(4), 328 (3), 191 (40), 177 (21), 166 (8), 165 (11), 149 (12), 135 (23), 123 (32), 121 (32), 109 (30), 107 (22), 105 (13), 95 (100), 93 (22), 91 (20), 81 (18), 79 (20), 77 (13).

Methylation of 3a To Give 3c. A mixture of 50 mg of **3a**, 5 mL of methyl iodide, and 20 mg of potassium carbonate in 4 mL of dry acetone was heated under reflux for 72 h. The reaction mixture was filtered, the solvent evaporated, and the residue partitioned between chloroform and water. The chloroform solution was dried (Na_2SO_4), the solvent evaporated, and the residue chromatographed over silica gel (hexane eluant) to give 10 mg of pure **3c**: mp 72–73 °C [from hexane–chloroform (1:1)]; ^1H NMR (300 MHz, $CDCl_3$) δ 0.90, 1.03 (each 3 H, s), 0.97 (3 H, d, J = 7 Hz), 2.45 (1 H, m), 2.55, 2.78 (each 1 H, d, $J \approx 13$ Hz), 3.72, 3.76 (each 3 H, s), 4.69, 4.71 (each 1 H, br s, $=CH_2$), 6.70–6.78 (3 H, m); MS (low resolution 70 eV), m/e (relative intensity) 342 (M^+, 2.7), 328 (5), 192 (3), 191 (15), 177 (5), 175 (3), 152 (44), 149 (6), 138 (68), 137 (20), 135 (17), 121 (28), 109 (26), 107 (15), 105 (7), 95 (100), 93 (12), 91 (10), 81 (15), 79 (13), 77 (9).

X-ray Analysis. Colorless crystals of the acetate **3b** were grown by evaporation from a chloroform–hexane solution. Data were collected by using a single crystal (0.08 × 0.15 × 0.35 mm) mounted on an Enraf-Nonius CAD-4 diffractometer with Ni-filtered Cu Kα radiation and a nitrogen-stream cooling device (temperature = 138 ± 2 K). Systematic absences established the space group $P2_12_12_1$. Lattice constants were determined from a least-squares fit to the $\pm 2\theta$ values of 48 intensity maxima (low temperature, a = 9.964 (5) Å, b = 6.81 (2) Å, c = 33.33 (5) Å, V = 2262.6 Å^3; room temperature, a = 10.133 (1) Å, b = 6.998 (2) Å, c = 32.971 (3) Å, V = 2337.9 Å^3, ρ_{calcd} = 1.132 for Z = 4). Data were collected by using the θ–2θ technique to a maximum 2θ = 150°. From 2720 unique data 2268 data were considered observed ($F > 4\sigma(F)$).

The structure was solved by the direct-methods program MULTAN.[12] The structure was refined by full-matrix least-squares techniques[13] using weights of $w = 1/\sigma^2(F)$. After initial anisotropic refinement, positions for all hydrogen atoms were determined from a difference electron density map. Hydrogen atoms were included in the structure factor calculations, but their position and temperature parameters were not refined. Refinement converged to R = 0.065, R_w = 0.066, and S = 2.51. The final difference map had a maximum density of 0.45 e/Å^3 near a methyl hydrogen. The next largest peak had a density of 0.25 e/Å^3. Neutral atom scattering factors for O and C were taken from Cromer and Mann.[14] Scattering factors for H were from Stewart, Davidson, and Simpson.[15]

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Registry No. **3a**, 87764-13-4; **3b**, 87764-14-5; **3c**, 87764-15-6; **4**, 87764-16-7.

Supplementary Material Available: Tables of atomic positions, thermal parameters, bond distances, and bond angles (6 pages). Ordering information is given on any current masthead page.

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Synthesis and Photochemical Studies of a Diazomalonyl-Containing Galactocerebroside Analogue. A Glycolipid Photolabeling Reagent[1]

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The synthesis of a galactocerebroside-based photolabeling reagent is described. The reagent is a structural analogue of the native lipid, containing a diazomalonamide group incorporated in the acyl chain. Photolysis studies in THF–H_2O resulted in the isolation and characterization of the water insertion product, demonstrating the molecule's capacity for intermolecular OH insertion. Photolysis of the reagent while incorporated into phospholipid vesicles gave the water insertion product, demonstrating that the bilayer membrane organization still permits access of water to the photogenerated carbene intermediate.

Galactocerebroside is a simple glycosphingolipid found in high concentrations in the myelin sheath, where it constitutes 20% by weight of the dry lipid.[2] In contrast to more complex glycosphingolipids, some of which have been proven to be cell receptors,[3] galactocerebroside seems to play a structural role in the neural membrane. Studies

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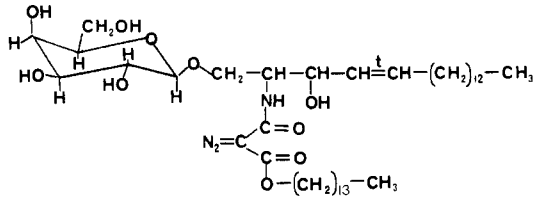
involving spin label,[4] ^{13}C NMR T_1 relaxation time,[5] thermotropic,[6] and bilayer exchange[7] techniques indicate that cerebroside interaction with phospholipids promotes stabilization of the bilayer system. There exists, however, no clear description of the interplay between cerebroside and neighboring phospholipid molecules.

