Design and Synthesis of Molecular Umbrellas

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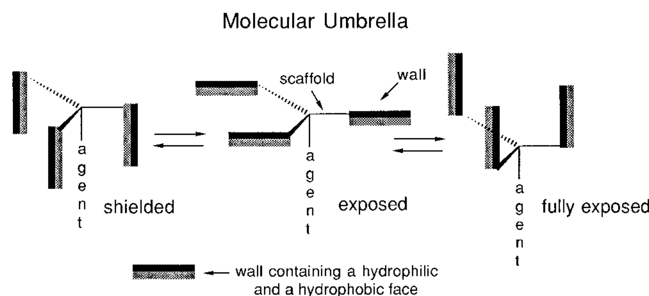
Abstract: This paper describes the design and synthesis of a series of conjugates derived from cholic acid, spermidine, and 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl), which effectively shield the dansyl moiety from water. Direct coupling of cholic acid to both terminal amino groups of spermidine, and attachment of the environmentally-sensitive dansyl moiety to the remaining secondary amine, yields a "molecular umbrella" (**Ia**) whose fluorescent properties (λ_{max} and emission intensity) reflect a nonpolar microenvironment in water and one that is relatively polar in intermediate dimethoxyethane/water mixtures. Comparison of **Ia** with analogous "single-walled" (**II**) and "no-walled" (**III**) umbrellas further indicates that a minimum of two walls is necessary in order to have "umbrella-like" properties. Examination of the fluorescent properties of a related double-walled umbrella, bearing a flexible (2-hydroxyethyl)-carbamate moiety at the C-3 position of the sterol (**Ib**), reveals that "umbrella-like" properties are present even when facial amphiphilicity is not rigorously maintained; however, the molecule's ability to shield the fluorophore, as judged by its relative emission intensity, is diminished. "Methyl-capping" of the (2-hydroxyethyl)carbamate (i.e., **Ic**) enhances the umbrella's ability to provide a hydrophobic shelter in water. A tetra-walled analogue of **Ia**, bearing four cholic acid units (i.e., **IV**), has been synthesized and its dansyl group found to have reduced exposure toward water. The potential utility of molecular umbrellas in the area of drug delivery is briefly discussed.

Introduction

Biological membranes play an essential role in cells by serving as selective barriers for transport.[1] Although they readily permit the passage of those molecules and ions that are necessary for maintaining the living state, they inhibit the entry of many classes of biologically-active molecules that have therapeutic potential, especially those that are highly hydrophilic and/or charged, e.g., antisense oligonucleotides, DNA, proteins, and certain peptides.[2−7] Finding ways to promote the passive transport of such agents across cellular membranes represents a formidable challenge, and one that has significant practical implications.

With this challenge in mind, we have conceived of a novel class of surfactant molecules that mimic the structure and function of umbrellas, i.e., molecules that can cover an attached agent and shield it from an incompatible environment.[8] In essence, our "molecular umbrella" concept may be summarized as follows: two or more "amphiphilic walls" (i.e., rigid hydrocarbon units that maintain a hydrophobic and a hydrophilic face) are coupled to a suitable scaffold that bears a biologically-active agent (Scheme 1, where the "darkened face" is hydrophobic and the agent is hydrophilic). When the agent is immersed in an aqueous environment, a fully-exposed conformation is favored such that intramolecular hydrophobic interactions are maximized and the external face of each wall is hydrated. When the agent is immersed in a hydrocarbon environment, the umbrella then favors a shielded conformation such that intramolecular dipole−dipole and hydrogen-bonding interactions are maximized and the hydrophobic faces are effectively solvated. Our working hypothesis is that such a molecule will permeate across a lipid membrane via the following sequence of events: (i) diffusion of the conjugate to the biomembrane surface in a fully exposed state, (ii) insertion into the outer monolayer leaflet by flipping into a shielded state, (iii) diffusion to the inner monolayer leaflet, and (iv) entry into the cytoplasm via the sequential reversal of steps ii and i.

Scheme 1



In addition to their potential for promoting the transport of polar agents across cellular membranes, we also envisioned that molecular umbrellas could improve the water solubility and stability of *hydrophobic drugs* of therapeutic interest. Specifically, attachment of a hydrophobic agent to an umbrella should result in a fully exposed conformation in hydrocarbon medium and a shielded conformation in an aqueous environment (Scheme 1, where the "darkened face" is hydrophilic and the agent is hydrophobic), i.e., conformational preferences that are opposite to those of an umbrella−polar agent conjugate. In principle, one can imagine that a nonpolar cross-linking agent that is susceptible toward hydrolysis (e.g., chlorambucil) might exhibit improved stability and solubility in water when seques-

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tered within an umbrella.[9] Subsequent binding to a biological membrane would then be expected to release the active agent by "flipping" from a shielded to a fully exposed state.

A primary aim of the work that is described herein was to demonstrate the feasibility of constructing a molecular umbrella.[8] In addition, we sought to define how the shielding effects of an umbrella depend upon the number of walls that are present and also on the wall's ability to maintain "facial amphiphilicity".[10,11] In order to test for umbrella action, we have selected the environmentally-sensitive fluorophore 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) as a "mock" agent. Previous studies have demonstrated that increased exposure of the dansyl group to an aqueous environment leads to a longer λ_{max} and to a decrease in fluorescence intensity.[12] Since the dansyl moiety is hydrophobic, derivative umbrellas were expected to favor exposed or fully exposed conformations in solvents of low polarity (i.e., dimethoxyethane and aqueous solutions that contain high concentrations of dimethoxyethane) and a shielded conformation in highly polar solvents (i.e., pure water). Dimethoxyethane was specifically chosen for this study because of its high miscibility with water and its low polarity.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. Cholic acid and cholic acid methyl ester were purchased from Sigma Chemical Co. Dansylglycine, monodansylcadaverine, and acetic acid *N*-hydroxysuccinimide ester (A-NHS) were commercially available (Sigma Chem. Co.) and used as obtained. *O*-(*N*-succinimidyl)-*N*,*N*,*N′N′*-tetramethyluronium tetrafluoroborate (TSU), 1-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIPEA) were obtained from Aldrich Chemical Co. *N*-(*O*-Succinimidyl)choleate was prepared using literature procedures.[13] EMS silica Gel 60 was used for column chromatrography; preparative thin layer chromatograpy employed EM Science silica gel 60 F-254. Detection by TLC was made by a combination of sulfuric acid 10% in water, I_2, and UV (254 and 365 nm). House-deionized water was purified using a Millipore Milli-Q filtering system containing one carbon and two ion-exchange stages. All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in parts per million and were referenced to residual solvents. High-resolution mass spectra (fast atom bombardment) were obtained at the Mass Spectrometry Facility of the University of California, Riverside. Surface tension measurements (23 °C) were made using a tensiometer/microbalance (NIMA, Model ST9000). Unless noted otherwise, all fluorescence measurements were made using a Perkin Elmer LS 50 luminescence spectrometer using 0.5 μM solutions of fluorophore. The excitation wavelength and spectral band width used in all cases were 330 and 7 nm, respectively.

