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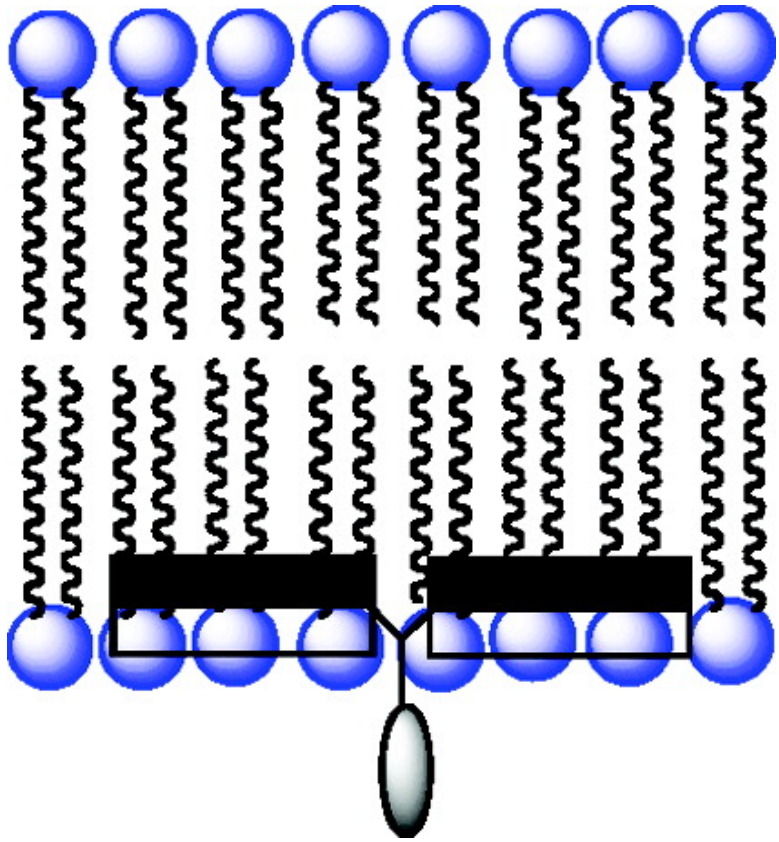

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# Viewing Membrane-Bound Molecular Umbrellas by Parallax Analyses

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***Abstract:*** Fluorescence quenching measurements have been made for a series of di-walled and tetra-walled molecular umbrellas having moderate (i.e., hydroxyl-) and strong (i.e., sulfate-) facial hydrophilicity, using Cascade Blue as the fluorophore. Through the use of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotempocholine, 1-palmitoyl-2-stearoyl-(5-DOXYL)-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-stearoyl-(12-DOXYL)-*sn*-glycero-3-phosphocholine as fluorescence quenchers, evidence has been obtained for a membrane-bound state in which the umbrella molecules lie on the surface of the lipid bilayer. In the case of the sulfated molecular umbrellas, evidence has also been obtained for a subpopulation in which the fluorophore lies deeper within the membrane. Probable structures for the shallow-lying and deep-lying molecular umbrellas are discussed.

## Introduction

One of the major goals of modern medicinal chemistry is to find ways of promoting the passive transport of polar drugs across hydrophobic barriers to improve their efficacy.[1−20] For example, it is now recognized that antisense oligonucleotides, which are being considered for the treatment of a wide range of diseases (e.g., nonsmall cell lung cancer, Crohn's disease, malignant melanoma, chronic lymphocytic leukemia, multiple myeloma, and cytomegalovirus retinitis) are limited by their inefficient delivery to mRNA in the cytoplasm of cells. Thus, most, if not all, methods that have been developed for oligonucleotide delivery appear to involve endocytotic pathways. Subsequent release from endosomal−lysosomal compartments results in only a the fraction of the oligonucleotide being delivered to mRNAs. In principle, the passive transport of oligonucleotides across plasma membranes should significantly improve their efficacy. Also, the enhanced transport of small polar drugs (e.g., peptides and nucleotides) across hydrophobic barriers is expected to result in improved efficacy. For example, the transport of opioids and antiviral agents to the brain, across the blood−brain barrier, has proven to be challenging.

With this goal in mind, we have introduced a unique class of compounds that we have termed, "molecular umbrellas". Such structures are composed of two or more facially amphiphilic units ("walls") that are covalently attached to a central scaffold. In a sense, these "amphomorphic" compounds mimic the structure and function of umbrellas by being able to cover an attached agent and shield it from an incompatible environment.[21−28]

† Responsible for all of the fluorescence measurements.
‡ Responsible for all of the organic synthesis.

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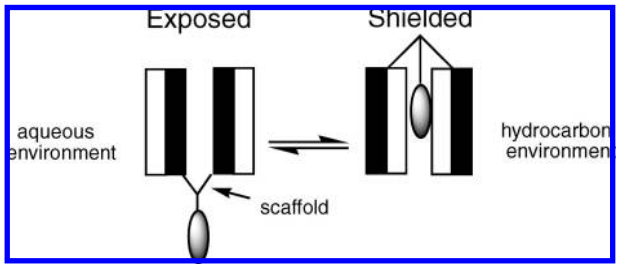
***Scheme 1***




Thus, when immersed in a hydrocarbon environment, a molecular umbrella can adopt a shielded conformation in which the facially amphiphilic units not only mask their own hydrophilicity, but also that of the attached agent. When immersed in an aqueous environment, these same molecules can create an exposed conformation that allows each of the hydrophilic faces and the bound hydrophilic agent to make direct contact with water. A stylized illustration of a molecular umbrella in an exposed and a shielded conformation is shown in Scheme 1. Here, the shaded and unshaded rectangles represent hydrophobic and hydrophilic faces of the amphiphilic units, respectively, and the lightly shaded oval represents a covalently attached, hydrophilic agent.

