Enhanced Dynamic Stability of Macrocyclic and Bolaamphiphilic Macrocyclic Lipids in Liposomes†

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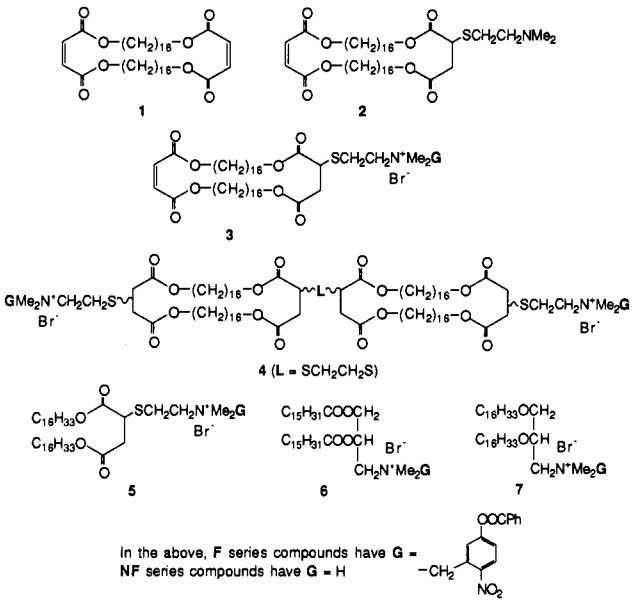
The unusual resistance to adverse environmental conditions displayed by the archaebacteria has stimulated interest in the synthesis and properties of macrocyclic and bolaamphiphilic membrane lipids similar to those found in the bacterial membranes. Yamauchi's group focused on this effort,[2] while, more recently, we[3] and Thompson[4] prepared bolaamphiphilic lipids that afforded liposomes in which the "bolas" assumed either U-shaped or bridging arrangements within the membrane.

Menger,[5] Yamauchi,[6] and others[7] concentrated on the synthesis of relevant macrocyclic lipids. Menger, in particular, noted that macrocyclization, achieved by "tethering" the termini of two-chained lipids, could affect "anti/gauche ratios, flip-flop rates, interdigitation, rotational, and translational motions."[5a] Here, we report our independent studies of macrocyclic and bis-macrocyclic bolaamphiphilic lipids. The intraliposomal dynamic properties of these novel lipids support Menger's predictions and complement his thermal stability studies.

Our choice of macrocyclic structure was based on the pioneering bolaamphiphile studies of Fuhrhop,[8] together with the synthetic modifications of Lo Nostro *et al.*[9] We thus began with the 44-membered macrocycle **1** (see Chart 1), derived from maleic anhydride and 1,16-hexadecanediol.[8,9] Michael addition[8,9] to **1** of 2-(dimethylamino)ethanethiol (0.96 equiv in 7:3 *i*-PrOH/H_2O, adjusted to pH 8 with Et_3N, 4 h, 80 °C, N_2 atm) afforded 19% of monoadduct **2**, which was purified by chromatography (SiO_2, 10.7:1 $CHCl_3$/MeOH) and characterized by NMR spectroscopy. It was then quaternized with 3-(bromomethyl)-4-nitrophenyl benzoate[10] (**G**-Br) (dry THF, 25 °C, 4 days), affording 23% of the 44-membered, functionalized, macrocyclic lipid **3-F**.[11,12]

Alternatively, 2 equiv of **2** and 1 equiv of 1,2-ethanedithiol (conditions as above) gave 79% of the bis-Michael adduct, which, after chromatography (as for **2**) and bis-quaternization with **G**-Br (dry THF, 25 °C, 2 days), gave 54% of the bis-macrocyclic bolaamphiphile, **4-F**. No doubt a mixture of structural isomers and diastereomers, **4-F** was characterized by NMR spectroscopy and elemental analysis (C, H, N).

Chart 1



In addition to the macrocyclic lipids, we prepared the open-chain models, **5-F** and **5-NF**, by Michael addition of 2-(dimethylamino)ethanethiol to dihexadecyl maleate,[8] followed by quaternization with either **G**-Br or MeBr (dry Et_2O, 25 °C, 3 days).[13] Finally, for comparison purposes, we made use of the previously described[14] ammonium ion lipids **6-F**, **6-NF**, and **7-NF**.

