Molecular Umbrella-Assisted Transport of Glutathione Across a Phospholipid Membrane

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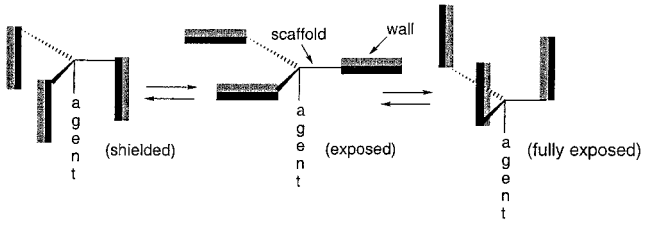
Abstract: A di-walled molecular umbrella (**1a**) has been synthesized by acylation of the terminal amino groups of spermidine with cholic acid, followed by condensation with bis{3-*O*-[*N*-1,2,3-benzotriazin-4(3*H*)-one]yl}-5,5′-dithiobis-2-nitrobenzoate (BDTNB), and displacement with glutathione (γ-Glu-Cys-Gly, GSH). Replacement of the sterol hydroxyls with sulfate groups, prior to displacement with GSH, afforded a hexasulfate analogue **1b**. Both conjugates have been found to enter large unilamellar vesicles (200 nm diameter, extrusion) of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and to react with entrapped GSH to form oxidized glutathione (GSSG). Evidence for vesicular entry has come from the formation of oxidized glutathione (GSSG) *within the interior of the vesicle*, the appearance of the thiol form of the umbrella (USH), and the absence of release of GSH into the external aqueous phase. Results that have been obtained from monolayer experiments, together with the fact that the heavily sulfated conjugate is able to cross the phospholipid bilayer, have yielded strong inferential evidence for an "umbrella-like" action of these molecules as they cross the lipid bilayer.

Introduction

One of the most significant challenges presently facing medicinal chemists is to find ways of promoting the passive transport of polar, biologically active agents across lipid membranes. This challenge exists not only for large- and intermediate-sized molecules (e.g., DNA and antisense oligonucleotides, respectively), but also for relatively small molecules (e.g., peptides).[1−10] In an attempt to devise a general solution to this problem, we have focused our attention on the synthesis of amphiphilic molecules that mimic the structure and function of umbrellas, i.e., "molecular umbrellas" that cover an attached agent and shield it from an incompatible environment.[11−14]

Chart 1



The design principle upon which our efforts have been based is illustrated in Chart 1. In brief, molecular umbrellas are composed of two or more amphiphilic "walls" (rigid hydrocarbon units that maintain a hydrophobic and a hydrophilic face), which are covalently coupled to a central scaffold. Our working hypothesis has been that such molecules will favor a shielded conformation, when taken up into the hydrocarbon region of a membrane, and that this conformation will mask the strong hydrophilicity of the attached agent, thereby facilitating its partitioning into, and diffusion across the membrane.

In previous studies, we have shown that molecular umbrellas exhibit "molecular amphomorphism", i.e., the ability to form a shielded or exposed conformation in response to changes in the hydrophilic/hydrophobic character of the microenvironment.[11,12] We have also shown that certain di-walled molecular umbrellas, as well as much larger tetra-walled analogues, can readily move from one side of a lipid bilayer to the other.[14] Whether or not molecular umbrellas are able to promote the passive transport of an attached polar agent across a membrane, however, has been a key question that we have sought to answer.

In this paper, we report the design and synthesis of two di-walled molecular umbrellas which bear glutathione (Glu-Cys-Gly) as the attached agent. We also report their transport properties with respect to phospholipid bilayers made from

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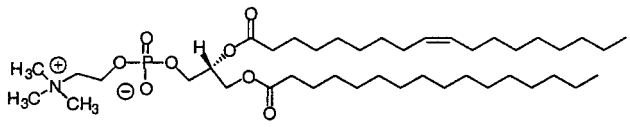
1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).[15] Monolayer studies that have been carried out with one of these conjugates in the absence, and in the presence, of POPC provide insight into its likely conformation within a phospholipid bilayer.



POPC

Experimental Section

General Methods. Unless stated otherwise, all reagents were obtained from commercial sources and used without further purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL). All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in ppm and are referenced to residual solvent. The borate buffer that was used in all experiments was composed of 0.1 M H_3BO_3, and 2 mM EDTA, where the pH was adjusted to 7.0 by use of 1 M NaOH. N_1,N_3-Dicholeamidospermidine was prepared using procedures similar to those previously described.[12]

Bis{3-*O*-[*N*-1,2,3-Benzotriazin-4(3*H*)-one]yl}-5,5′dithiobis-2-nitrobenzoate (BDTNB). To a suspension of 1.19 g (3.0 mmol) of 5,5′-dithiobis(2-nitrobenzoic acid and 1.078 g (6.52 mmol) of 3-hydroxy-1,2,3-benzotriazin-4*H*(3*H*)-one in 70 mL of CH_2Cl_2 was added 1.343 g (6.52 mmol) of dicyclohexylcarbodiimide. The heterogeneous mixture was stirred at room temperature under a nitrogen atmosphere for 12 h, and the solid separated by filtration and washed with CH_2Cl_2 (3 × 20 mL). The solid was dissolved in warm THF, and the solution then concentrated to 30 mL under reduced pressure to allow for crystalization, yielding 0.635 g (31%) of the desired diester having R_f 0.53 [$CHCl_3$/acetone, 6/1 (v/v)], mp 213−214 °C dec, and ^{1}H NMR ($CDCl_3$) δ 8.36 (dd, 1 H), 8.23 (m, 2 H), 8.18 (d, 1 H), 8.00 (dt, 1 H), 7.91 (dd, 1 H), 7.83 (dt, 1 H).

