36-Membered Macrocyclic Diether Lipid is Advantageous for Archaea to Thrive under the Extreme Thermal Environments

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Described in this paper is the biophysical significance of the archaeal 36-membered macrocyclic diether phospholipid 36MPC as a function of temperature, which was studied by measuring of membrane fluidity, liposomal proton permeability, and liposomal thermostability in comparison with its acyclic counterpart, DPhyPC, their glycolipid derivatives, 36MGen and DPhyGen, egg yolk lecithin eggPC, and a mixed lipid of DMPC–cholesterol (2:1). Fluorescence anisotropy measurements indicated that the macrocyclic structure led to a decrease in the fluidity in the inter-membrane hydrophobic part more than in the membrane surface by limiting the motional freedom of the alkyl chains. The proton permeability was also significantly reduced by introducing a macrocyclic structure. Liposomal thermostability measurements using 6-carboxyfluorescein (CF) suggested that 36MPC formed liposomes with greater thermal stability than these of DPhyPC. The presence of glycolipids to the corresponding phospholipids greatly reduced the CF leakage from liposomes. Most importantly, DMPC–cholesterol liposome showed less leakage than 36MPC at 40 °C. However, by raising the temperature, this situation was completely reversed. This suggested that the cyclic structure contributed to the formation of stable liposomes, especially at higher temperatures. These findings clearly demonstrate that the 36-membered macrocyclic lipid membrane plays an important role for the thermophilic archaea to adapt to extreme environments.

Archaea, including extreme halophiles, thermophiles, thermoacidophiles, alkalophiles, and methanogens, have attracted considerable attention for both biochemical and evolutionary studies. It is well established that archaea are distinct from bacteria and eucarya, and are now classified in a third independent domain.[1] Archaea usually inhabit extreme environments, such as high temperature, high acidic and/or salt-rich conditions, or complete absence of oxygen. One characteristic feature of archaea is the chemical structure of its core membrane lipids. The basic common core structure of the archaeal membrane is 2,3-di-*O*-phytanyl-*sn*-glycerol **1**, as illustrated in Fig. 1. The core lipids consist of hydrophobic isoprenoid chains connected to glycerol through ether linkages, which is in striking contrast to the ester linkage in bacterial and eucaryal membrane lipids.[2] The fatty acid components found in bacterial and eucaryal lipids are completely absent in the

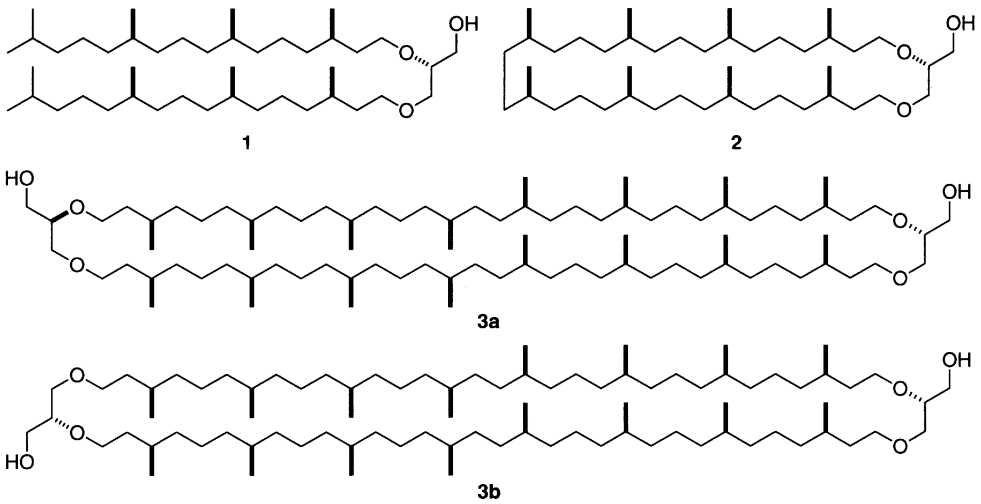


Fig. 1. Core structures of archaeal membrane lipids.

archaea. The second feature is the stereochemistry of the glycerol moiety in the archaeal lipids, i.e., the hydrophobic phytanyl groups are attached to the hydroxy groups at the *sn*-2- and 3-positions of glycerol. In contrast, the fatty acyl groups are substituted at the *sn*-1- and 2-positions in the bacterial and eucaryal lipids.[2] The third and most striking feature of archaeal membrane lipids is the presence of macrocyclic structures (**2** and **3ab**) as large as 36- or 72-membered rings of thermoacidophilic archaea and methanogens (Fig. 1).

Modeling and synthetic studies were previously reported in order to investigate the stability, fluidity, and permeability of the archaeal membrane in terms of the unusual archaeal lipid structures, such as ether linkage,[2b–d] isoprenoid,[3] bipolar structure,[4] and macrocyclic structure.[5,6] Further, the permeability and thermal stability of the reconstituted liposomes composed of the polar lipid extract (PLE) from thermophilic archaea, such as *Sulfolobus acidocaldarius, Thermoplasma acidophilum*, and several methanogens, were also described.[7] The constitution of the PLE, however, appeared to be various degrees of a mixture of **1**, **3ab**, and other tetraether lipids, some of which even contained up to four cyclopentane rings per C_{40} isoprenoid chain with a series of polar head groups such as phosphate, sugars, and nonitol. Therefore, little is known about the biophysical significance of the natural 36- and 72-membered macrocyclic lipids in its own state. Especially obscured are the thermal characteristics of liposomes composed of these macrocyclic natural lipids.

We are interested in studying the biophysical significance of the characteristic macrocyclic ring structures in the archaeal lipids, particularly the 36-membered ring. The 36-membered macrocyclic diether lipid **2** was first isolated from a deep-sea hydrothermal vent methanogen, *Methanococcus jannaschii*, by Comita et al.[8] and was subsequently found in other thermophilic methanogens, including *Methanococcus igneus* and *Methanohalobium evestigatum*.[9] We have already successfully synthesized the archaeal 36-membered macrocyclic lipid[10] and communicated the thermal analysis and monolayer studies of the phosphocholine derivative of archaeal 36-membered macrocyclic lipid (36MPC) in comparison with the acyclic counterpart (DPhyPC) (Fig. 2).[11] We also noted briefly the characterization of 36MPC by a microscopic analysis and water-permeability measurement at ambient temperature.[12]

In this paper, we address the question whether or not the structure of the archaeal 36-membered macrocyclic lipid is suited to the rather extreme environments in which they live. The biophysical significance of 36MPC is proved by measuring the membrane fluidity, liposomal proton permeability, and thermostability of liposomes in comparison with the open-chain analog DPhyPC, synthetic glycolipid derivatives 36MGen and DPhyGen, egg yolk L-α-lecithin (eggPC), and a mixed lipid of dimyristoyl-L-α-phosphatidylcholine (DMPC)–cholesterol (2:1). In addition, the thermostability of the reconstituted liposome of PLE from the thermophilic methanogen *M. jannaschii* is also discussed as a model of biomembranes and a reference for 36MPC.

