METHODS FOR THE SYNTHESIS OF MACROCYCLIC AND BOLAFORM PHOSPHATIDYLCHOLINES

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Abstract.

A general strategy for the synthesis of phospholipids was developed. The method was demonstrated using 3-O-allyl-sn-glycerol **33** which was regiospecifically converted to 1-palmitoyl 2-(5-O-TBDMS-stearoyl)-3-O-allyl-sn-glycerol **43** by the sequential acylation of the primary and secondary hydroxyls. The allyl group was removed in two steps by isomerization to a vinyl ether and hydrolysis with N-bromosuccinimide in aqueous tetrahydrofuran. A new method for the conversion of the 1,2-diacylglycerols to phosphatidylcholines under acid-catalyzed conditions was developed. The diacylglycerols were treated with (2-bromoethyl)-(2-cyanoethyl)-(N,N-diisopropylamino)-phosphoramidite **51** in the presence of tetrazole as catalyst, the phosphite triester was oxidized, and the neutral phosphate triester was treated with trimethylamine to simultaneously deprotect the phosphate and convert the bromoethyl substituent to the choline function. A modification was introduced to extend the synthetic strategy to unsaturated phosphatidylcholines. The starting material was 3-(4-methoxybenzyl)-sn-glycerol **30** (3-PMB-sn-glycerol). Diacylation of the hydroxyls with 13-tetradecynoic acid **64a** followed by Glaser oxidation gave 1,2-(13,15-octacosadiyn-1-28-dioyl)-3-PMB-sn-glycerol **66**. The 4-methoxybenzyl ether was cleaved using bromodimethylborane at -78 °C in methylene chloride. The diacylglycerol **67** was converted to the phosphatidylcholine as before. Sequential acylation of **30** with palmitic acid and 15-hexadecynoic acid **64b** followed by Glaser oxidation gave 2,2'-(15,17-dotriacontadiyn-1,32-dioyl)-bis-[1-palmitoyl-3-PMB-sn-glycerol] **72**, which was converted to the bis-phosphatidylcholine **75** in the same manner as **66**.

Résumé.

Une stratégie générale pour la synthèse des phospholipides a été développée. La méthode a été demontrée en utilisant le 3-O-allyl-sn-glycérol **33**, qui a été transformé régiospécifiquement en le 1-palmitoyl-2-(5-O-TBDMS-stéaroyl)-3-O-allyl-sn-glycérol **43** par l'acylation successive des alcools primaire et secondaire. Le groupe allyle a été enlevé en deux étapes par l'isomérization en éther vinyle, suivi par l'hydrolyse avec le N-bromosuccinimide dans du tétrahydrofurane aqueux. Une nouvelle méthode pour la transformation des 1,2-diacylglycérols en phosphatidylcholines avec catalyse acide a été développée. Les diacylglycérols ont été traités avec le (2-bromoéthyle)-(2-cyanoéthyle)-(N,N-diisopropyle)-phosphoramidite **51** en la présence de tétrazole comme catalyseur, le triester de phosphite a été oxidé, et le triester de phosphate traité avec de la triméthylamine pour déprotéger la fonction phosphate et transformer le groupe bromoéthyle en fonction choline simultanément. La stratégie a été modifiée pour permettre la synthèse de phospholipides insaturés. Le produit de départ a été le 3-(4-méthoxybenzyle)-sn-glycérol **30** (3-PMB-glycérol). La diacylation des alcools avec l'acide 13-tetradécynoique **64a** suivie de l'oxidation de Glaser a produit le 1,2-(13,15-octacosadiyn-1,28-dioyle)-3-PMB-sn-glycérol **66**. L'éther 4-méthoxybenzyle a été enlevé avec le bromodiméthylborane dans le dichlorométhane à -78 °C. Le diacylglycérol **67** a été transformé en phosphatidylcholine comme ci-dessus. L'acylation successive du glycérol **30** avec les acides palmitique et 15-hexadécynoique **64b** suivie de l'oxidation Glaser a donné le 2,2'-(15,17-dotriacontadiyn-1,32-dioyl)-bis-[1-palmitoyl-3-PMB-sn-glycérol] **72**, qui a été transformé en la bis-phosphatidylcholine **75** de façon analogue à **66** ci-dessus.

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Glossary of abbreviations.

α_{50}	Inhibitory power coefficient.
AA	Arachidonic acid.
acetyl CoA	Acetyl coenzyme A.
ADP	Adenosine 5'-diphosphate
A Kinase	Protein kinase A.
Anal	Combustion analysis.
ATP	Adenosine 5'-triphosphate.
BOC	t-Butyl carbamate.
BOM	Benzyloxymethyl.
br	Broad.
Bu_4N I	Tetra-n-butylammonium iodide.
Calcd	Calculated.
CL	Cardiolipin.
CMC	Critical micellar concentration.
c AMP	Adenosine 3',5'-cyclic-phosphate.
cGMP	Guanosine 3',5'-cyclic-phosphate.
C Kinase	Protein kinase C.
d	Doublet.
DABCO	Diazabicyclooctane.
DCC	Dicyclohexylcarbodiimide.
DDQ	2,3-dichloro-5.6-dicyanobenzoquinone.
DG	Diacylglycerol.
DMAP	N,N-dimethylaminopyridine.
DMF	N,N-dimethylformamide.
DNA	Deoxyribonucleic acid.
ER	Endoplasmic reticulum.
EtOAc	Ethyl acetate.
GC	Gas chromatography.
GDP	Guanosine 5'-diphosphate.
GPC	3-Glycerophosphatidylcholine.
GTP	Guanosine 5'-triphosphate.
HPETE	Hydroperoxyeicosatetraenoic acid.
hr	Hour.
IC_{50}	Inhibitory concentration for 50% of a population.
IP_3	Inositol triphosphate.
Lyso-PC	1-Acyl-glycero-3-phosphatidylcholine.
LO	Lipoxygenase.
LT	Leukotriene.
MEM	Methoxyethoxymethyl.
min	Minutes.
MOM	Methoxymethyl.
mRNA	Messenger ribonucleic acid.
MS	Mass spectrometry.
MTPA	(R)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid.
M/Z	Mass to charge ratio.
NADH	Nicotinamide adenine dinucleotide, reduced form.
NBS	N-bromosuccinimide.
NMR	Nuclear magnetic resonance.
PAF	Platelet activating factor.
PBP	Para-bromophenacylbromide.
PC	Phocphatidylcholine.

PE	Phosphatidylethanolamine.
PG	Phosphatidylglycerol.
PGX_2	Prostaglandin X_2.
PI	Phosphatidylinositol.
PIP	Phosphatidylinositol phosphate.
PIP_2	Phosphatidylinositol diphosphate.
PLA_2	Phospholipase A_2.
PLC	Phospholipase C.
PMB	4-Methoxymethyl.
$pPLA_2$	Pancreatic phospholipase A_2.
$ProPLA_2$	Phospholipase A_2 proenzyme.
PS	Phosphatidylserine.
Rf	Retention factor.
r t	Room temperature.
SDS	Sodium dodecyl sulfate.
sn-	Stereospecific numbering.
TBDMS	t-Butyldimethylsilyl.
TFA	Trifluoroacetic acid.
THF	Tetrahydrofuran.
TLC	Thin layer chromatography.
T_m	Main transition temperature,
TMS	Trimethylsilyl.
TNBS	Trinitrobenzenesulfonyl.
TXA_2	Thromboxane.

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INTRODUCTION.

Role of the membrane.

It has been stated that one of the earliest events in the development of living systems was their enclosure within a membrane[1]. Indeed, the organization which is inherent in cells only became possible through the agency of a structure the role of which is to create compartments where different conditions exist within and without. This barrier function is essential for the maintenance of cellular life, but the membrane also supports other functions which are equally important. These roles fall into three main categories which govern the interactions that the inner compartment has with its surroundings First, membranes allow control over the flow of matter. This is necessary for the maintenance of the essential environment within, as well as between different areas of the cell. The barrier permits the maintenance of local conditions so that certain processes may occur in a regulated fashion. Secondly, control of the flow of energy is accomplished by the creation of a potential barrier. This is associated with protein machinery which is capable of transducing energy from one form to another. In fact, although these two aspects are artificially differentiated, they are really related since the maintenance of compartments of different compositions is possible only at the expense of chemical energy. This in turn can only be accumulated with the help of a barrier where potential energy, arising from a concentration gradient, is harnessed. The third function of membranes is the regulation of the flow of information. This allows stimuli from an outside compartment to be recognized and to cause modifications of the inner conditions which give rise to the appropriate cellular response. The barrier properties of the membrane itself are insufficient to mediate all of these different activities; this is the role of the membrane proteins. In order to put the biological importance of phospholipids in perspective, the introduction will provide an overview of their functions *in vivo*.

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1) Kotyk, A.; Janacek, K.; Koryta, J. *Biophysical Chemistry of Membrane Functions*; John Wiley & Sons: New York, 1988, p 4.

Organization of the cell.

Plasma membrane.

In order to understand the function of the membrane, it is useful to examine the layout of a typical mammalian cell, in relation to the metabolic processes which occur. The obvious starting point is the plasma membrane which surrounds the cytosol and the internal compartments (organelles)(**Figure 1**). It forms the outermost barrier of most animal cells, and is supported by the cytoskeleton. Because of the need to maintain the contents of the cell, the plasma membrane is highly impermeable to most polar and ionic molecules[2], while allowing water to pass. This property allows the cell to generate and actively maintain an electrochemical gradient across the membrane. This results in a potential difference across the membrane of 70 to 90 mV, with the interior negative relative to the exterior. Specific mechanisms have evolved to allow the flow of desirable compounds in and out of the cell. These are of various types: solutes can flow into the cell down their concentration gradient through channel proteins, or through specific carrier proteins. Other compounds are carried in against their concentration gradient by cotransport with another molecule driven by the electrochemical gradient of the latter. The membrane potential is generated by the Na^+/K^+ATPase, which uses the energy of ATP hydrolysis to pump 3 sodium ions out and 2 potassium ions into the cell (net transfer of one positive charge out of the cell per ATP). Other solutes, e.g. calcium are also actively transported out of the cell with the energy generated by ATP hydrolysis.

Macromolecules cannot normally cross membranes, and special mechanisms have been developed to allow their passage. For example, secretory proteins, hormones, neurotransmitters etc. are released into the extracellular space by exocytosis. In this process, an intracellular vesicle containing the substance to be secreted fuses with the plasma membrane, and the contents are released. The reverse can also occur, and endocytosis is a normal mechanism for the ingestion of macromolecules to be digested. Both of these processes are dependent on the cytoskeleton and on the interactions of specific proteins with membranes. In certain cases, only the information carried by a molecule enters the cell. When an agonist binds to its receptor on the exterior of the cell, it causes a conformational change of the protein which leads to a sequence of reactions on the inside of the cell. For example, adrenaline binding to a liver cell

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2) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molecular Biology of the Cell*, Garland Publishing: New York, **1983**, p 287.

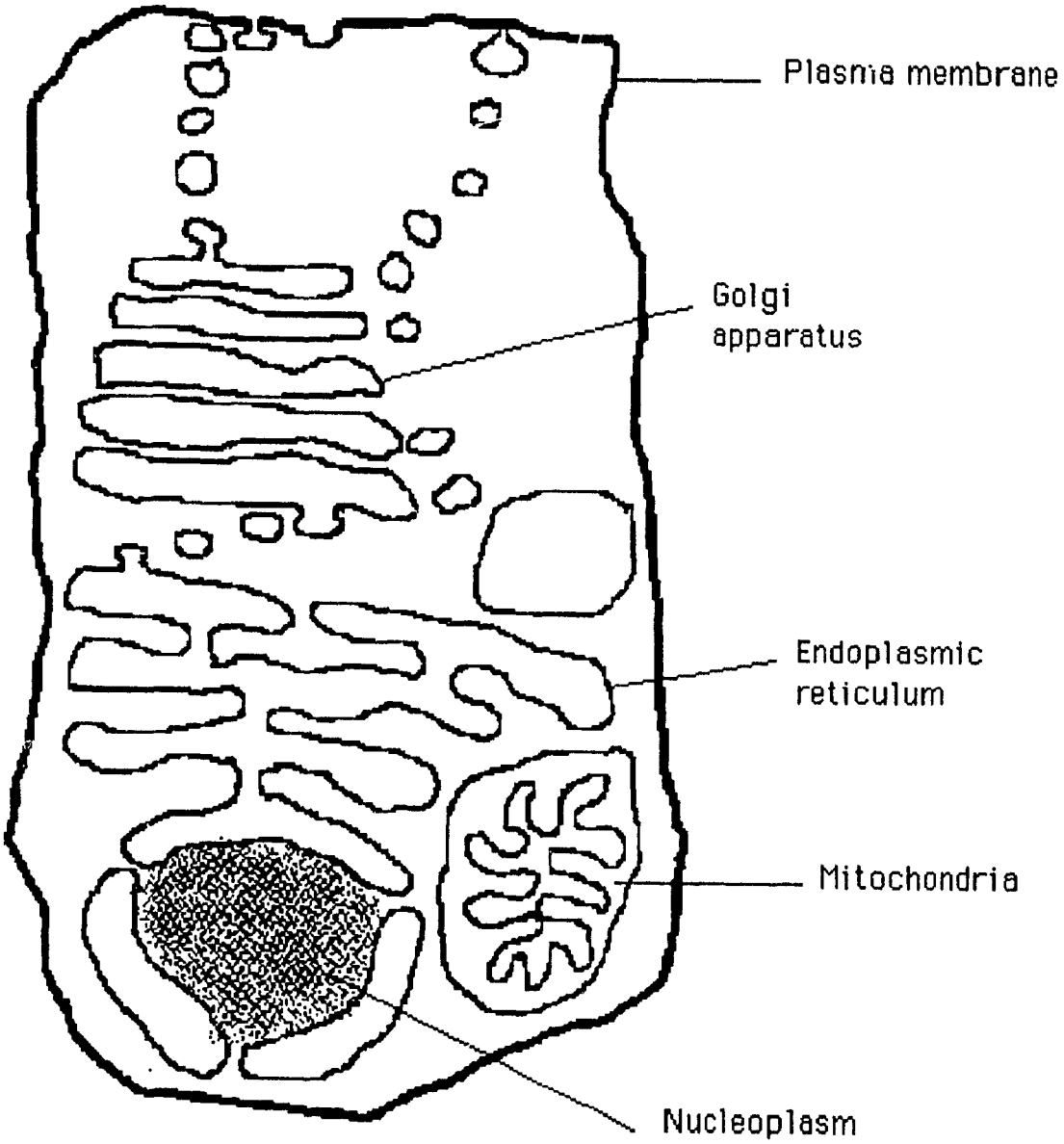

Figure 1 . Intracellular membrane compartments.

receptor causes the activation of adenylate cyclase[3] on the cytosolic side of the membrane. The cyclic AMP acts as a second messenger which causes the phosphorylation of specific proteins leading to the activation of glycogen hydrolysis. Thus, while the effector molecule is not internalized, it can have drastic effects on cellular metabolism and responses. Other examples of signal transduction will be discussed in later sections.

Endoplasmic reticulum.

The endoplasmic reticulum (ER) is a contiguous, highly convoluted membrane compartment separate from the rest of the cytosol (**Figure 1**). It provides cells with a mechanism for the synthesis and sorting of proteins and other molecules destined for specific locations in the cell or in particular organelles. Two distinct types of ER can be distinguished. Smooth ER is abundant in cells where lipid metabolism is prominent, and in liver cells responsible for the detoxification of drugs and other compounds. Rough ER is simply endoplasmic reticulum where ribosomes have bound and are synthesizing proteins which are to be secreted or are destined for a particular organelle. In this process the nascent polypeptide is actually threaded through the ER membrane. New proteins being synthesized in the rough ER have amino acid sequences at their N termini which serve as signals to direct the finished polypeptide to its destination in the cell. The synthesis of oligosaccharides for the glycosylation of proteins is accomplished in the luminal (interior) side of the ER. The endoplasmic reticulum is also the site of *de novo* phospholipid biosynthesis.

Golgi apparatus.

The Golgi apparatus is a series of flat disk shaped cisternae from which budding vesicles can often be observed. The Golgi serves as the control center where the fate of molecules synthesized in the ER is decided, and as the departure point for the delivery of proteins. Inside the cisternae the oligosaccharide components of plasma membrane glycoproteins are modified and trimmed, and the proteins destined for specific organelles or secretion are sorted and packaged in special vesicles containing the necessary proteins which deliver the cargo, unload the contents, and return to the point of origin[4]. It is also through this process that membrane components are recycled (**Figure 1**).

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[3]) Ibid ref 2, p 733.

[4]) Ibid ref 2, p 366

Nucleus.

Other structures in the cell are surrounded by a double membrane, which permits the functional differentiation of the two faces of the structure. This also has the additional complication that the intracellular sorting mechanisms must be able to deliver the appropriate proteins to each of the four subcompartments of these structures[5]. The cell nucleus is surrounded by the nuclear envelope. The inner nuclear membrane interacts with proteins in the nucleoplasm, while the outer nuclear membrane and the perinuclear space are contiguous with the endoplasmic reticulum[6] (**Figure 1**). The two membranes join at the nuclear pores which allow the passage of the various macromolecules. Ribosomes and nuclear proteins are transported in, while mRNA is transported out of the nucleus for translation.

Mitochondria.

Another double-membraned cellular structure is the mitochondrion (**Fig 1**)[7]. This topologically complicated organelle is the major site for energy production in the cell. The enzymes of the citric acid cycle are found in the mitochondrial matrix where they produce NADH by oxidation of acetyl CoA. The oxidation of NADH produces energy which is harnessed by the respiratory chain to pump protons out of the matrix and into the intermembrane space. This process generates a chemiosmotic gradient across the inner membrane. The backflow of protons across the membrane is used by the ATP synthetase complex to synthesize ATP from ADP and phosphate. The production of energy by oxidative phosphorylation is equally dependent on the impermeability of the membrane to protons as it is on the proteins of the electron transport chain.

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[5]) Ibid ref 2, p 376

[6]) Ibid ref 2, p 429

[7]) Ibid ref 2, p 483

Membrane structure.

Membrane proteins.

The ability of the cell to accomplish the various tasks outlined above depends on the structure of its membrane, which is in turn a consequence of its composition. Three main components have been mentioned: the cell wall, the cytoskeleton, and the membrane *per se*. The cell wall is composed of complex polysaccharides and protein, and is usually found only in yeast, fungi and plants, or in some animal cells such as protozoans[8]. In plants, the rigid cell wall is responsible for the functions that in animals are supplied by the skeleton, the circulatory system, and the skin[9].

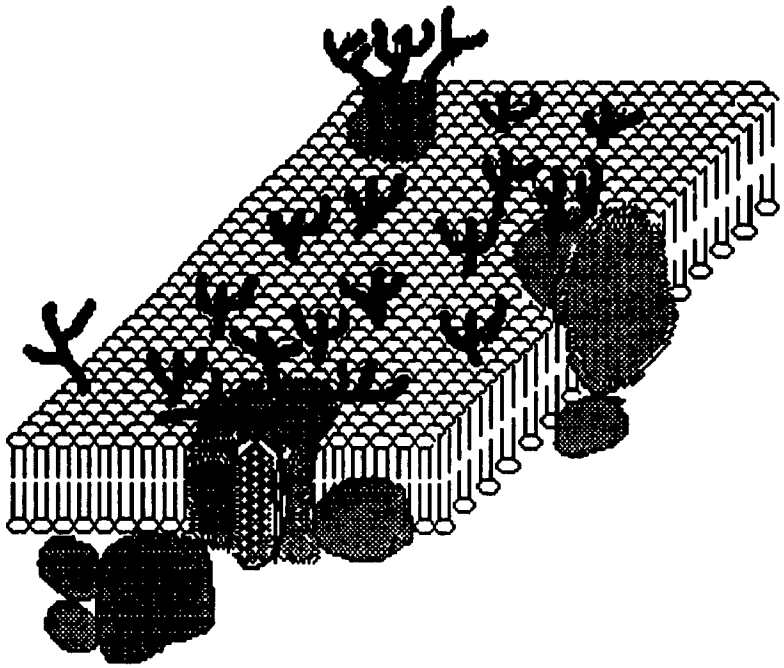

Figure 2 . Typical cellular membrane.

The membrane itself is a complex structure, composed of protein, cholesterol, and amphiphilic lipids with a variety of polar head groups arranged in a bilayer structure (**Figure 2**). The respective ratios of the components is dependent on the source and function of the membrane. Typical proportions of protein vary from 40 to 60% by weight, while cholesterol may account for 0 to 40% of the total membrane lipid in mammalian brain cells[10]. Membrane proteins are the mediators of the functions

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[8]) Ibid ref 1, p 41.

[9]) Ibid ref 2, p 1100.

[10]) Ibid ref 1, p 47.

described in the following sections. The role of the lipid bilayer is to serve as support and matrix for the protein, and to maintain a permeability barrier. Two main types of membrane protein have been described: some polypeptides have interactions with the hydrophobic portion of the membrane, and are called integral proteins. They may be imbedded on one side, or span the bilayer once or several times. All known proteins that accomplish a transport function are of this type. Other polypeptides interact with the hydrophilic portion of the membrane, and are called peripheral membrane proteins. The first type are responsible for the catalytic functions necessary for the flow of energy, matter and information, while the latter serve mainly as accessories to the integral proteins. The location and orientation of membrane proteins are decided and established during the course of their synthesis in the endoplasmic reticulum and the Golgi apparatus, based on information contained in the N-terminal sequences of the polypeptide. Details of the synthesis and targeting of proteins can be found in introductory biochemistry texts [11].

Membrane lipids.

Many different molecular species constitute the lipid component of the membrane, although they all share one common feature: all are amphiphilic molecules that spontaneously aggregate. This property arises from the structure of the various lipids. Typically, membrane lipids contain two long chain hydrocarbon residues esterified to a glycerol backbone which constitutes the hydrophobic portion, attached to an ionic or polar head group. The interaction of these molecules in water above a critical concentration characteristic of the compound (the CMC) causes a reorganization of the solute to a supramolecular assembly, driven by a minimization of the interaction energy of the non-polar region with the aqueous solution. The morphology of these assemblies depends on the amphiphile used[12,13]. Single chain ionic or zwitterionic surfactants typically form micellar structures due to their conical shape, while glycerol based double chain amphiphiles prefer to form lamellar bilayer structures such as vesicles. Inverted cone amphiphiles can adopt either lamellar or hexagonal phases. The bilayer arrangement in particlar is thermodynamically very stable. The arrangement of the individual glycerophospholipid molecules in biomembranes is dependent on the

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11) Ibid ref 2, p 269.

12) Fukuda, H.; Kawata, K.; Okuda, H.; Regen, S. L.; *J. Am. Chem. Soc.* **1990**, *112*, 1635.

13) Eibl, H.; *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 257.

temperature. The calorimetric behavior can be readily examined for a pure phospholipid. Below the main transition temperature (T_m), the hydrocarbon adopts a crystal-like, all-trans geometry, and individual molecules are tightly constrained in the bilayer. Above the melting temperature, the chains have a greater freedom of bond rotation and lateral movement. The acyl chains have an increased number of gauche conformations and take up almost twice the surface area in the fluid phase as in the crystalline state. The transition temperature is influenced by the structure of the phospholipid: longer acyl chains esterified to the glycerol increase the T_m, while branched chains and unsaturated chains decrease it. In addition, shorter or unsaturated chains at the 2-position cause a larger decrease of T_m than the corresponding 1-acyl isomers. While the transition temperature for a single lipid may be relatively well defined, mixtures of phospholipids show considerably broadened melting transitions. This is due to the presence of defects in the crystalline lattice where melting occurs, and to the non-ideal behavior of the lipid mixture[14]. This results in islands of crystalline lipid floating in a sea of fluid phase, surrounded by a layer of interphase lipid. In summary, mixtures of lipids tend to show preferences for their neighbors, and this affects the properties of the assembly. The leakiness of artificial membranes around the transition temperature is attributed to the formation of interfacial lipid, and to the mismatch of the phases[15]. Biological membranes show very broad phase transitions, and little leakiness as a consequence of the complexity of the lipid mixture which reduces the amount of interfacial lipid. This has also been ascribed to the presence of cholesterol which imbeds itself in the phospholipid bilayer.

Cholesterol.

Cholesterol interacts with the acyl chains in the interior of the bilayer and due to its rigid structure, prevents them from assuming gauche conformations as in the fluid phase of pure lipid above its T_m. Conversely, when the membrane freezes the cholesterol does not phase separate, and prevents the phospholipid from forming a regular crystalline lattice. This has the effect of reducing the surface area and the permeability of the phospholipid, while increasing the flexibility and fluidity of the

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[14]) Gennis, R. B.; *Biomembranes: Molecular Structure and Function*, **1989**, Springer-Verlag, New York.

[15]) Davies, D. A.; Critchlow, M.; Grainger, D. W.; Reichert, A.; Ringsdorf, H.; Lloyd, J. B.; *Biochim. Biophys. Acta*, **1991**, *1084*, 29.

membrane[16]. When sufficient cholesterol is present, the phase transition disappears altogether. Thus the role of cholesterol *in vivo* is to maintain the fluidity of the membrane over a broad range of temperature.

Glycerophospholipids.

The individual molecular species in biological membranes are most conveniently classified according to the type of structural backbone. The glycerophospholipids are the most common in most mammalian cells[17]. The basic structure of this class is a 1,2-diacylglycerol-3-phosphate, or phosphatidic acid (**Figure 3**). In addition, 1-O-alkyl (ether) and 1-O-alk-1-enyl (plasmalogen) derivatives are known, and are produced in peroxisomes by reduction of the ester function to an ether. Only the R enantiomer of the

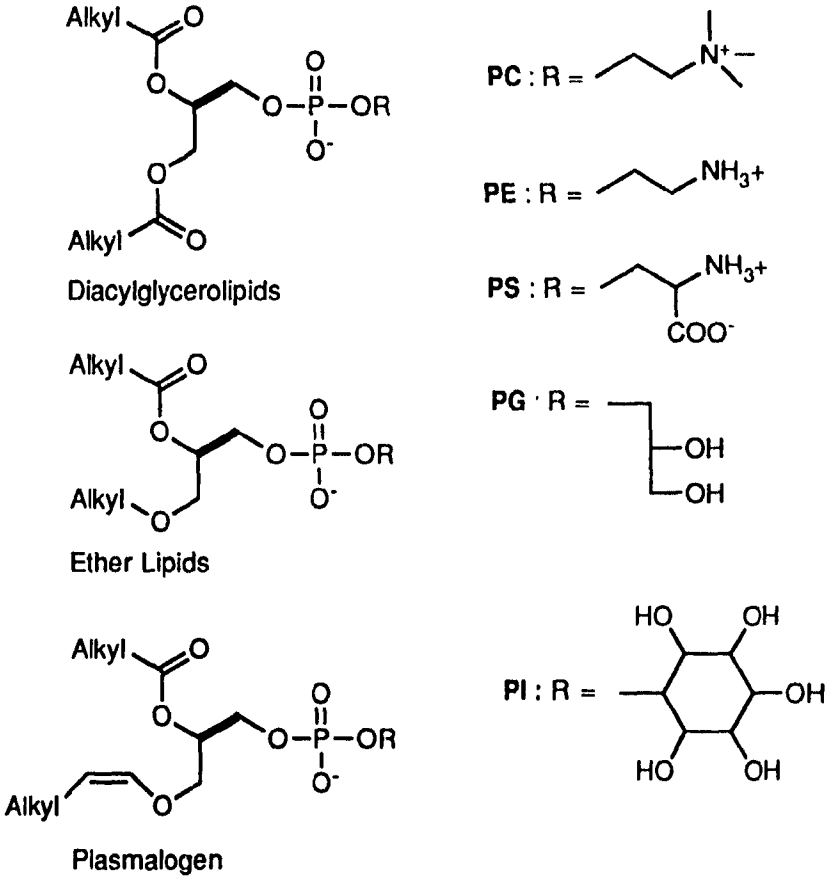

Figure 3

glycerol is found in biological membranes[13]. The different populations of phosphoglycerides are distinguished by the group esterified to the phosphate. When

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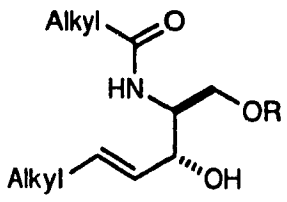
16) Djerassi, C.; Lam, W.; *Acc. Chem. Res.* **1991**, *24*, 69.

17) Ibid ref 1, p 50.

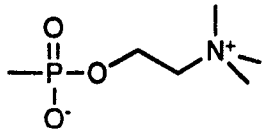
alcohols containing a cationic group are present (e.g. choline or protonated ethanolamine) the result is a zwitterionic molecule (PC or PE) with no net charge at physiological pH. Alternatively, polar groups such as serine or diacylglycerol derivatives occur, as well as neutral alcohols like glycerol or inositol. Under physiological conditions, phosphatidylserine (PS), cardiolipin (CL), phosphatidylglycerol (PG) and phosphatidylinositol (PI) carry one or more negative charges. In addition, PI can undergo additional phosphorylation in the membrane which gives rise to PI phosphate and diphosphate (PIP and PIP_2). The importance of these phospholipids in intracellular signaling will be discussed in a later section.

Sphingolipids.

The second type of backbone structure for membrane lipids is based on the long chain amino alcohol sphingosine (**Figure 4**), which is derived from palmitoyl CoA and serine. The amino group is amidated with a long chain fatty acid to give a ceramide. The primary alcohol can be coupled to a phosphocholine moiety as in sphingomyelin, or to simple or complex oligosaccharides to form the glycolipids. Cerebrosides have a single sugar residue attached to the ceramide, and are the major component of myelin. Gangliosides are an example of the latter. They are believed to be important determinants in intracellular recognition processes, behaving as cell surface markers. For example, the ABO blood type antigens are gangliosides[18].



Ceramide : R = H

Sphingomyelin : R = 

Cerebroside : R = Sugar

Ganglioside : R = Polysaccharide

Figure 4

Biosynthesis of membranes.

While there are many lipid and protein species in any biological membrane, they are not randomly distributed. The composition of a membrane is adapted to fulfil its role as

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[18]) Ibid ref 1 p 47.

efficiently as possible, and therefore varies from one cell type to another and from one organelle to another[19]. In many instances the two faces of the same bilayer have very different phospholipid compositions (**Table 1**). This is of particular importance in certain cases where the presence of a functional group on a membrane surface influences its function. Some examples are the glycolipids mentioned in the previous section, which are found exclusively on the exterior surface of cells[20]. Some of these function as antigenic markers. Another well studied case is that of phosphatidylserine in platelets[21], which is found exclusively in the inner leaflet of the plasma membrane, but is translocated to the outer leaflet after stimulation of the cell with thrombin or collagen. The movement of PS to the platelet surface facilitates blood clotting. The appearance of PS on the surface of aging red blood cells appears to affect their clearance by macrophages[22].

Type of membrane	PC	PE	PS	PI	PG	CL	Glyco-glycero lipids	Sphingo lipids	Sterols	Other
Sarcina lutea		-	—	8	90	1				1
Micrococcus roseus	--	—	--	—	66	32				2
Salmonella typhimurium										
Cytoplasmic	--	60	—	-	32	7	-			1
Outer	--	81	-	—	15	2				2
Bacillus subtilis	-	—	-		78	3				19
Anacystis nidulans	—	—		--	14		70			16
Saccharomyces cerevisiae										
Plasma membrane	27	10	14		—	—		—	40	9
Tonoplast	33	15	43							
Rat hepatocyte										
Plasma membrane	28	17	6	6		1	—	12	27	3
Smooth endoplasmic reticulum	41	16	5	10	--	1		7	17	3
Erythrocyte										
Rat	30	13	7	2	—	--	2	16	25	5
Pig	15	18	11	1	—	-	10	18	27	
Lobster nerve plasma membrane	29	18	8	—		—		9	36	
Rabbit muscle plasma membrane	23	9	1	—	-	-		4	57	6
Human myelin sheath	11	14	5	1				32	25	12
Inner mitochondrial membrane										
Rat liver	40	35	1	5	2	17	—	1	1	1
Cauliflower	37	34	—	4	3	13	--	—	5	1
Baker's yeast	36	29	2	8	1	19	-		1	4
Outer mitochondrial membrane										
Rat liver	46	21	1	10	2	5	—	2	10	3
Cauliflower	36	21	—	17	8	2			12	4
Baker's yeast	43	22	3	20	1	6	-		1	4
Spinach chloroplast										
Outer membrane	22	—	—	1	9	--	68	—		
Inner membrane	6	—	—	1	8	—	79	—		6
Thylakoid	5	—	—	1	9	-	78	-		7

Table 1: Lipid compositions of selected membranes (Taken from Ref 1, p 50).

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19) Zubay, G. L. *Biochemistry*, Addison-Wesley: Reading, Mass. **1983**, p 585.

20) Ibid ref 2, p 262.

21) Nozawa, Y.; Nakashima, S.; Nagata, K.; *Biochim. Biophys. Acta*, **1991**, *1082*, 219.

22) Bishop, W. R.; Bell, R. M.; *Ann. Rev. Cell Biol.* **1988**, *4*, 579.

