High-Stability Liposomes from Macrocyclic Diyne Phospholipids

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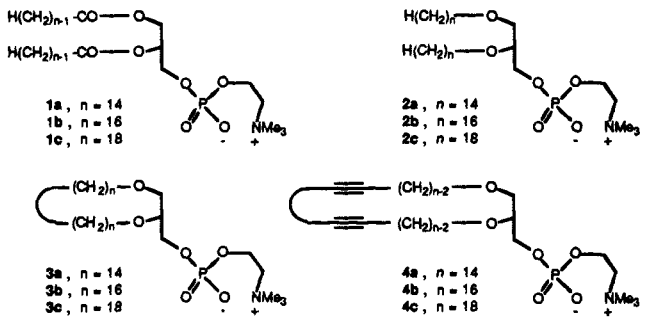
Phospholipids typically possess two chains (alkyl or alkenyl) which are linked to the glycerol backbone by means of ester functionalities (see, e.g., **1**), or less often by ether functionalities (see, e.g., **2**).[1] Such compounds are stable at ambient temperature and neutral pH, but readily decompose at elevated temperatures and in highly acidic media.[2] In contrast, extreme thermoacidophilic bacteria are fully functional at temperatures up to 90 °C and external pH values as low as 0.5.[3] Lipids of those bacteria have hydrocarbon chains linked to the glycerol backbone by means of ether groups, and those chains are frequently joined to form macrocycles.[3b,4] These lipids would appear to be good candidates for liposomes with increased stability. The rare occurrence of these natural materials has prompted efforts toward preparation of synthetic equivalents and exploration of their properties.



Recently Menger et al. developed a synthesis of macrocyclic ether-type lipids **3** with 32−44 atoms in a ring.[5,6] Here we report a synthesis of macrocyclic ether-type lipids **4** which possess a diyne moiety (Scheme 1).[7] The synthetic methodology is similar to that used in Menger's approach, but two additional goals were achieved: the precursor ω-alkyn-1-ols **8** were prepared in a more economical way, and the diyne functionality was preserved in final products **4**. In our synthesis the sources of alkyl chains are 1-bromoalkanes, which are more readily available than 1-alkynes and ω-bromo-1-alkanols used in Menger's scheme. When 1-bromoalkanes were submitted to the nucleophilic attack of a lithium salt of **6**, 2-alkyn-1-ols **7** were obtained. These alcohols were isomerized with NaH in 1,3-diaminopropane into ω-alkyn-1-ols **8** using the "acetylenic zipper" reaction.[8] Furthermore, in Menger's synthesis of saturated macrocyclic phospholipids **3** the C-3 hydroxyl functionality of glycerol was protected with a benzyl group. Our target alcohol **12** requires a diacetylene functionality, and therefore it was not possible to use hydrogenation to cleave the benzyl−ether linkage in the precursor **11**. Therefore, in our synthesis the C-3 hydroxyl functionality of the glycerol backbone was protected with a 4-methoxybenzyl group, which allowed acidic deprotection in cyclic ether **11** without affecting the diyne moiety. All reactions shown in Scheme 1 occur in fair to quantitative yield, and **4b** was synthesized from 1-bromotridecane in 24% overall yield.[9]

Previous study[5] of thermotropic properties of hydrated lipids **3** showed that chain "tethering"[4] raises gel-to-liquid phase-transition temperatures (T_c) compared to those in "untethered" lipids **1** and **2**. Our study shows a further increase in the T_c of lipids **4** relative to lipids **3**. While saturated lipids **3a**, **3b**, and **3c** have T_cs at 21.0, 50.4, and 70.1 °C, respectively, their diyne analogs **4a**, **4b**, and **4c** have T_cs at 76.2, 81.3, and 87.6 °C, respectively. These large increases in T_c are probably caused by the stiffening effect of the diyne moiety, which reduces the mobility of the macrocycle.

Sonication of lipids **4** using probe[10] or bath sonication gave liposomes with a unimodal distribution of particle diameters and an average size of about 100 nm, as determined by light-scattering techniques[11] and electron microscopy.[12] Liposome size was shown to be a function of the lipid concentration, lipid ring size, sonication time, sonication temperature, and heat history. Liposomes prepared with lipid **4b** showed a minimal average size (94 nm) at a lipid concentration of 2 mg/mL, while lower and higher lipid concentrations gave liposomes with larger diameters.

