Bolalipid Membrane Structure Revealed by Solid-State ^{2}H NMR Spectroscopy

David P. Holland[1], Andrey V. Struts[2], Michael F. Brown[2], and David H. Thompson[1*]

[1]*Department of Chemistry, Purdue University, West Lafayette Indiana 47907 and*
[2]*Department of Chemistry, University of Arizona, Tucson, Arizona 85721*

Supporting Information

Sample Preparation: Bolalipids were lyophilized from water prior to their addition to an NMR tube. ^{2}H-depleted ^1H_2O was then added to make a 50% w/w mixture. This sample was subjected to 10 freeze (liquid nitrogen), thaw (50 ^{o}C), and fracture (30 sec vortex) cycles before recording the ^{2}H NMR spectra. Sample tubes were closed with Teflon plugs.

^{2}H-NMR Spectroscopy: Solid-state ^{2}H NMR spectra were recorded on Bruker AMX300 spectrometer with a wide bore magnet (7 T) equipped with custom-built high-power probe. The ^{2}H NMR probe had an 8-mm diameter x 20-mm long transverse solenoidal coil with high-voltage capacitors, and provided a 3-3.5 μs 90^o pulse at 46.06 MHz. A quadrupolar echo sequence with composite pulses ($135^\circ_{-x}\,90^\circ_{x}45^\circ_{-x} - \tau - 135^\circ_{y}90^\circ_{-y}45^\circ_{y} - \tau - \text{acquisition}$) was applied with appropriate phase-cycling to accumulate the ^{2}H NMR signals. To minimize acoustical ringing of the radiofrequency coil, the length of the delay τ varied from 10 to 100 μs depending on the sample temperature. The data were processed with software written in-house in the MATLAB (The MathWorks, Inc., Natick, MA) environment.

Data Analysis: The ^{2}H NMR powder-type spectrum were numerically deconvoluted, or de-Paked, to generate spectra for the $\theta = 0°$ orientation as described elsewhere.[1] The measured quadrupolar splittings from the de-Paked ^{2}H NMR spectra were used to calculate the C-D bond order parameters, $S_{\mathrm{CD}}^{(i)}$ using the relation:

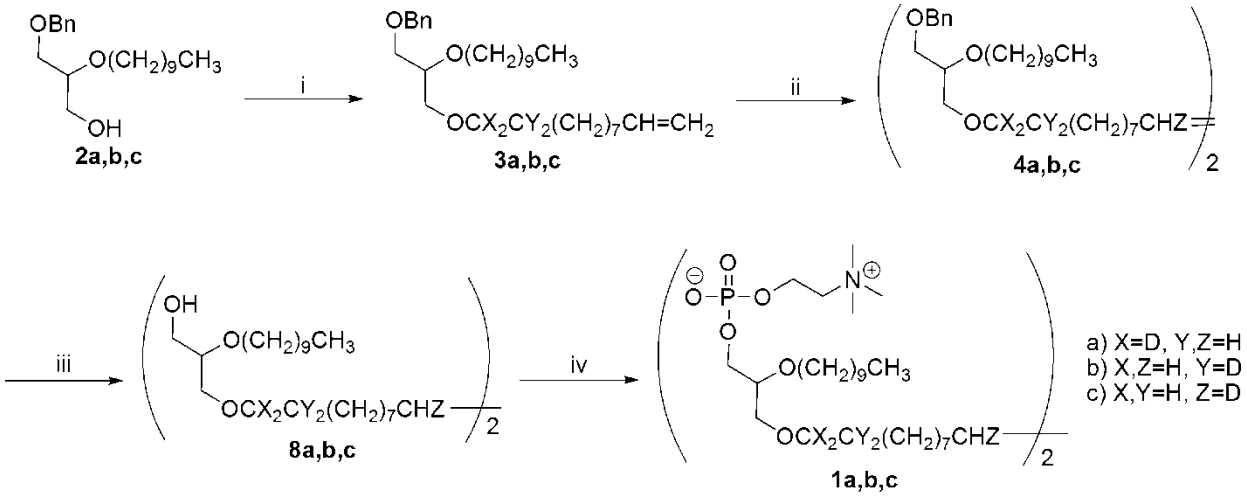
$$|\Delta\nu_Q^{(i)}| = \tfrac{3}{2}\,\chi_Q |S_{\mathrm{CD}}^{(i)}||P_2(\cos\theta)|,$$

