Archaebacterial Lipids: Stable Anionic Membranes from 1,2-Bis(dihydrophytyl)glycero-3-phosphate[1)]

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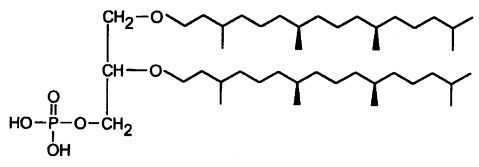
1,2-Bis(dihydrophytyl)glycero-3-phosphate (DPhyPA) was synthesized as an analog of archaebacterial lipids. Upon sonicating in amine-containing buffers like 0.05 M Tris–HCl (pH 7.8), the lipid gave rise to the stable liposomal assemblies, which could store 5(6)-carboxyfluorescein at high temperature as high as 70 °C in the interior. The assembly also had a large negative zeta-potential and showed an absence of gel-to-liquid crystalline phase transition from −20 to 80 °C. A Langmuir monolayer at an air/water interface suggested that the maximum molecular area of the lipid was 0.9—1.0 nm^2. These properties were compared with the assemblies prepared from conventional 1,2-dipalmitoyl-*sn*-glycero-3-phosphate and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

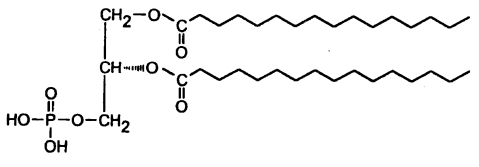
Many archaebacteria grow optimally in such extreme environments as volcanic hot springs, salt lakes, and acidic spots.[2—5)] At a molecular level, the most distinguishing feature of the microorganisms appears in the unusual lipid structures. Namely, while most membrane lipids of eubacteria and eukaryotes consist chiefly of straight-chain fatty acids and glycerol with an ester bonding between them, archaebacterial lipids are characterized by isoprenoid groups of 20 or 40 carbon atoms and an ether bonding between the hydrocarbon and polyols (glycerol or nonitol). Recently Lanzotti et al.[6)] isolated 2,3-bis(dihydrophytyl)-*sn*-glycero-1-phosphate from a halophilic bacterium strain 54R in the amount of 16% of the total lipids. In this paper we wish to report the synthesis and the properties of bilayers and monolayers of an diastereomeric mixture (**1**: DPhyPA), where the isoprenoid skelton is a (3*RS*,7*R*, 11*R*)-3,7,11,15-tetramethylhexadecyl group (Scheme 1). The lipids may be considered as analogs of the conventional phosphatidic acid such as DPPA (**2**).

Experimental

Materials and Instruments. DPPC and DPPA (purity at least 99%) were kindly donated by Nippon Fine Chemical Co. TLC was carried out on a silica-gel-precoated sheet (Merck Art. 5735). Column chromatography was performed using silica gel (Merck 7734, 70—230 mesh). ^{1}H NMR spectra were recorded on a JEOL GX-400 spectrometer using a dilute solution in $CDCl_3$ and CD_3OD (2:1, v/v) and tetramethylsilane as an internal standard. Fast atom bombardment mass spectra (FABMS) were obtained using a JEOL HX-100, whereby a sample was mixed with glycerol on a standard FAB target, then subjected to a beam of xenon atom produced at 8 kV and 2 mA. Ultrasonication was performed by means of a prove-type ultrasonic disintegrator, Ohtake Works Co., Model 5201. A microprocessor-controlled film balance, Sun-etsu, FDS-20, was utilized for the monolayer studies. Microphotographs were obtained by the use of a Hitachi H-7000 electron microscope. T_m values were examined employing a Rigaku DSC-8240 and a Microcal MC-2 scanning calorimeters. Zeta potentials of liposomes were measured using an Ohtsuka ELS-800 light scattering meter, which was equipped with an electrophoretic mobility cell.



1: DPhyPA



2: DPPA

Scheme 1.