Mixed Disulfide Derived from 2-Nitro-5 mercapto-*N*,*N*-dimethylbenzamide and Glutathione (2). To a suspension of 54.0 mg (0.079 mmol) of BDTNB in 1 mL of anhydrous DMF was added 0.022 mL of 40% dimethylamine/H_2O (0.197 mmol) plus 50 μL of *N*,*N*-diisopropyl-*N*-ethylamine (0.29 mmol). The reaction mixture was then stirred for 3 h at room temperature and diluted with 20 mL of CH_2Cl_2. Subsequent washing with 3 × 10 mL of saturated aqueous $NaHCO_3$ and 3 × 20 mL of H_2O, drying over Na_2SO_4, concentration under reduced pressure, and purification by column chromatography (SiO_2, $CHCl_3$/acetone, 6/1, v/v) afforded 35 mg (99%) of 5,5′-dithiobis(2-nitro-*N*,*N*-dimethylbenzamide, having R_f 0.46 and ^{1}H NMR ($CDCl_3$) δ 8.12 (d, 2 H), 7.57 (d, 2 H), 7.40 (s, 2 H), 2.82−3.12 (d, 12 H).

To a solution of 20.0 mg of 5,5′-dithiobis(2-nitro-*N*,*N*-dimethylbenzamide) (44.4 μmol) in 1.4 mL of MeOH was slowly added a solution of 13.6 mg of glutathione (44.3 μmol) in 0.4 mL of H_2O and the mixture then stirred under an argon atmosphere for 1 h at room temperature. The mixture was then concentrated under reduced pressure and the residue purified by flash chromatography (SiO_2,$CHCl_3$/MeOH/H_2O; 60/40/10; v/v/v) to give 8.1 mg (60%) of **2** having R_f 0.36 and ^{1}H NMR (CD_3OD) d 8.22 (d, 1 H), 7.82 (m, 1 H), 7.60 (s, 1 H), 4.66 (m, 1 H), 3.60−3.65 (m, 3 H), 2.80−3.11 (d, 6 H), 2.56 (m, 2 H), 2.14 (m, 2 H), 1.36 (m, 2 H).

N_2,N_2′-Bis[N_1,N_3-dicholeamidospermidineyl]-5,5′-dithiobis(2-nitrobenzamide) (4). To a stirred solution of 100 mg (0.108 mmol) of N_1,N_3-dicholeamidospermidine (**3**) in 0.8 mL of anhydrous DMF and 0.1 mL of triethylamine was added 33.0 mg (0.048 mmol) of BDTNB. The mixture was then stirred at room temperature under a nitrogen atmosphere for 14 h, and the solvents removed under reduced pressure (45 °C). The crude product was dissolved in 2 mL of methanol and purified by precipitating into 35 mL of 10% $NaHCO_3$. The solid was separated, washed with water (3 × 20 mL), and purified by preparative TLC [silica, $CHCl_3$/CH_3OH/H_2O, 103/27/2 (v/v/v)] to give 66.0 mg (61%) of **3** having R_f 0.60 and ^{1}H NMR (CD_3OD) δ 8.22 (m, 2 H), 7.83 (m, 2 H), 7.56 (d, 2 H), 3.92 (s, 4 H), 3.78 (s, 4 H), 3.54 (m, 4 H), 3.35 (m, 4 H), 3.30 (m, 4 H), 3.10 (m, 4 H), 2.95 (m, 4 H), 0.90−2.27 (m, 120 H), 0.90 (s, 12 H), 0.70 (s, 12 H).

Mixed Disulfide of GSH and N_1,N_3-Bis[dicholeamidospermidineyl]-N_2-(2-nitro-5-mercaptobenzamide) (1a). To a solution of 65.0 mg (25.9 μmol) of **4** in 1.5 mL of methanol was added, dropwise, a solution of 7.9 mg (25.9 μmol) of GSH in SHE buffer (8 mM NaCl, 2 mM HEPES, 0.05 mM EDTA, pH 7.0) and the mixture stirred for 12 h at room temperature. After concentration under reduced pressure (35 °C), the product was dissolved in a minimum volume of CH_3OH and purified by preparative TLC [silica, $CHCl_3$/CH_3OH/H_2O, 60/40/10 (v/v/v)] to give **1a** (53%) having R_f 0.37 and ^{1}H NMR (CD_3OD) δ 8.25 (m, 1 H), 7.84 (m, 1 H), 7.61 (d, 1 H), 4.69 (m, 1 H), 3.94 (s, 2 H), 3.78 (s, 2 H), 3.70 (m, 3 H), 3.35 (m, 2 H), 3.30 (m, 6 H), 3.15 (m, 2 H), 3.05 (m, 2 H), 2.50 (m, 2 H), 0.70−2.29 (m, 68 H), 0.69 (s, 6 H). HRMS for $C_{72}H_{113}N_7O_{17}S_2$ (MNa^+): calcd 1434.7532, found 1434.7603.

