Archaebacterial Lipid Models. Highly Thermostable Membranes from 1,1'-(1,32-Dotriacontamethylene)-bis(2-phytanyl-*sn*-glycero-3-phosphocholine)

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Abstract: 1,1'-(1,32-Dotriacontamethylene)bis[2-(3'*RS*,7'*R*,11'*R*)-phytanyl-*sn*-glycero-3-phosphocholine] was synthesized as a model of archaebacterial thermophile lipids. Upon sonication, the bipolar lipid furnished stable unilamellar vesicles of 400–1000-Å diameter and about 50-Å thickness. The liposomes, despite the low gel-to-liquid crystalline phase transition temperature (8 °C), could retain 5(6)-carboxyfluorescein and phospholipase A_2–fluorescein isothiocyanate conjugates in the interior remarkably well even at 80 °C.

Archaebacterial thermophiles thrive at high temperature in the range 50–80 °C or higher, in such extreme environments as hot springs and submarine volcanic fields.[1-4] The plasma membranes may provide a clue to preparation of heat-resistant lipid membranes. All lipids of the thermophiles so far identified feature the unusual structure of C_{20}- or C_{40}-isoprenoid chains bonded through ether linkages to two polar heads (Figure 1, types A and B). Thus, unlike the double-layer plasma membranes from organisms in normal temperate environments, the thermophiles have been considered to possess a thick single layer in the cell membrane.[5-7] However, it is difficult to obtain the bacterial lipids from natural sources. Chemical synthesis may not be an easy task, either, unless one obtains the complex bifunctional C_{40}-isoprenoid segments.[8] We hence have attempted to obtain thermostable membranes from 1,1'-(1,32-dotriacontamethylene)bis[2-(3'*RS*,7'*R*,11'*R*)-phytanyl-*sn*-glycero-3-phosphocholine] (L-32-Phy) that mimic the unique structure of type B.

Experimental Section

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; purity, at least 99%) was kindly donated by Nippon Fine Chemical Co. 1,2-Dicetyl-*sn*-glycero-3-phosphocholine (DCPC) was prepared previously.[9] Phospholipase A_2 (PLA_2) from *Naja mocambique* (product no. 7778) and fluorescein isothiocyanate on Celite (product no. F 1628) were purchased from Sigma. 5(6)-Carboxyfluorescein (CF) was from Eastman Kodak; it was purified by dissolving in 1 M sodium hydroxide, treating with active carbon, acidifying a filtered solution with 1 M hydrochloric acid, centrifuging, washing the resulting precipitate with distilled water, and finally drying in a desiccator. Thin-layer, column, and gel permeation chromatographies were performed with the supports described previously.[10] Compound spots in TLC were visualized by spraying with 0.0012% aqueous Rhodamine 6G and the Dittmer–Lester reagent.[11] The solvent systems for the chromatographies were as follows [system code (volume ratio)]: for hexane/ethyl acetate, A (10/1), B (4/1), C (2/1); for chloroform/methanol, D (2/1); for chloroform/methanol/water, E (65/25/1); and for chloroform/acetone/methanol/concentrated ammonia, F (8/6/6/3). ^{1}H NMR spectra were recorded on Jeol PS-100 and GX-400 spectrometers from a dilute solution in either $CDCl_3$ or a mixture of $CDCl_3$ and CD_3OD (2/1 v/v) with tetramethylsilane as an internal standard. Fast atom bombardment mass spectra (FABMS) were obtained by using a Jeol HX-100, whereby a sample in a mixture of glycerol and 1-thioglycerol (1/1 v/v) on a standard FAB target was subjected to a beam of xenon atoms produced at 8 kV and 2 mA. Ultrasonication was performed with a probe-type ultrasonic disintegrator, Ohtake Works Co., Model 5201. Other instruments used were a Hitachi H-300 transmission electron microscope, a Shimadzu RF-502A spectrofluorometer, an Ohtsuka DLS-700 light scattering spectrometer, and a Rigaku DSC-8240 differential scanning calorimeter.

