Synthesis of Macrocyclic Diacyl/Dialkyl Glycerols Containing Disulfide Tether and Studies of Their Effects upon Incorporation in DPPC Membranes. Implications in the Design of Phospholipase A_2 Modulators

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A general method for the preparation of novel disulfide-tethered macrocyclic diacylglycerols (DAGs) has been described. Overall synthesis involved stepwise protection, acylation, and deprotection to yield the bis(ω-bromoacyl) glycerols. In the crucial macrocyclization step, a unique reagent, benzyltriethylammonium tetrathiomolybdate (BTAT), has been used to convert individual bis(ω-bromoacyl) glycerols to their respective macrocyclic disulfides. DAG **6**, which had ether linkages between hydrocarbon chains and the glycerol backbone, was also synthesized from an appropriate precursor using a similar protocol. One of the DAGs (DAG **5**) had a carbon−carbon tether instead of a disulfide one and was synthesized using modified Glaser coupling. Preparation of α-disulfide-tethered DAG (DAG **4**) required an alternative method, as treatment of the bisbromo precursor with BTAT gave a mixture of several compounds from which separation of the target molecule was cumbersome. To avoid this problem, the bisbromide was converted to its corresponding dithiocyanate, which on further treatment with BTAT yielded the desired DAG (DAG **4**) in good yield. Upon treatment with the reducing agent dithiothreitol (DTT), the DAGs that contain a disulfide tether could be quantitatively converted to their "open-chain" thiol analogues. These macrocyclic DAGs and their reduced "open-chain" analogues have been incorporated in DPPC vesicles to study their effect on model membranes. Upon incorporation of DAG **1** in DPPC vesicles, formation of new isotropic phases was observed by ^{31}P NMR. These isotropic phases disappeared completely on opening the macrocyclic ring by a reducing agent. The thermotropic properties of DPPC bilayers having DAGs (**1**−**6**) incorporated at various concentrations were studied by differential scanning calorimetry. Incorporation of DAGs in general reduced the cooperativity unit (CU) of the vesicles. Similar experiments with reduced "open-chain" DAGs incorporated in a DPPC bilayer indicated a recovery of CU with respect to their macrocyclic "disulfide" counterparts. The effect of inclusion of these DAGs on the activity of phospholipase A_2 (PLA_2) was studied in vitro. Incorporation of DAG **1** in DPPC membranes potentiated both bee venom and cobra venom PLA_2 activities.

Introduction

Diacylglycerols (DAGs) are the precursors of phospholipids and have been shown to play an important role in controlling the activity of membrane-bound enzymes such as protein kinase C (PKC) and phospholipase A_2 (PLA_2).[1] Certain DAGs have the ability to modify the organizational properties of the membrane, such as inducing bilayer to hexagonal or other nonbilayer phase formation[2] or membrane fusion[3] affecting membrane curvature.[4] These changes within the membrane are thought to create "defects" within the bilayer,[5] which in turn make the substrate (phospholipids) more accessible to the membrane-bound enzymes.[6] Such processes may therefore be relevant to the understanding of the pathways through which the DAGs exert their biological activities.

Previous studies have shown that selected structural modifications in DAGs lead to pronounced changes in their membrane associated regulatory enzymic properties in vitro.[6d] All naturally occurring DAGs containing two saturated acyl chains are known to perturb the membrane at the lipid−water interface, while their unsatur-

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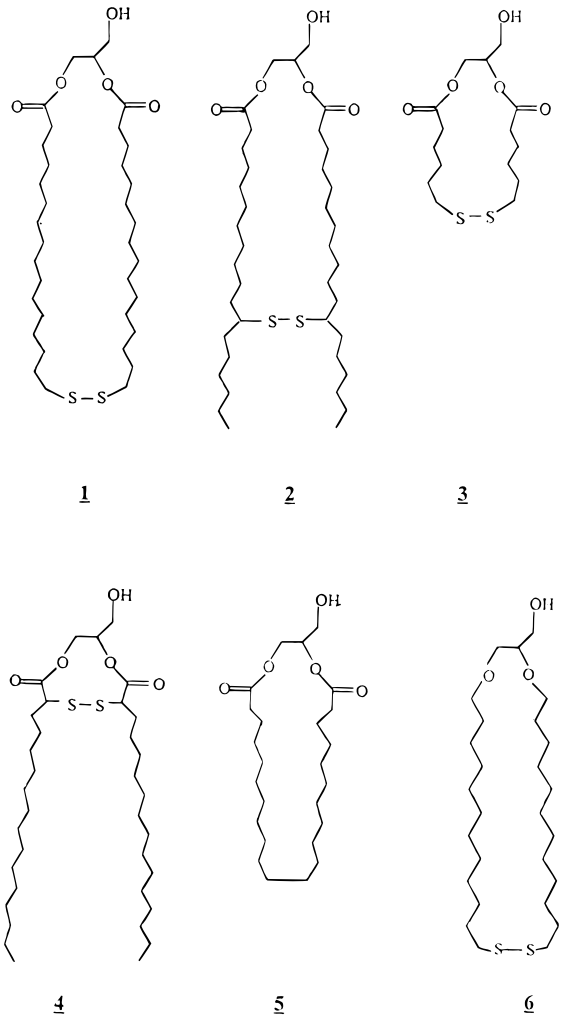
Chart 1



ated counterparts such as dioleoylglycerol (DOG) perturb the membrane only deep within the bilayer. Zidovetzki et al.[6d] have also shown that activation of PLA_2 due to DAG incorporation in model membranes depends on subtle perturbations at different depths of the membrane bilayer. Hence it would be desirable to develop systems in which the hydrocarbon part is modified to cause perturbation at a specific position within the bilayer. With such systems, one can look for the effect of such a perturbation to find out if any correlation could be made between PLA_2 activation and the exact location within the membrane at which perturbation is required for an enhanced activity of the enzyme.

Keeping these facts in mind, we synthesized macrocyclic 1,2-DAG analogues (**1**−**6**) in which the acyl/alkyl chains are connected at different positions to restrict the extents of chain motions within the membrane (Chart 1). Because of the use of a disulfide connection, these macrocycles can be conveniently opened up from disulfide to the corresponding DAG-containing bisthiols via an in situ reductive step.[7] This also enables us to compare the effect of macrocyclization with respect to that for its analogue in which chain motions are not restricted.

In a preliminary communication,[8] we reported the first synthesis of three disulfide-tethered macrocyclic diacylglycerols. In the present study, we present the full details of the syntheses of altogether six macrocyclic DAGs, including the ones described in earlier communication. Newly synthesized DAGs include one with a short chain (C_6) macrocyclic disulfide unit. Another one contains an ether type of hydrocarbon chain−glyceryl backbone connector (dialkylglycerol), and the third contains a carbon−carbon tethering at the chain termini instead of a disulfide connection. Upon completion of the synthesis and adequate characterization of the newly described DAG analogues, we included a few of them in DPPC membranes and determined their abilities to modulate the activities of cobra venom and bee venom PLA_2 enzymes. We also describe herein the results of incorporation of a few of these macrocyclic DAGs into DPPC membranes as revealed from the examination of calorimetric and ^{31}P spectroscopic studies of various DAG−DPPC mixed coaggregates.

Results and Discussion

Background. The DAGs (**1**−**6**) possess unique structural features as the acyl or alkyl chains are connected to form a macrocyclic ring and could therefore be considered as putative precursors of lipids of archaebacterial origin. The role of macrocyclic rings in archaebacterial lipids in providing thermal stability to the membranes has not been established yet but are expected to provide a very different lipid packing and overall membrane property, which might have bearing in membrane stability and permeability and in the activities of various membrane-bound enzymes.

Very few reports of macrocyclic models of archaebacterial lipids are known[9] because any macrocyclization reaction is inevitably accompanied by unwanted intermolecular reactions thereby giving polymer products. Menger et al. synthesized[9d] a number of macrocyclic phospholipids in which the two acyl chains are joined at the end by a carbon−carbon single bond and showed that such restrictions in chain mobility result in the formation of thermally stable membranes. To understand the effect of restriction of chain mobility on overall membrane structure and its correlation to enzyme activity, we chose a disulfide connector between the acyl chains of the diacylglycerols in the present study.

To make sure that the effect of incorporation of these DAGs (if any) is due to the imposed restriction in chain mobility and not due to the presence of bulkier sulfur atoms, which might lead to size incompatibility and therefore packing differences when incorporated within the membrane bilayers, we also synthesized a control macrocyclic DAG **5** where instead of a disulfide linkage a carbon−carbon tether is introduced by following modified Glaser coupling conditions developed by Menger et al.[9d]

Synthesis. When we initiated this study, a survey of the available methods for the synthesis of lipids revealed that there were limitations inherent in macrocyclization

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Table 1. Summary of Salient Features of Diacylglycerols Synthesized and Isolated Yields of the Final Macrocyclization Step

DAG[a]	macrocycle ring size	depth[b] (Å)	time (h)	% yield
1	38	20.5	3	55
2	30	14.7	5	62
3	18	7.0	4	65
4	10	2.8	2	70
5	26	12.7		75
6	30	16.5	6	45

[a] For actual structure, see Chart 1. [b] Depth indicates the length at which the tether is likely to be present within the membrane.

procedures.[10] Therefore, development of new procedures for giant ring closures was necessary as a prerequisite to the studies described herein.