In previous studies, we have shown that molecular umbrellas can transport small polar molecules, such as glutathione across fluid liposomal membranes.[28] Although the precise mechanism of this transport remains to be established, we have postulated one in which the umbrella first approaches a lipid bilayer in a fully exposed conformation (structure **A** in Scheme 2).[25] Hydrophobic interactions with the membrane interior then lead to an adsorbed state in which the hydrophilic faces are in contact with the polar headgroup region and the hydrophobic faces are in intimate contact with the hydrocarbon region of the lipid bilayer (structure **B**). Subsequent absorption into the interior of the membrane, being driven by hydrophobic forces, then affords structure **C**. Translocation to the adjoining leaflet, 180° rotation, and reversal of steps **B** and **A** (not shown) then release the conjugate from the other side of the membrane. In essence, we have postulated that the molecular umbrella masks the hydrophilicity of the polar agent as it crosses the hydrocarbon core of the bilayer. Also shown in Scheme 2 are three additional membrane-bound states that are possible. Here, **D** is analogous to **C**, except that the molecular umbrella has an inverted orientation, where the scaffold is in intimate contact with the aqueous phase. Structure **E** is similar to that of **D**, except that the ligand is now fully immersed in the aqueous phase. Finally, **F** depicts a state in which the molecular umbrella occupies an intermediate depth within a thinned region of the bilayer. Of these possibilities, only **C**, **D**, and **F** represent shielded states of the type that is indicated in Scheme 1, and **A** and **E** represent exposed states. The "flattened" structure that is shown as **B** can viewed as a "hybrid" state, where the pendant ligand if fully exposed to the aqueous phase, but is shielded from neighboring lipids by the amphiphilic walls that are attached to it.

In the work that is reported herein, we sought to gain insight into the interaction of molecular umbrellas with phospholipid membranes by identifying *the favored state(s) of membrane-bound molecular umbrellas*. Specifically, we sought to determine: (i) the favored depth of penetration of molecular umbrellas in fluid phospholipid bilayers, (ii) the consequences of umbrella size (i.e., the number of amphiphilic units) and facial hydrophilicity on such penetration, and (iii) the probable structure(s) of the membrane-bound state.

## Results and Discussion

**The Parallax Method.** In this work, we have made extensive use of the parallax method of analysis.[29−34] In brief, this technique measures relative fluorescence quenching efficiencies of a series of nitroxide-labeled phospholipids that lie at shallow, medium, and deep locations within a lipid bilayer with respect to a membrane-bound fluorophore. Comparison of these quenching efficiencies then allows one to estimate the average depth of the fluorophore. Three nitroxide-labeled phospholipids that have been frequently used for this purpose are shown in Chart 1. A shallow quencher, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotempocholine (Tempo PC) has a fluorescence-quenching nitroxide that is positioned in its headgroup. A medium quencher, 1-palmitoyl-2-stearoyl-(5-DOXYL)-*sn*-glycero-3-phosphocholine (5-DOXYL PC) has the nitroxide attached to the *sn*-2 chain just below the headgroup region. Finally, a deep quencher, 1-palmitoyl-2-stearoyl-(12-DOXYL)-*sn*-glycero-3-phosphocholine (12-DOXYL PC) has the nitroxide further down the *sn*-2 chain. Thus, when a membrane-bound fluorophore lies at or near the *surface* of a phospholipid bilayer, the fluorescence quenching efficiency for Tempo PC is expected to be greater than for that of 5-DOXYL PC and 12-DOXYL PC. In contrast, if the fluorophore is buried within the hydrocarbon interior of the bilayer, then greater fluorescence quenching is expected with 5-DOXYL PC and 12-DOXYL PC. Analysis by use of these three nitroxides allows calculation of the depth of a fluorophore at the 1−2 Å level of resolution.[31]

**Molecular Umbrella Design.** In this study, we have chosen Cascade Blue as a "surrogate agent" because of its high polarity and strong fluorescence. Specific conjugates that were selected as synthetic targets included a di-walled and a tetra-walled molecular umbrella having moderate (i.e., hydroxyl-) facial hydrophilicity (i.e., **1a** and **2a**, respectively), and analogs having strong (i.e., sulfate-) facial hydrophilicity (i.e., **1b** and **2b**, respectively). In addition, a nonumbrella derivative (**3**) was chosen as a control compound (Chart 2). To enhance their conformational flexibility, a glycine "handle" was also included in each of the molecular umbrellas.