Preparation of L-32-Phy: 1,1'-(1,32-Dotriacontamethylene)bis[2-(3'*RS*,7'*R*,11'*R*)-phytanyl]-*sn*-glycerol] (3). A tetrahydrofuran solution (30 mL) of 1,1'-(1,32-dotriacontamethylene)bis(3-benzyl-*sn*-glycerol) (1)[10] (1.17 g, 1.45 mmol) was stirred with sodium hydride (60% in oil, 0.2 g, 5 mmol) for 1 h at room temperature. The resulting alkoxide was refluxed in the presence of (3*RS*,7*R*,11*R*)-phytanyl bromide (1.6 g, 4.3 mmol) for 84 h, concentrated, and fractionated between water and chloroform. The organic layer, after being dried on anhydrous sodium sulfate, was subjected to silica gel column chromatography. Elution with a mixture of hexane and ethyl acetate (the volume ratio was changed gradually from 25/1 to 15/1) gave a viscous substance, which was homogeneous by silica gel TLC and had a ^{1}H NMR spectrum agreeing with the structure of 1,1'-(1,32-dotriacontamethylene)bis[2-(3'*RS*,7'*R*,11'*R*)-phytanyl-3-benzyl-*sn*-glycerol] (**2**): 0.40 g (20%); R_f 0.56 (solvent A); $[\alpha]^{25}_D$ −0.6° (*c* 3.6, chloroform); 100-MHz ^{1}H NMR ($CDCl_3$) δ 0.74–0.82 (complex, 30 H, $10CH_3$), 1.15 [coherent, 100 H, $(CH_2)_{28}$ plus $2CH(CH_2CH_2CH_2CH)_3$], ca. 1.2–1.7 (br, 8 H, $4OCH_2CH_2$), 4.51 (s, 4 H, $2CH_2C_6H_5$), and 7.25 (coherent, 10 H, $2C_6H_5$). Boron trifluoride etherate (47% ether solution, 1.5 mL, 4.9 mmol) and ethanethiol (1.5 mL, 20 mmol) were added to a dry carbon tetrachloride solution (10 mL) of **2** (0.36 g, 0.26 mmol), and the mixture was stirred overnight at room temperature. The resulting solution was concentrated by passing nitrogen gas through the solution, which was warmed in a water bath; the process was conducted in a draft chamber. A chloroform solution of the residue was washed with a saturated aqueous solution of sodium chloride, dried on anhydrous sodium sulfate, concentrated, and applied to a silica gel column. Elution with solvent C afforded **3**: 0.16 g (77%); mp 35–37 °C (sublimed); R_f 0.34 (solvent B); $[\alpha]^{25}_D$ −8° (*c* 2.4, $CHCl_3$); 100-MHz ^{1}H NMR ($CDCl_3$) δ 0.76–0.83 (complex, 30 H, $10CH_3$), 1.19 [coherent, 100 H, $(CH_2)_{28}$ plus $2CH(CH_2CH_2CH_2CH)_3$], ca. 1.2–1.7 (br, 8 H, $4OCH_2CH_2$), 2.16 (t, 2 H, *J* = 6 Hz, 2OH), and 3.22–3.69 [complex m, 18 H, $2CH_2CH(OCH_2)CH_2OCH_2$]; FABMS *m/z* 1191 (M + H). Anal. Calcd: C, 78.59; H, 13.36. Found: C, 78.28; H, 13.76.

L-32-Phy. A mixture of anhydrous **3** (0.10 g, 82 μmol) and excess (2-bromoethyl)phosphorodichloridate (1.0 mL) in a sealed container was stirred magnetically at 110 °C (oil bath temperature) for 40 min. The cooled reaction mixture was agitated with water (about 10 mL) for a short while and allowed to stand in a refrigerator overnight. The re-

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(1) Langworthy, T. A. In *The Bacteria. The Treatise on Structure and Function*; Woese, C. R., Wolfe, R. S., Eds.; Academic: Orlando, FL, 1985; pp 459–497.
(2) De Rosa, M.; Gambacorta, A.; Gliozzi, A. *Microbiol. Rev.* **1986**, *50*, 70–80.
(3) Setter, K. O. *Nature* **1982**, *300*, 258–260.
(4) Brock, T. D.; Brock, K. M.; Belley, R. T.; Weiss, R. L. *Arch. Mikrobiol.* **1972**, *84*, 54–68.
(5) De Rosa, M.; Gambacorta, A. *J. Mol. Biol.* **1985**, *182*, 131–149.
(6) Luzzati, V.; Gulik, A. *Syst. Appl. Microbiol.* **1986**, *7*, 262–265.
(7) Gliozzi, A.; Rolandi, R.; De Rosa, M.; Gambacorta, A. *J. Membr. Biol.* **1983**, *75*, 45–56.
(8) Heathcock, C. H.; Finkelstein, B. L.; Jarvi, E. T.; Radel, P. A.; Hadley, C. R. *J. Org. Chem.* **1988**, *53*, 1922–1942.
(9) Yamauchi, K.; Une, F.; Kinoshita, M. *J. Chem. Soc., Perkin Trans. 1* **1986**, 765–770.
(10) Yamauchi, K.; Moriya, A.; Kinoshita, M. *Biochim. Biophys. Acta* **1989**, *1003*, 151–160.
(11) Dittmer, J. C.; Lester, R. L. *J. Lipid Res.* **1964**, *5*, 126–127.