where $\chi_Q \equiv (e^2qQ/h)$ = 167 kHz and θ is the angle between the static magnetic field and the bilayer director axis. The first-order mean-torque model was applied to calculate the apparent partial specific area at the aqueous interface of the 20-carbon chain at the *sn*-1 position, together with the hydrophobic layer thickness (D_C) of the membrane. This model utilizes the plateau region of the order profile close to the glycerol backbone of the lipid, where all of the methylene segments have a similar degree of order and give rise to unresolved splittings, to approximate D_C.[2] We can apply this model to bolalipids which behave similarly to monopolar lipids in this region. We note the assumption that the membrane-spanning *sn*-1 chain and the shorter *sn*-2 chain both detect the same volumetric membrane thickness. The total area per lipid (<A>) is approximated from D_C and the estimated volumetric data for the methylene segments (V_{CH_2}=

26.5 Å^3) and terminal methyl groups ($2V_{CH_2} \approx V_{CH_3}$) through the relationship <A> = Hydrocarbon Volume/ D_C.

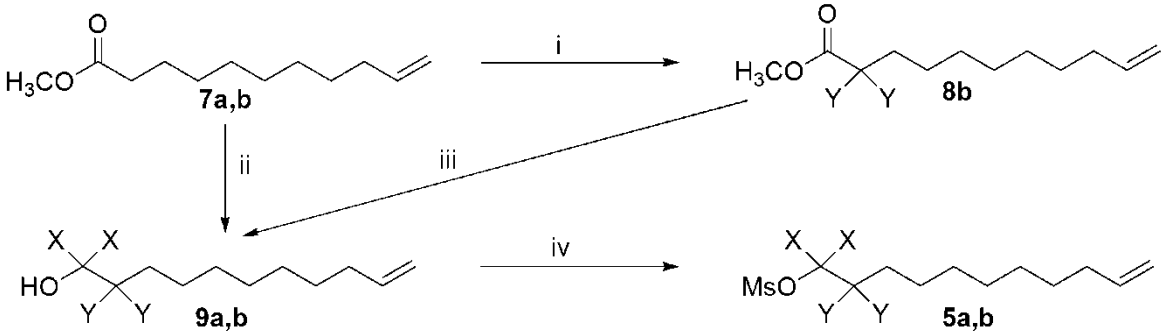
Synthesis. All chemicals were purchased from Sigma-Aldrich. All solvents except DMF were reagent grade and were distilled under argon before use: tetrahydrofuran (THF) from benzophenone ketyl, dichloromethane and triethylamine (Et_3N) from calcium hydride (CaH_2). Dimethylformamide (DMF) was purified by passage through an activated column prior to use (activated alumina and supported copper redox catalyst reactant). All reactions were performed under inert (argon or nitrogen) gas unless otherwise stated. NMR spectra were recorded on either Varian 300 MHz or Bruker 400 or 500 MHz spectrometers. Column chromatography was typically performed on 60 Å silica gel using HPLC grade eluents. Thin-layer chromatography was performed using Silicycle ultra pure silica gel thin layer chromatography plates and visualized using UV, I_2 adsorption, and/or sulfuric acid (20%) with heat. Compounds **2** and **5c** have been previously synthesized.[3]

Scheme 1: Synthesis of [1′,1′,20′,20′-$^2H_4$]C_{20}BAS-PC, [2′,2′,19′,19′-$^2H_4$]C_{20}BAS-PC, and [10′,11′-$^2H_2$]C_{20}BAS-PC.



(i) NaH,THF, (**5a**) $CH{=}CH_2(CH_2)_8CH_2CD_2OMs$ for **1a**, (**5b**) $CH{=}CH_2(CH_2)_8CD_2CH_2OMs$ for **1b**, and (**5c**) $CH{=}CH_2(CH_2)_8CH_2CH_2OMs$ for **1c**; (ii) $[(PCy_3)_2Ru{=}CHPh]Cl_2$, CH_2Cl_2, reflux; (iii) (**8a,8b**) H_2/Pd(OH)$_2$, 4:1 THF:ETOH (**8c**) D_2 H_2/Pd(OH)$_2$ 4:1 THF:ETOH; (iv) a. $ClP(O)(CH_2O)_2$, THF, Et_3N, b. DMF, Me_3N, 65 ^oC, 3 days.