Thus, the crucial step was the intramolecular disulfide formation to give the macrocyclic product. The major problem in this regard is the competing *intermolecular* disulfide formation resulting in polymeric products. Mindful of this limitation in widely adopted procedures for disulfide formation, we chose to employ a unique reagent, benzyl triethylammonium tetrathiomolybdate (BTAT),[11] in the key macrocyclization step. This reagent acts as a sulfur transfer agent and, due to its templating mechanism, unambiguously gives the desired intramolecular disulfide-containing DAG in good yields in a single step (Table 1). Although a number of procedures for preparing simple saturated 1,2-DAGs are known, the approach we adopted is direct, and the protection and deprotection steps are less cumbersome. At the same time, this affords good yields of the intermediates before the last macrocyclization step is reached.

Multistep syntheses of various disulfide-tethered diacylglycerols (Scheme 1) are detailed in the Experimental Section. Following this protocol, the bis(*ω*-bromoacyl) glycerols that were candidates for macrocyclization were obtained in good yield in only three steps starting from the commercially available starting material 1,2-isopropylideneglycerol. Once these bis(*ω*-bromoacyl)glycerols were synthesized, treatment with 2 equiv of the reagent benzyl triethylammonium tetrathiomolybdate (BTAT) gave us the desired *intramolecular* disulfide as the only isolable product. The reaction mixture in the sulfur-transfer step was first subjected to a workup. Upon workup, the material on a silica gel coated TLC plate showed a major (>90%) spot at $R_f \approx 0.5$ in addition to a minor spot at the base in hexane/EtOAc (4:1). By column chromatography, the material corresponding to the $R_f \approx 0.5$ was isolated. Subsequent spectral and analytical characterization established the identity of this material as the *intramolecular* disulfide. The material corresponding to the minor spot at the base of the TLC plate originated from the formation of inorganic tetrathiomolybdate related material.

It is noteworthy that the MEM deprotection was also equally successful on the macrocyclic disulfide-containing MEM-protected DAG. For DAG **4**, the corresponding bromide being at the α carbon of the carbonyl group gave, however, an inseparable mixture of mono- and disulfides upon treatment with BTAT. This problem was overcome by converting the dibromo precursor to the corresponding dithiocyanate in quantitative yields by stirring **11d** with excess NH_4SCN in acetone at ambient temperature. Bis-(2-thiocyanatopalmitoyl)glycerol was subsequently converted to the corresponding DAG α,α-disulfide by treating the bisthiocyanate with 2.2 equiv of the same reagent (BTAT), which in this case acts via reductive dimerization rather than by simple sulfur transfer.[12]

DAG **5**, which consists of a macrocycle with an all-carbon backbone, was synthesized as shown in Scheme 2. Briefly, benzylglycerol was obtained from isopropylidene glycerol. This was then acylated with the corresponding α,*ω*-alkynoic acid to yield bis(10,10′-undecynoyl)benzylglycerol. This was then subjected to Glaser coupling conditions (CuCl, TMEDA, xylene, O_2) to obtain bis(10,11-docosandiyne-1,22-dioyl) benzylglycerol **16** in ∼75% yield. This was then hydrogenated to remove the benzyl group to afford DAG **5** in excellent yield. The other DAG **6** that has been reported here has a different structural feature. This contains an ether type hydrocarbon−glycerol backbone linkage instead of a diester connection, and the two chains in this molecule are tethered via a disulfide bridge at the chain termini. The macrocyclization step was similar to that adopted in Scheme 1 for other disulfide formation. However, synthesis of the precursor bis(*ω*-bromoalkyl)glycerol required etherification as shown in Scheme 2.

Reduction of the Disulfides. Since our idea was to see the effect of restriction of chain mobility within the membrane bilayers of DPPC, it became imperative for us to convert the disulfide tether to the corresponding free chain DAG (containing thiols) by opening the macrocycle and look for any change in the properties at the membrane level. This would involve first the reduction of the intramolecular disulfide bridge of the diacylglycerols and then its incorporation in DPPC membranes. Hence, we decided to perform prior reduction of the macrocyclic disulfides in solution and characterize the resulting product to ensure a quantitative extent of disulfide to thiol conversion. First, reduction of DAG **3** (0.6 mmol) was attempted in ethanol upon incubation with dithiothreitol (DTT) (13.2 mmol) for 30 min. A qualitative inspection of thin layer chromatographic plates (silica gel) eluted in hexane/EtOAc (4:1) indicated the presence of components lying at $R_f \approx 0.2$, with complete disappearance of spot at $R_f \approx 0.5$, which corresponds to unreduced disulfide DAG. Their ^{1}H NMR spectra also confirmed the conversion of disulfide to thiol (Figure 1D). Thus the $-CH_2-CH_2-S-S-$ peak which appears in the ^{1}H NMR spectrum at 2.7 ppm as a triplet in the disulfide DAG changes to a multiplet at 2.55 ppm due to formation of $-CH_2-CH_2-SH$ upon treatment with the reducing agent. To further confirm and quantify the extent of reduction of the disulfide DAG to the corresponding dithiol analogue, Ellman's reaction[13] was also performed on the product isolated after DTT treatment. The disulfide DAG at a concentration of 0.07 mM showed a residual absorbance of 0.043 with no maximum at 412 nm, whereas the corresponding reduced product showed an absorbance of ∼0.6 with the maximum at 412 nm (Figure 1B). All other disulfide-containing macrocyclic DAGs could be reduced to their open chain thiol-containing analogues by the above procedure.

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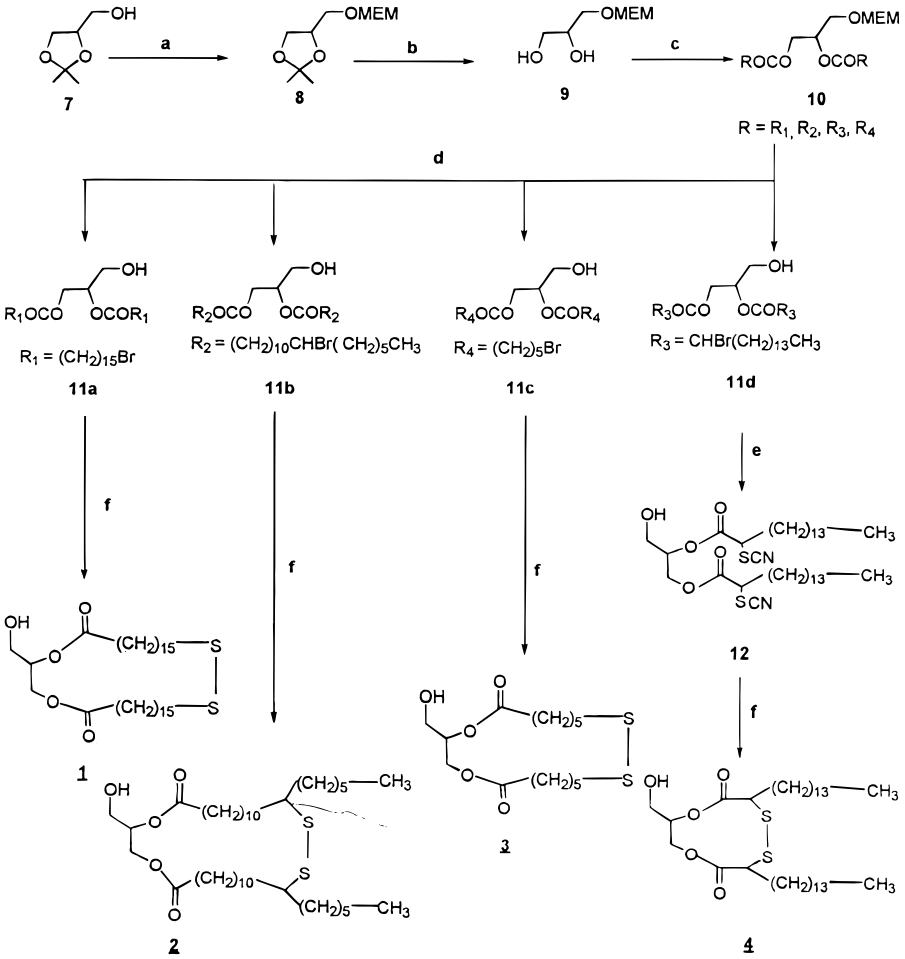
Scheme 1[a]



[a] Reagents and conditions: (a) NaH, THF, MEMCl, 0 °C, N_2, 1.5 h, 80%; (b) *p*-TsOH, MeOH−H_2O, RT, 2 h, 91%; (c) RCO_2H, DCC, DMAP, CCl_4, 4 h, [R = R_1, 65%; R = R_2, 87%; R = R_3, 60%; R = R4, 93%]; (d) $TiCl_4$, CH_2Cl_2, N_2, 0 °C, 1 h, 80%; (e) NH_4SCN, acetone, RT, 6h, 75%; (f) $(BnNEt_3)_2MoS_4$, DMF, RT, [R = R_1, 3 h, 55%; R = R_2, 3 h, 62%; R = R3, 5 h, 62%; R = R_4, 2 h, 69%].

