Title: Cyclohexane Rings Reduce Small Ion Membrane Permeability in Archaea-Inspired Tetraether Lipids

Authors: Takaoki Koyanagi; Geoffray Leriche; David Onofrei; Gregory Holland; Michael Mayer; Jerry Yang, Ph.D..

To be cited as: 10.1002/ange.201510445

Link to VoR: http://dx.doi.org/10.1002/ange.201510445

Cyclohexane Rings Reduce Small Ion Membrane Permeability in Archaea-Inspired Tetraether Lipids**

Takaoki Koyanagi,[a] Geoffray Leriche,[a] David Onofrei,[b] Gregory P. Holland,[b] Michael Mayer,[c] and Jerry Yang*[a]

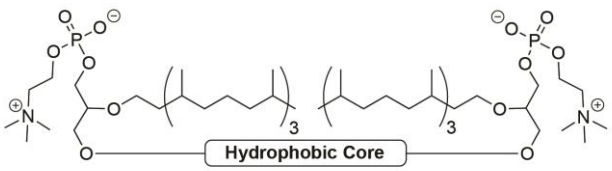
Abstract: Extremophile Archaea organisms overcome problems of membrane permeability by producing lipids with structural elements that putatively improve membrane integrity compared to lipids from other life forms. Here, we describe a series of lipids that mimic some key structural features of Archaea lipids such as: 1) single tethering of lipid tails to create fully transmembrane tetraether lipids and 2) small rings incorporated into these tethered segments. We found that membranes formed from pure tetraether lipids leaked small ions at a rate that was ~2 orders of magnitude slower than common, bilayer-forming lipids. Interestingly, incorporation of cyclopentane rings into the tetraether lipids did not affect membrane leakage, whereas a cyclohexane ring reduced leakage by an additional 40%. These results show that mimicking certain structural features of natural Archaea lipids results in improve membrane integrity, which may help overcome limitations of many current lipid-based technologies.

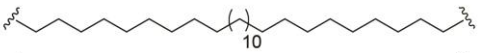
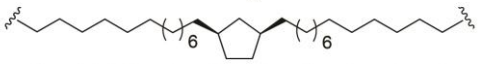
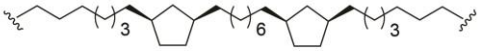
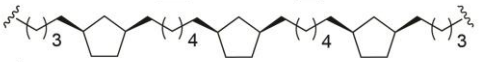
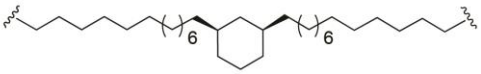
In Nature, ion pumps, molecular transporters, and alterations of membrane composition are used to reduce membrane leakage and maintain gradients.[1–4] Archaeal organisms (halophiles, thermophiles, acidophiles, nitrifiers and methanogens), one of the three domains of life, have evolved mechanically and chemically robust membrane compositions that allow survival in extreme environments.[5] For instance, Crenarchaeota, a kingdom of Archaea, have an optimal survival temperature above 80°C.[6] Interestingly, the membranes of these hyperthermophiles are comprised of lipids containing cyclopentane rings, with a positive correlation found between the number of cyclopentane rings integrated to their lipid membrane and environmental growth temperature.[1,7] This unique structural feature has been proposed to decrease membrane leakage by increasing lipid packing.[8] Additionally, Thaumarchaeota have cyclohexane rings incorporated into their lipids,[7] which have also been suggested to affect membrane packing.[9]

Modification of membranes by addition with cholesterol, PEG-lipids, and lipids with high phase transition temperature are a common strategy to reduce membrane leakage in laboratory settings.[10] However, incorporation of additives to membranes can be problematic due to, for instance, leaching,[11] potential long-term toxicity,[12–14] or difficulty with liposome preparation.[15]

Several attempts have been made to address problems with membrane leakage by using Archaea extracted lipids or through chemical synthesis of Archaea-inspired lipids. For instance, polar lipid fraction E (PLFE) extracted from *Sulfobus acidocaldarius* exhibit low permeability, tight membrane packing, and high stability.[16,17] However, harvesting reproducible and large quantities of specific lipid compositions from cultured Archaea can be challenging.[18] While a few groups have also reported the synthesis of singly tethered transmembrane spanning tetraether lipids,[19–23] the relationship between structure and function of these lipids remains unclear due to the limited data available on their membrane permeability properties. A systematic study of the effect of specific structural elements inspired from Archaea lipids on membrane leakage could make it possible to design lipids with improved integrity under a variety of environmental conditions.

