Orientation of the Benzophenone Group at Various Depths in Bilayers

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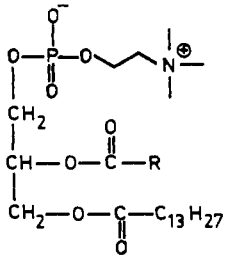
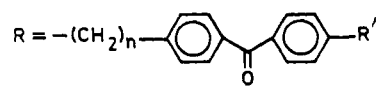
Abstract: The hydrophobic core of biological membranes is primarily composed of fatty acyl chains of lipids and side chains of nonpolar amino acids belonging to membrane-spanning domains of transmembrane proteins. Electron transport across the 35–40-Å membrane dielectric takes place via suitably oriented electron-transfer groups associated with transmembrane domains of membrane-bound proteins. We propose here that the design of lipids bearing electron-transport groups oriented at different depths can provide the necessary supramolecular assembly in the form of a monolayer or a bilayer to carry out electron transfer. The design of these modified lipids is crucial to the success of such a molecular device. We report here the design and synthesis of three benzophenone-based phospholipids capable of orienting the benzophenone group at different depths in a bilayer. The orientation of the benzophenone group was determined by photochemical cross-linking of these lipids with dimyristoylphosphatidylcholine in single bilayer vesicles followed by mass spectral analyses of the cross-linked products. The actual site of cross-linking on the myristoyl chain was determined, and it was observed that a range of carbon atoms are functionalized. The range of carbon atoms functionalized was found to be centered around the position expected from the transverse location of the benzophenone-based phospholipid in the bilayer. The data could be best interpreted in terms of zones of carbon atoms functionalized rather than any discreet site. This is in keeping with the current models of membranes which suggest the presence of a fluid gradient as one goes down the fatty acyl chain in the membrane. However, the range of carbon atoms functionalized was narrowed with probes reported here. The use of a hydrophobic tail attached to the benzophenone group assisted in directing the orientation of the photoactive group at different depths. Besides providing an effective design strategy for the orientation of electron-transfer groups at different depths in a bilayer, the high insertion yield and the depth-dependent labeling observed in artificial membranes suggest that the benzophenone-based phospholipids reported here could also prove useful for studying the structure of single and multiple spanning transmembrane proteins.

Biological systems utilize proteins oriented in membranes to carry out electron transfer via suitably oriented organic cofactors associated with amino acid side chains of transmembrane proteins.[1] Thus electron-transfer steps in respiration and photosynthesis are directed across the 35–40-Å span of the membrane dielectric.[2] The membrane-associated segments of such transmembrane proteins reside in the bulk hydrophobic milieu provided by the fatty acyl chains of the lipid in the bilayer structure. The role of the fatty acyl chain is primarily physical as it provides an organized medium conducive to electron transfer. However, appropriate molecular design can lead to lipids bearing suitably oriented functional groups capable of electron transfer. The availability of such self-assembling modified lipids can lead to molecular electronic devices.

In order to achieve this objective, it is important to design a suitable functional group bearing amphiphilic molecules capable of (a) self-assembly with other amphiphiles to organized media in the form of Langmuir–Blodgett (LB) films or bilayers, (b) orienting the functional groups at different depths, and (c) ensuring that the functional groups do not severely perturb the hydrophobic milieu of the organized media. The supramolecular architecture resulting from self-assembly of such amphiphiles can provide a relay along which information transfer can take place via a photon transfer or an electron transfer. Phospholipids, the major components of biological membranes, prepared from fatty acids bearing the desired functional group can provide the main building blocks for an LB film or a single bilayer vesicle, the supramolecular assembly for such a molecular device. However, for such a proposal to be effective it is important to evaluate the orientation of the desired functional group and the possible perturbation caused by it of the highly organized monolayer or bilayer structure. Toward this end we have used the benzophenone group as a conformational probe,[3] and we report here three phospholipids (**1–3**) prepared from benzophenone-based fatty acids and a study of their cross-linking behavior in single bilayer vesicles. While **1** incorporates a terminal ben-



DML (R = $C_{13}H_{27}$)



1 (n = 5, R′ = H)

2 (n = 5, R′ = C_6H_{13})

3 (n = 7, R′ = C_4H_9)

zophenone (Bz) group, **2** has a hydrophobic tail linked to the Bz

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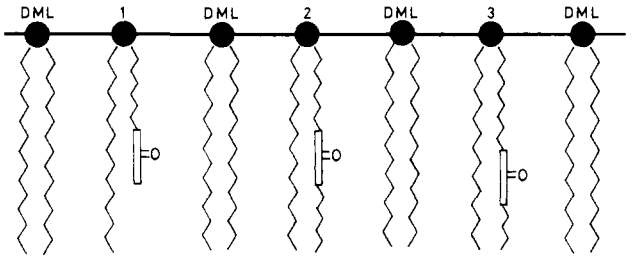

Figure 1. Schematic representation of the orientation of **1–3** in membranes prepared from them and dimyristoylphosphatidylcholine (DML). The flat rectangular box represents the benzophenone group.

group, and **3** in the same design has a more distal location of the Bz group. The attachment of a hydrophobic tail to the chromophoric group has been found to be extremely effective in immobilizing and orienting fluorescent probes designed for depth-dependent analysis of membranes.[4] Figure 1 gives a schematic representation of the orientation of **1–3** in membranes.