Calculation of Energy-Minimized Conformations. Molecular mechanics calculations using the INSIGHT II 2.3.5 package (DISCOVER, Biosym. Technologies) show energy-minimized, preferred conformations of the macrocyclic DAGs and also give a reasonable idea about the depth at which the tether is positioned within the membrane (Table 1).

For the purpose of calculation of energy-minimized conformations of the DAGs, the basic glyceryl backbone−hydrocarbon skeleton of each of these DAGs has been taken from the known crystal structure of phospholipids.[14] This structure was then modified by inserting a disulfide bond at an appropriate position on the hydrocarbon chains along with removal of the phosphatidylcholine portion of the phospholipid. Then each of these structure was subjected to the minimization as described above. After several iterations, the energy of the conformation did not vary, and we considered this minimized structure for measuring the depth of the tether even if this might not be the exact global minima for the DAG. It is gratifying to mention here that the energy-minimized structure for the corresponding dilauryl glycerol obtained by the above-described procedure is in close agreement with that of the reported crystal structure[14b] of the same compound. Since the rationale behind this calculation was to have an idea about the comparative positions of the tether, we feel that this calculation serves our purpose.

Figure 2 shows energy-minimized structure of DAGs (**1**−**6**) along with the structures of naturally occurring dipalmitoylglycerol (DPG). In the case of the open chain DPG, the two acyl (palmitoyl) chains are seen to move away from each other. Joining the two chains at the end makes the molecule adopt a rodlike structure. At the same time, tethering introduces a "bulge" at the level of the disulfide linkage at a depth of ca. 20.5 Å. A similar rodlike appearance is also observed for the $-CH_2-CH_2-$ end-linked structure in DAG **5**, although in this molecule there is no significant distortion at the tethering sites. However, this DAG being the one with shorter acyl chains, the tethering is at a depth of ∼12.7 Å, which when incorporated in DPPC membranes should be around the middle of the hydrocarbon part of the bilayer leaflets. In the case of DAG **2**, where chain tethering is around the middle (∼14.7 Å) of the chain (C-12), a rodlike shape is formed up to the level where the macrocyclic ring is joined. The portions of the two acyl chains that are not tethered remain free and move away from each other. For DAG **4**, in which the disulfide is located at the α position with respect to the ester carbonyl group, the

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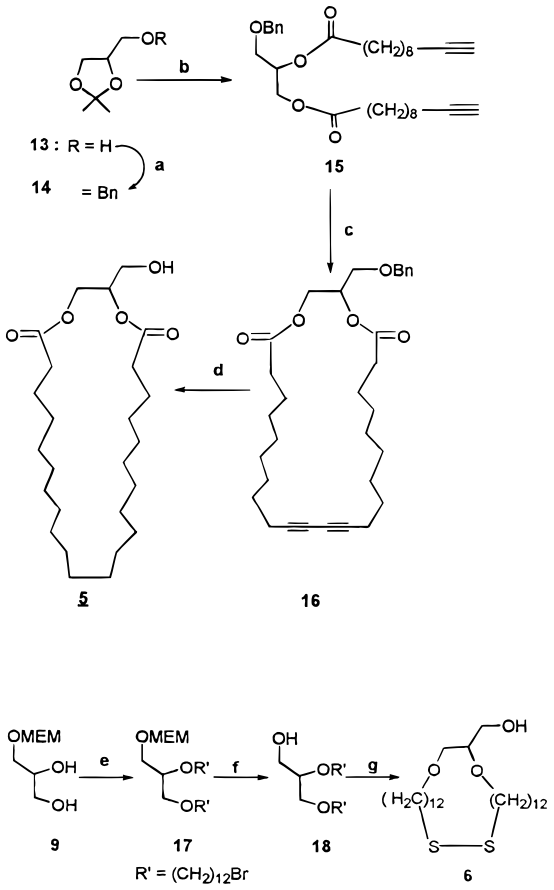
Scheme 2[a]



[a] Reagents and conditions: (a) (i) $PhCH_2Cl$, KOH, toluene, reflux, 16 h, 56%; (ii) *p*-TsOH, $MeOH-H_2O$, RT, 2 h, 95%; (b) $\equiv-(CH_2)_8CO_2H$, DCC, DMAP, CCl_4, RT, 4 h, 80%; (c) CuCl, TMEDA, xylene, 140 °C, O_2, 6 h, 75%; (d) Pd/C, THF−EtOH, H_2, 40 psi, 3 h, 60%; (e) NaH, THF, R/Br, reflux, 24 h, 45%; (f) $TiCl_4$, CH_2Cl_2, N_2, 0 °C, 58%; (g) $(BnNEt_3)_2MoS_4$, DMF, RT, 7 h, 45%.

energy-minimized structure is quite different from those of the other DAGs. Here, the disulfide linkage, in order to accommodate itself, adopts a totally different conformation around the glycerol backbone region.

^{31}P NMR Spectroscopy. Naturally occurring DAGs when incorporated within bilayer membranes produce nonlamellar phases, which appear as isotropic signals in ^{31}P NMR.[6c,15,16] It is these nonlamellar phases that are believed to expose the substrates (phospholipids) to the membrane-bound enzyme PLA_2. To ascertain the effect of the macrocyclic DAGs on the phase polymorphism of dipalmitoylphosphocholine (DPPC) membranes, we investigated specific DAG/DPPC mixed aggregates by ^{31}P NMR spectroscopy. The ^{31}P NMR spectra were recorded both above (50 °C) and below (25 °C) the phase transition temperatures for aqueous dispersions of DPPC codispersed with different concentrations of DAG **1** (Figure 3). These studies show that in the gel phase, i.e., below the phase transition temperature of DPPC membranes, the spectra are similar for concentrations ranging from 1 to 30 mol % of DAG **1** and consist of broad, axially anisotropic line shape characteristic of phospholipids in a typical bilayer arrangement. As the temperature was increased beyond T_m to ∼50 °C, the asymmetric line shape narrowed indicating that a gel to liquid-crystalline phase transition had taken place.[16] A noteworthy feature of this spectrum is the appearance of an additional isotropic peak even with a very small concentration of DAG (∼1 mol %), which is also relatively small in terms of total integrated area. The presence of this isotropic component is indicative of a situation where, due to the presence of DAGs, some of the DPPC molecules undergo rapid motion thereby completely averaging the chemical shift anisotropy (csa). This peak corresponds to either formation of regions of higher curvature or nonlamellar phases within the bilayer surface.[17] This means that the incorporation of macrocyclic DAG having tethering at the end of the hydrocarbon chain within DPPC bilayers produces local disturbances in packing, which appear as different entities only on melting of the main bilayer. As mentioned earlier, it might be these regions of nonlamellar phases that play a role in increasing the susceptibility of the bilayer to PLA_2 attack. However, on incorporation of a high concentration of DAG (>20 mol %), the line shape narrows showing reduced anisotropy with a decrease in the $\Delta\sigma_{csa}$ to 16 ppm from that of 30 ppm in case of parent DPPC vesicle (not shown). Interestingly, on incorporation of the corresponding reduced DAG at the same concentration (1−3 mol %) in the DPPC bilayer, no such isotropic phases were observed even beyond the phase transition temperature. Reduction of the disulfide opens up the macrocycle and thereby relieves the molecules of the gauche conformations, which are created as a consequence of chain tethering in macrocycles. This renders the reduced DAGs more "acceptable" to the parent DPPC bilayer, indicating that a packing disturbance in the hydrocarbon part of the bilayer determines the formation of nonamellar phases. The results of incorporation of most of the other disulfide DAGs in DPPC membranes in their disulfide were similar. Thus, upon reduction to their open chain nonmacrocyclic forms, no isotropic phases could be seen. All the other macrocylic disulfide-tethered DAGs showed isotropic phases above the phase transition temperature. Such isotropic phases disappeared when similar experiments were done with their reduced analogues. It is tempting to speculate that these macrocyclic DAGs might be potential activators of phospholipase A_2. It was, however, intriguing to find that DAG **5**, which had an all-carbon macrocycle, did not show an isotropic peak at 50 °C. Since the length of the hydrocarbon chain is smaller in this DAG **5**, it may be possible that the packing arrangement of this DAG within the DPPC membrane is totally different from that of DAG **1**, which is unable to induce isotropic phase.[15]

Thermotropic Phase Behavior. The temperature at which a phospholipid bilayer is half converted from a solid gel phase into a fluid phase is the gel to liquid crystalline phase transition temperature (T_m). Such phase transitions are in general a highly cooperative event. The cooperativity unit (CU)[18] gives an idea about the number of lipid molecules that undergo melting

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(18) The cooperative unit is defined as follows: cooperative unit = $(\Delta H_v/\Delta H_c)$, where ΔH_v is the van't Hoff enthalpy and ΔH_c is the calorimetric enthalpy.