Results

Synthesis of Glycolipids. In addition to the phosphocholine lipids, various polar head groups (PHGs) are also interesting, because archaeal lipids contain a number of PHGs such as phosphoethanolamine, phosphoinositol, glucose, and gentiobiose.[2a] Especially, the glycolipids represented as gentiobioside are major components in thermophilic archaea. The content of PHGs from *M. jannaschii* was reported to be 38% 6′-*O*-[(2-aminoethoxy)oxidophosphoryl]gentiobiose, 17% gentiobiose, 16% phosphoethanolamine, and 5% glucose by Ferrante et al.[13] We thus pursued the synthesis of 36MGen to explore the effect of PHG on the physicochemical membrane properties. Glycosidation of the TMS derivative of **2** with gentiobiose octaacetate[14] in the presence of TMSOTf[15] was performed, followed by deprotection with sodium methoxide and neutralization with Amberlyst® 15 (H^+ form) to afford 36MGen in 76% yields (Scheme 1). In addition, DPhyGen was also synthesized by similar manipulation.

Fluorescence Anisotropy. Membrane fluidity is an important factor in transport across a biological membrane. We used fluorescence anisotropy measurements to assess the membrane fluidity and the effect of the macrocyclic structure as a function of temperature.[3i,6,16] In this study, eggPC was used as a reference lipid found in eucarya and bacteria. A DMPC–cholesterol mixture (2:1) was also used as a reference of animal lipid.[17] These lipids are known to exist in the liquid–crystalline state at ambient temperature,[11,17c,18] which was further confirmed by the absence of a marked change in

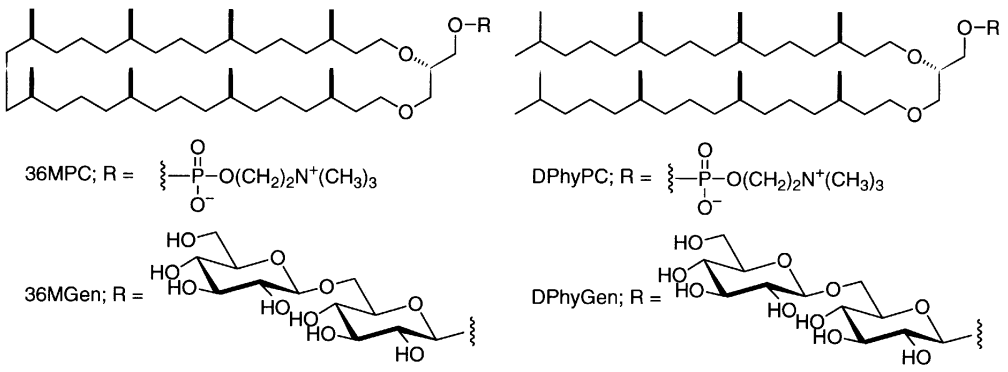
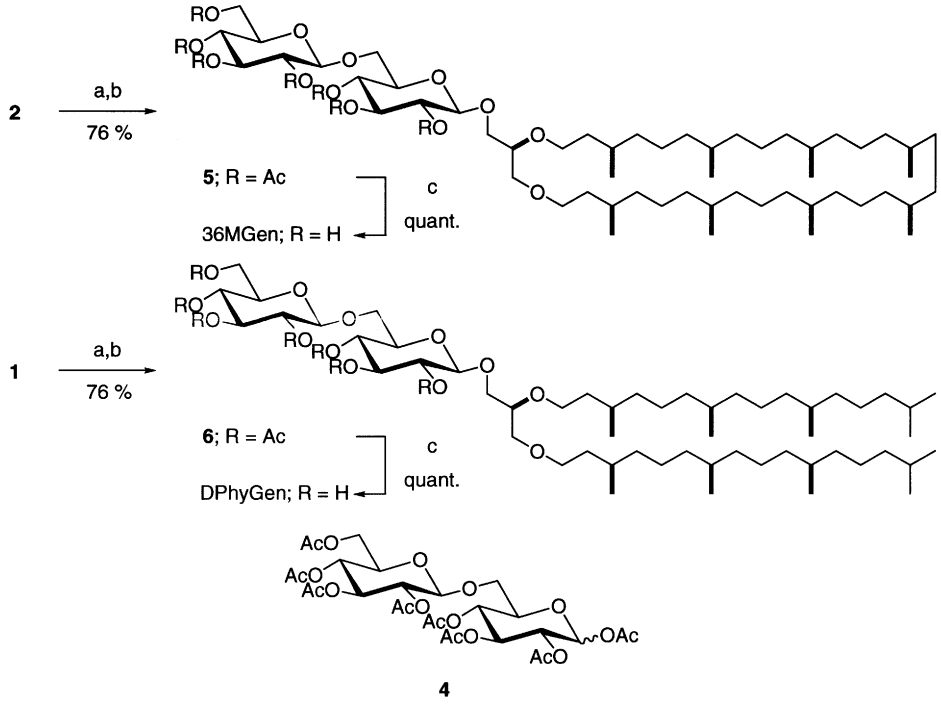


Fig. 2. Structures of synthetic archaeal diether phospholipids (36MPC, DPhyPC) and glycolipids (36MGen, DPhyGen).



(a) TMSCl, imidazole/DMF, (b) **4**, TMSOTf, MS4A/Cl(CH$_2$)$_2$Cl, (c) NaOMe/THF–MeOH; Amberlyst® 15 (H$^+$-form).

Scheme 1. Synthesis of archaeal diether glycolipids 36MGen and DPhyGen.

the fluorescence anisotropy, *r*. The observed fluorescence anisotropy of the lipid bilayers embedded with either 1,6-diphenyl-1,3,5-hexatriene (DPH) or trimethyl-4-(6-phenyl-hexa-1,3,5-trien-1-yl)phenylammonium iodide (tma-DPH) at 25–70 °C are shown in Fig. 3, and the *r* values are summarized in Table 1.