To explore the influence of macrocyclization on intraliposomal dynamics, we prepared coliposomes of the various functional lipids with **5-NF** or **7-NF** "background" lipids. The F/NF ratio was generally 1:7, except with bolaamphiphile **4-F**, where it was 1:14. The coliposomes (cf. Table 1) were created[10] by immersion probe sonication (60 W, 3 min, 55 °C) of $CHCl_3$-cast films of the lipid mixtures in pH 4 aqueous HCl, containing 0.01 M KCl. Coliposome solutions were cooled to 25 °C and filtered through 0.8-μm Millex filters before use.

Characterization of the coliposomes included determination of their hydrodynamic diameters by dynamic light scattering[10] and measurements of their gel-to-liquid crystal phase transition temperatures (T_c) from the temperature-dependent discontinuities in the fluorescence polarization of solubilized 1,6-diphenyl-1,3,5-hexatriene.[15] These data appear in Table 1. The observed mean diameters are compatible with the expectation that unilamellar coliposomes are created by the sonications.[10,14] Note that the coliposomes' T_c values are largely dominated by the NF components that are present in excess.

Dynamic studies of transbilayer lipid migration ("flip-flop") were performed using our normal protocol.[10,14] The coliposomes were first surface-differentiated by exposure to 1×10^{-4} M glutathione in 0.01 M pH 8 Tris buffer (0.01 M in KCl) at 25 °C. *Exoliposomal p*-nitrophenyl benzoate moieties (**G**) of the functional lipids were thus monitored spectrophotometrically at 400 nm. Subsequent *endoliposomal* cleavage occurred more slowly (k_s) as the exo/endo pH 8/4 gradient collapsed via permeative processes.[16] Values of k_f and k_s appear in Table 1, together with exo/endo distributions of the functional lipids ("phasing"), as deduced from the absorption changes accompa-

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(11) **3-F** was washed with ether and characterized (as a monohydrate) by NMR and elemental analysis (C, H, N).

(12) Quaternization of **2** with MeBr gave 35% of **3-NF** (characterized by elemental analysis, C, H, N).

(13) Characterizations included NMR spectroscopy and elemental analysis (C, H, N, Br, S).

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Table 1. Dynamics of Coliposomes

case	coliposome[a]	d,[b] nm	T_c, °C	k_f,[c] s^{-1}	$10^4\ k_s$,[c] s^{-1}	phasing[d]	$t_{1/2}$ flip[e] 40 °C	$t_{1/2}$ flip[e] 45 °C
1	**5-F/5-NF**	39.5	39	0.098	2.85	73:27	5	2
2	**6-F/5-NF**	38.6	41	0.12	2.55	78:22	8	2
3	**3-F/5-NF**	42.2	41	0.045	2.87	74:26	>30	7
4	**5-F/7-NF**	32.3	37	0.14	1.81	60:40	4	1
5	**3-F/7-NF**	36.0	38	0.12	1.26	59:41	>30	9
6	**4-F/5-NF**[f]	43.6	42	0.033	5.90	68:32	>30	>30

[a] See text for structures and conditions; F/NF = 1:7, [F] = 5×10^{-5} M, [NF] = 3.5×10^{-4} M unless otherwise stated. [b] Diameters from dynamic light scattering at pH 4, 0.01 M KCl. [c] Rate constants for exoliposomal (k_f) and endoliposomal (k_s) esterolyses were determined at 25 °C; see text for details. [d] Exo/endo distribution of functional lipid, from absorption changes corresponding to k_f and k_s. [e] Time (min) required for half-reequilibration of surface-differentiated coliposomes at 40 °C or 45 °C. [f] F/NF = 1:14 (2.5×10^{-5} M:3.5×10^{-4} M).

nying k_f and k_s. As usual, we observe an excess of the larger head group F-lipids in exoliposomal loci.