Synthesis of 1,2-Bis[(3*RS*,7*R*,11*R*)-dihydrophytyl]-glycero-3-phosphate (DPhyPA). 1,2-Bis[(3*RS*,7*R*, 11*R*)-dihydrophytyl]glycerol (0.31 g, 0.46 mmol),[7)] which was dried by azeotropic removal of a trace of water with benzene, was dissolved in anhydrous tetrahydrofuran (15 ml), cooled in ice-water, then mixed with phosphoryl chloride (2.0 ml, 21.5 mmol) (Fig. 1). After stirring magnetically at ambient temperature for 72 h, the reaction mixture was agitated with 0.1 M (1 M=1 mol dm^{-3}) potassium chloride (10 ml) for a few hours, neutralized with potassium carbonate, then extracted with chloroform. The organic solution was dried over anhydrous sodium sulfate, concentrated to give the residue, then applied to a silica gel column. Elution with a mixture of chloroform, methanol, and concentrated ammonia (17:35:10, v/v) gave the fraction which stained positive to 0.25 mM Rhodamin 6G (for the long chain aliphatic group) and the Dittmer–Lester reagent (for phosphoric ester).[8)] The concentrated fraction was further purified by Sephadex LH-20 column chromatography using a mixture of chloroform and methanol (2:1, v/v) to afford analytically pure DPhyPA, which was freeze-dried as a colorless powder; 0.22 g (65%); mp 152—157 °C; R_f 0.65 (chloroform/methanol/concentrated aq ammonia (17/35/10, v/v);

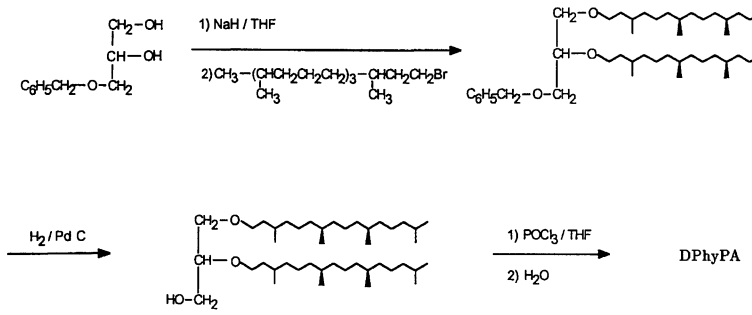


Fig. 1. Synthetic pathways of DPhyPA.

IR (NaCl) 2950(s), 1463(m), 1065(s), and 950(m) cm^{-1}; 400 MHz-^{1}H NMR δ=0.86 (m, 30H, $10CH_3$), 1.0—2.7 (m, 48H, $2CH_2CH(CH_2CH_2CH_2CH_1)_3$), 3.49 and 3.64 (m, 7H, $CH(OCH_2-)CH_2OCH_2-$), and 3.91 (m, 2H, CH_2-O-P); FABMS m/z (rel intensity, %) positive ion-detection mode 733 (M+H; 50) and 755 (M+Na; 15), negative ion-detection mode 731 (M-1; 35). Found: C 66.85; H, 11.71%. Calcd for $C_{43}H_{88}KO_6P$: C, 66.96; H, 11.50%.

Sonication of Lipids in Aqueous Media. DPhyPA, DPPA, or DPPC in an aqueous solvent (0.5 ml mg^{-1} of lipid) [solvent: distilled water, aqueous NaCl such as 0.1 M solution, 0.1 M sodium acetate–HCl (pH 2.0), 0.05 M Tris–HCl buffer (pH 7.8), 0.05 M glycine–NaCl (pH 9.4)] was sonicated at 35 W and at 25 °C (for DPhyPA) and 50 °C (for DPPA and DPPC) for 25 min, centrifuged at about 2000 g for 15 min. The resulting aqueous suspension was subjected to transmission electron microscopic observation using 1.5 wt% aqueous phosphotungstic acid/sodium hydroxide (pH 7) as a staining agent.[9] Typical micrographs of the assemblies are displayed in Fig. 2.