Experimental Section

Chemical Synthesis. All general chemicals and solvents were commercial grades of the highest purity available and were further purified and dried, if required. Dicyclohexylcarbodiimide, 4-(dimethylamino)-pyridine, adipic acid, suberic acid, myristic acid, 4-*n*-butylbenzoic acid, and 4-*n*-hexylbenzoic acid were procured from Fluka. *sn*-Glycero-3-phosphocholine·$CdCl_2$ complex and rattlesnake *Crotalus adamanteus* venom were procured from Sigma. Benzoyl chloride (E. Merck) was distilled before using, bp 197 °C. Nitrobenzene (Ranbaxy) was distilled under reduced pressure before using, bp 85–86 °C/7 mmHg. Thionyl chloride (E. Merck) was distilled over linseed oil, bp 72–74 °C. Methyl hydrogen adipate was prepared from adipic acid and isolated by vacuum distillation, bp 174–180 °C/10 mmHg.[5] It was converted to the acid chloride using thionyl chloride and isolated by vacuum distillation, bp 105–107 °C/10 mmHg. Ethyl hydrogen suberate was prepared from suberic acid and diethyl suberate[6] and converted to the acid chloride using thionyl chloride. Ethyl suberoyl chloride was then isolated by vacuum distillation, bp 137–40 °C/4 mmHg.[7] 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DML) was prepared in 80% yield from *sn*-glycero-3-phosphocholine·$CdCl_2$ complex and myristic acid using dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine in a one-pot reaction according to Regen et al.[8]

UV–Visible spectra were recorded on a Shimadzu UV-265 spectrometer. IR spectra were recorded on a Perkin-Elmer 691 spectrophotometer. ^{1}H NMR spectra were recorded on either a Varian 300-MHz or a Bruker 500-MHz spectrometer. Mass spectra were recorded on a Shimadzu QP-1000 spectrometer. High-performance liquid chromatographic analysis (HPLC) was carried out on a Shimadzu LC-4A system, and the eluant was monitored at 260 nm using a Shimadzu SPD-2AS UV detector. The flow rate used in all cases was 1 mL/min. HPLC analysis of all benzophenone-based fatty acids (**1–3**) was carried out on an Alltech silica column (4.6 × 250 mm) using hexane:2-propanol:acetic acid (70:30:1) as the mobile phase. HPLC of the esters of these acids was carried out on a Shimpack CLC-ODS column (6 × 150 mm) using acetonitrile:water (80:20) as the mobile phase unless specified otherwise. Sephadex LH-20 column chromatography was carried out on a Pharmacia SR column (2.5 × 45 cm) using chloroform:methanol (1:1) as the mobile phase.

Syntheses of Benzophenone-Based Fatty Acids. 6-[4-(4-*n*-Hexylbenzoyl)phenyl]hexanoic Acid. Phenylhexanoic acid was prepared by Friedel–Crafts acylation of methyl adipoyl chloride with benzene to give 5-oxo-6-phenylhexanoic acid methyl ester in 53% yield. This keto ester was then reduced by Wolff–Kishner reduction using hydrazine hydrate and base to give 6-phenylhexanoic acid in 56% yield, which was then converted to its methyl ester: HPLC R_t 5.76 min; IR 1730 cm^{-1} (ester carbonyl). *n*-Hexylbenzoic acid (3.2 g, 15.5 mmol) was dissolved in excess thionyl chloride and refluxed on a water bath. After 4 h, excess thionyl chloride was distilled off and the crude acid chloride was used as such. Anhydrous aluminium chloride (2.9 g, 21.7 mmol) was suspended in dry nitrobenzene (25 mL). To this suspension was added 6-phenylhexanoic acid methyl ester (3 g, 14.5 mmol) with stirring, and the reaction mixture was cooled to 4 °C. This was followed by dropwise addition of freshly prepared 4-*n*-hexylbenzoyl chloride (3.25 g, 14.4 mmol) in dry nitrobenzene over a period of 30 min, and stirring was continued for 45 min. During the addition the temperature was maintained at 4 °C. Finally the suspension was kept at 4 °C for a further 48 h. The aluminium chloride complex was then hydrolyzed by pouring the reaction mixture into ice-cold dilute hydrochloric acid. The organic layer was separated, and nitrobenzene was removed by steam distillation. The residue was extracted with solvent ether and washed sequentially with 10% sodium bicarbonate solution, water, and a saturated solution of sodium chloride. The residue was dried over anhydrous sodium sulfate and filtered. Ether was distilled off, and the crude material so obtained was subjected to column chromatography over silica gel (270 g). The desired acylated compound was eluted with 75% benzene in petroleum ether to give pure 6-[4-(4-*n*-hexylbenzoyl)phenyl]hexanoic acid methyl ester (2.8 g) in 50% yield: HPLC R_t 20.72 min (mobile phase methanol:water, 92:8); IR 1730 (ester carbonyl) and 1650 cm^{-1} (diaryl carbonyl). This ester (2 g, 5 mmol) was dissolved in 15% methanolic potassium hydroxide and refluxed on a water bath for 4 h. The reaction mixture was poured into crushed ice, neutralized with concentrated hydrochloric acid, and extracted with solvent ether. The ether layer was washed with water, dried over anhydrous sodium sulfate, and filtered. Ether was distilled off to give 6-[4-(4-*n*-hexylbenzoyl)phenyl]hexanoic acid (1.73 g) in 90% yield: HPLC R_t 3.4 min; UV (methanol) λ_{max} 264.9 nm; IR 1700 (acid carbonyl) and 1650 cm^{-1} (diaryl carbonyl); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3 H, J = 6.95 Hz, $-(CH_2)_5CH_3$), 1.32 (m, 8 H, $CH_3(CH_2)_3(CH_2)_2-$ and $-C_6H_4(CH_2)_2CH_2(CH_2)_2CO$), 1.69 (m, 6 H, $-C_6H_4CH_2CH_2(CH_2)_3CH_3$ and $-C_6H_4CH_2CH_2CH_2CH_2CH_2CH_2COOH$), 2.36 (t, 2 H, J = 7.5 Hz, $-C_6H_4(CH_2)_4CH_2COO$), 2.68 (m, 4 H, $-C_6H_4CH_2(CH_2)_4CO$ and $-C_6H_4CH_2(CH_2)_4CH_3$), 7.25 and 7.71 (d each, 4 H each, aromatic H); HRMS for $C_{25}H_{32}O_3$ calcd 380.2353, found 380.2351.

