Synthesis of macrocyclic phosphates as models of archæal membrane lipids. Monolayer and bilayer studies

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A novel macrocyclic phosphate, dotriacontan-1,32-diylphosphate (**C32P**), has been synthesized; its bilayer and monolayer properties have been investigated and compared with those of the nearly isomeric double-chain phosphate (**2C16P**). Examination by infrared spectroscopy, based on the temperature-dependent shift of a ν_{CH} stretching vibration band, suggested that, below T_c, the *n*-alkyl chain of **C32P** was mostly all *trans*, except for a few *gauche* conformers, which ensures a compact structure with a hairpin-like bend at the center of the chain. On the contrary, in the liquid crystal state, the *n*-alkyl chain of **C32P** retained more *trans* conformers (less *gauche* conformers) than that of **2C16P**. These features led, for the phase transition of **C32P**, to a lower enthalpy change and a lower entropy change. Fluorescence anisotropy measurements using diphenylhexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), indicated that the macrocylization lowered the membrane mobility in the liquid crystal state. In a monolayer of **C32P** on an air/water interface, the bend in the hydrocarbon chain did not impair a close-packed assembly, with a lower apparent molecular volume in the compressed monolayer, and probably also in the bilayer.

Synthèse de phosphates macrocycliques comme modèles de lipides membranaires archéobactériens. Etudes de mono- et de bicouches. Nous avons synthétisé un phosphate macrocyclique, le phosphate de dotriaconta-1,32-diyl (**C32P**), et comparé les propriétés de ses mono- et bi-couches avec celles du phosphate de dihexadécyle à deux chaînes (**2C16P**). En infra-rouge, la variation avec la température de la vibration d'élongation ν_{CH} suggère que, au-dessous de T_c, la chaîne alkyle de **C32P** est tout *trans*, mis à part un petit nombre de conformères *gauches*, ce qui assure une structure compacte avec un pli en épingle à cheveux au milieu de la chaîne. Dans l'état cristal liquide, la chaîne *n*-alkyle de **C32P** comporte davantage de conformères *trans* (donc moins de conformations *gauche*) que celle de **2C16P**. Ceci conduit pour la transition de phase de **C32P** à un plus faible changement d'enthalpie et à un plus faible changement d'entropie. Les mesures d'anisotropie de fluorescence, avec le diphénylhexatriène (DPH) et le 1-[4-(triméthylamino)phényl]-6-phénylhexa-1,3,5-triène (TMA-DPH), montrent que la macrocyclisation diminue la mobilité membanaire au-dessus de T_c. Dans les monocouches de **C32P** à l'interface eau/air, le cyclisation n'empêche pas un assemblage compact, avec un volume moléculaire apparent plus faible dans la monocouche comprimée; il en est probablement de même dans les bi-couches.

Archæa, the recently recognized third major kingdom of living organisms, have attracted much interest, since their cell structures and biocomponents are markedly different from those found in the usual bacteria and in eucaryotes. Archæal membrane lipids have in particular been studied extensively because of their characteristic chemical structures: the hydrophobic part is composed of C_{20-25} polyprenyl chains linked by ether functions to a variety of headgroups based on glycerol or other polyols and phosphoric acid derivatives; they sometimes comprise macrocyclic structures obtained by α, ω-cyclization of a dimeric C_{20} chain into a C_{40} one, the termini of which are linked either to the same or to both head groups, thus giving either 36- or 72-membered rings.[1,2] To clarify the roles of the characteristic chemical structures of the membrane lipids, a number of model lipids have been synthesized and their phase behavior has been studied by various methods, including electron microscopy, X-ray diffraction, differential thermal analysis, NMR and permeability measurements, and Langmuir monolayer studies.[3-12]

As simpler models of archæal lipids, we have now synthesized, by methods different from the McMurry coupling used before for a similar purpose[10] but identical to those used by Menger *et al.*[11] in the phosphatidylcholine series to introduce deuterium atoms conveniently, a macrocyclic lipid, dotriacontan-1,32-diyl phosphate (**C32P**), and compared its properties with those of its acyclic analog, dihexadecyl phosphate (**2C16P**). These model amphiphiles were chosen for their simplicity: their chains are not branched, which should simplify the interpretation of the monolayer studies and of thermal analysis and, furthermore, the simpler polar heads should facilitate spectral studies. For infrared spectroscopy, particularly, a simple chemical structure is a prerequisite to avoid overlaps of absorption bands, as condensed materials

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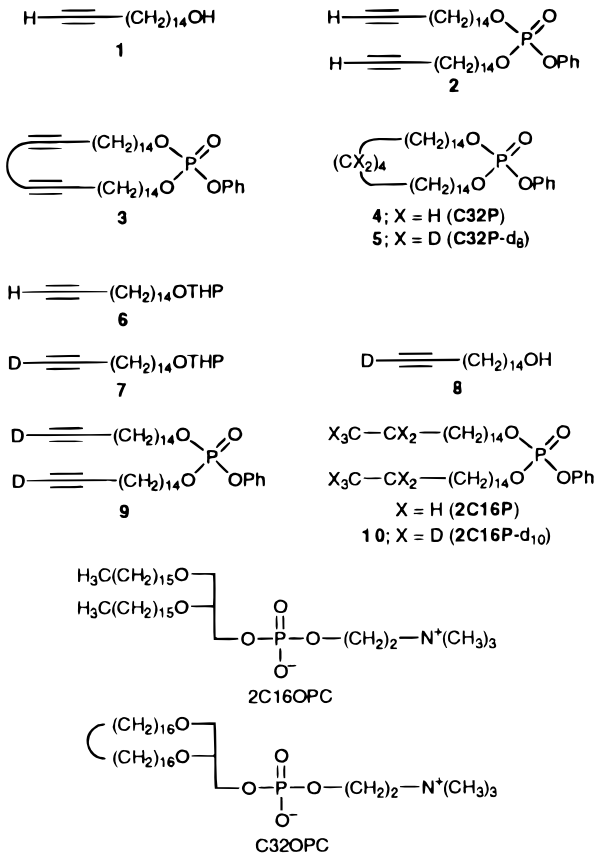

Scheme 1

show usually broad bands and their variation is small.