Leakage Study of CF from Liposomal Assemblies. A mixture of lipid (DPhyPA, DPPA, or DPPC) (4.0 mg), 0.05 M Tris-HCl (pH 7.8) (0.5 ml), and 0.2 M aqueous solution of CF (0.5 ml) was sonicated at 35 W and at 50 °C for 30 min and centrifuged at 2000 g for 6 min to give the supernatant, which was then applied to a gel column (2 cm×25 cm) [Sephadex G-50 (medium)] equilibrated with the same solvent. The assembles bearing CF probes in the inner domain were eluted first (1.5—3 ml) and separated from the latter band containing free probes. The aqueous suspension (15 μl) of the assemblies was then diluted with 0.05 M Tris–HCl (pH 7.8) (3.0 ml), and the fluorescence intensity (I_t) at 520 nm with excitation at 495 nm (band width: excitation, 10 nm; emission 5 nm) was measured at a suitable temperature (CF-leakage from the DPPA-aggregates was examinable below 40 °C only). The I_t increased proportionally with the amount of the probe released into the outer aqueous phase. After the measurement, the sample solution was mixed with a 10 vol% aqueous Triton X-100 solution (5 μl), and the maximum intensity (I^*) resulting from instantaneous rupture of the membranes was measured. The leakage extent (%) is given by the $100\times I_t/I^*$, and the rate (% per min) plotted against incubation temperature is shown in Fig. 3. The observed intensity was calibrated to the value at 25 °C. A separate experiment showed that the quenching of CF was eliminated by diluting the dye below about 10 mM.

Surface Pressure and Surface Area Isotherm. The film balance system consisted of a Teflon-coated trough of 506 nm (length)×150 mm (width), a Teflon-barrier, and a microprocessor (NEC, PC-9801) which controls the bar movement, collecting the pressure vs. area data. The temperature of the subphase (water) was thermostated with circulating water within the trough. A benzene/ethanol solution (30—45 μl) of lipid (2—3 mg ml^{-1}) was placed on the surface of the subphase, then the barrier was swept to compress the film at speed (1 point/s or 60 mm^2/s). The pressure was calibrated by means of stearic acid monolayer. The results are shown in Fig. 4.

Results and Discussion

DPhyPA furnished only undefined aggregates when the lipid was sonicated in 0—0.1 M aqueous sodium chloride (neutral), 0.01 M HCl and 0.1 M sodium acetate–HCl (pH 2). However, when DPhyPA was sonicated in amine-containing buffers such as 0.05 M Tris–HCl (pH 7.8) and 0.1 M glycine–HCl (pH 8.4), the lipid gave rise to an aqueous suspension of the multilamellar liposomal assemblies. The representative electron micrograph is displayed in Fig. 2a. The morphology was somewhat similar to that observed for the liposomes prepared from the polar lipid components in *Halobacterium cutirubrum*.[10] It was remarkable that the aqueous suspensions were very stable at ambient temperature for at least a week, and despite a highly fluid state (vide infra) the assemblies retained CF well in the interior at high temperature as 70 °C (Fig. 3, line -●-). Other archaebacterial lipids such as DPhyPC produced also liposomal membranes with a barrier property as good as DPhyPA.[7,11] By contrast, DPPA furnished the undefined assemblies in any aqueous media (Fig. 2b), which released readily CF at temperatures above ca. 20 °C (Fig. 3, line -○-). A liposomal membrane of DPPC became also leaky at around the T_m value (42 °C) (line -▲-).

Formation of stable membranes from DPhyPA in the amine buffers has not been explained, but it might be rationalized by a complex formation of the small phosphate head with the amine, NRH_2; viz., the resulting large polar head [$-OP(O)(OH)O^-\cdot{}^+NRH_3$] was balanced with a large cross section of isoprenoid chains (vide infra). A hampering effect of the NaCl solution and the acidic buffer on a formation of the membranes would be ascribed to a small polar head [$-OP(O)(OH)_2$