Upon raising the temperature, the *r* value gradually decreased in all samples using both fluorescent probes (Figs. 3A and 3B). Among the lipids measured, the liposome from the eggPC showed the lowest *r* value, that is, the highest fluidity. In contrast, DMPC–cholesterol formed the membrane with the lowest fluidity under these conditions. It is well known that cholesterol is a strong reinforcer, which significantly reduces the flexibility of a membrane composed of ester-type lipids, such as DMPC and eggPC. These results are consistent with the previous observations of Demel et al.[17c]

The archaeal phospholipids 36MPC and DPhyPC were compared, in which 36MPC appeared to show higher *r* values than DPhyPC over the entire temperature range. In other words, the membrane fluidity of 36MPC was found to be lower than that of DPhyPC. Since the difference in the *r* values for DPH between 36MPC and DPhyPC was larger than that for tma-DPH, the macrocyclic structure led to decrease the fluidity more efficiently in the hydrophobic part than in the membrane surface, probably due to a limitation of the mobility in the alkyl chain region.

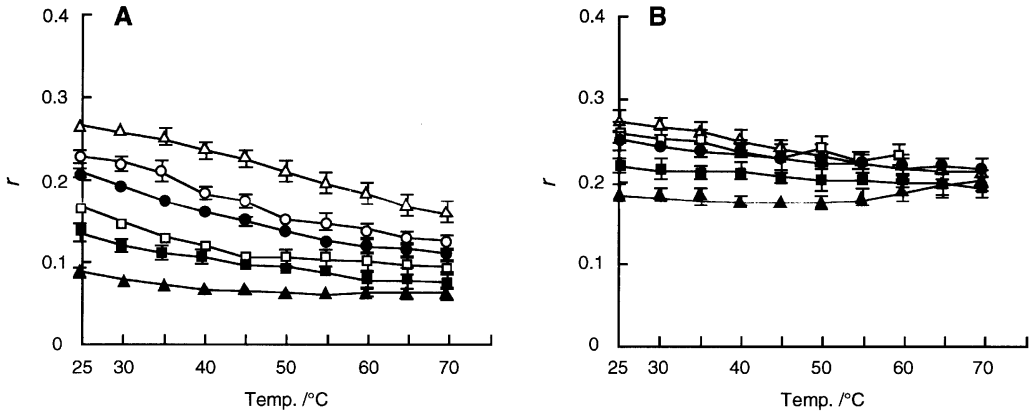


Fig. 3. Temperature dependence of the fluorescence anisotropy of A, DPH, B; tma-DPH incorporated in the bilayers of the lipids; 36MPC, ●; DPhyPC, ■; 36MGen, ○; DPhyGen, □; eggPC, ▲; DMPC–cholesterol (2 : 1); △. The concentrations of lipid and probe were 0.5 mM and 0.5 μM, respectively.

Table 1. Fluorescence Anisotropy

Lipid	*r* at hydrophobic region[a),c)]			*r* at hydrophilic region[b),c)]		
	25 °C	50 °C	70 °C	25 °C	50 °C	70 °C
36MPC	0.204	0.133	0.104	0.247	0.223	0.216
DPhyPC	0.128	0.0906	0.0694	0.222	0.210	0.194
36MGen	0.216	0.139	0.111	0.257	N.D.[d)]	N.D.[d)]
DPhyGen	0.162	0.101	0.0886	0.259	0.241	N.D.[d)]
eggPC	0.0923	0.0699	0.0668	0.186	0.174	0.205
DMPC–cholesterol	0.264	0.210	0.158	0.274	0.234	0.213

a) The lipid bilayers including DPH in buffer A were prepared by sonication methods, followed by addition of DPH in DMF as described in the Materials and Methods. b) The lipid bilayers including tma-DPH in buffer A were prepared by sonication methods, followed by addition of tma-DPH in DMF as mentioned above. c) The *r* values were average of at least three independent measurements. d) Not determined due to the low fluorescence intensity.

The same feature was observed between the macrocyclic glycolipid 36MGen and the acyclic glycolipid DPhyGen. In addition, the glycolipids interestingly showed larger *r* values in all temperature range than the corresponding phospholipids. These results probably imply that the glycolipids interact intermolecularly to each other by strong hydrogen bonding, resulting in a decrease in the fluidity. Fluorescence anisotropy measurements clearly indicated that the macrocyclic structure reduced the membrane fluidity in the entire lipid bilayer.

Proton Permeability of Liposomes. The CF fluorophore is known to exhibit pH-dependent quenching with lowering the pH.[19] The influx of H^+ into CF entrapped vesicles causes a decrease in the intensity of the fluorescence. This phenomenon was applied to an estimation of the proton permeability.[7e,h] The influx of protons into liposomes is usually very fast; therefore, equilibrium is reached in a few minutes. In an analysis of the proton permeability, it can be assumed that CF initially entrapped in the liposomes does not leak out to the bulk aqueous phase because the permeation rate of protons is two orders of magnitude faster than that of CF.[7h] The concentration of ions between the inside and outside of liposomes was set to the same value in this study; therefore, the effect of osmotic