**Umbrella Synthesis.** Schemes 3, 4 and 5 show the synthetic approach that we have used in preparing these target compounds. Thus, acylation of the methyl ester of glycine with bromoacetic anhydride to give **4**, followed by nucleophilic displacement by pyranine afforded **5** (scheme 3). Subsequent saponification and conversion to the corresponding triethylammonium salt gave **6** and **7**, respectively. The conversion to the triethylammonium salt was found necessary to solubilize this trisulfate in organic solvents. Condensation of **7** with $N^1$,$N^3$-spermidinebis[cholic acid amide] and $N^1$,$N^3$-spermidinebis(L-lysine-dicholylamide) yielded conjugates **1a** and **2a**, respectively (scheme 4). Persul-

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***Scheme 2***

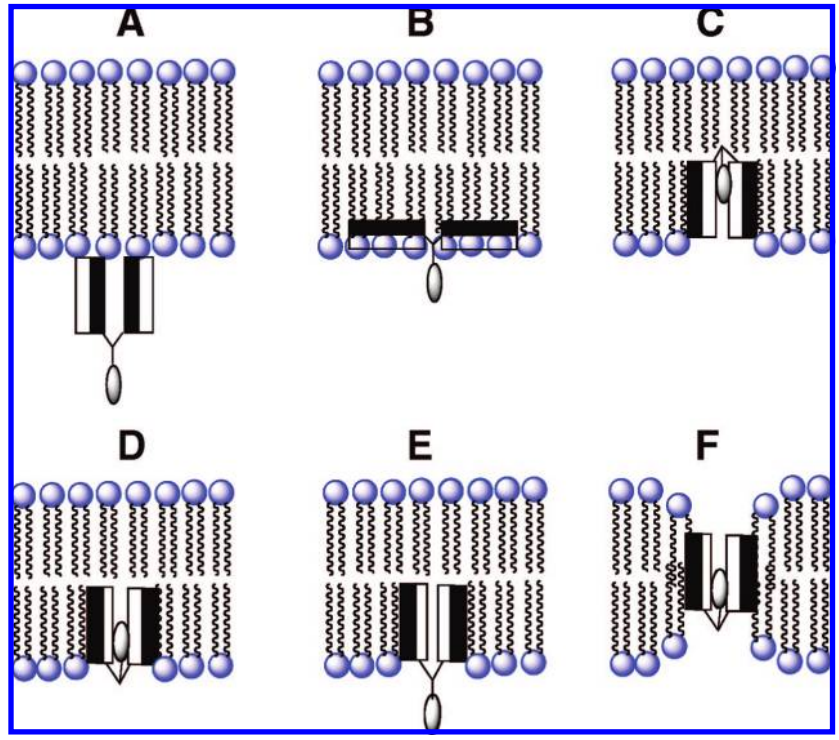
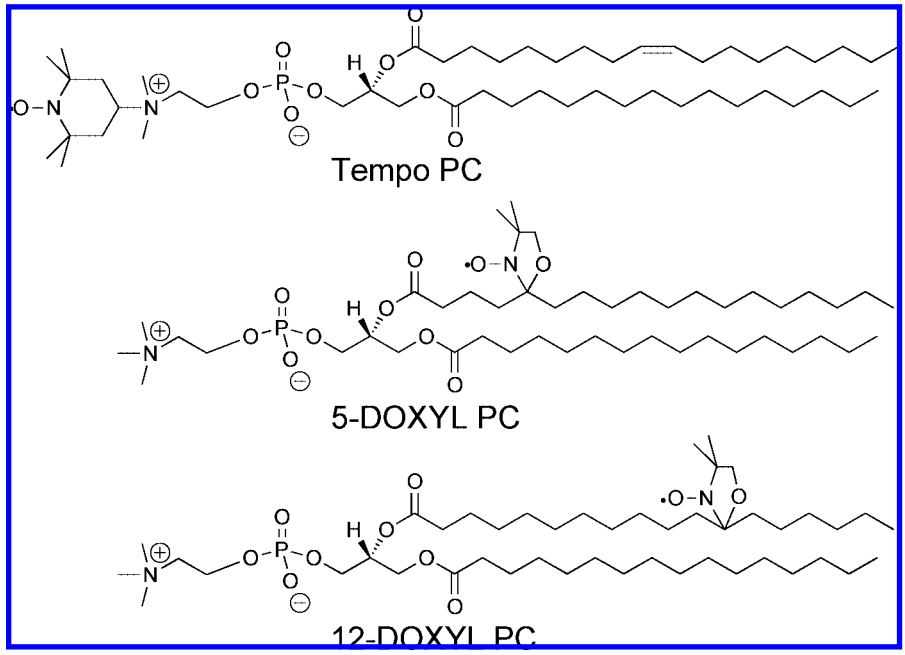