One effective means of studying lipid organization in micelles[8a,b] and phospholipid bilayers[8c,9] consists of incorporating a lipid molecule containing a photoactivable group into the vesicles. Photolysis of the vesicles yields cross-linked photoproducts whose structure is indicative of the position of the photoreactive molecule with respect to other mebrane components.

With the intent to explore lipid organization in cerebroside-containing bilayer vesicles, we designed the galactocerebroside photolabeling reagent **1**, and we now report its synthesis.



1

The reagent is a close structural analogue of galactocerebroside and should result in relatively small perturbation of the bilayer packing. The photoactivable group, a diazomalonamide system incorporated into the fatty acyl chain, is situated close to the polar region of the molecule. As a result, the reagent should sample the preferred interactions near the bilayer surface. In addition, sequestering the photoactivable system in the rigid polar region of the bilayer retards diffusion of the reactive intermediate from the site of photolytic generation.

A highly reactive carbene is expected to be the major intermediate generated.[10] The diazomalonamide[11,12] system is not prone to the intramolecular rearrangements that have plagued previous attempts[13] to incorporate other photoactivable groups into an acyl chain. Thus, intermolecular covalent linkages should ensue upon photolysis. Initial photolysis studies using THF/H_2O as the solvent and phospholipid vesicles resulted in the isolation of significant amounts of water insertion product, confirming the probe's capacity for intermolecular insertion.

Experimental Section

General Methods. All reagents were ACS grade or better and were used as obtained from the commercial supplier unless otherwise noted. Infrared spectra were recorded on a Perkin-Elmer Model 735 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on one of the following spectrometers: Bruker WH-360 (360 MHz for ^1H, 90.6 MHz for ^{13}C), Bruker WM-250 (250 MHz for ^1H, 62.9 MHz for ^{13}C), or IBM WP-200 (200 MHz for ^1H, 50.3 MHz for ^{13}C). Direct chemical-ionization mass spectra were recorded on a VG 7070 instrument with ammonia as the ionizing gas. Ultraviolet spectra were recorded on a Beckman DU-8 spectrophotometer. Sonication of lipid dispersions was accomplished by using a Sonicator Cell Disruptor (Heat Systems Inc., Plainview, NY). Silica gel chromatographic separations were done by using a medium-presssure liquid chromatographic system (MPLC).[14] The columns were packed with Baker silica gel (40-μm average particle diameter). High-performance liquid chromatography (HPLC) was accomplished by using a Waters system. Semipreparative reverse-phase separations were performed on a Waters C_{18} μBondapak column (4.5 × 300 mm). Methanol solvent used in the HPLC system was once distilled. Distilled water used in this system was further purified by passage through a Barnstead water purification cartridge (Barnstead Co., Boston, MA). TLC plates were obtained either from E. Merck or Baker. Spots on the TLC plates were visualized with either phosphomolybdic acid/ethanol,[15] a general hydrocarbon oxidant, or molybdenum blue reagent,[16] a stain which is specific for phosphorus-containing compounds. Elemental microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Lipid Vesicle Components. Cholesterol was purchased from Eastman. Dipalmitoylphosphatidylglycerol, NH_4^+ salt (DPPG), was purchased from Sigma. Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC) were synthesized by procedures developed by Gupta et al.[17]

Psychosine (*trans*-D-*erythro*-$CH_3(CH_2)_{12}CH{=}CHCH(OH)CH(NH_2)CH_2O$-1-$\beta$-D-Galactose, 7). Galactocerebroside was isolated from bovine brain by using the method of Radin[18] except for the following changes in procedure. The lower phase after the final extraction was evaporated to dryness. The solid was redissolved in 1 L of $CHCl_3$–CH_3OH (98:2), filtered, and washed with water (2 × 1 L). The lower phase was again collected and evaporated to dryness. The solid material was chromatographed by using a 25 × 500 mm MPLC column containing 110 g of silica gel linked to a 25 × 1000 mm MPLC column containing 220 g of silica gel. The system was eluted with 1 L of $CHCl_3$–CH_3OH (98:2), followed by 3–4 L of $CHCl_3$–CH_3OH (86:14). Quantities of galactocerebroside obtained in this manner (5–6 g) were comparable to those reported by Radin[18] for pig brain isolations. Psychosine was obtained by basic hydrolysis of galactocerebroside by using the procedure of Radin[19] with the following modifications. The final extraction was omitted. The reaction mixture was adjusted to pH 7 with 5 N $HClO_4$, and the precipitate was removed by filtration. Evaporation of the solvent under vacuum yielded a solid material which was redissolved in 250 mL of $CHCl_3$–CH_3OH (98:2). This solution was washed with water (250 mL). The lower phase was filtered and condensed under reduced pressure. Purification was achieved using a 25 × 1000 mm MPLC column, eluting with 1 L of $CHCl_3$–CH_3OH (98:2), followed by 2.5 L of $CHCl_3$–CH_3OH (70:30). The yields of purified psychosine (3.0–3.6 g) were somewhat lower than those reported by Radin.[19]