Lipids **1**−**4** were sonicated in the presence of calcein, a self-quenching fluorescent dye, to give liposomes with encapsulated calcein. Release of dye was followed by an increase in fluorescence intensity.[13] Dye release from liposomes synthesized with lipids **4** was significantly reduced compared to liposomes prepared with lipids **1**−**3**. All four classes of liposomes (prepared from lipids **1**−**4**) have comparable size, excluding the dependence of dye release on the size of liposomes. When calcein-containing liposomes prepared with acyclic ester-type lipid **1b** were treated with acid (pH 2), the dye quantitatively escaped from the liposomes during a 10-day period. However, liposomes prepared with macrocyclic ether-type lipid **4b** retained over 92% of calcein under the same conditions. Liposomes prepared with lipids **1b**, **2b**, or **3b** and heated at 50 °C for 24 h released over 85% of calcein, while liposomes prepared with **4b** released less than 15% of calcein under the same conditions. Similarly, when calcein-containing liposomes prepared with lipids **1b**, **2b**, or **3b** were treated with 33 wt % ethanol (Figure 1A) or 0.1 wt % Igepal 620 (Figure 1B), they all quantitatively released calcein, while the leakage of calcein from liposomes prepared with **4b** is negligible under the same conditions. Wheat germ lipase induces leakage in liposomes prepared with acyclic lipids **1b** and **2b**, while it had

(1) For a review, see: Cevc, G., Ed. *Phospholipids Handbook*; Marcel Dekker: New York, 1993.
(2) Crommelin, D. J. A.; Talsma, H.; Grit, M.; Zuidam, N. J. In ref 1, pp 335−348.
(3) (a) De Rosa, M.; Gambacorta, A.; Minale, L.; Bu'Lock, J. D. *J. Chem. Soc., Chem. Commun.* **1974**, 543. (b) De Rosa, M.; De Rosa, S.; Gambacorta, A.; Bu'Lock, J. D. *J. Chem. Soc., Chem. Commun.* **1977**, 514.
(4) The linkage of lipid chain ends to form a macrocycle is often referred to as "tethering".
(5) (a) Menger, F. M.; Brocchini, S.; Chen, X. Y. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1492. (b) Menger, F. M.; Chen, X. Y.; Brocchini, S.; Hopkins, H. P.; Hamilton, D. *J. Am. Chem. Soc.* **1993**, *115*, 6600.
(6) Supported in part by a Cooperative Research grant of The Dow Chemical Company.
(7) The Dow Chemical Company has a pending patent on the class of materials relating to compositions **3** and **4**.
(8) Brown, C. A.; Yamashita, A. *J. Am. Chem. Soc.* **1975**, *97*, 891.
(9) All new compounds show analytical and spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) data consistent with the assigned structures.
(10) A high-intensity ultrasonic homogenizer (Model 36260; Cole Parmer Int.) with a 3 mm (1/8 in.) microtip probe was used. The probe was immersed 3−4 mm below the surface of the lipid suspension, and the sample was sonicated with an input energy of 15−16 W, an on cycle of 3 s, and an off cycle of 0.1 s.
(11) Submicron particle size analyzer COULTER N4MD (Coulter Corp.) was used in dynamic light scattering measurements. For each sample, data were collected over 1000 s at 20 °C.
(12) A drop of a liposome suspension was applied onto the carbon-coated copper grid, the excess solution was blotted off, and phosphotungstic acid was used in negative staining. A Philips CM12 electron microscope was used at an operating voltage of 120 kV.
(13) Measurements were done on a Perkin-Elmer 650-10S fluorescence spectrophotometer using an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