Scheme 2: Synthesis of intermediates 5a and 5b.



(i) NaO^{t}Bu,DO^{t}Bu; (ii) LAH, THF 0 $^oC \rightarrow 25$ ^oC; (iii) LAD, THF 0 $^oC \rightarrow 25$ ^oC; (iv) MsCl, Et_3N, CH_2Cl_2.

1-O-Benzyl-2-decoxy-3-O-(10′undecenoxy)-rac-glycerol (**3c**). To a 1 L flask were added NaH (2.7 g, 106.9 mmol) and dispersed in 400 mL of THF and cooled to 0 ^{o}C. **2** (18.0 g, 55.9 mmol) was added and allowed to mix for 1 hour (once all the hydrogen gas had evolved). **5c** (17.6 g, 73.8 mmol) was then added to the flask and stirred overnight while heating under reflux. The mixture was quenched with water (200 mL) after cooling to room temperature. The mixture was extracted with ether (3 x 200 mL), dried over anhydrous $MgSO_4$ and filtered, and the solvent evaporated. The crude oil was then purified using silica gel column chromatography eluting with 9:1 hexane/Et_2O as eluent. Fractions collected at R_f = 0.76 were pooled, the solvent was removed via rotary evaporation, and then the residue was dried under vacuum to give 20.3 g (77% yield) of **3c**. ^{1}H (300 MHz, $CDCl_3$): 0.88 (t, 3H, CH_3), 1.26 (m, 26H, CH_2), 1.57 (m, 4H, β-CH_2), 2.02 (m, 4H, CH_2-C=C), 3.40-3.60 (m, 9H, -OCH_2, -OCH), 4.54 (s, 2H, -OCH_2Ph), 4.90-4.96 (m, 2H, C=CH_2), 5.78 (m, 1H, CH=C), 7.28 (m, 5H, C_6H_5).

1-O-Benzyl-2-decoxy-3-O-(1′,1′,20′,20′-$^2H_4$-10′-undecenoxy)-rac-glycerol (**3a**). This product was prepared in 50% yield as described for **3c** using **2** (2.68 g, 8.32 mmol), 95% NaH (238 mg, 9.42 mmol), and **5b** (2.11 g, 8.77 mmol). ^{1}H (300 MHz, $CDCl_3$): 0.99 (t, 3H, C2H_3), 1.38 (m, 26H, CH_2), 1.66 (m, 4H, β-CH_2), 2.15 (m, 2H, CH_2-C=C), 3.58-3.70 (m, 7H, -OCH_2, -OCH), 4.65 (s, 2H, -OCH_2Ph), 5.01-5.12 (m, 2H, CH_2=C), 5.89 (m, 1H, CH=C), 7.39 (m, 5H, C_6H_5).

1-O-Benzyl-2-decoxy-3-O-(2′,2′,19′,19′-$^2H_4$-10′-undecenoxy)-rac-glycerol (**3b**). This product was prepared in 52% yield as described for **3c** using **2** (3.78 g, 11.71 mmol), 95% NaH (440 mg, 17.42 mmol), and **5b** (3.84 g, 15.98 mmol). ^{1}H (300 MHz, $CDCl_3$): 0.95 (t, 3H, CH_3), 1.34 (m, 26H, CH_2), 1.66 (m, 2H, β-CH_2), 2.12 (m, 2H, CH_2-C=C), 3.48 (s, 2H, -OCH_2CD_2), 3.50-3.69 (m, 7H, -OCH_2, OCH), 4.62 (s, 2H, OCH_2Ph), 4.98-5.09 (m, 2H, C=CH_2), 5.81-5.92 (m, 1H, CH=C), 7.37 (m, 5H, C_6H_5).