Tetradecyl *N*-(Carbobenzyloxy)glycinate (3). *N*-(Carbobenzyloxy)glycine (20.9 g, 100 mmol) was dissolved in 800 mL

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of diethyl ether. To this solution was added dicyclohexylcarbodiimide (10.3 g, 50 mmol).[20] The solution was stirred for 20 min, and the precipitate was collected by filtration. Approximately 20 g of the white powder containing a mixture of the urea and the anhydride, **2**, was recovered after air drying of the precipitate. To a solution consisting of tetradecanol (1.0 g, 4.6 mmol) and 4-(dimethylamino)pyridine (0.57 g, 4 mmol) dissolved in 50 mL of acetonitrile was added 8 g of the anhydride/urea powder.[21] The reaction mixture was stirred for 30 min, and the undissolved solid was removed by filtration. The solvent was removed under vacuum, giving 3.5 g of crude, solid material. Purification was achieved by using a 15 × 500 mm MPLC column containing 40 g of silica gel. The column was eluted with CH_2Cl_2–diethyl ether (99:1) to yield 0.91 g (50% based on added tetradecanol) of the pure ester **3**: mp 61–62 °C; IR (CH_2Cl_2) 3425, 2900, 2825, 1725, 1510, 1460, 1390, 1360, 1205, 1160 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 6.5 Hz), 1.26 (m, 24 H), 3.97 (d, 2 H, J = 5.5 Hz), 4.14 (t, 2 H, J = 6.6 Hz), 5.12 (s, 2 H), 5.29 (br s, 1 H), 7.34 (s, 5 H). Anal. Calcd for $C_{24}H_{39}NO_4$: C, 71.07; H, 9.69; N, 3.45; O, 15.78. Found: C, 71.13; H, 9.74; N, 3.37; O, 15.71.

Tetradecyl Glycinate Hydrobromide (4). Tetradecyl *N*-(carbobenzyloxy)glycinate (3.70 g, 9.4 mmol) was dissolved in 25 mL of glacial acetic acid. Anhydrous hydrogen bromide gas[22] was bubbled through the solution until a white precipitate formed, and no further change could be detected. Diethyl ether (100 mL) was added to the solution followed by filtration. The precipitate was washed extensively with ca. 200 mL of diethyl ether and air-dried for 12 h, yielding 2.3 g (69%) of the crystalline hydrobromide salt. Recrystallization from diethyl ether–CH_3OH (1:1) provided an analytically pure sample of **4**: mp 88–89 °C. Anal. Calcd for $C_{16}H_{34}NO_2Br$: C, 54.54; H, 9.73; N, 3.97; O, 9.08. Found: C, 54.61; H, 9.75; N, 4.00; O, 8.93.

Tetradecyl Diazoacetate (5). The hydrobromide salt of tetradecyl glycinate (4.2 g, 12 mmol) was added to a K_2CO_3 solution (100 mL, 0.2 M) and stirred until the solid was converted to the liquid amine. The solution was extracted with $CHCl_3$ (2 × 50 mL) and the organic phase was dried over $MgSO_4$, filtered, and condensed in volume to ca. 50 mL. To this solution were added 1.2 equiv of isoamyl nitrite (1.8 mL, 14 mmol) and 0.2 equiv of acetic acid (0.14 mL, 2.4 mmol) followed by refluxing for 1 h.[23] The solution was washed with 10% K_2CO_3 (2 × 50 mL) and water (1 × 50 mL). The organic phase was dried over $MgSO_4$, filtered, and condensed. The residue was subjected to silica gel chromatography by using a 15 × 500 mm MPLC column containing 40 g of silica gel. The column was eluted with hexanes–CH_2Cl_2 (60:40) to yield 0.81 g (24% based on the HBr salt) of the green liquid diazo ester **5**: IR (CH_2Cl_2) 2900, 2825, 2075, 1690, 1460, 1400, 1360, 1190, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 6.5 Hz), 1.26 (m, 24 H), 4.15 (t, 2 H, J = 6.7 Hz), 4.73 (s, 1 H). Anal. Calcd for $C_{16}H_{30}N_2O_2$: C, 68.04; H, 10.71; N, 9.92; O, 11.33. Found: C, 68.06; H, 10.73; N, 9.96; O, 11.36.