The cyclic phosphate could be successfully synthesized from a simpler one, carrying two C_{16} chains terminated by acetylene groups, by oxidative coupling between these acetylene groups with a copper reagent at high temperature.[11] The resulting cyclic diyne could be hydrogenated with a PtO_2 catalyst, which simultaneously hydrogenolyzed the phenyl group used temporarily to protect the phosphate headgroup through this sequence. Furthermore, two partially deuterated lipids, corresponding to the macrocyclic and the acylic skeletons, could also be prepared by reducing the corresponding diynes with $^2H_2$ instead of $^1H_2$. The deuterated lipids could be used as appropriate references for the infrared spectroscopy since deuteration induces band shifts for the CH/CD vibrations, without any other significant change in the spectra or the phase properties (Scheme 1).

To study the bilayer mobility in the liquid crystal state, conventional fluorescence anisotropy measurements were made with two fluorescence probes, diphenylhexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH). As TMA-DPH will be located in a different region of the bilayer than DPH, because of its amphiphilic character and of the electrostatic interaction of the ammonium group with the phosphate headgroup, it was expected that the use of both probes would provide preliminary information on the depth profile of membrane mobility.

Monolayer studies on an aqueous phase were performed in order to determine whether the macrocyclic phosphate could be maintained in a close-packed conformation in spite of the additional C—C-linkage introduced at the termini of the alkyl chains. The phase observed in the surface pressure–surface area (π–A) isotherm and the calculated limiting area of the lipid molecule were expected to give information about the extent of expansion or disturbance of the monolayer caused by the macrocyclization.

Experimental

Materials

All the chemicals used were commercially available guaranteed reagents unless otherwise stated. The fluorescence probes, DPH and TMA-DPH, were obtained from Nakarai Chemical Co. and Dojin Chemical Co., respectively. Dihexadecyl phosphate and deuterium oxide (deuterium content 99.9%) were products of Fluka and Aldrich, respectively. Dioctadecylphosphate (**2C18P**) was prepared from 1-octadecanol and phosphorus oxychloride according to the previously reported method.[13]

Melting points were measured with a Yanagimoto BY-1 melting point apparatus and are uncorrected. IR spectra were taken on a Hitachi 285 infrared spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on Jeol La-300 and/or EX-400 spectrometers. Deuterochloroform (99.8% atom enriched, Merck) or CD_3OD (99.5% atom enriched, Merck) were used as NMR solvents. The NMR chemical shifts are reported in δ (ppm) based on internal TMS ($\delta_H = 0$) or the solvent signal ($CDCl_3$, $\delta_C = 77.0$) as references. Electron impact and FAB mass spectra were measured on a Jeol JMS-AX 505HA spectrometer. Column chromatography was carried out on Kieselgel 60 (70-230 mesh, Merck). All reactions, except for the catalytic hydrogenation, were carried out in an inert (Ar or N_2) atmosphere. Tetrahydrofuran (THF) was distilled from sodium–benzophenone; triethylamine was distilled from potassium hydroxide, and benzene and xylene were distilled from P_2O_5.

Di-15-hexadecynyl phenyl phosphate 2. Phenylphosphoryl dichloride (0.29 mL, 2.0 mmol) was added dropwise to a mixture of 15-hexadecyn-1-ol (931 mg, 3.9 mmol), triethylamine (0.71 mL, 5.1 mmol) and 4-dimethylaminopyridine (DMAP) (25 mg, 5.2 mol%) in benzene (10 mL) at 0 °C, and the mixture was stirred for 15 h at 0 °C. Water was added and the mixture was extracted with AcOEt. The combined organic layers were washed with 2N HCl, saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered, and concentrated to dryness. The residue was chromatographed over silica gel (150 g) with hexane–AcOEt (7 : 1, v : v) to give the phosphotriester **2** (1.12 g, 93%) as a wax. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.25–1.40 (br, 40H), 1.48–1.57 (m, 4H), 1.63–1.72 (m, 4H), 1.93 (t, $J = 2.7$ Hz, 2H), 2.18 (dt, $J = 7.1$, 2.7 Hz, 4H), 4.09–4.17 (m, 4H), 7.14–7.36 (m, 5H). ^{13}C-NMR (100 MHz, $CDCl_3$): 18.35, 25.34, 28.45, 28.72, 28.88, 29.07, 29.47, 29.51, 29.58, 30.13, 30.20, 68.00, 68.51 (d, $J = 5.5$ Hz), 84.74, 119.92 (d, $J = 3.6$ Hz), 124.84, 129.60, 150.76 (d, $J = 7.3$ Hz). IR ($CHCl_3$): 630, 950, 1020, 1270, 1460, 1485, 1590, 2850, 2925, 3300 cm^{-1}. Anal. calcd for $C_{38}H_{63}O_4P$: C, 74.23; H, 10.33. Found: C, 74.07; H, 10.10%.