1,1′-Di-O-benzyl-2,2′-di-O-decyl-3,3′-di-O-(10′-eicosenenyl)-bis-(rac-glycerol) (**4c**). To a 500 mL flask was added **3c** (20.3 g, 42.7 mmol) and dissolved in dry CH_2Cl_2 (200 mL). Bis(tricyclohexylphosphine) benzylidine ruthenium(IV) dichloride ([$(PCy_3)_2$Ru=CHPh]Cl_2) (1.1 g, 1.3 mmol) was added to the flask. The reaction mixture was heated under reflux for 12 hours, cooled, and then quenched with water. The mixture was extracted with diethyl ether (3 x 100 mL), and the combined organic phases were dried over anhydrous $MgSO_4$, filtered, and concentrated on a rotary evaporatory. The resulting brown oil was separated using silica gel chromatography with a 19:1 hexanes/Et_2O as the initial eluent, followed by 9:1. Fractions at R_f = 0.27 were pooled, the solvent was removed via rotary evaporation, and then the residue was dried under vacuum to give 15.2 g (77% yield) of **4c**. ^{1}H (300 MHz, $CDCl_3$): 0.98 (t, 6H, CH_3), 1.37 (m, 52H, CH_2), 1.65 (m, 8H, β-CH_2), 2.06 (m, 4H, CH_2-C=C), 3.50-3.69 (m, 18H, -OCH_2, -OCH), 5.48 (m, 2H, CH=CH), 7.39 (m, 10H, C_5H_6).

1,1′-Di-O-benzyl-2,2′-di-O-decyl-3,3′-di-O-(1′,1′,20′,20′-$^2H_4$-10′-eicosenenyl)-bis-(rac-glycerol) (**4a**). This product was prepared in 81% yield as described for **4c** using **3a** (1.98 g, 4.16 mmol) and ([$(PCy_3)_2$Ru=CHPh]Cl_2) (102 mg, 0.70 mmol). ^{1}H (400 MHz, $CDCl_3$): 0.88 (t, 6H, CH_3), 1.27 (m, 52H, CH_2), 1.56 (m, 8H, β-CH_2), 1.96 (m, 4H, CH_2-C=C), 3.47-3.63 (m, 14H, -OCH_2, OCH), 4.56 (s, 4H, OCH_2Ph), 5.39 (m, 2H, CH=CH), 7.29 (m, 10H, C_6H_5).

1,1′-Di-O-benzyl-2,2′-di-O-decyl-3,3′-di-O-(2′,2′,19′,19′-$^2H_4$-10′-eicosenenyl)-bis-(rac-glycerol) (**4b**). This product was prepared in 67% yield as described for **4c** using **3b** (2.70 g, 5.67 mmol) and ([$(PCy_3)_2$Ru=CHPh]Cl_2) (177 mg, 1.22 mmol). ^{1}H (300 MHz, $CDCl_3$): 0.89 (t, 6H, CH_3), 1.27 (m, 52H, CH_2), 1.58 (m, 4H, β-CH_2), 1.98 (m, 4H, CH_2C=C), 3.42 (s, 4H, -OCH_2CD_2), 3.44-3.62 (m, 14H, -OCH_2, OCH), 4.56 (s, 4H, -OCH_2Ph), 5.39 (m, 2H, CH=C), 7.33 (m, 10H, C_6H_5).

2,2′-*Di-O-decyl-3,3′-di-O-(10′,11′-$^2H_2$-eicosanyl)-bis-(rac-glycerol)* (**6c**). To a 500 mL flask was added **4c** (4.85 g, 5.26 mmol), dissolved in 4:1 THF:EtOH (250 mL); $Pd(OH)_2$ (438 mg 20% Pd-C) was then added. The system was evacuated and a double balloon filled with deuterium gas was added and allowed to bubble through the mixture, while stirring. The balloon was replenished three times and allowed to stir for 8 hours. The solution was filtered through a pad of celite and evaporated to give 1.16 g of product (29%) of **5c**. ^{1}H (300 MHz, $CDCl_3$): 0.88 (t, 6H, CH_3), 1.26 (m, 58H, CH_2), 1.55 (m, 8H, β-CH_2), 2.29 (broad s, 2H, -OH), 3.41-3.71 (m, 18H, -OCH_2, -OCH).

2,2′-*Di-O-decyl-3,3′-di-O-(1′,1′,20′,20′-$^2H_4$-eicosanyl)-bis-(rac-glycerol)* (**6a**). This product was prepared in 83% yield as described for **6c** using **4a** (1.56 g 1.69 mmol), $Pd(OH)_2$ (257 mg 20% Pd-C), and hydrogen gas to yield 1.26 g. ^{1}H (300 MHz, $CDCl_3$): 0.96 (t, 6H, CH_3), 1.34 (m, 60H, CH_2), 1.65 (m, 8H, β-CH_2), 2.27 (t, 2H, -OH), 3.54-3.81 (m, 14H, -OCH_2, -OCH).