6-(4-Benzoylphenyl)hexanoic Acid. 6-(4-Benzoylphenyl)hexanoic acid was prepared by a procedure similar to the one given above. Friedel–Crafts acylation of 6-phenylhexanoic acid methyl ester with benzoyl chloride gave the methyl ester of the target compound in 60% yield. Hydrolysis of this ester gave 6-(4-benzoylphenyl)hexanoic acid: HPLC R_t 3.8 min; UV (methanol) λ_{max} 260 nm; IR 1700 (acid carbonyl) and 1650 cm^{-1} (diaryl carbonyl); ^{1}H NMR ($CDCl_3$) δ 1.44 (m, 2 H, $-C_6H_4(CH_2)_2CH_2(CH_2)_2CO$), 1.68 (p, 4 H, $-C_6H_4CH_2CH_2CH_2CH_2CH_2CO$), 2.36 (t, 2 H, J = 7.2 Hz, $-C_6H_4(CH_2)_4CH_2CO$), 2.69 (t, 2 H, J = 7.6 Hz, $-C_6H_4CH_2(CH_2)_4CO$), 7.26–7.81 ppm (m, 9 H, aromatic H); HRMS for $C_{19}H_{20}O_3$ calcd 296.1412, found 296.1415.

8-[4-(4-*n*-Butylbenzoyl)phenyl]octanoic Acid. The preparation of this compound is similar to the one described above. 4-*n*-Butylbenzoic acid (3.0 g, 0.02 mol) was dissolved in excess thionyl chloride and refluxed on a water bath. After 3 h, excess thionyl chloride was distilled off and the crude acid chloride was distilled under reduced pressure at 80–82 °C/3 mmHg. 8-Phenyloctanoic acid methyl ester (1.7 g, 7.2 mmol), prepared by a method similar to that used for 6-phenylhexanoic acid methyl ester, was then acylated with freshly prepared 4-*n*-butylbenzoyl chloride (1.4 g, 7.2 mmol) in dry carbon tetrachloride at 4 °C. The crude material was subjected to column chromatography over silica gel (120 g), and the desired compound was eluted with 75% benzene in petroleum ether to give pure 8-[4-(4-*n*-butylbenzoyl)phenyl]octanoic acid methyl ester (1.0 g) in 36% yield: HPLC R_t 15.3 min; IR 1730 (ester carbonyl) and 1650 cm^{-1} (diaryl carbonyl). The ester was hydrolyzed to give 8-[4-*n*-butylbenzoyl)phenyl]octanoic acid in 94% yield: HPLC R_t 3.45 min; UV (methanol) λ_{max} 264.0 nm; IR 1700 (acid carbonyl) and 1650 cm^{-1} (diaryl carbonyl); ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3 H, J = 7.2 Hz, $-(CH_2)_3CH_3$), 1.35 (m, 8 H, $CH_3CH_2(CH_2)_2-$ and $-C_6H_4CH_2(CH_2)_3(CH_2)_2CO$), 1.65 (m, 6 H, $-C_6H_4CH_2CH_2CH_2CH_3$ and $-C_6H_4CH_2CH_2(CH_2)_3CH_2CH_2COOH$), 2.37 (t, 2 H, J = 7.5 Hz, $-C_6H_4(CH_2)_6CH_2COO$), 2.67 (t, 4 H, J = 7.5 Hz, $-C_6H_4CH_2(CH_2)_5CH_2CO$ and $-C_6H_4CH_2(CH_2)_2CH_3$), 7.29 and 8.02 ppm (d each, 4 H each, aromatic H); HRMS (methyl ester) for $C_{26}H_{34}O_3$ calcd 394.2508, found 394.2515.

Synthesis of Benzophenone-Based Phospholipids 1–3. All the phospholipids reported here were prepared from DML. DML was first hydrolyzed to lyso-DML using rattlesnake venom. The lyso-DML had to be purified by Sephadex LH-20 column chromatography to ensure a high yield of the final phospholipid. Attempts to prepare the final

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Table I. ^{1}H NMR Chemical Shift (δ) Assignments for Benzophenone-Based Phospholipids[a]

	proton									
compd	A (2 H)	B (2 H)	C (2 H)	D (2 H)	E (2 H)	F (2 H)	A2 (2 H)	A3 (2 H)	A4–A13 (20 H)	A14 (3 H)
1	4.11, 4.30	5.20	3.93	4.30	3.77	3.32	2.29	1.62	1.25	0.86 (t), J = 7.5
2	4.11, 4.37	5.20	3.97	4.33	3.67	3.30	2.24	1.59	1.24	0.92 (t), J = 7.3
3	4.10, 4.37	5.21	3.96	4.33	3.67	3.32	2.24	1.59	1.24	0.89 (t), J = 6.4

	proton						
compd	B2 (2 H)	B3 (2 H)	B4 (2 H)	B5 (2 H)	B6 (2 H)	B7 (2 H)	B8 (2 H)
1	2.41 (t) J = 7.5		1.38–1.77 (B3–B5)		2.70 (t) J = 7.7		
2	2.34 (t) J = 7.4		1.32–1.69 (B3–B5)		2.69 (t) J = 7.6		
3	2.41 (t) J = 7.8			1.34–1.64 (B3–B7)			2.65 (t) J = 7.2

	protons						
compd	arom H (9 H/4 H)	B1′ (2 H)	B2′ (4 H)	B3′ (3 H/2 H)	B4′ (2 H)	B5′ (3 H)	B6′
1	7.27–7.80						
2	7.25, 7.73	2.69, J = 7.6		1.32–1.69 (B2′–B5′)			0.94 (t), J = 7.1
3	7.23, 7.73	2.69, J = 7.5	1.34–1.64 (B2′–B3′)		0.94 (t), J = 7.2		