Bis [N_1,N_3-Dicholeamidospermidineyl]-5,5′-dithiobis(2-nitrobenzamide), Persulfate, Sodium Salt (5). To a solution of 41.0 mg (18.5 μmol) of **4** in 1.20 mL of anhydrous DMF was added 143 mg (0.9 mmol) of SO_3·Py and the mixture stirred at for 16 h at room temperature. Removal of solvent under reduced pressure (55 °C, 10 Torr), followed by drying [23 °C, 2 h (1 Torr)], afforded a solid that was redissolved in 2.50 mL of a saturated aqueous solution of $NaHCO_3$. The water was then removed under reduced pressure (10 Torr, 45°), and the resulting solid extracted with 3 × 5 mL of CH_3OH. The solution was concentrated under reduced pressure to give 71 mg of crude product, which was purified by preparative TLC (silica gel, $CHCl_3$/CH_3OH/H_2O, 34/28/8, v/v/v) to give 60 mg (94%) of **5** having R_f 0.60, and ^{1}H NMR (D_2O) δ 8.28 (m, 2 H), 7.86 (m, 2 H), 7.46 (bs, 2 H), 4.64 (s, 4 H), 4.45 (s, 4 H), 4.18 (m, 4 H), 3.35 (m, 8 H), 3.16 (m, 8 H), 0.92−2.35 (m, 132 H), 0.73 (s, 12 H).

Mixed Disulfide of GSH and Persulfated N_1,N_3-Bis[dicholeamidospermidineyl]-N_2-(2-nitro-5-mercaptobenzamide) (1b). To a stirred solution of 50.0 mg (14.5 μmol) of **5** in 0.80 mL of MeOH was added 0.84 mL of an aqueous solution of 4.46 mg (14.5 μmol) of glutathione (pH of which was adjusted to 7.16 by 1 N NaOH). After being stirred for 60 min under an argon atmosphere, the solution was freeze-dried, and the resulting solid was redisolved in 0.5 mL of MeOH/H_2O (5/2,v/v). The crude product was purified by preparative TLC (SiO_2; $CHCl_3$/MeOH/H_2O (34/28/8,v/v/v) under an argon atmosphere [solvents were removed by a combination of rotatary evaporation (35 °C, 10 Torr) and freeze-drying] to give 21 mg (72%) of **1b** with R_f 0.45 and ^{1}H NMR (D_2O) δ 8.26 (d, 1 H), 7.87 (d, 1 H), 7.53 (d, 1 H), 4.71 (m, 1 H), 4.68 (s, 2 H), 4.43 (s, 2 H), 3.91 (m, 2 H), 3.76 (t, 1 H), 3.50 (m, 2 H), 2.80−3.35 (m, 10 H), 2.42 (t, 2 H), 3.30−0.90 (m, 68 H), 0.71 (s, 6 H). Anal. Calcd for $C_{72}H_{107}N_7O_{35}S_8Na_8$·10 H_2O: C, 38.44; H, 5.60; N, 4.36. Found: C, 38.06; H, 5.52; N, 4.24.

Vesicles for Transport Experiments. Typically, 1.6 mL of a 25 mg/mL solution of POPC in chloroform was transferred to a Pyrex test tube, and the solvent evaporated by rotating the tube under a stream of nitrogen, resulting in a thin film of POPC. The last traces of solvent were removed under reduced pressure (25°, 48 h, <0.2 Torr). To the dried film was added 1.75 mL of a 1.20 mM solution of glutathione (GSH) that was prepared in borate buffer, which had been adjusted to pH 7.0 by addition of a few drops of 1 M NaOH. The mixture was vortexed for 30 s, incubated at room temperature for 5 min, vortexed for another 30 s, and incubated for 30 min, while being maintained under an argon atmosphere. The dispersion was then subjected to 5 freeze/thaw cycles (−196 °C/30 °C), followed by extrusion through a 400 nm Nuclepore membrane (12 times) and a 200 nm membrane (15 times).

Gel Filtration Procedure. All gel filtrations were carried out via minicolumn centrifugation using pre-packed Sephadex G-25, PD-10 (Pharmacia Biotech). Prior to use, the Sephadex G-25 was extensively rinsed with borate buffer. Typically, 1.5 mL of the dispersion were gel filtered (two times) using 4.0 g of Sephadex G-25 (fine), by expelling the void volume of the column via centrifugation (4 min). To ensure the complete removal of external GSH, the dispersion was dialyzed using a bag made from SpectraPor #7 dialysis tubing (MWCO 50 000),

(15) A preliminary account of this work has previoiusly appeared: Janout, V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2671.

where dialysis was made against 400 (1 h), 600 (20 h), and 500 mL (3 h) of borate buffer under a nitrogen atmosphere.