Table 1. Structure of synthesized tetraether lipids



Lipid	Hydrophobic Core
GMGTPC	
GMGTPC-CP1	
GMGTPC-CP2	
GMGTPC-CP3	
GMGTPC-CH1	

Herein, we describe a series of Archaea-inspired synthetic lipids, Glycerol Monoalkyl Glycerol Tetraether lipid with PhosphoCholine head groups (**GMGTPC**), which exhibit excellent membrane integrity without the necessity of additives (shown in Table 1). Collectively, the structural elements of these new synthetic lipids attempt to mimic lipids derived from Crenarchaeota or Thaumarchaeota.[6] By incorporating the essence of some key structural features (e.g., ether glycerol linkage, tethering of lipids, and incorporation of rings) found in natural Archaeal lipids, we generated a set of synthetic lipids that formed stable liposomal membranes at room temperature with reduced leakage properties compared to commercially available EggPC lipids.

[a] T. Koyanagi, Dr. G. Leriche, Prof. J. Yang
Department of Chemistry and Biochemistry
University of California
San Diego, La Jolla, California 92093-0358
E-mail: Jerryyang@ucsd.edu
[b] D. Onofrei, Prof. G. P. Holland
Department of Chemistry and Biochemistry
San Diego State University,
San Diego, California 92182-1030, United States
[c] Prof. M. Mayer
Department of Biomedical Engineering
University of Michigan, Ann Arbor
Michigan 48109, United States

[**] T. K. and G. L. contributed equally to this work.

Supporting information for this article is given via a link at the end of the document.

We designed the series of synthetic lipids shown in Table 1 to evaluate the effects of two important structural elements found in many Archaea lipids on membrane permeability: 1) the effect of tethering of alkyl tails to create bolaform amphiphiles capable of spanning the length of the membranes, and 2) the effect of incorporation of cyclopentane or cyclohexane rings within the tethered lipid chain. In all lipids synthesized, we included ether linkages instead of ester groups between the lipid tails and the head groups because ether functional groups are expected to be more chemically stable than the ester groups commonly found in eukaryotic and prokaryotic lipid membranes.

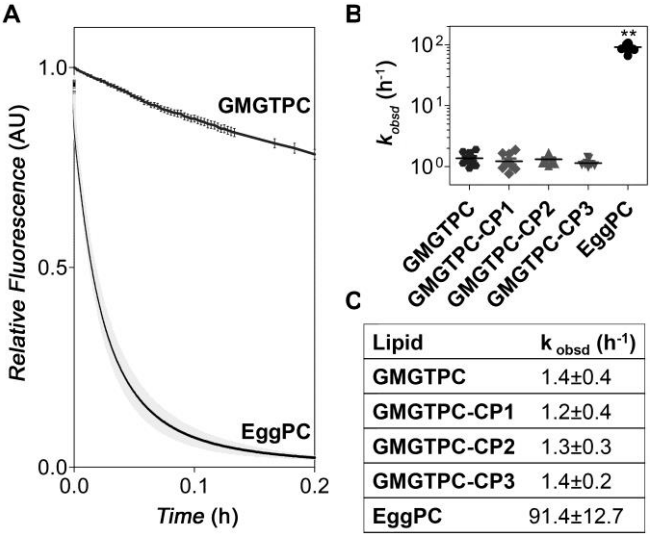
Tetraether Archaea lipids found in Nature contain either a single transmembrane tether or are macrocyclic (i.e. both transmembrane lipid tails are tethered).[24] While one example of a macrocyclic tetraether lipid has been prepared by total synthesis,[25] the preparation required over 20 synthetic steps (without incorporation of rings).[26,27] Hence, accessibility of a series of synthetic macrocyclic tetraether lipids comprising rings was not practical. We, therefore, synthesized lipids containing a single tether, which made it possible to prepare a series of transmembrane spanning lipids containing 0 to 3 rings in sufficient quantities (150-420 mg) to evaluate their leakage properties. Phosphocholine head groups were incorporated into all lipids because these zwitterionic groups are known to produce stable liposomes.[28]

We used a series of Wittig and S_N2 reactions[29,30] to generate a set of five **GMGTPC** tethered lipids (Table 1) that differ by the number and type of rings in the hydrophobic core in ~10 synthetic steps each (see Supporting Figures S1-S6). Here, phytanyl groups were incorporated as the untethered lipid chain in all **GMGTPC** lipids in order to avoid phase transition temperatures of the lipids occurring near room temperature. Differential Scanning Calorimetry (DSC) measurements support that all newly synthesized lipids in liposome form do not undergo a phase transition between 5-70°C (see Supporting Figure S7).