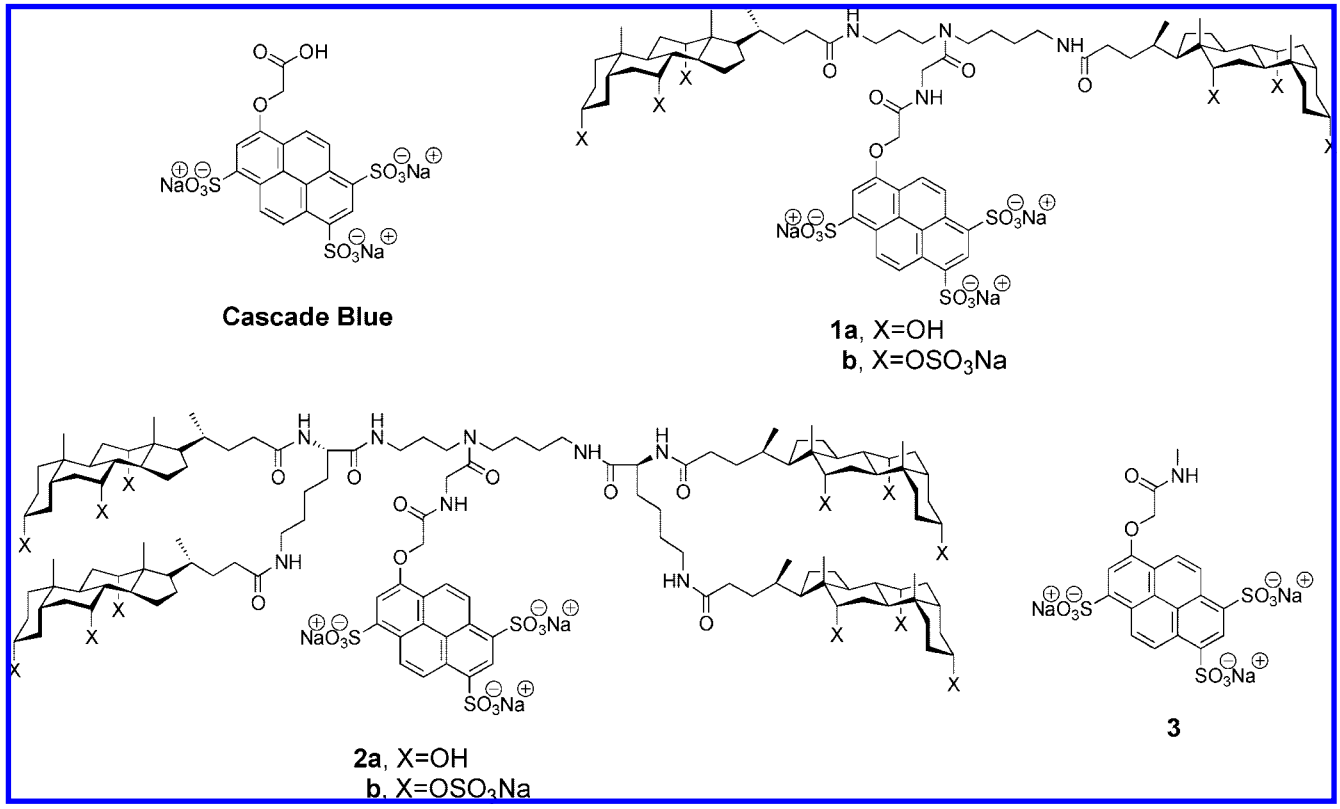

***Chart 1***




fation of **1a** and **2a** then afforded the corresponding sulfate derivatives, **2a** and **2b**. The synthesis of the nonumbrella analog was carried out in a similar way by first alkylating pyranine with methyl bromoacetate, followed by substitution with methylamine, to give **8** and **3**, respectively.

**Membrane Binding.** Before examining the relative quenching efficiencies of Tempo PC, 5-DOXYL PC, and 12-DOXYL PC, we first measured the affinity of **1a**, **1b**, **2a**, **2b**, and **3** to small unilamellar vesicles (SUVs) made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) via ethanol injection. Experimental conditions that were used for these measurements were identical to those employed for the fluorescence quenching experiments that were carried out, where an umbrella/phospholipid ratio of 1/300 was employed.[35] For these affinity measurements, an equilibrium dialysis cell was used to determine the fraction of umbrella that was bound to the liposomes and the fraction that remained free in solution. Because the molecular umbrellas were required to pass through a dialysis membrane, typically, 24 h was required to reach equilibrium. In contrast, equilibrium was rapidly reached when the umbrellas were mixed, directly, with the liposomes in a fluorimeter cell; that is,

*Chart 2*




fluorescence intensities that were measured after 10 min were the same as that found after 4 h. Based on these affinity measurements, the mole fractions of **1a**, **1b**, **2b**, and **3** that became bound to the liposomes were 80%, 52%, 80%, 0%, respectively. With **2a**, its adsorption to the walls of the dialysis cell and to the dialysis membrane precluded such analysis. When a 1 $\mu$M solution of **2a** was incubated with a 300 $\mu$M liposomal dispersion of POPC, the fluoresence intensity was essentially unchanged. Because the association of **2a** with these liposomes did not significantly alter its fluorescence intensity, we could not vary the liposome concentration to judge whether any of the molecular umbrella remained free in solution, as has often been done in parallax measurements. For this reason, fluorescence quenching experiments with **2a** were carried out by using a stock solution of this umbrella, adding equal quantities to pure POPC liposomes and POPC liposomes containing 15 mol% of Tempo PC, 5-DOXYL PC or 12-DOXYL PC, and assuming that binding is essentially complete.

Although one would not expect that substitution of only 15 mol% of POPC with a nitroxide-labeled phosphocholine would significantly affect membrane binding, similar measurements that were carried out with **2b** and liposomes made form POPC/Tempo PC (85/15, mol/mol) validated this assumption; that is, no difference in binding was found relative to liposomes made from 100% POPC.