Reaction of Vesicle-Entrapped GSH with 1a. In a typical experiment, 200 μL of the vesicle dispersion was mixed with 300 μL of a 20 μM solution of **1a** in borate buffer. Mixing was carried out in the "source" side of a 1.5 mL equilibrium dialysis cell containing an equal volume of borate buffer in the "receiving" side (SpectraPor #7 dialysis tubing, MWCO 50 000), using a Burrell wrist-action shaker. The extent of thiolate−disulfide interchange was then monitored in the source side by the appearance of USH at λ_{max} 427. Pseudo-first-order rate constants, k_{obsd}, were determined from linear plots of $\ln[1 - (C/C_o)]_{USH}$ versus time. Analysis of the residual GSH in the source at the end of the reaction was made by diluting a 30 μL aliquot of the dispersion with 360 μL of a 10% buffer solution of sodium dodecyl sulfate (SDS) and then adding 20 μL of a 2 mM buffer solution of 5,5′dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Measurement of the UV spectrum (350−500 nm) was recorded after 5 min, and compared with appropriate calibration curves. Note: Care must be taken in subtracting the absorbance due to USH from the absorbance produced upon reaction with Ellman's reagent. Analysis of the receiving side of the dialysis cell for GSH was made by withdrawing a 30 μL aliquot and reacting it with Ellman's reagent, in a manner that was similar to that used for the source side.

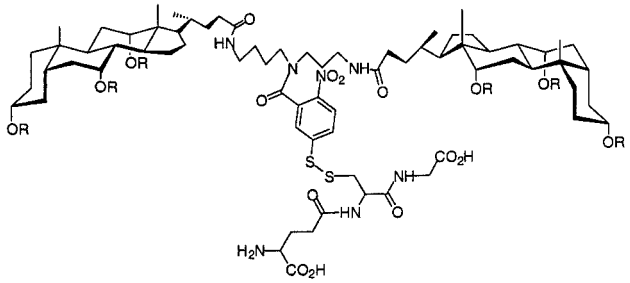
Analysis of Oxidized Glutathione (GSSG). For analysis of GSSG from the source side, 400 μL of the dispersion were diluted with 300 μL of ethanol in a 12-mL test tube. To this solution was added 60 μL of a 1.0 mM buffer solution of tris(2-carboxyethyl)phosphine (TCEP), followed by vortex mixing. After allowing this mixture to react for 24 h under an argon atmosphere, 50 μL of 6 M HCl was added, followed by addition of 50 μL of an aqueous solution that was 4 mM in 4,4′-dipyridyl disulfide (Aldrithiol-4) and 1 M in HCl. The UV spectrum (300−380 nm) was then recorded after 5 min of reaction; 4-mercaptopyridine has λ_{max} 330 nm ($\epsilon = 1.819 \times 10^3$ M^{-1} cm^{-1}, borate buffer). A baseline that was used in this analysis was obtained from an analogous sample, where the addition of TCEP was omitted. These results were also compared with a blank determination of TCEP, in which the vesicles were devoid of GSSG, i.e., vesicles containing entrapped GSH, which were not subjected to reaction with **1a**. Analysis of GSSG in the receiving side (after 8 h of reaction with **1a**) was made by use of a standard fluorescamine assay.[16,17]

Affinity of 1a and 1b toward POPC Membranes. Multilamellar vesicles of POPC were prepared using 40 mg of lipid (dried thin film) plus 2 mL of borate buffer via vortex mixing. To this dispersion was added 6 nmol of **1a** or **1b** in 0.3 mL of borate buffer. After incubation for 120 min at room temperature, the vesicles were pelleted by centrifugation. The percentage of the umbrella that remained in the supernatant was then quantified by adding 0.6 μmol of TCEP in 120 μL of buffer and analyzing the solution for the reduced form of the conjugate (USH) by UV at λ_{max} 427 nm.

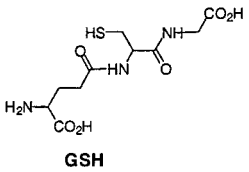
Surface Pressure−Area Isotherms. Surface pressure−area isotherms were recorded by use of an MGW Lauda film balance, which was equipped with a computerized data acquisition station. All isotherms were measured at 25 °C. Water (ca. 1 L), which was used in preparing a subphase, was purified via a Milli-Q filtration system and purged with nitrogen for 15 min. Before addition to the film balance, the surface of the aqueous solution was removed via aspiration to eliminate surface-active contaminants. A chloroform solution of **1a** was spread onto the aqueous subphase having a surface area of 600 cm^2, using a gastight, 50 μL Hamilton syringe. Actual concentrations were determined by direct weighing of aliquots after evaporation of solvent using a Cahn 35 electrobalance. In all cases, spreading solvents were allowed to evaporate for at least 30 min prior to compression under a flow of nitrogen. Monolayers were compressed at the rate of 25 cm^2/min.

Results and Discussion

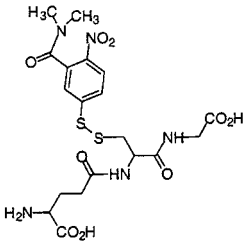
Umbrella Design. Two molecular umbrella-glutathione conjugates (**1a** and **1b**) and one nonumbrella analogue (**2**) were specifically designed as synthetic targets for this study. Our choice of **1a** was based on five considerations. First, glutathione (GSH) was viewed as a reasonable drug model based on its strong hydrophilicity. Thus, one would not expect GSH to readily cross lipid bilayers. The fact that mammalian cells