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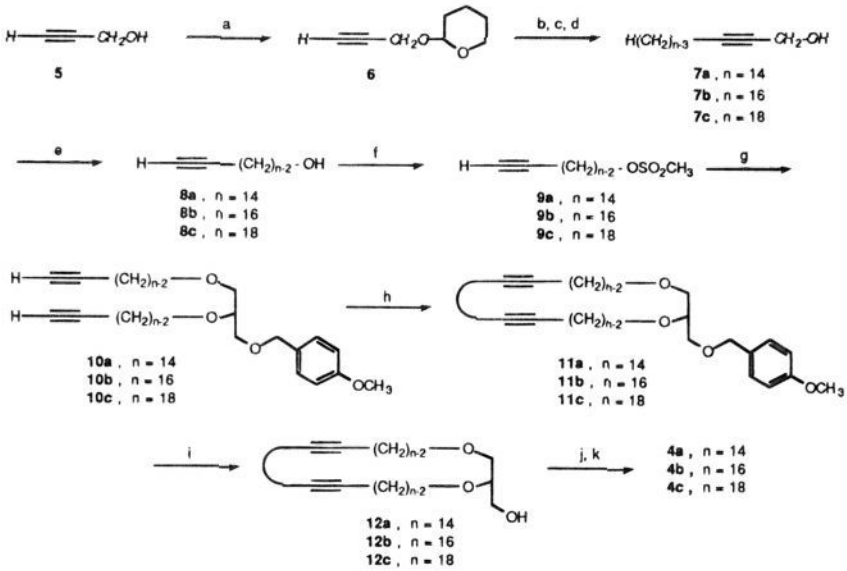
Scheme 1. Synthesis of Phospholipids **4**[a]



[a] (a) 2,3-Dihydropyran, TsOH−pyridine, CH_2Cl_2; (b) n-BuLi, THF; (c) $H(CH_2)_mBr$ (m = 11, 13, 15), HMPT; (d) TsOH, CH_3OH; (e) NaH, 1,3-diaminopropane; (f) Et_3N, CH_3SO_2Cl, CH_2Cl_2; (g) 3-(4-methoxybenzyl)glycerol, KOH, DMSO; (h) O_2, CuCl, 1,3-diaminopropane, *o*-xylene; (i) concentrated HCl, CH_3OH; (j) 2-chloro-1,3,2-dioxaphospholane 2-oxide, Et_3N, benzene; (k) Me_3N, CH_3CN.

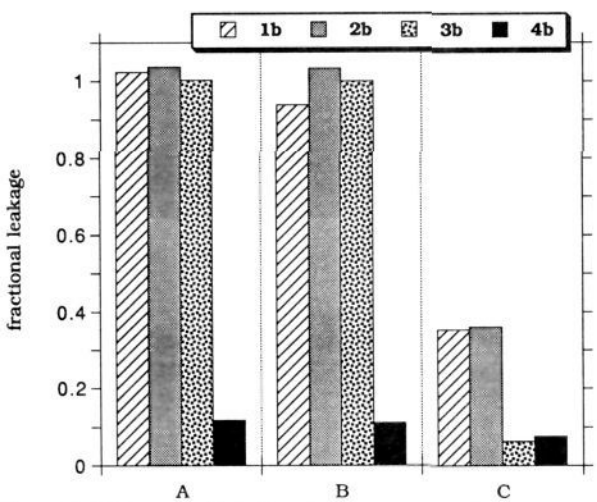


Figure 1. Release of calcein from liposomes prepared with lipids **1b**, **2b**, **3b**, and **4b**: (A) ethanol (33 wt %), 3 days at 20 °C; (B) Igepal 620 (0.1 wt %), 3 days at 20 °C; (C) wheat germ lipase (0.59 wt %), 3 days at 20 °C.

little effect on the dye release from liposomes prepared with macrocyclic lipids **3b** and **4b** (Figure 1C).

These results suggest that the stability of liposomes prepared from diyne-containing lipids **4** is significantly increased relative to their saturated macrocyclic analogs **3**, and to liposomes prepared with acyclic ester-type lipids **1**.[14] The remarkable liposome stability is worthy of particular note owing to the widespread interest in liposomes as delivery systems.

UV irradiation of liposomes from lipid **4b** with monochromatic light of 254 nm resulted in polymerization. Raman absorbances of a diyne moiety at 2249 and 2111 cm^{-1} decreased during UV irradiation, while a new Raman band corresponding to an allenic moiety appeared at 2077 cm^{-1}. The progress of polymerization also can be followed by UV spectroscopy and thin-layer chromatography. Upon exposure of liposomes to ethanol for 18 h, the particle size of nonpolymerized liposomes increased from 94 nm to 133 nm, while the size of polymerized liposomes under the same conditions increased to only 109 nm. Investigation of these diyne-based liposomes and their potential for controlled release is in progress.

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Supplementary Material Available: Experimental procedures, spectral data, and elemental analytical data on reported compounds (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(14) Alternatively, entrapment of calcein within the diacetylene-containing hydrophobic bilayer of **4** might allow liposome lysis without dye release.