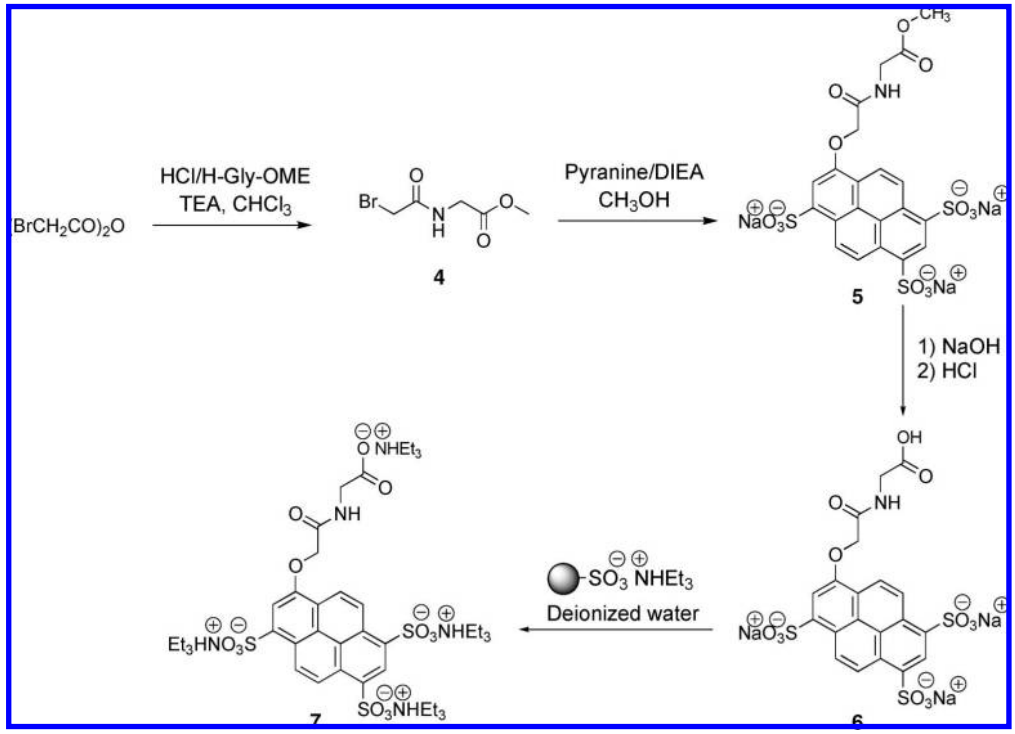
**Quenching Efficiencies.** Fluorescence intensities were measured for **1a**, **1b**, **2a** and **2b** after being exposed to 300 $\mu$M dispersions of lipsomes (30−40 nm, ethanol injection) made from (i) POPC, (ii) POPC/Tempo PC (85/15, mol/mol), (iii) POPC/5-DOXYL PC (85/15, mol/mol) and (iv) POPC/12-DOXYL PC (85/15, mol/mol). A typical series of fluorescence emission spectra are shown in Figure 1 for **2a**.

In order to compare the quenching efficiencies of Tempo PC, 5-DOXYL PC and 12-DOXYL PC for each *membrane-bound* molecular umbrella, it was necessary to correct for fluoresence contributions from that fraction of the umbrella that remained in the solution phase. In Table 1 are shown two sets of quenching efficiencies for each molecular umbrella. One set represents the raw data in which fluorescence intensity ratios (i.e., the ratio of the fluorescence intenisty in the presence of quencher/the fluorescence intensity in the absence of quencher) have not been corrected. These values are in rows that are designated as **1a** (apparent), **1b** (apparent), **2a** (apparent) and **2b** (apparent). The values that are in the rows designated as **1a** (bound), **1b** (bound), **2a** (bound) and **2b** (bound) refer to fluorescence intensity ratios that have been corrected for the solution-phase umbrella; that is, solution phase contributions have been subtracted from fluorescence intensities in the absence, and in the presence, of the nitroxide quencher.

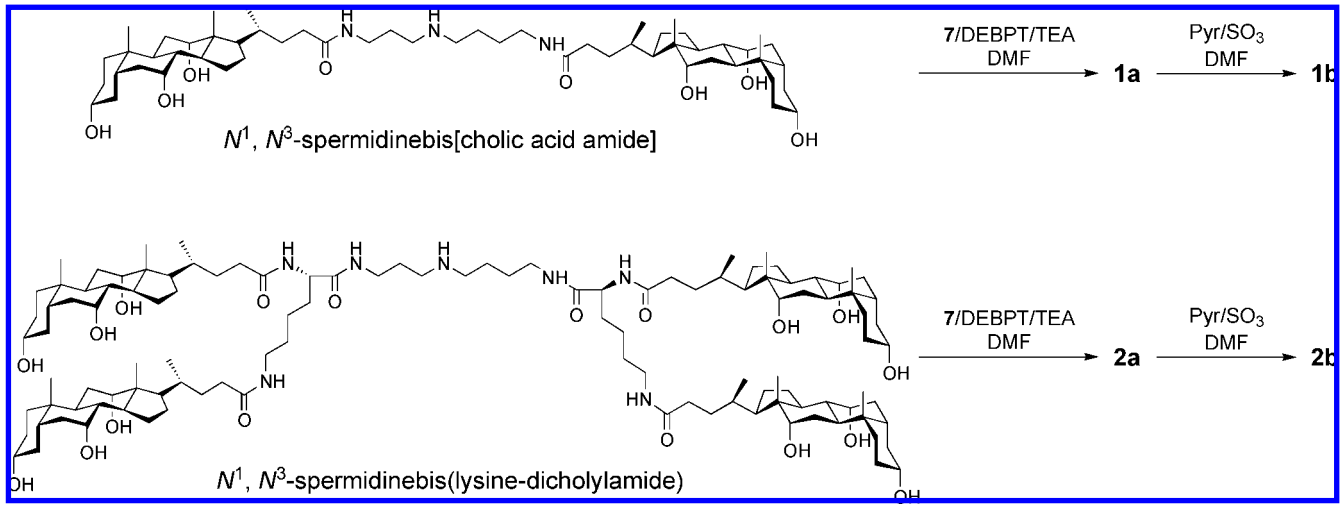
Since **3** was found to have negligible binding to liposomal membranes, one would not expect to observed any significant fluorescence quenching in the presence of liposomes containing Tempo PC, 5-DOXYL PC or 12-DOXYL PC. Consistent with this prediction, the values that are listed in Table 1 for **3** were