15,17-Dotriacontadiyne-1,32-diyl phenyl phosphate 3. A mixture of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) (0.51 mL, 3.4 mmol) and CuCl (319 mg, 3.23 mmol) in xylene (250 mL) was heated at 140 °C, while oxygen gas was bubbled into the mixture.[11] After 2 h, a solution of diyne **2** (793 mg, 1.29 mmol) in xylene (40 mL) was added dropwise to the mixture by a syringe pump over 5 h. After addition was complete, the mixture was further refluxed for 30 min. After cooling to room temperature, bubbling of oxygen gas was stopped. Almost all of the xylene was removed *in vacuo*. The resulting residue was diluted with AcOEt and 2 N HCl. The aqueous phase was extracted with AcOEt. The combined organic phase was washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was chromatographed over silica gel (100 g) with hexane–AcOEt (7 : 1, v : v) to give the cyclic phosphotriester **3** (363 mg, 46%) as a colorless solid. Mp 64.3–65.1 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.20–1.46 (br, 40H), 1.46–1.55 (m, 4H),

1.64–1.73 (m, 4H), 2.26 (t, $J = 6.7$ Hz, 4H), 4.08–4.21 (m, 4H), 7.14–7.36 (m, 5H). ^{13}C-NMR (75 MHz, $CDCl_3$): 19.04, 25.25, 27.93, 28.38, 28.88, 28.91, 29.18, 29.22, 29.27, 29.32, 29.36, 30.02, 30.11, 65.36, 68.43 (d, $J = 6.8$), 77.35, 119.84 (d, $J = 5.0$), 124.80, 129.55, 150.67 (d, $J = 7.5$ Hz). IR ($CHCl_3$): 955, 1030, 1275, 1465, 1490, 1600, 2850, 2925 cm^{-1}. MS: *m/z* 612 (M^+), 438, 175. Anal. calcd for $C_{38}H_{61}O_4P$: C, 74.47; H, 10.03. Found: C, 74.64; H, 10.20%.

Dotriacontan-1,32-diyl phenyl phosphate 4. A mixture of phosphotriester **3** (310 mg, 0.506 mmol) and PtO_2 (100 mg) in EtOH (40 mL) was stirred under a hydrogen atmosphere for 48 h. The catalyst was filtered off through a pad of Celite, and washed with $CHCl_3$–ethanol (1 : 1, v : v). The filtrate and washings were combined and concentrated to dryness. The residue was recrystallized from hexane to give cyclic phosphodiester **4** (213 mg, 79%) as a colorless solid. Mp 87.2–88.3 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.25–1.45 (br, 56H), 1.64–1.72 (br, 4H), 3.96–4.03 (br, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$–CD_3OD = 2 : 1): 25.12, 28.23, 28.28, 28.41, 28.52, 28.68, 28.72, 28.83, 28.94, 29.09, 29.12, 29.84, 29.89, 66.84 (d, $J = 5.4$ Hz). IR ($CHCl_3$): 955, 1030, 1275, 1465, 1490, 1600, 2850, 2925 cm^{-1}. Negative FAB-MS: *m/z*: 543 ($M^+ - 1$), 79. Anal. calcd for $C_{32}H_{65}O_4P$: C, 70.54; H, 12.03. Found: C, 70.78; H, 12.00%.

[15,16,17,18-^{2}H]Dotriacontan-1,32-diyl phenyl phosphate 5. A mixture of phosphotriester **3** (106 mg, 0.506 mmol) and PtO_2 (31 mg) in EtOD (4 mL) was stirred under a deuterium gas atmosphere for 64 h. The catalyst was filtered off through a pad of Celite and washed with $CHCl_3$–ethanol (1 : 1, v : v). The filtrate and washings were combined and concentrated to dryness. The residue was recrystallized from hexane to cyclic phosphodiester-d_8 **5** (55 mg, 57%) as a colorless solid. Mp 84.0–85.2 °C. ^{1}H-NMR (300 MHz, $CDCl_3$–CD_3OD = 2 : 1): δ 1.25–1.40 (br, 48H), 1.63–1.72 (br, 4H), 3.96–4.03 (br, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$–CD_3OD = 2 : 1): 25.18, 28.24, 28.57, 28.78, 28.79, 28.91, 29.01, 29.12, 29.15, 29.20, 29.25, 29.26, 29.31, 66.90 (d, $J = 3.2$ Hz). IR ($CHCl_3$): 1020, 1220, 1460, 2080, 2180, 2850, 2925 cm^{-1}. Negative FAB MS: *m/z* 551 ($M^+ - 1$). Mass calcd for $C_{32}H_{56}{}^2H_8O_4P$: 551.5044. Found: 551.5058.

2-(15-Hexadecynyloxy)tetrahydro-2*H*-pyran 6. A mixture of 15-hexadecyn-1-ol (61 mg, 0.26 mmol), 3,4-dihydro-2*H*-pyran (35 μL, 0.387 mmol) and *p*-toluenesulfonic acid (5.0 mg) in CH_2Cl_2 was stirred at room temperature for 2 h. Water (5 mL) was added and the mixture was extracted with diethyl ether. The ether extract was washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was chromatographed over silica gel (30 g) with hexane–AcOEt (20 : 1, v : v) to give the THP ether **6** (69.9 mg, 85%) as a wax. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25–1.45 (br, 20H), 1.48–1.90 (m, 10H), 1.94 (t, $J = 2.7$ Hz, 1H), 2.18 (dt, $J = 2.7$, 6.9 Hz, 2H), 3.34–3.42 (dt, $J = 6.7$, 9.5 Hz, 1H), 3.46–3.54 (m, 1H), 3.69–3.76 (dt, $J = 6.7$, 9.5 Hz, 1H), 3.84–3.91 (m, 1H), 4.56–4.59 (m, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): 18.39, 19.70, 25.50, 26.23, 28.48, 28.75, 29.10, 29.49, 29.59, 29.62, 29.74, 30.78, 62.34, 67.70, 68.00, 84.81, 98.83. IR ($CHCl_3$): 640, 720, 1025, 1080, 1125, 1140, 1460, 2860, 2935, 3320 cm^{-1}. Anal. calcd for $C_{21}H_{38}O_2$: C, 78.20; H, 11.88. Found: C, 78.47; H, 12.02%.