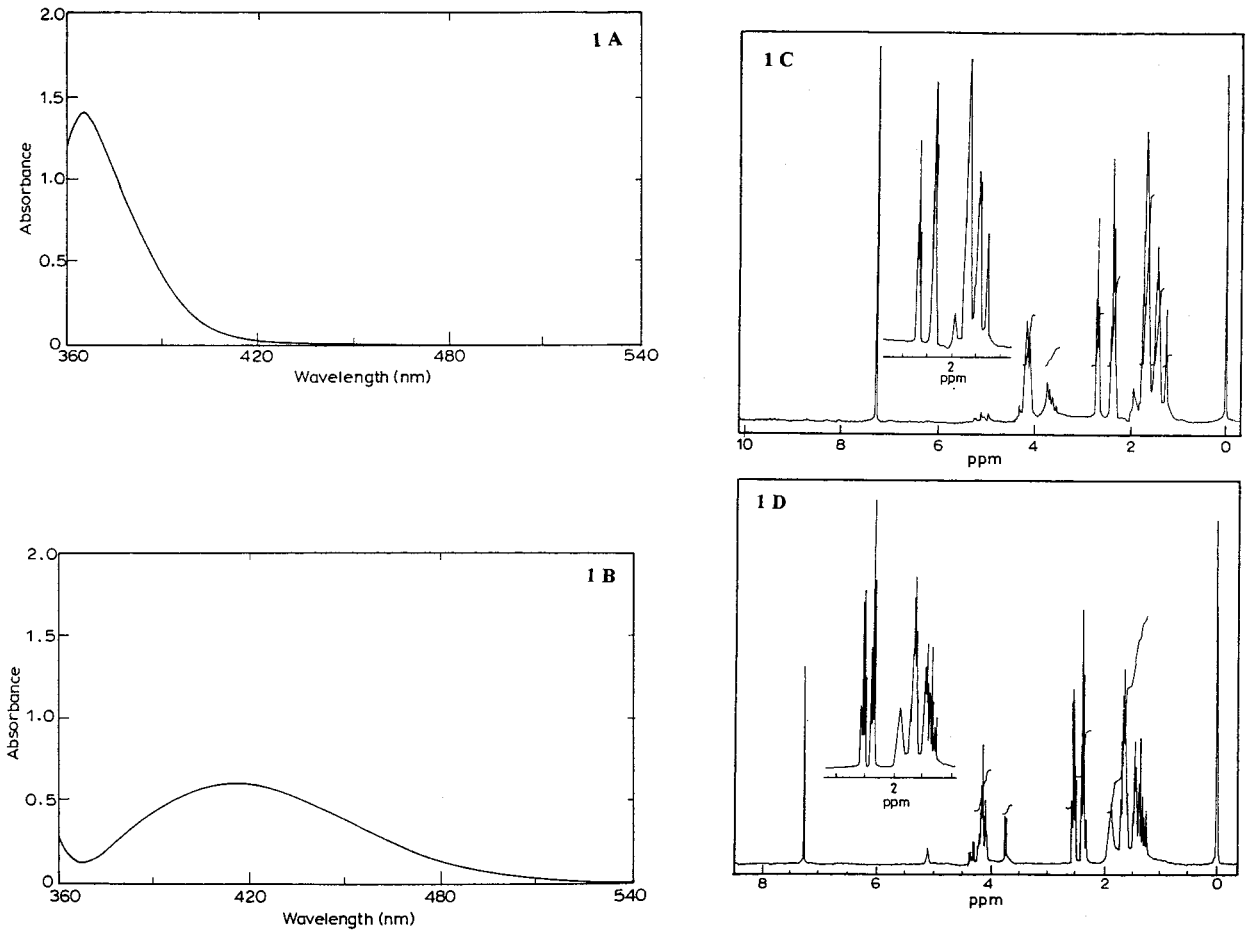

Figure 1. Comparison of the UV−vis and ^{1}H-NMR (300 MHz) spectra of DAG **3** and its reduced analog. Panel A shows the UV−vis spectrum of DAG **3** in ethanol upon treatment with Ellman's reagent. Panel B shows the UV−vis spectrum of DAG **3** in ethanol after treatment with DTT followed by addition of Ellman's reagent. Panel C shows the ^{1}H-NMR spectrum of DAG **3**. Panel D shows the ^{1}H-NMR spectrum of DAG **3** upon DTT treatment followed by purification of the product.

together. Hence, when the DAGs are more homogeneously distributed, the CU of transition should be high. A summary of the calorimetric data that characterize these phase transitions for various DPPC/DAG mixed aggregates is presented in Table 2. It is clear that the solid to fluid bilayer melting temperatures of DPPC in different coaggregates are little affected upon incorporation of various DAGs, even up to ~10 mol %. Comparison of the enthalpies of transition as well as the cooperativity unit of a DAG **1**/DPPC bilayer with its reduced DAG/DPPC bilayer clearly indicates a recovery of these parameters on reduction of the macrocyclic DAG to its corresponding free chain thiol analogue. The same is the case for other disulfide DAGs and their reduced analogues. This observation again substantiates our observation in ^{31}P NMR spectroscopy that opening of the macrocylic DAG by reduction makes it more "adaptable" to the DPPC bilayer thereby making a more homogeneous bilayer and hence showing the higher cooperativity value. The observation of disappearance of the isotropic peak at 50 °C on adding higher concentrations of DAG is also substantiated by the increased cooperativity value at higher DAG concentrations.

Phospholipase A_2 Assay. We also present herein the results on PLA_2 activity due to incorporation of one representative tethered DAG **1** into DPPC bilayer membrane. This result has been shown in terms of relative activities of the PLA_2s, which is actually a ratio of the initial rates of reaction obtained in the absence or in the presence of DAGs. In Figure 4, a comparison of the relative activities of two different PLA_2s in DAG **1** doped DPPC membranes is shown. As is seen from the figure, increasing concentrations of DAG **1** within DPPC membranes steadily enhances the activity of the two venom PLA_2s. Incorporation of 30 mol % DAG **1** enhanced the activity of bee venom ~6-fold and the activity of cobra venom ~4.5-fold with respect to that of the parent DPPC membrane. These results are similar to those for other macrocylic DAGs.

Many investigators propose that certain activators perturb membrane structure such that phospholipid substrates become more "exposed" to the membrane-bound PLA_2 enzymes. Most activators of PLA_2 that have been already reported in the literature act not by activating the membrane bound PLA_2 but by allowing more of the enzyme in the water layer to bind to the membrane interface. Generally, most of the PLA_2s, including the ones examined in the present study, bind not so strongly to the vesicles of phosphatidylcholine. Thus, most of the PLA_2 remains in the water layer, and the reaction velocity is slow. Under such conditions, many compounds that can partition into the vesicle interface can lead to more enzyme−membrane binding, and such agents will function as putative activators of PLA_2. This type of activation may not be specific activation in the usual sense of the activator binding directly to the enzyme and

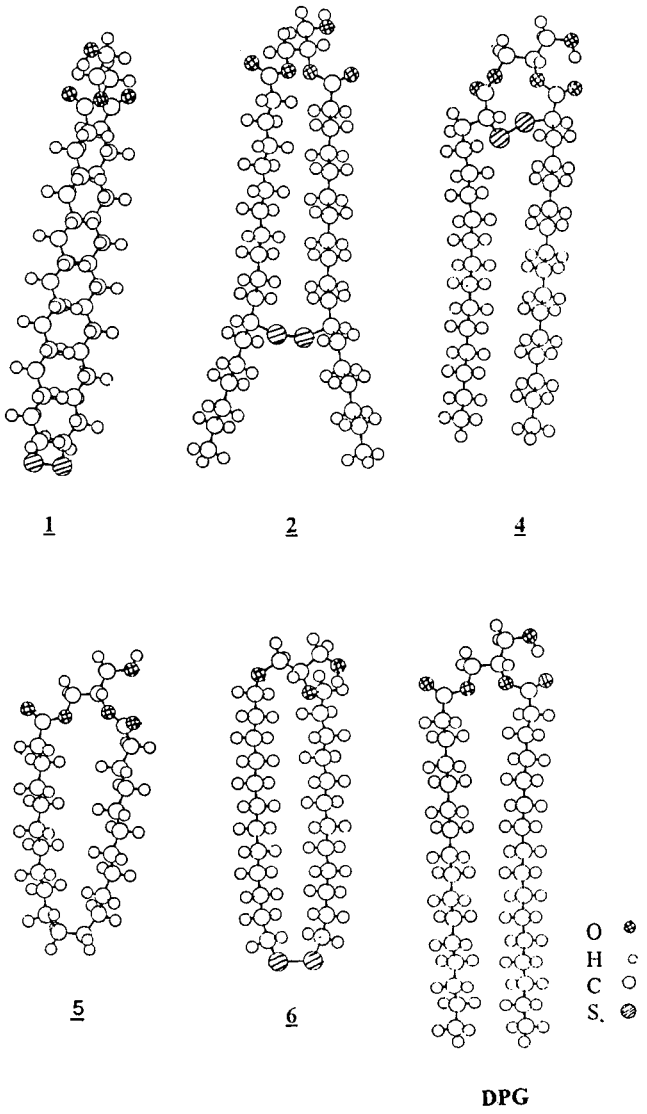

Figure 2. Ball and stick model representations of energy-minimized (INSIGHT) structures of the macrocyclic DAGs (**1**,**2** and **4**−**6**). Also shown is the structure of naturally occurring DAG, dipalmitoylglycerol (DPG), for comparison.

altering its catalytic properties. It is likely that the DAG analogues prepared in the present study cause activation of PLA_2 indirectly by making the phosphatidylcholine substrates more available to the PLA_2. Hence, it is evident that macrocyclization in the DAGs does perturb the DPPC membrane bilayer in a way such that increasing amounts of the PLA_2 enzyme can now be at the lipid−water interface, thereby showing enhanced activity.