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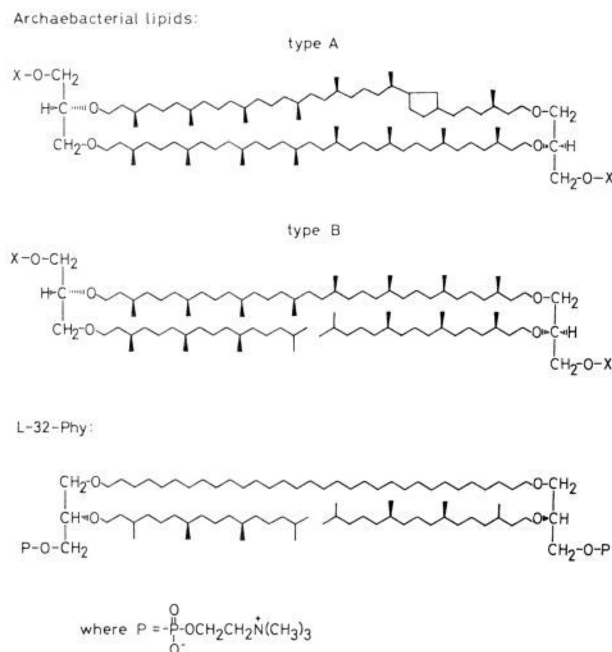


Figure 1. Archaebacterial lipids and L-32-Phy. The natural lipids have several related variants for the isoprenoid C_{40} chains. Type A often possesses a nonitol in place of one of the two glycerols. The X residues include H, sugars, phosphoethanolamine, and phosphosugars.

sulting solid was washed with cold water, dried, and then applied to a silica gel column. Elution with a mixture of chloroform, methanol, and concentrated ammonia (the volume ratio was changed gradually from 65/10/1 to 65/25/1) gave the lipid precursor **4** in the fraction that had R_f = 0.44 (solvent E) in TLC and was positive to both the Dittmer-Lester reagent and Rhodamine 6G sprayings; the yield was 45 mg (38%). Next, a mixture of **4** and 2 M trimethylamine in dimethylformamide (30 mL) was warmed in a sealed container at 55 °C for 3 days. The reaction mixture was then concentrated by means of a rotary evaporator to give the residue, which was applied to a silica gel column and eluted with a mixture of chloroform, acetone, methanol, and concentrated ammonia (the volume ratio was changed gradually from 8/6/4/1 to 8/6/4/3). The fraction, which showed a homogeneous spot at R_f 0.24 (solvent F) and was positive to both the Dittmer-Lester reagent and Rhodamine 6G sprayings, was treated with silver carbonate and then applied to a Sephadex LH-20 gel column equilibrated with solvent D. Elution with the same solvent afforded L-32-Phy as a colorless powder (after freeze-drying the benzene/methanol solution): 15 mg (35%); mp 152-160 °C; R_f 0.24 (solvent F); $[\alpha]^{25}_D$ +2° (*c* 0.2, chloroform/methanol, 2/1 v/v); 400-MHz ^{1}H NMR ($CDCl_3/CD_3OD$, 2/1 v/v) δ 0.86-0.91 (a mixture of d, 30 H, J = 6.7 Hz, $10CH_3$), 1.03-1.64 [complex m, 100 H, $(CH_2)_{28}$ plus $2CH(CH_2CH_2CH_2CH)_3$], 3.25 [s, 18 H, $2N(CH_3)_3$], 3.97 (m, 4 H, $2CH_2N$), and 4.31 (m, 4 H, $2OCH_2CH_2N$); FABMS *m/z* (rel intensity) 1522 (M + H, 6), 1463 (2), and 538 (10).