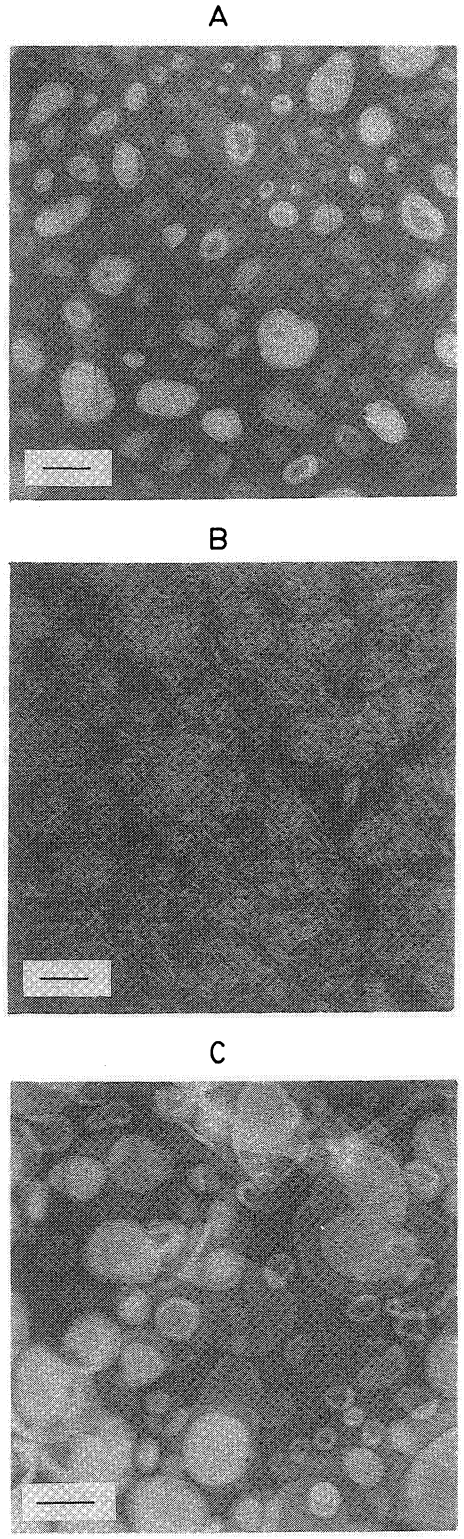


Fig. 2. Transmission electron micrographs, a bar is 1000 Å; negative-stained with phosphotungstic acid–NaOH (pH 7). A: DPhyPA (sonication in 0.05 M Tris–HCl at 25 °C); B: DPPA (sonication in 0.05 M Tris–HCl at 50 °C); C: a 1:2 molar mixture of DPhyPA and DPhyPC (sonication in 0.05 M Tris–HCl at 25 °C). Preparations are mentioned in detail in the experimental section.

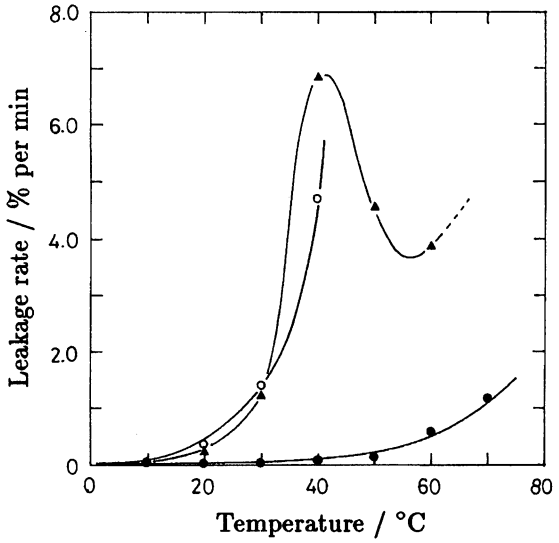


Fig. 3. Initial leakage extent (%) of CF per min from the liposomes of DPhyPA (●), DPPA (○), and DPPC (▲) as a function of temperature. Solvent, 0.05 M Tris–HCl (pH 7.8).