The biosynthetic origins of all phospholipids in mammalian cells are interconnected to a large extent, as can be seen from **Figure 5**[22]. The majority of the reactions in these pathways are catalysed by enzymes on the cytoplasmic side of the smooth endoplasmic reticulum. This asymmetric synthesis of membrane components implies a mechanism for the transfer of some phospholipid monomers to the other face of the bilayer to ensure coordinated growth. However, the rates of spontaneous phospholipid flip-flop are extremely slow, because the passage of the polar headgroup through the hydrophobic interior of the membrane is thermodynamically unfavorable. Recent evidence points to the existence of specific phospholipid flippases. These transport proteins have affinity for a particular phospholipid head group, and may be a part of the mechanism of lipid sorting. It is still unclear where the differences in lipid composition of the different membranes originate. They may be generated by differential activity of the individual flippases. Other evidence suggests that, while the transmembrane (flipping) movement of phospholipids at biogenic membranes in the endoplasmic reticulum does not require energy (i.e. occurs by facilitated transport), the movement of the aminophospholipids PE and PS across the plasma membrane of erythrocytes is ATP dependent. It is certain that several sorting steps are operative during membrane flow since the phospholipid composition in the ER is not conserved in the target membrane. In addition, certain steps in phospholipid biosynthesis are catalysed only in specific organelles such as the mitochondria or the peroxisomes. Mechanisms for the delivery of the substrates and the removal of the products must exist and imply sorting steps. In general the following processes are responsible for the delivery of membrane components. First, vesicular transport occurs by budding from a donor followed by fusion with an acceptor membrane. This process is well understood for the delivery of secretory proteins directed by the Golgi apparatus. It appears that a similar sorting function is operative for membrane components also. Phospholipids and proteins are synthesized in the endoplasmic reticulum, delivered to the Golgi apparatus by vesicular transport, and are then sorted and sent to their final destination. Furthermore, membrane recycling is made possible by the return of material to the Golgi via endocytosis at structures called coated pits[23]. Secondly, lateral diffusion is possible in the membrane plane, but this probably does not account for any of the specific sorting since there are few bridges

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[23]) Ibid ref 2, p 362, 366

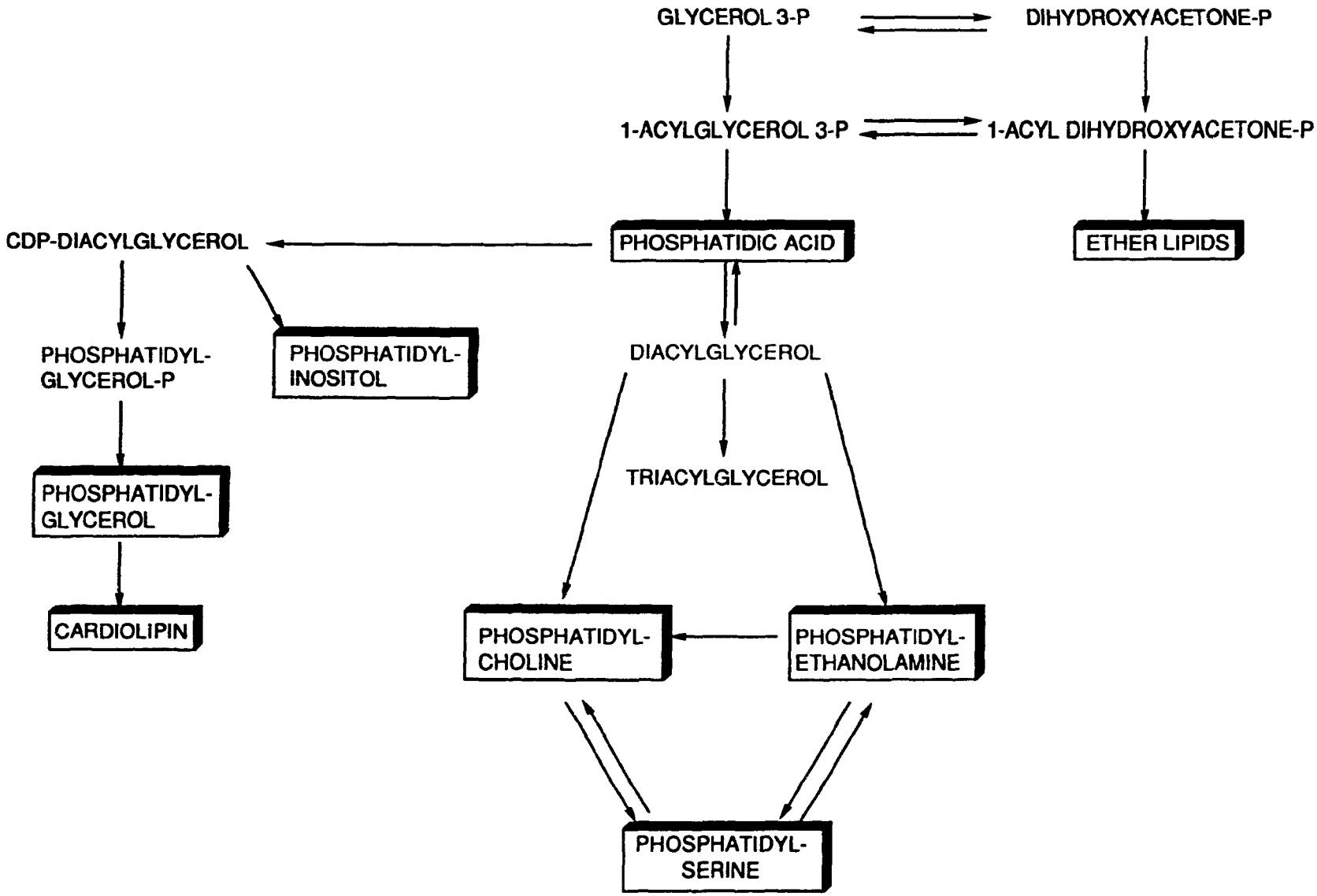
GLYCEROL 3-P
DIHYDROXYACETONE-P
1-ACYLGLYCEROL 3-P
1-ACYL DIHYDROXYACETONE-P
CDP-DIACYLGLYCEROL
PHOSPHATIDIC ACID
ETHER LIPIDS
PHOSPHATIDYL-GLYCEROL-P
PHOSPHATIDYL-INOSITOL
DIACYLGLYCEROL
TRIACYLGLYCEROL
PHOSPHATIDYL-GLYCEROL
CARDIOLIPIN
PHOSPHATIDYL-CHOLINE
PHOSPHATIDYL-ETHANOLAMINE
PHOSPHATIDYL-SERINE

Figure 5

between organelles, and because diffusion would tend towards maximum entropy. This mechanism may be of importance for the transfer of material in double membraned organelles, and from the endoplasmic reticulum to the nucleus. Thirdly, phospholipid monomers are transferred through the cytosol by specific carrier proteins. These may also mediate the sorting process by selective retrieval of phospholipids from the Golgi apparatus. It appears however that the main method for phospholipid movement is via vesicular transport and membrane fusion.

Fatty acid distribution in phospholipids.

Thus far, the various phospholipid classes have been discussed as if they were a homogeneous population. In fact, for a given species, e.g. phosphatidylcholine, the fatty acids at the 1- and 2-position are rarely identical. In general, the acyl chain at the 1-position is saturated, usually palmitic or stearic acid, while the sn-2 acyl chain is almost always unsaturated or polyunsaturated[21] (**Table 2**). The lengths of the fatty acid chains vary from 14 to 22 carbons. This rather broad composition is due to the enzymes which acylate the sn-1 and sn-2 positions of the glycerol in the biosynthetic pathways[24]. In addition, there is a great deal of phospholipid turnover in the plasma membrane, catalysed by a variety of phospholipases and transacylases. These enzymes modify the fatty acid distribution in the membrane. This process allows the acyl chain composition of a given membrane to be tuned to meet its functional needs. The variability in the acyl chain lengths and degree of unsaturation coupled to the variability

Fatty acids	Phosphatidylcholine			Phosphatidylserine			Phosphatidylinositol			Phosphatidylethanolamine		
	C-1	C-2	Total	C-1	C-2	Total	C-1	C-2	Total	C-1	C-2	Total
16:0 DMA[a]	–	–	–	tr[b]	–	–	–	–	–	15.3	–	7.5
16:0	49.5	16.5	28.5	6.0	8.0	3.0	15.3	7.0	6.1	12.4	2.4	7.0
18:0 DMA	–	–	–	–	–	–	–	–	–	15.7	–	7.4
18:0	29.5	tr	16.8	79.5	7.0	44.3	69.7	5.1	40.7	40.6	1.4	24.0
18:1	9.5	32.5	23.9	5.1	43.1	23.3	5.4	7.2	8.1	8.2	9.9	8.5
18:2	1.0	17.8	10.3	–	3.3	1.0	–	tr	–	tr	7.2	1.6
20:4	–	22.9	12.0	–	34.1	22.0	–	77.6	40.1	–	62.2	32.0
22:5	–	tr	tr	–	–	–	–	–	–	–	6.1	2.0
22:6	0.7	1.9	1.7	–	–	–	–	–	–	–	7.1	4.7

[a] DMA dimethylacetal derivatives
[b] tr trace

Table 2: Positional distribution of fatty acids in phospholipids from non-activated human platelets.(from ref 21, p 221).

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[24]) Ibid ref 19, p 511.

of headgroups means that a typical membrane is composed of hundreds of different molecular species. The reasons behind this complexity are not well understood, but the lowering of the phase transition temperatures and the increased fluidity of the membrane seem to be important factors. Conversely, it seems that microdomains with a concentration of specific phospholipid species are also maintained in the membrane as pools with particular metabolic functions.

Physiological importance of phospholipids.

Until now we have only considered the role of phospholipids as components of the various subcellular membranes. However a number of cellular mechanisms have evolved where phospholipids or their degradation products act to modify the metabolism and responses of a cell or its neighbors. In this section we will examine the role of phospholipids in the production of the second messengers inositol triphosphate (IP_3) and diacylglycerol (DG), and in the production of eicosanoids and platelet activating factor (PAF). Both of these activities depend on a class of enzymes known as phospholipases, the catalytic activity of which involves the hydrolysis of the various ester bonds in glycerophospholipids[25]. In subsequent sections we will focus on phospholipase A_2 (PLA_2) as a potential target for pharmacological control of eicosanoid production.

Phospholipids as second messengers.

The sequence of events following the binding of an agonist (e.g. a hormone) to its receptor on the cell surface involves the activation of specific proteins on the inside of the cell, and the modification of the cell's metabolism (**Figure 6**). This can occur by several different mechanisms: phosphorylation of intracellular proteins, internalization of the receptor complex, opening of ion channels in the membrane, or production of intracellular signal compounds (second messengers)[26]. Depending on the nature of the agonist and its receptor, stimulation or inhibition of metabolic reactions occurs by one of these four processes. Different receptors activate specific effector proteins which produce distinct second messengers (e.g. cAMP, cGMP, K^+, Ca^{2+}, IP_3, DG, AA) which initiate opposing responses. The first such compound to be identified was cyclic AMP (cAMP). Activation of the appropriate receptor causes the stimulation or inhibition of

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[25])Lapetina, E. G.; *Ann. Rep. Med. Chem.* **1984**, *19*, 213.

[26]) Dixon, R. A. F.; Strader, C. D.; Sigal, I. S.; *Ann. Rep. Med. Chem.* **1988**, *23*, 221.

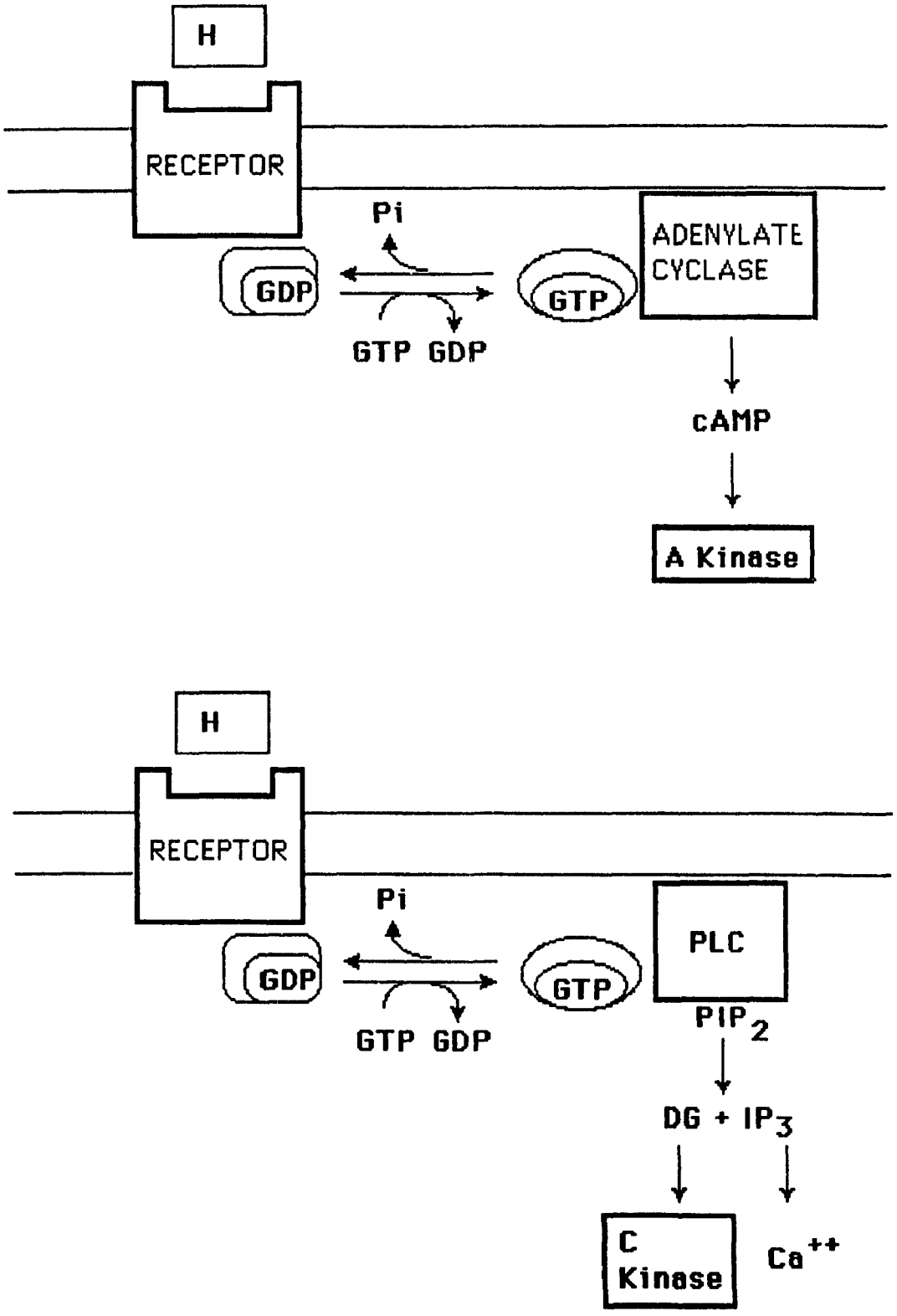
H
RECEPTOR
Pi
GDP
GTP
GTP GDP
ADENYLATE CYCLASE
cAMP
A Kinase
H
RECEPTOR
Pi
GDP
GTP
GTP GDP
PLC
PIP2
DG + IP3
C Kinase
Ca++

Figure 6

adenylate cyclase through an intermediary GTP binding protein[27]. G proteins are normally present in an inactive state with bound GDP. When they receive a stimulus from the receptor, a conformational change occurs, GTP is exchanged for GDP, and the active G protein is able to activate the effector, in this case adenylate cyclase. Hydrolysis of the GTP returns the G protein to an inactive state. The level of intracellular cAMP controls the activity of protein kinase A (A-kinase) which in turn regulates the activity of other proteins by phosphorylation. Downregulation occurs by dephosphorylation when levels of cAMP fall[28].

The other mechanism for signal transduction which concerns us here is the variation of intracellular calcium concentration, which is controlled by changes in phosphatidylinositol metabolism. The role of PI as a precursor to the intracellular second messengers inositol triphosphate (IP_3) and diacylglycerol (DG) is particularly well understood in platelets[21]. These are small enucleate cells which circulate in the blood and respond to damage in the endothelial lining of the blood vessels. Platelets are also sensitive to a variety of chemical and tactical stimuli, which cause them to aggregate, change shape, and secrete various substances which include eicosanoids and platelet aggregating factor (PAF). These activities result in termination of the hemorrhage, the initiation of tissue repair, and the attraction of cells of the immune system which prevent infection.

Phophatidylinositol in the cytosolic face of the plasma membrane undergoes phosphorylation to the mono and diphospho derivatives PIP and PIP_2. When cells such as platelets are stimulated, a cascade of events is initiated: the binding of an agonist to a surface receptor causes the activation of membrane bound phospholipase C (PLC)(**Figure 6**) through an intermediate G protein (distinct from those responsible for cAMP modulation). Activated PLC hydrolyses the phosphate ester bond between diacylglycerol and the inositol phosphates PIP and PIP_2 which are preferentially localized in the plasma membrane. The DG and IP_3 released then diffuse through the cytosol to activate protein kinase C (C-kinase) and release calcium from intracellular stores, or allow it to enter through calcium channels in the plasma membrane[29]. Calcium mobilization and C-kinase activity are then responsible for the amplification of

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27) Spiegel, A. M.; *Ann. Rep. Med. Chem.* **1988**, *23*, 235.

28) Ibid ref 2, p 733.

29) Jaken, S.; Leach, K. L.; *Ann. Rep. Med. Chem.* **1988**, *23*, 243.

the cellular response by activating many different effector proteins. C-kinase is also responsible for feedback inhibition of PLC by an indirect mechanism which is still undetermined. Among the many responses, we are mainly concerned with the liberation of arachidonic acid from phospholipids for the secretion of eicosanoids.

Biological effects of eicosanoids.

The concentration of free arachidonic acid (AA) in intact cells is normally very low, and it is generally accepted that the rate limiting step of eicosanoid biosynthesis is the release of AA from membrane phospholipids[30]. Once liberated, AA is rapidly converted to biologically active metabolites via two pathways (**Figure 7**)[31]: through cyclooxygenase, AA gives rise to the cyclic endoperoxides PGG_2 and PGH_2, which are the precursors of the prostaglandins PGD_2, PGE_2 and PGF_2, as well as prostacyclin (PGI_2) and thromboxane (TXA_2). The lipoxygenase pathway leads to the various AA hydroperoxides (HPETEs). The most important of these enzymes is 5-lipoxygenase which generates the precursor of the leukotrienes (LTs). All cells in the body with the exception of red blood cells are capable of secreting one or several PGs or LTs, and all of these substances exert profound (and often opposing) effects on their target cells[32]. Among the major roles for eicosanoids are the regulation of blood clotting. Endothelial cells secrete prostacyclin which inhibits aggregation, but when platelet receptors detect damage to the blood vessels they secrete TXA_2 which is a potent stimulator of platelet function. The other prostaglandins have major effects on vascular permeability and smooth muscle tone, as well as on reproductive functions. Leukotrienes are potent modulators of immune system function. They are responsible for inflammation by attracting macrophage, mast cells, neutrophils etc. and for causing large changes in vascular permeability. The involvement of LTs in allergic reactions such as asthma is also well documented.

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30) Samuelsson, B.; *Drugs*, **1987**, *33(Supl. 1)*, p2.

31) Moncada, S.; Higgs, E. A.; *Ann. New York Acad. Sci.* **1988**, *522*, 454.

32) Kirtland, S. J.; *Prost. Leuk. Ess. Fatty Acids-Rev.* **1988**, *32*, 165; Samuelsson B.; *Angew. Chem Int. Ed. Engl.* **1983**, *22*, 805; Bergström, S.; *Angew. Chem Int. Ed. Engl.* **1983**, *22*, 858; Vane, J. R.; *Angew. Chem Int. Ed. Engl.* **1983**, *22*, 741; Giles, H.; Leff, P.; *Prostaglandins*, **1988**, *35*, 277.

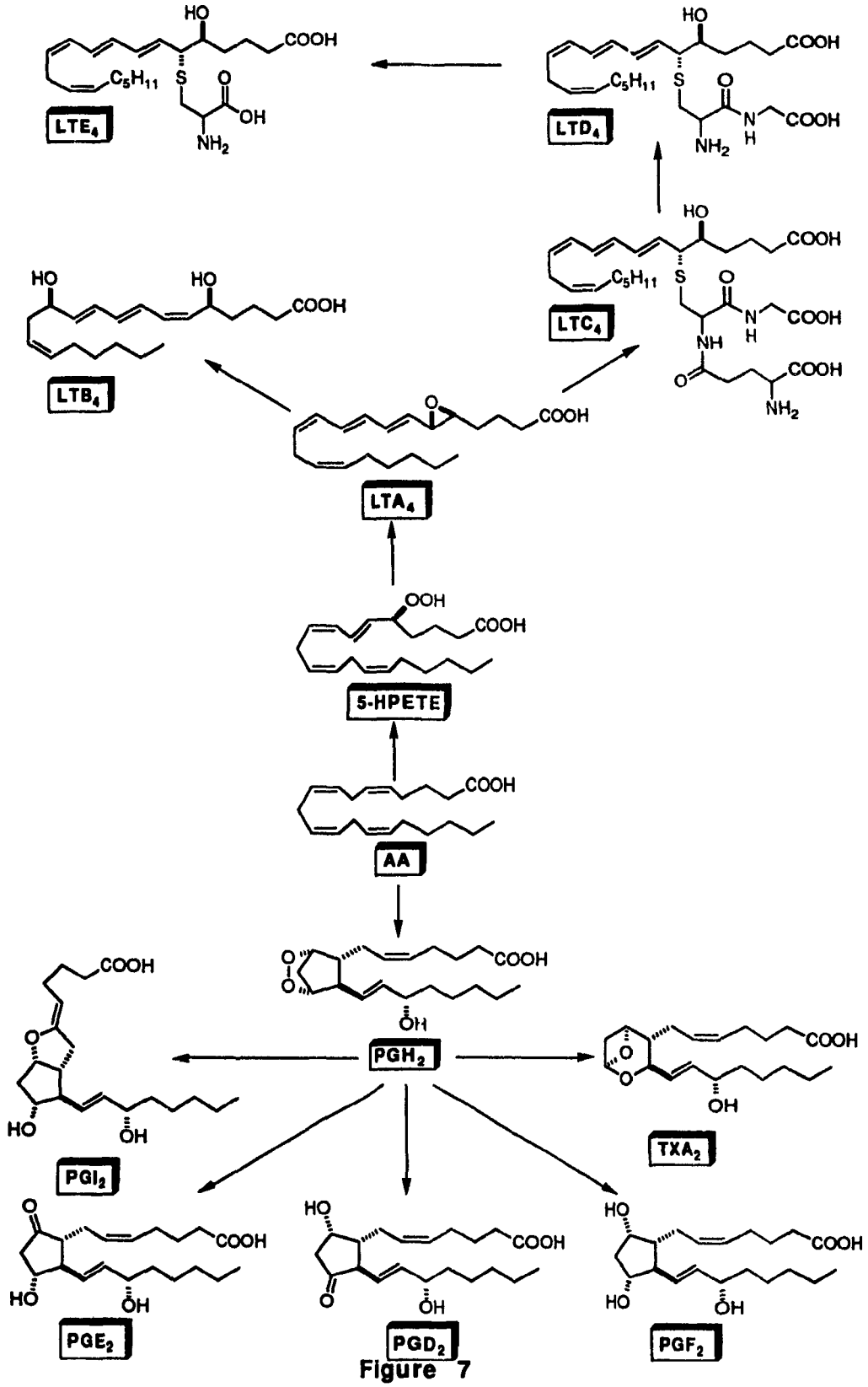
LTE4
LTD4
LTC4
LTB4
LTA4
5-HPETE
AA
PGH2
PGI2
TXA2
PGE2
PGD2
PGF2

Figure 7

Control of arachidonic acid release.

In a previous section, it was stated that arachidonic acid is stored mainly at the 2-position of membrane phospholipids, and that its release is the rate limiting step of the eicosanoid cascade. Because AA metabolites are often the regulators of physiological responses, and since they are responsible for the appearance of disease states such as arthritis and asthma, much effort has been devoted to understanding and controlling eicosanoid metabolism. This search has led to the discovery of cyclooxygenase inhibitors, 5-LO inhibitors, various PG and LT antagonists etc[33].

The control of AA release from membrane phospholipids is still poorly understood[21]. It is known that PI hydrolysis is one of the first events to occur in receptor activation of platelets. The diacylglycerol generated is a potential source of AA through hydrolysis by cellular lipases[34], but the major function of DG is the activation of C-kinase. When cells are incubated with labelled arachidonic acid however, it is preferentially incorporated into and released from phosphatidylcholine (PC), indicating that specialized pools of PC exist. In stimulated platelets the major source of AA is from PC. It is cleaved by the action of phospholipase A_2, although PC is not the largest reservoir of this fatty acid. The specific PLA_2 responsible for eicosanoid production has not yet been unequivocally identified since multiple enzymes with this activity can be detected, and the precise details of its regulation are still controversial[21]. It is clear that mechanisms which cause calcium mobilization (ionophores, IP_3) are instrumental in initiating AA release, but calcium alone is insufficient. There is evidence that specific receptors initiate PLA_2 activity by a G protein-linked mechanism which is independent of the PLC activation sequence described earlier. The involvement of C-kinase in PLA_2 activation appears to be indirect. Lipocortins which were earlier implicated in the modulation of PLA_2 activity are now known to be substrates of C-kinase, and that phosphorylation removes their inhibitory activity. This inhibition appears to be due to the binding of lipocortins to phospholipids which prevents access of PLA_2 to its

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33) Peplow, P. V.; *Prost. Leuk. Ess. Fatty Acids-Rev.* **1988**, *33*, 239; Brooks, D. W.; Bell, R. L.; Carter, G. W.; *Ann. Rep. Med. Chem.* **1988**, *23*, 69; Bauer, R. F.; Collins, P. W.; Jones, P. H.; *Ann. Rep. Med. Chem.* **1987**, *22*, 191; Cross, P. E.; Dickinson, R. P.; *Ann. Rep. Med. Chem.* **1987**, *22*, 95.

34) Dennis, E. A.; *Bio/Technology*, **1987**, *5*, 1294.

substrate[35]. The physical state of the membrane is also viewed as an important factor modulating PLA_2 activity, although the precise role of membrane perturbation in the regulation of the enzyme is still under investigation. At present it appears that DG and lipocortins modify the state of the phospholipid interface and facilitates the penetration of PLA_2 into the bilayer. This aspect will be discussed further in a subsequent section. Free arachidonic acid is itself an activator of both PLC and C-kinase, and causes Ca^{2+} release from intracellular stores independently and synergistically with IP_3. The general conclusion which is drawn from these data is that activation of PLA_2 and PLC through the participation of receptor mediated signal transduction mechanisms are independent although simultaneous processes (depending on the agonist). The two processes serve to facilitate and accelerate each other, leading to an amplification of the cellular response following receptor activation. At present, although there is much interest in this area, it is still unknown by which mechanism PLA_2 is 'turned on' and 'off': whether there is covalent or allosteric modification of the enzyme, or whether there is a specific activator/inhibitor protein interaction.

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[35]) Davidson, F. F.; Dennis, E. A.; Powell, M.; Glenney, J. R., Jr.; *J. Biol. Chem.* **1987**, *262*, 1698.

Mechanism and properties of phospholipase A_2 catalysis.

Most of the current knowledge about the structural and mechanistic aspects of phospholipase A_2 catalysis was obtained through the study of extracellular enzymes obtained from pancreatic secretions (bovine or porcine) or from the venoms of rattlesnake (*Crotalus*), water mocassin (*Agkistrodon*) or cobra (*Naja*)[36]. Bee venom is also a source of PLA_2, although this enzyme is evolutionarily distant and structurally quite different from proteins of snake venom or pancreatic origin. These small robust proteins (MW≈14000 Da) with seven disulfide bridges are abundant and are readily purified. All require Ca^{2+} for activity, and show a great deal of sequence homology. These have been divided into two classes based on the presence of a particular disulfide bond between residues 11 and 69: class I enzymes include PLA_2s from mammalian pancreas and elapid snakes (*Naja naja*), while class II is comprised of enzymes isolated from the venoms of the *Crotalus* and *Agkistrodon* families, as well as from human arthritic synovial fluid, placenta, and human and rat platelets. The class II snake venom enzymes may be a better model for the development of inhibitors because of their greater similarity to the synovial fluid PLA_2s found in arthritic inflammation sites[37]. They are therefore more relevant to therapeutic targets. Enzymes of both classes may exist as monomers or dimers. Pancreatic PLA_2 ($pPLA_2$) is secreted as a proenzyme ($proPLA_2$) which is cleaved by trypsin to the fully active species. ProPLA2 shows only low catalytic activity at all substrate concentrations. After tryptic removal of seven amino acids at the N-terminal, the enzyme shows markedly different kinetics than the $proPLA_2$. At low substrate concentrations, pPLA has the same activity as the proenzyme. Above the critical micellar concentration (CMC) of the substrate, i.e. the concentration at which monomeric phospholipid forms large aggregate structures, pPLA2 activity jumps by several orders of magnitude. This new form of the enzyme is therefore specific for aggregated substrate. The preference for phospholipid/water interfaces is made possible by the conformation of the new N-terminal of the enzyme, and is prevented in $proPLA_2$ by the secretory signal sequence. The specificity of all known PLA_2 for aggregates, and the increased catalytic activity compared to monomeric substrate, is known as interfacial recognition.

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[36]) Achari, A.; Scott, D.; Barlow, P.; Vidal, J. C.; Otwinowski, Z.; Brunie, S.; Sigler, P. B.; *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 441.

[37]) Mobilio, D.; Marshall, L. A.; *Ann. Rep. Med. Chem.* **1989**, *24*, 157.

Another particular feature of $pPLA_2$ catalysis is the so-called "lag phase" phenomenon. Under certain conditions, the rate of hydrolysis of saturated phospholipid vesicles or monolayers by $pPLA_2$ reaches its maximal value after a latency period of variable duration[38]. The length of this latency is dependent on many factors which include the physical state of the lipid/water interface and the presence of negatively charged lipid in the bilayer. This latter effect has been termed interfacial activation, and several different mechanisms have been proposed to explain it: penetration of PLA_2 into the monolayer[39], conformational changes induced by the substrate or by the interface[40], enzyme-activator complex formation[41], or dimerization of the enzyme[34]. Although these theories explain various aspects of PLA_2 catalysis, a more satisfactory explanation is provided by the data of Kézdy *et al.*[42] and Tsai *et al.*[43] They report that $pPLA_2$ and the enzyme from *A. piscivorus* undergo autocatalytic acylation at specific lysine residues by the substrate, and that this results in a dimeric species which displays full catalytic activity without any lag phase. A similar acylation with palmitic anhydride has the same effect. In addition, the replacement of this lysine by methionine in a genetically engineered PLA_2 increases the catalytic efficiency of the enzyme and removes the lag phase. Conversely, derivatization of lysine in *N. naja atra* PLA_2 with trinitrobenzene sulphonic acid completely deactivates the enzyme[44], although the inhibition can be partially reversed by removal of the TNBS groups with hydrazine. The derivatized Lys residues in all these cases surround the active site, and are in close proximity to the N-terminus of the enzyme. Human synovial fluid PLA_2 has several positively charged residues in the area of the hydrophobic channel around the active site as well[45]. In

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38) Bell, J. D.; Biltonen, R. L.; *J. Biol. Chem.* **1989**, *264*, 12194.

39) Verger, R.; Mieras, M. C. E.; De Haas, G. H.; *J. Biol. Chem.* **1973**, *248*, 4023.

40) Verheij, H. M.; Slotboom, A. J.; De Haas, G. H.; *Rev. Physiol. Biochem. Pharmacol.* **1981**, *91*, 91.

41) Adamich, M.; Roberts, M. F.; Dennis, E. A.; *Biochemistry*, **1979**, *18*, 3308.

42) Cho, W.; Tomasselli, A. G.; Heinrikson, R. L.; Kézdy, F. J.; *J. Biol. Chem.* **1988**, *263*, 11237;

43) Noel, J. P.; Deng, T.; Hamilton, K. J.; Tsai, M.-D.; *J. Am. Chem. Soc.* **1990**, *112*, 3704.

44) Yang, C.-C.; Chang, L.-S.; *Biochem. J.* **1989**, *262*, 855.

45) Wery, J. P.; Schevitz, R. W.; Clawson, D. K.; Bobbitt, J. L.; Dow, E. R.; Gamboa, G.; Goodson, T. Jr.; Hermann, R. B.; Kramer, R. M.; McClure, D. B.; Mihelich, E. D.;

addition, the N-terminal signal peptide of proPLA$_2$ must be cleaved before the enzyme is fully active against aggregated substrates. This additional sequence interferes with the binding of PLA$_2$ to aggregates by creating steric hindrance in the region of the active site channel, and preventing the access of Lys residues near the N-terminus of the protein to the interface. These data implicate the basic residues around the catalytic site of the enzyme in the process of interfacial recognition and activation.

In the presence of negatively charged phospholipids or fatty acids, PLA$_2$ does not show any lag phase, and does not display a jump in the rate of hydrolysis at the CMC of the lipid[46]. It has been stated that this is due to the ability of the enzyme to form an aggregate complex with anionic lipids below the CMC of the monomer. It is reasonable to consider that the role of the interaction with anionic lipids is to allow the enzyme to imbed itself in the interface and to allow the substrate access to the active site. The membrane substrate for PLA$_2$ *in vivo* is negatively charged, and the formation of ion pairs between anionic lipids and the cationic side chains around the active site channel may be part of the "interfacial activation" mechanism. Desolvation of the interface region and insertion into the lipid headgroups would effectively allow the diffusion of substrate into the active site as is proposed by Scott *et al.* (**Figure 8**) [47] This picture

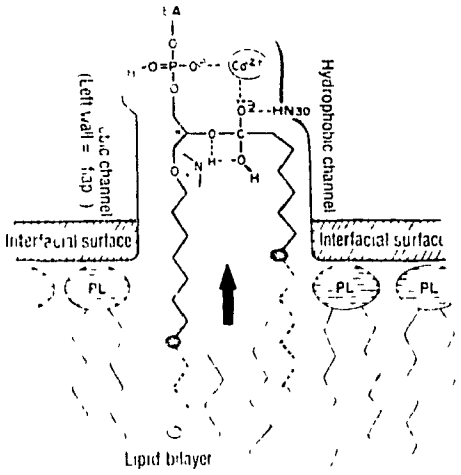

Figure 8 (From ref. 47)

Putnam, J. E.; Sharp, J. D.; Stark, D. H.; Teater, C.; Warrick, M. W.; Jones N. D.; *Nature*, **1991**, *352*, 79.

46) Jain, M. K.; Rogers, J.; *Biochim. Biophys. Acta*, **1989**, *1003*, 91.

47) Scott, D. L.; White, S. P.; Otwinowski, Z.; Yuan, W.; Gelb, M. H.; Sigler, P. B.; *Science*, **1990**, *250*, 1541.

is consistent with the structure of the enzyme, which is quite resistant and stable due to the many disulfide bonds. The initiation of catalysis may not be due to a conformational change in the enzyme, but rather to "allosteric" changes in the phospholipid interface. We believe this process may be dependant on anionic lipid. When PLA_2 encounters a zwitterionic surface, it cannot penetrate efficiently and can only hydrolyze a few phospholipid monomers in the hopping mode. After a number of turnovers, the density of anions on the surface becomes sufficient to allow the formation of ion pairs between product fatty acid and the positively charged surface of the enzyme. Once this occurs, the protein has an anchor in the bilayer, and a desolvation process (which we describe as the fusion of the enzyme with the surface of the membrane) inserts the opening of the active site channel into the interface. We believe this forms the basis of the hydrophobic channel and the seal provided by interfacial binding as proposed by Scott, and allows the enzyme to display full catalytic activity. Such a picture is consistent with the fluorescence data of the time course of PLA_2 activation[38].

The mechanism of PLA_2 catalysis has also been the subject of controversy and debate, but recent X-ray crystallography data helps shed some light on the conformational details[48]. The authors describe the interaction between a transition state analogue and the enzyme, and infer the role of the active site side chains and of the essential calcium ion in the esterolysis. It is suggested that the enzyme provides a hydrophobic channel which allows the substrate phospholipid to penetrate the active site by facilitated diffusion, without exposure to the surrounding aqueous environment. This greatly reduces the energy required to transfer a phospholipid molecule from its environment in the membrane and position it for esterolysis, as well as to remove the products from the active site. The calcium ion serves to bind the phosphate group and as an electrophile to facilitate the nucleophilic attack by water on the sn-2 carbonyl (**Figure 9**)[47]. In the latter respect, the mechanism of PLA_2 hydrolysis is very reminiscent of serine proteases[49] with water replacing the serine hydroxyl as the nucleophile.

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[48]) White, S. P.; Scott, D. L.; Otwinowski, Z.; Gelb, M. H.; Sigler, P. B.; *Science*, **1990**, *250*, 1560.

[49]) Lehninger, A. L.; *Principles of Biochemistry*, Worth: New York, **1982**, p 229.