Flip-flop was assessed from separate experiments in which the external pH was reduced to 2.1 (HCl) immediately after *exoliposomal* esterolysis, thus quenching further reaction. The surface-differentiated coliposomes were warmed to selected incubation temperatures (35–55 °C) for specific times to induce flip-flop, cooled back to 25 °C, and readjusted to pH 8 (NaOH), generating a new, fast (k_f) appearance of *p*-nitrophenylate that represented the esterolysis of functional lipids that had "flipped" from endo- to exoliposomal loci during incubation. Subsequent residual esterolysis (k_s) was due to the still-intact *endovesicular* functional lipids. The extent (%) of flip-flop induced by incubation was revealed by the G′ absorptions attending the postincubation k_f and k_s reactions. By varying the incubation conditions, we obtained correlations of % flip-flop vs time at various temperatures and thence approximate half-times ($t_{1/2}$) for the flip-flop equilibrations. Data for the $t_{1/2}$ values at 40 °C and 45 °C (*i.e.*, temperatures in the vicinity of the T_c values) appear in Table 1.

The thermally-driven flip-flop of the macrocyclic lipid **3-F** is markedly slowed, relative to either "open"-chain model **5-F** or the previously examined[14] pseudoglyceryl lipid **6-F**. The $t_{1/2}$ data show that, at 40 °C (near the T_c), surface-differentiated coliposomes containing **3-F** reequilibrate by flip-flop at least 6 times more slowly than coliposomes containing **5-F**, with either **5-NF** or **7-NF** background lipids (*cf.* cases 3 vs 1 and 5 vs 4 in Table 1). The macrocyclic/pseudoglyceryl lipid comparison (cases 3 vs 2) displays a factor of >3, in the same sense.

Is the additional thermal stability of **3** due to interdigitation across the bilayer midplanes, into the opposing leaflets?[17] For example, interdigitation affords resistance to flip-flop in the 20-carbon acyl chain analogue of **6**.[18] In the case of **3** vs **5**, however, we consider interdigitation an unlikely cause of the observed differences. Thus, the *octadecyl* ester analogues of **5** have been prepared.[19] In a comparable F/NF coliposomal system, the octadecyl lipids show $t_{1/2} \sim 8$ min at 45 °C (5 °C *below* their T_c of 50 °C).[19] Thus, this 20(21)-chain model lipid exhibits no effect of interdigitation on flip-flop. We attribute the enhanced flip-flop resistance of macrocyclic lipid **3** to "tethering", as proposed by Menger.[5a]

Macrocycle **3** is more resistant to flip-flop than its open-chain analogues in the T_c region at 40 °C, but this advantage largely disappears at 45 °C (Table 1). On the other hand, the bolaamphiphilic, bis-macrocyclic lipid **4-F** maintains its resistance to flip-flop at both 40 °C and 45 °C (case 6). This is not because it predominantly adopts an extended, bilayer-bridging conformation across the background bilayer of lipids **5-NF**; from the esterolysis results, we note that the exo–endo "phasing" or distribution of **4-F** in **5-NF** is 68:32, not the 50:50 demanded by exclusive bilayer bridging.[3a] As in earlier examples of flexible bolaamphiphilic lipids, **4-F** must, at least in part, adopt U-shaped conformations in which both head groups reside at the same leaflet–water interface.[3b] Nevertheless, flip-flop of the endo **4-F** head groups in the exo/endo differentiated **4-F/5-NF** coliposomes is very slow, even above the T_c. We attribute this both to the "freezing out" of independent chain motion[5a] in bis-macrocyclic lipid **4-F** and to substantial resistance to relocation on the part of neighboring lipids.

Our kinetic results were obtained within a few degrees of T_c [and these transitions are broad (±6 °C)]. However, dramatic changes in lipid/liposomal dynamics occur very close to the mean T_c.[14,16] We observe (Table 1) that the kinetic differences between macrocyclic lipids **3-F** and **4-F** and open-chain models **5-F** and **6-F** are independent of background lipid (**6-NF** or **7-NF**), responsive to temperature at the mean T_c, and persistant above the T_c. This supports interpretations based on structural differences between the lipids rather than unexpected peculiarities of bilayer structure.

The sluggish dynamic properties of macrocyclic lipids **3-F** and **4-F**, as revealed in this study, complement previous observations of unusual thermotropic properties[5a] and enhanced liposomal stability[8,9] associated with macrocyclic lipids, accord with speculations about the archaebacterial membrane lipids,[2–5] and offer further encouragement to those preparing "designer lipids" for potential pharmaceutical applications.

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