[16-^{2}H$_1$]2-(15-Hexadecynyloxy)tetrahydro-2*H*-pyran 7. A solution of ethyl magnesium bromide in THF (13 mL, 1.9 M, 24.7 mmol) was added dropwise to a solution of THP ether **6** (1.28 g, 4.03 mmol) in THF (15 mL) at 0 °C and the mixture was stirred at room temperature for 2.5 h. Deuterium oxide (3.0 mL, 148 mmol) was added to the solution at 0 °C and the mixture was stirred at room temperature for 2.5 h. The mixture was diluted with diethyl ether and 2 N HCl was immediately added. The aqueous phase was extracted with ether. The combined organic phase was washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was chromatographed over silica gel (130 g) with hexane–AcOEt (10 : 1 v : v) to give the THP ether-d_1 (**7**) (1.25 g, 98%) as a wax. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25–1.45 (br, 20H), 1.48–1.90 (m, 10H), 2.18 (t, $J = 6.9$ Hz, 2H), 3.34–3.42 (dt, $J = 6.7$, 9.5 Hz, 1H), 3.46–3.54 (m, 1H), 3.69–3.76 (dt, $J = 6.7$, 9.5 Hz, 1H), 3.84–3.91 (m, 1H), 4.56–4.59 (m, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): 18.29, 19.62, 25.46, 26.19, 28.44, 28.70, 29.05, 29.43, 29.44, 29.54, 29.58, 29.69, 30.72, 62.21, 67.60, 67.74 (t, $J = 37.5$ Hz), 84.18 (t, $J = 7.5$ Hz), 98.73. IR ($CHCl_3$): 1025, 1075, 1120, 1135, 1460, 2590, 2850, 2920 cm^{-1}. Anal. calcd for $C_{21}H_{37}{}^2H_1O_2$: C, 77.96; H + ^{2}H, 11.84. Found: C, 77.77; H + ^{2}H, 11.86%.

[16-^{2}H$_1$]15-Hexadecyn-1-ol 8. A mixture of THP ether-d_1 **7** (1.25 g, 3.86 mmol) and 2 N HCl (1 mL) in THF–MeOH (1 : 1, 10 mL, v : v) was stirred at room temperature for 17 h. The mixture was concentrated *in vacuo* and the resulting residue was chromatographed over silica gel (70 g) with hexane–AcOEt (4 : 1, v : v) to give alkynyl alcohol-d_1 **8** (919 mg, 99%) as a colorless powder. Mp 48.4–50.3 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25–1.47 (br, 20H), 1.48–1.61 (m, 4H), 2.18 (t, $J = 6.9$ Hz, 2H), 3.64 (t, $J = 6.6$ Hz, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): 18.35, 25.72, 28.48, 28.75, 29.09, 29.41, 29.49, 29.58, 29.61, 32.78, 63.07, 67.76 (t, $J = 37.5$ Hz), 84.34 (t, $J = 7.5$ Hz). IR ($CHCl_3$): 1460, 2590, 2850, 2920 cm^{-1}. Anal. calcd for $C_{16}H_{29}{}^2H_1O$: C, 80.27; H + $^2H_1$, 12.63. Found: C, 80.30; H + $^2H_1$, 12.63%.

Di-[16-^{2}H$_1$]15-hexadecynyl phenyl phosphate 9. Phenyl phosphoryl dichloride (0.21 mL, 1.37 mmol) was added dropwise to a mixture of alkynyl alcohol-d_1 **8** (654 mg, 2.73 mmol), triethylamine (0.50 mL, 3.55 mmol) and DMAP (29 mg, 8.81 mol%) in benzene (15 mL) at 0 °C and the mixture was stirred at room temperature for 11 h. Water was added and the mixture was extracted with AcOEt. The organic layer was washed with 2 N HCl, saturated $NaHCO_3$ and brine, dried (Na_2SO_4), filtered and concentrated to dryness. The residue was chromatographed over silica gel (140 g) with hexane–AcOEt (7 : 1 v : v) to give phosphotriester-d_2 **9** (622 mg, 74%) as a wax. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25–1.40 (br, 40H), 1.48–1.57 (m, 4H), 1.63–1.72 (m, 4H), 2.17 (t, $J = 7.1$ Hz, 4H), 4.09–4.17 (m, 4H), 7.14–7.36 (m, 5H). ^{13}C-NMR (75 MHz, $CDCl_3$): 18.31, 25.32, 28.44, 28.70, 29.04, 29.06, 29.44, 29.45, 29.49, 29.57, 30.12, 30.21, 67.76 (t, $J = 36.8$ Hz), 68.50 (d, $J = 6.2$ Hz), 84.23 (t, $J = 7.4$ Hz), 119.92 (d, $J = 5.0$ Hz), 124.84, 129.58, 150.78 (d, $J = 6.8$ Hz). IR ($CHCl_3$): 950, 1020, 1280, 1460, 1490, 1590, 2590, 2850, 2920 cm^{-1}. Anal. calcd for $C_{38}H_{61}{}^2H_2O_4P$: C, 73.99; H + ^{2}H, 10.29. Found: C, 73.71; H + ^{2}H, 10.48%.

Di-[15-^{2}H$_2$,16-^{2}H$_3$]hexadecyl phenyl phosphate 10. A mixture of phosphotriester-d_2 **9** (452 mg, 0.733 mmol) and PtO_2(70 mg) in CH_3OD (4 mL) was stirred under a deuterium gas atmosphere for 46 h. The catalyst was removed by filtration and washed with $CHCl_3$–methanol (1 : 1, v : v). The filtrate and washings were combined and concentrated to dryness. The residue was recrystallized from hexane to afford phosphodiester-d_{10} **10** (207 mg, 51%) as a colorless solid. Mp 69.8–70.6 °C. ^{1}H-NMR (300 MHz, $CDCl_3$–CD_3OD = 2 : 1): δ 1.25–1.45 (br, 48H), 1.64–1.73 (br, 4H), 3.96–4.02 (br, 4H). ^{13}C-NMR (75 MHz, $CDCl_3$–CD_3OD = 2 : 1): 25.00, 28.66, 28.79, 29.03, 29.06, 29.12, 29.15, 29.74, 29.83, 31.11, 66.71 (d, $J = 6.2$ Hz). IR ($CHCl_3$): 1020, 1230, 1460, 2200, 2850, 2925

cm^{-1}. MS (negative FAB): m/z 555 ($M^+ - 1$). Anal. calcd for $C_{32}H_{57}{}^2H_{10}O_4P$: C, 69.03; H + ^{2}H, 12.13. Found: C, 69.30; H + ^{2}H, 12.25%.