Conclusions

In summary, we have synthesized a series of macrocyclic diacylglycerols (**1**−**6**) where a disulfide linkage has been used to "stitch" the two hydrocarbon chains. Our synthetic strategy is general and involves convenient protection and deprotection reactions as well as mild reaction conditions. We have been able to join the two hydrocarbon chains by disulfide linkage at different depths thereby providing systems with different extents of restriction in chain mobility. Interchain linking through a disulfide tether also provided the extra advantage of converting these macrocycles into the corresponding open chain thiol analogues via a simple dithiothreitol-mediated reduction step. The access to the open chain analogue allows further investigation to systematically compare the effects of macrocyclization. The synthesis outlined herein convincingly establish the utility of this intramolecular disulfide coupling protocol for the efficient synthesis of this important class of molecules. Though the overall yield for each of these macrocyclic DAGs are moderate to good, we feel that this method would find application in the synthesis of related macrocyclic compounds. One notable strength of the syn-

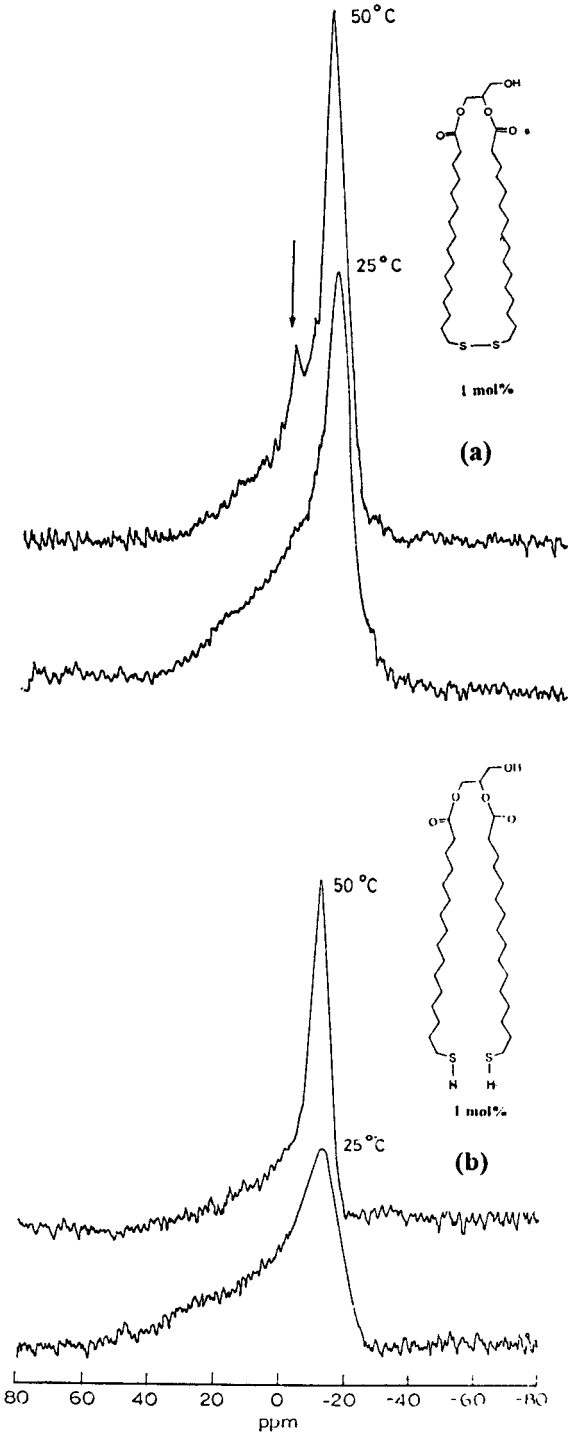

Figure 3. ^{31}P-NMR spectra of DPPC bilayer membranes incorporated with (a) DAG **1** and (b) DAG **1** that has been reduced by prior DTT treatment.

Table 2. Thermotropic Transition Data on the DAG-Doped DPPC Coaggregates[a]

DAG	conc (mol %)	T_m (°C)[b]	$\Delta H_c \times$ E3[c]	$\Delta H_v \times$ E3[c]	C. U. ($\Delta H_v/\Delta H_c$)
1	3	40.9	5.08	421	82.9
	10	40.9	4.57	282	61.7
reduced	3	41.3	3.6	330	91.7
	10	41.2	3.8	257	67.8
2	10	39.8	5.32	213	40.04
reduced	10	39.3	6.15	262	42.6
3	5	39.04	6.9	617	90.07
	10	38.9	6.6	423	64.1
reduced	5	40.1	7.06	809	114.6
	10	39.6	6.9	546	78.7
4	5	39.5	7.5	254	33.7
	15	39.5	7.2	170	23.7
reduced	5	41.6	7.5	359	48.2
	15	44.6	8.3	230	27.8

[a] See text for preparation of individual coaggregates. [b] T_m refers to the main transition temperature. [c] ΔH_c and ΔH_v values are expressed as kcal mol^{-1}.

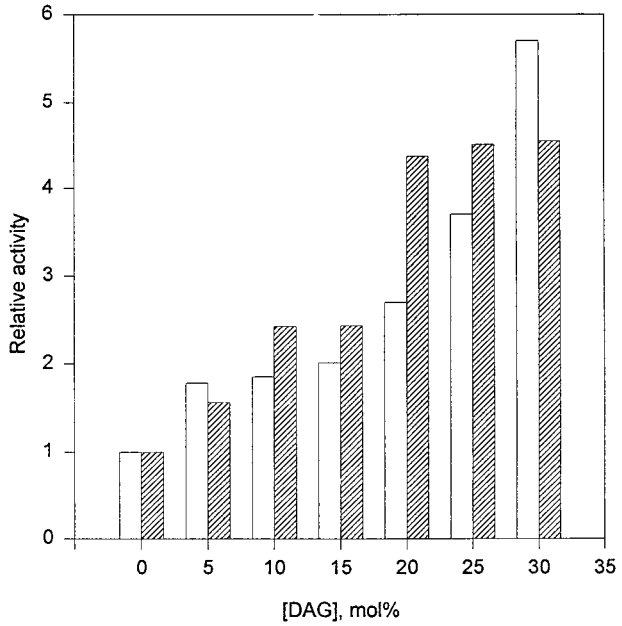

Figure 4. Histogram showing the activity of PLA_2s on DAG **1** incorporated DPPC vesicles. Hollow bar corresponds to bee venom PLA_2, while shaded bar corresponds to cobra venom PLA_2.

thetic strategy adopted herein is that this unambiguously gives the *intramolecular macrocyclic disulfide* as the only isolable product, thus making the separation and the purification a much easier task.

The macrocyclic DAGs (**1**−**6**) and their reduced open chain analogues have been used to investigate the effect of imposing chain restriction within the membrane bilayer. Preliminary results of these investigation have been reported here. ^{31}P NMR of DAG **1** incorporated DPPC vesicles indicates formation of isotropic phases. These phases disappear upon the opening of the macrocycle by the reduction of the disulfide tether. Moreover, thermotropic properties have been studied for DPPC membranes containing various amounts of individual restricted DAGs (**1**−**6**) as well as their nonmacrocyclic analogues. These studies indicate that the cooperativity of the vesicular system is enhanced upon relaxation of the macrocyclic DAG to the open chain version. Preliminary studies establish that macrocyclization of DAG **1** results in an *enhancement* of the activity of both types of PLA_2s examined herein. However, their effect on PLA_2 activation might be phenomenological. It is likely that the DAG **1** analogues function to activate PLA_2 by causing an increasing fraction of enzyme to be at the membrane surface.