1a, R=H
b, R=SO_3^-



GSH



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maintain millimolar concentrations of GSH within their cytoplasm, and that only micromolar levels of GSH can be detected in the extracellular milieu, fully supports this premise. Second, glutathione has the potential for being covalently attached to, as well as released from, a molecular umbrella by taking advantage of thiolate−disulfide interchange chemistry. Third, the 5-thiol(2-nitrobenzoyl) "handle" provides a sensitive means for monitoring the release of GSH through its strong UV absorption. Fourth, previous studies from our laboratory have shown that di-walled molecular umbrellas derived from cholic acid and spermidine exhibit molecular amphomorphism, which is a likely prerequisite for membrane transport.[11,12] Fifth, examination of **1a** by CPK molecular models (not shown) indicates that such an umbrella can provide significant coverage for an attached glutathione molecule.[15]

Our interest in the hexasulfate analogue of **1a** (i.e., **1b**) was derived largely from mechanistic considerations. Specifically, if **1a** were to enhance the transport of glutathione across a POPC bilayer, a question that could be asked is whether this enhanced permeability relative to GSH was due to an "umbrella-like action", or simply due to a net increase in overall hydrophobicity, resulting from the attached sterols. Since the permeability coefficient of a permeant is directly proportional to its membrane/water partition coefficient, an overall increase in hydrophobicity due to conjugation would increase its membrane permeability.[18−20]

By replacing all of the hydroxyl groups of the cholic acid moiety with sulfate groups, the likelihood of diffusion of **1b** across a lipid bilayer in an exposed or a fully exposed conformation is remote. Thus, in addition to the strong hydrophilicity that is expected for each of these sterol units, the

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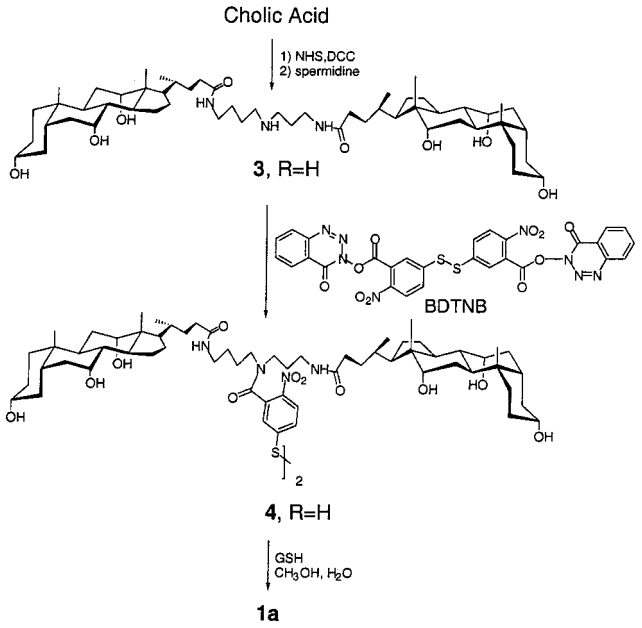
Scheme 1



presence of six sulfate groups should strongly inhibit transbilayer movement by anchoring the umbrella at the membrane−water interface. It is noteworthy, in this regard, that the half-life for the translocation of phosphatidylgycerols (bearing a single negative charge in the headgroup) between the inner and outer monolayer leaflet of a lipid bilayer is on the order of days to weeks.[21] Thus, the transport of an umbrella molecule such as **1b**, bearing *six* ionized sulfate groups, is expected to be considerably slower. If the molecule were to adopt a shielded conformation, however, then much, if not all, of its hydrophilicity could be masked, and movement across the bilayer would be facilitated. The observation of movement of **1b** across a lipid bilayer, therefore, would constitute strong inferential evidence for an umbrella-like action by the conjugate.

Finally, a nonumbrella analogue (**2**) was deemed worthy of investigation, to serve as a frame of reference in which to judge the effects of umbrella attachment on membrane transport.

Umbrella Synthesis. The synthetic strategy that was used to prepare **1a** is outlined in Scheme 1. Thus, cholic acid was first activated by forming the corresponding *N*-hydroxy succinimide ester, and then condensed with spermidine to give the di-walled molecular umbrella **3**. Subsequent reaction with bis{3-*O*-[*N*-1,2,3-benzotriazin-4(3*H*)-one]yl}-5,5′dithiobis-2-nitrobenzoate (BDTNB) afforded the corresponding symmetrical disulfide-based dimer (**4**), which was then cleaved with GSH to give the requisite umbrella−glutathione conjugate, **1a**.[22]

The hexasulfate analogue of **1a** was obtained by treating **4** with Pyr·SO_3 to give **5**, followed by nucleophilic displacement with GSH (Scheme 2). Finally, the nonumbrella analogue (**2**) was synthesized by reacting BDTNB with dimethylamine to give the corresponding bisamide, and then subjecting it to displacement by GSH (Scheme 3).