[a] The ^{1}H NMR spectra were recorded at 500 MHz. The chemical shifts values are expressed in ppm and J values in Hertz. Different protons are identified as follows: the glycerol backbone carbon atoms are referred to as A, B, and C. The A and B carbon atoms refer to the C1 and C2 chains bearing carbon atoms; the C carbon atom is linked to the phosphate group, which is further linked to the D and E carbon atoms in increasing order beginning from the phosphate group. The methyl protons corresponding to the $N(CH_3)_3^+$ group are labeled F. The individual acyl chain carbon atoms are referred to in increasing numerical order beginning from the acyl carbonyl carbon, prefixed by A or B. Thus, the A carbon atom of the glycerol backbone is linked to the myristoyl chain in all the phospholipids reported here (**1–3**). The benzophenone-based fatty acids are linked to the B carbon atom and are numbered in increasing numerical order. However, in the case of fatty acyl chains, which have a hydrophobic tail attached to the benzophenone group, the protons are referred to by the corresponding carbon atoms, numbered in increasing order beginning from the aryl ring. Thus, B1′–B4′ in the case of **3** and B1′–B6′ in the case of **2** refer to the tail carbon atoms with the terminal methyl group represented by B4′ and B6′, respectively. The number in parentheses under the alphabetical representation of carbon atoms bearing the protons in the column headings refers to the number of protons. The d or t written next to chemical shift value in some cases refers to doublet or triplet observed with coupling constants J given also.

phospholipid from lyso-DML, benzophenone-based fatty acid, dicyclohexylcarbodimide (DCC), and 4-(dimethylamino)pyridine (DMAP) by a procedure similar to that reported by Kallury et al.[9] were unsuccessful. Consequently, it was decided to first isolate the anhydride of the benzophenone-based fatty acid and then carry out the acylation as reported earlier for the syntheses of other photoactive phospholipids.[10] The anhydride was prepared using DCC in carbon tetrachloride. The yield of the final phospholipid varied from 50 to 85% in different preparations. The purity of the phospholipids **1–3** was established by TLC and HPLC. TLC was carried out on silica gel plates using chloroform:methanol:water (65:25:4 v/v) as the developing solvent system. HPLC was carried out on an Altech silica column (4.6 × 250 mm) using acetonitrile:methanol:water (65:21:14 v/v) as the mobile phase at a flow rate of 1 mL/min. Under these conditions, **1–3** appeared at 11.22, 11.52, and 11.62 min, respectively. The UV absorption spectra of **1–3** in methanol gave λ_{max} (ϵ) at 260.2 (19 200), 264.4 (27 900), and 263.4 nm (28 200), respectively. Molecular weights were determined by FAB-MS and were observed to correspond to the expected molecular weights of 746 for **1** and 830 for **2** and **3**. The phospholipids were also fully characterized by proton NMR (Table I). A typical procedure for the preparation of the benzophenone-based phospholipids **1–3** is given below.

1-Myristoyl-2-[6-(4-benzoylphenyl)hexanoyl]-*sn*-glycero-3-phosphocholine (1). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DML, 150 mg, 215 μmol) was dissolved in 35 mL of diethyl ether:methanol (99:1 v/v). This was followed by addition of 20 mL of 20 mM tris-HCl, pH 8, containing 40 mM calcium chloride. Finally, 3 mg of rattlesnake *Crotalus adamanteus* venom was added, and the reaction flask was flushed with nitrogen and sealed with a glass stopper. The reaction mixture was then stirred overnight. The reaction was monitored by TLC on silica gel plates using chloroform:methanol:water (65:25:4 v/v) as the developing solvent system. The mixture was taken into a separating funnel, and the two layers were separated. The ether layer was washed with water, and water was added to the aqueous layer. The combined aqueous layer was washed with ether. The aqueous layer was then evaporated to dryness by lyophilization. The lyophilized compound was extracted with chloroform:methanol (2:1 v/v) and centrifuged to separate out inorganic impurities. It was concentrated under reduced pressure. At this stage a major spot for lyso-DML could be observed by TLC analysis. This product was purified on a Sephadex LH-20 column to give pure 1-myristoyl-2-lyso-*sn*-glycero-3-phosphatidylcholine (90 mg) in 90% yield.

6-(4-Benzoylphenyl)hexanoic acid (114 mg, 0.38 mmol) was dissolved in dry chloroform (4 mL), and dicyclohexylcarbodiimide (44 mg, 0.21 mmol) was added. The flask was closed with a stopper under nitrogen atmosphere, and the mixture was stirred at room temperature overnight. From the reaction mixture precipitated dicyclohexyl urea, which was rapidly filtered through a sintered glass funnel. The formation of anhydride was checked by scanning the IR spectra in chloroform, which indicated characteristic bands at 1820 and 1730 cm^{-1}. This solution was used as such for further reaction.