We prepared liposomes from pure **GMGTPC** lipids by hydration of thin films of each lipid in buffer containing 5,6-Carboxyfluorescein (CF), followed by extrusion. This preparation afforded liposomes of ~130 nm average hydrodynamic diameter as determined by dynamic light scattering (DLS, see Supporting Figure S8). We expect the lipids in these liposomes to predominantly adopt a transmembrane configuration.[31,32] However, it is plausible that some fraction of the lipids can adopt a hairpin configuration to help stabilize the high curvature of the liposomes. The average size of these liposomes were stable over at least 6 hours, and we did not observe evidence of liposome aggregation or fusion when subjected to a FRET-based liposome fusion assay[33] (see Supporting Figures S8 and S9). The absence of a lipid phase transition or aggregation/fusion of liposomes near room temperature suggests that the **GMGTPC** lipids are suitable for evaluating the effects of small rings incorporated into tetraether lipids on membrane leakage.

In order to evaluate the relative permeability of membranes formed from these different lipids, we developed a modified pH equilibration assay that was previously reported by Kakinuma and coworkers.[20] In this assay, we encapsulated CF within the liposomes with an initial internal liposomal pH of 7.2. The liposomes were then incubated in a buffered solution with an external pH of 5.8, and the change in fluorescence intensity of CF was monitored over time as the internal liposome pH equilibrated to pH 5.8. We chose to use pH 5.8 and 7.2 as external and internal liposomal pH, respectively, since CF exhibits a linear correlation between its fluorescence and environmental pH within this pH range.[34] We also chose to use CF as the fluorescent reporter of pH since we did not observe any appreciable leakage of CF from these liposomes over the time required to observe pH equilibration at room temperature from any of the lipids used in this study (see Supporting Figure S10).

While we expect all of the membranes to be most permeable to protons over any other ionic species under these experimental conditions, it is possible the intraliposomal buffer and other ions such as OH^-, Na^+, or Cl^- could also contribute to the observed rate of pH equilibration.[35] We, therefore, consider the observed initial rates of pH equilibration from membranes comprised of the different lipids to represent an estimate of their overall permeability to small ions rather than an estimate of the permeability of a single, specific ionic species.



Lipid	k_{obsd} (h^{-1})
GMGTPC	1.4±0.4
GMGTPC-CP1	1.2±0.4
GMGTPC-CP2	1.3±0.3
GMGTPC-CP3	1.4±0.2
EggPC	91.4±12.7

Figure 1. Observed rate of pH equilibration from liposomes formed from EggPC or synthetic lipids. (A) Graph of the change in CF fluorescence from CF encapsulated **GMGTPC** or EggPC liposomes vs. time (h); (B) Comparison of the observed initial rates of decreased CF fluorescence from CF encapsulated liposomes comprised of different lipids; (C) Average observed initial rates of pH equilibration in liposomes comprised of different lipids. Standard errors of the mean are provided based on 9 measurements each. Statistical analyses were performed using a paired t-test. ** indicate a p-value < 0.01.

To understand whether tethering of lipids affected small ion membrane leakage, we evaluated the observed initial rates of pH equilibration of liposomes comprised of EggPC and **GMGTPC** (Figure 1A and Supporting Figure S11). Similar to previously reported trends comparing liposome permeability of lipids derived from Archaea vs. Bacteria,[17] **GMGTPC** liposomes exhibited ~2 orders of magnitude reduction in rate of leakage of small ions when compared to liposomes formed from EggPC. This result could arise, in part, by a combination of the absence of the ester linkage found in EggPC, the presence of the branched alkane network provided by the phytanyl group in **GMGTPC**, and the elimination of the small aqueous layer found in between the two lipid leaflets of a bilayer forming lipid (which, presumably, would not be present in a membrane comprised of pure tethered **GMGTPC** lipids).[16,36,37]

A common feature found in many natural Crenarchaeota lipids is the presence of cyclopentane rings within the tethered transmembrane core of tetraether lipids.[38-40] In order to examine the effect of cyclopentane integration in the lipid on small ion membrane leakage, we evaluated the rate of pH equilibration from liposomes formed from **GMGTPC-CP1-3** (Figure 1B and