Techniques

Monolayer studies. The surface pressure–surface area (π–A) isotherm of the lipid was recorded on a Surface Barostat, KSV2200 instrument with a Teflon trough 16.4 cm wide and 27.0 cm long. The buffer used as the aqueous subphase contained 100 mM NaCl, 10 mM Tris, and 0.1 mM EDTA; it was adjusted to pH 8.0 with HCl. The lipid, dissolved in chloroform (*ca.* 1 mg mL^{-1}), was spread on the aqueous subphase at 23 °C by a Hamilton microsyringe. After spreading, the monolayer was equilibrated for 5 min and compressed at a constant rate of 15 mm min^{-1}. The limiting area of each lipid was measured at film collapse. Each isotherm was measured at least in triplicate.

DSC thermograms. A thin lipid film was prepared on the inner wall of a round-bottom flask by evaporating a chloroform solution of the lipid with a stream of nitrogen and vacuum drying for 3 h. The lipid film was mixed with an equivalent amount of 1 M NaOH and 30 mM Tris-HCl buffer (pH 8.0) and was hydrated by a combination of freeze-thaws and vortex mixings above the temperature of the phase transition from gel to the liquid crystal phase (T_c). The lipid suspension (30 mM) was introduced into a 70 μL aluminum pan that had been washed with hot water prior to use. Thermograms were measured with a Seiko Electric 2000 differential scanning calorimeter at a heating rate of 1.0 °C min^{-1}. Scanning was repeated several times to reach a reproducible thermogram.

Fourier transform infrared spectroscopy. The thin lipid film prepared in a round-bottom flask was dispersed in a mixture of 100 mM Tris-HCl buffer (pH 8.0) and an amount of concentrated NaOH solution equivalent to the lipid by vortex mixing above the T_c for 5 min. The suspension was frozen and dried under vacuum; the resulting powder was re-suspended in deuterium oxide by vortex mixing. The final composition of the dispersion was 30 mM lipid and 100 mM Tris. The sample was introduced into a cell with CaF_2 windows (path length 0.035 mm). Infrared spectra were recorded in a nitrogen atmosphere on a Perkin Elmer 2000 FTIR spectrophotometer with an optical resolution of 1 cm^{-1}, a digital resolution of 0.5 cm^{-1} and an accumulation of 100 scans. The sample cell was thermostated by water circulation in a cell holder and monitored with a CA thermocouple located at the window edge. Absorbance spectra were obtained as the ratio of the signals from the sample and a reference (empty cell); peak maxima were measured with a resolution of 0.1 cm^{-1}.

Fluorescence anisotropy measurements. A thin lipid film was prepared in a round-bottom flask in the same way as above, except for the addition of a fluorescence probe before evaporation of the solvent. The sample was suspended in 100 mM Tris-HCl (pH 8.0) by sonication with a Tomy Seiko UR-200P sonicator above T_c for 5 min. The suspension contained 0.1 mM lipid and 0.5 mM probe, and had an optical density of 0.1 at 350 nm and of 0.06 at 450 nm. After purging the oxygen with a gentle nitrogen stream, the fluorescence intensity was measured, using a Shimadzu RF5300PC fluorescence spectrophotometer equipped with polarisers, at an excitation wavelength of 350 nm and an emission wavelength of 460 nm (bandpass 3 nm and 5 nm, respectively). The sample temperature was controlled by water circulation in the cell holder and gentle stirring with a magnetic bar and corrected by measurement with a thermocouple. The fluorescence anisotropy (r) was calculated from the following formula: $r = (I_{\parallel} G - I_{\perp})/(I_{\parallel} G + 2I_{\perp})$ where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities for the parallel and vertical directions, respectively, and G a correction factor for the instrument.

Results and Discussion

Monolayer studies

The π–A isotherm of the macrocyclic phosphate **C32P** shows a condensed phase but no apparent expanded phase (Fig. 1). The limiting surface area of **C32P** measured at film collapse (about 0.42 nm^2 $molecule^{-1}$) is slightly larger than that of **2C16P** (about 0.39 nm^2 $molecule^{-1}$). The slope of the curve for the condensed phase, which reflects the modulus of the monolayer, is almost the same, suggesting that the macrocyclic phosphate forms a close-packed aggregate in the compressed monolayer.

In the condensed phase, we can note that the macrocyclic phosphate **C32P** necessarily has at least two molecular bends in its n-alkyl chain: one at the hydrophilic phosphate group, which is preferentially located at the surface of the subphase, where the P—O—C linkage would appropriately form the bend, and the other in the alkyl chain to allow for ring formation. We had assumed that the molecular bend in the alkyl chain might prevent close packing of this chain because of the supplementary volume required; this postulate is clearly suggested by a consideration of a space-filling model. Consequently, lipid **C32P** should show a larger limiting area in the isotherm. In fact, amphiphiles with a kink, such as oleic acid, which has a Z double bond in the hydrophobic chain, show such features.[14] The limiting area of both lipids (**2C16P** and **C32P**, 0.39 and 0.42 nm^2 $molecule^{-1}$, respectively) is nearly the same as twice the value of the typical limiting area for an amphiphile with one n-alkyl chain (0.20 × 2 nm^2 $molecule^{-1}$)[15,16] and also as those of the phosphatidylcholines **DPPC** (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, **2C16OPC** in the presently used nomenclature: 0.40 nm^2 $molecule^{-1}$) and **C32OPC** (0.41 nm^2 $molecule^{-1}$) (this last substance was synthesized[30] for comparison by the alternate method described earlier.[10]) This implies that the longitudinal extension of the lipids would be more slanted towards the surface, even in the condensed phase; therefore, an expansion of the monolayer due to the macrocyclization would not be observed. The volume expansion of the macro-