1-Myristoyl-2-lyso-*sn*-glycero-3-phosphatidylcholine (30 mg, 64 μmol) was dried by addition of dry benzene and azeotropic removal of traces of water and benzene in vacuum. The residue was then dissolved in 10 mL of dry chloroform which had been freshly distilled over phosphorous pentoxide. This was followed by addition of a chloroform solution of the anhydride (190 μmol) of C6ABz mentioned above and 4-(dimethylamino)-pyridine (25.0 mg, 210 μmol). The reaction flask was then closed with a stopper after being flushed with argon, and the mixture was stirred at room temperature for 48 h. The reaction was monitored by TLC as in the preparation of lyso-DML mentioned above. After completion of the reaction, the solvent was removed under reduced pressure and the crude product was loaded over a column of Sephadex LH-20 and eluted with chloroform:methanol (1:1) at a flow rate of 0.5 mL/min. The desired phospholipid **1** eluted first, followed by the corresponding acid. The final isolated yield of **1** was 50%.

Membrane Associated Studies. A 5 mM phosphate buffer, pH 7.4, containing 150 mM NaCl (hereafter referred to as phosphate buffer), was used for the preparation of all vesicles described here. The buffer was deoxygenated under reduced pressure, followed by purging with argon. It is important to eliminate dissolved oxygen in these experiments. Oxygen, being a triplet in the ground state, tends to quench the intermediate benzophenone excited triplet state, leading to dissipation of the reactive intermediate. This results in low insertion yields and long photolysis time.

GC and GC-MS analyses were carried out on a Shimadzu QP-1000 gas chromatograph-mass spectrometer (GC-MS) on a Shimadzu OV-17 column (2% glass spiral column, 2.6 × 1100 mm, support gas Chrom Q 80–100 mesh). Helium was used as the carrier gas at a flow rate of 40 mL/min. A flame ionization detector was used for monitoring gas chromatograms. In both GC and GC-MS studies the injection port temperature was maintained at 300 °C. The column temperature was programmed as follows: 200 °C for the first 2 min and then increased at a rate of 3 °C/min to 240 °C. Stock solutions of the benzophenone-based phospholipids were prepared in chloroform, and the exact

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concentration was determined by phosphate assay.[11] Vesicles were prepared using a Branson B-30 Sonifier using a $^1/_4$-in. microtip at 25 °C. Photolysis was carried out in Corning glass tubes, which cut off light below 300 nm, using an Applied Photophysics Annular Photoreactor APQ 40 with a 400-W medium-pressure mercury lamp.

Photolysis of DML-1 (2:1) Vesicles. A chloroform solution of DML (65.3 mg, 94 μmol) and **1** (34 mg, 47 μmol) was taken in a pear-shaped flask. A thin film was formed using a rotary evaporator, and the film was dried under vacuum (1 mmHg) for 10 h. To this flask was added 18 mL of deoxygenated phosphate buffer, and the contents were vortexed for 2 min. The suspension was then sonicated for 25 min and centrifuged at 11 000*g* for 15 min to remove any titanium particles shredded from the probe. The supernate was collected and deoxygenated by bubbling argon through it for 15 min. It was then photolyzed for 2 h at 28 °C. The sample tube was kept at a distance of 4 cm from the lamp. The progress of photolysis was monitored by observing the decrease in the absorbance at 260 nm. After completion of photolysis the photolysate was extracted with chloroform:methanol (1:1 v/v; 2 × 18 mL). The combined organic extract was evaporated with a stream of nitrogen, and the crude product was loaded on a Sephadex LH-20 column (2.5 × 20 cm) and eluted with chloroform:methanol (1:1 v/v) at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected and analyzed for their phosphate content and absorbance at 245 nm. The high molecular weight fractions corresponding to the cross-linked product were pooled together and assayed for phosphate, which corresponded to 43 μmol.

Transesterification, Dehydration, and Ozonolysis of Cross-Linked Phospholipid DML-1. The cross-linked phospholipid (40 μmol) fraction was dissolved in anhydrous methanol (6 mL), and 200 μL of 0.5 M sodium methoxide in methanol was added to it. This solution was stirred at room temperature for 12 h. The pH was then adjusted to 7.0, and the crude product was extracted with *n*-hexane:ether (8:2 v/v). The organic layer was washed with water, and the solvent was distilled off to obtain a residue consisting of a mixture of fatty acid methyl esters. This material was further treated with an excess of diazomethane in order to ensure complete esterification.

The residue was dissolved in glacial acetic acid (3 mL), and iodine (2 mg) was added to it. This solution was refluxed for 2 h. Acetic acid was removed under vacuum, and the dehydrated mixture was dried under vacuum on potassium hydroxide. The dehydrated material was subjected to ozonolysis in methanol at −60 °C for 20 min using ozonized oxygen. During ozonolysis the oxygen flow was 20 L/h, corresponding to 1.06 g/h of ozone (0.35 g ozone, 7.29 mmol). Excess ozone was removed by passing nitrogen over the sample. A few drops of dimethyl sulfide was then added to cleave the ozonide to give a mixture of *n*-ketomyristic acid methyl ester and C6ABz. This material was dissolved in boron trifluoride etherate (1 mL), and 1,2-ethane dithiol (0.1 mL, 1.1 mmol) was added to it. The whole solution was stirred at room temperature for 1 h. The reaction mixture was extracted with solvent ether, and the ether layer was washed with dilute hydrochloric acid, 5% sodium bicarbonate solution, and water. The ether extract was dried over anhydrous sodium sulfate, and ether was distilled off to give the ethylene thioketal derivatives which were then subjected to mass spectral analysis.

Similarly, vesicles were prepared from DML (43.6 mg, 63 μmol) and **3** (26 mg, 31.3 μmol) and from DML (67 mg, 96 μmol) and **2** (40 mg, 48 μmol) in phosphate buffer and photolyzed as described above. These photolysates were extracted and passed over a Sephadex LH-20 column to separate the cross-linked phospholipids. The cross-linked phospholipids were then transesterified (DML-**3**, 28 μmol and DML-**2**, 18 μmol) and further processed as described above.

