active ingredients that are contained in said liposomes and cannot usually be absorbed into the bloodstream after oral administration.

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