3-(2-Hydroxyethyl)cholic Acid Carbamate (1). To a cooled solution (0 °C) of 500 mg (1.2 mmol) of cholic acid methyl ester in 15 mL of anhydrous CH_2Cl_2 was added 4.3 mL of 1.9 M phosgene in toluene. The mixture was then warmed to room temperature and stirred for 3 h (selective chloroformylation of the C-3 hydroxyl group shifts its methine proton from 3.45 to 4.62 ppm). Concentration under reduced pressure afforded the monochloroformate as a white solid: ^{1}H NMR ($CDCl_3$) δ 0.68 (s, 3 H), 0.89 (s, 3 H), 0.97 (d, 3 H), 1.1−2.4 (m, 27 H), 3.65 (s, 3 H), 3.85 (br s, 1 H), 3.98 (br s, 1 H), 4.62 (m, 1 H). Dissolution of this sterol in 5 mL of anhydrous CH_2Cl_2, followed by dropwise addition of dry ethanolamine (8 mmol) and stirring for 3 h at room temperature, afforded a product mixture that was then washed with 10% aqueous HCl. Concentration under reduced pressure and purification by column chromatography (silica, $CHCl_3/CH_3OH$, 10/1, v/v) afforded 489 mg (80%) of the methyl ester of 3-(2-hydroxyethyl)-cholic acid carbamate as a white solid: mp 99−100 °C; $R_f = 0.5$; ^{1}H NMR ($CDCl_3$) δ 0.65 (s, 3 H), 0.76 (s, 3 H), 0.86 (d, 3 H), 0.94−1.87 (m, 19 H), 2.15−2.34 (m, 5 H), 3.26 (bs, 3 H), 3.39−3.41 (m, 4 H), 3.63 (s, 3 H), 3.81 (br s, 1 H), 3.94 (br s, 1 H), 4.42(m, 1 H), 6.00 (bs, 1 H); HRMS for $(C_{28}H_{47}O_7NNa)^+$ calcd 532.3250, found 532.3223. Selective methyl ester hydrolysis was carried out by dissolving the sterol (489 mg, 0.9 mmol) in 15 mL of methanol plus 3 mL of a 10% aqueous Na_2CO_3 and refluxing the mixture for 12 h. Sequential cooling to room temperature, concentration under reduced pressure, dissolution in 20 mL of $CHCl_3$, washing with aqueous HCl, drying over anhydrous Na_2SO_4, and concentration under reduced pressure afforded crude **1**, which was purified by column chromatography (silica, $CHCl_3/CH_3OH$, 8/1, v/v). The desired product [0.375 g (79%)] was obtained as a white solid: mp 243−247 °C; $R_f = 0.3$; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3 H), 0.89 (s, 3 H), 0.97 (d, 3 H), 1.00−1.95 (m, 22 H), 2.15−2.44 (m, 5 H), 3.62−3.68 (m, 4 H), 3.83 (br s, 1 H), 3.96 (br s, 1 H), 4.43 (m, 1 H), 5.67 (m, 1 H).

3-(2-Methoxyethyl)cholic Acid Carbamate (2). Using procedures similar to those used to prepare **1**, the methyl ester of **2** was first synthesized (using 2-methoxyethylamine) in 82% yield: mp 74−76 °C; $R_f = 0.80$ (silica, $CHCl_3/CH_3OH$, 9/1, v/v); ^{1}H NMR ($CDCl_3$) δ 0.66 (s, 3 H), 0.87 (s, 3 H), 0.96 (d, 3 H), 1.01−1.95 (m, 21 H), 2.15−2.35 (m, 5 H), 3.30−3.41 (m, 7 H), 3.62 (s, 3 H), 3.78 (br s, 1 H), 3.94 (br s, 1 H), 4.39 (m, 1 H), 5.08 (m, 1 H); HRMS for $(C_{29}H_{50}O_7N)^+$ calcd 524.3587, found 524.3558. Mild ester hydrolysis yielded **2** (80%): mp 134−137 °C; $R_f = 0.55$ (silica, $CHCl_3/CH_3OH$, 9/1, v/v); ^{1}H NMR ($CDCl_3$) δ 0.69 (s, 3 H), 0.89 (s, 3 H), 1.01 (d, 3 H), 1.01−1.95 (m, 21 H), 2.18−2.43 (m, 5 H), 3.32−3.45 (m, 7 H), 3.84 (br s, 1 H), 3.99 (br s, 1 H), 4.46 (m, 1 H), 5.19 (m, 1 H).

***N*^{1},*N*^{3}-Spermidinebis[3-(2-hydroxyethyl)cholic acid carbamate amide] (3b)**. To a solution of 0.4 mmol of **2** in 5 mL of anhydrous DMF was added 0.44 mmol of DIPEA (dropwise addition), followed by the addition of 0.44 mmol of *O*-(*N*-succinimidyl)-*N*,*N*,*N′N′*-tetramethyluronium tetrafluoroborate (TSU) in one portion. The resulting solution was then stirred at room temperature for 3 h and then added directly to a separate solution that was made from 0.19 mmol of spermidine, 0.5 mmol of DIPEA, and 0.5 mL of dry DMF. After stirring for 12 h at room temperature, the product mixture was concentrated under reduced pressure and purified by preparative TLC (silica, $CHCl_3/CH_3OH/NH_4OH$, 65/25/4, v/v/v) to give 53 mg (27%) of **3b**: mp 195−198 °C; $R_f = 0.3$; ^{1}H NMR (CD_3OD) δ 0.68 (s, 6 H), 0.86 (s, 6 H), 0.97 (d, 6 H), 1.00−2.47 (m, 54 H), 2.63 (mult., 4H), 3.05−3.20 (m, 8 H), 3.52−3.56 (t, 4 H), 3.79 (br s, 2 H), 3.95 (br s, 2 H), 4.37 (m, 2 H); HRMS for $(C_{61}H_{106}O_{12}N_5)^+$ calcd 1100.7838, found 1100.7900.

N^1,*N*^{3}-Spermidinebis[3-(2-methoxyethyl)cholic acid carbamate amide] (3c). Using procedures similar to those used for the preparation of **3b**, **3c** was synthesized from **2** in 42% yield: mp 138−139 °C; $R_f = 0.1$ (silica, $CHCl_3/CH_3OH/NH_4OH$, 65/25/10); ^{1}H NMR (CD_3OD) δ 0.72 (s, 6 H), 0.94 (s, 6 H), 1.03 (d, 6 H), 1.08−2.26 (m, 54 H), 2.80 (m, 4 H), 3.09−3.45 (m, 18 H), 3.80 (br s, 2 H), 3.96 (br s, 2 H), 4.38 (m, 2 H); HRMS for $(C_{63}H_{110}O_{12}N_5)^+$ calcd 1128.8151, found 1128.8105.

***N*^{1},*N*^{3}-Spermidinebis[cholic acid amide] (3a).** The *N*-hydroxysuccinimide ester of cholic acid (NHS-DCC method)[13] was isolated prior to its coupling with spemidine. Reaction of this ester with spermidine was carried out in $CHCl_3/CH_3CN$. Using purification procedures similar to those used for the preparation of **3b**, **3a** was obtained from cholic acid in 50% yield: mp 164−166 °C; R_f 0.69 (silica, $CHCl_3/CH_3OH/NH_4OH$, 65/25/10, v/v/v); ^{1}H NMR (CD_3OD) δ 0.65 (s, 6 H), 0.78 (s, 6 H), 0.87 (d, 6 H), 0.85−2.20 (m, 54 H), 2.50 (m, 4 H), 3.09−3.18 (m, 4 H), 3.30 (m, 2 H), 3.70 (br s, 2 H), 3.83 (br s, 2 H); HRMS for $(C_{55}H_{96}O_8N_3)^+$ calcd 926.7197, found 926.7163.