2,2′-*Di-O-decyl-3,3′-di-O-(2′,2′,19′,19′-$^2H_4$-eicosanyl)-bis-(rac-glycerol)* (**6b**). This product was prepared in 84% yield as described for **6c** using **4b** (210 mg 0.23 mmol), $Pd(OH)_2$ (94 mg 20% Pd-C), and hydrogen gas to yield 143 mg. ^{1}H (300 MHz, $CDCl_3$): 0.89 (t, 6H, CH_3), 1.26 (m, 60H, CH_2), 1.58 (m, 4H, β-CH_2), 2.23 (t, 2H, -OH), 3.43-3.72 (m, 18H, -OCH_2, -OCH).

2,2′-*Di-O-decyl-3,3′-di-O-(10′,11′-$^2H_2$-eicosanyl)-bis-(rac-glycerol)-1,1′-diphosphocholine* (**1c**) ([10′,11′-$^2H_2$]C_{20}BAS-PC). **5c** (503 mg, 0.675 mmol), previously azeotroped in toluene and dried in *vacuo*, was dissolved in 5 mL THF and put on an ice bath. To this mixture, Et_3N (0.400 mL, 2.87 mmol) and $ClP(O)(CH_2O)_2$ (0.250 mL, 2.72 mmol) was added and allowed to stir at 0 ^{o}C for 15 minutes. This mixture was transferred into a flame-dried pressure tube with 5 mL dry DMF. Trimethylamine was condensed into the pressure tube at -78 ^{o}C; the pressure tube was then sealed and heated at 65 ^{o}C for 2 days. The mixture was dissolved in a 1:1 mixture of $CHCl_3$:MeOH and condensed. The resulting solid was purified using silica gel chromatography using the following series of eluents ($CHCl_3$, MeOH, H_2O): 80:20:0, 50:50:0, 0:100:0, 65:35:5, 60:40:10. Fractions at R_f = 0.2 (60:40:10 $CHCl_3$: MeOH: H_2O eluent) were pooled and condensed to yield 325 mg (45%) of **1c**. Note: The R_f of **1c** is highly dependent on the water concentration of the eluent. ^{1}H (400 MHz, $CDCl_3$): 0.88 (t, 6H, CH_3), 1.25 (m, 58H, CH_2), 1.51 (m, 8H, β-CH_2), 3.29-4.29 (m, 44H, $(CH_3)_3$-N^+, -OCH_2, -OCH, -$OCH_2CH_2NMe_3$).

2,2′-*Di-O-decyl-3,3′-di-O-(1′,1′,20′,20′-$^2H_4$-eicosanyl)-bis-(rac-glycerol)-1,1′-diphosphocholine* (**1a**) ([1′,1′,20′,20′-$^2H_4$]C_{20}BAS-PC). This product was prepared in 7% yield (33 mg) as described for **1c** using **5a** (340 mg, 0.455 mmol), $ClP(O)(CH_2O)_2$ (0.175 mL, 1.90 mmol), and Et_3N (0.300 mL, 2.15 mmol). ^{1}H (300 MHz, $CDCl_3$): 0.84 (t, 6H, CH_3), 1.22 (m, 60H, CH_2), 1.50 (m, 8H, β-CH_2), 3.22-4.24 (m, 40H, $(CH_3)_3$-N^+, -OCH_2, -OCH,

-$OCH_2CH_2NMe_3$).

2,2′-Di-O-decyl-3,3′-di-O-(2′,2′,19′,19′-$^2H_4$-eicosanyl)-bis-(rac-glycerol)-1,1′-diphosphocholine (**1b**) ([2′,2′,19′,19′-$^2H_4$]C_{20}BAS-PC). This product was prepared in 8% yield (30 mg) as described for **1c** using **5b** (260 mg, 0.348 mmol), $ClP(O)(CH_2O)_2$ (0.150 mL, 1.63 mmol), and Et_3N (0.250 mL, 1.79 mmol). ^1H (300 MHz, $CDCl_3$): 0.85 (t, 6H, CH_3), 1.23 (m, 60H, CH_2), 1.50 (m, 4H, β-CH_2), 3.23-4.25 (m, 44H, $(CH_3)_3$-N^+, -OCH_2, -OCH, -$OCH_2CH_2NMe_3$).