Mass Spectral Analysis. Analysis of the mixture of thioketal derivatives obtained in the section described above by direct inlet mass spectroscopy gave data which were difficult to analyze as other impurities present in the crude material masked the fragments arising from the thioketals. While this method has been reported to be successfully with insertion compounds obtained from long *n*-alkyl chain alcohol derivatives of benzophenone-based fatty acids[3b] and in micelles,[3c] subsequent studies[3d] by the same group in bilayers prepared from diacetyl phosphate indicated a need for purification of the thioketal derivatives by HPLC prior to mass spectral analysis. Basically the mass spectral analysis method reported by Breslow et al.[3b] works very well, but in cases where the cross-linking yields are low, it becomes essential to purify the thioketal derivatives. We used GC-MS instead of direct inlet mass spectroscopy to get over this problem. Consequently, three different keto acids, i.e., 12-ketostearic acid, 8-ketomyristic acid, and 10-ketomyristic acid, were prepared and converted to their thioketal derivatives. The methyl esters of these compounds, as well as mixtures of known composition, were analyzed by direct inlet mass spectroscopy and GC-MS at 20 eV. The fragment corresponding to thioketal cleavage of the terminal methyl of these esters formed the base peak in all three cases and was followed in all subsequent cases. Once the position of these model thioketal derivatives was known, GC-MS proved to be very effective. Mass spectra were recorded every 3 s over the whole range of the gas chromatogram, and the mass spectra for the peak corresponding to the thioketal derivatives of *n*-keto methyl myristate were recorded. Spectra of the fragments corresponding to the thioketal cleavage were recorded using a mass chromatogram. The data for these fragments were averaged over several data points, each data point referring to mass spectra recorded every 3 s across the peak corresponding to the thioketal derivatives of *n*-keto methyl myristate. The data were finally normalized by summing the intensity of ions corresponding to different thioketal derivatives and expressed as percent data. The terminal methyl group has a variable reactivity relative to the other methylene groups[3c,3a] and was therefore set to 0 prior to normalization of the data.

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Results

Synthesis of Benzophenone-Based Fatty Acids and Corresponding Phospholipids. The benzophenone-based fatty acids were prepared by Friedel–Crafts acylation of the corresponding phenyl alkanoates with *n*-alkylbenzoyl chloride. The phospholipids **1–3** were prepared by acylation of 1-myristoyl-2-lyso-*sn*-glycero-3-phosphatidylcholine (lyso-DML) with the anhydride of the corresponding benzophenone-based fatty acids using 4-(dimethylamino)pyridine as base. The NMR spectra of these phospholipids (Table I) gave characteristic signals corresponding to the glycerophosphocholine portion of the molecule as in the case of standard phospholipids like dimyristoylphosphatidylcholine.[12] In addition, signals corresponding to the benzophenone-based fatty acyl chain were also observed. All the NMR assignments are given in Table I. The phospholipids **2** and **3** were prepared so that the overall length of fatty acyl chains would be similar though the position of the benzophenone chromophore along the fatty acid chain would differ. Further, the alkyl chains were attached to the 4′ position of the benzophenone group in **2** and **3** in order to provide better alignment of phospholipids in the membranes prepared from them.

Cross-Linking Studies. Single bilayer vesicles were prepared from **1–3** and dimyristoylphosphatidylcholine (DML) taken in a molar ratio of 1:2 and photolyzed at 28 °C. After photolysis the vesicle preparation was extracted, and the dimeric cross-linked product was separated from the monomeric products by Sepahadex LH-20 column chromatography using phosphate assay and absorption at 245 nm for detection. The yield of cross-linked product for **1–3** was found to be in the range of 35–40%. The cross-linked product on transesterification with NaOMe gave the cross-linked product between myristic acid and the corresponding benzophenone-based fatty acid as a diester, as analyzed by MS. Little, if any, of the pinacol product that can be formed by dimerization of the benzophenone-based phospholipid was observed. In order to establish the site of cross-linking, we used the methodology developed by Breslow et al.[3b] Scheme I gives the basic strategy used for analysis of the cross-linked fraction obtained from Sephadex LH-20 chromatography. It involves dehydration of the cross-linked diester followed by ozonolysis. The wavy line in Scheme I indicates multiple sites of insertion. Oxidative cleavage of the resulting ozonide leads to two products, one of them being the parent benzophenone-based fatty acid. However, as the benzophenone chromophore inserts at more than one site in the neighboring myristoyl chain of DML, a series of *n*-keto acids are obtained and correspond to the second product obtained on cleavage of the ozonide. Conversion of these *n*-keto acids to the corresponding thioketal derivatives followed by mass spectral analysis permits one to establish the degree of cross-

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Scheme I

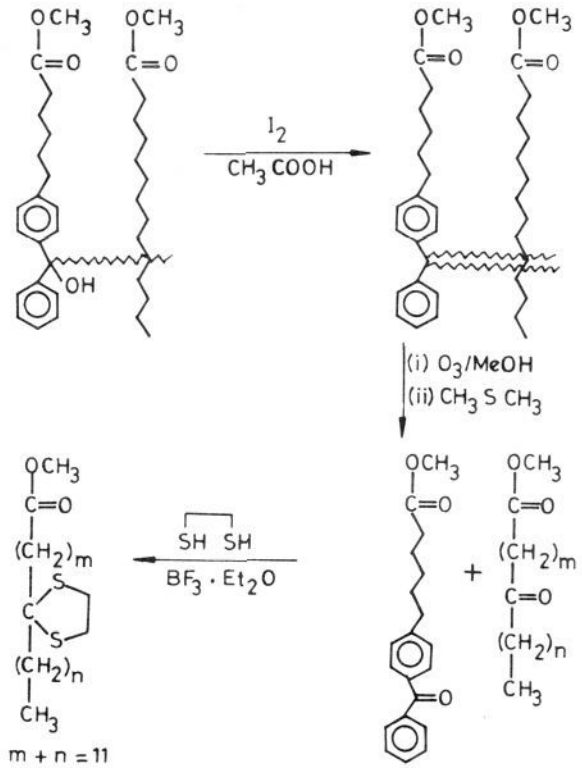

Table II. Percent Functionalization Data for Carbon Atoms of the Myristoyl Chain in the Cross-Linked Phospholipid Fraction Obtained from **1**–**3** and DML