***N*^{2}-(Dansylglycinamido)-*N*^{1},*N*^{3}-Spermidinebis[3-(2-hydroxyethyl)-cholic acid carbamate amide] (Ib).** To a stirred solution of 92.5 mg (0.3 mmol) of dansylglycine and 34 mg (0.25 mmol) of HOBt in 2 mL of anhydrous DMF were added 72 mg (0.35 mmol) of DCC at 0 °C and, after 15 min, 88 mg (0.08 mmol) of **3b**. After stirring for an additional 20 h at room temperature, the product mixture was concentrated under reduced pressure, dissolved in 1 mL of methanol, and precipitated in 20 mL of a saturated aqueous solution of $NaHCO_3$.

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The precipitate was redissolved in methanol and then precipitated in pure water. This last step was repeated another time. Purification by preparative TLC (silica, $CHCl_3/CH_3OH/H_2O$, 65/25/3, v/v/v) afforded 8.1 mg (9%) of **Ib**: mp 210−212 °C; R_f = 0.72; ^{1}H NMR (CD_3OD) δ 0.67 (s, 6 H), 0.88 (s, 6 H), 0.99 (d, 6 H), 1.05−2.20 (m, 54 H), 2.85 (br s, 6 H), 3.09−3.24 (m, 12 H), 3.55 (t, 4 H), 3.65 (s, 2 H), 3.78 (br s, 2 H), 3.93 (br s, 2 H), 4.32 (m, 2 H), 7.19 (d, 1H), 7.47−7.59 (m, 2H), 8.16 (d, 1H), 8.30 (d, 1H), 8.51 (d, 1H); HRMS for $(C_{75}H_{119}O_{15}N_7SNa)^+$ calcd 1412.8383, found 1412.8458.

***N*^{2}-(Dansylglycinamido)-*N*^{1},*N*^{3}-Spermidinebis[cholic acid amide] (Ia).** Using procedures similar to those used for the preparation of **Ib**, **Ia** was synthesized from **3a** in 14% yield: mp 228−231 °C; R_f 0.60 (silica, $CHCl_3/CH_3OH/H_2O$, 65/25/4, v/v/v); ^{1}H NMR ($CDCl_3$) δ 0.70 (s, 6 H), 0.90−2.20 (m, 66 H), 2.88 (s, 6 H), 3.00−3.25 (m, 8 H), 3.47 (m, 2 H), 3.81 (m, 2 H), 3.93 (m, 2 H), 4.40 (d, 2 H), 6.20 (t, 1 H), 6.90 (m, 1 H), 7.15−7.66 (m, 3 H), 8.28−8.62 (m, 3H); HRMS for $(C_{69}H_{110}O_{11}N_5S)^+$ calcd 1216.7923, found 1216.7983. Anal. Calcd for $C_{69}H_{109}O_{11}N_5S \cdot 2\ H_2O$: C, 66.11; H, 9.17; N, 5.59. Found: C, 66.14; H, 8.96; N, 5.65.

***N*^{2}-(Dansylglycinamido)-*N*^{1},*N*^{3}-Spermidinebis[3-(2-methoxyethyl)cholic acid carbamate amide] (Ic).** Using procedures similar to those used for the preparation of **Ib**, **Ic** was synthesized from **3c** in 29% yield: mp 210−2 °C; R_f = 0.83 (silica, $CHCl_3/CH_3OH$, 8/2, v/v); ^{1}H NMR (CD_3OD) δ 0.65 (s, 6 H), 0.87 (s, 6 H), 0.97 (d, 6 H), 1.08−2.32 (m, 54 H), 2.78 (s, 6 H), 3.09−3.45 (m, 24 H), 3.79 (br s, 2 H), 3.92 (br s, 2 H), 4.40 (m, 2 H), 7.19 (d, 1H), 7.47−7.59 (m, 2H), 8.16 (d, 1H), 8.30 (d, 1H), 8.51 (d, 1H); HRMS for $(C_{77}H_{123}O_{15}N_7SNa)^+$ calcd 1440.8696, found 1440.8770. Anal. Calcd for $C_{77}H_{123}O_{15}N_7S$: C, 65.18; H, 8.74; N, 6.91. Found: C, 65.42; H, 8.86; N, 6.81.

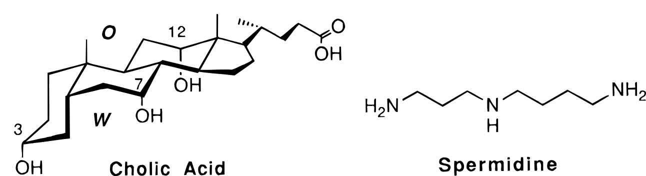
***N*-Dansyl, *N'*-Cholic Acid Amide Cadaverine (II).** To a stirred solution of 67.0 mg (2.0 mmol) of dansylcadaverine in 7 mL of CH_2Cl_2 was added 102 mg (2.0 mmol) of *N*-(*O*-succinimidyl)choleate in three equal portions at room temperature. After being stirred for 3 h, the product mixture was concentrated under reduced pressure and purified by preparative TLC (silica, $CHCl_3/CH_3OH$, 8/1, v/v) to give 81.0 mg (56%) of **II**: mp 126−8 °C; R_f = 0.48; ^{1}H NMR (CD_3OD) δ 0.68 (s, 3 H), 0.88 (s, 3 H), 1.19 (d, 3 H), 1.20−2.22 (m, 33 H), 2.90 (s, 6 H), 2.91 (t, 2 H), 3.09 (m, 2 H), 3.41 (br m, 1 H), 3.82 (br s, 1 H), 3.95 (br s, 1 H), 5.37 (t, 1 H), 6.05 (t, 1 H), 7.16−7.56 (m, 3 H), 8.19−8.53 (m, 3 H); HRMS for $(C_{41}H_{64}O_6N_3S)^+$ calcd 726.4516, found 726.4497.

N*^{2}-(Dansylglycinamido)-*N*^{1},*N*^{3}-Spermidinebis(acetamide) (III).** To a stirred solution of 0.25 g (1.72 mmol) of spermidine in 100 mL of CH_2Cl_2 was added, dropwise, a solution of 0.55 g (3.18 mmol) of acetic acid ***N-hydroxysuccinimide ester (A-NHS) in 30 mL of CH_2Cl_2 at 0 °C. After the reaction mixture was stirred for 1 h at 0 °C and for 22 h at room temperature, the solvent was removed under reduced pressure. Purification by column chromatography (silica, CH_3OH, NH_4OH, 100/8, v/v) afforded 0.10 g (27%) of *N*^{1},*N*^{3}-spermidinebis(acetamide): ^{1}H NMR ($CDCl_3$) δ 1.48−1.65 (m, 7 H), 1.93 (d, 6 H), 2.59 (t, 2 H), 2.66 (t, 2 H), 3.24 (t, 2 H), 3.30 (t, 2 H), 5.91 (br s, 1 H), 6.51 (m, 1 H). To a stirred solution of 67 mg (0.35 mmol) of 1,3-dicyclohexylcarbodiimide in 2 mL of anhydrous THF (cooled to 0 °C) was added 100 mg (0.32 mmol) of dansylglycine. After the mixture was stirred for 15 min, a solution of 70 mg (0.31 mmol) of *N*^{1},*N*^{3}-spermidinebis[acetamide] in 6 mL of THF/ethyl acetate/$CHCl_3$ (1/1/1, v/v/v) and 2 mg of DMAP was added. After the resulting solution was stirred at room temperature for 36 h, the solvents were removed under reduced pressure and the residue then dissolved in 5 mL of $CHCl_3$, washed with water, and purified by preparative TLC to afford 48 mg (31%) of **III**: R_f 0.40 (silica, $CHCl_3/CH_3OH$, 10/1, v/v); ^{1}H NMR ($CDCl_3$) δ 1.31−1.52 (m, 6 H), 1.87−1.91 (d, 6 H), 2.84 (s, 6 H), 2.80−2.85 (m, 2 H), 2.90-3.10 (m, 2 H), 3.13−3.31 (m, 4 H), 3.71 (d, 2 H), 5.84−6.30 (m, 3 H), 7.15−7.59 (m, 3 H), 8.19−8.53 (m, 3 H); HRMS for $(C_{25}H_{38}O_5N_5S)^+$ calcd 520.2594, found 520.2582.