***N*-(Tetradecyldiazomalonyl)-1-*O*-β-D-galactosyl-D-sphingosine (Galactocerebroside Photolabeling Reagent, 1).** To a solution of 12.5% phosgene in benzene (3 mL)[24] was added tetradecyl diazoacetate (0.282 g, 1 mmol), and the resulting solution was stirred at ca. 25 °C for 3 h. The excess phosgene and benzene were removed under vacuum, and the residue was added to 5 mL of $CHCl_3$–CH_3OH (9:1) containing psychosine (0.115 g, 0.25 mmol) and triethylamine (35 μL, 0.25 mmol). The reaction mixture was stirred for 15 min and condensed, and the residue was purified using a 9 × 500 mm MPLC column containing 16 g of silica gel. The column was eluted with $CHCl_3$–CH_3OH (86:14) to give 0.165 g (21% based on added diazo ester **5**) of a pale yellow solid (**1**). The photolabeling reagent was further purified for elemental analysis by using reverse-phase C_{18} HPLC, with CH_3OH–H_2O (87.5:12.5) as the mobile phase: IR (CH_2Cl_2) 3600, 3550–3300 (br), 2900, 2825, 2125, 1690, 1640, 1530, 1460, 1390, 1320, ($CDCl_3$–CD_3OD, 1120, 1070, 1040, 1010, 960, 870 cm^{-1}; UV (CH_2Cl_2) 257 nm (ϵ 6700); ^1H NMR (Me_2SO-d_6, 65 °C)[25] δ 0.87 (t, 6 H, J = 6.5 Hz), 1.10–1.36 (m, 44 H), 1.62 (m, 2 H), 1.96 (m, 2 H), 3.28–3.41 (m, 3 H), 3.43–3.59 (m, 3 H), 3.65 (br s, 1 H), 3.98–4.24 (m, 7 H), 4.39 (t, J = 5.8 Hz, OH),[26] 4.48 (m, OH),[26] 4.50 (m, OH),[26] 4.88 (d, J = 5.5 Hz, OH)[26] 5.40 (dd, 1 H, J = 5.8, 15.0 Hz), 5.53–5.71 (m, 1 H), 7.60 (d, 1 H, J = 8.6 Hz); ^{13}C NMR ($CDCl_3$–CD_3OD, 2:1) δ 14.18 (CH_2CH_3), 22.92 (CH_2CH_3), 26.04 ($OCH_2CH_2CH_2$), 28.85, 29.47, 29.61, 29.83, 29.91 (main CH_2), 32.19 (OCH_2CH_2), 32.64 (CH=CHCH$_2$), 54.63 (CHNH), 61.80 (Gal C-6), 66.31 (OCH_2CH_2), 67.48 (C=N$_2$), 69.21 (OCH_2CHNH, Gal C-4), 71.46 (Gal C-2), 72.04 (CHNHCHOH), 73.64 (Gal C-3), 75.44 (Gal C-5), 104.17 (Gal C-1), 128.79 (CH=CHCH$_2$), 134.99 (CH=CH-CH$_2$), 161.52 (CO_2), 164.45 (CONH). Anal. Calcd for $C_{41}H_{75}N_3O_{10}\cdot 1.5H_2O$: C, 61.78; H, 9.86; N, 5.27; O, 23.08. Found: C, 61.84; H, 9.60; N, 5.37; O, 23.39.