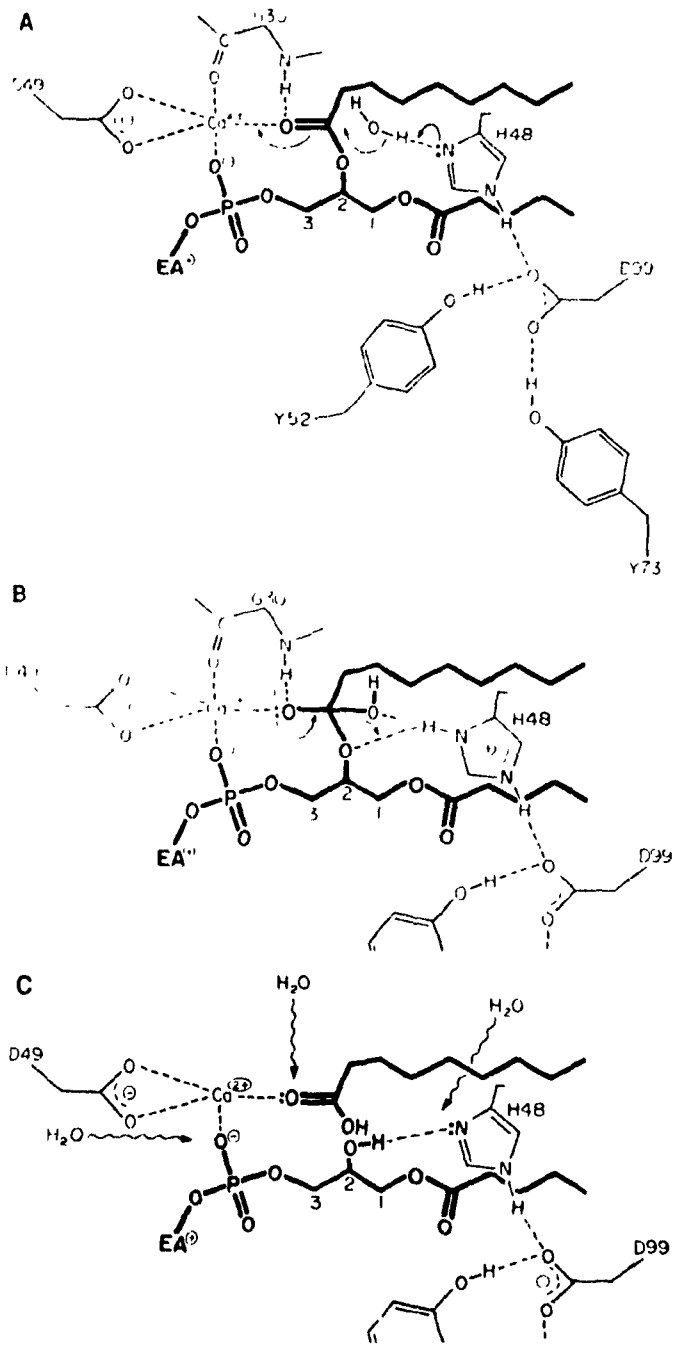

Figure 9 (Taken from ref.47)

Properties of lipids in monomolecular films

It is well known that phospholipase A_2 can only hydrolyze its substrate while it is in aggregates, but the physical state of the lipid is also an important factor[50]. With the use of a Langmuir film balance it is possible to create a monolayer of phospholipid at an air/water interface and to vary the interface by changing the lateral surface pressure exerted on the lipid. When a lipid solution is spread on the water surface, the polar headgroups interact with the aqueous compartment, while the hydrophobic chains prefer to face towards the air. The resulting two-dimensional lipid phase is functionally analogous to one half a bilayer. By plotting surface pressure vs. area per molecule, an isothermal two-dimensional phase diagram can be obtained (**Figure 10**). At low surface concentrations (gas-analogous) there is no interaction between the individual phospholipid monomers which lie essentially flat on the water. When the area is reduced, the molecules begin to interact, and the hydrophobic tails begin to orient themselves

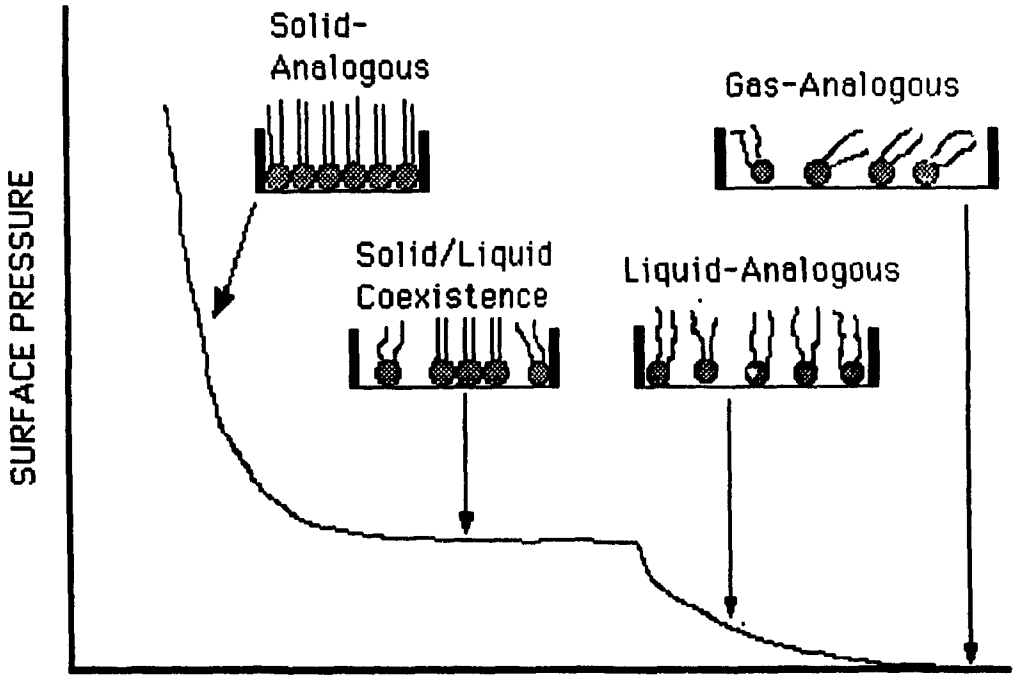

Figure 10

50) Ahlers, M.; Müller, W.; Reichhert, A.; Ringsdorf, H.; Venzmer, J.; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1269.

perpendicular to the plane of the water. In this liquid-analogous phase, there is still little order in the alkyl chains, and the monolayer resembles the state of a bilayer at a temperature above the phase transition of the lipid. Reducing the area per molecule further causes packing constraints, and regions of the monolayer begin to adopt more crystalline-like conformations: there are now islands of solid-analogous lipid floating in the liquid-analogous phase. This is a state similar to a bilayer assembly at its phase transition temperature. Reducing the area even more causes a rapid rise in surface pressure when all the phospholipid acyl chains have adopted a trans geometry as in the crystalline phase of a bilayer. The molecules can no longer come any closer, and any further increase in pressure causes film collapse. This type of pressure/area isotherm is used extensively to characterize amphipathic molecules, since it gives a measure of the packing properties of the compound at water interfaces.

Modulation of PLA_2 activity by the interface structure.

Other factors which affect the hydrolysis of phospholipid by PLA_2 are surface lateral packing defects and lipid electrostatic influences on the substrate interface. The latter were dealt with in a previous section in the discussion of anionic lipid vesicles. Through a series of elegant experiments[51], Ringsdorf and co-workers were able to show that PLA_2 initiates hydrolysis at the boundary region between the liquid- and solid-analogous phases in a monolayer. Apparently, the microstructure of the lipid in this interphase region is such that it favors enzyme binding. This phenomenon also explains why PLA_2 hydrolysis of vesicles is faster near the thermal phase transition of its phospholipid components: the amount of interphase lipid is greatest near the phase transition temperature[52]. It is quite likely that the boundary lipid presents to the enzyme a region with a high number of packing defects due to its partially disordered nature. In this context, a surface defect would be any structural factor which prevents the tight association of the phospholipid headgroups, and partially exposes the hydrophobic interior of the membrane, like a crack in a wall or a missing brick.

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51) Grainger, D. W.; Reichert, A.; Ringsdorf, H.; Salesse, C.; *FEBS Letters*, **1989**, *252*, 73; Grainger, D. W.; Reichert, A.; Ringsdorf, H.; Salesse, C.; Davies, D. A.; Lloyd, J. B.; *Biochim. Biophys. Acta*, **1990**,*1022*, 146.

52) Ibid ref 1 p 66.

Although there are conflicting reports of the existence of arachidonate specific PLA_2 enzymes in various cell preparations[53], most PLA_2s from various sources have a preference for unsaturated lipids. The presence of packing defects is believed to be a major cause of the increased rate of hydrolysis of chain-unsaturated phospholipids[54]. It is generally accepted that cis-unsaturated acyl chains reduce the phase transition temperature of phospholipids by preventing the tight packing of the neighboring chains. Monolayer studies also show a larger area/molecule ratio for phospholipids containing 2-oleoyl, linoleoyl or arachidonoyl chains[55]. These data are consistent with the idea that a phospholipase which encounters the surface of the bilayer undergoes an activation process involving adsorption or penetration into the interface. The unsaturated acyl chains would be a factor promoting this activation by creating a greater number of surface defects which the enzyme recognizes.

There are other examples which belong in this category. Sevanian and others have proposed another physiological role for PLA2 other than arachidonic acid release: participation in the detoxification of lipid hydroperoxides produced by free radical oxidation of polyunsaturated fatty acids[56] in membrane phospholipids. The breakdown of lipid hydroperoxides has been implicated in the process of aging by creating reactive intermediates which form adducts with amino functions *in vivo* [57]. Another group has shown that peroxidized phospholipids were preferentially hydrolysed by PLA_2, and that a phospholipid with an ω-carboxylate function on the 2-acyl substituent was cleaved 15 times faster than 2-linoleoyl-PC[58]. This preference of PLA_2 for phospholipids with a second polar function on the sn-2 acyl chain has been explained in terms of the presence of a second headgroup. These physiologically aberrant compounds may be better substrates for the enzyme because the polar function on the sn-2 chain can fold back to

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53) Kim, D. K.; Kudo, I.; Inoue, K.; *J. Biochem.* **1988**, *104*, 492.

54) Schalkwijk, C. G.; Märki, F.; Van den Bosch, H.; *Biochim. Biophys. Acta*, **1990**, *1044*, 139.

55) Demel, R. A.; Geurts van Kessel, W. S. M.; van Deenen, L. L. M.; *Biochim. Biophys. Acta*, **1972**, *266*, 26.

56) Van Kuijk, F. J. G. M.; Sevanian, A.; Handelman, G. J.; Dratz, E. A.; *Trends in Biol. Sci.* **1987**, *12*, 31.

57) Iio, T.; Yoden, K.; *Lipids*, **1988**, *23*, 937.

58) Itabe, H.; Kudo, I.; Inoue, K.; *Biochim. Biophys. Acta*, **1988**, *963*, 192.

the surface of the bilayer. This loop structure would prevent the tight packing of headgroups found in an undisrupted membrane, and would favor the recognition and hydrolysis of these reactive hydroperoxy fatty acids. The detoxification mechanism involves reduction of the hydroperoxide to an alcohol by glutathione peroxidase, an enzyme which accepts fatty acids but not phospholipids as substrates.

PLA_2 inhibitors.

Several reviews have appeared in the literature describing recent progress in the design and synthesis of PLA_2 inhibitors[34,37,59]. These fall into two main categories based on their structure and mode of action: covalent irreversible inhibitors and competitive inhibitors. There are only three known examples of the first type. Para-bromophenacylbromide (PBP), a non-selective PLA_2 inhibitor has been shown to react with the active site histidine which is involved in catalysis. This compound (**Figure 11**) is not being considered as a potential therapeutic agent due to its reactivity and lack of specificity. Thiols and amines for example are known to react with 1-bromoketones of this type. Manoalide, a sesterpene obtained from the *Luffariella variabilis* sponge, and a structurally simpler analogue, coined manoalogue[60] are known to react irreversibly with PLA_2. The inhibition has been shown to depend on the presence of the α,β-unsaturated aldehyde and the butenolide ring. Irreversible covalent modification of lysine residues near the N-terminus of cobra venom PLA_2 has been implicated in the inactivation of catalysis (also in ref 49). Finally, a suicide-type inhibitor (SIBLINKS) has been described which is purported to generate a cyclic anhydride in the active site of the enzyme[61]. Time-dependant inhibition is observed for derivatives where the rate of cyclization of the intermediate is greater than the rate of diffusion out of the active site. It should be pointed out that these compounds only inhibit the enzyme after a preincubation period in the absence of substrate, and do not compete with true substrate for the active site.

A second class of PLA_2 inhibitors is a group of phospholipid analogues which have an amide function (Amide-PC) at the 2-position instead of the ester as in the true

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59) Chang, J.; Musser, J. H.; McGregor, H.; *Biochem..Pharmac.* **1987**, *36*, 2429.

60) Reynolds, L. J.; Morgan B. P.; Hite, G. A.; Mihelich, E. D.; Dennis, E. A.; *J. Am. Chem. Soc.* **1988**, *110*, 5172.

61) Washburn, W. N.; Dennis, E. A.; *J. Am. Chem. Soc.* **1990**, *112*, 2040, 2042.

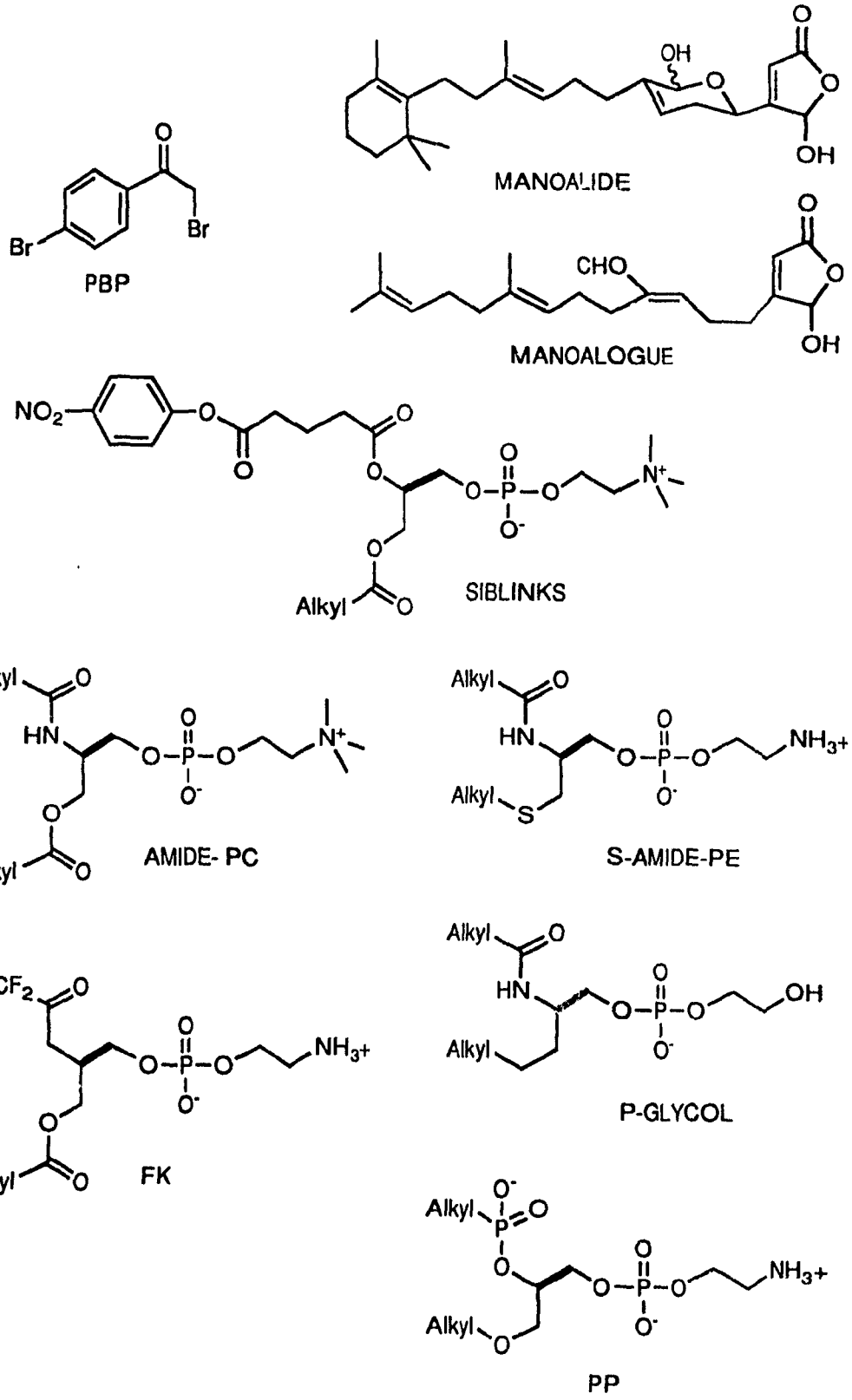
PBP
MANOALIDE
MANOALOGUE
SIBLINKS
AMIDE- PC
S-AMIDE-PE
FK
P-GLYCOL
PP

Figure 11

substrate[62,63]. These compounds are reported to be potent competitive inhibitors of PLA_2, due to their ability to form hydrogen bonds with unspecified active site residues, or due to the stability of the amide function to hydrolysis. The results of these studies are difficult to compare, and the relevance to physiological conditions are difficult to establish because the assay utilises a mixed detergent/ substrate/ inhibitor system. The inhibition of the enzyme is reported as an IC_{50}, or the concentration of inhibitor required to reduce the rate of PLA_2 hydrolysis by 50%. This number gives a misleading idea of the actual inhibitory power since these IC_{50} values refer to bulk concentrations, and not the concentration at the interface which would provide a relative measure of the affinity of the enzyme for the inhibitor. In addition, IC_{50} values vary with the substrate concentration used in the assay. Verger and de Haas have developed a kinetic model to quantify this parameter by measuring the rate of hydrolysis in the presence of an inhibitor, and deriving an inhibitory power coefficient which is relative to the mole fraction of inhibitor in the micellar system[64]. By comparing the α_{50} (the mole fraction of inhibitor giving 50% inhibition), we can obtain a measure of the inhibition by the analogues which is independent of the concentration. Dennis and co-workers found that the best inhibitor in their assays was the 1-thioalkyl-2-amido-3-sn-glycero-phosphatidylethanolamine (S-Amide-PE, $\alpha_{50} = 9 \times 10^{-4}$), and explained that a hydrophobic substituent at the 1-position gives better inhibition. Verger and de Haas found that they could improve inhibitory power by having a simple alkyl chain instead of an ether or thioether at the 1-position, and that anionic lipids such as phosphatidylglycols (P-Glycol) were more powerful inhibitors than their choline counterparts by one to two orders of magnitude ($\alpha_{50} = 6.5 \times 10^{-5}$).

Another approach towards inhibitors of PLA_2 has been the testing of compounds designed to mimic the putative tetrahedral intermediate of the hydrolytic reaction. This concept supposes that the binding of an enzyme to the transition state of the reaction is stronger than to either the substrate or the product, and that a compound which emulates this will

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62) Magolda, R. L.; Galbraith, W.; *J. Cell Biochem.* **1989**, *40*, 371; Yu, L.; Deems, R. A.; Hajdu, J.; Dennis, E. A.; *J. Biol. Chem.* **1990**, *265*, 2657; de Haas, G. H.; Dijkman, R.; van Oort, M. G.; Verger, R.; *Biochim. Biophys. Acta*, **1990**, *1043*, 75.

63) de Haas, G. H.; Dijkman, R.; Ransac, S.; Verger, R.; *Biochim. Biophys. Acta*, **1990**, *1046*, 249.

64) Ransac, S.; Rivière, C.; Soulié, J. M.; Gancet, C.; Verger, R.; de Haas, G. H.; *Biochim. Biophys. Acta*, **1990**, *1043*, 57.

be a good competitive inhibitor. This approach has been used with some success in the design of peptidase inhibitors, and in the generation of catalytic antibodies. Gelb's group has produced two such transition state analogues, the fluoroketone and the phosphonate phospholipids (FK and PP)[65]. There is good evidence that this reasoning is correct, since the latter compound has recently been observed in a crystal structure with PLA_2 in the expected orientation with respect to the catalytic side chains[45,47].

Although many different compounds have been synthesized and identified by screening (see also Mobilio and Marshall in ref 42), none have yet gone on to clinical trials. The compounds designed so far are not potent enough to be therapeutically useful, and there is no means of evaluating the selectivity of the potential drug for the targeted enzyme i.e. the PLA_2 responsible for arachidonic acid release.

Plan of Study.

Because of the crucial role played by PLA_2 in eicosanoid metabolism, we were interested in studying how its activity could be modified. The ultimate goal would be the design of an efficient inhibitor of the enzyme, but this is not yet possible for the following reasons. Firstly, there is no definite identification of the specific phospholipase responsible for arachidonate release *in vivo*. This is necessary for the development of an *in vitro* assay, which would determine the potency and specificity of any putative inhibitor. Secondly, the details of the regulation of PLA_2 activity are not known. In this respect, it may be safer to design a drug which prevents PLA_2 activation than to make mechanism based inhibitors, due to the necessity of phospholipase function for the maintenance of normal cellular metabolism. Thirdly, the structural details of phospholipids which govern the affinity of the enzyme for its substrate are not yet clearly understood.

This last point is the basis of the work described in this account. Because of the paucity of knowledge as to what makes a compound a better substrate for PLA_2, and since an understanding of this is essential for the design of an eventual inhibitor, we set out to examine the effect of substituents on phospholipid acyl chains on the rate of PLA_2 hydrolysis. The type of products we decided to study are those which can behave as lipids with two headgroups, in particular those with polar functions on the sn-2 chain.

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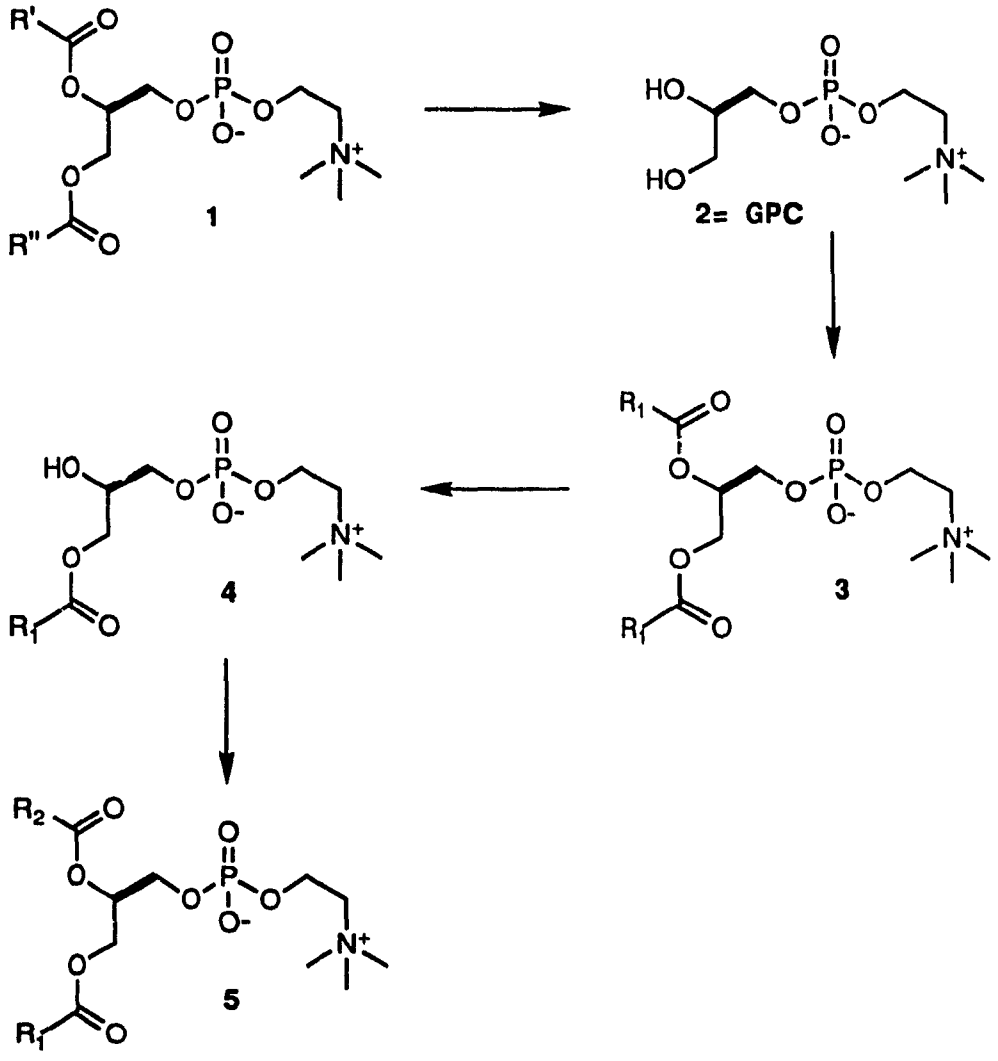
65) Gelb, M. H.; *J. Am. Chem. Soc.* **1986**, *106*, 3146; Yuan, W.; Berman, R. J.; Gelb, M. H.; *J. Am. Chem. Soc.* **1987**, *109*, 8071; Yuan, W.; Gelb, M. H.; *J. Am. Chem. Soc.* **1988**, *110*, 2665; Yuan, W.; Fearon, K.; Gelb, M. H.; *J. Org. Chem.* **1989**, *54*, 906.

Relatively little attention has been paid to the synthesis of phospholipids, and the methods so far described do not allow much structural variation in the products. The remainder of this work relates the details and considerations of new procedures for the total synthesis of phospholipids with chemically sensitive functional groups.

Discussion.

Hemisynthesis.

Since phospholipids are available from natural sources, techniques have been developed to utilize these compounds as precursors for analogues having a more complicated structure. Natural lipids from egg yolk or soybean contain different types of phospholipids as classified by their headgroup composition. While these are readily isolated, the separation according to acyl chain substitution is not usually possible by standard techniques such as recrystallization or chromatography. In order to obtain



Scheme 1

phosphatidylcholines of known chain composition, it is therefore necessary to saponify the ester groups, and to reacylate the glycerophosphatidyl choline (GPC) with the fatty

acid of interest (**Scheme 1**). This method is useful only for PCs with identical fatty acids at both the 1- and 2- positions of the glycerol[1].

Many investigations focus however on phospholipids with a single modified acyl chain, generally at the 2- position of the glycerol. These are accessible by the cleavage of the sn-2 chain of a phosphatidylcholine with phospholipase A_2, followed by reacylation of the lysophospholipid (lysoPC) with the desired fatty acid in the presence of a suitable activating reagent. If a modification only at the 1- position is desired, it must be introduced at both hydroxyls and replaced at the 2- position by PLA_2 cleavage and reacylation. Many esterification procedures have been developed for phospholipids using acid anhydrides[2], acid imidazolides[3], DCC and DMAP[1], etc. These methods however, suffer from a number of disadvantages. First, they employ a large excess of activated acid derivatives with lysoPC as limiting reagent[4]. The low efficiency of this procedure is prohibitive when the most valuable component is the carboxylic acid. Secondly, the yields of the acylation reaction are often low due to the low solubility of some carboxylic acid derivatives and especially of lysoPC in chloroform (the usual reaction solvent). Solubility is often a problem as well during chromatographic purification of the products: lysoPC is usually more polar than the diacyl product, but in the solvent systems employed (typically mixtures of chloroform, methanol and water), there is not always enough resolution to obtain pure product. Moreover, under more drastic acylation conditions, variable amounts of isomeric 1,3-diacyl-2-PC are produced by phosphate migration, as well as substantial quantities of products having undergone chain migration[5]. It is quite likely that a large part of the acyl migration reported during the esterification of lysoPC is caused by isomeric impurities in the samples of the starting material in addition to isomerization caused by the acylation conditions[6].

Certain modifications of the headgroup are possible by taking advantage of the reactivity of the amino group of phosphatidylethanolamines and serines[1]. In particular, protection of the amine as a t-butyl carbamate (BOC) has been used in the synthesis of a bolaform

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1) Bogomolov, O. V.; Kaplun, A. P.; Shvets, V. I.; *Russ. Chem. Rev.* **1988**, *57*, 382.
2) Gupta, C. M.; Radhakrishnan, R.; Khorana, H. G.; *Proc. Natl. Acad. Sci. USA*, **1977**, *74*, 4315.
3) Beck, A.; Heissler, D.; Duportail, G.; *J. Chem. Soc. Chem. Commun.* **1990**, 31.
4) Delfino, J. M.; Schreiber, S. L.; Richards, F. M.; *Tetrahedron Lett.* **1987**, *28*, 2327.
5) Ali, S.; Bittman, R.; *Chem. Phys. Lipids*, **1989**, *50*, 11.
6) Nicholas, A. W.; Khouri, L. G.; Ellington, J. C. Jr.; Porter, N. A.; *Lipids*, **1983**, *18*, 434.

phosphatidylethanolamine[7]. The BOC protected phosphate diester is still recognized as a PLA_2 substrate so that the sn-2 chain can be cleaved. Methylation of the phosphate provides a neutral lysoPE which is efficiently esterified with a dicarboxylic acid using DCC and DMAP. This strategy allows the esterification to proceed in high yield due to the increased solubility and absence of charge of the lyso derivative, and prevents any acylation of the phosphate. These two factors allow the coupling to proceed at a faster rate and effectively prevents acyl migration in the product. The scope of the method is however limited by the necessary deprotection reactions which require both NaI in 2-butanone, and trifluoroacetic acid.

Total synthesis of phospholipids.

There are relatively few reported total syntheses of diacylglycero phospholipids. Most investigators rely on chemo-enzymatic methods rather than the more labor intensive chemical procedures for the production of phospholipids with different acyl groups on the glycerol, in spite of the disadvantages outlined above. One notable exception to this rule is the assembly of ether lipids. Since many useful tactics used in the synthesis of these latter compounds are also applicable to diacyl lipids, we will examine them later in this section.

Diacyl glycero lipids.

The prototype for phospholipid total synthesis is described by Eibl[8]. This approach is based on the preparation of a neutral 1-acyl-2-benzyl-3-sn-glycerol-phosphotriester in eight steps from 3,4-isopropylidene-D-mannitol. Debenzylation followed by reacylation of the sn-2 hydroxyl and deprotection of the phosphate group furnishes the phospholipid. This strategy allows flexibility in the headgroup functionalities by changing the alcohol coupled to the phosphate, and in the sn-2 acyl chain since it is introduced in the next to last step. Some disadvantages can be noted. First, the synthesis is rather lengthy, requiring a minimum of eleven steps for a simple phosphatidylcholine. Many of the reactions are protection/deprotection steps required to introduce the three glycerol substituents in the correct position. Secondly, the preparation of the key intermediate involves the esterification of three different alcohols with phosphoryl chloride ($POCl_3$). This is expected to proceed with little specificity since there is not always a sufficient difference in reactivity towards

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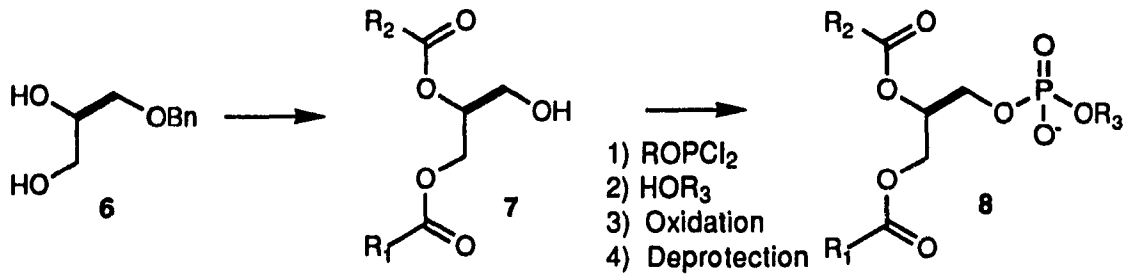
7) Delfino, J. M.; Stankovic, C. J.; Schreiber, S. L.; Richards, F. M.; *Tetrahedron Lett.* **1987**, *28*, 2323

8) Eibl, H.; *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 257.

alcohols between $POCl_3$, and phosphodichloro and monochloridates ($ROPOCl_2$ and $(RO)_2POCl$). In spite of these drawbacks, this route avoids the possibility of acyl migration.

The second landmark in phospholipid total synthesis is the report by Martin and Josey[9], who reported the assembly of 1,2-diacyl-3-benzyl-sn-glycerols and their subsequent conversion into phosphatidylethanolamines (**Scheme 2**). They showed



Scheme 2

that low temperature DCC catalysed esterification of a carboxylic acid with the 3-protected glycerol proceeds regiospecifically at the primary hydroxyl, and that a second acylation can be accomplished at room temperature without migration. They introduced the phosphate headgroup by the consecutive substitution of a dichlorophosphite. This suggests a possible lack of specificity as outlined before. The authors did not however extend their approach to lipids with unsaturated chains, preferring instead a multiple protection/deprotection scheme similar to that described by Eibl.

Dialkyl glycero lipids

Ether lipids are also important targets for synthesis since they exhibit many interesting characteristics. For example (**Scheme 3**), the highly potent physiological mediator platelet aggregation factor (PAF, **9**) is 1-alkyl-2-acetyl-sn-3-glycerophosphocholine[10]. Certain dialkyl phospholipids such as **10** are also of clinical interest, since they exhibit immunomodulating and antitumor activity[11,12]. Ether lipids such as **11** are also the main components in the membranes of archaebacteria, organisms which survive under extreme conditions of acidity, temperature and ionic

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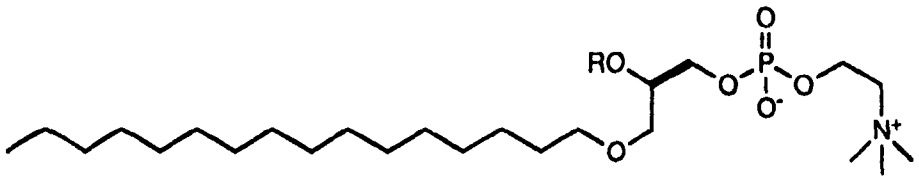
9) Martin, S. F.; Josey, J. A.; *Tetrahedron Lett.* **1988**, *29*, 3631.

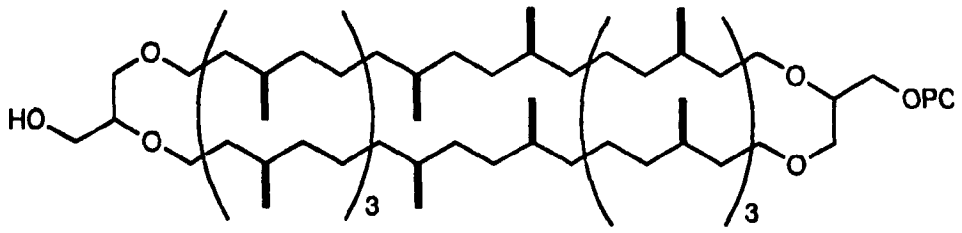
10) Synthesis: Guivisdalsky, P. N.; Bittman, R.; *J. Org. Chem.* **1989**, *54*, 4637, 4643. Isolation: Benveniste, J.; *Nature*, **1974**, *249*, 581.

11) Bhatia, S. K.; Hajdu, J.; *J. Org. Chem.* **1988**, *53*, 5034.

12) Ukawa, K.; Imamiya, E.; Yamamoto. H.; Mizuno, K.; Tasaka, A.; Terashita, Z.; Okutani, T.; Nomura, H.; Kasukabe, T.; Hozumi, M.; Kudo, I.; Inoue, K.; *Chem. Pharm. Bull.* **1989**, *37*, 1249.



9: R = CH_3CO
10: R = CH_3



11

Scheme 3

strength[13]. For example, *Sulfolobus solfataricus* growth is optimal at 85°C and pH2, conditions which can be found in hot springs and submarine volcanic fields. *Halobacterium halobium* is better known as the source of bacteriorhodopsin, a light driven proton pump. This organism is found only in salt marshes, where the salt concentration can exceed 2M. The special structure of the lipids in the membranes of these bacteria allows them to thrive in these extreme ecological niches. Examples of the lipids found in these organisms are macrocyclic tetraethers (also known as bolaform phospholipids) which span the membrane. Some species are further distinguished by having 2,3-dialkyl-sn-glycerol backbones instead of the more usual 1,2-substituted-sn-glycerols found in virtually every other species.