Relative Affinity toward POPC Bilayers. The relative affinities of **1a** and **1b** toward bilayers of POPC were determined by adding each conjugate to multilamellar vesicles (MLVs) of the phospholipid under identical conditions. After a 120-min incubation period, the MLVs were pelleted by centrifugation and the supernatant analyzed for free conjugate. Under the

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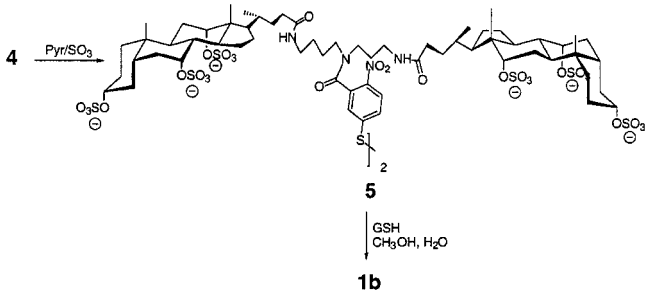
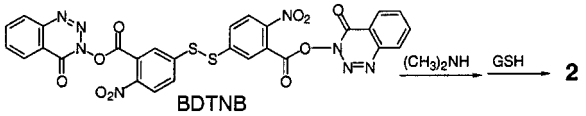
Scheme 2



Scheme 3



conditions used, 94% of **1a** and 50% of **1b** were removed from solution by the pelleted vesicles, i.e., **1a** exhibited significantly greater affinity toward POPC bilayers relative to **1b**.

Monolayer Properties of 1a at the Air−Water Interface. To gain insight into the likely conformation of **1a**, when bound to a lipid membrane, we examined its monolayer behavior in the absence and in the presence of POPC. This conjugate was chosen for these experiments because of its lower water-solubility, which decreases its tendency to dissolve in the aqueous subphase.[23]

In Figure 1 is shown the surface pressure−area isotherm that was obtained for **1a**, spread over an aqueous solution that was 0.14 M in NaCl and 0.01 M in phosphate buffer (pH 7.0) at 25 °C. Here, three distinct regions can be seen: a highly compressible region between 0 and 21 $mN \cdot m^{-1}$, a very broad first-order phase transition region, and a region of low compressibility. Extrapolation of the condensed portion of the highly compressible region to zero surface pressure yields a limiting area of 3.03 $nm^2 \cdot molecule^{-1}$. This value is consistent with a conformation in which the hydrophilic face of each sterol and the attached peptide are in intimate contact with water,; i.e., the entire molecule lies flat on the aqueous subphase. The high compressibility within this region can then be accounted for by a model in which the umbrella "folds up" into a shielded conformation as the area per molecule is reduced, i.e., both sterols lift up into air, and encase the pendant GSH, the resulting structure being anchored to the aqueous subphase via the C-3 hydroxyl group of each sterol.

The appearance of the broad first-order phase transition, together with the low surface area that **1a** occupies at the end of this transition (just before reaching the less compressible region), implies that a radical change in umbrella conformation has taken place. Such behavior is similar to that which we have recently reported for a conjugate derived from spermine and cholic acid.[24] One plausible explanation, which could account for this behavior, is that one of the sterol units has "flipped" up into air or down into water.

(23) Approximate water solubilities were estimated by injecting a small volume of a 34 mM methanolic solution of each conjugate into 0.750 mL of borate buffer (pH 7) and determining the concentrations at which the solutions became nontransparent at room temperature. In each case, the methanol content in the final solution was <2.5% (v/v). For **1a**, transparency was lost when the concentration reached ca. 400 μM; for **1b**, the solution remained transparent at concentrations >34 mM, which was the highest concentration tested. As expected, based on the greater hydrophilicity of **1b** relative to **1a**, the hexasulfate analogue exhibited substantially greater water-solubility.

(24) Bandyopadhyay, P.; Janout, V.; Zhang, L. H.; Sawko, J. A.; Regen, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 12888.

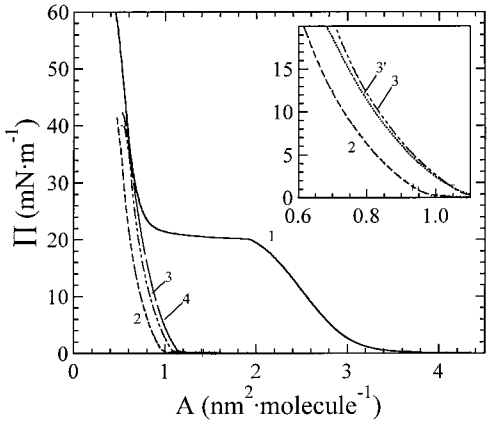


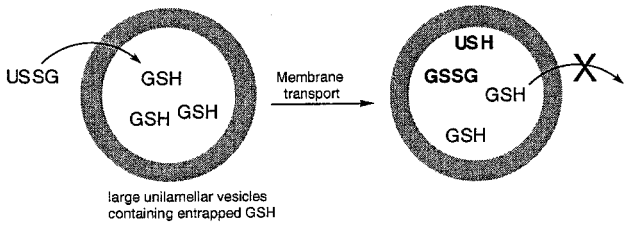
Figure 1. Surface pressure−area isotherms for **1a** and mixtures of **1a** plus POPC on the surface of 0.1 M NaCl plus 0.01 M Na_2HPO_4/NaH_2PO_4 solution (pH 7.0) at 25 °C: (1) **1a**; (2) POPC; (3) 5.0% **1a**/95% POPC; (3′) calculated isotherm for 5.0% **1a**/95% POPC, assuming ideal mixing and additivity between **1a** and POPC; (4) 9.4% **1a**/90.6% POPC.