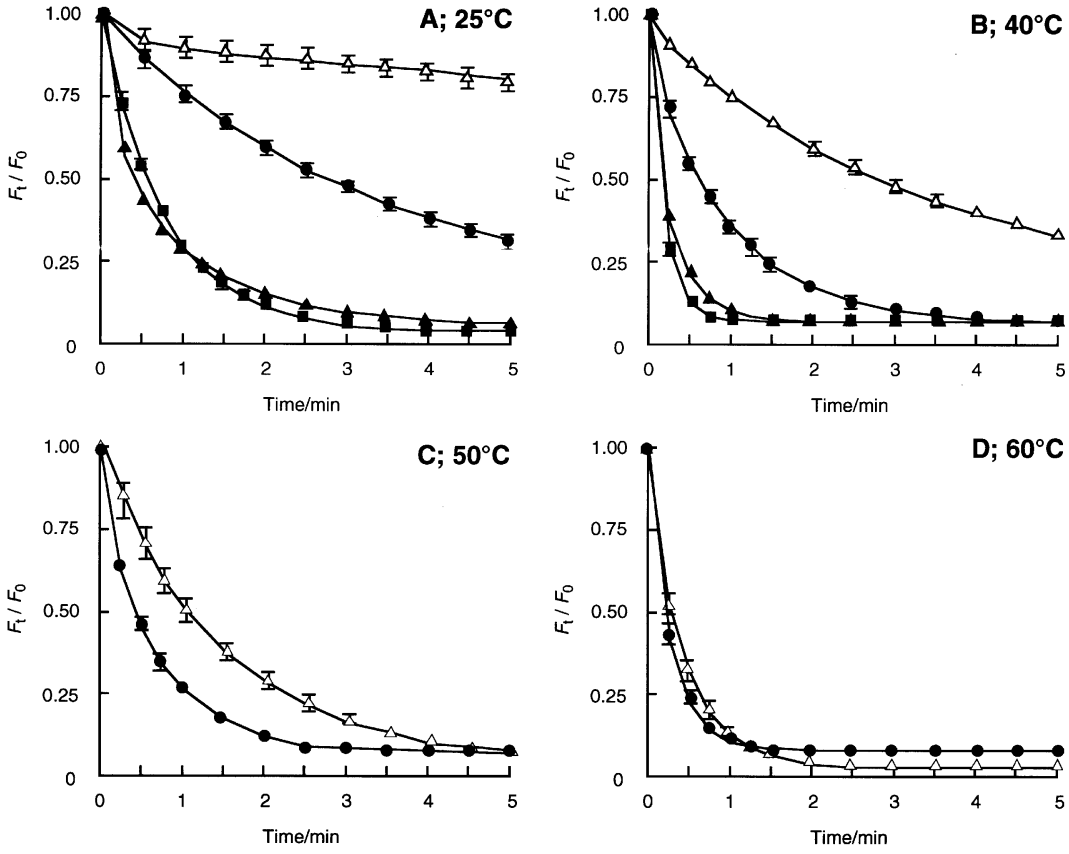


Fig. 4. Proton permeability of large unilamellar vesicles of the phospholipids (36MPC, DPhyPC, eggPC, DMPC–cholesterol). Plots of fluorescence intensity of CF F_t/F_0 vs. incubation time *t*. A, at 25 °C; B, at 40 °C; C, at 50 °C; D, at 60 °C; 36MPC, ●; DPhyPC, ■; eggPC, ▲; DMPC–cholesterol (2 : 1), △. Initial pH gradient; pH 7.5 (inner), pH 2.5 (outer).

change can also be neglected.

Figs. 4A–D show time-dependent plots of F_t/F_0 at 25, 40, 50, and 60 °C. At the temperatures above 40 °C, the rate of the proton permeability of DPhyPC and eggPC could not be estimated, since the fluorescence intensities of these lipids immediately decreased within a minute (data not shown). In particular, the fluorescence intensities of 36MGen and DPhyGen were too low to measure F_t/F_0, probably due to insufficient liposome formation.

The DMPC–cholesterol liposome exhibited the lowest proton permeability, while the liposomes of DPhyPC and eggPC almost equally demonstrated the highest proton permeability over the temperature range (Figs. 4A–D). These results suggest that the isoprenoid chains and the ether linkages do not affect the proton permeability. More importantly, within the archaeal phospholipids, the rate of proton leakage of the 36MPC liposome was slower than that of DPhyPC. Therefore, this strongly suggests that the macrocyclic structure in 36MPC is effective in forming less pH-sensitive membranes.

The order of the relative rates of the proton permeability among the measured lipids appears to be correlated well with the order of the lipid fluidity observed by the fluorescence anisotropy over the experimental temperature range. It therefore appeared that the proton permeability may be regulated by the presence of the cyclic structure.

Thermostability of Liposomes. High concentrations of fluorescence probe (above 100 mM) generally cause self-quenching, which prevents the dye from fluorescing. When entrapped CF in liposomes is leaked to outside, fluorescence appears due to the dilution of CF into a much larger volume of the external aqueous media.[20] Therefore, increasing the fluorescence intensity correlates to the stability of the liposome, thereby allowing an estimation of the thermostability of the liposomes at various temperature.[3d,f–i,4b,5c,7e–h]

The thermostability of vesicles is known to be affected by size.[7h] Therefore, sizing of the liposomes was carried out by extrusion at least ten times through a filter having a pore size of 200 nm prior to studies of the barrier properties. The average diameters of the liposomes of 36MPC, DPhyPC, eggPC, and DMPC–cholesterol, all of which included 200 mM CF in inner aqueous media, are given in Table 2.

Glycolipids 36MGen and DPhyGen formed insufficient liposome to measure their thermostability. Therefore, 36MGen and DPhyGen were mixed with their corresponding phospholipid 36MPC and DPhyPC in a ratio of 1:1 and 1:9, respectively. Although the reasons is unclear, liposome formation may depend upon a subtle balance between the hydrophobic and hydrophilic components. In addition to these samples, CF leakage from the liposomes of PLE of *M. jannaschii* was also examined as a model of biomembrane.

Table 2. Average Diameter of the Liposomes

Lipid	Average diameter/nm[a),b)]
36MPC	185 ± 65
DPhyPC	146 ± 17
eggPC	159 ± 35
DMPC–cholesterol	173 ± 79
PLE	205 ± 41

a) The liposomes containing 200 mM CF in buffer A were prepared by freezing–thawing and extrusion methods as described in the Materials and Methods. b) All measurements were carried out at 25 °C for in triplicate.

Time-dependent CF leakages at 40, 50, 60, and 70 °C are plotted in Figs. 5A–D for these lipids, and the extent of leakage at various temperature after 5 h are summarized in Table 3. The leakage extent of CF exhibited a gradual increase in the response to time for all of the liposomes examined. At 40 °C, DMPC–cholesterol liposome showed the least leakage, and thus the highest barrier among the liposomes examined, which was well related to the results of the fluorescence anisotropy and the proton permeability, as described above. However, by raising the experimental temperature, the result changed significantly. The PLE appeared to form the most thermostable liposomes at 70 °C, which correlates well with the previous results.[7e–h] Most importantly, the relative rate of CF leakage between DMPC–cholesterol and 36MPC was reversed at 70 °C from that at 40 °C, which strongly suggests that the cyclic structure of 36MPC maintained a relatively low fluidity of the liposome at elevated temperatures. In contrast, the rigid structure composed of cholesterol and fatty acyl phospholipid found at ambient temperature was destroyed, at least partially, at higher temperatures.

It should be noted that the addition of glycolipids 36MGen and DPhyGen to their corresponding phospholipid 36MPC and DPhyPC greatly reduced the CF leakage in both cases, as shown in Figs. 5A–D. It is well known that a mixture of lipids with different polar head groups frequently causes certain physico-chemical changes, such as lipid polymorphism,[21] molecular packing,[22] and water permeability.[23] The PLE of *M. jannaschii*, the most thermostable liposomes at 70 °C, was a mixture of various polar head groups.[13] These results suggest that the co-presence of different polar head groups also contributed to maintain a low fluidity and a high barrier of the membrane. However, to our knowledge, no report concerning the temperature-dependent variation of the composition of polar head groups in thermophilic archaea has appeared to date, in contrast to the hydophobic portions of the lipids, *vide infra*. Therefore, the presence of a variety of polar head groups in an archaeal membrane is not necessary for adaptation to thermal environments, but to maintain the low permeability of the membrane.