Tetra-Walled Umbrella IV. To a stirred solution of 260 mg (1.95 mmol) of iminodiacetic acid in 11 mL of water was added 530 mg (5 mmol) of Na_2CO_3, followed by 30 mL of acetone. To the resulting solution was added 530 mg (1.97 mmol) of dansylchloride in three equal portions, and the mixture then stirred at room temperature for 14 h. After the solvents were removed under reduced pressure, the residue was dissolved in 6 mL of 1 M HCl. Subsequent removal of water under reduced pressure and recrystallization from H_2O/CH_3OH (3/1, v/v) afforded 400 mg (56%) of *N*-dansyliminodiacetic acid: mp 134−137 °C; ^{1}H NMR ($CDCl_3$) δ 2.76 (s, 6 H), 4.16 (s, 4 H), 7.14−7.47 (m, 3 H), 8.16−8.45 (m, 3 H); HRMS for $(C_{16}H_{18}O_6N_2S)^+$ calcd 366.0886, found 366.0873. To a solution of 18.3 mg (54 μmol) of *N*-dansyliminodiacetic acid in 0.5 mL of dry DMF was added 18.7 μL (110 μmol) of DIPEA and 36 mg (110 μmol) of TSU. After 2 h at room temperature (the solution becomes bright yellow), 180 μL of DIPEA and 88 mg (95 μmol) of **3a** were added. The mixture was then stirred for 22 h at room temperature, 15 min at 50 °C, concentrated under reduced pressure, and purified by preparative TLC (silica gel, $CHCl_3/CH_3OH/H_2O$, 105/27/4, v/v/v) to give 51 mg (25%) of **IV**: mp 213 °C; R_f 0.65; ^{1}H NMR ($CDCl_3/CD_3OD$ 10/1, C:2.8 mM) δ 0.59 (m, 12 H), 0.79−2.06 (m, 130 H), 2.78 (s, 6 H), 3.09−3.31 (m, 16 H), 3.34 (m, 4 H), 3.72 (br s, 4 H), 3.85 (br s, 4 H), 4.38 (s, 4H), 7.10−7.45 (m, 3H), 8.50−8.86 (m, 3H); HRMS for $(C_{126}H_{204}O_{20}N_8SNa)^+$ calcd 2204.4810, found 2204.4954. Anal. Calcd for $C_{126}H_{204}N_8O_{20}S \cdot 4\ H_2O$: C, 67.11; H, 9.48; N, 4.92. Found: C, 66.91; H, 9.58; N, 4.92.

Results

Selection of Starting Materials. Starting materials that were of particular interest to us for constructing molecular umbrellas were those that are present in mammalian cells, i.e., we sought materials that would furnish umbrellas that are potentially biocompatible and biodegradable. Thus, cholic acid and spermidine were selected as amphiphilic wall and scaffold material, respectively. Cholic acid, a major component of bile acids, has a sterol nucleus that presents a hydroxylated, "water-loving" (***W***) face and also a face that is all-hydrocarbon and "oil-loving" (***O***).[10,14,15] Spermidine, which is also found in mammalian cells, has terminal amino groups that can be used for wall attachment, and a secondary amino group can serve as a "handle" for binding an agent.



Target Molecules. Specific target molecules that were chosen for this study included three double-walled umbrellas (**Ia**,**b**,**c**), one single-walled analogue (**II**), a dansylated spermidine derivative that was devoid of sterol (**III**), and one tetra-walled umbrella (**IV**). A comparison of the fluorescence properties of **Ia**, **II**, and **III** was expected to verify our hypothesis that a minimum of two amphiphilic walls is necessary for effective shielding. A comparison of **Ia** with **Ib** was expected to provide insight into the importance of rigorously maintaining facial amphilicity with respect to the umbrella's ability to provide an effective "cover" for the attached agent. Thus, in contrast to **Ia**, which has all three of its hydroxyl groups firmly positioned on the ***W*** face, the hydroxyl group that is tethered to the C-3 position in **Ib** can lie on *either* face of the

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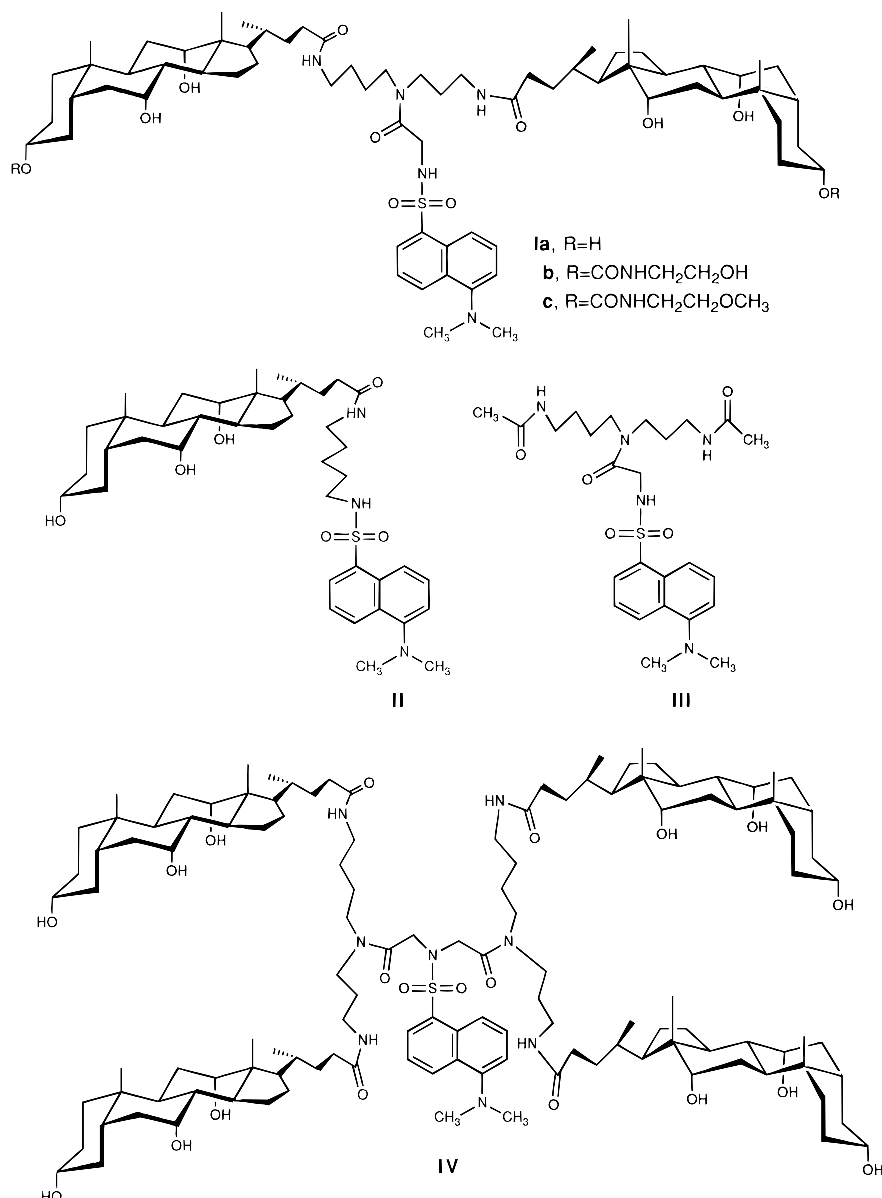
(15) Hjelmeland, L. M.; Nebert, D. W.; Osborne, J. C. *Anal. Biochem*, **1983**, *130*, 72.