Ether lipids must be prepared by total synthesis, since the phosphate diester does not survive the vigorous alkylation conditions necessary to form the ether bonds. However, the protecting group strategy is considerably simpler than for the corresponding acyl phospholipids due in large part to the hydrolytic stability of the ether bonds. Several starting materials can be used depending on the pattern of substitution desired. The method of Roy and co-workers (**Scheme 4**) is recommended when dialkyl lipids of

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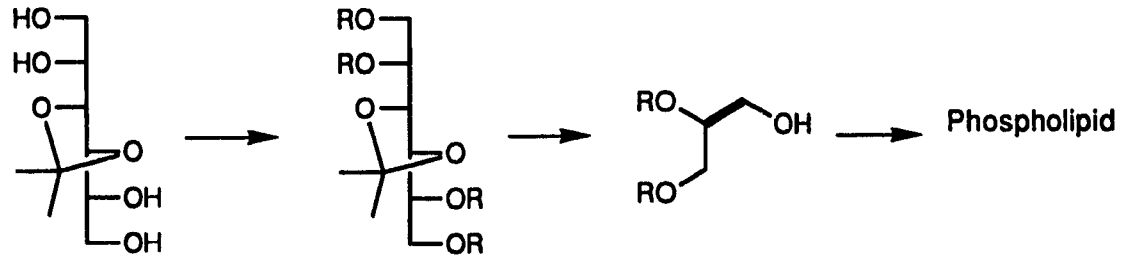
13) Yamauchi, K.; Sakamoto, Y.; Moriya, A.; Yamada, K.; Hosokawa, T.; Higuchi, T.; Kinoshita, M.; *J. Am. Chem. Soc.* **1990**, *112*, 3188; Kakinuma, K.; Obata, Y.; Matsuzawa, T.; Uzawa, T.; Oshima, T.; *J. Chem. Soc. Chem. Commun.* **1990**, 925; Fuhrhop, J.-H.; Fritsch, D.; *Acc. Chem. Res.* **1986**, *19*, 130.

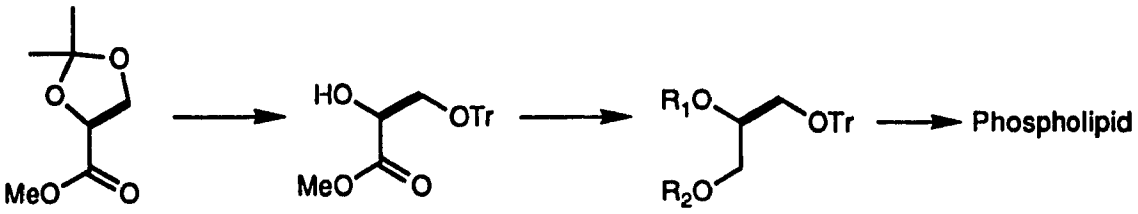
natural configuration are desired[14]. The 1,2-dialkyl-sn-glycerol is made by tetraalkylation of



12 **13**, R= Alkyl **14**

Scheme 4

3,4- isopropylidene mannitol, followed by acid hydrolysis of the acetal, periodate cleavage and borohydride reduction. Any convenient method can be used to convert the sn-3 hydroxyl into a phospholipid headgroup. For products with a different substituent at the sn-2 position, additional protection steps are required to differentiate the primary hydroxyls from each other and from the secondary OH[8,11,15]. This is exemplified by the method of Hajdu and Bhatia, which uses L-methyl glycerate as a starting material (**Scheme 5**). A trityl group is used to block the primary alcohol. The secondary alcohol is alkylated before reduction of the ester and its conversion into the primary ether. Detritylation unmasks the sn-3 alcohol which is then phosphorylated by conventional methods.



16 **17** **18**

Scheme 5

A final approach to ether lipids is worthy of mention. In recent years, many investigators have devoted their efforts to the synthesis of chiral natural products from achiral starting materials. Methods have been found to make carbon-carbon bonds with high levels of enantio- and diastereoselectivity. Others have devised procedures to

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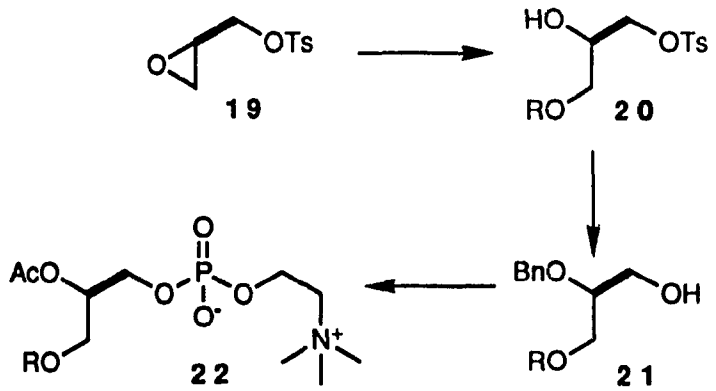
[14]) Roy, R.; Letellier, M.; Fenske, D.; Jarrell, H. C.; *J. Chem. Soc. Chem. Commun.* **1990**, 378.

[15]) Abdelmageed, O. H.; Duclos, R. I.; Abushanab, E.; Makriyannis, A.; *Chem. Phys. Lipids*, **1990**, *54*, 49; Bhatia, S. K.; Hajdu, J.; *Synthesis*, **1989**, 16.

introduce functional groups into prochiral substrates in a stereospecific fashion. Of particular interest in this respect is the Sharpless asymmetric epoxidation of allyl alcohol to give either R- or S- glycidol[16]. Previously, this method had been unsuccessful with allyl alcohol due to the difficulty in isolation of the product, and the lower conversions and enantiomeric excess. Fortunately, improvements in the protocol have been reported: *in situ* derivatization of the glycidol as arene sulfonates (tosylate, m-nitrobenzene sulfonate) improves the recovery of the product and allows the optical purity of the products to be increased by recrystallization[17]. These two arene sulfonate derivatives also allow complementary control over the regiospecificity of substitution on the glycidyl backbone by a variety of nucleophiles under basic conditions. Epoxide opening with long chain alcohols catalysed by boron trifluoride etherate has also been achieved in an application to the synthesis of ether phospholipids[10]. However, the introduction of a 2-acyl substituent can only be accomplished through transient protection of the sn-2 hydroxyl (**Scheme 6**). The intermediate **20** is benzylated at the 2-position under mild conditions with benzyl triflate in the presence of 2,6-di-t-butyl-4-methylpyridine. Removal of the tosylate is then done in two steps by displacement with cesium acetate, followed by reduction of the ester with lithium aluminium hydride to give **21**. These last conditions prevents this approach from being successfully used in the synthesis of diacyl phospholipids[18]. The synthesis of PAF is completed by phosphorylation, debenzylation, and acetylation. A partial remedy to this drawback was reported by



Scheme 6

16) Gao, Y.; Hanson, R. M.; Klunder, J. M.; Ko, S. Y.; Masamune, H.; Sharpless, K. B.; *J. Am. Chem. Soc.* **1987**, *109*, 5765.

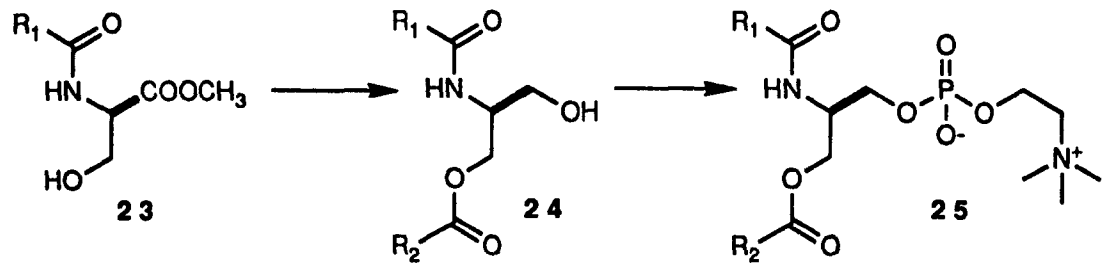
17) Klunder, J. M.; Onami, T.; Sharpless, K. B.; *J. Org. Chem.* **1989**, *54*, 1295.

18) Ali, S.; Bittman, R.; *J. Org. Chem.* **1988**, *53*, 5547; Burgos, C. E.; Ayer, D. A.; Johnson, R. A.; *J. Org. Chem.* **1987**, *52*, 4973.

Hendrickson, who introduced a trityl group instead of an arenesulfonate in the derivatization step of the Sharpless epoxidation[19]. The use of trityl glycidol for the synthesis of diacyl lipids is still limited by the low yield of epoxide opening using carboxylic acid nucleophiles, and by the possibility of isomerization during detritylation.

Amido lipids.

Another special case in the synthesis of phospholipids is the production of the analogues which have an amide group[20] or a phosphonate[21] replacing the sn-2 ester. These are currently being investigated as potential PLA_2 inhibitors. The 2- amido phospholipids are usually prepared from L-serine which is converted to an amido alcohol which is regiospecifically transformed to the corresponding phosphatidylcholine derivative.



Scheme 7

In this case, the stability of the sn-2 amide bond to hydrolysis and migration greatly simplifies the synthetic strategy: the structure of the products allows the use of more vigorous reaction and deprotection conditions than a 1,2-diacylglycerol could withstand (**Scheme 7**).

Synthetic strategy.

The goal of the following investigations was to develop synthetic methods for phospholipids which would allow the variation of the fatty acid acylated at the 2-position of the glycerol, to study the behaviour of the phosphatidylcholine species in micelles, monolayers and bilayer vesicles, and to study their susceptibility to phospholipase A_2 hydrolysis. In particular, we desired to study the properties of phosphatidylcholines

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19) Hendrickson, H. S.; Hendrickson, E. K.; *Chem. Phys. Lipids*, **1990**, *53*, 115.

20) de Haas, G. H.; Dijkman, R.; Ransac, S.; Verger, R.; *Biochim. Biophys. Acta*, **1990**, *1046*, 249; Dijkman, R.; Dekker, N.; de Haas, G. H.; *Biochim. Biophys. Acta*, **1990**, *1043*, 67; Yu, L.; Deems, R. A.; Hajdu, J.; Dennis, E.A.; *J. Biol. Chem.* **1990**, *265*, 2657; Magolda, R.L.; Galbraith, W.; *J. Cell. Biochem.* **1989**, *40*, 371; Chandrakumar, N.; Hajdu, J.; *J. Org. Chem.* **1983**, *48*, 1197; Chandrakumar, N.; Hajdu, J.; *J. Org. Chem.* **1982**, *47*, 2144.

21) Yuan, W.; Gelb, M. H.; *J. Am. Chem. Soc.* **1988**, *110*, 2665.

with a hydroxyl group at various positions along the sn-2 chain, and to prepare phospholipids with unsaturated acyl chains. It was sought to prepare the desired compounds by the most general possible method compatible with the functional groups present. Because of the problems inherent in the hemisynthetic methods, namely acyl migration, low yields and difficult purification of the products, as well as incompatibility with certain functional groups, we decided to devise a route to circumvent all of these potential obstacles.

The majority of the complications in the synthesis of phosphatidylcholines arise from the presence of the phosphodiester functionality itself[4]. It was therefore decided to introduce the zwitterionic moiety at the last step in the sequence (**Scheme 8**). This can be done by treating a suitable phosphate triester with trimethylamine[22,23,24]. Since phosphate triesters are neutral, they should be readily manipulated and purified if necessary. An array of methods exist to phosphorylate alcohols under different conditions which should be compatible with the present approach[25,26,27,28]. The report by Martin and Josey[9] inspired our strategy for the assembly of the glycerol moiety. They claimed that a 3- protected glycerol could be sequentially acylated with different fatty acids in a regiospecific manner without migration, deprotected and phosphorylated in high yield. This has the advantage of requiring fewer protection/deprotection steps than the products derived from 3,4- isopropylidene mannitol or other members of the chiral pool. Another benefit of Martin and Josey's method is that a common intermediate, the 1-acyl-3-protected glycerol can be used to synthesize many phospholipids simply by changing the carboxylic acid in the second coupling reaction. Since we needed to vary the acyl group at the 2- position this would allow us to introduce it as late in the sequence as possible so that the yield of final products could be maximized. The scope of the strategy is dictated by the choice of protecting group for the sn-3 hydroxyl. A glycerol with the sn-3 alcohol selectively protected is necessary to establish the stereochemistry of the products. This

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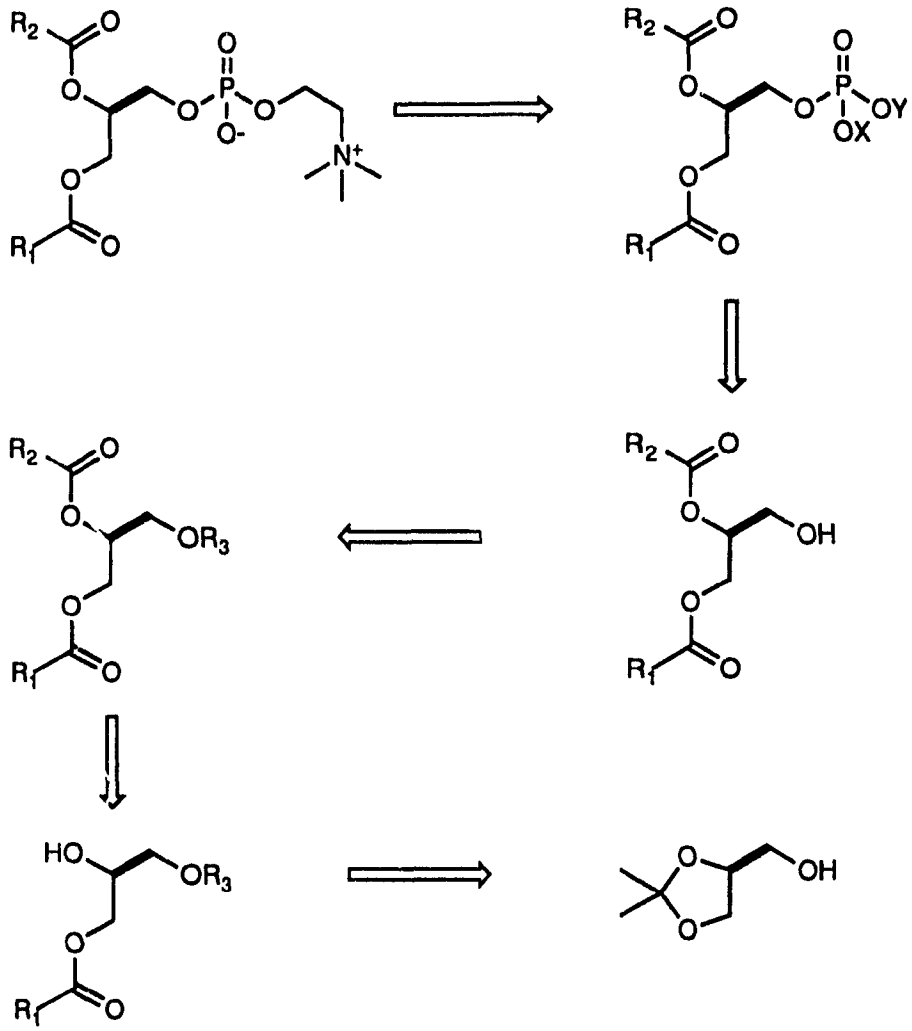
22) Eibl, H.; *Chem. Phys. Lipids*, **1980**, *26*, 239.
23) Magolda, R. L.; Johnson, P. R.; *Tetrahedron Lett.* **1985**, *26*, 1167.
24) Chandrakumar, N. S.; Hajdu, J.; *J. Org. Chem.* **1982**, *47*, 2144.
25) Ramirez, F.; Marecek, J. F.; *Synthesis*, **1985**, 449.
26) Stepanov, A. E.; Shvets, V. I.; *Chem. Phys. Lipids*, **1986**, *41*, 1.
27) Bannwarth, W.; Trzeciak, A.; *Helv. Chim. Acta*, **1987**, *70*, 175.
28) Bruzik, K. S.; Salamończyk, G.; Stec, W. J.; *J. Org. Chem.* **1986**, *51*, 2368.

intermediate is readily available from 1,2-isopropylidene-sn-glycerol by blocking the free hydroxyl and acidic hydrolysis of the 1,2-acetal.



Scheme 8

Protecting group strategy.

A critical difficulty resided in the choice of the protecting group for the sn-3 hydroxyl. In order to maximize the flexibility of the synthesis, the blocking group should be removed quantitatively under mild conditions, preferably in the absence of strong acid or base which are likely to catalyse acyl migration. Additionally, the deprotection should not affect whatever functional and protecting groups are present in the sn-2 chain. Since the possibility of studying unsaturated phospholipids was envisaged as well, the use of a benzyl ether as described by Martin was excluded. It was anticipated that palladium or platinum catalysed hydrogenolysis of the benzyl group would also reduce

any alkenes present on the acyl chains. Other ether protecting groups were considered[29] (MEM, MOM, BOM, Trityl), but rejected since they are commonly removed by acid hydrolysis, and the possibility of isomerization under these conditions was foreseen. Silyl ethers are commonly used as alcohol protecting groups, but their removal requires fluoride ion under basic conditions, or strong acidic conditions which are incompatible with the ester functions. Many other protecting groups such as the trityl group are also incompatible with our approach due to their instability to acid. We had planned to prepare the chiral intermediate 3-protected glycerol by hydrolysis of the corresponding acetonide. In many cases, it may be impossible to selectively remove the acetal function without affecting the sn-3 blocking group.

The choice was the 4-methoxybenzyl ether (PMB ether: *para*-methoxybenzyl) which was used by Martin and Josey to temporarily mask the sn-3 hydroxyl. In their scheme a BOM group (benzyloxymethyl ether) was used to block the sn-2 hydroxyl while the PMB ether was cleaved to allow the introduction of the phosphate triester. The BOM group was then removed, and a linoleate chain attached. They did not report the removal of the PMB ether in the presence of olefinic e.g. linoleoyl acyl chains, nor did they discuss any unsuccessful attempts to do so. This electron-rich benzyl ether is routinely removed by oxidation with DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) in aqueous methylene chloride[30]. In their original publication, the Japanese authors report that these neutral conditions do not affect many functional groups. Notably, the stability of internal and terminal double bonds, as well as esters such as acetyl and benzoyl was demonstrated. There was no *a priori* reason to believe that the deprotection conditions using DDQ would affect the functional groups on the linoleate chains. It is therefore curious that Martin and Josey did not report such a reaction or comment on this incongruity. The first step would be to verify the compatibility of this blocking group with easily oxidized unsaturated acyl groups such as linoleate esters.

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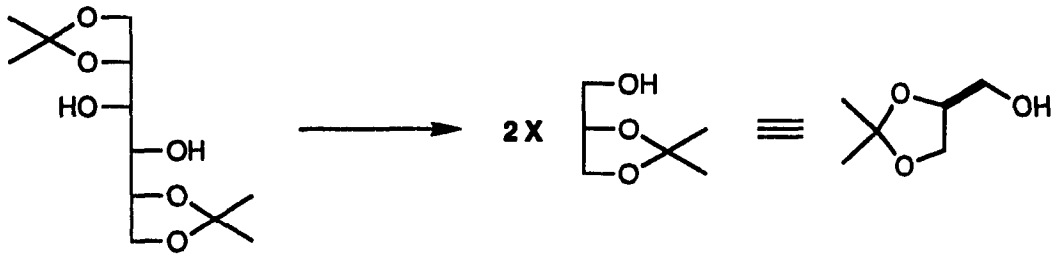
[29]) Greene, T. W.; *Protective Groups in Organic Synthesis*; Wiley Ed. New York, **1981**.
[30]) Oikawa, Y.; Yoshioka, T.; Yonemitsu, O.; *Tetrahedron Lett.* **1982**, *23*, 885.

Preparation of 3- protected glycerols.

Preparation of 1,2-isopropylidene-sn-glycerol.

Many different starting materials have been used as phospholipid precursors in the past. These choices have been dictated by the intermediates and targets in the synthetic approaches outlined in the previous sections. In many cases, numerous manipulations were used in order to control the regiochemistry of the products. Because of the report by Martin and Josey, we were convinced that differentiation of the sn-1 and 2-hydroxyls was unnecessary. The most rapid and convenient route to the desired 3-PMB-sn-glycerol is by alkylation of 1,2-isopropylidene-sn-glycerol followed by hydrolysis of the acetonide. Historically, the source of chiral glycerols has been 1,2:5,6-diisopropylidene mannitol (diacetone mannitol, **Scheme 9**)[31]. In the



26 **27**

Scheme 9

original procedure, lead tetraacetate was used to cleave the vicinal diol, followed by Raney nickel reduction of the aldehydes. It has been determined that residual acid in the product causes some isomerisation. Subsequently this procedure has been improved by Eibl[32], and Hirth and Walther[33]. We initially followed the conditions prescribed by the latter. In the first attempts, 1,2:5,6-diisopropylidene-D-mannitol was prepared by acetalation of D-mannitol with 2-methoxypropene according to the procedure by Horton *et al*[34]. Subsequently, diacetone mannitol was puchased from Aldrich. The diol was suspended in water at pH 5 to which was added an equivalent amount of sodium periodate. After complete cleavage of the 3,4-diol, the iodate salts were precipitated with methanol, the pH adjusted to 8, and excess sodium borohydride added. After extraction and distillation, pure 1,2-isopropylidene-sn-glycerol was obtained in 70% yield. The

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31) Baer, E.; Fischer, H. O. L.; *J. Biol. Chem.* **1939**, *128*, 463.
32) Eibl, H.; *Chem. Phys. Lipids*, **1981**, *28*, 1.
33) Hirth, G.; Walther, W.; *Helv. Chim. Acta*, **1985**, *68*, 1863.
34) Debost, J.-L.; Gelas, J.; Horton, D.; *J. Org. Chem.* **1983**, *48*, 1381.

product is reported to be optically pure when the manipulations are performed in the absence of acid and with careful control of the reaction temperatures (<30°C).

This preparation of 1,2-isopropylidene-sn-glycerol is quite tedious because of the difficulty in extraction of the product from aqueous solution, and the manipulations needed to change from the oxidative to reducing conditions. Alternative conditions were sought to accomplish the cleavage and reduction of diacetone mannitol which would be less time consuming. The solution to the problem was provided by the work of Bessodes and Antonakis, who reported the ring cleavage and reduction of the sugar portion of ribonucleotides to give acyclic trihydroxy derivatives[35]. For this purpose they used a mixture of periodate and borohydride anion exchange resins, which are used as a mixture in the same solvent[36]. Since these antagonistic reagents are tightly bound to the polymeric support they are not freely diffusible, and consequently do not react with each other.

The periodate and borohydride resins were prepared by stirring a suspension of Dowex 1X2 (quaternary ammonium type, Cl^- form) in water with a threefold excess of either sodium borohydride or sodium periodate, followed by filtering and rinsing with water and ethanol. The dried resins were then suspended in methanol/water (9:1), and a solution of diacetone mannitol in methanol added with stirring. Care was taken to keep the temperature below 30°C, and to have an excess of the resins present. Diacetone mannitol diffuses into the periodate resin where it is cleaved to two molecules of glyceraldehyde, which are then reduced immediately by the polymer supported borohydride. Water is necessary for the rate of the reaction to be appreciable. This requirement is probably due to the periodate reaction, which is normally conducted in water, rather than the reduction step which can be carried out in ethanol solution using the borohydride resin. The reaction is monitored by the disappearance of the mannitol peak by gas chromatography. The product was quite easily isolated by filtering the resins, and evaporating the solvent under reduced pressure. Residual water was removed by azeotropic distillation with toluene *in vacuo*. The purity of the 1,2-isopropylidene-sn-glycerol was established by NMR: no aldehydic protons could be detected, and only two peaks were recorded for the methyl groups of the dioxolane ring, indicating the absence of starting material. Distillation of the product was unnecessary.

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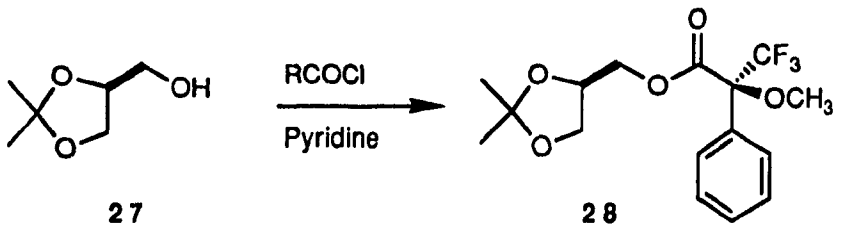
35) Bessodes, M.; Antonakis, K.; *Tetrahedron Lett.* **1985**, *26*, 1305.

36) Harrison, C. R.; Hodge, P.; *J. Chem. Soc. Perkin 1*, **1982**, 509; Gibson, H. W.; Bailey, F. C.; *J. Chem. Soc. Chem. Commun.* **1977**, 815.

Since this method constituted a new preparation of the glycerol acetonide from mannitol, it was necessary to establish the optical purity of the product. Hirth and Walther accomplished this by converting both enantiomers of 1,2-isopropylidene glycerol to the diastereomeric esters of (R)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (Mosher's acid, (R)-(+)-MTPA)[37]. The ratio of diastereomers can then be determined by gas chromatography, or more conveniently by integration of the methoxy or trifluoromethyl peaks in the ^{1}H or ^{19}F NMR spectra. Consequently, the Mosher acid chloride was prepared according to Bittman *et al.*[10]: the acid was treated with excess oxalyl chloride in benzene in the presence of a catalytic amount of N,N-dimethylformamide (DMF). The acid chloride was then added to a solution of **27** (prepared with the periodate and borohydride resins) in dry pyridine (**Scheme10**).



27 **28**

Scheme 10

As a control, racemic glycerol acetonide (sold commercially as Solketal) was also acylated with the Mosher acid chloride. Comparison of the 300 MHz ^{1}H NMR spectra of the racemic and chiral material showed that the material produced by the cleavage of diacetone mannitol was not contaminated with isomerized product. The 1.2-1.5 ppm region of the spectra was particularly significant since the racemic material showed 4 peaks in this region which were assigned to the methyl peaks on the diastereomeric dioxolane rings, while the product from the cleavage of mannitol showed only two peaks. Therefore, within the limits of detection of the instrument, the material is enantiomerically pure (>99% ee). This is in fact not surprising, since racemization of isopropylidene glycerol is catalysed by traces of acid through intramolecular transketalization, and the conditions used here were completely neutral.

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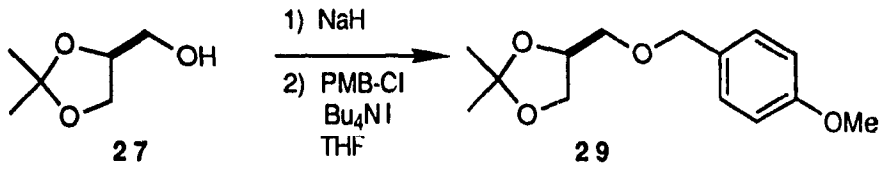
[37]) Dale, J. A.; Dull, D. L.; Mosher. H. S.; *J. Org. Chem.* **1969**, *34*, 2543.

Alkylation of 1,2-isopropylidene-sn-glycerol.

Because of the aforementioned possibility of racemization of the 1,2-isopropylidene-sn-glycerol in the presence of a trace of acid, the alkylation of the free hydroxyl was performed immediately. For the reasons explained earlier, a 4-methoxybenzyl ether had been chosen as protecting group[9]. A number of different methods exist to effect this transformation. Kates *et al.* (and references therein) report the alkylation of **27** with benzyl chloride in refluxing benzene in the presence of powdered KOH, with removal of the water produced[38]. Manley *et al.* introduced the PMB ether by treating the glycerol acetonide with potassium t-butoxide in tetrahydrofuran (THF) followed by addition of 4-methoxybenzyl chloride[39]. The group of Abushanab made the benzyl and allyl ethers of **27** by generating the alkoxide in DMF with sodium hydride, and adding the alkyl bromide[40]. Previous studies in our laboratory has demonstrated that this type of alkylation is best accomplished in THF with a catalytic amount of phase transfer catalyst (**Scheme 11**). Consequently, 1,2-isopropylidene-sn-glycerol was added to a



27 **29**

Scheme 11

slurry of sodium hydride in anhydrous THF. Once the alkoxide was generated, tetrabutylammonium iodide (10 mol%) was added, followed by 4-methoxybenzyl chloride. The use of a phase transfer catalyst in this type of reaction serves two purposes. First, the iodide ion is capable of displacing the chloride at the benzylic position, which enhances the reactivity of the reagent because of the better leaving ability of iodide. Whether this substitution is important in the ether formation or not, it is accepted that the quaternary ammonium ion greatly increases the rate of the alkylation by increasing the solubility of the alkoxide and leaving the anion more free to attack the alkyl halide[41]. The crude PMB ether was directly used in the next step without purification.

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[38]) Kates, M.; Chan, T. H.; Stanacev, N. Z.; *Biochemistry*, **1963**, *2*, 394.

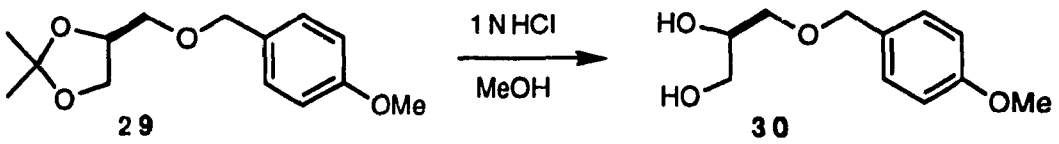
[39]) Manley, P. W.; Tuffin, D. P.; Allanson, N. M.; Buckle, P. E.; Lad, N.; Lai, S. M. F.; Lunt, D. O.; Porter, R. A.; Wade, P. J.; *J. Med. Chem.* **1987**, *30*, 1812.

[40]) Mikkilineni, A. B.; Kumar, P.; Abushanab, E.; *J. Org. Chem.* **1988**, *53*, 6005.

[41]) March, J.; *Advanced Organic Chemistry*, 3rd Ed.; Wiley, New York, **1985**, 320.

Hydrolysis of the isopropylidene group.

The standard conditions for the hydrolysis of acetonides involve transketalization in an alcohol solvent using an acid catalyst such as toluenesulfonic acid. In these studies, the method of Abushanab or of Manley *et al.* were used successfully. In the latter protocol (**Scheme 12**), the acetal **29** was dissolved in methanol and an equal volume of aqueous



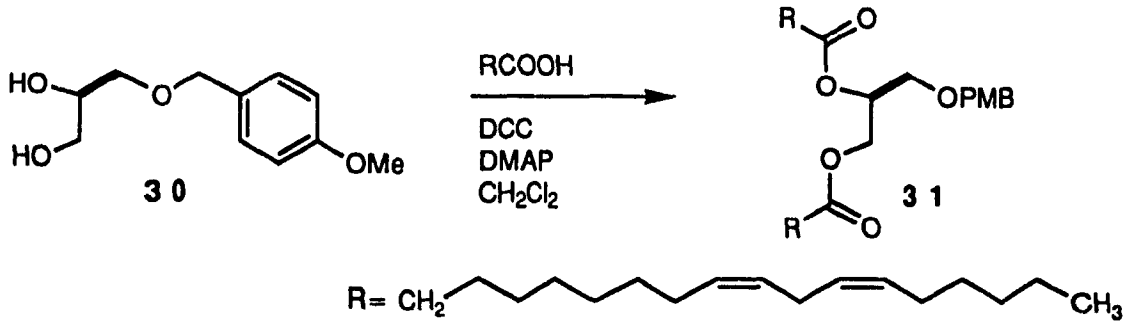
29 **30**

Scheme 12

1N HCl added. After stirring for one hour, the product was isolated by extracting with ethyl acetate and removing the acid with a bicarbonate wash. The crude product was isolated by chromatography on silica using 2% methanol in chloroform as eluant. Pure 3-PMB-sn-glycerol was obtained as a crystalline solid in 60% overall yield from diacetone mannitol.

Diacylation and deprotection of 3-PMB-sn-glycerol.

Since it was unclear whether the unsaturated acyl groups normally found in natural phospholipids would survive the removal of the PMB group using DDQ, it was decided to study the deprotection reaction on a model. For this purpose, it was decided to esterify both hydroxyls with linoleic acid, and attempt to remove the PMB ether. All of the acylations in this work were done under standard conditions by activating the carboxylic acid with dicyclohexyl carbodiimide (DCC) in the presence of a nucleophilic catalyst, dimethylaminopyridine(DMAP)[42]. The diol **30** and a slight excess (2.2 eq.) of linoleic acid (**Scheme 13**) were dissolved in anhydrous dichloromethane and a concentrated solution of DCC (2.5 eq) and DMAP (1.2 eq) was added dropwise with stirring at room



30 **31**

Scheme 13

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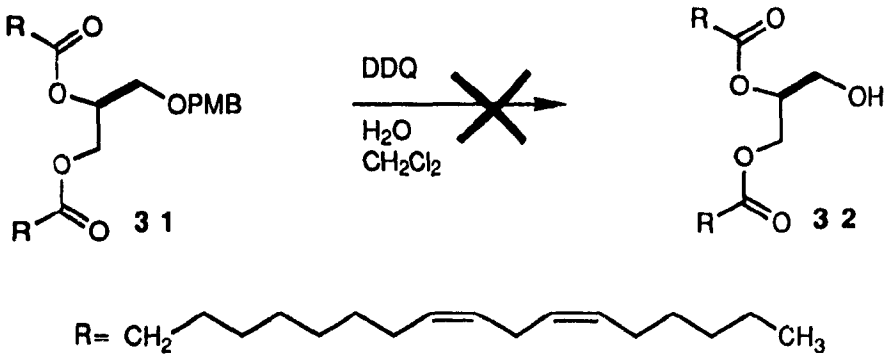
[42]) Neises, B.; Steglich, W.; *Angew. Chem. Int. Ed. Engl.* **1978**, *17*, 522; Hassner, A.; Alexanian, V.; *Tetrahedron Lett.* **1978**, *19*, 4475.

temperature. The diacylated product was isolated by precipitation of the urea side product and chromatography of the residue to remove excess linoleic acid and other side products. 1,2-Dilinoleoyl-3-PMB-sn-glycerol was obtained in 85-95% yield. The diacyl glycerol PMB ether was then subjected to the conditions described for the oxidative cleavage of the protecting group[30]. A solution of the PMB glycerol in methylene chloride/water (18:1) was treated with DDQ in the usual way (**Scheme 14**). The reaction was monitored by TLC over the course of several hours. Only small



Scheme 14

amounts of product could be isolated from the reaction mixture after chromatography. In the course of this work, a report on the use of the PMB protecting group in the synthesis of chain-unsaturated diacylglycerols from the mollusc *Umbraculum mediterraneum was* published[43]. These authors claimed a yield of 29% for the removal of a PMB ether using DDQ from an unsaturated diacylglycerol. It appears that the low yields are a general phenomenon in this type of system. The disappointing yield of this deprotection was a severe obstacle to our goals. Our short term goals were therefore reevaluated. It was decided to abandon the synthesis of unsaturated phospholipids for the time being, and to concentrate instead on the preparation of glycerolipids with polar substituents on the sn-2 acyl chain. It was clear that a new protecting group would be required for this purpose due to the poor results with the PMB ether. The various possibilities were reviewed, and the choice finally rested with an allyl ether. This group has been easily removed under mild conditions by isomerization of the allyl to a vinyl ether catalysed by transition metal complexes of rhodium[44] or iridium[45]. The

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43) de Medeiros, E. F.; Herbert, J. M.; Taylor, R. J. K.; *Tetrahedron Lett.* **1990**, *41*, 5843.

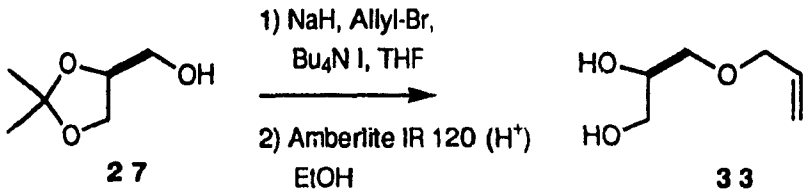
44) Corey,E. J.; Suggs, J. W.; *J. Org. Chem.* **1973**, *38*, 3224.