The fact that until now only a few "structure−activity" comparisons between appropriately designed synthetic DAGs with differing extents of hydrocarbon modifications have been carried out reflects the inaccessibility of the required hydrocarbon-modified DAGs. In the present work, we demonstrate how effective design and synthesis of large macrocyclic diacylglycerols influences phase transition and other properties of the DPPC membranes. We are currently examining further details of the effects of incorporation of these diacylglycerol derivatives in other membranes.

Experimental Section

General. Unless noted otherwise, all starting materials were obtained from the best known commercial suppliers and were used without further purification. THF was distilled from sodium benzophenone under N_2, and CH_2Cl_2 was distilled from P_2O_5 under N_2 immediately prior to use. DMF was distilled under reduced pressure over P_2O_5 and stored over molecular sieves (4 Å). Reactions involving air- and/or moisture-sensitive reagents were executed under an inert atmosphere of dry N_2. Column chromatography was performed using silica gel (Merck, 60−120 mesh). Benzyltriethylammonium tetrathiomolybdate was prepared as reported earlier.[19] *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoic acid (NBD-aminohexanoic acid), dipalmitoylphosphocholine (DPPC), 1-hexadecanoyl-2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC), phospholipases A_2 (cobra venom *Naja mocambique* and bee venom), and TRIZMA base were obtained from Sigma, and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) was purchased from Aldrich and used as received. Descriptions of analytical instruments, ^{1}H NMR, IR, fluoroscence, mass and UV−vis spectrometers have been previously published.[20] ^{1}H and ^{13}C NMR spectra were obtained at the indicated field for each compound as solutions in $CDCl_3$. Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in parts per million (ppm δ) downfield relative to the internal standard tetramethylsilane (TMS); for the ^{13}C spectra TMS was referenced to the central line of the $CDCl_3$ triplet (δ 77.0). Low-voltage ionization (30 eV) was used to record the mass spectra.

General Procedure for Preparing 1,2-*O*-Diacyl-3-*O*-(2-methoxyethoxymethyl)glycerol.[21] The diol, **9** (0.263 g, 1.49 mmol), was esterified with the requisite fatty acid by adding a solution of diol in dry CCl_4 (0.1 g/mL) to a solution of 2.2 mol equiv of the acid in dry CCl_4 (0.1 g/mL) and a catalytic amount of DMAP at 0 °C. To the resulting mixture was added *N*,*N*-dicyclohexylcarbodiimide (0.71 g, 2.99 mmol), and the mixture was stirred for 4 h. DCU formed during the reaction was filtered off, and the precipitate was washed with 2 mL of CCl_4. The filtrate and washings were taken together, and the solvent was evaporated to give a crude product, which was purified by column chromatography eluting with the solvent indicated for each specific compound.

1,2-Bis(16-bromohexadecanoyl)-3-*O*-(2-methoxyethoxymethyl)glycerol (10a). 10a was obtained as a colorless, viscous oil in 65% yield from **9** by column chromatography upon elution with hexane/EtOAc (12:1): IR (neat) ν 1740 cm^{-1};

(19) Ramesha, A. R.; Chandrasekaran, S. *Synth. Commun.* **1992**, *22*, 3277.
(20) (a) Bhattacharya, S.; Snehalatha, K.; George, S. K. *J. Org. Chem.* **1998**, *63*, 27. (b) Bhattacharya, S.; Snehalatha, K. *J. Org. Chem.* **1997**, *62*, 2198. (c) Bhattacharya, S.; Haldar, S. *Biochim. Biophys. Acta* **1996**, *1283*, 21−30.
(21) Yamauchi, K.; Hihara, M.; Kinoshita, M. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 2169−2172.

^{1}H NMR (200 MHz) δ 1.25 (s, 52H), 2.32 (t, 4H), 3.4−3.8 (complex m, 13H), 4.11−4.3 (m, 2H), 4.7 (s, 2H), 5.23 (m, 1H); ^{13}C NMR (22.5 MHz) δ 24.87, 28.12, 28.66, 29.53, 32.78,33.87, 58.89, 62.47, 65.94, 66.91, 69.94, 71.57, 95.5, 172.88, 173.2.

1,2-Bis(6-bromohexanoyl)-3-*O*-(2-methoxyethoxymethyl)glycerol (10c). 10c was obtained as a colorless, viscous oil in 92.4% yield from **9** by column chromatography eluting with hexane/EtOAc (12:1): IR (neat) ν 1740 cm^{-1}; ^{1}H NMR (80 MHz) δ 1.25 (m,12H), 2.25 (t, 4H), 3.25 (t, 4H), 3.5−3.75 (m, 9H), 4.0−4.3 (m, 2H), 4.65 (s, 2H), 5.0−5.2 (m, 1H); ^{13}C NMR (22.5 MHz) δ 23.9, 27.47, 32.24, 33.32, 33.76, 58.89, 62.58, 65.83, 70.05, 71.57, 95.51, 172.55, 172.88.

1,2-Bis(12-bromooctadecanoyl)-3-*O*-(2-methoxyethoxymethyl)glycerol (10b). 10b was obtained as a viscous oil in 86.5% yield from **9** by column chromatography eluting with hexane/EtOAc (10:1): IR (neat) ν 1730 cm^{-1}; ^{1}H NMR (200 MHz) δ 0.88 (t, 6H),1.25 (s, 56H), 2.30 (t, 4H), 3.39 (s, 3H), 3.1−4.31 (complex m, 10H), 4.71 (s, 2H), 5.22 (m, 1H); ^{13}C NMR (22.5 MHz) δ 13.82, 22.38, 24.65, 26.17, 27.36, 28.88, 29.42, 31.05, 31.70, 33.76, 38.96, 41.02, 58.24, 58.68, 62.25, 65.72, 66.69, 69.73, 71.46, 95.30, 172.447, 172.77.

1,2-Bis(2-bromohexadecanoyl)-3-*O*-(2-methoxyethoxymethyl)glycerol (10d). 10d was obtained as a viscous oil in 60% yield from **9** by column chromatography eluting with hexane/EtOAc (20:1): IR (neat) ν 1740 cm^{-1}; ^{1}H NMR (200 MHz) δ 0.88 (t, 6H), 1.25 (s, 52H), 3.36 (s, 3H), 3.3−3.7 (m, 6H), 4.0−4.35 (m, 4H), 4.6 (s, 2H), 5.2 (m, 1H).

1,2-Bis(12-bromododecyl)-3-*O*-(2-methoxyethoxymethyl)glycerol (17). NaH (0.34 g, 14.04 mmol) was added to a stirred dry THF solution (40 mL) of diol **9** (0.4 g, 2.27 mmol), and the solution was stirred at room temperature for 30 min. To it 1,12-dibromododecane (4.1 g, 12.5 mmol) was added, and the mixture was refluxed for 24 h. It was cooled and concentrated, and the residue was extracted with $CHCl_3$. The extract was washed with water, dried over anhyd Na_2SO_4, and concentrated. A colorless oil in 45% yield was obtained by column chromatography upon elution initially with hexane until excess dibromide was separated and then with hexane/EtOAc (6:1): ^{1}H NMR (90 MHz) δ 1.25 (s, 40H), 3.2−3.7 (complex m, 17H), 3.3 (s, 3H), 4.7 (s, 2H); ^{13}C NMR (22.5 MHz) δ 25.98, 28.06, 28.72, 29.5, 30.02, 32.75, 33.79, 70.46, 71.63, 75.66, 95.69, 114.04.

General Procedure for Deprotection of a Methoxyethoxymethyl (MEM) Group from 1,2-*O*-Diacyl/dialkyl-3-*O*-(2-methoxyethoxymethyl)glycerol. The MEM protecting group could be conveniently removed under aprotic conditions by stirring (0.713 mmol) of the appropriate 1,2-*O*-diacyl/dialkyl-3-*O*-(2-methoxyethoxymethyl)glycerol in dry CH_2Cl_2 with 2.1 mol equiv of $TiCl_4$ (1.4 mmol) under an N_2 blanket at 0 °C for 1 h. To it 1 M $NaHCO_3$ was added, and the resulting mixture was stirred vigorously and finally extracted with $CHCl_3$. The organic layer was washed with water, dried over anhyd Na_2SO_4, and concentrated.

1,2-Bis(16-bromohexadecanoyl)glycerol (11a). 11a was obtained as a white semisolid in 40% yield by column chromatography eluting with hexane/EtOAc (9:1): IR (neat) ν 3600−3300, 1740 cm^{-1}; ^{1}H NMR (80 MHz) δ 1.25 (s, 52H), 2.25 (t, 4H), 3.32 (t, 4H), 3.62 (d, 2H), 4.12(m, 2H), 5.0(m, 1H); ^{13}C NMR (22.5 MHz) δ 24.98, 28.23, 28.88, 29.64, 32.89, 34.08, 65.07, 68.43, 173.96.