1C), which contained 1, 2, or 3 *cis*-1,3-cyclopentane rings (Table 1). Interestingly, we found that the presence or number of rings had no observable effect at room temperature on the rate of pH equilibration compared to **GMGTPC** (which has zero rings within its tethered hydrophobic core). These results are in contrast to reported computational studies suggesting that cyclopentane rings[41] in tethered lipids increased lipid packing.[7,42] While the stereochemistry of the cyclopentane rings (*cis* versus *trans*) may account for the discrepancy between our experimental results and the reported computational studies, conformational analysis[43] and X-ray studies[44] of polymers containing multiple 1,3-cyclopentane rings support that the energy of inter-strand packing of these polymers is independent of the *cis* or *trans* configuration of the rings. Nevertheless, the results shown in Figure 1 demonstrate that any structural effects to the lipid through introduction of *cis* cyclopentane rings is not sufficient to significantly affect membrane leakage of small ions under the conditions used in these studies.

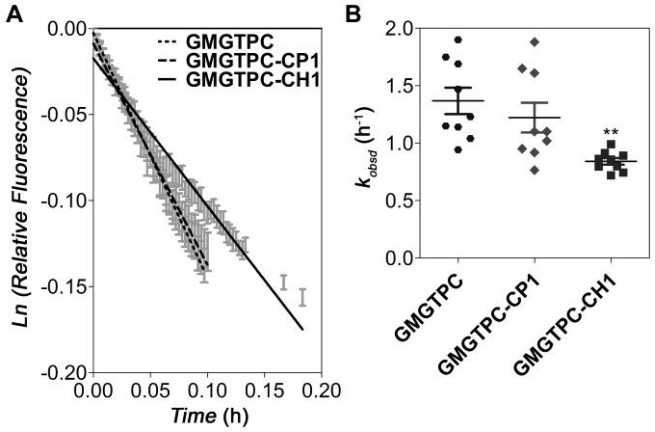


Figure 2. Comparison of observed initial rates of pH equilibration of liposomes comprising lipids with zero rings, one cyclopentane ring, or one cyclohexane ring. (A) Plot of natural log of relative fluorescence of CF (first 15% of pH-dependent fluorescence decrease of CF) vs. time (h) of **GMGTPC**, **GMGTPC-CP1**, **GMGTPC-CH1**; (B) Graph of the observed initial rates of pH equilibration from liposomes comprised of **GMGTPC**, **GMGTPC-CP1**, or **GMGTPC-CH1**. Statistical analyses were performed using a paired t-test. ** indicate a p-value < 0.01.

In order to examine the effects on membrane leakage of a cyclohexane ring incorporated into tethered lipids, we synthesized **GMGTPC-CH1** (Table 1) comprising a *cis*-1,3-cyclohexane group. Here, we maintained the same *cis*-1,3 stereochemistry as in the **GMGTPC-CP1-3** lipids to avoid introducing an additional variable when comparing their leakage properties. Interestingly, Figure 2 shows that liposomes comprised of **GMGTPC-CH1** lipids exhibited an additional ~40% reduction in the rate of small ion membrane leakage (k_{obsd} = 0.8 $\pm$ 0.1 h^{-1}) compared to liposomes comprised of lipids with no rings (**GMGTPC**) or with one cyclopentane ring (**GMGTPC-CP1**). Presumably, the difference in flexibility of the cyclohexane ring compared to a cyclopentane ring affects lipid packing, which leads to reduced membrane permeability to small ions in **GMGTPC-CH1** liposomes.[45] Additionally, intercalation of cyclohexane or cycloheptane between the hydrophobic tails of a bilayer lipid is believed to decrease proton permeability.[46] These results could help support the structural benefits of cyclohexane ring incorporation found in lipids derived from Thaumarchaeota, which grow optimally in environments between pH 5-8 and temperature of 20-45°C.[6]

We have, thus, presented a systematic study of the effects on membrane leakage of some key structural features inspired from natural Archaea lipids. As expected, the incorporation of phytanyl groups, the presence of tethering, and the incorporation of ether glycerol backbones in tetraether lipids substantially reduced membrane permeability of small ions when compared to commercially available EggPC lipids. Surprisingly, we found that incorporation of *cis*-1,3 cyclopentane rings had no effect on leakage of small ions in **GMGTPC** lipids. In contrast, incorporation of a *cis*-1,3 cyclohexane ring significantly reduced small ion membrane leakage compared to all other **GMGTPC** lipids studied. Such differences in small ion permeability between lipids containing cyclopentane versus cyclohexane rings could reflect differences in ring flexibility as it relates to lipid packing. This work represents an important step towards establishing some design principles inspired from Nature for generating lipids with low membrane permeability.