C no.	1	2	3
1	0	0	0
2	0	0	0.34
3	0	0	0
4	0	0	3.37
5	7.92	7.05	4.74
6	9.75	7.58	5.94
7	18.3	14.79	11.43
8	23.9	23.00	14.32
9	27.4	30.03	21.65
10	10.4	15.16	20.85
11	0	0	5.89
12	0.31	0	1.86
13	1.84	2.39	9.62
14	0	0	0

linking associated with a particular carbon atom. The data so obtained by analysis of the cross-linked product obtained from **1**–**3** and DML are given in Table II. These data indicate that the benzophenone group in **1** functionalizes the myristic acid chains in DML and the cross-linking site is largely centered around the C8 and C9 carbon atoms of the myristic acid chain (Figure 2). Other carbon atoms around the C7–C9 zone are also functionalized, albeit to a lower degree. Similar data were obtained for **2**, though the overall functionalization was slightly shifted to the right, i.e., centered around the C8–C10 zone but more toward the C10 carbon atom relative to the **1**-DML functionalization data. On the basis of the fully extended transverse location of **1** (or **2**) and DML in membranes, molecular modeling studies indicate that the benzophenone insertion site should be located between carbon atoms C7 and C10 of the myristoyl chain, the variation in insertion site being a result of the fatty acyl chain at C2 being shorter than the corresponding chain at the C1 carbon atom of the glycerol backbone.[13,14] The

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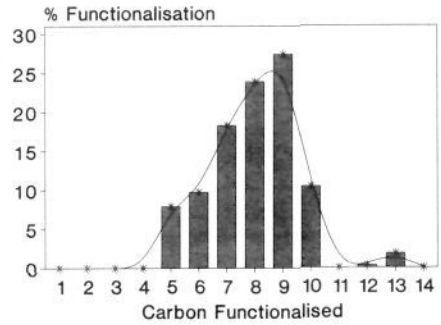

Figure 2. Bar diagram of percent functionalization of different carbon atoms of the myristoyl chain in the cross-linked phospholipid fraction obtained from **1** and DML. A best fit curve for the percent functionalization data is superimposed on the bar plot.

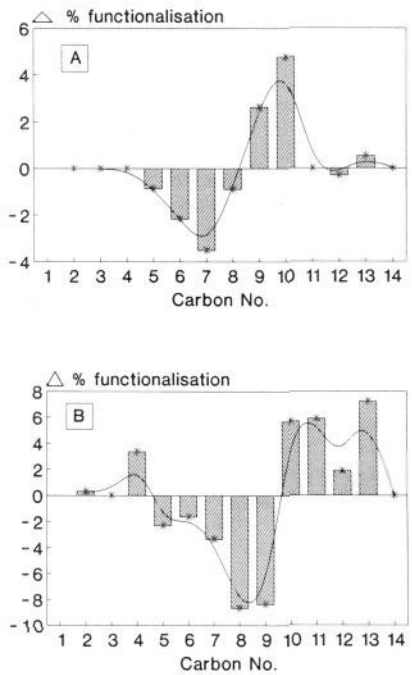

Figure 3. Bar diagram of the change in percent functionalization of different carbon atoms of the myristoyl chain in the cross-linked phospholipid fraction. (A) [**2** + DML] – [**1** + DML]. (B) [**3** + DML] – [**2** + DML]. The data were obtained by subtracting the percent functionalization data obtained with one phospholipid system from those of the other, as indicated. In each case a best fit curve for the change in percent functionalization data is superimposed on the bar plot.

observed carbon functionalization data are thus supported by the expected range of carbon atoms functionalized. The ability of **2** to probe deeper relative to **1** is easy to appreciate from the percent change in carbon functionalization at different carbon atoms, as given in Figure 3A. Thus, as compared to **1**, the hydrophobic tail bearing **2** labels the shallower C5–C8 carbon atoms to a lesser degree but labels the deeper C8–C9 carbon atoms to a larger degree. These results suggest a better packing of the hydrophobic tail bearing compounds within the bilayer. Carbon functionalization data obtained with **3** indicated that this probe has a rather broad functionalization range. While it did seem to probe deeper, i.e., more toward C10 relative to **2**, it also inserted in C4 and C13 carbon atoms of the myristoyl chain (Table II). Molecular modeling studies with **3** and DML indicate that the benzophenone insertion site should be located between carbon atoms C8 and C12 of the myristoyl chain. However, the observed labeling of even shallower range suggests that the deeper probe has a larger tendency to wobble. Figure 3B compares the

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percent change in carbon functionalization at different carbon atoms for **2** and **3**. It is interesting to note that the deeper probe **3** does insert preferentially in the deeper region of the myristoyl chain as compared to its shallower counterpart **2**.