(35) Fluorescence intensity measurements were made with **2a** as a function of umbrella/phospholipid ratio to test for self-quenching of membrane-bound umbrella. With an umbrella/phospholipid ratio of 1/30, the fluorescence intensity was ~50% of that measured for the same umbrella concentration in solution in the absence of liposomes. As this ratio was decreased to 1/100, the fluorescence intensity returned to its original solution value; a further decrease to 1/300 left the fluorescence intensity unchanged. We interpret these results as a partial reduction in fluorescence intensity via self-quenching (when the umbrella/phospholipid ratio is 1/30), and negligible self-quenching when this ratio is 1/100.

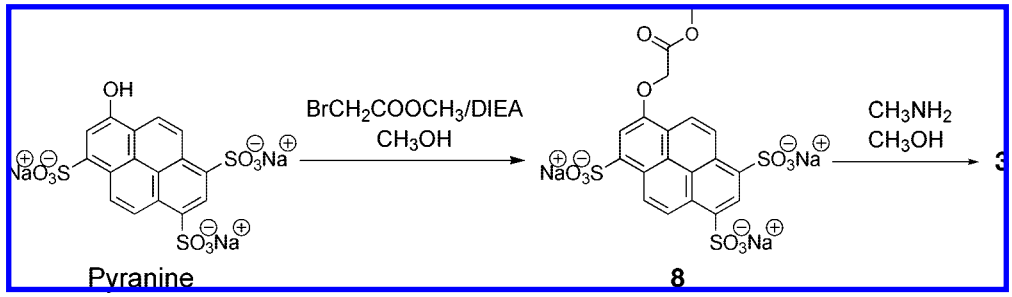
***Scheme 3***




***Scheme 4***




***Scheme 5***




the same, regardless of whether Tempo PC, 5-DOXYL PC or 12-DOXYL PC was included in the liposomes. To ensure that light scattering by the liposomes did not contribute, significantly, to the observed fluorescence intensities, we checked for any "apparent emission" by a dispersion of POPC liposomes that was similar in size and concentration to those used for the fluorescence quenching experiments. No apparent emission (i.e., <1%) was detected.

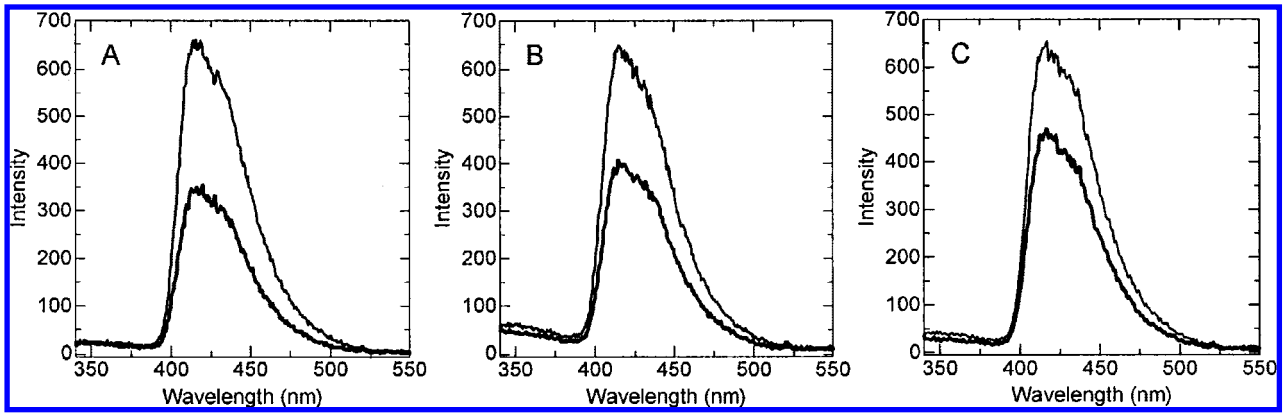



***Figure 1.*** Fluorescence emission spectra of **2a** in the presence of liposomes made from POPC (the stronger emission spectrum in parts A, B, and C). The weaker emission spectra, due to fluorescence quenching, were obtained using (A) POPC/Tempo PC (85/15, mol/mol), (B) POPC/5-DOXYL PC (85/15, mol/mol), and (C) POPC/12-DOXYL PC (85/15, mol/mol).