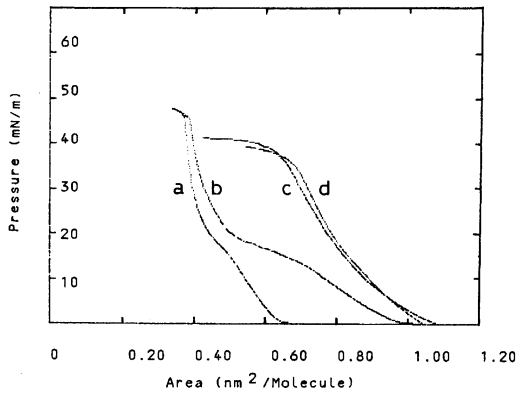


Fig. 4. Surface pressure-surface area isotherms; subphase=water. DPPA: curve a (10 °C) and b (20 °C); DPhyPA: curve c (10 °C) and d (20 °C).

or −OP(O)(OH)(ONa)] relative to the bulky hydrocarbon chains. As anticipated, DPhyPA, upon mixing with DPhyPC, furnished a stable suspension of liposomes in various aqueous media including 0.1 M NaCl and 0.01 HCl; for instance, Fig. 2c. It has been known that lipid structure determines primarily the morphology of the molecular assembly which is most stable thermodynamically.[12]

Other features of the DPhyPA membrane were itemized as follows.

(a) The assemblies did not exhibit any low gel-to-liquid crystalline phase-transition temperature (T_m) from −20 to 80 °C. Many archaebacterial lipids did not have T_m, either; otherwise, they underwent a phase transition at temperature as low as 8 °C or lower.[7,10,11,13–15] The absence of the transition or very low T_m may be explained by steric hindrance of the methyl side chains to the ordered packing of the isoprenoid chains, or a

small energy difference between the trans and gauche rotamers.

(b) The liposomes made of ester-type lipids such as DPPC were decomposed readily at pH 12; for instance, 41% of DPPC molecules in the liposomes was hydrolyzed at 30 °C after 24 h. By contrast, DPhyPA was hardly hydrolyzed even after 3 d at pH 12 and 50 °C. The stability must be due to the chemically inert ether bond between isoprenoid chains and glycerol backbone.

(c) The DPhyPA membrane possessed a large negative zeta-potential of −47.6 mv in comparison with the DPPC- and DPhyPC-liposomal membranes (about −2—−6 mV) [solvent: 0.05 M Tris–HCl buffer (pH 7.8); temperature: 20 °C]. This property may be useful to adjust the surface charge of archaebacterial lipid membranes. DPhyPA upon mixing with DPhyPC gave well-defined liposomes as mentioned above, and a preliminary study indicated that the potential decreased with increasing relative amount of DPhyPA. We have been studing liposomes from a mixture of the lipids in this direction.

(d) A surface pressure-surface area isotherm in a air-water interface revealed that DPhyPA occupied the maximum molecular area of about 0.9—1.0 nm^2 at 10—20 °C which was about two-fold larger than those of the lipids having straight hydrocarbon chains; e.g., DPPA, 0.4—0.5 (Fig. 4); DPPC, 0.45; egg yolk lecithin, 0.62 nm^2/molecule.[16] It seems that such a large molecular area is associated commonly with bis(dihydrophytyl ether) phospholipids.[7,17] The large area may be caused by the bulky dihydrophytyl chains.

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References

1) Abbreviations. CF, 5(6)-carboxyfluorescein; DPhyPA, compound **1** in Scheme 1; DPhyPC, 1,2-bis(dihydrophytyl)-glycero-3-phosphocholine; a lipid which has a $-OP(O_2^-)-OCH_2CH_2N^+(CH_3)_3$ group in place of the $-OPO_3H_2$ group of DPhyPA; DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (compound **2** in Scheme 1); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; FABMS, fast atom bombardment mass spectrum; R_f, relative mobility in thin layer-chromatography; T_m, the temperature at midpoint in gel-to-liquid crystalline phase transition; Tris, tris(hydroxymethyl)aminomethane.

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