DPhyPC showed less CF leakage than eggPC at 25 °C (Fig. 5A). However, at high temperatures, the liposomal thermostability of DPhyPC and eggPC were indistinguishable (Figs. 5B–D). The methyl branchings in the isoprenoid chains showed apparently no effect on the thermostability at higher temperatures. These results were consistent with the results of Chang et al.[7f] and Komatsu et al.[7h] Conversely, Yamauchi[3d] and Nishikawa[3g] independently reported that the isoprenoid chains play a significant role for the formation of thermostable liposomes, which may have been due to different experimental

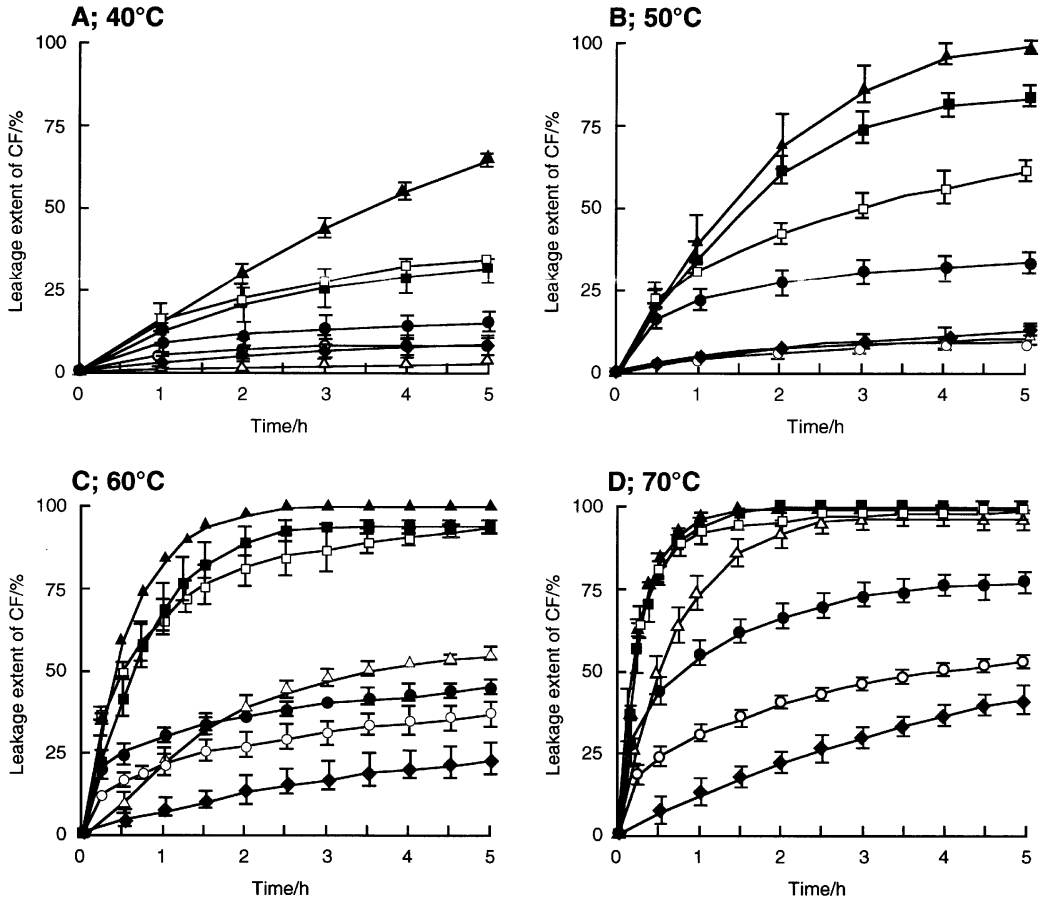


Fig. 5. Thermostability of large unilamellar vesicles of the lipids (36MPC, DPhyPC, eggPC, DMPC–cholesterol, 36MPC–36MGen) and polar lipid extract (PLE). Plots of the leakage extent of CF vs. incubation time *t*. A, at 40 °C; B, at 50 °C; C, at 60 °C; D, at 70 °C; 36MPC, ●; DPhyPC, ■; 36MPC and 36MGen (1 : 1), ○; DPhyPC and DPhyGen (9 : 1), □; eggPC, ▲; DMPC–cholesterol (2 : 1), △; and PLE, ◆.

Table 3. Thermostability of Liposomes

Lipid	Leakage extent of CF after 5 h/%[a),b)]			
	40 °C	50 °C	60 °C	70 °C
36MPC	14.4	32.6	43.4	75
DPhyPC	31.2	83.5	94.0	100
36MPC–36MGen	8.87	9.68	35.7	53.2
DPhyPC–DPhyGen	34.1	61.6	93.3	97.7
eggPC	66.5	98.7	100	100
DMPC–cholesterol	< 1	10.6	54.5	95.9
PLE	7.99	11.8	23.0	40.6

a) The liposomes including 200 mM CF in buffer A were prepared by freezing–thawing and extrusion methods as described in the Materials and Methods. b) These values were average of at least three independent measurements.

conditions, including liposome preparation.

Discussion

In general, the archaeal membranes appear to have properties that are well suited to the rather extreme environments in which these organisms live.[24] Thus, ether structure should impart resistance to the hydrolysis of the lipids over a wide range of pH, since an ether linkage is generally more resistant to acidic or alkaline hydrolysis than an ester linkage found in other organisms, bacteria and eucarya. The saturated alkyl chains can impart stability against oxidative degradation. The branched isoprenoid chains may be responsible for membrane fluidity by keeping the membrane in the liquid-crystalline state over a wide range of temperatures. Further, the unusual stereochemistry of the glycerol moiety could impart resistance to attack by other microorganisms. However, the effect of the macrocyclic structure has not yet been elucidated. The macrocyclic structure should affect the conformation (*trans*/*gauche* ratios), rotational motions, and lateral diffusion of the chains, which could consequently lead to changes in the structure, lipid packing, membrane permeability, and stability of the lipid bilayer composed of macrocyclic lipids.