45) Oltvoort, J. J.; van Boeckel, C. A. A.; de Koning, J. H.; van Boom. J. H.; *Synthesis*, **1981**, 305.

hydrolysis of the vinyl ether is then effected with mercury chloride and mercury oxide under essentially neutral conditions[46]. These conditions were judged compatible with the other functional groups on the diacylglycerol.

Studies with allyl protected glycerols.

The preparation of the central intermediate, 3-allyl-sn-glycerol, was performed using essentially the same conditions as for the PMB derivative. The sodium salt of isopropylidene glycerol was generated in anhydrous THF as previously described (**Scheme 15**) and an excess of allyl bromide added in the presence of a catalytic amount of tetra-(n-butyl)ammonium iodide. The alkylation proceeded at room temperature overnight, was quenched and the product extracted as before. The diol was produced by stirring a solution of the acetonide in 95% ethanol in the presence of a sulfonic acid resin (Amberlite IR-120 H^+) according to the method of Abushanab and co-workers[40]. The product 3-allyl-sn-glycerol was recovered in 90% yield .



27 **33**

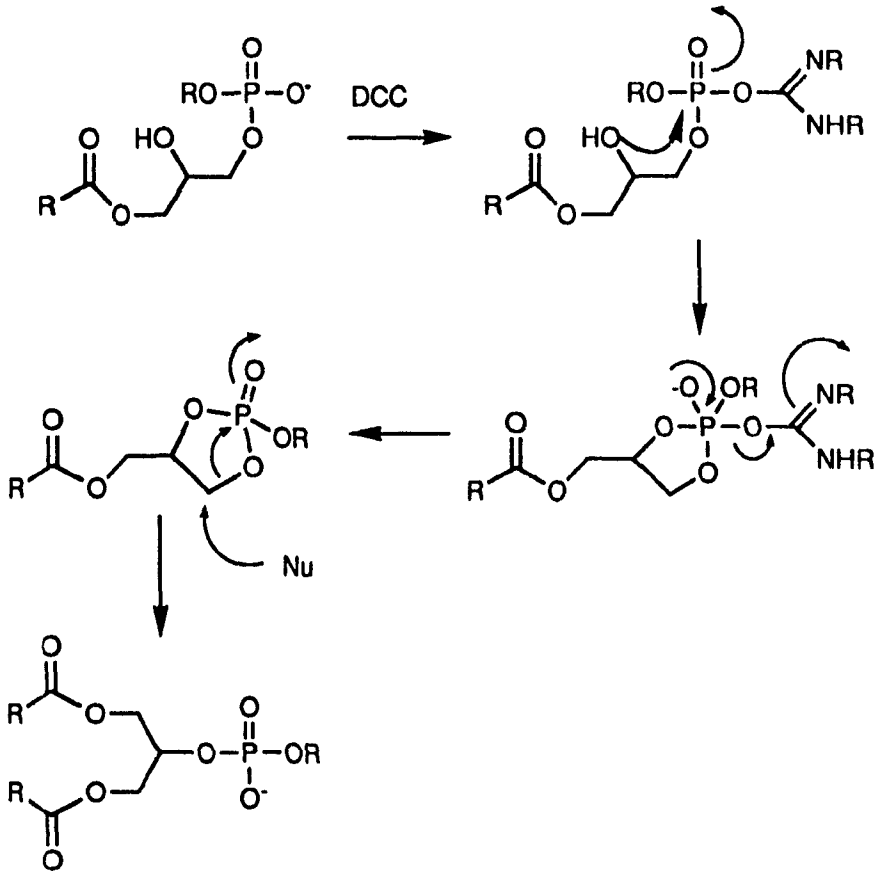
Scheme 15

Selective acylation of 3-allyl-sn-glycerol.

It was previously reported by Martin and Josey that when 3-benzyl-sn-glycerol was treated with palmitic acid, DCC and DMAP in methylene chloride at 0°C, the product obtained was the primary ester. The authors claimed that the secondary alcohol could then be acylated with a second carboxylic acid at room temperature without isomerization. This absence of migration of the acyl groups under these dehydrating conditions has been confirmed in virtually every example of this technique, with one notable exception. In the hemisynthesis of phospholipids starting with lyso-PC and an unreactive carboxylic acid, acyl chain and phosphate migration have been observed. However, this type of acylation requires heating over prolonged reaction times in the presence of excess DMAP. This nucleophilic catalyst is basic enough to cause transesterification at more elevated temperatures. Phosphate diesters also react with dehydrating reagents such as DCC (**Scheme 16**), trichloroacetonitrile and

[46]) Gigg, R.; Warren, C. D.; *J. Chem. Soc. (C)*, **1968**, 1903.

triisopropylbenzenesulfonylchloride[47]. This can result in intramolecular cyclic phosphate triester formation, followed by nucleophilic attack (by carboxylate anion or DMAP) on the less hindered primary carbon. The overall result is the migration of the phosphate and formation of a primary ester. In general, DCC and DMAP catalysed



Scheme 16

esterifications proceed without isomerization. It was decided to verify the claim by Martin and Josey that the monoacylation of 3-protected glycerols gives the primary ester regiospecifically. When a solution of DCC and DMAP was added to 3-allyl-sn-glycerol and 0.95 eq. of palmitic acid in dry methylene chloride at 0°C and stirred for several hours, analysis by thin layer chromatography showed a mixture of several products which were isolated by flash chromatography (**Scheme 17**). The least polar material proved to be the dipalmitate ester of the glycerol (16%). Two other more polar products with very similar mobilities were also isolated. The major less polar product was identified as 1-palmitoyl-3-allyl-sn-glycerol, and the minor more polar

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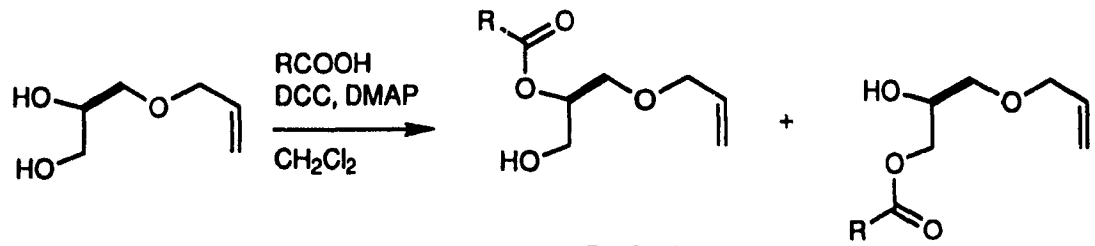
[47]) Dugas, H.; Penney, C.; *Bioorganic Chemistry*. Springer-Verlag, New York, **1981**, 110.

product was the 2-palmitoyl isomer. The yields of the primary and secondary esters were 68 and 13% respectively (based on the carboxylic acid). Several attempts were made to increase the selectivity of this step, since the efficiency of the approach would be considerably improved if the primary palmitate could be obtained as the only product.



33 **34**, R= $C_{15}H_{31}$ **35**, R= $C_{15}H_{31}$

Scheme 17

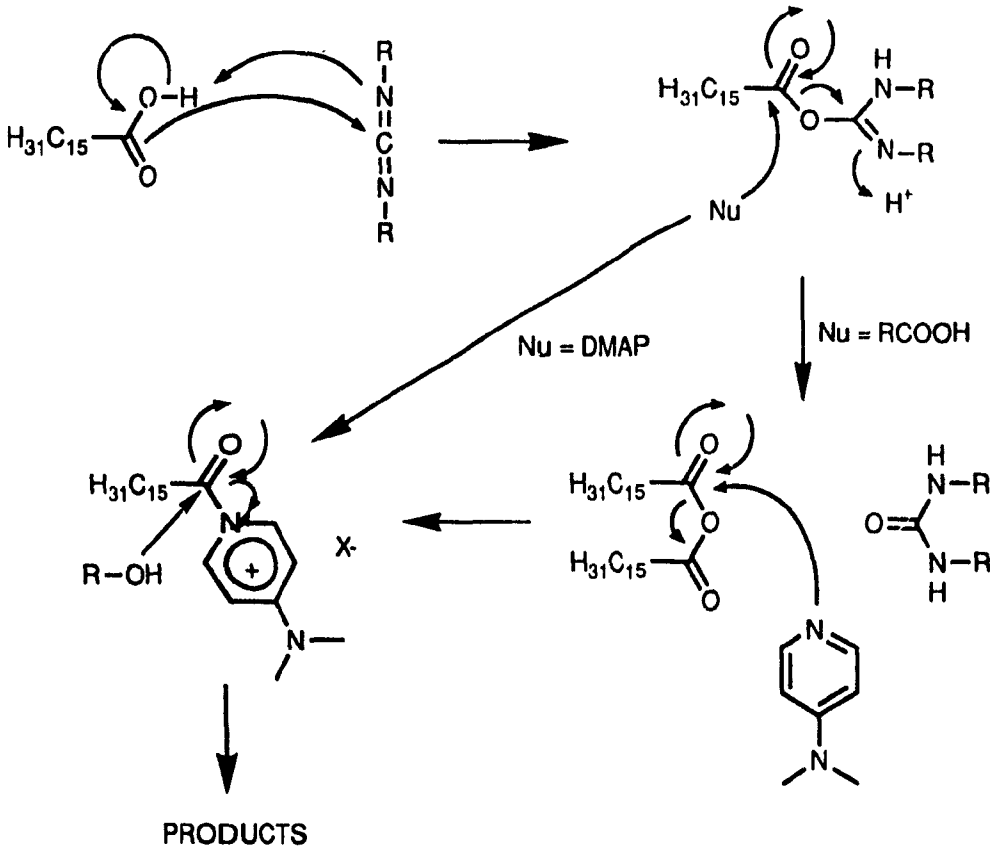
The preference for the primary ester can be rationalized by the following. First, a primary alcohol has only one other non-hydrogen substituent, and is therefore less hindered than a secondary carbinol. This fact alone could explain the selectivity of the reaction. In many well known cases, such as the preparation of tosylates and trityl or t-butyldimethylsilyl (TBDMS) ethers, the reaction proceeds with the formation of the primary product preferentially. In these cases and in the esterification reaction, the primary product is kinetically favored because of the lower steric crowding. In the case of trityl and TBDMS ethers, the substitution takes at an extremely crowded neopentyl center, so that the difference in energy between the primary and secondary derivatives is large. Such a difference is perhaps not expected for the formation of a palmitate ester, since there is only one alkyl substituent on the chain (e.g. compared with three for a pivalate, which is also selective for primary hydroxyls). However, long chain alkanoic acids such as palmitate are believed to coil in solution in organic solvents. The effective bulk around the carboxylate is therefore greater than would be expected for a monosubstituted carboxylic acid, and steric factors favor the smaller nucleophile.

In the case at hand, it seems that the thermodynamic equilibrium gives the same mixture of products as the kinetically controlled conditions. This was established by the following experiments. When the acylation of 3-allyl-sn-glycerol was performed at 0 ^{o}C in the presence of DCC and DMAP in dichloromethane, approximately a 5:1 ratio of primary to secondary product was obtained. When the purified 2-palmitoyl-3-allyl-sn-glycerol was heated to reflux in toluene in the presence of excess triethylamine for several hours, it was isomerized to a 5:1 mixture of isomers once again. Under these latter conditions, it is expected that the esters will reach equilibrium. Indeed, prolonged

refluxing in the presence of base had no effect on the final ratio of products after about 12 hours.

Since kinetic factors are at work, it would be expected that changing the temperature of the acylation reaction would favor the formation of primary ester. However, increasing the temperature of the DCC catalyzed reaction to 25°C had little effect on the ratio of primary to secondary esters, but increased the proportion of diacylated product. The result of increasing the temperature is therefore to provide enough energy to allow the more crowded monoester to react again with the activated acid derivative. If the reaction temperature was lowered much below 0°C, the rate of the reaction became impractically slow. The optimum balance between the yield of desired product and practicality was therefore achieved at 0°C.

Let us consider the mechanism of a DCC and DMAP catalysed esterification in this perspective (**Scheme 18**). When a carboxylic acid is allowed to react with carbodiimide, an O-acyl urea is the first intermediate. A second mole of carboxylate then attacks the first carbonyl, and an anhydride is produced. The driving force behind

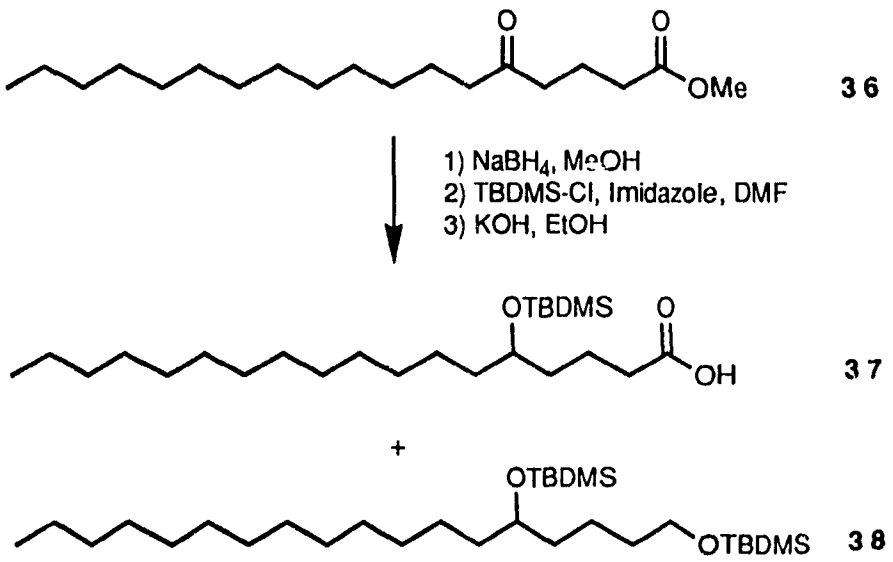


Scheme 18

this dehydration is the energy liberated by the formation of the urea in the second step. At this stage, it is proposed that DMAP attacks the anhydride to generate an acylpyridinium species. The latter may also arise directly by nucleophilic attack of DMAP on the O-acylurea. The acylpyridinium species forms due to the very high nucleophilicity of the aminopyridine. The acylpyridinium species is extremely electrophilic, and is the one that reacts with the alcohol to generate the ester. When the temperature was varied in the esterification of the 3-allyl-sn-glycerol, little difference was observed in the ratio of primary to secondary product. Attempts to increase the selectivity by lowering the reaction temperature were unsuccessful. Lowering the reaction temperature further only slowed the rate of reaction without affecting the product ratio. The Hammond postulate tells us that in an exothermic reaction the structure of the transition state resembles the reactants. An extension of this principle is that to increase the selectivity of a reaction one must generate a less reactive intermediate. Since the energy of the transition state is strongly affected by solvation, another non-polar solvent was used as medium in order to change the selectivity. When palmitic acid and diol **33** were treated with DCC and DMAP in THF however, the only product recovered was the palmitoyl N-acylurea derivative, which arises from isomerization of the N,N-dicyclohexyl-O-acylurea intermediate. Since there was little difference between the glycerol hydroxyls as nucleophiles, the possibility of increasing the selectivity by inverting the reaction polarity was investigated. In other words, transforming the hydroxyl into a leaving group and using the carboxylate as nucleophile might give better results. One good method of doing this is to treat the alcohol with triphenylphosphine and diethyl azodicarboxylate (DEAD) in THF, followed by the carboxylic acid. When the Mitsunobu conditions were used the reaction was extremely slow. In addition, both the primary and secondary ester products could be detected by TLC. This renders the method unsuitable, since any secondary product would arise form inversion during the reaction to give an acylglycerol of unnatural configuration. At this point, it was decided to abandon the search for completely selective acylation conditions. The requisite 1-palmitoyl-3-allyl-sn-glycerol could be isolated in 65% yield quite easily by chromatography, and an additional 15% of the 2-acyl isomer could be recycled by isomerization with triethylamine in refluxing toluene. With sufficient quantities of 1-palmitoyl-3-allyl-sn-glycerol now in hand, the task of introducing the functionalized acyl chains at the sn-2 position was undertaken. The method of preparation of the carboxylic acid derivatives will be discussed here, and the remainder of the preparation of the phospholipid resumed afterwards.

Preparation of substituted fatty acids.

As it was stated earlier, little information is available on the effect of polar functional groups on the sn-2 acyl chain of phospholipids on the rate of hydrolysis by PLA_2. The simplest type of functional group to introduce is the alcohol function. Consequently, it was decided to synthesize a number of phospholipids with a hydroxyl group at various positions along the sn-2 chain. Several fatty acid precursors are available from commercial sources such as the methyl esters of 5-, 7-, and 12-oxooctadecanoic acid (isomeric ketostearic acids), and ricinoleic acid ([R]-12-hydroxy-9-cis-octadecenoic acid). Most of the following work was carried out with the 5-hydroxy derivatives, so the discussion will be concentrated on these compounds. The alcohol derived from 5-ketostearic acid was obtained in high yield by sodium borohydride reduction in methanol (**Scheme 19**).



Scheme 19

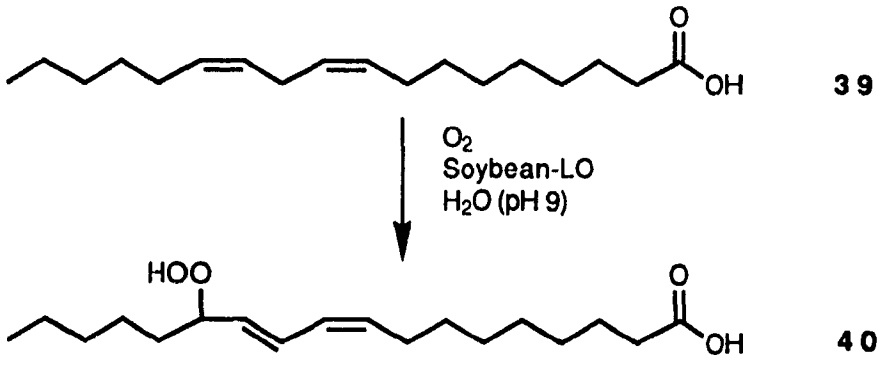
Since these hydroxy acids were to be esterified to the sn-2 hydroxyl of the glycerol, their alcohol function required protection. For this purpose a t-butyldimethylsilyl ether was chosen. The removal of this group under acidic conditions should be possible without affecting the rest of the phospholipid. Many precedents exist to support this assumption, notably in the synthesis of phosphatidylethanolamines. The amino function of PE's is often blocked as a t-butyl carbamate which is removed by treatment with trifluoroacetic acid in dichloromethane at low temperature. The silyl ether was introduced under standard conditions: the appropriate methyl ester alcohol was dissolved in anhydrous dimethylformamide (DMF), and excess t-butyldimethylsilyl chloride

(1.2-1.5 eq.) and imidazole (1.5 eq.) were added. The silyl ethers were generally isolated in quantitative yield. From this reaction mixture a product arising from overreduction of the ester was identified. Two products were isolated by chromatography after the silylation step. The proton NMR spectrum of the less polar compound showed doublets for the t-butyl and methyl peaks of the silyl ether (0.88, 0.87 ppm and 0.04, 0.03 ppm respectively), and integration indicated the presence of two silyl groups. The spectrum lacked the triplet characteristic of the methylene adjacent to the carbonyl, and the singlet corresponding to the methyl ester. Based on this information, the structure was deduced to be the bis-silyl ether of 1,5-octadecanediol (15% from the keto acid). The NMR spectrum of the more polar compound was consistent with the desired silyl ether of methyl 5-hydroxystearate (75% from **36**).

Usually, the mixture of mono and disilyl ethers was not separated, but used as such in the hydrolysis of the methyl ester. The crude product was dissolved in 95% ethanol containing 10 equivalents of potassium hydroxide and stirred overnight at room temperature. After acidic workup, the product was purified by flash chromatography. The carboxylic acid could be easily separated from the other side products which were much less polar. Thus, the three step sequence (reduction, silylation and ester hydrolysis) gave a 75% yield of the protected carboxylic acid after a single purification.

Enzymatic oxidation of linoleic acid.

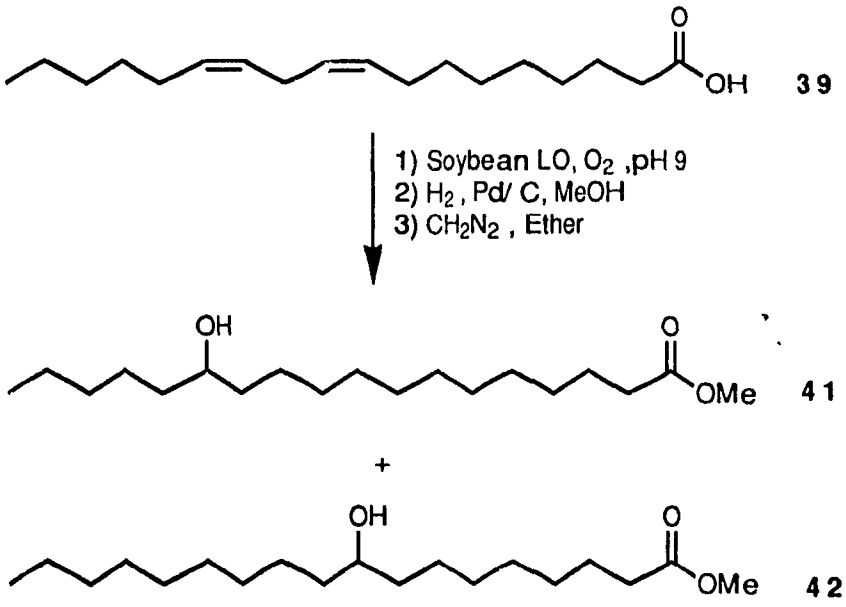
As was mentioned in an earlier section, the biosynthesis of leukotrienes and prostaglandins involves enzymes which catalyze the insertion of molecular oxygen into polyunsaturated fatty acids. In particular, the enzyme 5-lipoxygenase (5-LO) is



Scheme 20

responsible for the oxidation of arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE). Although this enzyme is not commercially available, another enzyme

of this class is produced in large quantities by soybeans[48]. Soybean lipoxygenase (soybean-LO), catalyzes the insertion of molecular oxygen into its preferred substrate, linoleic acid at the terminal double bond. The 1Z,4Z-pentadiene unit is transformed into a conjugated 2E,4Z-diene hydroperoxide (**Scheme 20**). The enzymatic reaction is reputed to be highly specific, yielding the 13-hydroperoxide. It has been shown that the hydroperoxide can be reduced to an alcohol in methanolic sodium borohydride, and tne conjugated diene can be readily hydrogenated with a platinum catalyst[49]. It was therefore envisaged that this chemo-enzymatic approach would provide a convenient source of 13-hydroxystearic acid. Baldwin and co-workers had previously optimized the procedure for the oxidation of arachidonic acid, and had determined that the addition of $NaBH_4$ directly to the enzymatic reaction (which is run at pH 9 in borate buffer) gave



Scheme 21

the highest yields of the 13-hydroxy acid[50]. More recently, Iacazio and coworkers have described the lipoxygenase catalysed hydroperoxidation of linoleic acid under 4 atmospheres of pure oxygen[51]. The authors report an 80% yield of diene hydroperoxide based on the UV absorption of the product, but do not give an isolated yield. Following

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[48]) Veldink, G. A.; Vliegenhart, J. F. G.; Boldingh, J.; *Prog. Chem. Fats other Lipids*, **1977**, *15*, 131.

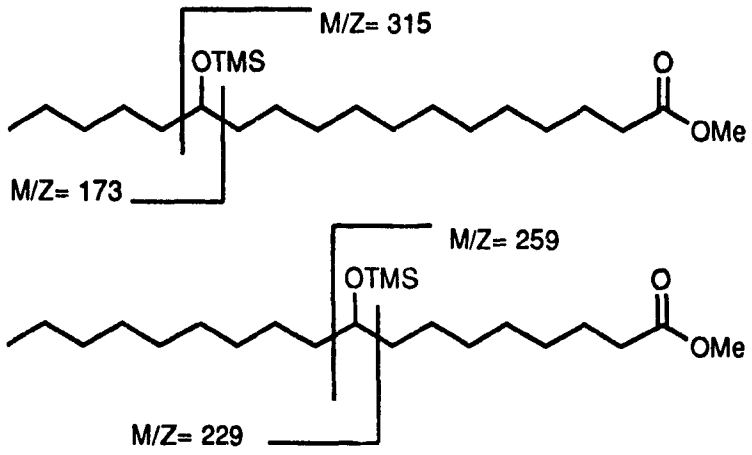
[49]) Hamberg, M; Samuelsson, B.; *J. Biol. Chem.* **1967**, *242*, 5329.

[50]) Baldwin, J. E.; Davies, D. I.; Hughes, L.; Gutteridge, N. J. A.; *J. Chem. Soc. Perkin Trans. 1*, **1979**, 115.

[51]) Iacazio, G.; Langrand, G.; Baratti, J.; Buono, G.; Triantaphylidès, C.; *J. Org. Chem.* **1990**, *55*, 1690.

Baldwin's protocol (**Scheme 21**), linoleic acid was dissolved in a minimal amount of methanol and added to a large amount of borate buffer (0.1M) adjusted to pH 9. Soybean-LO was then added, followed by sodium borohydride. The aqueous solution was then stirred in an open vessel until the starting material had disappeared, at which time concentrated HCl was added dropwise to adjust the pH to 4. The solution was extracted with ether, and the crude hydroxydienoic acid was immediately dissolved in methanol and hydrogenated under 4 atmospheres of hydrogen in the presence of a catalytic amount of 10% palladium on charcoal.

Analysis of the product by thin layer chromatography showed the presence of two compounds with very similar Rf. Careful separation by flash chromatography gave the pure products. Both compounds had virtually identical proton and carbon NMR spectra which were consistent with the structure of a hydroxystearic acid. It was known from the literature that soybean-LO prefers to insert the hydroperoxy group at the C-13 position (i.e at the sixth carbon from the end of the chain, or ω-6 position) of linoleic acid, but that significant amounts of oxidation at the ω-10 position (at C-9) could also occur, depending on the reaction conditions. In order to unambiguously determine the structure of the products, the methyl esters were produced by adding an excess of diazomethane to a solution of the carboxylic acids in ether. The methyl ester alcohols were then subjected to analysis by gas chromatography-mass spectrometry (GC-MS) after *in situ* derivatization into the trimethylsilyl ethers (**Scheme 22**). The silyl ethers of aliphatic alcohols have a characteristic fragmentation pattern in their electron impact mass spectra. The major cleavage occurs adjacent to the carbinol carbon. In the present case, the isomer arising from oxygenation at C-13 gives two fragments: cleavage



Scheme 22

of a pentyl radical on the ω- side of the silyl ether gives a fragment with M/Z = 315, while cleavage on the carboxylate side gives M/Z = 173. The C-9 isomer gives the analogous peaks with M/Z = 259 ($M^{+\cdot}$ - $C_9H_{19}\cdot$), and M/Z = 229 (TMS-O-$C_{10}H_{20}^{+}$) respectively. The major component of the enzymatic reaction (less polar on TLC) was identified as 13-hydroxystearic acid by analysis of its fragmentation pattern, and the minor, more polar product was shown to be the 9-hydroxy isomer. The most efficient preparative conditions were found to be the following. Linoleic acid was converted to the mixture of hydroxydienoic acids with soybean-LO in borate buffer (pH 9) in the presence of $NaBH_4$. The crude product was immediately dissolved in methanol and hydrogenated in the presence of catalytic palladium on charcoal. The mixture of hydroxy acids was then converted to the methyl esters by treatment with ethereal diazomethane. The separation of the isomers was best accomplished at this stage, and gave a 50% yield of 13-hydroxystearate methyl ester, along with 30% of methyl 9-hydroxystearate, based on linoleic acid. The purified isomeric hydroxy esters were then quantitatively converted to the TBDMS ethers by treatment with t-butyldimethylsilyl chloride and imidazole in anhydrous DMF. The hydrolysis of the methyl ester was done as previously described, with potassium hydroxide in ethanol at room temperature overnight. In this manner, half-gram quantities of O-protected 13-hydroxystearic acid could be produced.

Lipoxygenase catalysis in organic solvents.

In recent years the use of enzymes in organic transformations has increased dramatically due to the discovery that their catalytic activity is retained in non-aqueous media[52]. The features of this method are that only the essential water of hydration around the enzyme is necessary. Hydrophobic organic solvents are generally preferred over polar hydrophilic ones. In some cases, small quantities of water need to be added (up to 1%) for catalysis to occur. It is absolutely essential to prepare the enzyme by lyophilization from an aqueous solution at the pH optimum for catalysis. This ensures that the amino acid side-chain functional groups are in the ionization state required for catalytic activity and that the protein is in the active conformation. Both of these factors are strongly affected by the pH. Dissolving the enzyme in buffer followed by freeze-drying removes the bulk water, but the essential water of hydration remains strongly bound to the protein. This trace of water is often the only amount necessary to maintain catalytic activity in otherwise anhydrous solvent. A further limitation is the size of the

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52) Klibanov, A. M.; *Acc. Chem. Res.* **1990**, *23*, 114; Chen, C.-S.; Sih, C. J.; *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 695.

enzyme particles. When the protein forms crystals or large clusters, the number of active sites available is often diminished. One solution to this problem has been to adsorb the enzyme onto an inert surface like glass powder during the lyophilization. Most of the enzymes used to date as suspensions in organic solvents have been hydrolytic enzymes (i.e. lipases and proteases). The catalytic mechanism of these enzymes often involves nucleophilic attack by an active site residue on the scissile bond to form an acyl-enzyme intermediate. In organic solvents, this intermediate can be intercepted with nucleophiles other than water (alcohols, amines, thiols etc.). Most of the transformations accomplished thus far have been esterifications and transesterifications. Because of the intrinsic chirality of proteins and their active sites, this type of reaction is ideal for the resolution of alcohols or carboxylic acids, depending on the specificity of the enzyme.

Very few examples of the use of non-hydrolytic enzymes in an organic medium exist. Of these, the report of the oxidation of phenols to catechols was particularly significant[53]. The enzyme polyphenol oxidase catalyses the hydroxylation of phenols using molecular oxygen, and the subsequent oxidation of the catechols to *o*-quinones. In aqueous solution, these latter compounds are unstable and rapidly polymerize. In chloroform solution the quinones are stable, and can be chemically reduced back to the catechols. In this case, the use of the organic solvent prevents the rapid decomposition of the product which would otherwise occur in water. This prompted us to investigate the possible use of soybean lipoxygenase under similar conditions. Although the products of linoleic acid peroxidation are relatively stable in aqueous solution, the products of arachidonic acid (AA) oxidation are much more labile. The use of organic solvents as reaction medium might allow the preparative enzymatic transformation of AA into biologically active metabolites. The development of the methodology with soybean lipoxygenase may prove useful when mammalian lipoxygenases or cyclooxygenase become available from recombinant DNA techniques.

Samples of soybean LO were therefore prepared in a manner similar to that described by Klibanov for polyphenol oxidase. The protein was dissolved in 0.01M borate buffer at pH 9, as well as 0.01M Tris buffer at pH 9. To some samples, a large quantity of glass powder or sand (100 mg per mg protein), previously washed with the appropriate buffer were added. Each preparation was lyophilized. The following assays were carried

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[53]) Kazandjian, R. Z.; Klibanov, A. M.; *J. Am. Chem. Soc.* **1985**, *107*, 5448.

out with the protein adsorbed onto glass powder. The support was suspended in an organic solvent (diethyl ether, ethyl acetate, chloroform, hexane, benzene, acetone, butanone), an aliquot of water added (0%, 0.1%, 1.0%), and the suspension stirred for 15 minutes to ensure homogeneity. The substrate linoleic acid was then added, and the mixture stirred under an oxygen atmosphere. After incubation for one hour, no trace of product could be detected by TLC in any of the preparations. Similar treatment of the enzyme usually gave complete conversion of linoleate to hydroperoxide in aqueous solution. Although the enzyme was prepared at pH 9, it was thought that the addition of a large quantity of fatty acid caused a change in the ionization state of the enzyme. Consequently, the experiments were repeated with various linoleate salts: sodium, potassium and ammonium salts were all assayed, but in no case was product detected

Up to this point, it had been assumed that the enzyme was not adversely affected by the preparatory treatments, or deactivated by the organic solvents. This was verified by subjecting the enzyme to the usual conditions: adsorption onto glass powder in pH 9 buffer, followed by lyophilization. The enzyme powder was then incubated for one hour in an organic solvent alone (ether, ethyl acetate, benzene, hexane, chloroform), or in the presence of trace water. After this time, the suspensions were evaporated to dryness, redissolved in borate buffer, and substrate added under an oxygen atmosphere. Analysis of the reaction mixtures by TLC showed that all the samples incubated in the organic solvents had retained their catalytic activity and formed product in the aqueous assays, with the exception of those treated with chloroform. This solvent is known to undergo photochemical decomposition over time to give HCl and phosgene, and may explain the deactivation of soybean-LO. Once this result was obtained, the use of chloroform was discontinued.

Since the organic solvent does not destroy the catalytic activity, does the enzyme remain active in the presence of substrate? Soybean-LO preparations were once again incubated in non-polar solvents (ether, ethyl acetate, benzene, hexane) in the presence of linoleate salts under oxygen. After 30 minutes, the solvents were removed, and replaced with borate buffer. However, even after prolonged stirring, no trace of hydroperoxide products could be detected. Under the same conditions, the substrate had been completely consumed by the other enzyme samples not incubated in the presence of linoleate. It therefore appears that the substrate is responsible for the inactivation of the enzyme in organic solvents.

Two possible explanations can be invoked to explain this curious result. Proteins are known to be denatured by ionic detergents such as sodium dodecyl sulfate. SDS penetrates into the hydrophobic interior of the protein and causes it to unfold. It is possible that the linoleate salts are acting as soaps in the organic solvents, causing this type of denaturation. This is unlikely however, because the hydrophobic portion of detergents partition into the inside of proteins to minimize their interactions with the aqueous environment. The driving force for this should be minimal in non-polar organic solvents. The most likely explanation comes from the work of Smith and Lands[54]. It is known that lipoxygenases undergo self-catalyzed inactivation as part of their normal catalytic cycle. In other words, the enzyme can only perform a limited number of turnovers before becoming irreversibly inactivated. Under physiological conditions, such a mechanism may serve to limit the amount of product formed after enzyme activation. In the presence of a large amount of linoleic acid, lipoxygenase becomes inactive long before the substrate is depleted, in a process that is different from product inhibition. In view of this information it seems likely that, in organic solvents the enzyme undergoes self-catalyzed inactivation much faster than it does in water, to such an extent that no significant amount of product is ever formed. In fact, it is quite possible that in organic solvents the enzyme undergoes a single catalytic cycle and self destructs, since the activity could not be restored by incubation in water.

Preparation of 1,2-diacylglycerols.

Acylation at the sn-2 position.

With five protected hydroxystearic acids now readily available, we were now ready to optimize the remaining steps in the synthesis. Since the most easily prepared product was the 5-TBDMS-hydroxystearic acid, the conditions were worked out with this derivative. The manipulations were also carried out with the 9- and 13-hydroxystearic acids.

The second acyl chain could be attached to the glycerol very easily (**Scheme 23**). The carboxylic acid of interest (1.2 to 1.5 eq.) was dissolved in methylene chloride along with 1-palmitoyl-3-allyl-sn-glycerol. A solution of DCC (1.5 to 1.8 eq.) and DMAP (0.5 eq.) was then added dropwise at room temperature, and the resulting solution stirred for about one hour. At this time the starting alcohol had usually been consumed.