Liposomes. The lipid (L-32-Phy, DPPC, or DCPC; 2-3 mg) in distilled water or 0.05 M Tris-HCl buffer, pH 7 (1-2 mg/mL), was sonicated at 20-70 °C (L-32-Phy) or 55 °C (other lipids) at 30 W for 25 min. The resulting solution was centrifuged at 2000*g* for 15 min to give the supernatant, which was applied to a Sephadex G-25 (medium) gel column (1.7 cm × 20 cm) equilibrated with the same solvent. The initial fraction (about 3 mL) contained exclusively small unilamellar vesicles (SUVs), as seen by transmission electron microscopy. The majority of the vesicles range from 350 to 700 Å (DPPC), 650 to 1000 Å (DCPC), and 400 to 1000 Å (L-32-Phy) in diameter according to light scattering measurements. Figure 2 is a micrograph of the L-32-Phy liposomes. The specimen was prepared as described previously.[13]

Differential Scanning Calorimetry. L-32-Phy (10 mg) in a mixture of water and ethylene glycol (4/1 v/v; 1.0 g) was vortexed for a few minutes and sonicated at 50 °C for 15 min. The resulting aqueous dispersion (23 mg) was placed in an aluminum pan, and then endothermic T_m and ΔH values were measured by means of the highly sensitive calorimeter upon scanning temperatures from -10 to 60 °C; experimental error, T_m ±0.5 °C, ΔH, ±0.5 kcal/mol.

Phospholipase A_2-Fluorescein Isothiocyanate Conjugates (PLA_2-FITC). (a) Preparation. A mixture of PLA_2 (7 mg) and fluorescein isothiocyanate on Celite (10%, 40 mg, 10 μmol) in 0.1 M carbonate-bicarbonate buffer of pH 9.0 (4.0 mL) was stirred gently at room temperature for 3 h. The reaction mixture was then centrifuged at about 2000*g* for 15 min to give the supernatant, which was applied to a Sephadex G-25 (medium) column (1.5 cm × 20 cm) equilibrated with 0.067 M phosphate buffer (pH 7.4). Elution with the same solvent gave PLA_2-FITC in an initial yellow band (about 5 mL) that was well separated from the later band containing unreacted FITC and degradation products. All of the 1-mL fractions belonging to the initial bond had absorption peaks at 280 nm (for protein) and 490 nm (for the fluorescent probe) with an intensity ratio (280 nm/490 nm) in a narrow range, 1.4-1.5. After the solution was freeze-dried, the PLA_2-FITC conjugates were obtained as 110 mg of yellow voluminous powder containing inorganic salts from the buffer used. The powder was easy to handle and was used in the following experiments without desalting. Polyacrylamide gel electrophoresis [12.5% polyacrylamide; electrophoresis buffer, Tris (40 mM)-sodium acetate (20 mM)-acetic acid-EDTA (2 mM), pH 7.4; 10 mA/tube and 8 V/cm; tracking dye, Pyronin Y] was conducted according to the procedure reported;[12] the PLA_2-FITC powder showed a fluorescent yellow band with the same mobility (0.75) as that of PLA_2 (MW about 14000). The electrophoresis exhibited also two faint unknown bands (mobility 0.50 and 0.63); the amounts were <3% of the major PLA_2-FITC as judged by UV spectroscopic intensity of Coomassie Brilliant Blue R-250 staining. The FITC content in the conjugate was determined by measuring UV absorbance (0.72) at 490 nm (ϵ = 83400) of the aqueous solution (2.3 mg/mL of water). The protein content was determined by the Lowry method with a colorimetric microdetermination kit (Sigma, product no. p 5656). The quantitative analyses indicated that the amount of PLA_2-FITC in the powder was 8.0 mg/110 mg and that one molecule of the enzyme was coupled with 1.27 ± 0.05 molecules of the probe on average.

(b) Phospholipase Assay. A mixture of DPPC (10.0 mg), $CaCl_2 \cdot 2H_2O$ (0.5 mg), and 0.05 M Tris-HCl buffer (pH 7.8, 4 mL) was sonicated as mentioned above to provide a substrate solution. PLA_2 or PLA_2-FITC was dissolved in the same buffer to give the stock solution, which contained 0.35 mg of protein/mL or [protein] = 2×10^{-5} M. The protein stock solution (50 μL) was added to the substrate solution (4.0 mL), which was kept at 42 °C. Hydrolysis rates of DPPC by the enzyme and the conjugates were then followed by means of thin-layer chromatography according to the procedure reported.[13] The initial rate of hydrolysis by PLA_2-FITC was 60 ± 15% of the rate of PLA_2 itself. The activities of PLA_2 and PLA_2-FITC were 1800 and 1200 units/mg of protein. Under typical sonication conditions (55 °C for 15 min in 0.05 M Tris-HCl buffer, pH 7.8), PLA_2-FITC was deactivated by 10-30%.