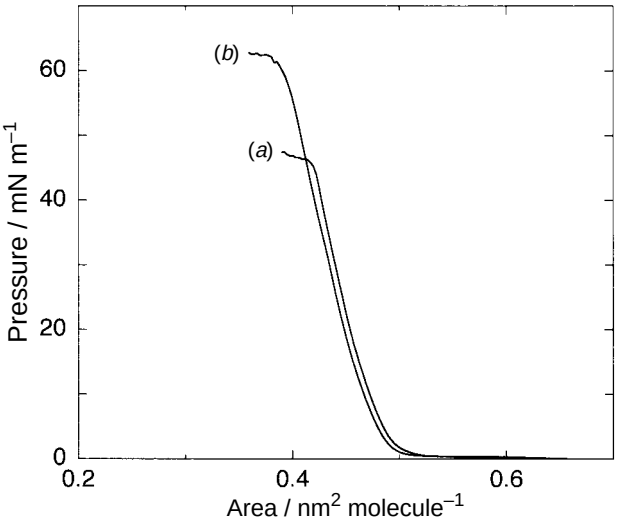

Fig. 1 Surface pressure–surface area isotherms of the phosphate lipids measured at 23 °C: (*a*) **C32P**; (*b*) **2C16P**. The buffer used for the subphase contained 100 mM NaCl, 10 mM Tris, and 1 mM EDTA and was adjusted with HCl to pH 8.0

cyclic lipid is therefore not a large one; however, the slightly lower collapse pressure of **C32P** (46 mN molecule^{-1}), compared to those of **2C16P** (58 mN molecule^{-1}) or of **C32OPC** (53 mN molecule^{-1}), may be due to a lower stability of the condensed phase, caused by the distal bend in the alkyl chain.

Thermal analysis

The phase transition from the gel to the liquid crystal phase of **C32P** was observed at a higher temperature, with lower enthalpy change (73.2 °C, 12.9 kJ mol^{-1}, ΔS 37 J mol^{-1} K^{-1}) than for **2C16P** (55.4 °C, 31.0 kJ mol^{-1}, ΔS 94 J mol^{-1} K^{-1}). Generally, amphiphiles with a phosphate as the hydrophilic group show a higher T_c among a series of amphiphiles with the same hydrophobic part;[13] however, we had not predicted that the macrocyclic **C32P** would show such a high T_c (19 °C higher than that of **2C16P**). It was supposed that macrocyclization would perturb the bilayer assembly due to the existence of the molecular bend and consequently decrease the phase transition temperature, as the introduction of a double bond or a methyl branch into alkyl chains generally lowers T_c.[17,18] The observation made in the present study, however, is consistent with the results of Menger and collaborators on a series of macrocyclic lipids with a phosphocholine head-group.[11,17]

Fourier transform infrared spectroscopy

Fig. 2 shows the C—H and C—D stretching bands of the FTIR spectra of the lipids, measured in a KBr tablet. The stretching bands are rather plain, owing to the simple chemical structures without a group that would cause spectral overlap. In the spectra of the macrocyclic lipid [Fig. 2(*a*) and (*b*)], symmetric and asymmetric CH_2 stretching vibration bands appear at 2850 cm^{-1} and 2917 cm^{-1}, respectively, while in the spectrum of **C32P**-d_8 two additional minor peaks, derived from the C—D bonds, appear at 2106 cm^{-1} and 2195 cm^{-1}. In addition to the CH_2 bands, the non-cyclic lipid, **2C16P** shows symmetric and asymmetric CH_3 stretching bands around 2870 cm^{-1} and 2960 cm^{-1} [Fig. 2(*c*)]. On the other hand, in the case of **2C16P**-d_{10}, some minor peaks appear in the range 2070–2120 cm^{-1}. These are assigned to CD_3 groups instead of CH_3, demonstrating that the deuterium exchange of the acetylenic group and the catalytic deuteration of the diacetylene has successfully proceeded. The absence of CH_3 bands in the spectrum of **2C16P**-d_{10} means that migration of hydrogen or deuterium into an undesired position of the hydrocarbon chain is negligible. In both lipids, deuteration makes the stretching bands sharper.

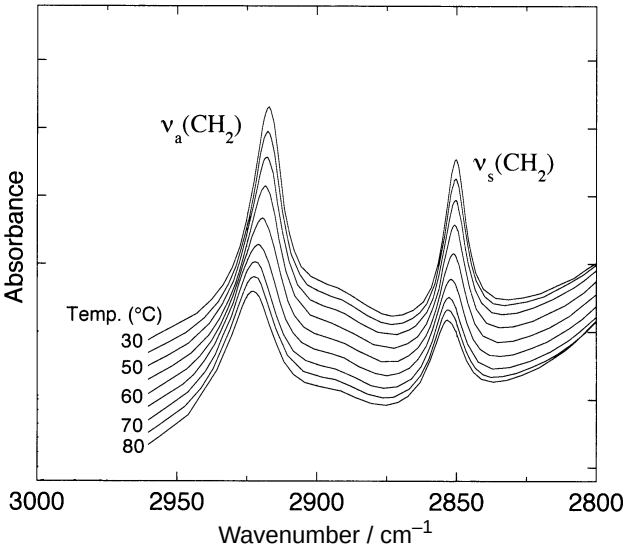

Fig. 3 Temperature dependence of the C—H stretching vibration bands of a dispersion of **C32P** in Tris-deuterium oxide buffer (pH 8.0)

Fig. 3 shows an example of the temperature dependence of the C—H stretching vibration bands of the aqueous dispersion of the lipids. At higher temperatures, the wave numbers, widths, and heights of the C—H stretching bands changed remarkably around the phase transition temperature observed by the DSC measurement. Such spectral changes have often been reported in previous studies of membrane fluidity by infrared spectroscopy.[19-22] The band maximum wavenumber of the CH_2 stretching vibration has been employed as a simple index to estimate the ratio of *gauche*/*trans* conformers or the extent of hydrocarbon chains disorder. Fig. 4 shows the temperature dependence of the band maximum wavenumbers of the CH_2 asymmetric stretching vibration of **C32P** and **2C16P**. The symmetric stretching vibrations around 2850 cm^{-1} give practically the same results, except for a smaller variation of the wavenumber (2.5 cm^{-1}). The band maxima of the lipids shift gradually to higher wavenumbers in both the gel and liquid crystal states, and abruptly from 2919 to 2923 cm^{-1} around the phase transition temperatures (55 for **2C16P** and 73 °C for **C32P**),[23] showing that the content of *gauche* con-