Experimental Section

Additional details for the synthesis and characterization of **GMGTPC** lipids, for formation and characterization of liposomes, and for kinetic analysis for the pH equilibration studies can be found in the supplemental information.

Acknowledgements

Financial support from the Air Force Office of Scientific Research (FA9550-12-1-0435 and FA9550-14-1-0014) is gratefully acknowledged. We thank Thomas B. H. Schroeder for helpful discussions during development of the pH equilibration assay. We would like to thank Daichi Koyanagi for designing the table of contents Figure. We thank Dr. Kendra Hailey for training of the stopped-flow experiments.

Keywords: Archaea • Tetraether lipid • Permeability • Biomembrane • Liposome • Ring

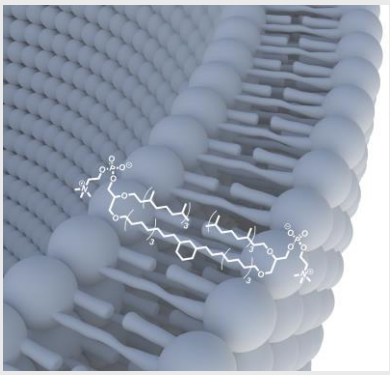
[1] D. L. Valentine, *Nat. Rev. Microbiol.* **2007**, *5*, 316–323.
[2] D. W. Deamer, J. Nichols, W. *Proc. Natl. Acad. Sci. U. S. A.* **1983**, *80* (1), 165–168.
[3] Y. Nozaki, C. Tanford, *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78* (7), 4324–4328.
[4] J. W. Nichols, D. W. Deamer, *Proc. Natl. Acad. Sci. U. S. A.* **1980**, *77* (4), 2038–2042.
[5] C. R. Woese, L. J. Magrum, G. E. Fox, *J. Mol. Evol.* **1978**, *11* (3), 245–252.
[6] L. Villanueva, J. S. S. Damsté, S. Schouten, *Nat. Rev. Microbiol.* **2014**, *12* (6), 438–448.
[7] P. L. G. Chong, U. Ayesa, V. P. Daswani, E. C. Hur, *Archaea* **2012**, *2012*, 1–11.
[8] J. L. Gabriel, P. L. G. Chong, *Chem. Phys. Lipids* **2000**, *105* (2), 193–200.
[9] P. L. G. Chong, *Chem. Phys. Lipids* **2010**, *163* (3), 253–265.
[10] M. Sipai Altaf Bhai, Y. Vandana, Y. Mamatha, V. V. Prasanth, *J. Pharm. Sci. Innov.* **2012**, *1* (1), 13–21.
[11] M. C. Phillips, W. J. Johnson, G. H. Rothblat, *Biochim. Biophys. Acta* **1987**, *906* (2), 223–276.
[12] D. Lorusso, A. Di Stefano, V. Carone, A. Fagotti, S. Pisconti, G. Scambia, *Ann. Oncol.* **2007**, *18* (7), 1159–1164.