Photolysis of 1 in THF–H_2O Solvent. Isolation of the Water Insertion Product 8. The galactocerebroside photolabeling reagent **1** (50 mg, 63 μmol) was dissolved in 50 mL of THF–H_2O (80:20), and the resulting solution was warmed in a water bath (33 °C) while N_2 was bubbled through the liquid for 10 min prior to photolysis. The solution was irradiated under argon in a quartz tube equipped with a magnetic stirring bar at 254 nm for 30 min at 33 °C with continuous stirring (Rayonet RPR-100 photoreactor equipped with 16 RPR 2537-Å bulbs). The solvent was condensed under reduced pressure, and the photoproduct mixture was examined by TLC ($CHCl_3$–CH_3OH–H_2O, 70:30:5). The water insertion product, **8**, was separated from the mixture by using a 6 × 500 mm MPLC column containing 8 g of silica gel. The column was eluted with $CHCl_3$–CH_3OH (86:14) to yield 20 mg of **8** (40%). The isolated material was found to be a mixture of two unresolved epimers at the newly created chiral center, the malonyl α-carbon: IR (CH_2Cl_2) 3600–3050 (br), 2900, 2825, 1730, 1680, 1660, 1520, 1460, 1360, 1080 cm^{-1}; ^1H NMR (Me_2SO-d_6, 35 °C)[25] δ 0.87 (t, 6 H, J = 6.5 Hz), 1.24 (m, 44 H), 1.54 (m, 2 H), 1.95 (m, 2 H), 3.10–3.35 (H_2O, 3 Gal CH), 3.38–3.58 (m, 3 H), 3.63 (br s, 1 H), 3.72–3.88 (m, 1 H), 3.93–4.23 (m, 5 H), 4.32 (d, J = 4.0 Hz, OH),[26] 4.49 (br s, 1 H, COCH(OH)CO),[26] 4.54 (m, OH),[26] 4.65 (m, OH),[26] 4.78 (m, OH),[26] 4.89 (m, OH),[26] 5.40 (dd, 1 H, J = 5.8, 15.0 Hz), 5.52–5.70 (m, 1 H), 6.18 (d, J = 7.0 Hz, COCH(OH)CO epimer),[27] 6.37 (d, J = 7.0 Hz, COCH(OH)CO epimer),[27] 7.51–7.69 (m, 1 H); ^{13}C NMR ($CDCl_3$–CD_3OD, 2:1) δ 14.17 (CH_2CH_3), 22.97 (CH_2CH_3), 26.08 ($OCH_2CH_2CH_2$), 29.61, 29.67, 29.77, 29.89, 30.00 (main CH_2), 32.26 (OCH_2CH_2), 32.72 (CH=CHCH$_2$), 54.28 (CHNH, epimer), 54.36 (CHNH, epimer), 61.92 (Gal C-6), 66.65 (OCH_2CH_2), 68.39 (OCH_2CHNH, epimer), 68.53 (OCH_2CHNH, epimer), 69.49 (Gal C-4), 71.65 (Gal C-2, epimer), 71.71 (Gal C-2, epimer), 72.01 (CHNHCHOH, epimer), 72.12 (CHNHCHOH, epimer), 72.74 (COCH(OH)CO, epimer), 72.88 (COCH(OH)CO, epimer), 73.86 (Gal C-3, epimer), 73.91 (Gal C-3, epimer), 75.58 (Gal C-5), 104.18 (Gal C-1), 129.02 (CH=CHCH$_2$, epimer), 129.14 (CH=CHCH$_2$, epimer), 134.42, (CH=CHCH$_2$, epimer), 134.49 (CH=CHCH$_2$, epimer), 168.90 (CO_2, epimer), 169.90 (CO_2, epimer), 170.11 (CONH, epimer), 170.40 (CONH, epimer); DCI mass spectrum (NH_4^+), m/e (relative intensity) 777 (M^+ + 18, 2.0) 742 (M^+ – OH, 4.5), 598 (4.0), 580 (3.7), 564 (2.7), 472 (28.4), 343 (31.9), 334 (22.5), 310 (23.1), 290 (90.9), 264 (65.9), 256 (77.2), 198 (97.5), 179 (100.0), 162 (72.7), 144 (51.3). Anal. Calcd for $C_{41}H_{77}NO_{11}$: C, 64.79; H, 10.21; N, 1.84; O, 23.15. Found: C, 64.71; H, 10.37; N, 1.65; O, 23.44.

Small Unilamellar Vesicles (SUV) and Multilamellar Vesicles (MLV). SUV were prepared similarly to the method of Huang.[28] The lipid components (including GCP; see Table I) of the vesicle were dissolved in $CHCl_3$–CH_3OH (2:1), and the solvent was removed under vacuum. The resulting lipid film was dispersed in buffer solution (0.1 M KCl, 2 mM K_2HPO_4) by a combination of mechanical shaking and vortexing. The final concentration of lipid was ca. 10 mg/mL. The suspension was

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(26) Integration was less than 1 H due to exchange with residual water in the solvent.
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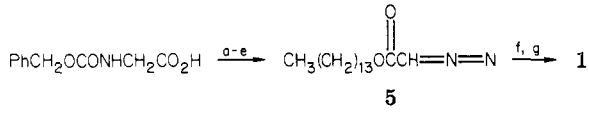
Scheme I. Preparation of Photolabeling Reagent 1[a]



[a] (a) Dicyclohexylcarbodiimide; (b) $CH_3(CH_2)_{13}OH$, 4-(dimethylamino)pyridine; (c) HBr, CH_3CO_2H; (d) K_2CO_3; (e) $(CH_3)_2CHCH_2CH_2ONO$; (f) $COCl_2$, C_6H_6; (g) *trans*-D-*erythro*-$CH_3(CH_2)_{12}CH{=}CHCHOHCHNH_2CH_2O$-1-$\beta$-D-galactose (7), Et_3N, $CHCl_3$-CH_3OH.

sonicated for 30–60 min during which the temperature of the suspension was allowed to rise to ca. 10 °C above the T_c of the highest melting phospholipid. The vesicle suspension was centrifuged at 27 000 g for 20 min, and the resulting supernatant was put through a 0.4-μm Gelman filter.

MLV were prepared by the same procedure as for SUV, except that the procedure was terminated after the combination of vortexing and mechanical shaking, and the final concentration was adjusted to ca. 5 mg/mL. During vortexing and shaking, the temperature of the suspension was raised to ca. 10 °C above the T_c of the highest melting phospholipid in the mixture by warming the vessel in a water bath. (No sonication was carried out.) The resulting suspension was used directly for the photolysis studies.