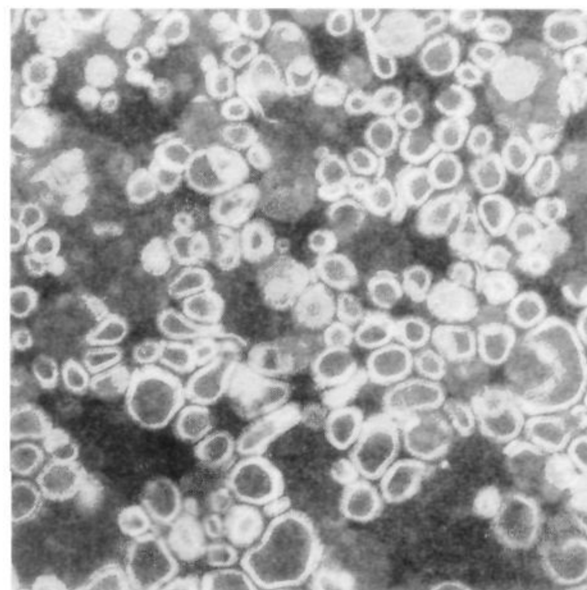


Figure 2. Transmission electron micrograph of L-32-Phy liposomes negatively stained with phosphotungstic acid/sodium hydroxide (pH 7). Magnification 90000×. The micrograph implies that the liposomes are about 50 Å in thickness. It appears that some vesicles in the specimen are flattened and some unilamellar walls are overlapped to display an apparent thickness of about 100 Å.

(12) Fairbanks, G.; Steck, T. L.; Wallach, D. F. H. *Biochemistry* **1971**, *10*, 2606-2617.
(13) Yamauchi, K.; Yamamoto, I.; Kinoshita, M. *Biochim. Biophys. Acta* **1988**, *938*, 51-60.

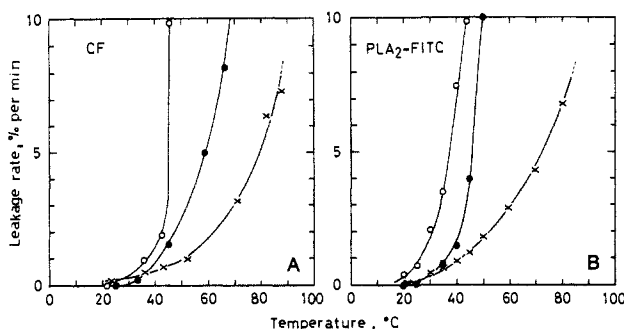


Figure 3. Leakage rates of 5(6)-carboxyfluorescein (CF; panel A) and phospholipase A_2–fluorescein isothiocyanate conjugates (PLA_2–FITC; panel B) from the liposomes of DPPC (○), DCPC (●), and L-32-Phy (×) as a function of temperature.

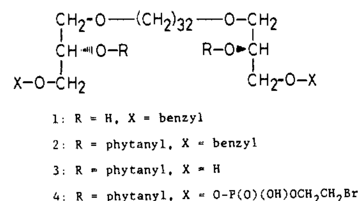
Leakage Rates of CF and PLA_2–FITC from Liposomes. A mixture of lipid (3 mg) and 0.2 M CF or 0.17–0.25 mM PLA_2–FITC solution (solvent, 0.05 M Tris-HCl buffer, pH 7.8; 1.0 mL) was sonicated at 50 °C for 15 min. In the preparation of DPPC liposomes bearing PLA_2–FITC, the buffer contained 1 wt % of EDTA, which was an inhibitor of PLA_2. The sonicated dispersion was centrifuged at 2000*g* for 15 min to give the supernatant, which was applied to a gel column [2 cm × 25 cm; Sephadex G-50 (fine) and G-100 (superfine) for the dispersions having CF and PLA_2–FITC, respectively] equilibrated with the same buffer. The SUVs bearing the fluorescent probes were eluted first (1.5–3 mL) and separated from the latter band containing free probes.[23] The presence of CF in the aqueous interior of liposomes has been established.[14] Encapsulation of PLA_2–FITC, on the other hand, was confirmed by (i) increased fluorescence upon rupturing the liposome membrane by addition of aqueous Triton X-100 (vide infra) and (ii) latency of the phospholipase activity of the PLA_2–FITC probes that were trapped inside the liposomes; the activity was observed when the DCPC liposomes were exposed to an egg lecithin emulsion and ruptured by addition of Triton X-100 (the detailed results will be reported later). However, it seemed that most PLA_2–FITC probes inside the liposomes were concentrated on the inner interface, because despite a small partition coefficient (1-octanol/water) of the probe—0.16 (30 °C) and 0.22 (50 °C)—the apparent probe concentrations in the DPPC liposomes were 3.1 and 4.6 mM when the original aqueous probe concentrations (to be sonicated with DPPC) were 0.17 and 0.25 mM, respectively, viz., a concentration ratio (interface/aqueous phase) of 18. Here, the apparent concentration was estimated by quantitative phosphorus assay[15] (for the lipid) and UV absorption at 495 nm (for the probe) of the SUV solutions, assuming the liposomes were 500 Å in diameter and the limiting area was 50 Å^2/molecule. By the same method, an apparent CF concentration in the more permeable DPPC liposomes was estimated to be 0.22 M, compared with 0.20 M in the original aqueous solution; the closeness between the two values for the leaky DPPC liposomes supports the present method for estimating the probe concentration inside liposomes.