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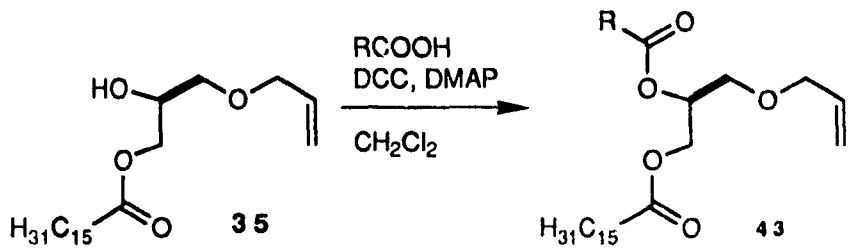
[54]) Smith, W. L.; Lands, W. E. M.; *Biochem. Biophys. Res. Commun.* **1970**, *41*, 846; Smith, W. L.; Lands, W. E. M.; *J. Biol. Chem.* **1972**, *247*, 1038.



Scheme 23

A wide variety of fatty acids were used in this reaction with equal success: linoleic acid, palmitic acid, all of the isomeric silylated hydroxystearates, and even a 32 carbon dicarboxylic acid (*vide infra*). In the case where the acid is poorly soluble in dichloromethane it was found that adding excess DMAP prior to the DCC addition helped dissolution. The reaction was worked up by filtering the dicyclohexylurea and applying the resulting oil to a silica column. Elution with ethyl acetate in hexanes gave the pure product in 75 to 99% yield. The yield was found to depend on the quality of the DCC: material which was stored for long periods tended to deteriorate, and produce turbid solutions in dichloromethane. Although it was not verified at this point in time, all reports agree that acylations under these conditions do not cause migration of the acyl chains on the glycerol. The diacylglycerols appeared as homogeneous single spots on TLC. The proton and carbon NMR spectra did not show any additional peaks which would indicate the presence of isomeric products.

Isomerization of the 2-propenyl group.

The next part of this work was the optimization of the conditions for the removal of the allyl ether, and the introduction of the phosphate function. The original procedure for this transformation required the isomerization of the allyl group to a vinyl ether (i.e a 2-propenyl ether to a 1-propenyl ether) using potassium t-butoxide in dimethyl sulfoxide at 100°C [55]. The enol ether was then hydrolyzed in acetone containing 1N HCl, or mercuric chloride and mercuric oxide[46]. These isomerization conditions were very harsh, and an alternate method was developed by Corey and Suggs using Wilkinson's catalyst to accomplish this step[44]. After this work was completed, a report appeared which described the removal of allyl ethers using tetrakis(triphenylphosphine) rhodium hydride as catalyst in the presence of one equivalent of trifluoroacetic acid[56]. Under these conditions the hydrolysis of the resulting vinyl ether occurs immediately. This procedure may be applicable to the present synthesis, provided that 1,2-

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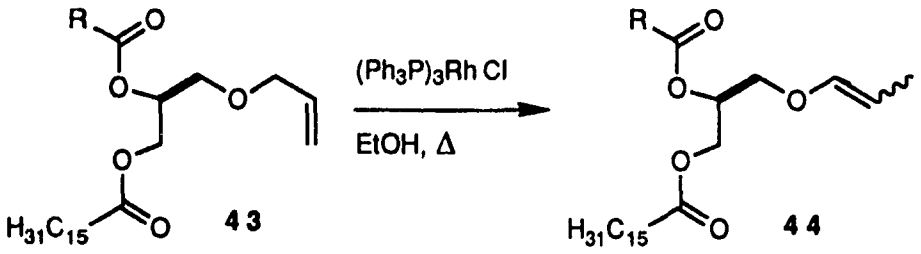
[55]) Gigg, J.; Gigg, R.; *J. Chem. Soc. (C)*, **1966**, 82.

[56]) Ziegler, F. E.; Brown, E. G.; Sobolov, S. B.; *J. Org. Chem.* **1990**, *55*, 3691.

diacylglycerols are not isomerized in the presence of one equivalent of TFA. The use of π-allyl-palladium chemistry has recently provided additional methods for the removal of other allyl derivatives. These involve the activation of the allyl group as a palladium complex, and trapping with a nucleophile such as an amine or hydride[57]. For example, amino acid allyl esters[58] and oligonucleotides bearing N-allyloxycarbonyl and allyl groups to block the nucleoside bases and phosphates respectively[59], can be deprotected using palladium catalysis and a large excess of an amine such as morpholine or butylamine. Allyl esters and allyl carbamates can also be removed with Pd^0 and tributyltin hydride[60]. These methods are inapplicable to the present case however, because they only function with strong nucleophiles (hydride, amines), and with good leaving groups (phosphate, carboxylate, carbonate). For our allyl ethers, the corresponding leaving group would be an alkoxide which would surely affect the ester groups adjacent to it.

Following Corey's protocol (**Scheme 24**), the allyl ether was dissolved in ethanol/water (9:1) containing 0.25 eq. diazabicyclooctane (DABCO, to prevent liberation of propionaldehyde and poisoning of the catalyst). A catalytic amount of tris(triphenylphosphine) rhodium chloride (0.05 eq.) was then added, and the solution heated to reflux for one hour under an inert atmosphere. The starting material was completely converted into a less polar product, the proton NMR spectrum of which showed complete disappearance of the characteristic allyl peaks. In their place, two sets



Scheme 24

of resonances could be seen: two doublets of quartets at 6.2 and 5.9 ppm, two sextets at 4.8 and 4.4 ppm, and a multiplet at 2.6 ppm obscured by the ß protons on the acyl

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57) Hutchins, R. O.; Learn, K.; *J. Org. Chem.* **1982**, *47*, 4381.

58) Friedrich-Bochnitschek, S.; Waldmann, H.; Kunz, H.; *J. Org. Chem.* **1989**, *54*, 751.

59) Hayakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R.; *J. Am. Chem. Soc.* **1990**, *112*, 1691.

60) Dangles, O.; Guibé, F.; Balavoine, G.; Lavielle, S.; Marquet, A.; *J. Org. Chem.* **1987**, *52*, 4984.

chains. The first peaks were assigned to the proton on C1 of the propenyl group which couples to the C2 proton, and shows long range coupling to the methyl group. The sextets are actually an overlapping doublet of quartets, which correspond to the C2 proton between the C1 proton and the methyl group. These resonances indicated that the allyl group had indeed been isomerized to the vinyl ether, but the reaction showed no selectivity for either the cis or trans isomer which were produced in equal amounts. This was confirmed by the two sets of peaks for the C1 protons which were in a 1:1 ratio. Some difficulty was encountered in this reaction. Initially, the reaction went to completion within one hour, but subsequent runs needed prolonged heating and additional amounts of catalyst. This problem of lack of reproducibility has been described before[61], and was ascribed to deactivated catalyst, caused either by oxidation or complexation with aldehydic impurities in the solvent. An expedient solution to this problem was to reactivate the catalyst before adding the allyl ether: this could be easily done following the original preparation of Wilkinson's catalyst[62]. The catalyst was first suspended in the ethanol solvent, and 2 equivalents of triphenylphosphine added under nitrogen. The solution was heated to reflux for 30 minutes before adding DABCO and the ally. ether. Under these conditions the isomerization was generally complete within one hour. It has been reported that one common side reaction during this process is reduction of the allyl to a propyl ether, ethanol acting as hydrogen donor, but this was not observed in these studies.

Although no such case had been reported, it was decided to verify whether the rhodium catalyst could be used to selectively isomerize the allyl ether in the presence of unsaturated acyl chains. For this purpose, linoleic acid was introduced as the carboxylic acid in the second coupling reaction. The resulting 1-palmitoyl-2-linoleoyl-3-allyl-glycerol was treated with Wilkinson's catalyst in refluxing ethanol as described. Examination of the product showed that the allyl group had been isomerized, but that the linoleate portion had also been affected. The characteristic pattern of the 1,4-cis-diene in the ^{1}H NMR had been replaced by a group of overlapping peaks consistent with a conjugated diene structure. The product also appeared to be a mixture of positional and geometric isomers.

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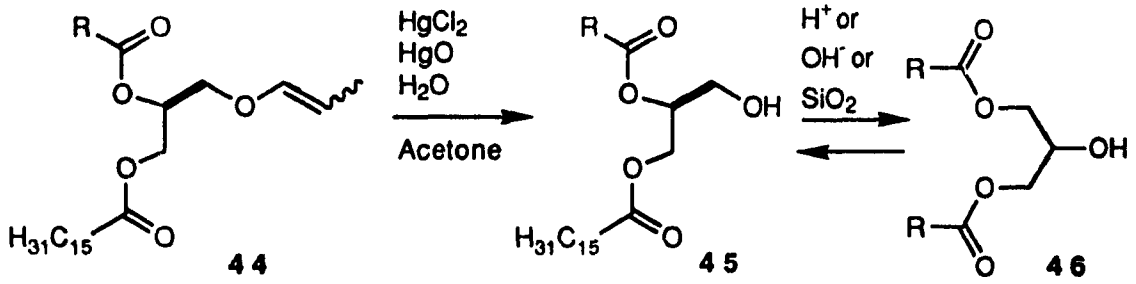
61) Moreau, B.; Lavielle, S.; Marquet, A.; *Tetrahedron Lett.* **1979**, *30*, 2591.
62) Osborn, J. A.; Jardine, F. H.; Young, J. F.; Wilkinson, G.; *J. Chem. Soc. (A)*, **1966**, 1711.

It was then sought to effect the isomerization of the allyl moiety before introducing the unsaturated acyl group. However, for unknown reasons the 1-palmitoyl-3-allyl-glycerol could not be isomerized under these conditions, and was recovered unchanged. The failure of this reaction is not readily explicable since there is no obvious reason why the presence of a free hydroxyl instead of an ester should prevent it, particularly when the solvent is itself an alcohol. The sn-2 hydroxyl was therefore blocked as the t-butyldimethylsilyl ether and subjected to the isomerization conditions. Once again the reaction was unsuccessful for unknown reasons, and this approach to unsaturated lipids was abandoned.

Hydrolysis of the 1-propenyl group.

Hydrolysis of the diacylglycerol enol ether was done following the procedure by Gigg and Warren (**Scheme 25**): mercuric chloride was added to a solution of vinyl ether and mercuric oxide in acetone/water (10:1). After complete disappearance of starting material, a single polar spot could be observed. The mercuric salts were removed by washing an ethereal extract of the product with an aqueous solution of potassium iodide. The complete removal of the mercury contaminants was not possible however, even after chromatography. Furthermore, it was observed that the 1,2-diacylglycerol product was extremely prone to acyl migration. This was easy to detect, since the 1,3 isomer was slightly less polar than the 1,2-diacylglycerol. Their proton NMR spectra were also quite distinctive: the 1,3 isomer showed a single multiplet for all the glycerol protons, indicative of its more symmetrical structure. The 1,2-diacylglycerol showed a multiplet at 5.2 ppm characteristic of the proton at the sn-2 position, and the typical

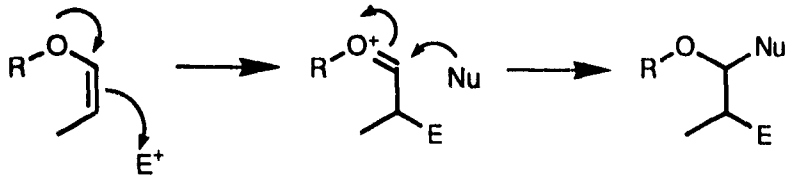


Scheme 25

AB portion of an ABX system for the sn-1 methylene protons. Simply washing the ether extracts with such weakly basic solutions as potassium iodide (pH 7-9), or 5% sodium bicarbonate caused extensive isomerization. The use of these solutions was discontinued, and the crude extracts filtered through silica to remove the mercury salts, but it was then observed that chromatography itself causes a severe amount of isomerization. It

became quite clear that a more efficient and mild method for the hydrolysis of these enol ethers was needed. A number of acid catalyzed hydrolyses were attempted, but these also resulted in variable amounts of migration.

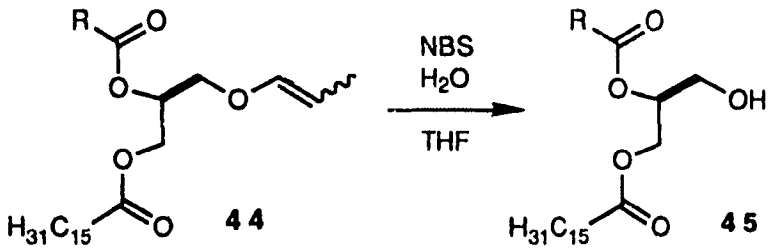
One property of enol ethers which has not been discussed is their affinity for electrophiles. For example (**Scheme 26**), the treatment of an enol ether with acid results in protonation at the ß-carbon, resulting in an oxocarbonium ion which is stabilized by resonance, and can then react at the α position with a nucleophile. This



Scheme 26

property is exploited in the synthesis of tetrahydropyranyl ethers for the protection of alcohols. Other electrophiles also react readily with enol ethers in a similar way. In fact, the mercuric chloride catalyzed hydrolysis described earlier is an example of this: chloromercuration at the ß position, followed by attack of water and breakdown of the hemiacetal produces the free alcohol. Bromine adds in a 1,2 fashion to enol ethers in non-polar solvents to give the unstable α,ß-dibromoacetals. These can then be converted into 2-bromoacetals by treatment with an alcohol and triethylamine.

It was decided to examine this type of conditions for the removal of the diacylglycerol propenyl ether. It was believed that the addition of bromine to a solution of enol in the presence of water would yield the free alcohol directly by a mechanism similar to that described above. These conditions however, would generate one equivalent of HBr during the course of the reaction, and the stability of the product to acid was uncertain. It was decided instead to use the neutral electrophile N-bromosuccinimide (NBS) (**Scheme 27**). The enol ether was dissolved in THF or acetone, and water added until the solution became slightly turbid. NBS was then added. The reaction proceeded very quickly, and



Scheme 27

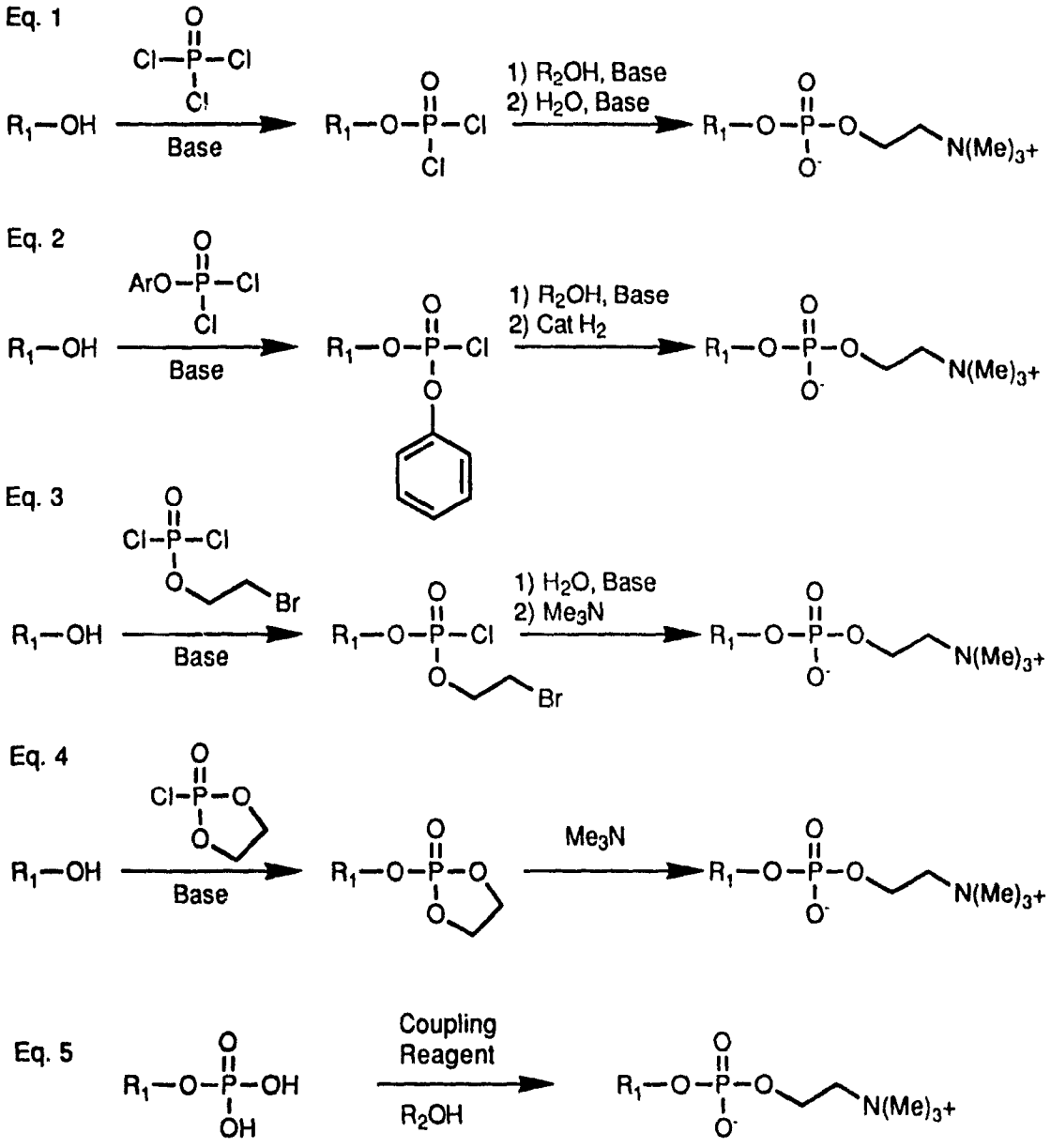
was terminated by dilution with water and extraction. The succinimide side product and any excess NBS were washed away in the aqueous layer. The use of NBS has the advantage that it does not generate any acid during the reaction. Thus, if the workup is done with the complete exclusion of any base, only pure 1,2-diacylglycerol is isolated in essentially quantitative yield. Chromatography was avoided since silica catalyzes the migration of the acyl groups. The NMR spectra and TLC analysis showed the presence of the 1,2 isomer only, and no trace of chain migration was observed. Since there was reason to believe that isomerization could occur on standing, the diacylglycerols were used immediately in the following step.

Methods for the synthesis of phosphodiesters.

Coupling of phosphates.

The introduction of a phospholipid headgroup can be viewed as the formation of two phosphate ester bonds. Numerous approaches to this problem have been proposed, and have been reviewed recently[25,26]. The majority of the methods employ an activated phosphate derivative (i.e. at the P^V oxidation level) which is coupled sequentially to the glycerol and choline moieties. For example (**Scheme 28**, Eq 1), the coupling of a diacylglycerol with phosphoryl chloride in the presence of triethylamine gives a glycerol dichlorophosphate, which is not isolated but treated *in situ* with a soluble choline salt, followed by addition of water to hydrolyze the remaining P-Cl bond. This type of reaction seldom gives more than 40-50% yield of the desired product due to the lack of selectivity. One possible improvement comes from the use of protected reagents such as phenyl dichlorophosphate (Eq 2). This produces phosphate triesters which must then be deprotected by hydrogenation, which obviates the use of this reagent for the synthesis of unsaturated lipids. Several other different protecting groups for phosphorus have been used, but these have many of the disadvantages already mentioned. This approach has been improved by using a dichlorophosphate which already contains one phosphate ester bond present in the final phospholipid. For example (Eq 3), a diacylglycerol is coupled to 2-bromoethyl phosphoryl dichloride, followed by addition of water. The bromoethyl group can then be efficiently converted to the choline by treatment with trimethylamine. This approach has been extended to other phospholipid classes by using the appropriately substituted phosphoryl dichloride. All these methods suffer from the disadvantage that they use bifunctional reagents which are not selectively substituted under the reaction conditions. The use of certain reagents necessitates deprotection steps which may be incompatible with the functionalities in the final products (hydrogenolysis in particular).

One particularly elegant way to circumvent these disadvantages is in the use of 2-chloro-2-oxo-1,3,2-dioxaphospholane (Eq 4)[63]. The problem of selectivity is solved because this reagent is monofunctional, and thus cannot give mixtures of products. Protecting groups for the phosphate are avoided since the dioxaphospholane ring blocks the two other oxygens. The diester is generated by nucleophilic opening of the strained ring with trimethylamine. The protection of the phosphate and the use of a choline.



Scheme 28

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63) Thuong, N. T.; Chabrier, P.; *Bull. Soc. Chim. Fr.* **1974**, 667.

precursor are thus accomplished with a single reagent. Another versatile way of introducing phospholipid headgroups is to synthesize a diacylglycerol phosphomonoester (phosphatidic acid), and to couple the second hydroxylic component in the presence of a suitable activating agent (Eq 5). Among the reagents used are DCC, trichloroacetonitrile, and triisopropylbenzene sulfonyl chloride (TPS). Phosphatidic acids can be generated by phosphorylation of a diacylglycerol, or by phospholipase D-catalysed hydrolysis of a precursor phospholipid. The coupling proceeds by the reaction of phosphatidic acid with the activating agent, and nucleophilic attack by the second hydroxylic component on the activated intermediate. This strategy has been used with success for most of the phospholipid classes with the exception of phosphatidylcholines[25,26]. This is due to the low solubility of choline salts in pyridine, the usual reaction solvent. However, the method succeeds when a haloethyl group is coupled followed by treatment with trimethylamine. Secondary alcohols generally give poor yields of product under these conditions.

Numerous other methods have been developed based on the formation of ester bonds with an activated phosphate, but they differ in detail rather than substance: most are modifications of the leaving group or protecting groups on the phosphorus center.

Coupling of phosphites.

More recently, the field of polynucleotide chemistry has been the area of innovation in phosphate diester synthesis. In particular, many methods have been developed for automated DNA synthesis, and have been subsequently applied to phospholipid synthesis. Many different approaches based on the properties of phosphites with different heteroatom substituents have been successfully used. One approach which is being used with increasing frequency is the so-called H-phosphonate method[64]. According to this protocol, a diacylglycerol (R_1-OH) is treated with PCl_3/imidazole in the presence of base followed by hydrolysis to give the H-phosphonate (**Scheme 29**). This phosphite monoester intermediate is then coupled to a second alcohol (choline, or a protected ethanolamine or serine) in the presence of an activating agent such as pivaloyl chloride. Oxidation gives the desired phosphate, or treatment with sulfur or selenium provides the thio- or selenophosphate. The method is particularly useful when the second coupling component is a secondary alcohol such as in the synthesis of glycolipids, since it gives

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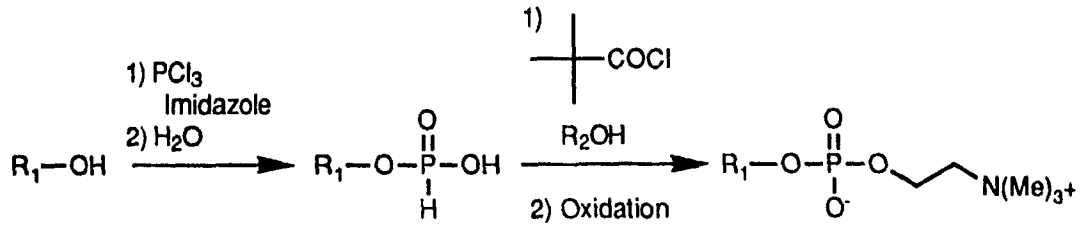
64) Lindh, I.; Stawiński, J.; *J. Org. Chem.* **1989**,*54*, 1338.



Scheme 29

good yields of product[65]. It can be anticipated that the H-phosphonate approach will see many uses in the synthesis of phosphatidylinositides for example.

The other class of reagents originally developed for DNA synthesis and utilized for making phospholipids are the phosphoramidites[66]. These compounds are particularly interesting because of their reactivity. For example, an alkoxy-amido-chlorophosphite is easily made by treating PCl_3 sequentially with an alcohol and a secondary amine. The purified alkyl chlorophosphoramidite then reacts specifically with another alcohol in the presence of base exclusively by substitution of the chloride to give a dialkyl phosphoramidite. This product is then inert to base, but can be induced to react with another alcohol by activation with a weak acid such as tetrazole. The two stages of esterification are thus totally selective by virtue of the opposing conditions used. This is in contrast to the methods using chlorophosphates, where the substitution at phosphorus occurs successively under the same reaction conditions, and where the selectivity is based on the slight difference of reactivity between the phosphomono-, di- and trichlorides. The phosphite triesters can then be oxidized to the phosphates by treatment with a variety of reagents, and deprotected to give the desired phosphodiesters. This strategy was adopted by Stec *et al.* [28] and Willson *et al.* [67] who treated a diacylglycerol with chloro-(N,N-diisopropylamino)-methoxyphosphine to give the dialkylphosphoramidites, which were coupled with a choline salt, oxidized and deprotected (**Scheme 30**). Stec and co-workers also demonstrated that other phospholipid headgroups could be introduced using this technique by varying the coupling partner in the tetrazole reaction.

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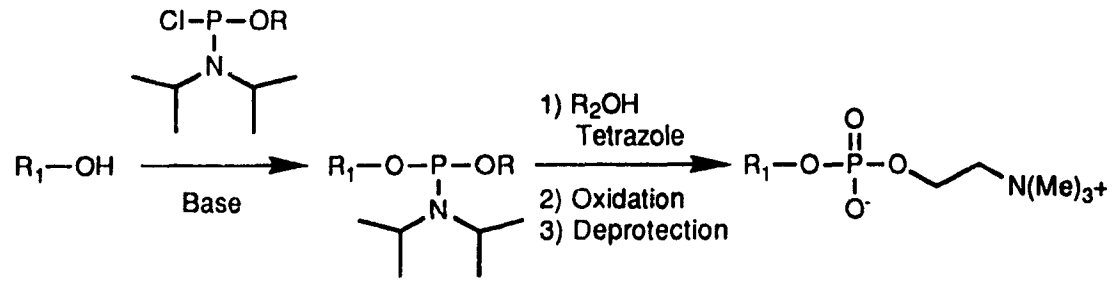
65) Murakata, C.; Ogawa, T.; *Tetrahedron Lett.* **1991**, *32*, 671; ibid. p. 101.

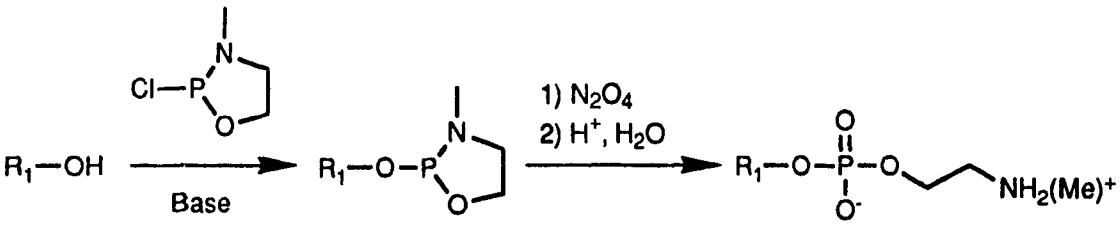
66) Matteucci, M. D.; Caruthers, M. H.; *J. Am. Chem. Soc.* **1981**, *103*, 3185; Melnick, B. P.; Letsinger, R. L.; *J. Org. Chem.* **1980**,*45*, 2715.

67) Lamant, V.; Chap, H.; Klaébé, A.; Périé, J. J.; Willson, M.; *J. Chem. Soc. Chem. Commun.* **1987**, 1608.



Scheme 30

McGuigan and co-workers have devised an elegant route to N-methylethanolamine phospholipids[68]. Starting with 2-chloro-3-methyl-1,3,2-oxazaphospholane, a series of dialkylphosphoramidites were made by displacement of the chloride with a long chain alcohol in the presence of base (**Scheme 31**). After oxidation, the heterocycle is opened in the presence of aqueous acid to give the N-methylethanolamines. The advantage of such a scheme is that there is no need to use protecting groups for phosphorus because the heterocycle serves as precursor for the entire zwitterionic portion of the product. This strategy has not been used in the synthesis of glycerophospholipids yet, and will require additional steps to convert the unnatural N-methylethanolamines to cholines.



Scheme 31

Phosphorylation model studies.

Since the synthesis of the diacylglycerols was a labo ious endeavor, it was decided to conserve precious material and to optimize the co tions for the introduction of the phosphocholine moiety using a more readily available substrate. The model chosen for this was the monolinoleate ester of ethylene glycol. This product was chosen because the linoleate portion would prove the compatibility with unsaturated acyl groups, and because the phosphorylation would occur adjacent to an ester group as in the diacylglycerol case. The starting material was easily made by dissolving linoleic acid in a large excess of dry ethylene glycol and stirring in the presence of 2.2 eq. of

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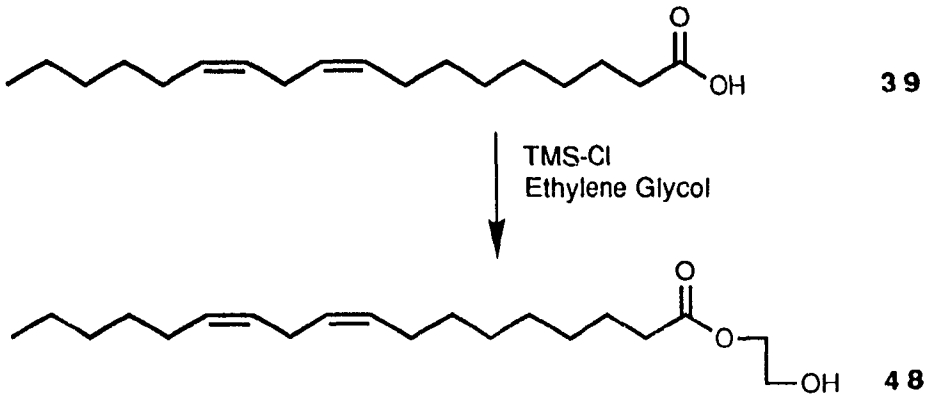
[68]) McGuigan, C.; *J. Chem. Soc. Chem. Commun.* **1986**, 533; McGuigan, C.; Swords, B.; *J. Chem. Soc. Perkin Trans. 1*, **1990**, 783; Anson, M. S.; McGuigan, C.; *J. Chem. Soc. Perkin Trans. 1*, **1989**, 715.

chlorotrimethylsilane until the acid was consumed (**Scheme 32**). The reaction was quenched by adding a large volume of water, and extracting the linoleate ester with ethyl acetate. These workup conditions often gave a side product which was identified as the trimethylsilyl ether of the desired product. Pure monoester could be obtained in 93% yield by simple chromatography, and the TMS ether could be hydrolyzed quantitatively in acetic acid/THF/water.



Scheme 32

It had been reported by Martin and Josey that a dichlorophosphite could be converted into a trisubstituted phosphate triester by the sequential addition of two alcohols and oxidation, with better selectivity than phosphoryl chloride. They suggested that there is a greater difference in reactivity between di- and trisubstituted derivatives at the P^{III} than the P^{V} oxidation level. They added octadecanol to a THF solution of methyl dichlorophosphite and a base at -78°C, followed by N-BOC-ethanolamine. After adding hydrogen peroxide to the resulting phosphite, they obtained a 85-92% yield of phosphate triester. This procedure was repeated with the ethylene glycol monolinoleate, and either methanol or N-BOC-ethanolamine as the second coupling partner. In both cases, substantial amounts of homocoupling could be detected, producing bis(2-linoleoylethyl)methyl phosphate. In fact, after the work described below was completed, a Japanese group reported similar problems controlling the selectivity of phosphorylation using Martin and Josey's method during the synthesis of a lysophosphatidylserine[69]. Since the selectivity could not be controlled satisfactorily, it was decided to examine other methods to accomplish the desired transformation.

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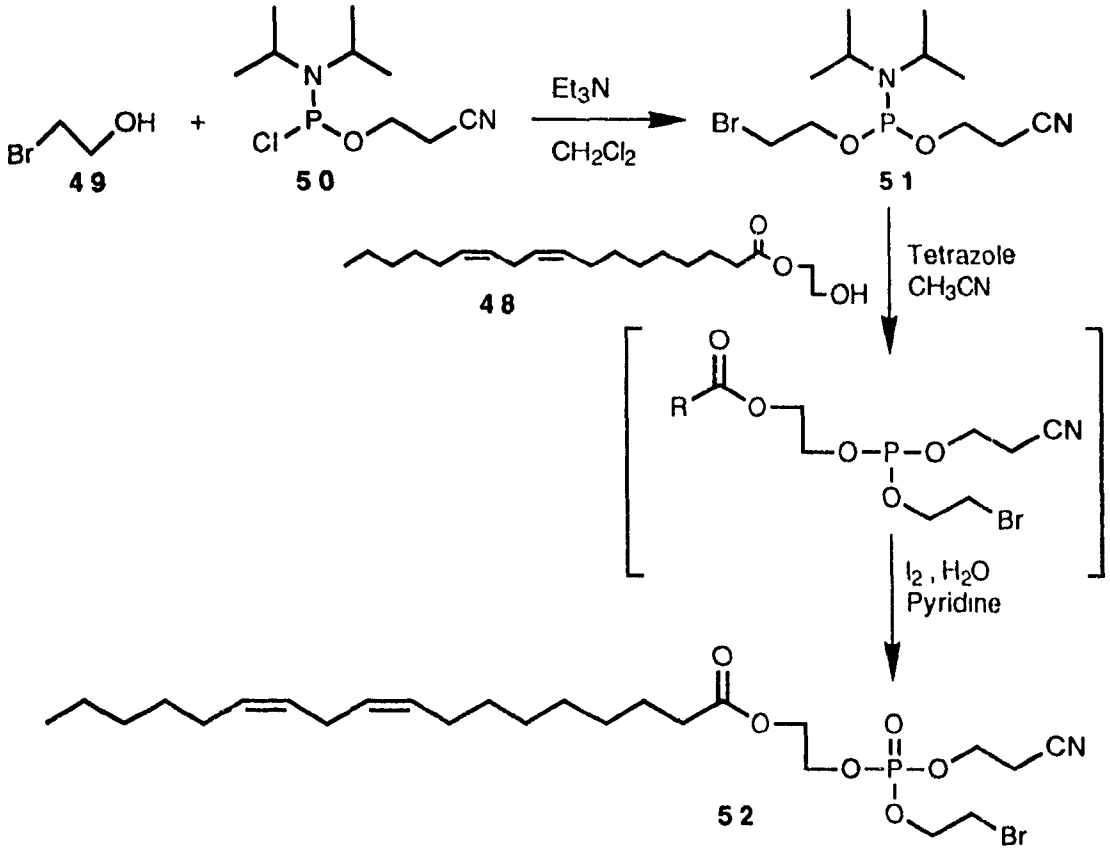
[69]) Inami, K.; Teshima, T.; Emura, J.; Shiba, T.; *Tetrahedron Lett.* **1990**, *31*, 4033.

The experiments with the dichlorophosphites were conducted concurrently with the studies on the deprotection of the allylglycerols. At the same time that the problems with the selective phosphitylation were being encountered, the extreme sensitivity of the diacylglycerols to base were also being discovered. Considerable concern over the stability of the diacylglycerols in the presence of base developed as a result of our experience during the hydrolysis of the allyl ether. At this time, the available phosphorylation methods were reexamined, and it was discovered that all, without exception, used basic conditions to form the glycerol phosphate ester. It was believed that many reports of acyl migration in the synthesis of phospholipids could be attributed to the phosphorylation conditions, and consequently a method was sought to accomplish this transformation under non-basic conditions.

It was decided to explore the chlorophosphoramidite approach to introduce the phosphate group into the diacylglycerol, since there is no problem of selectivity with these reagents. The first alcohol displaces the chloride under basic conditions. The intermediate dialkylphosphoramidites are stable to base and water, and can be purified by chromatography if necessary. The second alcohol is then introduced by displacement of the phosphoramide under acidic conditions. Oxidation of the phosphite to the phosphate triester is then done using a variety of reagents. It is then necessary to selectively remove the phosphorus protecting group, which is the original substituent on the phosphite. As discussed earlier, the previous syntheses of phosphatidylcholines with this methodology were performed by using the glycerol portion as the first coupling partner under basic conditions, followed by a choline salt in the second coupling reaction. In retrospect, it seems obvious that our requirement for non-basic conditions can be met by reversing the usual order of coupling to the chlorophosphoramidite, with respect to the procedure proposed by Stec[28].