(16) We have found that the fluorescence emission intensities are sensitive to the onset of micelle formation, but the corresponding λ_{max} values are not. Thus, a 2-fold increase in the concentration of **Ia** from 0.5 to 1.0 μM, which places it just below its cmc of 1.1 μM, results in a doubling of its fluorescence intensity. In contrast, a 4-fold increase to 2.0 μM leads to a 6.4-fold increase in intensity. The λ_{max} values that were observed at 0.5 and 2.0 μM, however, were unchanged, i.e., 495.2 ± 0.6 and 495.6 ± 0.3 nm, respectively. Similarly, as the cmc of **Ib** is exceeded (cmc = 0.8 μM), its emission intensity increases disproportionally, i.e., on going from 0.5 μM to 1.0 and 2.0 μM, the fluorescence intensity increased by factors of 4.3 and 12.7, respectively; the λ_{max} values that were observed at 0.5 and 2.0 μM were 493.5 ± 0.5 and 493.5 ± 0.3 nm, respectively. Although we do not yet understand this phenomenon, we suspect that changes in fluorescent lifetimes and/or aggregation (turbidity) are primary factors that are responsible for this behavior.



sterol. A methyl-capped analogue of **Ib** (i.e., **Ic**) was also of interest since it bears the same ethylcarbamate spacer at the C-3 position but lacks the strongly hydrophilic hydroxyl group; an increased level of facial amphiphilicity was, therefore, expected. Finally, the tetra-walled umbrella (**IV**) was considered to be a worthy target since the dansyl group may now, in principle, be enclosed from three and/or four sides; a situation that could result in even greater shielding efficiency.

Umbrella Synthesis. Schemes 2 and 3 summarize the synthetic routes that were used to prepare **Ia**−**c**, **II**, and **III**. In brief, selective functionalization of cholic acid methyl ester at the C-3 position using an excess of phosgene, followed by condensation with 2-hydroxyethylamine and mild ester hydrolysis afforded **1**; alternative conjugation with 2-methoxyethylamine and hydrolysis yielded **2**. Conversion of **1** and **2** to their *N*-hydroxysuccinimide esters, followed by condensation with the primary amino groups of spermidine (to give **3b** and **3c**, respectively) and coupling with dansylglycine, afforded the requisite double-walled umbrellas **Ib**,**Ic**; similar transformations that were carried out using unmodified cholic acid afforded **Ia**. The analogous single-walled umbrella, **II**, was prepared by similar methods using monodansylcadaverine as starting material. Compound **III** was synthesized by acetylation of spermidine with acetic acid *N*-hydroxysuccinimide ester (A-NHS), followed by condensation with dansylglycine.

The synthetic sequence that was used to prepare the tetra-walled umbrella is shown in Scheme 4. Thus, dansylation of iminodiacetic acid, followed by activation with *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and coupling with **3a**, afforded **IV**.

Critical Micelle Concentrations. In order to ensure that shielding effects in pure water reflect the monomeric state of each umbrella, critical micelle concentrations (cmc's) were determined by use of standard surface tension methods. Thus, plots of the surface tension versus amphiphilic concentrations yielded cmc values of 1.1, 0.8, 2.1, and 3.3 μM for **Ia**, **Ib**, **Ic**, and **IV**, respectively (Figure 1); the cmc of **II** was 3.0 μM (data not shown).

Umbrella Properties. The fluorescence emission spectra that were measured for 0.5 μM solutions of **Ia** in varying dimethoxyethane/water mixtures are shown in Figure 2. Incremental replacement of DME with water resulted in a continuous decrease in fluorescence intensity until a composition of ca. 10/90 (DME/water, v/v) was reached (Figure 2A). Further increases in water content resulted in a *significant increase* in emission intensity (Figure 2B). A plot of the relative emission intensity at λ_{max} for **Ia** as a function of water content (v/v) is presented in Figure 2C. The fluorescence intensity that was measured in pure DME was similar to that which was found in pure water. The specific value of λ_{max} was also sensitive to

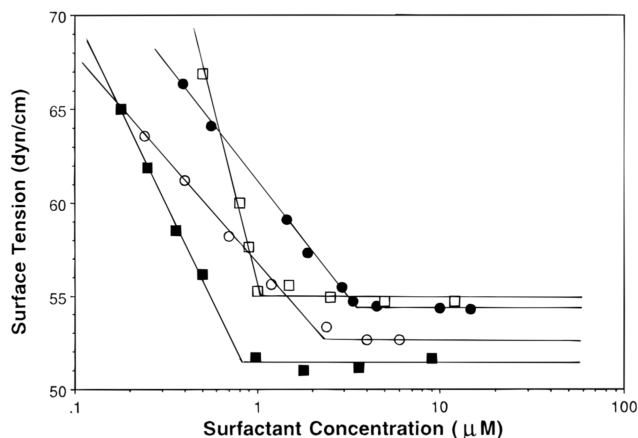
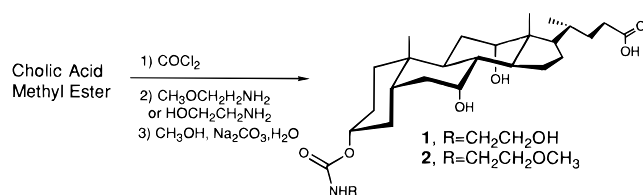
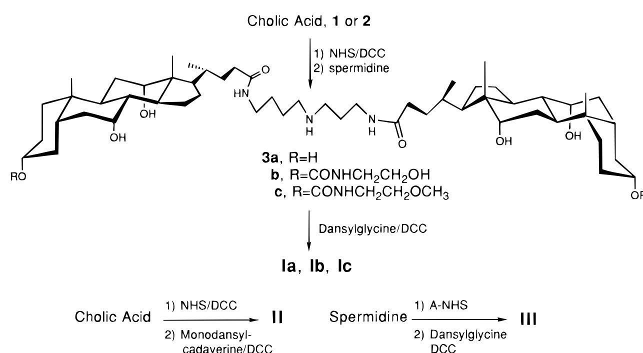


Figure 1. Surface tension as a function of umbrella concentration for **Ia** (□), **Ib** (■), **Ic** (○), and **IV** (●), where each data point represents an average of ca. 10 independent measurements.