Methyl [2′,2′-$^2H_2$]-undec-10-enoate (**8b**). To a 50 mL flask was added **7** (4.97 g, 21.1 mmol) and diluted with deuterated *tert*-butanol (20 mL) followed by addition of NaO^tBu (333 mg). The mixture was allowed to stir overnight at 30 °C. The mixture was diluted with water and extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers was washed with brine, dried over anhydrous $MgSO_4$, filtered and condensed. This reaction was repeated twice to yield 3.63 g (72%) of material (>90% deuterated by NMR integration). ^1H (300 MHz, $CDCl_3$): 1.32 (m, 10H, CH_2), 1.55 (m, 2H, $C(O)CD_2CH_2$), 3.60 (s, 2H, OSO_2CH_3), 4.84-4.96 (m, 2H, $C=CH_2$), 5.67-5.81 (s, 1H, CH=C).

[1′,1′-$^2H_2$]-Undec-10-en-1-ol (**9a**). To a 50 mL flask was added **7** (1.37 g, 6.91 mmol) and diluted with dry THF (25 mL). Lithium aluminum deuteride (LAD, 611 mg) was dispersed with THF (25 mL) in a 100 mL flask and cooled to 0 °C. The **7**/THF solution was transferred to the LAD/THF solution and allowed to stir overnight, slowly reaching room temperature. The mixture was quenched with water and 1 M HCl was added. The product was extracted with diethyl ether (3 x 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and condensed to produce 1.15 g of **9a** (96%). ^1H (300 MHz, $CDCl_3$): 1.25 (m, 12H, CH_2), 1.49 (m, 2H, β-CH_2), 2.01 (m, 2H, $CH_2C=C$), 3.04 (s, 1H, -OH), 4.87-4.98 (m, 2H, $C=CH_2$), 5.75 (m, 1H, CH=C).

[2′,2′-$^2H_2$]-Undec-10-en-1-ol (**9b**)**.** This product was prepared in 89% yield (2.77 g) as described for **9a** using **7** (3.63 g, 18.13 mmol) and 95% LAH (990 mg, 22.40 mmol). ^1H (300 MHz, $CDCl_3$): 1.37 (m, 12H, CH_2), 2.06 (m, 2H, CH_2-C=C), 2.80 (broad singlet, 1H, -OH), 3.60 (s, 2H, -OCH_2CD_2), 4.92-5.03 (m, 2H, $C=CH_2$), 5.76-5.89 (m, 1H, CH=C).

[1′,1′-$^2H_2$]-Undec-10-enyl methanesulfonate **(5a).** To a 250 mL flask was added **9a** (1.46 g, 8.48 mmol) in 125 mL of dry CH_2Cl_2. Et_3N (1.5 mL, 10.76 mmol) followed by methane sulfonyl chloride (0.80 mL, 10.33 mmol) were then added. The mixture was allowed to stir for 4 hours at room temperature before quenching with water and extraction of the product with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated to produce 2.04 g (100%) of **5a**. ^1H (300 MHz, $CDCl_3$): 1.29-1.37 (m, 12H, CH_2), 1.73 (t, 2H, -OCD_2CH_2), 2.05 (m, 2H, CH_2-C=C), 3.00 (s, 3H, OSO_2CH_3), 4.91-5.02 (m, 2H, $C=CH_2$), 5.79 (m, 1H, CH=C).

[2′,2′-$^2H_2$]-Undec-10-enyl methanesulfonate (**5b**). This product was prepared in 100% yield (3.84 g) as described for **5a** using **9b** (2.77 g, 16.04 mmol), Et_3N (3 mL, 21.62 mmol), and methane sulfonyl chloride (1.5 mL, 19.38 mmol). ^1H (300 MHz, $CDCl_3$): 1.23 (m, 12H, CH_2),

1.97 (m, 2H, CH_2=C), 2.93 (s, 3H, OSO_2CH_3), 4.14 (s, 2H, -OCH_2CD_2), 4.84-4.95 (m, 2H, C=CH_2), 5.70-5.75 (m, 1H, CH=C).

(1) Mccabe, M. A.; Wassall, S. R., *J Magn. Reson. B* **1995,** *106*, 80-82.
(2) Petrache, H. I.; Dodd, S. W.; Brown, M. F., *Biophys. J.* **2000,** *79*, 3172-3192.
(3) Patwardhan, A. P.; Thompson, D. H., *Langmuir* **2000,** *16*, 10340-10350.