Menger et al.[5c] and we[6] reported using model compounds of the archaeal 36-membered lipid that the macrocyclic structure induced the following effects: (i) elevation of the phase transition temperature, T_c, (ii) decrease of a phase-transition enthalpy change, ΔH_c, due to reducing the number of *gauche* C–C bonds, and (iii) decrease of a phase-transition entropy change, ΔS_c, by restricting the motional freedom of the alkyl chains. Further, in a preliminary communication, we reported that 36MPC appeared to aggregate into a more closely packed

structure than DPhyPC in the liquid-crystalline state.[11] Komatsu et al. reported that the lipid packing was a major contributor in proton permeability.[7h] It is known that cholesterol in animals, phytosterol in plants, and hopanoids and α,ω-dipolar carotenoids in bacteria interact with fatty acyl phospholipids by van der Waals interactions and hydrogen bonding, and are membrane reinforcers in each lipid by their relatively rigid structures.[3a,17,25] These molecules reduce the membrane fluidity with a tightly packed lipid structure to gain the higher barrier properties at ambient temperature.[25] To demonstrate this, the limiting areas in monolayer of DMPC–cholesterol,[17] eggPC,[26] 36MPC, and DPhyPC are shown in Table 4. DMPC–cholesterol forms the most tightly packed lipid structure and eggPC, 36MPC, and DPhyPC follow in that order, as shown in Table 4. The high barrier observed in DMPC–cholesterol lipid at ambient temperature appears to correlate well with the tightly packed lipid structure. The differences in fluidity, proton permeability, and thermal stability between 36MPC and DPhyPC over all temperature ranges, *vide ante*, can also be explained in terms of a tightly packed lipid structure. This tendency of the macrocyclic lipids may be maintained even at elevated temperatures, probably due to the less temperature-sensitive entropical factor induced by the restricted motional freedom of the alkyl chains, compared with those of the acyclic lipids and DMPC–cholesterol, and consequently the macrocyclic lipid exhibits the high barrier properties of its liposomes.

The results obtained in the present study correlate well with the following biological observations: the content of the cyclic lipids in *M. jannaschii*[27] and the number of the cyclopentane rings per isoprenoid chain in thermoacidophilic archaea[28] increase with the elevation of the incubation temperature, and thermal stress induces a structural modification of the lipids, such as covalent bond formation at the terminal region of the hydrophobic alkyl chains in anaerobic bacteria *Butyrivibrio fibrisolvens*.[29] Therefore, it seems that certain enzyme systems responsible for the coupling reaction of hydrophobic alkyl chains in these microorganisms may be regulated for adaptation to thermal environments.

In conclusion, the present studies clearly demonstrate that the 36-membered macrocyclic lipid membrane plays an important role in the thermophilic archaea adapting to extreme environments. Because the macrocyclic lipids appear to impart high barrier properties at higher temperature to thermophilic archaea, the macrocyclic structure is a major contributor and advantageous to tolerance against the extreme thermal conditions.

Table 4. Limiting Area Derived from π–A Isotherms of the Lipids

Lipid	Limiting area/nm^2 molecule^{-1}
36MPC	0.70[a)]
DPhyPC	0.92[a)]
eggPC	0.62[b)]
DMPC–cholesterol (2 : 1)	0.46[c)]

a) From Ref. 11. b) From Ref. 26. c) From Ref. 17.

Experimental

Materials. Phospholipids 36MPC and DPhyPC were those previously synthesized,[10] which were further purified by Sephadex® LH-20 with $CHCl_3$–methanol (2:1). Glycolipids 36MGen and DPhyGen were synthesized in this study. DMPC and eggPC [acyl compositions: 16:0 (38%), 16:1 (3%), 18:0 (9%), 18:1 (33%), 18:2 (17%)] were purchased from Sigma. Cholesterol was from Kanto Chemical Co., Inc., which was recrystallized from ethanol prior to use. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and trimethyl-4-(6-phenylhexa-1,3,5-trien-1-yl)phenylammonium iodide (tma-DPH) were purchased from Aldrich and Dojindo, respectively. 6-Carboxyfluorescein (CF) was from Eastman Kodak, which was further purified according to a method of Yamauchi et al.[4b]

Synthesis of 36MGen and DPhyGen. [(2*R*,7*R*,11*R*,15*S*,19*S*,22*S*,26*S*,30*R*,34*R*)-7,11,15,19,22,26,30,34-Octamethyl-1,4-dioxacyclohexatriacontan-2-yl]methyl 6-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (5): After trimethylsilyl chloride (80 μl, 0.63 mmol) was added to a mixture of **2**[10] (56.8 mg, 87.2 μmol) and imidazole (55.5 mg, 815 μmol) in DMF (4 ml) at 0 °C, the mixture was stirred at room temperature for 1.5 h. Water (10 ml) was added and the mixture was extracted with hexane. The organic phase was washed with water, dried (Na_2SO_4), filtered, and concentrated to dryness to afford a crude silyl ether. A mixture of the silyl ether, gentiobiose octaacetate **4**[14] (91.2 mg, 134 μmol), and molecular sieves 4A powder (196 mg) in 1,2-dichloroethane (8 ml) was stirred at room temperature for 15 min. To the mixture was added trimethylsilyl triflate (16.0 μl, 88.5 μmol), and the resulting mixture was stirred at room temperature for 2.5 h. The reaction was quenched by the addition of Et_3N (18.0 μl, 129 μmol), and filtered through a pad of Celite®, which was thoroughly washed with $CHCl_3$. The filtrate and washings were combined and concentrated to dryness. The residue was chromatographed over silica gel with $CHCl_3$ to give glycoside heptaacetate **5** (84 mg, 76%) as hygroscopic wax. $[\alpha]_D^{28}$ – 6.92° (*c* 0.760, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84–0.88 (m, 24H), 1.00–1.75 (m, 52H), 1.99–2.10 (7 × s, 21H, C*H*$_3$C=O), 3.37–3.70 (m, 11H), 3.89 (m, 2H), 4.12 (dd, *J* = 2.2, 12.2 Hz, 1H, H-6′), 4.27 (dd, *J* = 4.6, 12.2 Hz, 1H, H-6′), 4.55 (d, *J* = 7.8 Hz, 1H, H-1′), 4.59 (d, *J* = 8.1 Hz, 1H, H-1), 4.90 (t, *J* = 9.5 Hz, 1H, H-4), 4.96 (dd, *J* = 7.8, 9.5 Hz, 1H, H-2′), 4.99 (t, *J* = 8.1, 9.5 Hz, 1H, H-2), 5.07 (t, *J* = 9.5 Hz, 1H, H-4′), 5.18 (t, *J* = 9.5 Hz, 2H, H-3,3′). ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.77, 19.81, 19.84, 19.86, 20.00, 20.04, 20.15, 20.56, 20.60, 20.64, 20.65, 20.71, 24.13, 24.17, 24.41, 29.70, 29.84, 32.61, 32.65, 32.79, 32.99, 33.33, 34.05, 34.08, 36.59, 37.12, 37.19, 37.25, 37.29, 37.33, 37.35, 37.39, 37.42, 61.80, 68.16, 68.27, 69.07, 69.15, 69.95, 70.35, 70.84, 71.04, 71.32, 71.95, 72.76, 72.85, 73.23, 77.92, 100.77, 100.85, 169.19, 169.28, 169.37, 169.57, 170.11, 170.21, 170.60. IR ($CHCl_3$): 1039, 1230, 1377, 1462, 1755, 2858, 2927, 2954 cm^{-1}. Found: C, 65.39; H, 9.74%. Calcd for $C_{69}H_{120}O_{20}$: C, 65.27; H, 9.53%.