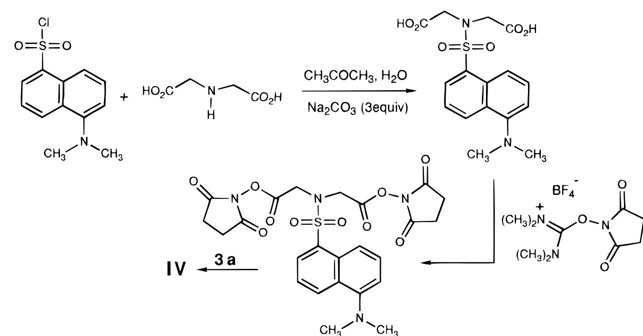
Scheme 2



Scheme 3



Scheme 4



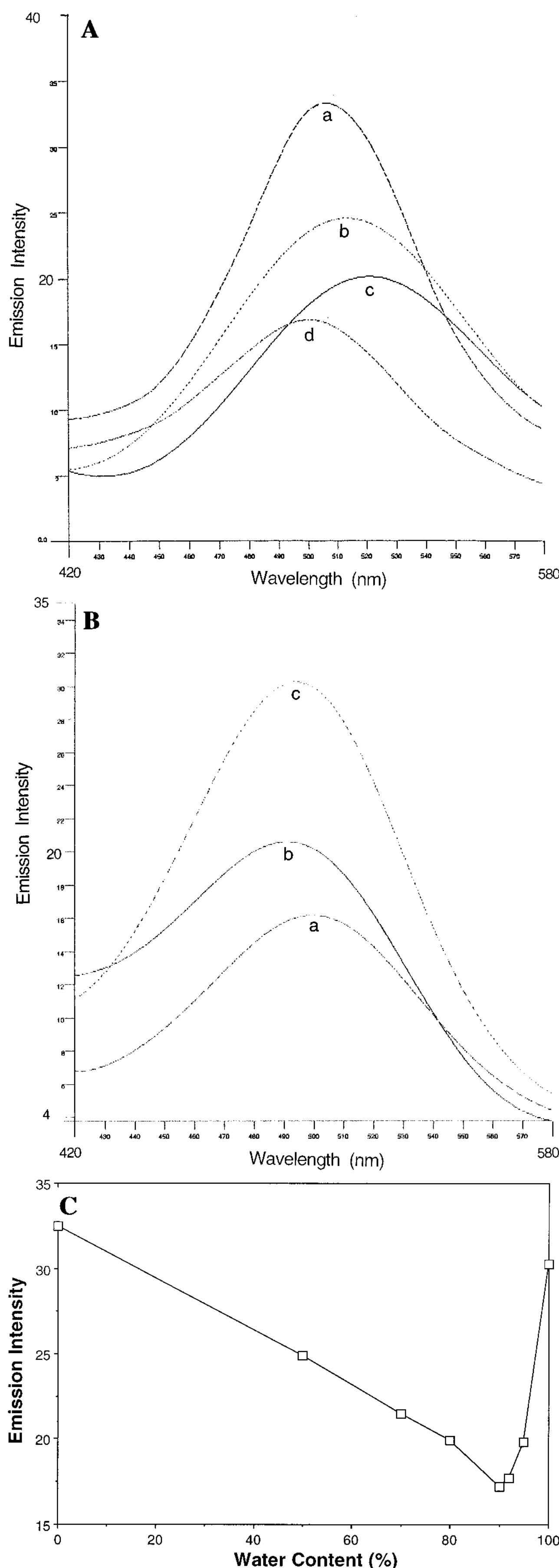


Figure 2. (A) Fluorescence emission spectra of 0.5 μM solutions of **Ia** in DME/water (v/v) mixtures: (A) (a) 100/0 (pure DME), (b) 50/50, (c) 20/80, (d)10/90. (B) (a) 10/90, (b) 5/95, (c) 0/100 (pure water). (C) Plot of relative emission intensity at λ_{max} for **Ia** as a function of water content (v/v).

the solvent compostion that was used. Thus, a maximum value of 516.1.6 ± 1.6 nm was reached at ca. 20/80 (DME/water, v/v). In pure water, the λ_{max} sharply decreased to 495.5 ± 0.4 nm; a value that is essentially the same as that which has been found in hexane (496 nm). In sharp contrast, an incremental increase in water content for the single-walled analogue, **II**, produced a continuous shift in λ_{max} to longer wavelengths and a continuous decrease in fluorescence intensity *over the entire range of solvent mixtures used* (Figure 3A); exactly analogous behavior was observed for **III** (Figure 3B).

Introduction of a (hydroxyethyl)carbamate group at the C-3 position of cholic acid component of the umbrella significantly

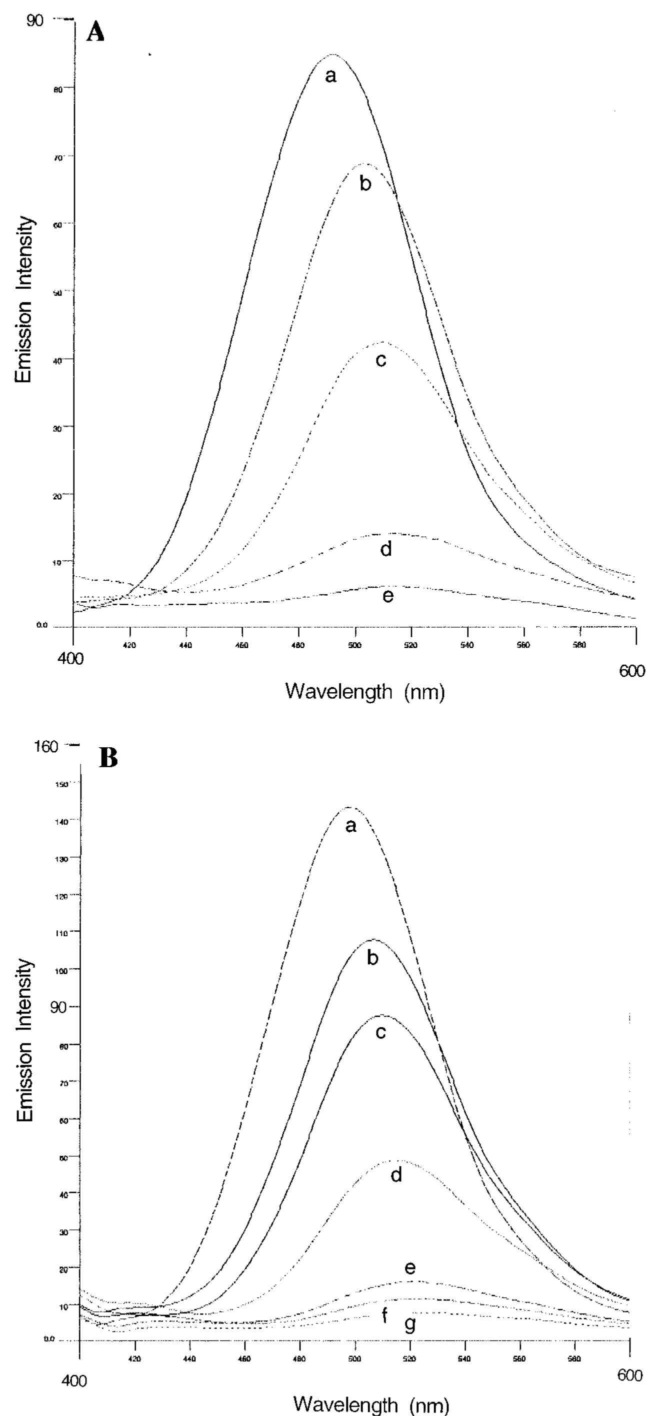


Figure 3. Fluorescence emission spectra of 0.5 μM solutions in DME/water (v/v) mixtures of (A) **II**: (a) 100/0 (pure DME), (b) 80/20, (c) 50/50, (d) 20/80, (e) 0/100 (pure water) and (B) **III**: (a) 100/0 (pure DME), (b) 90/10, (c) 80/20, (d) 50/50, (e) 20/80, (f) 10/90, (g) 0/100 (pure water).