1,2-Bis(6-bromohexanoyl)glycerol (11c). 11c was obtained as an oil in 50% yield by column chromatography eluting with hexane/EtOAc (9:1): IR (neat) ν 3600−3200, 1740 cm^{-1}; ^{1}H NMR (80 MHz) δ 1.25−1.5 (m, 12H), 2.3 (t, 4H), 3.25 (t, 4H), 3.65 (d, 2H), 4.0−4.25 (m, 2H), 5.0(m, 1H); ^{13}C NMR (22.5 MHz) δ 23.79, 25.31, 27.36, 32.13, 33.32, 33.65, 60.95, 62.25, 64.85, 67.67, 72.00, 173.31, 176.88.

1,2-Bis(12-bromooctadecanoyl)glycerol (11b). 11b was obtained as an oil in 72% yield by column chromatography eluting with hexane/EtOAc (9:1): IR (neat) ν 3400−3160, 1760 cm^{-1}; ^{1}H NMR (200 MHz) δ 0.88 (t, 6H), 1.25 (s, 52H), 3.65 (d, 2H), 3.8−4.2 (m, 2H), 5.0 (m, 1H); ^{13}C NMR (22.5 MHz) δ 14.04, 22.6, 24.76, 27.15, 27.47, 28.99, 29.42, 31.16, 31.81, 33.11, 33.97, 39.07, 61.28, 62.14, 64.96, 68.10, 72.00, 173.31, 173.74.

1,2-Bis(2-bromohexadecanoyl)glycerol (11d). 11d was obtained as a gum in 57% yield by column chromatography eluting with hexane/EtOAc (8:1): IR (neat) ν 3600−3300, 1780 cm^{-1}; ^{1}H NMR (80 MHz) δ 0.88 (t, 6H), 1.25 (s, 52H), 3.75 (d, 2H), 4.0−4.4 (m, 4H), 5.0 (m, 1H).

1,2-Bis(12-bromododecyl)glycerol (18). 18 was obtained as a viscous oil in 58% yield by column chromatography eluting with hexane/EtOAc (8:1): IR (neat) ν 3600−3400 cm^{-1}; ^{1}H NMR (80 MHz) δ 1.25 (s, 40H), 3.25−3.6 (complex m, 13H).

1,2-Bis(2-thiocyanatohexadecanoyl)glycerol (12). Compound **11d** (0.592 g, 0.868 mmol) was dissolved in 5 mL of acetone and to it was added a ∼20-fold excess of NH_4SCN (1.345 g, 17.6 mmol), and the mixture was stirred for 6 h. The acetone was evaporated, and the residue after aqueous workup gave a light-yellow oil in 75% yield via column chromatography using hexane/EtOAc (9:1): IR (neat) ν 3600−3300, 2140, 1760 cm^{-1}; ^{1}H NMR (80 MHz) δ 0.88 (t, 6H), 1.25 (s, 48H), 3.6−3.75 (m, 4H), 4.25 (m, 6H), 5.1 (m, 1H); LRMS, EI, *m*/*z* (%) 682 (M+)(2).

General Procedure for Preparing Macrocyclic Disulfide-Containing 1,2-Diacylglycerol. To a solution of the appropriate bis(ω-bromo) or bis(α-thiocyanato)acylglycerol (0.277 mmol) in DMF (20 mL) was added benzyltriethylammonium tetrathiomolybdate (0.4 g, 0.657 mmol), and the mixture was stirred for a requisite time (Table 1). The resulting mixture was concentrated, poured into water, and extracted with $CHCl_3$. The organic layer was washed with water, and the solvent was evaporated.

1,2-Bis(16-thiotetratriaconta-1,34-dioyl)glycerol (1). 1 was obtained as a white semisolid in 55% yield by column chromatography eluting with hexane/EtOAc (6:1): IR (neat) ν 3500−3100, 1720 cm^{-1}; ^{1}H NMR (200 MHz) δ 1.25 (s, 52H), 2.23 (t, 4H), 2.69 (t, 4H), 3.72 (d, 2H), 4.1−4.37 (m, 2H), 5.11-(m, 1H);^{13}C NMR (22.5 MHz) δ 24.4, 24.8, 27.4, 28.4, 28.8, 33.14, 33.89, 39.55, 61.44, 67.7, 73.11, 173.45, 173.7; LRMS, EI, *m*/*z* (%) 630 (M+) (85); HRMS, EI, calcd for $C_{35}H_{66}O_5S_2$ 630.4351, found 630.435

1,2-Bis(6-thiotetradeca-1,14-dioyl)glycerol (3). 3 was obtained as a colorless oil in 65% yield by column chromatography eluting with hexane/EtOAc (6:1): IR (neat) ν 3500−3100, 1720 cm^{-1}; ^{1}H NMR (80 MHz) δ 1.25−1.75 (m, 12H), 2.25 (t, 4H), 2.65 (t, 4H), 3.6 (d, 2H), 4.0−4.3 (m, 2H), 5.1 (m, 1H); ^{13}C NMR (22.5 MHz,) δ 24.44, 24. 76, 25.41, 27.47, 28.45, 28.66, 33.11, 33.87, 39.28, 39.50, 61.39, 62.79, 64.53, 67.67, 72.22, 173.53; LRMS, EI, *m*/*z* (%) 350 (M+) (85); HRMS, EI, calcd for $C_{15}H_{26}O_5S_2$ 350.1215, found 350.1208.

1,2-Bis(12-thiooctadecanoyl)glycerol (2). 2 was obtained as a colorless oil in 62% yield by column chromatography eluting with hexane/EtOAc (6:1): IR (neat) ν 3500−3100 cm^{-1}, 1740; ^{1}H NMR (200 MHz) δ 0.88 (t, 6H), 1.25(s, 56H), 2.26 (t, 4H), 2.6 (m, 1H), 2.8 (m, 1H), 3.72 (d, 2H), 4.1−4.4 (complex m, 2H), 5.06 (m, 1H); ^{13}C NMR (22.5 MHz) δ 14.15, 22.7, 24.98, 26.82, 27.15, 29.53, 31.92, 34.19, 39.072, 41.23, 65.07, 173.42, 173.85; LRMS, EI, *m*/*z* (%) 686 (M+) (20); HRMS, EI, calcd for $C_{39}H_{74}O_5S_2$ 686.4995, found 686.4978.

1,2-Bis(12-thiohexacosyl)glycerol (6). 6 was obtained as a white semisolid in 45% yield by column chromatography eluting with hexane/EtOAc (6:1): IR (neat) ν 3600−3240 cm^{-1}; ^{1}H NMR (200 MHz) δ 1.21 (s, 20H), 2.63 (t, 2H, CH_2−S−S−, J = 7.47 Hz), 3.25−3.6 (complex m, 8H); ^{13}C NMR (22.5 MHz) δ 14.15, 22.7, 27.26, 28.89, 29.43, 29.75, 32.03, 34.74, 45.57, 65.94, 169.52, 169.85; LRMS, EI, *m*/*z* (%) 490 (M+) (10); HRMS, EI, calcd for $C_{27}H_{54}O_3S_2$ requires 490.4111, found 490.3516.

1,2-Bis(2-thiohexadecanoyl)glycerol (4). 4 was obtained as a colorless oil in 70% yield by column chromatography eluting with hexane/EtOAc (8:1): IR (neat) ν 3600−3300, 1740 cm^{-1}; ^{1}H NMR (400 MHz) δ 0.81 (t, 6H), 1.18 (m, 52H), 3.2−3.4 (complex m, 2H), 3.7−4.6 (complex m, 4H), 5.25 (m, 1H); ^{13}C NMR (22.5 MHz) δ 14.04, 22.7, 27.25, 28.77, 29.31, 29.64, 31.92, 34.62, 45.57, 60.95, 63.23, 65.83, 67.67, 73.3, 169.73; LRMS, EI, *m*/*z* (%) 630 (M+) (5); HRMS, EI, calcd for $C_{35}H_{66}O_5S_2$ requires 630.4384, found 630.4352.

1,2-*O*-Diacyl-3-*O*-benzylglycerol. The diol[22] (0.263 g, 1.49 mmol) was condensed with the requisite acid by adding a solution of diol in dry CCl_4 (0.1 g/mL) to 2.2 mol equiv of the acid in dry CCl_4 (0.1 g/mL) and a catalytic amount of DMAP at 0 °C. To the resulting mixture was added *N*,*N*-dicyclohexylcarbodiimide (0.71 g, 2.99 mmol), and the mixture was stirred for 4 h. DCU formed during the reaction was filtered off, and the precipitate was washed with 2 mL of CCl_4. The solvent was evaporated from the filtrate and washings to give a crude product, which was purified by column chromatography eluting with a solvent mixture as indicated in the following.