In the initial attempts, the coupling of a choline salt to either methyl- or (2-cyanoethyl)-(N,N-diisopropylamino)-chlorophosphite in the presence of base was attempted. Unfortunately, the putative product was totally insoluble in organic solvents, making it impossible to separate it from the amine hydrochloride byproduct. The crude product could not be coupled to another alcohol in acetonitrile in the presence of tetrazole. It was therefore necessary to introduce the choline group via an indirect method. It has long been known that 2-haloethylphosphates can easily be converted into

cholines by treatment with trimethylamine[70]. It was therefore proposed to use this group as a choline precursor in the first coupling reaction, since the intermediates derived from it would be neutral until the last step, and consequently more easily handled.



Scheme 33

Therefore (**Scheme 33**), 2-chloro- or 2-bromoethanol was condensed with (2-cyanoethyl)-(N,N-diisopropylamino)-chlorophosphite in methylene chloride in the presence of triethylamine. After one hour, the solvent was removed under reduced pressure, and the amine hydrochloride salts precipitated with ether and removed by filtration. The oily residue obtained by evaporation of the ether extract was redissolved in anhydrous acetonitrile to which were added 2-linoleoyloxyethanol followed by tetrazole. Within 15 minutes an ammonium tetrazolide precipitate had formed, and TLC showed complete disappearance of the alcohol. The phosphite triester was oxidized *in situ* by adding a solution of iodine in 1:1 pyridine/water, or with 30% hydrogen

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[70]) Hirt, R.; Berchthold, R.; *Pharm. Acta Helv.* **1958**, *33*, 349; Eibl, H.; Nicksch, A.; *Chem. Phys. Lipids*, **1978**, *22*, 1; Diembeck, W.; Eibl, H.; *Chem. Phys. Lipids*, **1979**, *24*, 237.

peroxide, giving an 86% yield of product based on the alcohol. The product could be easily purified by flash chromatography since the phosphate triester is neutral. Optimal yield of phosphate triester was ensured by preparing two equivalents of dialkylphosphoramidite per alcohol to be coupled. It was also essential to prepare the phosphoramidite by using equimolar amounts of bromoethanol and chlorophosphite, since the excess alcohol was carried through to the next step and reacted with the dialkylphosphoramidite in competition with the ethylene glycol monoester. The dialkylphosphoramidite was used immediately, since it was discovered that the yield of the subsequent reaction decreased if the product was stored more than a few hours. Distillation of the product was also attempted, but this led only to complete decomposition of the product. It seems quite likely that the thermal instability of these products is due to the possibility of an inter or intramolecular attack by phosphorus on the carbon bearing the bromide, followed by rearrangement to a phosphonate in a process similar to the well known Arbuzov reaction.

The previously cited syntheses using P^{III} reagents have used the chloro-amino-methoxyphosphine reagent as phosphitylating reagent. The methyl phosphate derived from it can be removed by nucleophilic displacement with either trimethylamine or sodium iodide. An alternative phosphorus protecting group which has been used in oligonucleotide synthesis is the cyanoethyl group. It can be removed by ß-elimination with ammonia, or with an amine base[71]. This group was believed to be a good alternative to the methyl phosphate since it was anticipated that trimethylamine treatment of the trisubstituted phosphate would effect both the conversion of the bromoethyl to the choline and remove the cyanoethyl protecting group. When the model compound was treated with trimethylamine in acetonitrile or toluene at 65 °C for 18 hours (**Scheme 34**), both transformations occurred simultaneously, giving the phosphatidylcholine product in 96% yield. These experiments confirmed that the phosphatidylcholine headgroup could be introduced under acid catalysed conditions in high yield. The dialkyl phosphoramidite reagent ensures that the coupling reaction is completely selective i.e. that no homocoupling occurs. The phosphite can be oxidized with a wide variety of reagents (iodine, hydrogen peroxide, t-butyl hydroperoxide, peracids etc.), so that an appropriate one can be chosen when there is a doubt about the stability of functional groups on the acyl chains. An additional advantage stems from the formation of neutral

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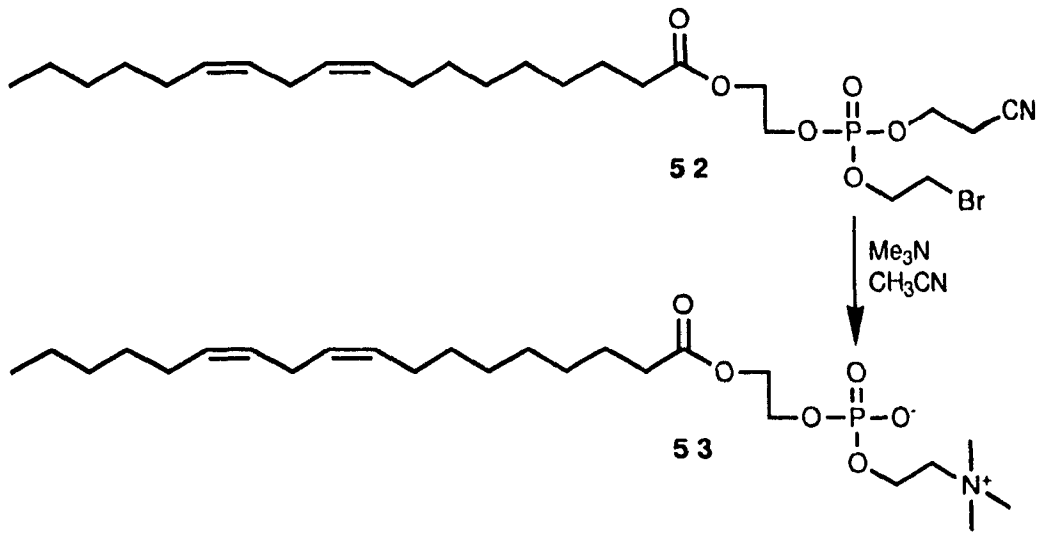
[71]) Bhongle, N. N.; Notter, R. H.; Turcott, J. G.; *Synth. Comm.* **1987**,*13*, 1071; van der Klein, P. A. M.; Dreef, C. E.; van der Marel, G. A.; van Boom, J. H.; *Tetrahedron Lett.* **1989**, *30*, 5473.



Scheme 34

phosphate triester intermediates. Should there be the need for purification, it will be much more easily done at this stage rather than on the zwitterionic phospholipids, which are notoriously difficult to separate from isomeric impurities. Once the pure phosphotriester was obtained, it was shown that it was deprotected and converted in high yield to the phosphatidylcholine in a single step.

It should be noted that after the completion of this work[72] a similar strategy was reported by Lemmen *et al.* for the synthesis of phosphatidylethanolamines and serines[73]. They coupled a protected ethanolamine or serine to the chlorophosphoramidite, and the resulting dialkylphosphoramidite to the diacylglycerol in the second step. They chose their protecting groups for nitrogen and phosphorus (and for the carboxylate of serine) so that they could all be removed in the final step by reduction using cobalt[II] phthalocyanine.

Phosphorylation of diacylglycerols.

The only question remaining unanswered about the validity of this method for introducing the headgroup into phospholipids was whether acyl migration occurred during the process. The initial attempts at using this protocol were conducted on mixtures of 1,2- and 1,3-diacylglycerols since the optimal conditions for the removal of the allyl group had not yet been worked out. When the mixture of isomers obtained from mercuric chloride catalyzed hydrolysis of the 1-propenyl ether was coupled with

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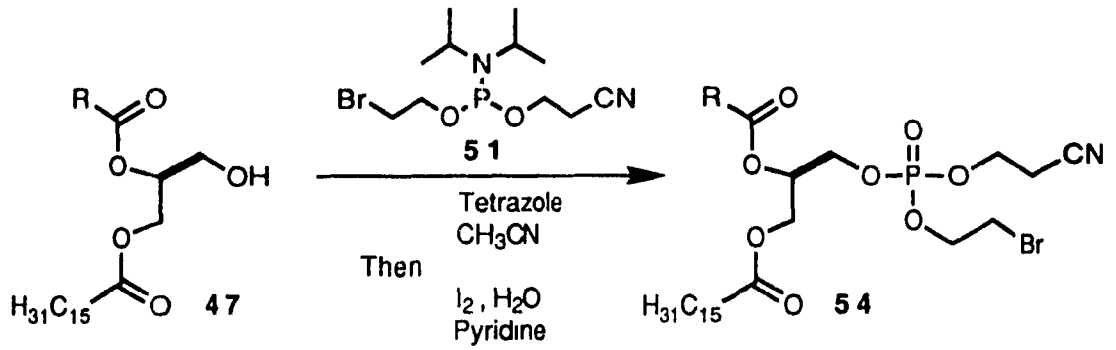
[72]) Hébert, N.; Just, G.; *J. Chem. Soc. Chem. Commun.* **1990**, 1497.

[73]) Lemmen, P.; Buchweitz, K. M.; Stumpf, R.; *Chem. Phys. Lipids*, **1990**, *53*, 65.

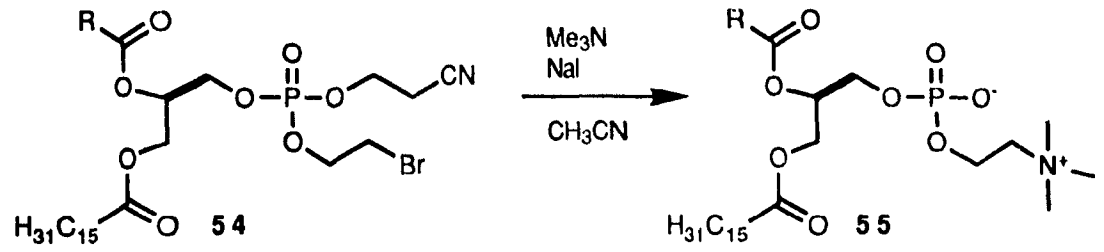
the phosphoramidite and oxidized, the respective phosphate triesters could be resolved by TLC. The 1,3-diacyl phospholipid was found to be slightly less polar, but cleanly separable from its isomer with the natural configuration. Although the NMR spectra of both compounds were quite similar, they could easily be distinguished by comparing the shape of the peak assigned to the α protons of the acyl chains and the chemical shift of the glycerol H_2 proton. In the 1,3- isomer the α methylenes are chemically equivalent and appear as a triplet at 2.33 ppm, while the glycerol H2 multiplet is found at 4.8 ppm. In the natural 1,2-diacylglycerol phosphate the α methylenes are nonequivalent by virtue of being attached to a primary and secondary alcohol. Therefore the protons adjacent to the esters appear as a quartet (actually overlapping triplets), and the carbon spectrum shows two resonances for the carbonyl carbons. The glycerol H_2 also has a chemical shift of 5.2 ppm, as in all diacylglycerol derivatives observed during this work.



Scheme 35

Eventually, 1,2-diacylglycerol was obtained free of any 1,3-diacyl isomer by NBS catalyzed hydrolysis of the 1-propenyl ether. This pure material was therefore coupled with the dialkylphosphoramidite in the presence of tetrazole, and oxidized *in situ* with iodine in pyridine/water (**Scheme 35**). Analysis of the crude product by TLC showed that no isomerization of the acyl chains had taken place during the reaction. Chromatography was performed to remove some residual impurities, and a 70% yield of pure phosphate triester was obtained based on the protected glycerol. The phosphate triester was then dissolved in acetonitrile containing a catalytic amount of sodium iodide and added to trimethylamine in a pressure vessel and stirred at 65 °C overnight (**Scheme 36**). The trimethylamine converted the bromoethyl group to the choline, and eliminated the cyanoethyl group in 92% yield after chromatography. The overall yield of phosphatidylcholine from its protected precursor was 65%. No trace of isomerization could be detected during the deprotection or the introduction of the phosphocholine headgroup. The protocol is more efficient than the procedure proposed by Stec[28], since

it gives a better yield despite an additional step. In addition the purification is simplified since it is done on neutral intermediates which are more



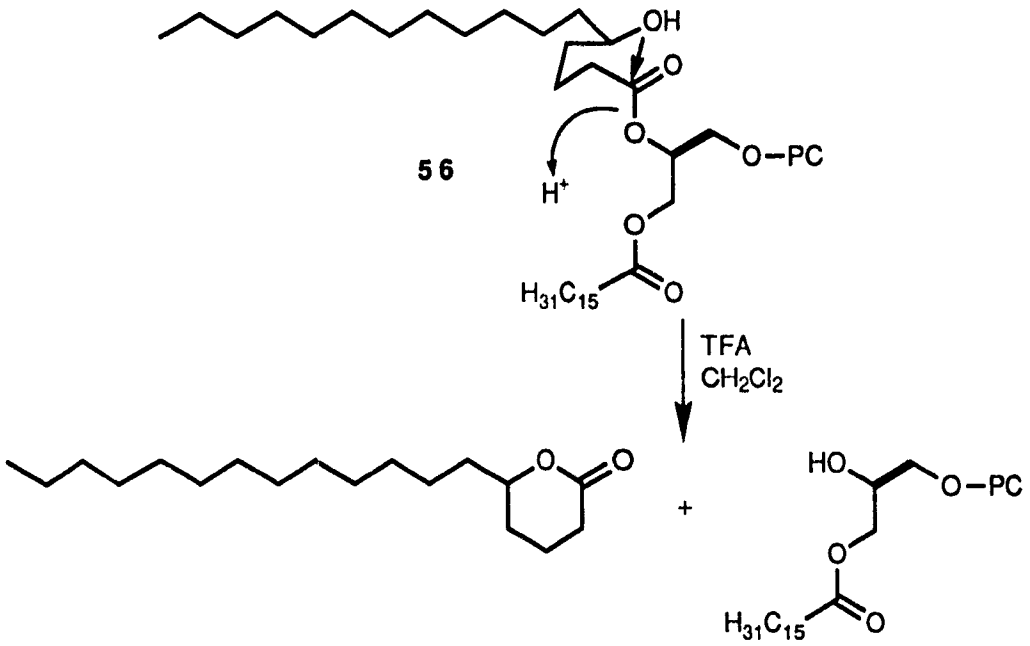
Scheme 36

amenable to flash chromatography than zwitterionic phospholipids. In fact, the final purification may be omitted in certain cases, or replaced by ion exchange chromatography to remove the trace of unreacted starting materials.

Removal of acyl chain protecting groups.

The final step which remained was the deprotection of the hydroxyl functions along the sn-2 chain. Since phospholipids are more stable to acidic than basic conditions due to the presence of the esters, it was decided to employ the former for the hydrolysis of the TBDMS ethers. The phosphatidylcholine with a 5-TBDMS ether on the sn-2 chain was dissolved at 0 ^{o}C in methylene chloride/trifluoroacetic acid (9:1). After 30 minutes at this temperature the solution was diluted with a large volume of hexanes and evaporated. These conditions caused the complete hydrolysis of the sn-2 ester bond, and only lyso-PC and the six-membered ring lactone were recovered (**Scheme 37**). None or the 5-hydroxy phospholipid could be obtained. It seems likely that the silyl group is first removed, and that the ester bond is cleaved very quickly by acid-catalyzed nucleophilic attack of the hydroxyl group. This unusual reactivity was noted earlier in the context of the sodium borohydride reduction of methyl 5-ketostearate, where the methyl ester was also reduced. This seems to be a property of the 5-substituted fatty acids, since other phosphatidylcholines with the TBDMS ether at the 7-, 9- or 13- position could be quantitatively converted to the alcohols without any ester hydrolysis in the presence of TFA in methylene chloride. The high reactivity of the ester group in these 5-hydroxy fatty acids can be in part explained by a tendency to form aggregates in solution, with the ester and hydroxyl groups in close proximity at the boundary of the micelle. This would favor the intramolecular reactions observed with these compounds. Of course, the formation of 5 and 6 membered ring lactones is known to be much faster than larger rings, but there may be an important contribution from the surfactant properties of the compounds as well. In contrast, the fatty acids with a hydroxyl group more to the middle

of the chain may tend to form monodisperse solutions because of the relative position of the polar functions along the chain, and the tendency for intramolecular reactions are



Scheme 37

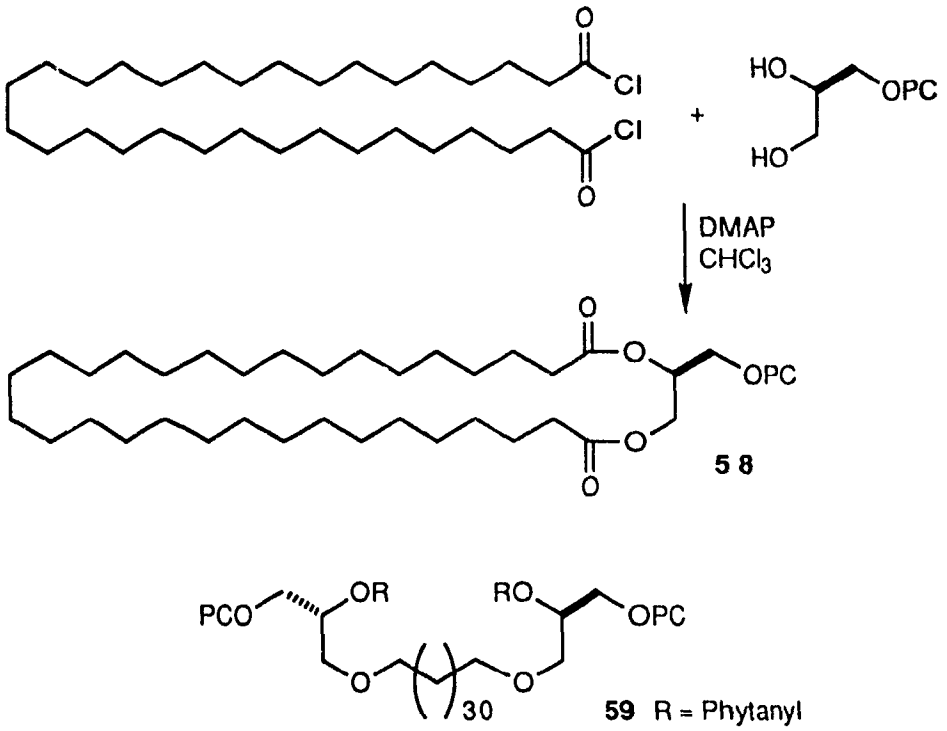
proportionally reduced. The desired phosphatidylcholine with a 5-hydroxyl on the sn-2 chain was obtained rather serendipitously in spite of the failure of the hydrolysis reaction. While other work was in progress, the 5-silyl phospholipid was left standing at room temperature on the bench. It seems that there was enough water and acid remaining in the solid product after the extraction of the phospholipid from the trimethylamine reaction to catalyze the hydrolysis of the TBDMS ether. This was discovered due to the characteristic smell of silanol which developed over time. The product was chromatographed again, and a 25% yield of desilylated phospholipid **56** was recovered. The NMR spectrum was consistent with the proposed structure which lacks the t-butyldimethylsilyl group.

Synthesis of bolaform and macrocyclic phosphatidylcholines

Rationale.

Once the synthesis of hydroxylated phospholipids had been completed, it was decided to extend the methods to compounds bearing unsaturated acyl groups, since these are more common in biological membranes. At the same time, we became interested in making products which would have increased steric bulk in the hydrophobic region, since this was believed to be an important factor for the recognition of the lipid surface as a substrate by phospholipase A_2. The type of products which would show an increased area

while remaining PLA_2 substrates that were chosen for study were phosphatidylcholines with the hydrophobic portion consisting of a macrocyclic ring. One such compound, 1,2-dotriacontanedioyl-sn-glycero-3-phosphocholine **58** has been reported[74].



58

59 R = Phytanyl

Scheme 38

It was synthesized by performing the diacylation of glycerophosphatidylcholine with a 32 carbon dicarboxylic acid chloride in the presence of DMAP in 8% yield (**Scheme 38**). It was reported that small unilamellar vesicles of this compound were indeed substrates for PLA_2, and that their hydrolysis proceeded faster than that of vesicles made of dipalmitoyl-PC at temperatures above and below the phase transition temperature. The goal was to make compounds of this type which would have an even larger loop structure. One way to accomplish this has been suggested by a recent report by Regen *et al.*[75] They have made a series of polyethers with a central hydrophobic segment composed of a long dicarboxylic diester with an acetylene or alkene in the middle. The result is a bolaamphiphile bearing a rigid central portion. By incorporating this type of inflexible segment into the macrocycle, the resulting lipid should show an unusually large surface area and interesting physical properties.

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[74]) Yamauchi, K.; Yamamoto, I.; Kinoshita, M.; *J. Chem. Soc. Chem. Commun.* **1988**, 445.

[75]) Jayasuriya, N.; Bosak, S.; Regen, S. L.; *J. Am. Chem. Soc.* **1990**, *112*, 5844, 5851.

The prospect of making long dicarboxylic acids also suggested the possibility of synthesizing another class of compounds, collectively known as the bolaamphiphiles, or bolaforms already mentioned. The ether-linked phospholipids found in archaebacteria are members of this class. The bolaform phospholipids are interesting because they form vesicular structures with extremely high thermal and chemical stability[76]. The archaebacterial bolaform glycerolipids are known to form monolayer membranes in sharp contrast to diacyl or dialkyl glycerophospholipids which form bilayers[77]. The synthesis of an archeabacterial lipid analogue has recently been reported: compound **59**, 1,1'-(1,32-dotriacontamethylene)bis(2-phytanyl-sn-glycero-3-phosphocholine) was made by assembling the dialkylglycerol, and introducing the phosphocholine in the last step in 15% yield. We were mainly interested in the behaviour of PLA_2 in the presence of similar compounds with ester linkages which would still be potential substrates. To our knowledge, these studies have not yet been done possibly due to the unavailability of such bolaform phospholipids.

Synthetic strategy.

The first synthetic target was a macrocyclic phosphatidylcholine with a hydrophobic portion of approximately the same length as a normal bilayer forming phospholipid, and bearing a rigid central portion. A macrolactonization approach is not very efficient in terms of starting material for several reasons. First, long chain dicarboxylic acid derivatives are poorly soluble in most solvents since they are bolaamphiphiles themselves. Secondly, the statistical probability of the closure of such a large ring is small because of the large number of degrees of freedom of the chain. The work of Sauvage has suggested a strategy for the preparation of such large rings[78]. In his work on the synthesis of catenands, interlocked macrocyclic rings, much use has been made of the template effect of transition metals for directing ring closures. In particular, the cupric ion-mediated oxidative coupling of terminal acetylenes (Glaser coupling) has been used for the formation of a 44 membered ring in 58% yield. Further examination of this methodology revealed a procedure for forming the 16 membered diyne lactone **60** from an acyclic precursor in 88% yield (**Scheme 39**)[79]. This suggested that an approach based on the ring closure at high dilution of a protected diacylglycerol bearing

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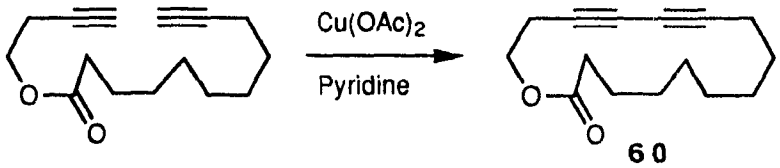
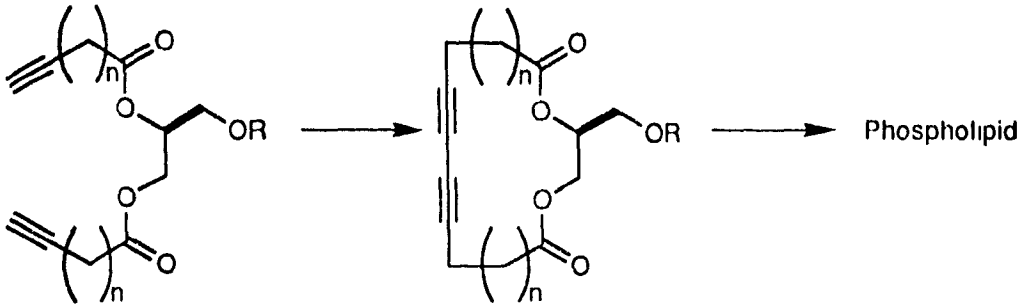
76) Yamauchi, K.; Sakamoto, Y.; Moriya, A.; Yamada, K.; Hosokawa, T.; Higuchi, T.; Kinoshita, M.; *J. Am. Chem. Soc.* **1990**, *112*, 3188.
77) Fuhrhop, J. H.; Fritsch, D.; *Acc. Chem. Res.* **1986**, *19*, 130.
78) Sauvage, J.-P.; *Acc. Chem. Res.* **1990**, *23*, 319.
79) Eglinton, G.; McCrae, W.; *Adv. Org. Chem.* **1964**, *4*, 225.

two terminal acetylenic fatty acids should give acceptable yields of the desired macrocycle.



60



Scheme 39

Subsquent introduction of the phosphatidylcholine headgroup would then be performed using the previously described conditions. This approach could be easily modified to give the bolaform phospholipid as well. Since methods to introduce acyl chains specifically at the sn-2 position had also been worked out, one could envisage the Glaser coupling of two protected diacylglycerols with an ω-alkynoic acid residue, followed by deprotection and phosphorylation. In the event of failure of this approach, the diynedicarboxylic acid could be made and esterified at both ends to a suitable glycerol. However, successful execution of this plan would require the solution of the fundamental problem of phospholipid chemistry: finding a glycerol protecting group which can be removed in the presence of unsaturated acyl chains without causing any isomerization.

4-Methoxybenzyl ether revisited.

During the course of the search for a protecting group which fulfilled the aforementioned criteria, a study on the isomerization of diacylglycerols under various conditions appeared[80]. The authors reported without giving experimental conditions that 1,2-dipalmitoyl-3-benzyl-sn-glycerol could be debenzylated using dimethylboron bromide at -10 °C without producing any 1,3- isomer. This prompted an examination of the literature on this reagent. Dimethylboron bromide has been developed as a reagent for

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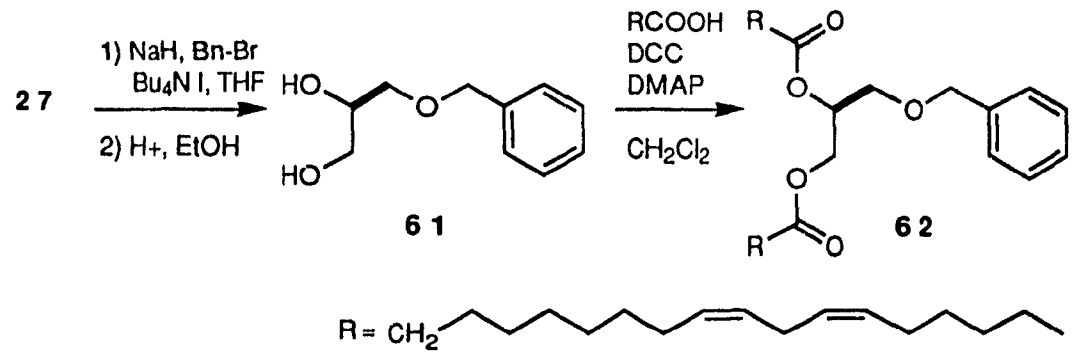
80) Kodali, D. R.; Tercyak, A.; Fahey, D. A.; Small, D. M.; *Chem. Phys. Lipids*, **1990**, *52*, 163.

the cleavage of acetals and ketals at low temperature (-78 °C)[81]. It has also been used for the hydrolysis of ethers at more elevated temperatures (0 to 25 °C)[82]. In particular, ester groups are reported stable under conditions in which benzyl ethers are cleaved. Since no experimental detail was provided by Kodali *et al.*, it was decided to verify whether the result could be duplicated and if the method was efficient enough for use in a preparative scheme. It was necessary to prepare 1,2-dilinoleoyl-3-benzyl-glycerol for this purpose (**Scheme 40**). In a manner analogous to that previously described for the PMB analogue, 1,2-isopropylidene glycerol was alkylated by treatment with sodium hydride, followed by benzyl bromide and tetrabutylammonium iodide in THF. The acetonide was cleaved in ethanol using Amberlite IR 120(H+) as acid catalyst. The diol was then acylated with 2.2 equivalents of linoleic acid using DCC and DMAP as catalysts



Scheme 40

in methylene chloride. The purified 1,2-diacyl-3-benzyl-glycerol was then dissolved in methylene chloride and cooled to -10 °C, and a twofold excess of dimethylboron bromide was added to the solution. The original procedure by Guindon calls for the addition of 10 mol% of triethylamine to neutralize any acid arising from hydrolysis of the boron reagent, and the quenching of the reaction mixture with saturated aqueous sodium bicarbonate. These steps were omitted because they have been shown to be the main causes of acyl migration in the previous studies. After strirring overnight at -10 °C, TLC analysis indicated that starting material still remained and that some decomposition of the product had occurred. Although some cleavage of the benzyl ether was observed, the reaction was neither quantitative nor clean.

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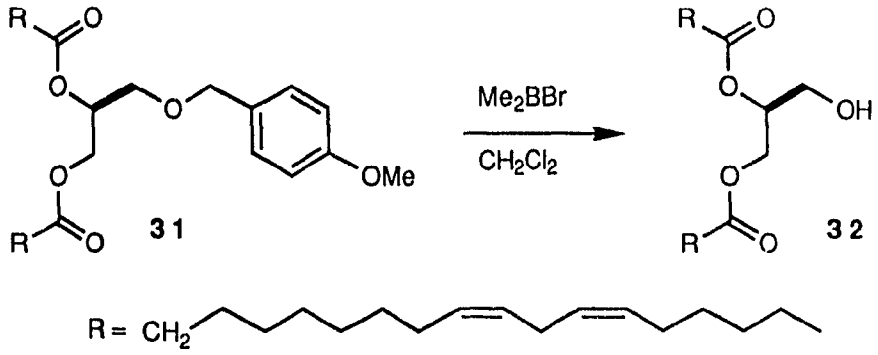
81) Guindon, Y.; Yoakim, C.; Morton, H. E.; *J. Org. Chem.* **1984**, *49*, 3912; Guindon, Y.; Morton, H. E.; Yoakim, C.; *Tetrahedron Lett.* **1983**, *24*, 3969.
82) Guindon, Y.; Yoakim, C.; Morton, H. E.; *Tetrahedron Lett.* **1983**, *24*, 2969.

Although the reaction conditions were not optimal, there was no evidence of acyl migration. In an effort to improve the synthesis, a way of using dimethylboron bromide for the deprotection step was sought. One route which was considered was to make the protecting group more easy to cleave. The mechanism proposed for the cleavage of ethers by dimethylboron bromide involves coordination of the ether oxygen to the boron reagent, followed by nucleophilic attack of bromide on the ether carbon[81]. It is claimed that the reaction proceeds through an S_N2 rather than an S_N1 mechanism because of the electronic properties of the boron reagent. If this is the case, then the position which is the least hindered and develops the largest positive charge should be the favored site of attack. The stabilization of the positive charge by the aromatic ring explains why cleavage occurs preferentially at a benzylic rather than an alkyl carbon. It was reasoned that a more electron rich benzyl ether would help stabilize the positive charge better, and be more reactive towards dimethylboron bromide cleavage.



Scheme 41

The hypothesis was verified by treating 1,2-dilinoleoyl-3-(4-methoxybenzyl)-sn-glycerol **31** with dimethylboron bromide in methylene chloride solution at -78 °C (**Scheme 41**). TLC analysis showed the cleavage to be complete in less than 5 minutes. The reaction was quenched by addition of a large volume of diethyl ether to the solution at -78 °C, followed by washing the resulting solution with portions of distilled water until the washings were neutral. Quenching with sodium bicarbonate solution as recommended by Guindon was omitted, since this was previously shown to be the major cause of acyl migration. After removal of the solvent, only 1,2-dilinoleoylglycerol and the methoxybenzyl side products could be detected by TLC.

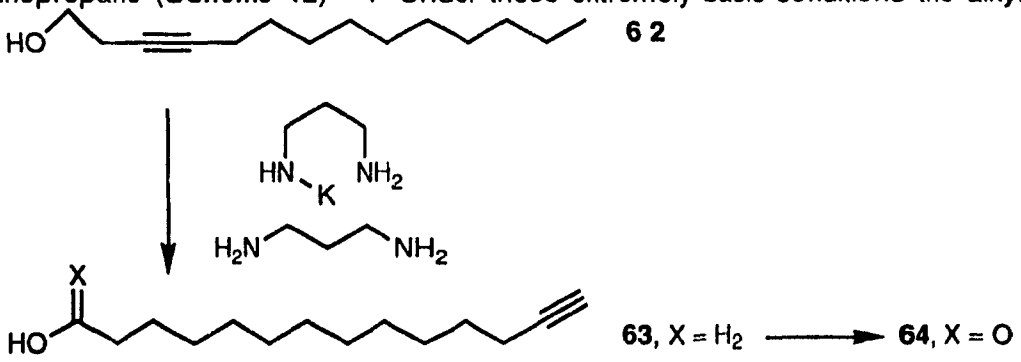
One of the initial concerns was the possibility that dimethylboron bromide might act as a Lewis acid and catalyze acyl migration, but no isomerization could be detected. It was somewhat surprising that the reaction went to completion so rapidly. However, the 4-

methoxybenzyl ether can be considered to be a "benzylogous" acetal (by analogy to a vinylogous function[83]). In other words, a positive charge at the benzylic carbon can be stabilized by resonance through the aromatic ring by the electron donating methoxy group. It is therefore not surprizing that the PMB ether is cleaved even faster than a methyl acetal (MOM group, which requires 1 hour at -78 °C), since the positive charge in the PMB ether receives additional stabilization from the aromatic ring.

The discovery of the use of dimethylboron bromide for the cleavage of PMB ethers should be very useful in the synthesis of other phospholipids and analogues. The reaction conditions are very mild, and with a non-basic workup no trace of acyl migration is detectable. In addition, many commonly used protecting groups are stable to the deprotection conditions (e.g. benzoyl, tetrahydropyranyl, silyl, esters[81] etc.). Since olefinic groups are not affected, the PMB group may now be the most versatile protecting group for the total synthesis of phospholipids.

Preparation of the macrocyclic phosphatidylcholine.

With the problem of finding a glycerol protecting group compatible with unsaturated acyl chains now solved, work on the preparation of the macrocycle was initiated. The synthesis of ω-alkynoic acids was accomplished according to the procedure of Shak *et al.*[84] Commercially available acetylenic alcohols such as 3-tetradecyn-1-ol or 7-hexadecyn-1-ol were added to a solution of potassium aminopropylamide in 1,3-diaminopropane (**Scheme 42**)[85] . Under these extremely basic conditions the alkyne



Scheme 42

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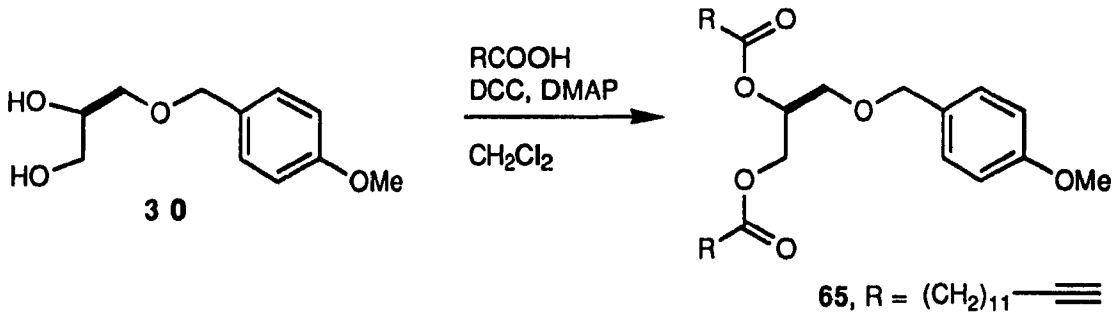
83) March, J.; *Advanced Organic Chemistry*, John Wiley, New York, **1985**, p 418

84) Shak, S.; Reich, N. O.; Goldstein, I. M.; Ortiz de Montellano, P. R.; *J. Biol. Chem.* **1985**, *260*, 13023.