Now, the aforementioned SUV solution (100–200 μL) was diluted with the same buffer to 4 mL in a quartz cell, 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 at 10–15-min intervals. The intensity increased proportionally with the amount of probe released into the outer aqueous phase. After the measurement, the sample solution was mixed with a 10 vol % aqueous Triton X-100 solution (10 μL), and the maximum fluorescence intensity (I_*) resulting from instantaneous rupture of the liposomes was measured. The extent of leakage is given by the I_t/I_* and plotted against time as shown in Figure 3. All of the fluorescent intensity data were calibrated to the value at 25 °C by use of a calibration curve for temperature effects on intensity. It was found in separate experiments that the quenching of CF and PLA_2–FITC was eliminated by diluting the fluorophores below about 10 and 0.017 mM, respectively.

Results and Discussion

L-32-Phy was synthesized in a manner similar to that previously reported;[10] see Chart I. The sodium salt of 1,1′-(1,32-dotriacontamethylene)bis(3-benzyl-*sn*-glycerol) (**1**) was reacted with (3*RS*,7*R*,11*R*)-phytanyl bromide. The resulting ether **2** was debenzylated easily with boron trifluoride/ethanethiol[16] to give the diglycerol **3**. Phosphocholination of **3** at both hydroxyl groups was best performed by melting a mixture of **3** and excess (2-bromoethyl)phosphorodichloridate at about 100 °C for 20–30 min, followed by treatment of the resulting **4** with trimethylamine. Analytically pure L-32-Phy was isolated from the reaction mixture by means of silica gel and Sephadex LH-20 column chromatographies; the overall yield from **1** was 3–8%.

Chart I



Sonication of L-32-Phy in distilled water or 0.05 M Tris-HCl buffer (pH 7) at 20–70 °C and 30 W for 25 min afforded small unilamellar vesicles (SUVs) of 400–1000-Å diameter and about 50-Å thickness (Figure 2).[24] The aqueous dispersion was stable for weeks at ambient temperature. Differential scanning calorimetry indicated that the membranes underwent gel-to-liquid crystalline phase transition at only 8 ± 1 °C (T_m = the temperature at the midpoint in the transition) with $\Delta H = 9 \pm 2$ kcal/mol and ΔS = 0.4–0.6 eu/methylene unit. By contrast, 1,1′-(1,32-dotriacontamethylene)bis(2-hexadecyl-*sn*-glycero-3-phosphocholine), which possesses *n*-$C_{16}H_{33}$ residues in place of the phytanyl residues of L-32-Phy, gave always planar membranes in water with T_m = 62 °C, ΔH = 16.5 kcal/mol, and ΔS = 0.8 eu/methylene unit and was devoid of encapsulating ability.[10] The low T_m and vesiculation of the L-32-Phy membranes may thus be due to the phytanyl groups. The isoprenoid group has often been found as one of the structural elements of archaebacterial lipids, and its high fluidity is one of their characteristics.[1,2,20]

Thermostability of the L-32-Phy membranes was studied by the use of 5(6)-carboxyfluorescein (CF) and the phospholipase A_2 (*N. mocambique*) labeled with 1.27 molar equiv of fluorescein isothiocyanate (PLA_2–FITC). Namely, a mixture of L-32-Phy and 0.2 M CF in 0.05 M Tris-HCl buffer (pH 7) was sonicated under the aforementioned conditions and passed through a gel column to furnish the SUV dispersion that had the water-soluble fluorescent probes in the vesicle interior exclusively.[14] The aqueous dispersion was then subjected to heating, and leakage rates of the trapped compounds from the vesicles were measured fluorometrically (Figure 3, panel A). For the sake of comparison, SUVs made of ordinary 1,2-dipalmitoyl- and 1,2-dicetyl-*sn*-glycero-3-phosphocholines (DPPC and DCPC, respectively; palmitoyl = $C_{15}H_{31}CO$, cetyl = $C_{16}H_{33}$) were also examined for permeability. It was remarkable that the L-32-Phy liposomes, despite the low T_m (8 °C), could retain the carboxyfluorescein remarkably well even at 80 °C. By contrast, the DPPC liposomes became leaky with increasing temperature and particularly so beyond the T_m (42 °C).[10] DCPC furnished liposomes that were fairly tight containers at the T_m (45 °C);[10] so were liposomes of the dioctadecyl analogue of DCPC.