[(2*R*,7*R*,11*R*,15*S*,19*S*,22*S*,26*S*,30*R*,34*R*)-7,11,15,19,22,26,30,34-Octamethyl-1,4-dioxacyclohexatriacontan-2-yl]methyl 6-*O*-β-D-Glucopyranosyl-β-D-glucopyranoside (36MGen): A mixture of heptaacetate **5** (38.2 mg, 30.1 μmol), 0.1 M (1 M = 1 mol dm^{-3}) sodium methoxide solution in methanol (305 μl, 30.5 μmol), THF (2 ml), and methanol (2 ml) was stirred at room temperature for 21 h. Methanol (3 ml) was added and the mixture was

treated with Amberlyst-15 ion exchange resin (51 mg, H^+ form). The resulting neutralized mixture was filtered through a pad of Celite®, and the resin washed with $CHCl_3$. The filtrate and washings were combined and concentrated to dryness. The residue was purified by a gel permeation chromatography over Sephadex® LH-20 with $CHCl_3$–methanol (2:1) to give glycolipid 36MGen (30 mg, quant.) as hygroscopic wax. $[\alpha]_D^{27}$ – 22.4° (*c* 0.710, $CHCl_3$) (Ref. 13 $[\alpha]_D$ – 18.16°). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 7:1) δ 0.85–0.89 (8 × d, 24H), 1.00–1.70 (m, 52H), 3.25–4.15 (m, 21H), 4.28 (d, *J* = 7.8 Hz, 1H, H-1), 4.33 (d, *J* = 6.8 Hz, 1H, H-1′). ^{13}C NMR (100 MHz, C_6D_6-CD_3OD = 7:1) δ 19.84, 19.91, 20.02, 20.20, 20.24, 20.42, 24.54, 24.59, 24.91, 30.12, 30.25, 33.03, 33.08, 33.22, 33.25, 33.44, 34.38, 34.43, 37.13, 37.45, 37.48, 37.53, 37.55, 37.65, 37.77, 37.81, 37.84, 37.90, 61.93, 68.76, 68.85, 69.77, 70.02, 70.11, 70.49, 71.38, 74.10, 74.17, 75.74, 76.84, 76.88, 76.96, 78.49, 103.96, 104.21. IR ($CHCl_3$): 1072, 1377, 1464, 2870, 2927, 2954, 3384 cm^{-1}. Found: HR FABMS *m*/*z* 997.7485. Calcd for $C_{55}H_{106}O_{13}$ [M^++Na]: 997.7531.

(2*R*)-2,3-Bis[(3*R*,7*R*,11*S*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 6-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (6): Compound **1**[10] (54.5 mg, 83.4 μmol) was treated in the same manner as described for **5** to give glycoside heptaacetate **6** (58 mg, 54%) as a hygroscopic wax. $[\alpha]_D^{26}$ – 5.04° (*c* 0.413, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84–0.87 (10 × d, 30H), 1.00–1.70 (m, 48H), 1.99–2.09 (7 × s, 21H, C*H*$_3$C=O), 3.38–3.70 (m, 11H), 3.89 (m, 2H), 4.12 (dd, *J* = 2.2, 12.2 Hz, 1H, H-6′), 4.27 (dd, *J* = 4.6, 12.2 Hz, 1H, H-6′), 4.56 (d, *J* = 7.8 Hz, 1H, H-1′), 4.60 (d, *J* = 8.0 Hz, 1H, H-1), 4.90 (t, *J* = 9.5 Hz, 1H, H-4), 4.96 (dd, *J* = 7.8, 9.5 Hz, 1H, H-2′), 4.99 (t, *J* = 8.1, 9.5 Hz, 1H, H-2), 5.07 (t, *J* = 9.5 Hz, 1H, H-4′), 5.18 (t, *J* = 9.5 Hz, 2H, H-3,3′). ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.68, 19.73, 20.57, 20.60, 20.65, 20.72, 22.61, 22.71, 24.36, 24.46, 24.77, 27.95, 29.85, 29.92, 32.78, 32.80, 36.62, 37.16, 37.27, 37.41, 37.44, 37.49, 37.54, 39.34, 61.76, 68.15, 68.23, 69.14, 69.18, 70.07, 70.29, 70.48, 71.01, 71.32, 71.93, 72.76, 72.85, 73.21, 77.95, 100.75, 100.87, 169.17, 169.28, 169.37, 169.57, 170.12, 170.21, 170.61. IR ($CHCl_3$): 1039, 1230, 1377, 1466, 1755, 2871, 2927, 2958 cm^{-1}. Found: C, 64.88; H, 9.95%. Calcd for $C_{69}H_{122}O_{20}$: C, 65.17; H 9.67%.

(2*R*)-2,3-Bis[(3*R*,7*R*,11*S*)-3,7,11,15-tetramethylhexadecyloxy]propan-1-yl 6-*O*-β-D-Glucopyranosyl-β-D-glucopyranoside (DPhyGen): Compound **6** (30.7 mg, 24.1 μmol) was treated in the same manner as described for 36MGen to give glycolipid DPhyGen (24 mg, quant.) as a hygroscopic wax. $[\alpha]_D^{26}$ – 20.4° (*c* 0.523, $CHCl_3$) (Ref. 30 $[\alpha]_D$ – 25.10°). ^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD = 7:1) δ 0.82–0.89 (10 × d, 30H), 1.00–1.70 (m, 48H), 3.25–4.15 (m, 21H), 4.28 (d, *J* = 7.6 Hz, 1H, H-1), 4.33 (d, *J* = 7.6 Hz, 1H, H-1′). ^{13}C NMR (100 MHz, C_6D_6–CD_3OD = 8:1) δ 19.81, 19.85, 19.94, 22.76, 22.86, 24.89, 24.98, 25.26, 28.33, 30.14, 30.25, 30.35, 33.22, 33.27, 33.29, 37.19, 37.52, 37.72, 37.93, 38.02, 39.74, 61.94, 68.93, 69.79, 69.96, 70.25, 70.45, 71.21, 74.10, 74.15, 75.67, 76.80, 76.90, 78.51, 104.03, 104.31. IR ($CHCl_3$): 1072, 1377, 1458, 2858, 2927, 2952, 3396 cm^{-1}. Found: HR FABMS *m*/*z* 999.7629. Calcd for $C_{55}H_{108}O_{13}$ [M^++Na]: 999.7688.