1,2-Bis(10-undecynoyl)-3-*O*-benzylglycerol (15). 15 was obtained as a colorless oil in 80% yield by column chromatography eluting with hexane/EtOAc (8:1): IR (neat) ν 3300, 2120, 1760 cm^{-1}; ^1H NMR (80 MHz) δ 1.25 (s, 24H), 1.93 (t, 2H), 2.15−2.3 (complex m, 8H), 3.5 (d, 2H), 4.15−4.25 (m, 2H), 4.5 (s, 2H), 5.2 (q like, 1H), 7.3 (s, 5H); ^{13}C NMR (22.5 MHz) δ 18.06, 24.58, 27.56, 28.34, 28.67, 32.65, 33.76, 33.98, 62.39, 68.03, 69.80, 73.01, 84.28, 103.85, 116.01, 127.29, 128.06, 134.59, 137.46, 137.79, 172.62, 172.84; LRMS, EI, *m*/*z* (%) 510 (M^+) (5).

1,2-(10,11-Docosandiyne-1,22-dioyl)-3-*O*-benzylglycerol (16). To a three-necked 500 mL round-bottom flask fitted with a condenser and a rubber stopper with a glass tube reaching the bottom of the flask were added CuCl (1.0 g, 10 mmol), *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (20 mmol), and 300 mL of xylene, and the mixture was stirred. This was then heated gradually to 140 °C with the passage of bubbling O_2 through the reaction mixture. When the reaction mixture reached 140 °C, 3 mL of a solution of compound **15** (1.4 g, 2.75 mmol) in 50 mL of xylene was added to the reaction mixture slowly. The solution finally became dark brown in color. After this, the heating and O_2 bubbling were stopped, xylene was filtered off, the reaction mixture was acidified with concentrated HCl and extracted with $CHCl_3$ solvent was evaporated. The pure product from this material was isolated as a semisolid in 75% yield by column chromatography upon eluting with hexane/EtOAc (16:1): IR (neat) ν 1720 cm^{-1}; ^1H NMR (80 MHz) δ 1.25 (s, 24H), 1.6 (m, 4H), 2.25 (t, 4H), 3.5 (d, 2H), 4.0−4.25 (m, 3H), 4.5 (s, 2H), 7.3 (s, 5H); ^{13}C NMR (22.5 MHz) δ 18.82, 25.67, 28.49, 28.92, 29.36, 34.78, 34.99, 63.71, 66.74, 68.91, 70.75, 74.00, 128.39, 129.15, 130.45, 138.47, 173.79, 174.11; LRMS, EI, *m*/*z* (%) 508 (M+) (27).

1,2-(Docosane-1,22-dioyl)glycerol (5). A solution of compound **24** (0.5 g, 0.98 mmol) in THF−ethanol (20 mL, 4:1 v/v) was hydrogenated in the presence of 10% Pd/C (0.25 g, 50% w/w) at 40 psi for 3 h. The catalyst was filtered off, the solvent was evaporated, and the pure product was obtained as a solid in 60% yield by column chromatography eluting with hexane/EtOAc (5:1): IR (neat) ν 3600−3200, 1760 cm^{-1}; ^1H NMR (80 MHz) δ 1.25 (s, 36H), 2.25 (t, 4H), 3.6 (d, 2H), 4.1−4.25 (m, 2H), 5.0−5.1 (m, 1H); ^{13}C NMR (22.5 MHz) δ 24.87, 27.69, 28.12, 28.88, 34.08, 34.30, 61.17, 62.47, 64.85, 67.89, 72.12,-173.42, 173.64; LRMS, EI, *m*/*z* (%) 426 (M^+) (40); HRMS, EI, calcd for $C_{25}H_{46}O_5S_2$ 426.3341, found 426.3337.

Thiol Analysis. To a solution of a given disulfide containing DAG (1 equiv) in ethanol was added 2.2 equiv of dithiotreitol (DTT), and the mixture was stirred for 30 min at ambient temperature. Ethanol was evaporated, and the product was extracted with $CHCl_3$, washed with water, and dried with anhydrous Na_2SO_4. TLC indicated complete removal of DTT from the reaction mixture after four extractions. The resulting solution was concentrated upon evaporation of $CHCl_3$, and the residue was examined spectroscopically to ensure the quantitative conversion of disulfide to thiols. A representative ^1H NMR spectra for the product obtained upon treatment of DAG **3** with DTT is shown in Figure 1. A 0.5 M Tris buffer (pH 8.3) was prepared and diluted with an equal volume of ethanol containing 1% EDTA. To 20 mL of this solution was added 40 mg (0.1 mmol) of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to prepare a stock reagent solution. An amount of 0.04 mg of the DTT-induced reaction product was taken in 3 mL of the same buffer solution, and 0.1 mL of the DTNB reagent solution was added and kept for 30 min at room temperature with stirring. The absorbance of the resulting mixture was then measured at 412 nm. However, when the parent disulfide-containing DAG was treated with Ellman reagent without prior treatment with DTT, no absorption maximum (λ_{max}) at 412 nm was observed. This is consistent with the conclusion that more than a 2-fold molar excess of DTT ensures quantitative reduction of disulfide.

Vesicle Preparation. In a typical protocol used in this study, several mixtures of DPPC and an appropriate amount of DAG were mixed in a final volume of 0.3 mL of chloroform in a small glass tube. In these mixtures, the concentrations of DAG varied from 1 to 30 mol %. These solutions were evaporated to dryness under a stream of oxygen-free dry N_2. For the preparation of vesicles containing reduced DAGs, 2.2 equiv of DTT was also added as an ethanolic solution along with the DAG and DPPC. The remaining traces of the solvent were removed by keeping the samples under high vacuum for 3−4 h. The lipid films were then hydrated with water (Milli-Q) and were subjected to three freeze−thaw cycles. The resulting suspension was used for DSC experiments.

^{31}P NMR Spectroscopy. D_2O (0.5 mL) was added to the dry lipid film as described in the preceding section, and the samples were heated at 70 °C for 30 min to ensure optimal hydration of the samples. The samples were then vortexed and put through three freeze−thaw cycles. The samples were then transferred to 5 mm NMR tubes. The final concentration of the phospholipid was 80 mM. Proton dipolar decoupled 242.8 MHz ^{31}P NMR spectra were recorded in Fourier transform mode using a Varian Unity plus 600 MHz spectrometer interfaced with a SPARC LX station computer. Temperature was controlled using a temperature control unit. All chemical shift values are quoted in parts per million (ppm), positive values referring to low-field shifts. Accumulated free induction decays were obtained from 5000 transients. A spectral width of 100 kHz, a memory of 8000 data points, a 1 s interpulse time, and a 90° radio frequency pulse were the parameters used to record the spectra. Before Fourier transformation, an exponential multiplication was applied resulting in a 100 Hz line broadening.

Differential Scanning Calorimetry (DSC). Multilamellar vesicles were prepared by dispersing a thin lipid film of DPPC (1.47 mg) containing different amounts of DAGs in 2 mL of water (Milli-Q). DSC measurements were carried out using a Microcal MC-2 model heat conduction differential scanning microcalorimeter that consisted of a reference cell and a sample cell. The melting behavior was measured using the same water as a reference. Heating scans were recorded between 30 and 60 °C at a scan rate of 1.5 deg/min. The calorimetric data were analyzed using Origin data analysis software for DSC supplied by the manufacturer. T_m is the temperature of the half of the transition peak area. The ratio of the van't Hoff enthalpy (ΔH_v) to the calorimetric enthalpy (ΔH_c), i.e, $\Delta H_v/\Delta H_c$ provides the cooperativity (CU) of the transition. Analysis of DPPC using this protocol gave thermodynamic data, which were in agreement with those that were previously reported.[23]

Phospholipase A_2 Assay. Multilamellar vesicles consisting of substrates DPPC and NBD−PC and doped with different concentrations of DAG were prepared in the same manner as decribed earlier. The concentration of each substrate in the vesicular suspension was 5 mM and 5 μM, respectively. A specific DAG derivative was incorporated in the MLVs at concentrations ranging from 5 to 30 mol %. In the case of phospholipase A_2 assay, however, a buffer was used to make the vesicular dispersions instead of water, which contained 50 mM Tris-HCl, 10 mM Ca^{2+}, and 1 mM NaN_3 at pH 7.4. Phospholipase A_2 activity was measured according to the method of Stubbs et al.[24]

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Typically, to a 0.4 mL vesicular solution was added 100 μL of phospholipase A_2 (0.5 μg of either cobra venom or bee venom) to start the reaction. The reaction mixtures were kept for 5 min with respective venom PLA_2s at 45 °C, the temperature at which host lipid matrix, DPPC, remains in its fluid state ($> T_m$ of DPPC). After the reaction mixture was incubated for the requisite time, the reaction was stopped by the addition of 1.88 mL of a mixture of $CHCl_3$/MeOH (1:2 v/v). The resulting suspension was separated into discrete aqueous and organic phases by further addition of 625 μL each of $CHCl_3$ and water. An aliquot of the aqueous phase was then removed, and its fluorescence intensity at 530 nm with excitation at 470 nm was measured. A standard curve was obtained using known quantities of NBD−hexanoic acid. Background fluorescence was determined from the identical measurements in the absence of PLA_2.

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Supporting Information Available: Copies of ^1H, ^{13}C NMR, and mass spectra for compounds **1**−**6** (23 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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