By a similar sonication–gel chromatography method, SUVs encapsulating PLA_2–FITC were prepared.[23] It seemed here that most of the PLA_2–FITC probes resided close to the inner interface of the liposomes at 20–50 °C because despite a small partition coefficient of the probe in a system of 1-octanol/water—0.16 (30 °C) and 0.22 (50 °C)—the ratio $[\text{probe}]_v/[\text{probe}]_w$ was as much as 18 ± 1 at 30 °C and remained constant against varying $[\text{probe}]_w$ (from 0.17 to 0.25 mM) at 30 °C, where $[\text{probe}]_v$ is an apparent

(14) Lelkes, P. I. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC Press: Boca Raton, FL, 1984; pp 51–61.
(15) Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466.
(16) Fuji, K.; Ichikawa, K.; Node, M.; Fujita, E. *J. Org. Chem.* **1979**, *44*, 1661–1664.
(17) Franks, N. P.; Lieb, W. R. In *Liposomes: from Physical Structure to Therapeutic Applications*; Knight, C. G., Ed.; Elsevier/North-Holland Biochemical Press: Amsterdam, The Netherlands, 1981; Chapter 8.
(18) Fuhrhop, J.-H.; Fritsch, D. *Acc. Chem. Res.* **1986**, *19*, 130–137.
(19) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 113–158 (the related references are cited on p 132).
(20) Yang, L. L.; Haug, A. *Biochim. Biophys. Acta* **1979**, *573*, 308–320.

concentration of PLA_2–FITC in the DPPC liposomes ("vesicles") and $[probe]_w$ is the concentration in the aqueous bulk phase ("water"). Such a mode of association of PLA_2–FITC may accord with the presence of an interfacial recognition site in PLA_2.[21] Figure 3, panel B, reveals that the L-32-Phy liposomes retained PLA_2–FITC more firmly than did the DPPC and DCPC liposomes at 50 °C and above. Although proteinaceous PLA_2–FITC is different from CF in size and affinity for the membranes, temperature similarly affected the leakage of both kinds of fluorophores (Figure 3).

The results presented here could be taken as substantiating the heat-resistant property of the L-32-Phy membranes. Since the L-32-Phy, DPPC, and DCPC membranes possess similar phosphorylcholine interfaces, the slow leakage in the L-32-Phy liposomes may be regarded phenomenologically as originating in the isoprenoid residues, the glyceryl ether linkage, and perhaps the prime monolayer membrane. It would be considered likely that the branched isoprenoid chains offer greater intramembrane resistance to the diffusing probes than the *n*-alkyl chains of DPPC and DCPC. This, in a sense, accords with the finding that the branched molecules diffuse via a lipid pathway more slowly than do small and simple compounds.[22] Biocompatible use of the newly synthesized membrane is under investigation.

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(21) Dennis, E. A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: Orlando, FL, 1983; Vol. XVI, Chapter 9.

(22) Wright, E. M.; Diamond, J. M. *Proc. R. Soc. London, B* **1969**, *172*, 203–225, 227–271, and 273–316.

(23) The gel chromatography efficiently removed the PLA_2–FITC probes that were located outside the liposomes. Namely, the fluorescence intensity ratio (eluents/Triton X-100 treated eluents) was 4/10 (emission at 520 nm). The fluorescence from the probes stored inside the vesicles is completely quenched because of the high concentration (>0.17 mM). The ratio, then, equals approximately a number ratio of the probes (outer aqueous phase/total probes in the eluents). Considering that the aqueous phase is about 5000 times the combined volume of the liposomes, the concentrations of the probes in the outer aqueous phase and interface were calculated to be negligibly low in comparison with the probe concentrations in the inner aqueous phase and interface, respectively. A concentration ratio (interface/aqueous phase) is about 18 at 25 °C as described in the Experimental Section.