[13] A. G. Kohli, P. H. Kierstead, V. J. Venditto, C. L. Walsh, F. C. Szoka, *J. Control. Release* **2014**, *190*, 274–287.
[14] R. Van der Meel, M. H. A. M. Fens, P. Vader, W. W. Van Solinge, O. Eniola-Adefeso, R. M. Schiffelers, *J. Control. Release* **2014**, *195*, 72–85.
[15] M. R. Toh, G. N. C. Chiu, *Asian J. Pharm. Sci. (Singapore, Singapore)* **2013**, *8* (2), 88–95.
[16] M. G. L. Elferink, J. G. de Wit, A. J. M. Driessen, W. N. Konings, *Biochim. Biophys. Acta, Biomembr.* **1994**, *1193* (2), 247–254.
[17] E. L. Chang, *Biochem. Biophys. Res. Commun.* **1994**, *202* (2), 673–679.
[18] I. Uda, A. Sugai, Y. H. Itoh, T. Itoh, *Lipids* **2001**, *36* (1), 103–105.
[19] K. Arakawa, T. Eguchi, K. Kakinuma, *Chem. Lett.* **2001**, 5, 440–441.
[20] K. Arakawa, T. Eguchi, K. Kakinuma, *Bull. Chem. Soc. Jpn.* **2001**, *74* (2), 347–356.
[21] B. Raguse, P. N. Culshaw, J. K. Prashar, K. Raval, *Tetrahedron Lett.* **2000**, *41* (16), 2971–2974.
[22] A. P. Patwardhan, D. H. Thompson, *Org. Lett.* **1999**, *1* (2), 241–243.
[23] M. Brard, C. Lainé, G. Réthoré, I. Laurent, C. Neveu, L. Lemiègre, T. Benvegnu, *J. Org. Chem.* **2007**, *72* (22), 8267–8279.
[24] M. De Rosa, A. Gambacorta, B. Nicolaus, B. Chappe, P. Albrecht, *Biochim. Biophys. Acta - Lipids Lipid Metab.* **1983**, *753* (2), 249–256.
[25] K. Arakawa, T. Eguchi, K. Kakinuma, *J. Org. Chem.* **1998**, *63* (14), 4741–4745.
[26] T. Eguchi, K. Arakawa, T. Terachi, K. Kakinuma, *J. Org. Chem.* **1997**, *62* (7), 1924–1933.
[27] T. Eguchi, K. Ibaragi, K. Kakinuma, *J. Org. Chem.* **1998**, *63* (8), 2689–2698.
[28] *Cell and Model Membrane Interactions*, S. Ohki, Ed., Plenum Press: New York, **1991**.
[29] M. Brard, W. Richter, T. Benvegnu, D. Plusquellec, *J. Am. Chem. Soc.* **2004**, *126* (32), 10003–10012.
[30] Z. Huang, F. C. Szoka, *J. Am. Chem. Soc.* **2008**, *130* (46), 15702–15712.
[31] L. A. Cuccia, F. Morin, A. Beck, N. Hébert, G. Just, R. B. Lennox, *Chem. - A Eur. J.* **2000**, *6* (23), 4379–4384.
[32] D. P. Holland, A. V. Struts, M. F. Brown, D. H. Thompson, *J. Am. Chem. Soc.* **2008**, *130* (14), 4584–4585.
[33] D. K. Struck, D. Hoekstra, R. E. Pagano, *Biochemistry* **1981**, *20* (14), 4093–4099.
[34] S. Massou, R. Albigot, M. Prats, *Biochem. Educ.* **2000**, *28* (3), 171–173.
[35] S. Paula, A. G. Volkov, A. N. Van Hoek, T. H. Haines, D. W. Deamer, *Biophys. J.* **1996**, *70* (1), 339–348.
[36] W. Shinoda, K. Shinoda, T. Baba, M. Mikami, *Biophys. J.* **2005**, *89* (5), 3195–3202.
[37] K. Yamauchi, K. Doi, Y. Yoshida, M. Kinoshita, *Biochim. Biophys. Acta, Biomembr.* **1993**, *1146* (2), 178–182.
[38] K. L. Hershberger, S. M. Barns, A. L. Reysenbach, S. C. Dawson, N. R. Pace, *Nature*. **1996**, *384*, 420.
[39] Z. Q. Song, J. Q. Chen, H. C. Jiang, E. M. Zhou, S. K. Tang, X. Y. Zhi, L. X. Zhang, C. L. L. Zhang, W. J. Li, *Extremophiles* **2010**, *14* (3), 287–296.
[40] J. A. Fuhrman, K. McCallum, A. A. Davis, *Nature* **1992**, *356*, 148–149.
[41] In these computational studies, the stereochemistry of the cyclopentane rings was not explicitly shown.
[42] A. Gliozzi, A. Relini, P. L. G. Chong, *J. Membr. Sci.* **2002**, *206*, 131–147.
[43] O. Ruiz de Ballesteros, L. Cavallo, F. Auriemma, G. Guerra, *Macromolecules* **1995**, *28*, 7355–7362.
[44] O. Ruiz de Ballesteros, V. Venditto, F. Auriemma, G. Guerra, L. Resconi, R. Waymouth, A. L. Mogstad, *Macromolecules* **1995**, *28* (7), 2383–2388.
[45] H. Kwart, M. C. Rock, R. Sanchez-Obregon, F. Walls, *J. Am. Chem. Soc.* **1972**, *94* (5), 1759–1760.
[46] T. H. Haines, *Prog. Lipid Res.* **2001**, 40 (4), 299–324.

Entry for the Table of Contents (Please choose one layout)

COMMUNICATION

Extremophile Archaea organisms thrive in harsh environments due, in part, to the unusual structural features present in their lipids. A systematic study of synthetic lipids reveals that membrane tethering and incorporation of cyclohexane rings into the tethered segment are two key structural features present in natural Archaea lipids that can improve membrane integrity by reducing membrane permeability to small ions.



*Author(s), Corresponding Author(s)**

Page No. – Page No.

Title