***Table 1.*** Fluorescence Quenching Efficiencies by Nitroxide-Labeled Phospholipids[a]

| fluorescent species | $F_T/F_o$ | $F_5/F_o$ | $F_{12}/F_o$ |
|---|---|---|---|
| **1a** (apparent) | 0.44 ± 0.02 | 0.55 ± 0.05 | 0.61 ± 0.04 |
| **1a** (bound) | 0.30 ± 0.03 | 0.43 ± 0.04 | 0.51 ± 0.07 |
| **1b** (apparent) | 0.65 ± 0.07 | 0.84 ± 0.03 | 0.86 ± 0.04 |
| **1b** (bound) | 0.33 ± 0.10 | 0.71 ± 0.06 | 0.74 ± 0.07 |
| **2a** (apparent) | 0.51 ± 0.02 | 0.63 ± 0.02 | 0.70 ± 0.02 |
| **2a** (bound)[b] | 0.51 ± 0.02 | 0.63 ± 0.02 | 0.70 ± 0.02 |
| **2b** (apparent) | 0.68 ± 0.03 | 0.86 ± 0.03 | 0.85 ± 0.04 |
| **2b** (bound) | 0.62 ± 0.03 | 0.83 ± 0.04 | 0.80 ± 0.05 |
| **3**[c] | 0.95 ± 0.02 | 0.95 ± 0.04 | 0.96 ± 0.01 |

[a] All fluorescence experiments were carried out at 23 °C using 300 $\mu$M SUV dispersions that were incubated with a 1 $\mu$M solution of a given fluorophore. $F_T/F_o$, $F_5/F_o$ and $F_{12}/F_o$ values represent the ratio of observed fluorescence intensities with POPC liposomes containing 15 mol% Tempo PC, 5-DOXYL PC, and 12-DOXYL PC, respectively, to those found in liposomes made from 100% POPC. All values are the average ± 1 SD from a minimum of six independent experiments, where three experiments were made using two different batches of liposomes. Fluorescence quenching efficiencies that are uncorrected for contributions from solution-phase fluorophore are in rows designated as "apparent". Fluorescence quenching efficiencies that have been corrected for contributions from the solution-phase fluorophore are in rows that are designated as "bound". [b] Complete binding has been assumed (see text). [c] Values for **3** were the same, regardless of whether Tempo PC, 5-DOXYL PC, or 12-DOXYL PC was included in the liposomes.

**Depth of Bilayer Penetration.** To estimate the depth of membrane penetration by the Cascade Blue moiety for each molecular umbrella, we have used the parallax equation 1:

$$z_{cf} = L_{c1} + [-\ln(F_1/F_2)/\pi C - L_{21}^2]/2L_{21} \quad (1)$$

Here, $z_{cf}$ is the distance of the fluorophore from the center of the bilayer, $F_1$ is the fluorescence intensity ($F/F_o$) in the presence of the "shallow" quencher (i.e., quencher 1), $F_2$ is the fluorescence intensity ($F/F_o$) in the presence of the "deeper" quencher (i.e., quencher 2), $L_{c1}$ is the distance of the shallow quencher from the center of the bilayer, $L_{21}$ is the distance between the shallow and deep quenchers, and $C$ is the concentration of quencher in molecules/Å$^2$ = (mole fraction of nitroxide-labeled phospholipid/area per phospholipid) = (mole fraction of nitroxide/70 Å$^2$).[30] To obtain the best estimates for $z_{cf}$ values (in Å), we used the strongest quenching pair of nitroxide-labeled phospholipids; that is Tempo PC and 5-DOXYL PC.[30] Distances of the nitroxide moiety from the bilayer center that were used in these calculations were 12.2 Å for 5-DOXYL PC and 19.5 Å for Tempo PC.[31,36] On the basis of the observed quenching efficiencies for these membrane-bound molecular umbrellas, we estimate average distances from the bilayer center for the Cascade Blue moiety ($z_{cf}$) to be the following: 19.3 Å for **1a**, 23.5 Å for **1b**, 18.0 Å for **2a**, and 19.0 Å for **2b**. Thus, the pendant fluorophore for each molecular umbrella favors a "shallow" location, and the more hydrophobic, di-walled molecular umbrella (**1a**), as well as the more hydrophobic tetra-walled molecular umbrella (**2a**), lie slightly deeper in the membrane relative to their more hydrophilic counterparts (i.e., **1b** and **2b**, respectively).

It is noteworthy that for the two sulfated umbrellas, **1b** and **2b**, the quenching efficiencies of 5-DOXYL PC and 12-DOXYL PC) are similar. This finding implies that a subpopulation of these umbrellas exist, which is far from the main population.[30] Specifically, it implies that a minor subpopulation of these umbrellas lie deeper within the membrane, possibly in a region that is intermediate between the 5- and 12-positions of the stearoyl chain.[30]