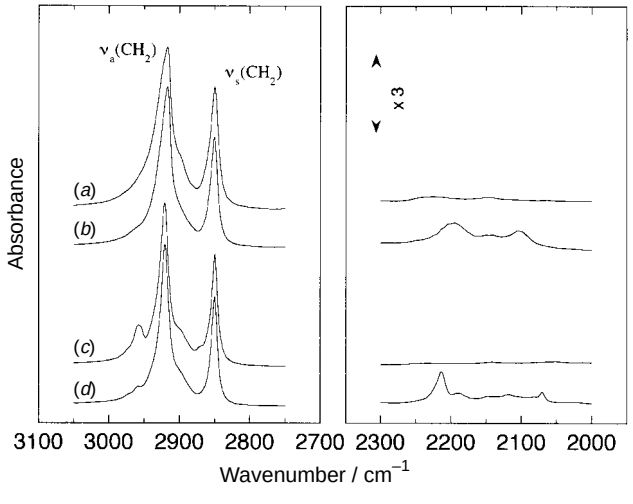

Fig. 2 C—H and C—D stretching vibration bands in FTIR spectra of the phosphate lipids measured in a KBr tablet: (*a*) **C32P**; (*b*) **C32P**-d_8; (*c*) **2C16P**; (*d*) **2C16P**-d_{10}

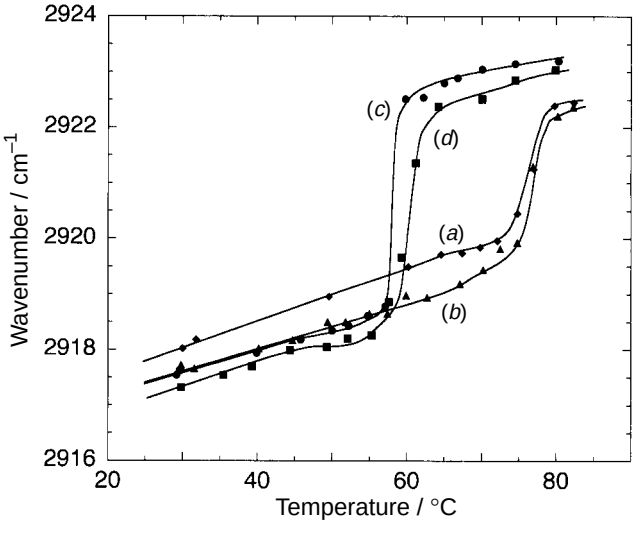

Fig. 4 Temperature dependence of the wavelength of the C—H asymmetric stretching band maximum: (*a*) **C32P**; (*b*) **C32P**-d_8; (*c*) **2C16P**; (*d*) **2C16P**-d_{10}

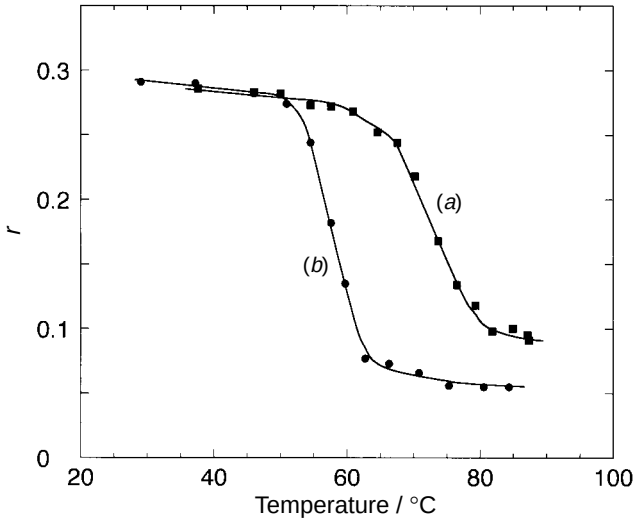

Fig. 5 Temperature dependence of the fluorescence anisotropy of DPH incorporated in the bilayers of the phosphate lipids: (*a*) **C32P**; (*b*) **2C16P**. The concentration of the lipids and probes were 0.1 mM and 0.5 μM, respectively

formers of the methylene chain increases at the phase transition, that is, at the melting point of the chains.

In the gel state of deuterated **C32P**, the band maximum wavenumber of the asymmetric stretching is always about 1 cm^{-1} larger than in the non-deuterated substance at the same temperature. As in the monolayer system, the macrocyclic phosphate should accommodate a molecular bend in the hydrocarbon chain to build a close-packed aggregate. Therefore, even in the gel state, the macrocyclic phosphate should have some *gauche* conformers, probably with at least two adjacent *gauche* conformations to obtain a sharp turn. Assuming that the *gauche* conformers would be located in the central region of the C_{32} chain, far away from the phosphate, the C_4 methylenes of **C32P**-d_8, masked by the deuteration, would not contribute to the stretching band at 2920 cm^{-1}. As it was estimated that, at the phase transition, 2 to 6 *gauche* conformers are present per chain to obtain a disordered packing, accompanied by a wavelength change of about 5 cm^{-1},[11,24] it would be a rational explanation that the larger (1 cm^{-1}) band maximum wavelength of the non-deuterated lipid, compared to that of **C_{32}**-d_8, would arise from several *gauche* conformers at the molecular bend. In fact, the band