Table 1. Values of λ_{max} for Molecular Umbrellas in DME and Water

umbrella	DME	DME/water[a]	water
Ia	501.0 ± 0.6	516.6 ± 1.6	495.5 ±0.4
Ib	497.9 ± 0.4	511.1 ± 0.9	493.8 ± 0.9
Ic	501.4 ± 0.4	507.5 ± 2.5	490.7 ± 0.7
IV	507.1 ± 1.0	516.5 ± 0.8	492.8 ± 0.2

[a] Maximum λ_{max} for **Ia**,**b**,**c** and **IV** occurring at ca. 20/80, 30/70, 40/60, and 50/50 DME/water (v/v), respectively.

affected its fluorescence properties. Thus, the fluorescence intensity of **Ib** was found to continuously decrease with increasing water content, until a composition of ca. 10/90 (DME/water, v/v) was reached (Figure 4); further increases in water content resulted in only a *slight increase* in intensity. In pure water, the emission intensity of **Ib** was ca. 15% of that which was measured in pure DME;[17] values of λ_{max} for **Ib** that were measured in pure DME and pure water are listed in Table 1, along with a maximum value in a 40/60 mixture of DME/water. The methyl-capped analogue, **Ic**, also showed a minimum in fluorescence intensity and a maximum in λ_{max} at intermediate DME/water mixtures. In pure water, the emission intensity was significantly greater than that of **Ib** (Figure 5); also, the λ_{max} was significantly lower (Table 1).

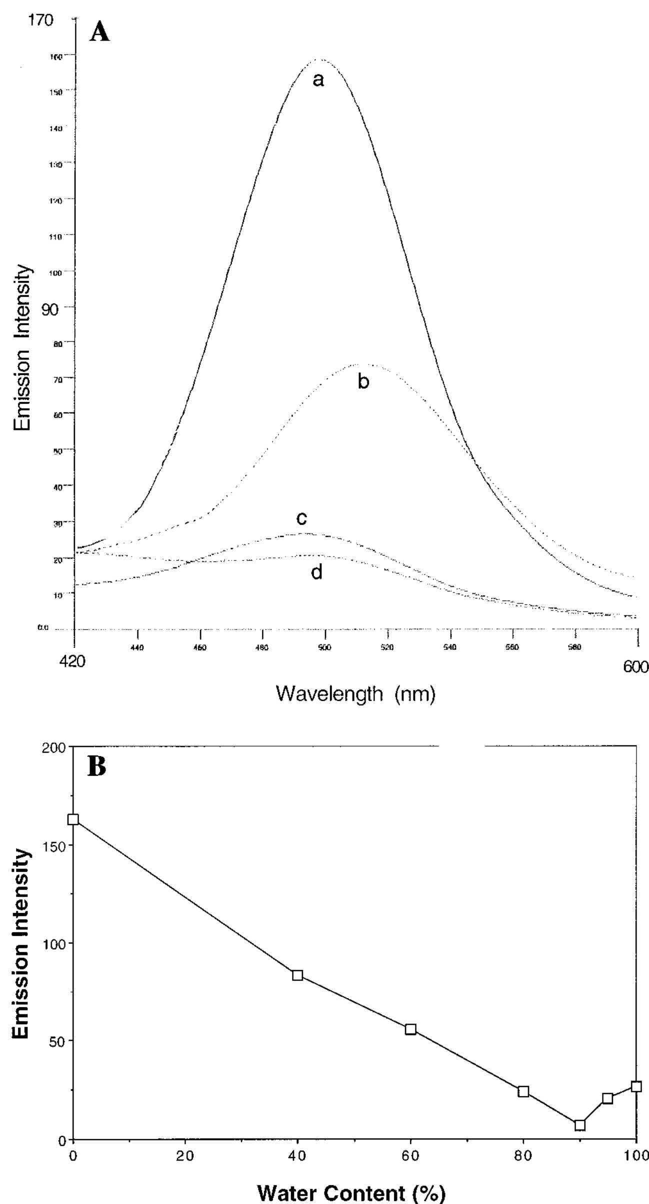


Figure 4. (A) Fluorescence emission spectra of 0.5 μM solutions of **Ib** in DME/water (v/v) mixtures: (a) 100/0 (pure DME), (b) 60/40, (c) 0/100 (pure water), (d) 10/90. For purposes of clarity, not all of the data are shown. (B) Plot of relative emission intensity at λ_{max} for **Ib** as a function of water content (v/v).

The fluorescence properties of the tetra-walled umbrella (**IV**) were qualitatively similar to those of its double-walled analogue (**Ia**), except that its minimum intensity extended over a much

(17) A widely held view in the drug delivery field is that molecular weight is an important determinant of a molecule's ability to cross a membrane (e.g., Pidgeon, C.; Ong, S.; Liu, H.; Qiu, X.; Pidgeon, M.; Dantzig, A. H.; Munroe, J.; Hornback, W. J.; Kasher, J. S.; Glunz, L.; Szczerba, T. *J. Med. Chem.* **1995**, *38*, 590. A comparison of the permeation properties of single-, double-, and tetra-walled umbrellaconjugates should either lend support or dispel this notion.