Photolysis of Vesicle Suspensions. The vesicle suspension was incubated in a water bath at the photolysis temperature for 15 min; during this period N_2 was bubbled through the suspension to remove any dissolved O_2. The vesicle suspension was then photolyzed in quartz tubes under argon, as described for the THF–water photolysis above, for a period of 30 min in the case of SUV and 1–2 h in the case of MLV. The reaction progress was followed by monitoring the disappearance of photolabeling reagent by TLC ($CHCl_3$–CH_3OH–14.8 M NH_4OH, 70:30:5). After photolysis, the vesicle suspension was extracted three times with a volume of $CHCl_3$–CH_3OH (2:1) equal to the volume of the buffer, and the organic layer was condensed to yield the crude photoproduct mixture. The mixture was examined by TLC using four solvent systems: CH_2Cl_2–acetone (9:1), $CHCl_3$–CH_3OH (9:1), $CHCl_3$–CH_3OH–14.8 M NH_4OH (70:30:5), $CHCl_3$–CH_3OH–H_2O (65.25:4). Chromatographic separation and isolation of the water insertion product were strictly analogous to the methods described in the THF–H_2O photolysis section.

Results

Synthesis of the Galactocerebroside Photolabeling Reagent 1. The pivotal step in the synthesis of 1 (Scheme I) was the preparation of tetradecyl diazoacetate (**5**), which could then be converted to the corresponding malonyl chloride and subsequently coupled to the deacylated cerebroside derivative **7**. *N*-(Carbobenzyloxy)glycine was allowed to react with dicyclohexylcarbodiimide to give the corresponding anhydride, **2**, that coprecipitated with dicyclohexylurea. The urea/anhydride powder was allowed to react with tetradecanol in the presence of (dimethylamino)pyridine, affording the *N*-carbobenzyloxyglycinate ester **3**. Removal of the *N*-Cbz protecting group (HBr, acetic acid)[22] resulted in the isolation of the HBr salt of the amino ester, **4**. Neutralization of the salt followed by reaction with isoamyl nitrite–acetic acid ($CHCl_3$)[23] gave tetradecyl diazoacetate (**5**) in 20–25% yield from **4**.

Conversion of **5** to the malonyl chloride **6** was accomplished by allowing the diazo ester to react with excess phosgene.[24] The final coupling reaction to yield the photolabeling reagent was accomplished by combining the malonyl chloride (**6**) with the deacylated cerebroside derivative, psychosine (**7**). Selective acylation of the amino residue was achieved as a result of the deactivating influence of the conjugated diazomalonyl system on the reactivity of the acid chloride.

Photolysis of 1 in THF–H_2O Solvent. Isolation and Characterization of the Water Insertion Product 8. Photolysis of **1** in THF-H_2O solvent resulted in a photoproduct mixture which resolved into five components when examined by TLC. The water insertion product, accounting for 40% of the mass of the photolabeling reagent, was the only photoproduct that proved to be stable enough for structural characterization. Attempts to isolate the remaining photoproducts proved futile, as these disintegrated into mixtures of new components when exposed to preparative silica gel chromatography.

The proposed structure of the isolated product is the product of water addition to the carbene **8**, having a CO–CHOH–CO group in place of the diazomalonyl group in **1**. The proof of structure was based largely on direct comparisons of both the ^1H NMR and ^{13}C NMR spectra of **8** with the spectra of **1**, galactocerebroside, and model compounds. As indicated by a pair of doublets at 6.18 and 6.37 ppm in the ^1H NMR spectrum (Me_2SO-d_6[25]), the isolated water insertion product consisted of a mixture of two unresolved epimers corresponding to the two possible stereoisomers resulting from OH insertion into water. The 6.18- and 6.37-ppm resonances were assigned to the epimeric hydroxy protons since these resonances did not appear in the spectrum of **1** or galactocerebroside. Further substantiation of the assignment was achieved by addition of D_2O to the NMR sample containing **8**, resulting in the disappearance of both of these resonances from the spectrum. The malonyl CH proton was assigned to a broad singlet resonance at 4.49 ppm on the basis of decoupling studies which demonstrated the collapse of the epimer OH doublet resonances to singlets when the 4.49-ppm resonance was irradiated. Both the direct chemical-ionization mass spectrum and the elemental analysis were completely consistent with the proposed structure.

Photolysis of 1 in Phospholipid Vesicles. Given that the water-insertion product predominated in THF–H_2O but that other (unstable) products were also observed, preliminary photolyses of **1** in lipid bilayer vesicles were carried out. We wanted to determine (a) whether the bilayer organization would create an environment around **1** capable of generating CH as well as OH insertion and (b) whether other OH insertion products would form in competition with water insertion when **1** is photolyzed in vesicles composed of lipids containing OH groups positioned near the bilayer surface.

Vesicles containing **1** were, therefore, photolyzed at temperatures above the T_c of the constituent phospholipid. The resulting photoproduct mixtures were analyzed by TLC using several solvent systems spanning a large polarity range. The TLC plates were cospotted with samples of the original lipid components to aid in the identification of the photoproducts. Preparative separation of the lipid components was accomplished by using MPLC silica gel chromatography. The vesicle photolysis experiments are shown in Table I.