(24) L-32-Phy was amorphous. The X-ray diffraction pattern from a film of L-32-Phy, which was prepared on the surface of a thin glass tube according to a reported procedure,[17] gave a layer repeat distance of 48.5 ± 0.5 Å. Though the molecular structure in the layer was not clear, it would be most likely that the dipolar lipid furnished a membrane that is a monolayer, by analogy with other dipolar amphiphiles.[5–7,10,18,19]

Oxygenation of Dialkyl Sulfides by a Modified Sharpless Reagent: A Model System for the Flavin-Containing Monooxygenase

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Abstract: The chemical and enzymatic S-oxygenation of 2-(*p*-methoxyphenyl)-1,3-dithiolane and 2-(*p*-cyanophenyl)-1,3-oxathiolane has been investigated. In the presence of chemical oxidizing agents (i.e., $NaIO_4$ or H_2O_2), modest diastereoselective formation of the trans *S*-oxide is formed with 0% enantioselectivity. In the presence of bovine serum albumin, the diastereoselectivity and enantioselectivity of $NaIO_4$-catalyzed S-oxygenation are increased. The maximum level of diastereoselective and enantioselective S-oxygenation was obtained in the presence of the Kagan modification of the Sharpless oxidation reagent, and the stereoselectivity mimicked that of the microsomal flavin-containing monooxygenase from hog liver. Thus, a marked preference for *pro-R* S-oxygenation of 2-(*p*-methoxyphenyl)-1,3-dithiolane was observed. Rat and mouse liver cytochrome P-$450_{PB\text{-}B}$ catalyzed the S-oxygenation of 2-(*p*-methoxyphenyl)-1,3-dithiolane preferentially at the *pro-S* sulfur atom. For 2-(*p*-cyanophenyl)-1,3-oxathiolane, a marked preference for *pro-S* S-oxygenation was observed for catalysis by the modified Sharpless reagent, hog liver microsomes, and highly purified hog liver flavin-containing monooxygenase, whereas for rat and mouse liver cytochrome P-$450_{PB\text{-}B}$, S-oxygenation of 2-(*p*-cyanophenyl)-1,3-oxathiolane occurs by attack on the *pro-R* sulfur atom. The modified Sharpless reagent is an efficient S-oxygenating catalyst that mimics the hog liver flavin-containing monooxygenase in diastereoselectivity and enantioselectivity of dialkyl sulfide S-oxygenation.

The dialkyl sulfide functionality is present in many important drugs, chemicals, and pesticides.[1] In principle, dialkyl sulfide containing drugs can be metabolized by S-dealkylation[2] or by S-oxygenation, but it is the latter biotransformation that is the main metabolic pathway for the dialkyl sulfide group. *S*-Oxide metabolites of sulfides are known to have interesting and useful biological and pharmacodynamic properties.[3] Among the various mammalian *S*-oxygenase enzymes responsible for sulfide oxidation,[4] there are three major monooxygenase enzyme systems. The flavin-containing monooxygenase (FMO),[1a] the cytochromes P-450,[5] and prostaglandin cyclooxygenase peroxidase[6] have been shown to be capable of S-oxygenating dialkyl sulfides. The hepatic flavin-containing monooxygenase from hog liver is the most carefully studied form of FMO, and the enzyme mechanism has

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(1) (a) Ziegler, D. M. In *Metabolic Basis of Detoxication*; Jacoby, W. B., Bend, J. R., Caldwell, J., Eds.; Academic Press: New York, 1982. (b) *Sulfur in Pesticide Action and Metabolism*; Rosen, J. D., Magee, P. S., Casida, J. E., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1981. (c) Damani, L. A. In *Sulfur-Containing Drugs and Related Organic Compounds*; Damani, L. A., Ed.; Ellis Horwood: Chichester, England, 1989; Vol. 1, Part A, pp 135–150.

(2) Mazel, P.; Henderson, J. F.; Axelrod, J. *J. Pharmacol. Exp. Ther.* **1964**, *143*, 1–6.

(3) (a) Duggan, D. E. *Drug Metab. Rev.* **1981**, *12*, 325–337. (b) Renwick, A. G. In *Sulfur-Containing Drugs and Related Organic Compounds*; Damani, L. A., Ed.; Ellis Horwood: Chichester, England, 1989; Vol. 1, Part B, pp 133–154.

(4) Ziegler, D. M. In *Sulfur-Containing Drugs and Related Organic Compounds*; Damani, L. A., Ed.; Ellis Horwood: Chichester, England, 1989; Vol. 2, Part A, pp 53–62.

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