In sharp contrast, monolayers formed from POPC, and also from two different mixtures of POPC/**1a** (i.e., 95/5 mol/mol and 90.6/9.4 mol/mol), produced isotherms of low compressibility *with no discernible phase transition* (Figure 1). If one assumes that POPC and **1a** mix ideally, then the calculated isotherm for the POPC/**1a** (95/5, mol/mol) mixture (from 0 to 20 mN·m^{-1}, based on simple additivity) is in excellent agreement with what is observed. This finding implies that **1a** adopts the *same* conformation as that found in monolayers made from the conjugate itself, within this range of surface pressures. In addition, the absence of a distinct phase transition implies that this compressed structure, which we assign to a shielded conformation, is stabilized in these POPC-rich films. Since the internal surface pressure of fluid phospholipid bilayers is generally thought to be ca. 30 mN·m^{-1}, these monolayer results are fully consistent with a shielded umbrella conformation of **1a** in POPC bilayers.[25]

Umbrella-Assisted Transport of Glutathione. One of the more common experimental methods that has been used to quantify transport rates of water-soluble permeants across phospholipid bilayers has been to encapsulate the permeant within liposomes, and to then measure their efflux rates.[18] Because of the high affinity of **1a**, and to a lesser extent **1b**, toward POPC membranes, we chose a different protocol for this study. Specifically, we loaded the aqueous interior of POPC liposomes with glutathione and then added **1a** or **1b**, externally, to the bulk aqueous phase. In principle, if the conjugate crosses the bilayer without disturbing it, one should then observe (i) the formation of oxidized glutathione (GSSG) *within the interior of the vesicle*, (ii) the appearance of the thiol form of the umbrella (USH), and (iii) the absence of release of GSH into the external aqueous phase (Scheme 4).

An additional reason that we were interested in carrying out membrane transport experiments in this manner was because it simulates what could be a drug delivery process. Specifically, one can envision taking advantage of intracellular glutathione to cleave an umbrella−drug conjugate on the inner surface of a cell membrane, thereby releasing the drug into the cytoplasm. The umbrella−drug conjugate would then function as a prodrug. One disadvantage of this approach, however, is that it relies on a chemical reaction to detect membrane transport. If the rate of chemical reaction is slow relative to permeation across the lipid bilayer, then the observed half-life represents an upper limit for the half-life of membrane transport.

Scheme 4



Experimentally, large unilamellar vesicles of POPC (200 nm diameter) were prepared in borate buffer (pH 7.0) containing 1.2 mM GSH via extrusion.[26] Nonentrapped GSH was then removed by gel filtering the dispersion. To ensure the complete removal of nonentrapped GSH, the dispersion was then subjected to dialysis. In a typical transport experiment, 200 μL of a 26 mM dispersion of POPC liposomes was mixed with 300 μL of a 20 μM solution of **1a** in borate buffer. Here, a submicellar concentration of the conjugate was used to simplify the interpretation of the results.[27] Mixing was then carried out in the "source" side of a 1.5-mL equilibrium dialysis cell, which contained an equal volume of borate buffer in the "receiving "side. [Note: Scheme 4 shows the chemistry that is expected for this umbrella-assisted transport experiment. Although an equilibrium dialysis cell was used to confirm that GSH did not leak out of the vesicles, and also that all of the GSSG remained entrapped in the vesicles, it has not been included in this scheme for simplicity.] Agitation of the cell was made by use of a wrist-action shaker. The extent of thiolate−disulfide interchange was monitored in the source side by measuring the appearance of the strongly UV absorbing, reduced form of the umbrella, USH (λ_{max} 427 nm; ϵ 7.007 × 10^3 M^{-1} cm^{-1}; borate buffer). An analogous experiment that was performed using a higher concentration of entrapped GSH (i.e., 2.0 mM) resulted in a proportional increase in the observed pseudo-first-order rate constant (Figure 2). These results indicate that the rate of formation of GSH is determined by chemical reaction and not by permeation across the POPC bilayer, meaning that diffusion across the membrane is relatively fast (eq 1), i.e., the half-life for membrane transport must be ≪22 min.

$$\text{USSG} + \text{GSH} \rightarrow \text{USH} + \text{GSSG} \qquad (1)$$

To ensure that GSH did not leak out of the liposomes and promote cleavage of the conjugate on the vesicle exterior, the receiving side of the cell was analyzed for GSH (Ellman's reagent) after a 24 h period. This analysis showed that less than 0.1 μM was present. Since the half-life for permeation of GSH across the dialysis membrane is 200 min, complete release of GSH would have corresponded to a thiol concentration of ca. 28.5 μM in both the source and the receiving side of the cell after 24 h. In addition, an analysis of the source side of the cell for residual thiol content, after the liposomes were first destroyed with excess sodium dodecyl sulfate, confirmed the presence of 22.6 nmol of GSH. Thus, the mass balance is excellent since complete reaction of GSH with **1** would, theoretically, leave 23.5 nmol of the tripeptide within the vesicles.

(25) Georgallas, A.; Hunter, D. L.; Lookman, T.; Zuckerman, M. J.; Pink, D. A. *Eur. Biophys. J.* **1984**, *11*, 79.

(26) Hope, M. J.; Bally, M. B.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55.

(27) The critical micelle concentration of **1a** in buffer is 60 μM (surface tension method). For **1b**, the cmc is ≫1000 μM, which was the highest concentration tested; the cmc for **2** is 180 μM.