Although photolysis studies of model compounds[11] had demonstrated that the malonyl carbene favored OH over CH insertion by a factor of ca. 10^4, CH insertion had been found when the carbene was sequestered in a hydrophobic environment, e.g., in cyclohexane solution. Therefore, DPPC vesicles were chosen to determine whether CH groups situated near the head-group region of the molecule would shield the carbene contained in the GCP from water molecules at the bilayer surface. A cross-link between the GCP and a phospholipid molecule could then ensue. It was found that only the water insertion product could be isolated from photolysis of GCP in small unilamellar vesicles (SUV). Since SUV have a substantial bilayer curvature, it was possible that multilamellar vesicles (MLV), which are larger and have little bilayer curvature,

Table I. Photolysis of Galactocerebroside Photolabeling Reagent 1 in Bilayer Vesicles

vesicle composition,[a] mol/mol	vesicle type	photolysis temp, °C	isolated photoproduct
9:1 DPPC-GCP	SUV	53	water insertion, 8
9:1 DPPC-GCP	MLV	53	8
9:1 DOPC-GCP	SUV	33	8
4.5:4.5:1 DPPC-cholesterol-GCP	SUV	53	8
9:1 DPPG-GCP	SUV	53	8

[a] DPPC = dipalmitoylphosphatidylcholine, DOPC = dioleoylphosphatidylcholine, DPPG = dipalmitoylphosphatidylglycerol, and GCP = galactocerebroside photolabeling reagent, 1.

would exclude water. However, photolysis of GCP in DPPC MLV also gave 8 as the only isolable photoproduct. Since GCP has an acyl chain, containing the diazo group, that is 18 atoms long, its incorporation into vesicles composed of phospholipids having acyl chains composed of 16 carbon atoms might be disruptive to the packing of the lipids within the vesicle. Therefore, incorporation of GCP into vesicles containing longer chain phospholipids would be less disruptive to the lipid packing and could result in a more effective exclusion of water from the bilayer interior. Nevertheless, photolysis of GCP incorporated into DOPC vesicles, having 18-carbon chains, again yielded only 8.

The predominance of OH insertion suggested that incorporation of hydroxy lipids into vesicles containing GCP might result in a cross-link between the lipid and the GCP via insertion into the lipid OH. DPPG and cholesterol were incorporated into vesicles with 1 to test this hypothesis. Both of these lipids have hydroxy groups situated in the polar region of the bilayer. While alcohols[11] have been proven to be effective competitors with water for the carbene, careful examination of the photoproduct mixture in both instances again demonstrated the water insertion product 8 to be the only isolable product.

Discussion

The effectiveness of a photolabeling reagent rests in its ability to generate covalent cross-links to other molecules in its immediate environment. A complex molecule such as GCP, containing several functional groups, could conceivably give only intramolecular photoproducts, but the isolation of the water insertion product clearly establishes GCP's capacity for forming intermolecular, covalent links to hydroxy groups. Model studies[11] have demonstrated that an alcohol hydroxy group competes effectively with the water hydroxy group for the carbene; therefore, the potential exists for GCP to form intermolecular cross-links to other hydroxy-containing substrates.

The model studies[11] also demonstrated the malonamide carbene's capacity for CH insertion. However, the large OH/CH selectivity ratio dictates that the yield of CH insertion product will remain undetectably low on using TLC or spectroscopic methods when OH residues are also present in the environment. An exception to this conclusion results if the CH bond is α to an oxygen, in which case the CH residue is activated toward insertion.[29] Insertion into the activated α-methylene residues probably accounts for some of the unstable photoproducts found when GCP was photolyzed in THF/water as the solvent.

From the phospholipid vesicles studied so far only the water insertion product could be isolated after photolysis, demonstrating that membrane bilayer organization does not preclude access of water to the photogenerated carbene intermediate. Increasing the lipid chain length (DPPC vs. DOPC) did not preclude water access, nor did the addition of cholesterol, which is known to have a condensing effect upon bilayer organization,[30] nor did the use of MLV. MLV are expected to have tighter lipid packing because of their lower curvature, and in one instance, they have been shown to give increased specificity in photolysis of incorporated molecules containing a benzophenone moiety.[8c]

The high OH/CH insertion selectivity observed in our model studies[11] suggests that appropriately situated hydroxy or other reactive functional groups should be capable of competing with water for the photogenerated carbene. For this reason, cholesterol was added to GCP–DPPC SUV, and GCP was incorporated into phosphatidylglycerol SUV, but, again in each case, only the water insertion product could be isolated after photolysis. Therefore, it must be concluded that these lipid hydroxy groups are not "properly situated" to trap the carbene. Four possible explanations can be proposed to account for the photochemical behavior of the glycolipid probe inside the bilayer. (1) Water is present in the bilayer to a depth that permits it to trap the carbene or can penetrate to that depth rapidly enough to trap the carbene after it is formed. (2) The hydroxy groups of cholesterol and DPPG are so highly solvated by water that the carbene tends to be trapped by water even if these lipid hydroxy groups are nearby. (3) The carbene is actually generated in, or capable of moving into, a water-rich region at the bilayer surface. (4) The reagent GCP is laterally phase separated from other lipids on the bilayer surface, so that the carbene has minimal contact (only at phase boundaries) with the other lipid(s) present in the bilayer.