**Probable Structures of Membrane-Bound Molecular Umbrellas.** Examination of the data in Table 1 reveals a significant difference between the di-walled and tetra-walled molecular umbrellas, in general. Specifically, the fluorophore in the di-walled molecular umbrellas is more quenchable by all three of the quenchers, relative to the tetra-walled analogs. Thus, the $F_T/F_o$, $F_5/F_o$ and $F_{12}/F_o$ values show greater quenching for **1a** as compared with **2a**. Similar behavior can be seen for the sulfated analogs; that is, **1b** relative to **2b**. These results indicate that an increase in the number of facial amphiphiles increases the umbrella's ability to shield the attached fluorophore from neighboring lipids. They argue, therefore, against structure **A** as being a plausible candidate for a shallow umbrella. They are, however, fully consistent with structures **B**, **C**, **D**, **E**, and **F**. The shallowness of the pendant Cascade Blue moiety in the membrane, which is indicated by the estimated $z_{cf}$ values, point toward **B**, **D**, and **E** as being the most probable candidates. Although we cannot distinguish, unambiguously, among these three possibilities, we presently favor **B**. In the case of **B**, one can envision the fluorophore being "pulled" into the membrane to a slightly greater extent by the more hydrophobic amphiphilic walls of **1a** and **2a**. Also consistent with structure **B** is the fact that the sulfated molecular umbrellas, bearing the same number of amphiphilic walls as the hydroxylated analogs (i.e., **1a** versus

(36) Abrams, F. S.; London, E. *Biochemistry* **1993**, *32*, 10826–10831.

**1b**, and **2a** versus **2b**) are less quenchable. Thus, in state **B**, one would expect less quenching from the sulfated umbrella molecules because even in the surface state, the stronger hydrophilicity of the sulfate moieties should cause the umbrella to position less deeply in the membrane. As a result, the fluorescent group would be pushed away from the membrane somewhat, and become less quenchable. In the case of structure **E**, one *would not* expect to observe a significant difference between the sulfated and nonsulfated molecular umbrellas, because the umbrella walls are already fully inserted into the membrane. For this reason, **E** seems unlikely.

The existence of a predominant surface state in preference to a minor deeply inserted state, as is indicated for **1b** and **2b**, has precedent. Thus, in a previous investigation, it was found that a very hydrophobic pore-forming helical peptide, with polar groups being hydroxyl-bearing serines on one face of the helix, predominantly formed a surface state. It is likely that the more deeply inserted pore-forming state for that peptide was only formed by a minor subpopulation.[34] In terms of probable structure for deeply inserted molecular umbrellas **1b** and **2b**, structure **F** can be ruled out since the fluorescent group would then be at the level of the deepest quencher (i.e., the 12-DOXYL PC), and should be quenched most by that quencher, which is clearly not the case. This leaves us with **C** and **D** as the two most probable candidates. In both of these shielded conformations, a relatively nonpolar microenvironment (low water content) would favor tight ion pairing between $Na^+$ ions and the sulfate ions of the sterols, as well as between $Na^+$ ions and the sulfonate groups of the Cascade Blue moiety. This tight ion pairing should be expected to minimize charge repulsion and allow for shielded conformations for **1b** and **2b**.

**Fluorescence Quenching by A Water-Soluble Nitroxide.** Although the primary aim of this investigation was to gain insight into the favored depth of penetration and probable structure(s) of **1a**, **1b**, **2a**, and **2b** in the membrane-bound state, it was also of interest to measure the quenching properties of these same conjugates *in solution* using a water-soluble nitroxide. Since a molecular umbrella in an exposed conformation should exhibit quenching that is similar to that found for the nonumbrella analog, **3**, we sought to examine this possibility. As expected, the fluorescence quenching of **1a**, **1b**, **2a**, **2b**, and **3** in phosphate buffered saline by 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy (i.e., 4-hydroxy Tempo) was found to be similar over a range of nitroxide concentrations (see Supporting Information).[37]

## Conclusions

Fluorescence measurements that have been carried out for membrane-bound **1a**, **1b**, **2a**, and **2b**, using Tempo PC, 5-DOXYL PC and 12-DOXYL PC as quenchers, indicate that these molecular umbrellas favor a shallow membrane-bound state, and that the sulfated molecular umbrellas (**1b** and **2b**) lie further away from the membrane surface relative to their more hydrophobic counterparts (i.e., **1a** and **2a**, respectively). The most probable structure for this shallow-lying state appears to be structure **B** (Scheme 2). These same fluorescence measurements also indicate that a subpopulation of deeper-lying sulfated molecular umbrellas exists, which are likely to be in the form of shielded structures **C** or **D** (Scheme 2). Results that have been obtained from fluorescence measurements for **1a**, **1b**, **2a**, **2b**, and **3** in phosphate buffered saline, using a water-soluble quencher (i.e., 4-hydroxy Tempo), are consistent with fully exposed conformations in solution.

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**Supporting Information Available:** Experimental procedures plus fluorescence quenching data in homogeneous solution by 4-hydroxy Tempo. This material is available free of charge via the Internet at http://pubs.acs.org.

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(37) In an attempt to obtain further support for a minor subpopulation of deep-lying umbrellas for **1b** and **2b**, we compared the fluorescence quenching of **1a**, **1b**, **2a**, and **2b** by 4-hydroxy Tempo in liposomal dispersions. Since deep-lying molecular umbrellas would be expected to exhibit different quenching behavior by 4-hydroxy Tempo relative to ones present in the aqueous phase and those lying at the surface of liposomes, this might be detectable by discontinuity in a Stern−Volmer plot. Such plots, however, did not reveal any discontinuities (Supporting Information). The absence of a discontinuity in the case of **1b** and **2b** is presumed to be due to insufficient sensitivity of this approach as compared with the parallax method of analysis.