Discussion

In evaluating the data obtained on sites of cross-linking using the probes described here, it is important to note that any attempt toward determination of the actual site of intermolecular cross-linking in artificial membranes can at best provide a zone of carbon atoms functionalized in the neighboring chain. This is a result of the inherent mobility associated with the fatty acyl chains. Thus, Breslow et al., in their studies with micelles[3c] and bilayers[3d] prepared from dicetyl phosphate, reported functionalization of a broad range of carbon atoms in benzophenone-based fatty acids, e.g., C4 to C12, in sodium dodecyl sulfate using benzophenone-4-carboxylate. However, these studies were not carried out in single bilayer vesicles prepared from phospholipids. Using phospholipid probes based on 2-diazo-3,3,3-trifluoropropionoxy-ω-substituted fatty acids, Gupta et al.[15] described intermolecular cross-linking in single bilayer vesicles. Once again the carbon functionalization data indicated an extremely broad (C6–C17) range of functionalized carbon atoms. The relatively high functionalization of the terminal and penultimate carbon atoms reported in these studies is of particular interest. This is not surprising in view of the fluidity gradient observed in membranes wherein mobility increases on going deeper into the membranes. Several membrane order determinations based on ESR and deuterium NMR studies have substantiated that the membrane order decreases as one goes deeper in the membrane.[14] Consequently, the terminal parts of the fatty acyl chains in bilayer membranes are extremely mobile. The high degree of freedom associated with terminal carbon atoms of the fatty acyl chains, relative to, e.g., methylene groups in the middle of the chain, increases the probability of photolabeling the terminal carbon atoms by the reactive intermediates formed from the photoactive reagents. Thus, intermolecular cross-linking studies with photoactive reagents in lipid bilayers can at best provide a narrow zone of carbon atoms functionalized. Thus the effectiveness of a reagent depends on the range of carbon atoms functionalized and the carbon atom around which the functionalization is centered.

It is in this context that the results obtained here become interesting. Thus, **1** labels primarily the carbon atoms C5–C10, and the labeling is centered around C8–C9 (Figure 2). On the basis of simple distance measurements one would predict the hydrophobic tail bearing phospholipid **2**, with the benzophenone group separated from the acyl carbon by five methylene groups exactly as in **1**, to provide a labeling pattern similar to that of **1**. However, one observes that the labeling pattern in **2**, though still covering the C5–C10 zone, is now centered around C8–C10. A difference plot of the carbon functionalization data brings out this point quite clearly (Figure 3A). Thus, as compared to **1**, the hydrophobic tail bearing **2** labels the shallower C5–C8 carbon atoms to a lesser degree but labels the deeper C8–C9 carbon atoms to a larger degree. These results clearly point out the advantage of using the hydrophobic tail bearing compounds **2** and **3** for better alignment of the probe in membranes for effective depth-dependent photolabeling. Similar results have been obtained using fluorene-based fluorescent probes for depth-dependent probing of membranes.[4] A noticeable point in labeling by both **1** and **2** is that both label the C13 carbon atom, though to a very small extent. However, the deeper probe **3** labels the C13 carbon atom to a relatively larger degree, indicating that this probe, as expected from the deeper position of benzophenone in this probe, labels carbon atoms deeper than the **1** or **2** probes. The higher labeling of C13 in **3** is a result of not only deeper location of this probe but also greater mobility associated with the terminal carbon atoms of the fatty acyl chain. However, the broad range, C4–C13, of labeling by **3** indicates that probes penetrating deep into the membrane have a tendency to undergo a larger wobbling motion, leading to a broad range of carbon functionalization. The labeling centered around C9–C10 indicates a shift in the labeling toward deeper carbon atoms on the fatty acyl chains. A comparison between carbon functionalization data for **3** and **2** indicates clearly that **3** preferentially labels carbon atoms toward the center of the bilayer (Figure 3B).

Photolabeling of lipids at different depths in bilayers can thus provide zones of labeling rather than a discreet site at a typical depth. However, this problem is likely to be minimized if the probes reported here are used to identify membrane-spanning domains in single or multiple spanning transmembrane proteins due to the relatively lower mobility associated with the peptide backbone of a large protein. The use of depth-dependent photolabeling to determine topography of membrane-bound proteins, which are difficult to crystallize relative to their soluble counterparts, has been reported earlier.[16,17] Despite the looping back problem reported with such probes in the past,[18] current attempts toward better design and syntheses of phospholipid probes[19] have led to useful results.[20] The benzophenone-based phospholipid probes reported here can thus be used for membrane-associated photolabeling studies. At this stage it is important to point out that the major advantages associated with use of benzophenones as photoactive reagents are their ability to form a benzophenone excited triplet state and their inertness toward water. The electrophillic nature of other commonly used photoactive reagents like carbenes or nitrenes and the resulting high reactivity toward water is probably one of the main drawbacks associated with these reagents. While benzophenone-based phospholipids have been reported in the past,[21] the low labeling yields associated with them, i.e., <0.1%,[21a] have been discouraging, especially in view of the fact that other benzophenone-based photoactive reagents give rise to quite reasonable cross-linking yields.[22] Further, these phospholipid probes were prepared using only 4-carboxybenzophenone, which strongly limits the use of these probes for depth-dependent labeling of membrane hydrophobic cores. It is quite possible that such shallow probes may only label proteins near the membrane surface, and it is not surprising that a recent report on a similar phosphatidyl ethanolamine-based azide probe indicated selective labeling of spectrin in erythrocytes.[23] The present work indicates that benzophenone-based phospholipids can be conveniently prepared and can give rise to fairly high cross-linking yields, i.e., 35–40%.

In conclusion, the results obtained here using **1–3** indicate a sharper range of carbon functionalization and highlight the advantages associated with use of phospholipids with a fatty acyl chain bearing a hydrophobic tail. A tail longer than 4–6 carbon

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atoms can be utilized so that it interdigitates[24] with the opposite bilayer, thus providing a more specific orientation of the photoactive group. The current work thus demonstrates that with effective molecular design it should be possible to make amphiphiles with suitable functional groups capable of orienting at different depths in bilayers so as to form a relay. Electron transfer by such a relay provides an alternative to the rather rigid molecular wires reported recently.[25]

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