Lateral phase separation seems unlikely in view of data on cerebrosides, including our ^{13}C NMR T_1 studies[5] as well as thermotropic studies,[6] which show that they do not undergo lateral phase separation in lipid vesicles at the 10 mol % concentrations we have employed. Even complexes of two GCP molecules might inhibit access of the carbene to other lipid molecules, however, and further evidence would be needed to establish the membrane properties of GCP more fully.

Although explanation 2 or 3 cannot be ruled out at present, we expected to obtain *some* evidence of OH insertion into cholesterol or DPPG if the carbene were actually able to be present at the membrane surface where these two lipid OH groups are located. Since no evidence for these other OH insertion products has been found as yet, the most reasonable interpretation seems to be explanation 1, water penetration into the bilayer. Such water penetration might be enhanced by the polarity of the diazomalonyl group, but thermotropic studies[31] of galactocerebroside have suggested that a lateral hydrogen bonding network containing water molecules exists in the headgroup region of cerebroside bilayers.

If water does penetrate bilayers this far, it is still possible that biomembranes have other components which prevent or lessen water penetration. Our reagent could be radio-

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labeled to permit more sensitivity in detection of cross-linking in the present bilayer systems as well as those containing other lipid types. Cross-linking to specific integral or peripheral membrane proteins could also be studied; peptide segments residing near the membrane surface could be identified.[32] In fact, protein amino or thiol functional groups may be reactive enough relative to OH[33] to compete effectively with water, a possibility that needs to be tested in model studies analogous to those already reported.[11]

Conclusion

We were particularly interested in synthesizing and determining the properties of a membrane photolabeling reagent which has a diazo group *within* a fatty acid chain analogue, and we chose initially to place the diazo group near the highly organized polar–nonpolar interface region of lipid bilayers. Our initial experiments with the galactocerebroside photolabeling reagent we have synthesized showed (a) that the bilayer systems so far studied did not exclude water from the photogenerated carbene, so that water insertion was predominant, and (b) that the OH groups of cholesterol or phosphatidylglycerol incorporated into bilayers still did not compete with water. Water would not necessarily be capable of penetrating a bilayer membrane in sufficient quantity or at a sufficient rate to give such a predominance of water insertion. Though further proof is needed, the present results suggest that water may actually be incorporated in the membrane structure to the depth of the photogenerated carbene.

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Cyclization of 2-[*N*-(Methylsulfonyl)anilino]acetaldehyde Diethyl Acetals to Indoles. Evidence for Stereoelectronic Effects in Intramolecular Electrophilic Aromatic Substitution

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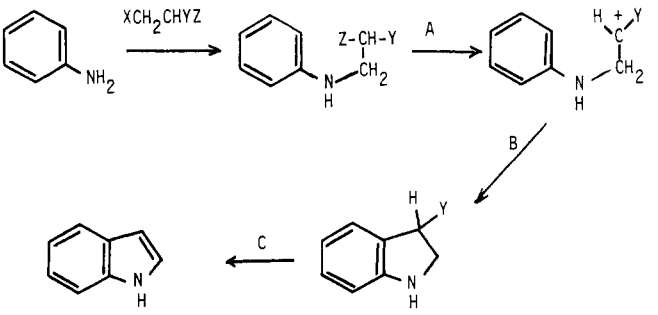
Methanesulfonamides of *N*-(2,2-diethoxyethyl)anilines can be cyclized to indoles in aromatic solvents by reaction with titanium tetrachloride. The temperature of the cyclization is substituent dependent, occurring at 0 °C for the *m*-methoxy derivative but requiring 130 °C for the *p*-bromo compound. Yields are good for various alkoxy-, alkyl-, and haloindoles, ranging from 60% to 90%. Meta-substituted reactants give rise to mixtures of 4- and 6-substituted indoles in which the 6-substituted product dominates by 2–4:1. The cyclization fails for ortho-substituted reactants. The major reaction process is N-dealkylation in the case of ortho-substituted compounds. An analogous cyclization occurs with the methanesulfonamides of *N*-(3,3-diethoxypropyl)anilines to give 1-(methylsulfonyl)-4-chloro-1,2,3,4-tetrahydroquinolines. This cyclization is much more rapid than for the five-membered ring closure leading to indoles and indicates a substantial rate retardation due to stereoelectronic effects in the indole cyclization. Ortho substitution also prevents cyclization in the six-membered-ring case.

The efficient conversion of anilines to indoles is a useful synthetic objective. The classical Fischer synthesis via the diazonium ion and aryl hydrazones is quite general but is usually not applicable for 2,3-unsubstituted indoles.[1] Gassman developed a method for conversion of substituted anilines to indoles that has good generality for 2,3-unsubstituted indoles and is based on the Sommelet rearrangement of anilinosulfonium ions.[2] Both of these methods depend on a sigmatropic rearrangement to effect the crucial ortho substitution. Most other syntheses of 2,3-unsubstituted indoles depend upon having appropriate ortho disubstitution built in to the reactant.[3]

Scheme I



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