Liposome Preparation.[31] Each lipid (3–5 mg, ca. 4.5 mmol) was dissolved in 1 ml of $CHCl_3$–methanol (1:1). The organic solvent was removed by a gentle stream of argon gas, and the resulting film was dried under vacuum for at least 10 h.

For fluorescence anisotropy measurements, liposomes were prepared by the sonication method as follows: a lipid film prepared as above was hydrated with 9 ml of buffer A (20 mM Tris, 200 mM NaCl; pH = 7.5) by vortexing at room temperature. The resulting suspension was sonicated at room temperature at 70 W with a Branson sonifier 250 featuring a titanium microtip for 10 min. The dispersion was centrifuged at room temperature at 2000 × *g* for 15 min to give vesicles dispersion.

For proton permeability and thermostability measurements, CF-entrapped large unilamellar vesicles were prepared by the freezing–thawing and extrusion methods. For proton permeability measurements, a lipid film prepared as above was hydrated with 1 ml of buffer B (50 mM sodium diphosphate/citrate, 150 mM NaCl; pH = 7.5) including 4 mM CF by vortexing at room temperature for 5 min. The mixture was then frozen at – 78 °C and slowly thawed to room temperature. This cycle was repeated five times. The resulting dispersion was passed through a polycarbonate membrane filter (Nucleopore®, ϕ = 200 nm) with the Extruder™ (Lipex Biomembranes Inc.)[32] ten times under 0.8–1.0 MPa nitrogen gas pressure. The external CF was removed by a gel permeation chromatography over Sephadex® G-50 fine (ϕ 1.5 × 30 cm) by eluting with the same buffer at 5 °C to give a dispersion of large unilamellar vesicles including CF. For thermostability measurements, the liposome was prepared as the same method except for using buffer A and 200 mM CF instead of buffer B and 4 mM CF.

The average diameters of the prepared liposomes were measured as intensities of laser light-scattering by a photon correlation spectroscopy on a Coulter-Counter® N4SD instrument with a 90° scattering angle at 25 °C. The vesicular solutions were diluted to ca. 10^{-4} M with the same buffer.

Fluorescence Anisotropy. The membrane fluidity was estimated using fluorescence anisotropy of a fluorescence probe embedded in a lipid bilayer.[3i,6d,16,33] DPH and tma-DPH were used as fluorescence probes for the fluidity at hydrophobic and hydrophilic regions in the lipid bilayer, respectively.

To a liposome dispersion, prepared as above (3 ml), was added either DPH or tma-DPH (3 μl, 0.5 mM in DMF). In the case of DPH, the mixture was incubated at room temperature for at least 1 h prior to the measurement, while the fluorescence measurement of tma-DPH was started immediately. The fluorescence intensity was measured at a temperature range from 25 to 70 °C using a Jasco FP-750 spectrofluorometer equipped with two polarizers at an excitation and emission wavelength of 350 and 460 nm (each band width = 5 nm), respectively. The temperature was controlled by the circulation of water in the cell holder, and the sample was gently stirred with a magnetic stirrer.

The fluorescence anisotropy (*r*) was calculated from the following formula:[16]

$$r = (F_{II} - G \cdot F_{\perp})/(F_{II} + 2G \cdot F_{\perp}),$$

where F_{II} and $F_{\perp}$ are the fluorescence intensities of the parallel and vertical directions, respectively. *G* is a correlation factor for the instrument.

Proton Permeability of Liposomes. A vesicle dispersion (20 μl) including CF (4 mM) was added to 3 ml of acidic buffer C (50 mM sodium diphosphate/citrate, 150 mM NaCl; pH = 2.5), preincubated at 25–70 °C. At time zero, the inner pH was 7.5, and the outer was 2.5. The fluorescence intensity of CF at time *t*(F_t) was measured at an excitation and emission wavelength of 480 and 520 nm (each band width = 5 nm), respectively. The proton permeability inward toward the vesicles was quantitated as a value of F_t/F_0 (F_0; initial fluorescence at pH = 7.5).

Thermostability of Liposomes. A vesicle dispersion (5–40 μl) including a high concentration of CF, described as above, was added to 3 ml of buffer A in a fluorescence cuvette, which was pre-

incubated at various temperatures (40, 50, 60, and 70 °C). The fluorescence intensities of CF at time zero (F_0; the value of initial background) and time t(F_t) were measured at an excitation and emission wavelength of 480 and 520 nm (each band width = 5 nm), respectively. The maximum level of the CF fluorescence (F_{max}) was measured after the addition of 10% Triton X-100 (10 µl). The leakage extent of CF at time t was estimated from the following formula:[7f]

$$\text{Leakage extent of CF (\%)} = (F_t - F_0)/(F_{max} - F_0) \times 100$$

Isolation and Purification of PLE from *Methanococcus jannaschii*: *M. jannaschii* JCM10045^T (= DSM 2661) was obtained from RIKEN (The Institute of Physical and Chemical Research, Japan). The medium, containing K_2HPO_4 0.14 g; $CaCl_2{\cdot}2H_2O$ 0.14 g; NH_4Cl 0.25 g; $MgSO_4{\cdot}7H_2O$ 3.4 g; $MgCl_2{\cdot}2H_2O$ 2.7 g; KCl 0.33 g; $NiCl_2{\cdot}6H_2O$ 0.75 mg; $Na_2SeO_3{\cdot}5H_2O$ 0.5 mg; NaCl 30.0 g; $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ 0.01 g; trace mineral solution 10.0 ml; vitamin solution 10.0 ml; resazurin 1.0 mg; $NaHCO_3$ 1.0 g; cysteine·HCl 0.5 g; $Na_2S{\cdot}9H_2O$ 0.5 g in 1 liter of water (pH = 6.0), was prepared anaerobically under a H_2–CO_2 gas mixture (4:1) and autoclaved. The cultivation was carried out for 5 h at 65 °C in the presence of H_2–CO_2 gas (4:1).[7g,13,27a,34] Cells were harvested by centrifugation at 6,000 rpm for 30 min. The total lipids were extracted with $CHCl_3$–methanol–5% aqueous trichloroacetic acid (5:10:4) according to a procedure of Nishihara and Koga.[35] The resulting residue was dissolved in a small amount of $CHCl_3$–methanol (2:1), followed by the addition of 20-fold of chilled acetone to precipitate a white powder.[7g,27b] The precipitate was collected by centrifugation to afford the polar lipid fraction (4 mg from 3 liter of medium).

The lipid composition of the above PLE preparation was estimated as follows. A portion of PLE was treated with acetic anhydride in acetic acid, and was then subjected to acid hydrolysis.[36] The hydrolysate was applied to a thin-layer chromatographic analysis, and the ratio of the core lipids was observed to be similar to that of Sprott et al.[27] (data not shown).

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