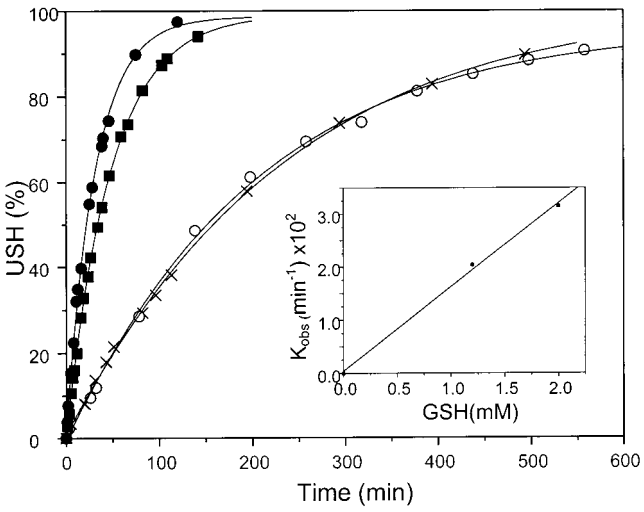


Figure 2. Rate of appearance of USH for reaction of **1a** with 0.2-μm POPC vesicles containing 1.2 mM GSH (■) and 2.0 mM GSH (●) at 23 °C; the inset shows the pseudo-first-order rate constant, k_{obsd}, versus the internal concentration of GSH. Analogous reactions carried out with **1b** using 1.2 (○) and 2.0 mM GSH (×).

To obtain further evidence for umbrella entry, as is depicted in Scheme 4, we analyzed both the source and receiving side of the dialysis cell for GSSG after a 28 h reaction period. The question here was whether all of the oxidized glutathione, which was formed from the displacement reaction, was entrapped *within* the vesicles. It should be noted that GSSG crosses the dialysis membrane with a half-life of ca. 8 h. Analysis for GSSG in the source side was performed by a combination of disulfide reduction via excess tris(2-carboxyethyl)phosphine (TCEP) and back-titration with 4,4′-dipyridyl disulfide (Aldrithiol-4), i.e., the excess of TCEP was quantified by reaction with Aldrithiol-4. To prevent GSH, itself, from reacting with the Aldrithiol-4 reagent, strongly acidic conditions were used. Thus, when 12 μM (6 nmol) **1a** and 57 mM (28.5 nmol) vesicle-entrapped GSH were introduced into the source side, and the vesicles allowed to react for 28 h, subsequent analysis of the source side revealed the presence of 10.9 μM (5.45 nmol) GSSG and 45.2 μM (22.6 nmol) GSH. A separate analysis of the receiving side for GSSG, using a more sensitive fluorescamine assay, indicated the presence of less than 0.24 μM (0.12 nmol) peptide. As a final control, GSSG was added, *externally*, to the vesicle dispersion (corresponding to 10 μM of external GSSG in the source side), and the receiving side analyzed for GSSG. After 8 h of dialysis, the concentration of GSSG that appeared in the receiving side was 2.2 μM. Thus, adsorption of GSSG to the lipid membrane is negligible. Taken together, these results establish that **1a** enters the POPC vesicles and reacts with the GSH to produce entrapped GSSG.

Analogous transport experiments that were carried out with the hexasulfate, **1b**, using similar experimental conditions as that employed for **1a**, gave similar results (Figure 2). Again, umbrella entry was confirmed by the appearance of USH in the vesicle dispersion, the formation of entrapped GSSG, and the absence of release of GSH into the external aqueous phase. The only notable differences were that the rate of appearance of USH was signficantly less than that observed with **1a**, and this rate was unchanged when the entrapped GSH concentration was increased from 1.2 to 2.0 mM. The insensitivity of the rate of formation of USH to the concentration of entrapped GSH indicates that chemical reaction is not rate-limiting. At present, we can only speculate on what may be rate-controlling. Two possibilities that appear likely are partial dehydration of the sulfate groups upon entry into the lipid membrane and diffusion across the bilayer. Nonetheless, the ability of **1b** to cross POPC bilayers and to react with internalized GSH is signficant. It provides inferential evidence that this conjugate is crossing the bilayer in a shielded conformation, which minimizes exposure of its strongly hydrophilic sulfate groups (vide ante).

Addition of **2** to liposomal dispersions of POPC that contained entrapped GSH (1.2 mM), using experimental conditions that were similar to those used for **1a** and **1b**, did not produce a detectable reaction after 5 h. This result further demonstrates the important role that the sterols in **1a** and **1b** have in promoting the transport of the attached glutathione molecule across POPC bilayers.

Conclusions

The results reported herein provide clear evidence that molecular umbrellas can transport polar peptides such as glutathione across POPC bilayer membranes. Monolayer results that have been obtained with **1a**, in the absence and in the presence of POPC, together with the fact that a hexasulfate analogue (**1b**) can also cross POPC bilayer membranes, constitute strong inferential evidence for an "umbrella-like" action of these molecules during the transport process. In a broader context, these findings provide a solid foundation (i.e., proof-of-principle) for the molecular umbrella concept for the transport of polar molecules across lipid bilayers.

Efforts currently in progress are aimed at synthesizing appropriate umbrella−peptide and umbrella−antisense oligonucleotide conjugates for in vitro studies.

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