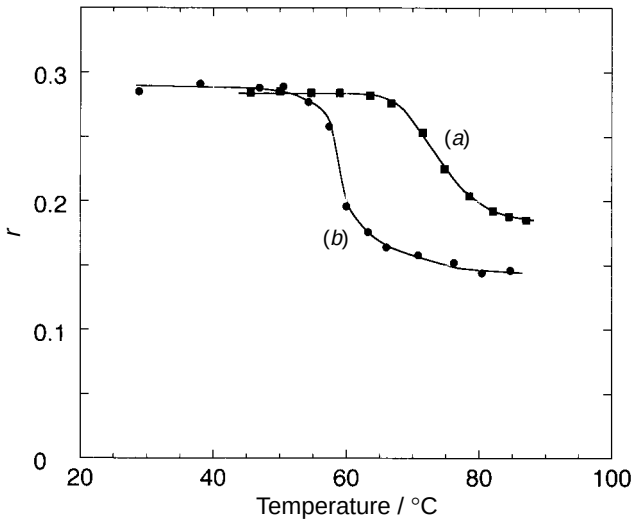

Fig. 6 Temperature dependence of the fluorescence anisotropy of TMA-DPH incorporated in the bilayers of the phosphate lipids: (*a*) **C32P**; (*b*) **2C16P**

maximum wavelength of **C32P**-d_8 is almost comparable to that of the double-chain lipids, **2C16P** or **2C16P**-d_{10}, in which few *gauche* conformations exist in the gel state.

On the contrary, in the liquid crystal state, the band maximum wavenumbers of the macrocyclic lipids are lower than those of the double-chain lipids: at the same temperature (80 °C), they are lower by 1.0 cm^{-1}–1.5 cm^{-1}, and at temperatures above T_c plus 10 °C, they are lower by 0.2 cm^{-1}– 0.5 cm^{-1} (2922.5 cm^{-1} for **C32P** and 2922.3 cm^{-1} for **C32P**-d_8 at 83 °C, 2923.0 cm^{-1} for **2C16P** and 2922.5 cm^{-1} for **2C16P**-d_{10} at 65 °C). The differences mean that in the liquid crystal state the macrocyclic lipid has fewer *gauche* conformers than the double-chain lipid, though the macrocyclic lipid already has some *gauche* conformers before the phase transition.

Fluorescence anisotropy

Because of experimental difficulties at temperatures above 90 °C, we proceeded to fluorescence anisotropy measurements[25,26] to assess properly the membrane fluidity and to confirm the effect of macrocyclization in the liquid crystal state. Fig. 5 shows the temperature variation in the fluorescence anisotropy for DPH embedded in **C32P** and **2C16P** bilayers. With a temperature increase, the fluorescence anisotropy r for **C32P** gradually decreases in the gel state from 0.29 and then drops markedly around T_c to reach a constant value of 0.09. The r value for **2C16P** decreases from 0.29 around T_c to a lower final value (0.05). Another double-chain lipid with longer chains, **2C18P**, which has almost the same T_c as **C32P** (75 °C), shows an r value of 0.06 at 85 °C and the typical phospholipid, DPPC, has an r value of 0.07 at 55 °C (T_c 43°). The abnormally large r value of the macrocyclic lipid in the liquid crystal state means that the viscosity of the milieu where the probe was embedded in the hydrophobic core of the membrane should be higher than that of common lipids.

The same feature is observed between **C32P** and **2C16P** when the fluorescence probe is TMA-DPH, which has a similar chemical structure and fluorescence properties as DPH, except for the additional trimethylamino group at a terminal phenyl ring (Fig. 6). However, the difference in the anisotropy in the liquid crystal state seems to be insignificant compared to that for DPH: the r values for **C32P** and **2C16P** are 0.19 at 83 °C and 0.16 at 65 °C, respectively. TMA-DPH should be located closer to the bilayer surface and the longitudinal axis of the probe should be better fixed along the normal to the bilayer plane, to place the hydrophilic group attached to the probe in the water phase. For this reason, the r value for TMA-DPH decreases less markedly above T_c than that of DPH, which is distributed more randomly in the hydrophobic core of the membrane. The difference between DPH and TMA-DPH may come from the difference between the effects due to the macrocyclization in the region of the core and in the region close to the membrane surface. Macrocyclization appears to decrease the fluidity more efficiently in the core of the membrane than in the region close to the membrane surface. To understand the difference in the fluorescence anisotropy quantitatively and discuss the depth profile of the membrane fluidity, further detailed studies are needed.[27]

Conclusion

The comparative experiments using the deuterated macrocyclic lipid indicate that in the gel state the molecular bend exists in the central region of the methylene chain as some *gauche* conformers, which would not be far away from each other (not more than four carbons of the methylene chain) so as to form a sharp bend in the molecule. The unexpected π–A isotherm of the macrocyclic lipid, similar to that of the double-chain lipid, suggests that the molecular bend does not require a larger volume in the monolayer and does not

prevent the lipid from forming a close-packed assembly. About the conformation of the methylene chain, the situation would be the same. This corresponds to the fact that the T_c of the macrocyclic lipid is not lower, but rather higher than that of the corresponding double-chain lipid. The higher T_c corresponds to the additional linkage, which apparently does not impair the close-packing in the gel state and inhibits the disorder resulting from the destruction of intermolecular and intramolecular chain-chain associations at the phase transition. FTIR results also support the hypothesis that the lower enthalpy change upon cyclization is due to some *gauche* conformers in the gel state and fewer *gauche* conformers in the liquid crystal state, and that the lower entropy change comes from fewer *gauche* conformers due to restriction of independent thermal movements of the two chains in the liquid crystal state. The fluorescence anisotropy measurements gave a preliminary proof of a restriction effect of the macrocyclization on the membrane fluidity, which will be more accurately measured by appropriate spectroscopic methods (ESR[28] and NMR[29]), and permeability measurements.[5]

From the present study, it is obvious that the linkage at the termini of the two chains strikingly affects not only the conformation of the isolated lipid molecule, but also the properties of the whole membrane. The newly designed lipid should also be valuable as a membrane material with excellent physical stability and high barrier ability.

Acknowledgements

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