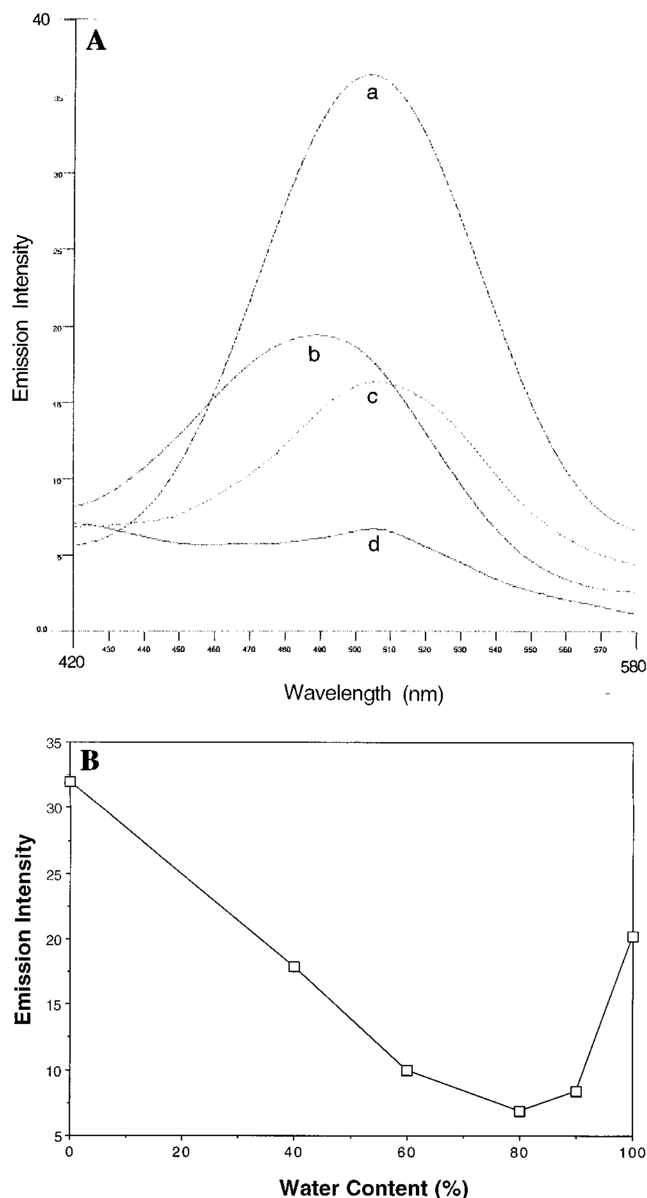


Figure 5. (A) Fluorescence emission spectra of 0.5 μM solutions of **Ic** in DME/water (v/v) mixtures: (a) 100/0 (pure DME), (b) 0/100 (pure water), (c) 60/40, (d) 20/80. For purposes of clarity, not all of the data are shown. (B) Plot of relative emission intensity at λ_{max} for **Ic** as a function of water content (v/v).

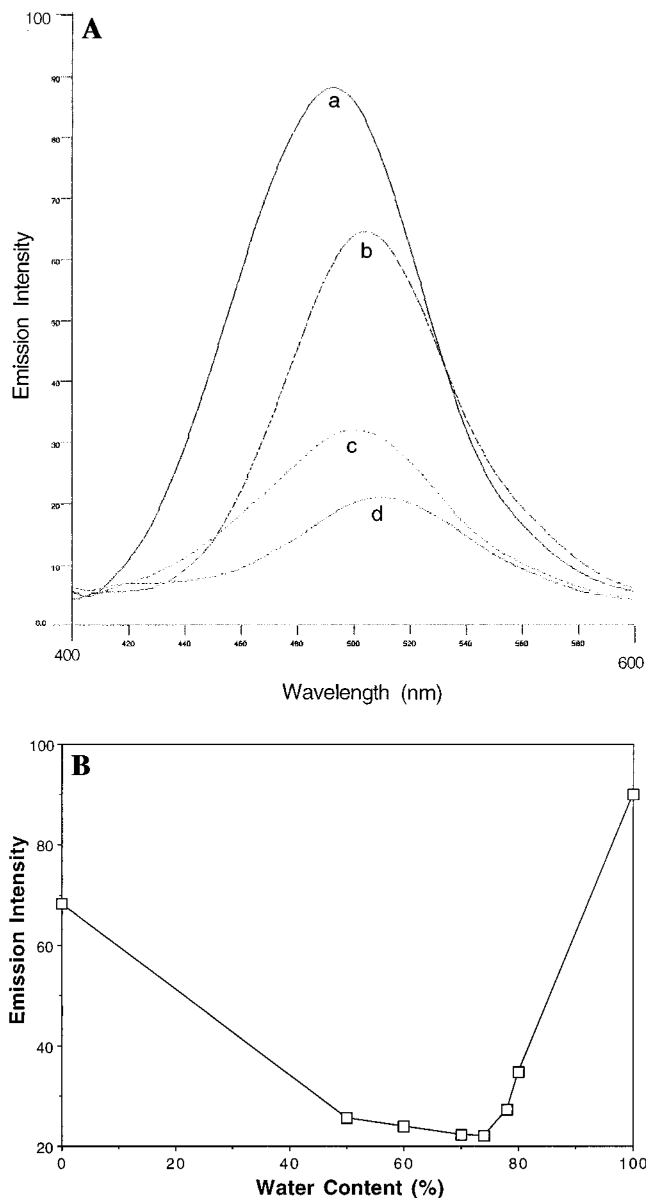


Figure 6. (A) Fluorescence emission spectra of 0.5 μM solutions of **IV** in DME/water (v/v) mixtures: (a) 0/100 (pure water), (b) 100/0 (pure DME), (c) 22/78, (d) 50/50. For purposes of clarity, not all of the data are shown. (B) Plot of relative emission intensity at λ_{max} for **IV** as a function of water content (v/v).

broader range (Figures 6). In addition, its relative intensity in pure water was significantly greater, and its λ_{max} was substantially lower than that of **Ia** (Table 1).

Discussion

The fluorescence properties of **Ia** in varying mixtures of water/DME provide strong evidence that it acts like a molecular umbrella. Specifically, the changes in its fluorescence intensity and λ_{max}, on going from pure DME to intermediate DME/water mixtures, clearly reflect an increase in the polarity surrounding the dansyl moiety and the presence of exposed and/or fully-exposed conformations. The sharp reversal in these spectral changes that accompanies further increases in water content demonstrates that **Ia** functions like an umbrella by providing a hydrophobic shield over the dansyl moiety. The fact that a single-walled analogue (**II**) and a dansylated bis-acetylated spermidine (**III**) that is devoid of amphiphilic walls do not show such a reversal provides strong support for our hypothesis that *a minimum of two umbrella walls is necessary for effective shielding of an attached agent.*

Although **Ib** also provides a shield for the dansyl group, as judged by its relatively low λ_{max} value in pure water, its low emission intensity implies that it is less effective than **Ia**. The improvement in shielding efficiency that results from methyl capping (i.e., **Ic**), as judged by both its λ_{max} and emission intensity, is a likely consequence of enhanced facial amphiphilicity due to the removal of the highly polar hydroxyl group in the C-3 position. Finally, a comparison of the λ_{max} and relative emission intensity that are associated with **Ia** and **IV** in pure water indicates that the tetra-walled umbrella provides a much more hydrophobic shelter for the fluorophore. The simplest explanation for this result, we believe, is that **IV** encloses the dansyl group from three and/or four sides, thereby further reducing the exposure of the dansyl group toward water.

Conclusions

The feasibility of creating molecules that can cover an attached agent and shield it from an incompatible environment has been demonstrated through the use of double- and tetra-walled "molecular umbrellas". These umbrellas have been

constructed from cholic acid, spermidine, and dansyl chloride. A minimum of two walls has been found necessary in order to provide an effective cover for the dansyl moiety; increasing the number of walls to four enhances its shielding efficiency. Although the rigorous maintenance of facial amphiphilicity is not essential in order to observe molecular umbrella-like properties, its presence does appear to improve its effectiveness.

Studies that are currently in progress are aimed at (i) defining the interactions of molecular umbrellas with lipid bilayers, (ii) synthesizing umbrella–drug conjugates of therapeutic interest, (iii) defining the permeation properties of umbrella–drug conjugates with respect to phospholipid bilayers, and (iv) exploring the therapeutic potential of umbrella-drug conjugates *in vitro*.[17] The results of these studies will be reported in due course.

Acknowledgment. We are grateful the National Institutes of Health (PHS Grant GM51814) for support of this research.

Supporting Information Available: ^{1}H NMR spectra for **Ia**,**b**,**c** and **II**–**IV** and a complete set of surface tension measurements (9 pages). See any current masthead page for ordering and Internet access instructions.

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