85) Brown, C. A.; Yamashita, A.; *J. Am. Chem. Soc.* **1975**,*97*, 891; Macauley, S. R.; *J. Org. Chem.* **1980**, *45*, 734; Abrams, S. R.; Shaw, A. C.; *Org. Synth.* **1987**, *66*, 127.

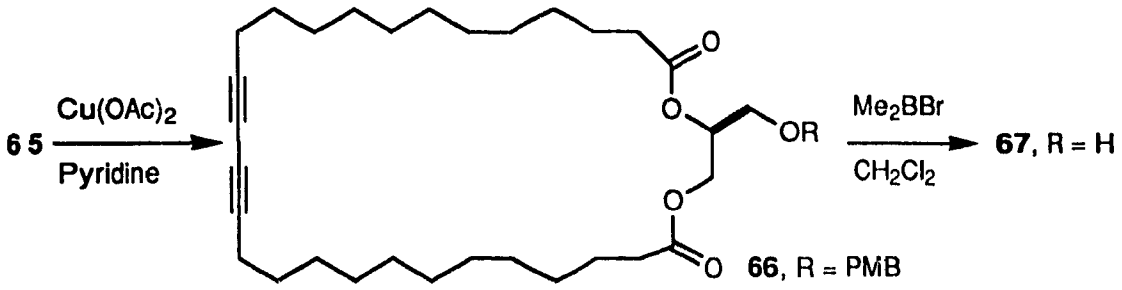
migrates to the terminal position of the chain through a series of allene-alkyne isomerizations. The "zipper" reaction terminates when the alkyne reaches the terminal position because this acetylide salt is less basic than the allene such that the reaction cannot be reversed. The resulting alkyn-1-ols were then oxidized to the carboxylic acids using Jones reagent in acetone. The overall yield of ω-alkynoic acids was 61% for two steps after recrystallization of the product.

With 13-tetradecynoic acid in hand, the strategy for the formation of the macrocycle was tested. Using the usual DCC and DMAP catalyzed esterification conditions, 1,2-di(13-tetradecyn-1-oyl)-3-(4-methoxybenzyl)-sn-glycerol was assembled in 77% yield using 1.1 equivalents of carboxylic acid per hydroxyl (**Scheme 43**).



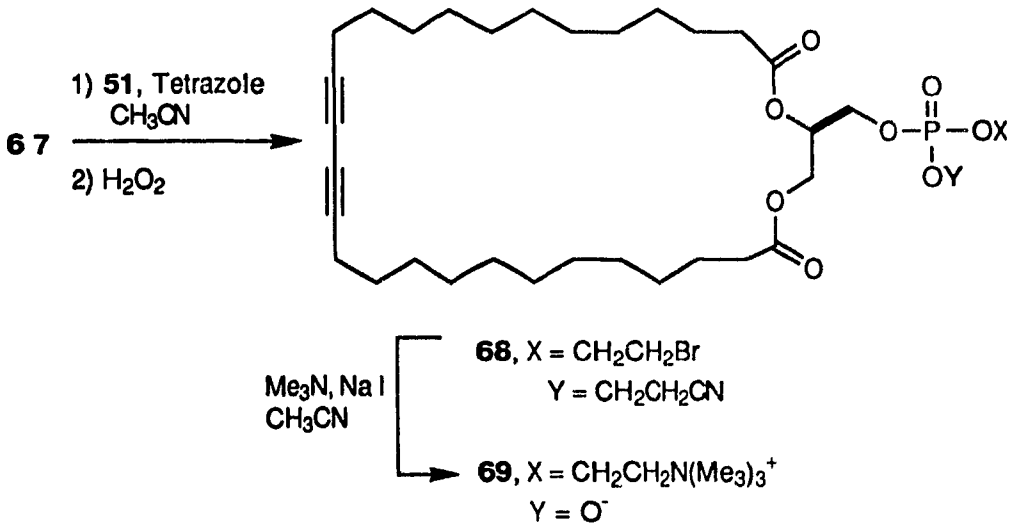
Scheme 43

The acetylenic diacylglycerol was then added over a 3 hour period to a refluxing solution of $Cu(OAc)_2$ monohydrate in refluxing pyridine. After chromatography, a 50 to 60% yield of macrocyclic glycerol was obtained (**Scheme 44**). The product which was slightly more polar than the uncyclized material, was identified by its proton NMR spectrum which showed the disappearance of the acetylenic proton signals. The chemical ionization mass spectrum of the macrocycle also showed a molecular ion two mass units lower than that of the uncyclized precursor. Further proof of the cyclic diacetylene structure came from the slow transformation of the white product to a blue solid when stored in the light. The photopolymerization of diacetylenes in the solid state is known to produce blue or red solids depending on the physical state, due to the formation of conjugated polyene-ynes. The Glaser oxidation proved to be more temperamental than originally believed. It was necessary to use dry, freshly distilled pyridine since solvent which had been left standing gave lower yields of product, presumably due to the presence of oxidation products. Even with careful preparation, the yields of the coupling were variable. It had been feared that the PMB group might not survive the coupling of the alkynes since the methoxybenzyl ether is known to be sensitive to oxidation, but this proved to be an unfounded assumption.



Scheme 44

Next, the macrocyclic PMB- protected glycerol was converted into the phosphatidylcholine. The PMB ether was dissolved in methylene chloride and cooled to -78 °C, and dimethylboron bromide was added neat (**Scheme 44**). TLC indicated full disappearance of the starting material within 5 minutes. The reaction was quenched with ether and the extracts washed with water as before. The PMB side products were not removed from the crude material, which otherwise contained none of the product arising from migration of the sn-2 ester to the primary position. Removal of the aromatic side products would have required chromatography and this has been shown to be a major cause of isomerization in other cases. The crude diacylglycerol was redissolved in anhydrous acetonitrile and added to a solution of phosphocholine precursor, (2-cyanoethyl)-(2-bromoethyl)-N,N-diisopropylphosphoramidite (**Scheme 45**). Solid tetrazole was added, and the formation of the phosphite triester proceeded smoothly as before. Aqueous hydrogen peroxide (30%) was then added to oxidize the product to the neutral phosphate triester. Two equivalents of freshly



Scheme 45

prepared dialkylphosphoramidite were used to ensure complete phosphitylation of the glycerol macrolactone, and the side products of the deprotection did not interfere with

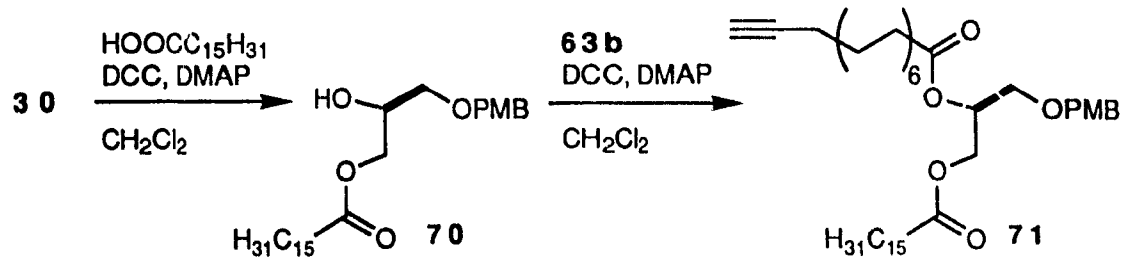
the reaction. Chromatography was done after the oxidation to remove excess phosphitylating reagent and the aromatic byproducts of the deprotecting reaction. The phosphate triester was isolated in 81% yield, based on the protected glycerol. No trace of isomerized product could be detected either during the deprotection or after the phosphorylation. The final step in the synthesis, the conversion of the phosphate triester into the phosphocholine, was performed using the previously established procedure. The neutral phosphate was dissolved in acetonitrile and added to condensed trimethylamine in a pressure vessel. Stirring overnight converted the bromoethyl group into the choline, and eliminated the cyanoethyl group to give the phosphate diester. This last transformation proceeded in 85% yield after chromatography, corresponding to a 69% yield of phosphatidylcholine based on the PMB protected glycerol. The identity of the product was established by ^{1}H and ^{13}C NMR, as well as fast atom bombardment (FAB) mass spectrometry.

As a further proof of the structure, the diacetylenic phospholipid was subjected to exhaustive hydrogenation. The product was dissolved in methanol containing a catalytic amount of 10% palladium on charcoal, and shaken under 2 atmospheres of hydrogen. The proton NMR of the product was identical in all respects to that of the starting material, except in the region around 2.2 ppm. The diacetylenic phospholipid shows a multiplet in this region corresponding to the signals of the 4 protons adjacent to the carbonyl carbons, and the 4 protons adjacent to the diacetylene. The spectrum of the reduced product **76** shows only a quartet (actually two overlapping triplets) corresponding to 4 protons. This quartet structure is a characteristic of the C2 protons on the acyl chains in phosphatidylcholines. No signals were observed in the olefinic region of the spectrum, indicating complete reduction of the product in 97% yield.

Preparation of a bolaform phosphatidylcholine.

The second desired product was a phosphatidylcholine which could either span the membrane, or form a hairpin structure on one side of the bilayer. The synthetic methods described in this account allow the easy preparation of phospholipid with modified acyl chains at the sn-2 position. Consequently, it was planned to link two monomers together through a diacetylene linkage in the middle of the sn-2 chains through a modification of the procedure described for the macrocyclic PC. The 3-PMB-sn-glycerol was selectively acylated at the primary position with palmitic acid in the manner previously described (**SCheme 46**). The diol and 0.95 mole palmitic acid were dissolved in anhydrous methylene chloride at 0 ^{o}C, and DCC and DMAP added. After

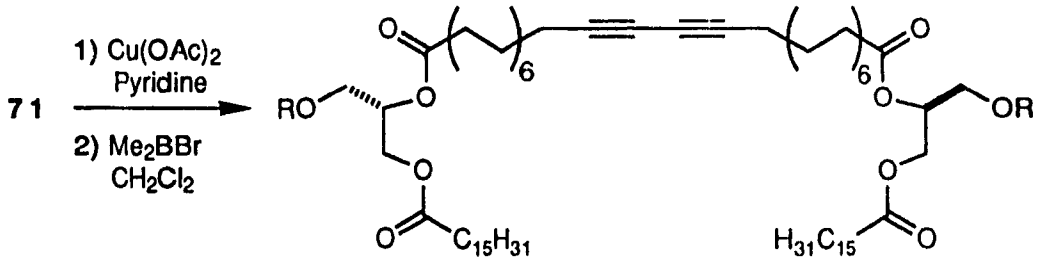
stirring several hours, the urea byproduct was filtered, the solvent removed, and the residue chromatographed on silica gel. A 65% yield of pure product acylated at the primary position was obtained after purification. The secondary alcohol



Scheme 46

was acylated again with 15-hexadecynoic acid using DCC and DMAP at room temperature, giving a 95% yield of 1-palmitoyl-2-(15-hexadecyn-1-oyl)-3-PMB-sn-glycerol **71**.

This diacylglycerol with a terminal alkyne introduced regiospecifically on the sn-2 acyl chain was then subjected to the Glaser oxidation conditions to produce the protected symmetrical dimeric diacylglycerol **72** (**Scheme 47**). The coupling of the acetylenes proved to be more difficult than anticipated. Once again the yield of product varied depending on the exact conditions used. A number of alternate catalyst systems were tried. For example, the complex formed by reaction of cuprous salts with N,N,N',N'-tetramethyl ethylenediamine (TMEDA) in acetone is reported to be an effective catalyst



72, R = PMB
73, R = H

Scheme 47

for the dimerization of acetylenes in the presence of oxygen[86], but no reaction was observed with this reagent in the present case. More recently, the complex formed from cuprous iodide and two moles of TMEDA has also been used in this context[87], but this was again ineffective in this case. It is known that alkynes at the end of a long alkyl chain are

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86) Hay, A. S.; *J. Org. Chem.* **1962**, *27*, 3320.
87) Valentí, E.; Pericàs, M. A.; Serratosa, F.; *J. Am. Chem. Soc.* **1990**, *112*, 7405.

less reactive than other substrates. The best result obtained was a 68% yield of diacetylene dimer, produced by warming a solution of terminal alkyne in pyridine containing 10 equivalents of cupric acetate to 80 °C (oil bath) under an inert atmosphere for 2 hours. The product was again identified on the basis of its ^{1}H NMR spectrum which showed the absence of the alkyne proton signals. The FAB mass spectrum also showed a weak molecular ion for the dimer.

It was decided to verify whether some advantage could be gained by performing the Glaser coupling first to give the 32 carbon dialkynoic diacid, and esterifying the latter to two moles of 1-palmitoyl-3-PMB-sn-glycerol. Treatment of 15-hexadecynoic acid with cupric acetate in aqueous pyridine at 45 °C gave an 89% yield of diacid after workup. The diacid and the glycerol were suspended in dichloromethane at room temperature, and were treated with DCC and DMAP. However, the diyne diacid is poorly soluble in organic solvents and tends to polymerize fairly rapidly in the solid state when exposed to light. The purified diacid polymerizes even more rapidly than slightly impure material. This is because the presence of trace impurities prevents the proper packing required for the topotactic polymerization of the diacetylene[88]. At any rate, because of poor solubility and partial polymerization of the diacid, only a 69% yield of PMB-bolaform could be obtained. The product could be obtained in equal yield with less difficulty by the previous method, since polymerization was not a serious problem with the bolaform when minimal precautions were taken.

The introduction of the phosphatidylcholine functions was accomplished following the usual procedure. The 4-methoxybenzyl ethers were cleaved by treatment with dimethylboron bromide in dichloromethane at -78 °C. It was necessary to warm the solution slightly since at dry-ice/acetone bath temperature the bis-PMB ether precipitated. The reaction was quenched with a large volume of ether as soon as the stirred suspension became homogeneous, and the ethereal solution of the bis-alcohol washed with distilled water until neutral. The dried bis-diacylglycerol was then redissolved in anhydrous acetonitrile. A solution of freshly prepared (2-bromoethyl)-(2-cyanoethyl)-phosphoramidite (4 equivalents per mole of bolaform) was added, followed by tetrazole. After disappearance of the alcohol starting material, 30% aqueous hydrogen peroxide was added and the solution stirred until all the phosphite had been oxidized to the phosphate triester. There was obtained after chromatography a 65%

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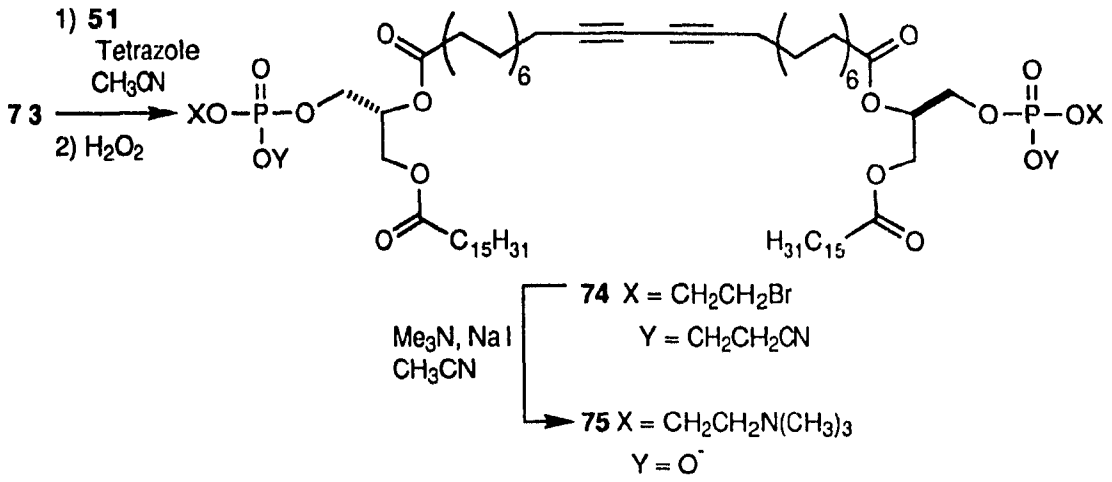
88) Kuo, T.; O'Brien, D. F.; *J. Am. Chem. Soc.* **1988**, *110*, 7571 and references therein.

yield of the bolaform bisphosphate triester. The lower yield is apparently a result of the lower solubility of the bolaform materials.



Scheme 48

The phosphate triesters were once again converted to the phosphatidylcholines by dissolving the bolaform in toluene (since the starting material is poorly soluble in acetonitrile) and adding it to a solution of trimethylamine and sodium iodide in acetonitrile at -78 °C. The pressure bottle was sealed, and the mixture heated to 65 °C overnight. After workup and chromatography, the extremely polar and poorly soluble product was obtained in 56% yield (36% overall from the PMB protected precursor). The low yield can be explained by the low solubility of the material. once the phosphatidylcholine is generated at one end of the molecule, the product becomes much less soluble and tends to precipitate from the acetonitrile/trimethylamine solution. This decreases the availability of the other phosphate triester function for reaction. In addition, the product may bind more tightly to the silica column due to its very high polarity. The structure of the product was again established by its ^1H NMR spectrum, and the FAB mass spectrum which showed a weak parent ion.

The diacetylene portion of this bolaform phosphatidylcholine was also subjected to catalytic hydrogenation. A solution of diacetylene-PC was dissolved in methanol containing a catalytic amount of 10% Pd on carbon, and the suspension shaken under 3 atmospheres of hydrogen. The reaction was incomplete after several hours, and the ^1H NMR spectrum showed that some of the product was only partially reduced. Consequently, a fresh portion of diacetylene in methanol was shaken under 3 atmospheres of H_2 in the presence of platinum oxide. This catalyst is less likely than palladium to be poisoned and to stop at the olefin stage. After removal of the catalyst an

85% yield of saturated bolaform **77** was obtained. The lower yield than for the analogous macrocyclic PC can be ascribed to the more difficult recovery of the bolaform PC.

Film balance and Kinetic studies with modified phospholipids.

Both Langmuir film balance and enzyme kinetic studies have been performed on the phospholipid analogues described in this thesis. Langmuir film balance studies provide details of phospholipid orientation and phase properties at the air-water interface. These details can be related in turn to the properties of the compounds in the aqueous dispersed (i.e. bilayer) state. Compression isotherms (25 °C) of the 1-acyl-2-(**X**-hydroxystearoyl)-sn-glycero-3-phosphatidylcholine (**X**-OH-PC) and the corresponding 2-(ketostearoyl)-PCs are particularly sensitive to the position of the substituent[89]. As shown in **Figure 1**, the area at which the molecules first begin to interact (A_{ON}) is dependent on the position **X**, and follows the trend **X** = (5)<(9)<(12). The A_{ON} trends suggest that the 12-OH-PC occupies ≈35% more area than the 5-OH-PC in the dilute surface state. A simple interpretation of this phenomenon would be one where the secondary alcohol acts as an auxiliary polar headgroup so that the A_{ON} is the sum of the areas of the PC headgroup, the hydroxyl group, and the hydrocarbon link between the two.

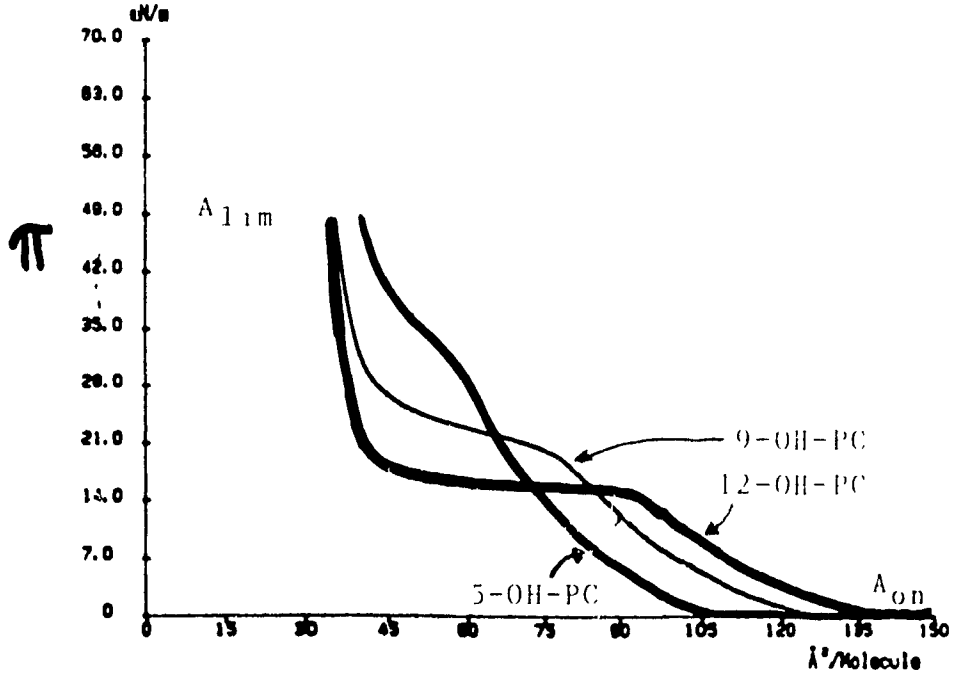


Figure 1; Pressure/Area isotherms for 5-OH-PC, 9-OH-PC and 12-OH-PC (see text) on pure water (18 mΩ, MilliQ) at 25 °C.

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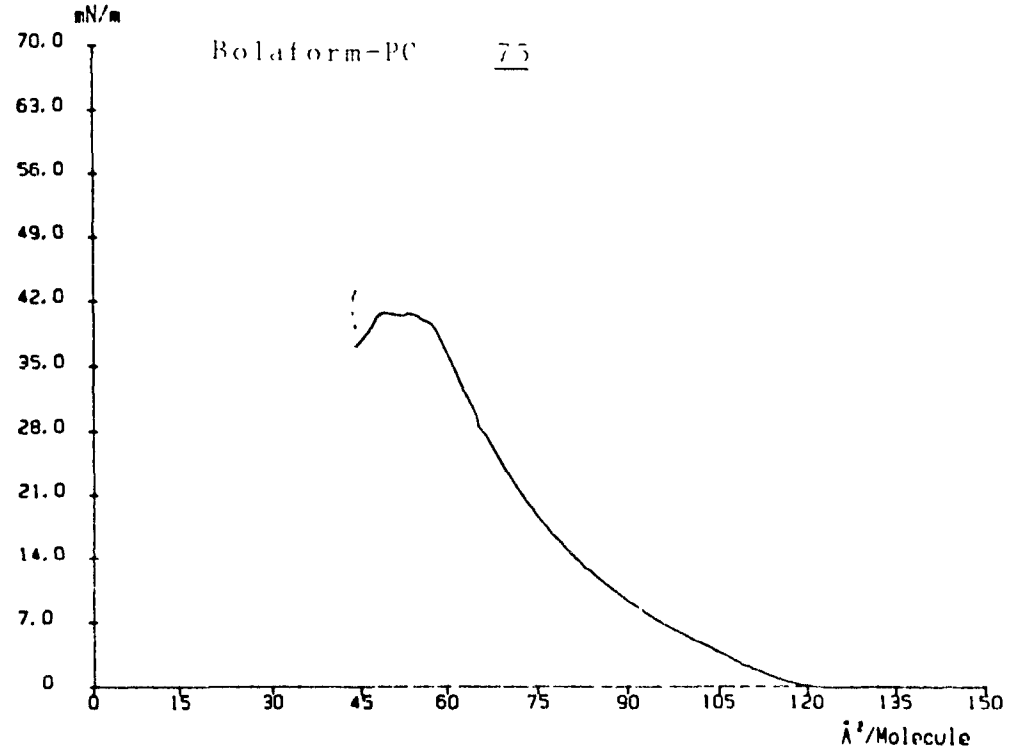
89) The products mentioned in this section were not necessarily synthesized by the methods described earlier, but were chosen to illustrate the concepts presented above. The data and discussion are from the work of M. Louis Cuccia and Ms. Ling Wei.

Compression of the 12-OH-PC for example, beyond A_{ON} results in monotonically increasing surface pressure up to 16 mN/m, at which point the isotherm passes through a discontinuity (Π_t) and a highly compressible, first order region. This behaviour is repeated for the 9-OH-PC and 5-OH-PC, where the transition occurs at smaller areas, Π_t is larger, and the transition plateau is less flat. The limiting areas (A_{LIM}) at high Π correspond to a close-packed state of PCs whose area per molecule is about 50 $Å^2$. At high lateral pressures all three compounds adopt similar surface densities and chain packing properties.

Both the diacetylenic and saturated bolaform phosphatidylcholines appear to form stable monolayers on a pure water surface, but exhibit a different type of isotherm from those described above (**Figure 2a** and **2b**). For the saturated bolaform-PC the A_{ON} value is 175 $Å^2$/molecule, indicating that both headgroups and their hydrocarbon links occupy a surface site when the surface density is small. Compression of these molecules leads to a surface pressure increase, followed by a sharp discontinuity (Π_t) leading to a very flat plateau. This plateau is remarkable in that it proceeds to extremely low areas without exhibiting the surface pressure increase typical of films in a highly compressed state. Moreover , the Π value of the transition is large (35 mN/$Å^2$), compared to dipalmitoyl-PC (14 mN/$Å^2$). This isotherm has been interpreted in the following manner. From A_{ON} to the discontinuity, both PC headgroups and much of the hydrocarbon linking them are surface adsorbed. Up to 100 $Å^2$ per molecule this hydrocarbon link forms an air-localized loop during compression. 100 $Å^2$ roughly corresponds to the combined area of the two phosphorylcholine headgroups. Any compression beyond this point would cause displacement of one headgroup from the water surface. Past 100 $Å^2$ per molecule, the lateral surface pressure remains constant as further compression causes incremental displacement from the surface of one PC headgroup per molecule. However, at the area corresponding to one PC headgroup per bolaform molecule at the surface, the isotherm remains flat. This indicates that eventually most bolaform molecules do not express any surface area at mean areas less than 50 $Å^2$/molecule. This arises if the molecules are driven into the aqueous subphase, or are forced above the original monolayer. The latter is energetically more likely, and would result in the formation of well defined multilayers of phospholipid molecules.

2a



2b

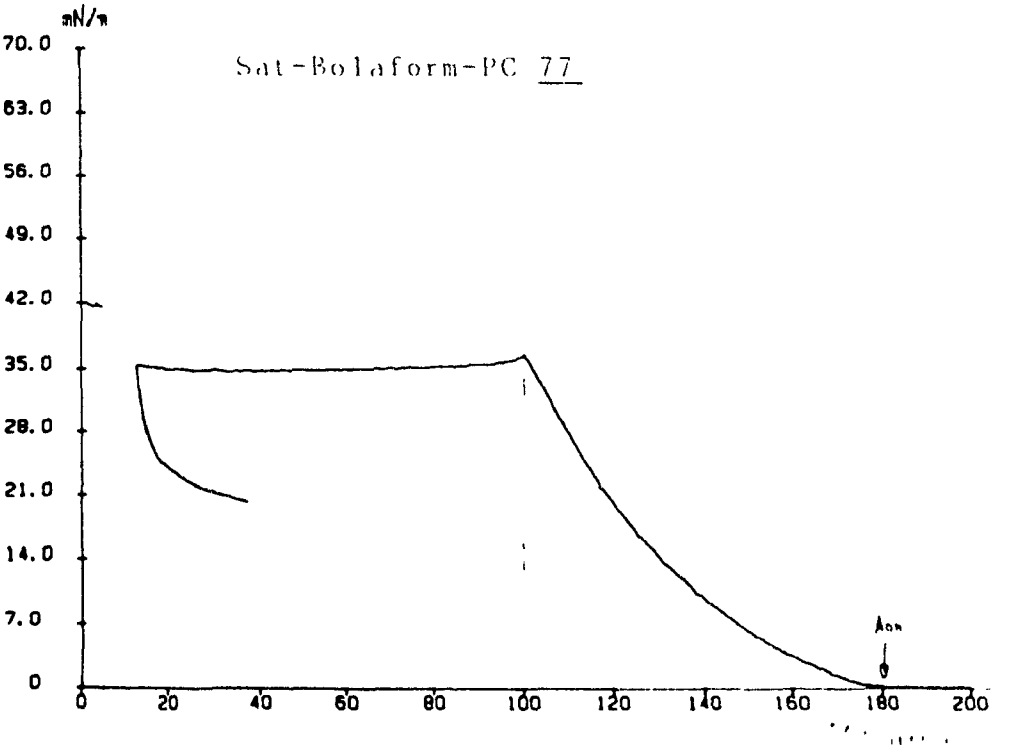


Figure 2a; Pressure/Area isotherms for diacetylenic bolaform **75** (**Fig 2a**) and the saturated derivative **77** (**Fig 2b**)(see text) on pure water (18 mΩ, Millic) at 25 °C.

The bipolar chain-substituted phospholipids were also subjected to PLA_2 hydrolytic conditions. In one study, the phospholipids were dispersed in a non-hydrolysable, non-inhibitory detergent micelle matrix of Triton-X100. As shown in **Table 1**, relatively small reactivity differences are observed with respect to the saturated dimyristoyl-PC. These reactivity differences may reflect either k_{cat} or K_m differences, or both. What is notable is that all of these molecules are relatively good substrates for PLA_2. When dispersed in a disorganized interface such as provided by Triton-X100, polar groups further than five carbons from the scissile ester bond have little effect on the hydrolytic process. A different picture arises for the saturated bolaform-PC. In both a Triton-X100 matrix and in a pure aqueous dispersion (presumably a vesicle), virtually no hydrolysis is observed. While detailed studies are underway to determine the origin of this lack of activity, it is interesting to speculate that the saturated bolaform is not hydrolyzed because one headgroup cannot diffuse into the enzyme active site without pulling the other headgroup into the hydrophobic interior of the micelle or bilayer. This is consistent with the suggestion that the enzyme active site lies at the end of a long hydrophobic channel into which the substrate must diffuse for catalysis to occur. In this case, different bolaform chain lengths may provide an indirect measure of the channel depth.

Table 1: Relative rates of hydrolysis of phospholipids (1 mM) dispersed in 40 mM Triton X-100 [a]

	k_{rel}[(b)]
1-Stearoyl-2-(5-ketostearoyl)-glycero-PC:	2.5
1-Stearoyl-2-(12-ketostearoyl)-glycero-PC:	2.0
1,2-Di-(12-hydroxystearoyl)-glycero-PC:	0.4
1,2-Di-(12-O-TBDMS-stearoyl)-glycero-PC:	0.3
1-Palmitoyl-2-(5-hydroxystearoyl)-glycero-PC[d]:	0.2
1-Stearoyl-2-(9-hydroxystearoyl)-glycero-PC[d]:	0.3
1-Stearoyl-2-(12-hydroxystearoyl)-glycero-PC[d]:	0.2
Saturated Bolaform PC:	<0.03[c]

a) Measured by pH Stat technique at pH 8.0, 35 °C. b) Relative to rate measured for dimyristoyl-PC. c) Upper limit estimate of sensitivity of pH Stat technique. d) 5mM in 40mM Triton X-100.

Contributions to knowledge.

- The use of an allyl ether as a protecting for the synthesis of phosphatidylcholines was demonstrated. The allyl ether was cleaved using rhodium-catalyzed isomerization to a vinyl ether followed by hydrolysis in the presence of N-bromosuccinimide. This procedure allows the preparation of isomerically pure 1,2-diacylglycerols.

- A new strategy for the introduction of the phosphocholine headgroup into phospholipids was developed. The diacylglycerol is converted to a phosphite triester under mildly acidic conditions using a dialkylphosphoramidite, and oxidized to the phosphate triester. This allows the introduction of the phosphate group in high yield without any acyl migration during the reaction. By a judicious choice of substituents on the phosphoramidite, a neutral phosphate triester is produced and can be purified readily. The phosphorus protecting group is then removed and the choline function generated in one step using trimethylamine.

- A new method for the cleavage of 4-methoxybenzyl ethers using dimethylboron bromide in methylene chloride at -78 °C, and the use of the method for the deprotection of 1,2-diacylglycerols without any acyl migration is reported. This allowed the easy preparation of diacetylenic macrocyclic and bolaform phosphatidylcholines by the dialkylphosphoramidite method.

- The autocatalytic deactivation of soybean lipoxygenase by substrate in organic solvents was demonstrated.

EXPERIMENTAL PROCEDURES.

General Procedures: Chemicals were obtained from Aldrich Chemical Corporation and Farchan Ltd, and were used without prior purification. Solvents used were of reagent grade, and were distilled before use: THF from sodium benzophenone ketyl, CH_2Cl_2 from P_2O_5, acetonitrile, pyridine and triethylamine from calcium hydride. $CHCl_3$ was washed with water, dried with $CaCl_2$, distilled from P_2O_5 and stored in the dark. Reactions were performed under an inert atmosphere (N_2 or Ar) unless otherwise indicated. Thin layer chromatography was done using Merck Kieselgel 60 F_{254} aluminum backed plates, and visualised by dipping in a solution of $(NH_4)_2MoO_4$ and Ce_2SO_4 in dilute H_2SO_4 and charring. Flash chromatography was done on Merck silica gel 60 (230-400 mesh). Melting points were determined on a Gallenkamp Block, and are uncorrected. All proton and carbon NMR spectra were obtained on a Varian XL-200 or XL-300, or a Varian Gemini-200. ^1H NMR spectra are reported in ppm downfield from TMS, using the residual H resonance of the solvent as reference (7.24 ppm for $CDCl_3$). ^{13}C NMR is reported in ppm downfield from TMS using the $CDCl_3$ triplet at 77.00 ppm as internal reference. Fast atom bombardment mass spectra were measured at the McGill Biomedical Mass Spectrometry Unit on a ZAB 2F HS spectrometer with a Xenon gun (8 kV, 1mA beam current), and chemical ionization mass spectra by direct inlet on a Hewlett-Packard 5980A instrument using NH_3 as ionization gas ($5X10^{-4}$ torr). Elemental analyses were performed at Guelph Chemical Laboratories Ltd. All compounds were judged pure by examination of their ^1H and ^{13}C NMR spectra.

1,2-Isopropylidene-sn-glycerol. (27).

A solution of 3.00g (12 mmol) of 1,2:5,6-Di-O-isopropylidene-D-mannitol in 10 ml MeOH was added to a suspension of 36 ml Amberlite A27 borohydride form and 40 ml Amberlite A27 periodate form in 150 ml of MeOH/H_2O 9:1. After stirring for 2h, the starting material had been consumed (GC monitoring), and the spent resins were filtered off. The solvent was evaporated under reduced pressure, 100 ml of brine was added and the emulsion was extracted with CH_2Cl_2 (2 X 150 ml), dried with $MgSO_4$, and evaporated. ^1H NMR (200 MHz, $CDCl_3$): δ 4.22 (m, 1H, H2), 4.02 (t, 1H, H1a, J=8.2 Hz), 3.77 (t, 1H, H1b, J=8.2 Hz), 3.71 (dd, 1H, H3a, J=8Hz J=5Hz), 3.56 (dd, 1H, H3b, J=11Hz, J=5Hz), 1.42 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

1,2-Isopropylidene-3-[(R)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetoyl]-sn-glycerol. (28)

The (R)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (0.050g, 0.20 mmol) is added to a solution of alcohol **27** (0.018g, 0.14 mmol) in dry pyridine. After 2 hrs the mixture was poured into water and extracted with ethyl acetate (3 X 25 ml), and washed with water, satd $NaHCO_3$, brine, dried with $MgSO_4$ and evaporated. ^1H NMR (200 MHz, CDCl3): δ 7.4 (m, 5H, Ar), 4.43-4.24 (m, 3H, H2, H3a and H3b), 4.02 (dd, 1H, H1a, J=6 Hz, J= 9 Hz), 3.72 (dd, 1H, H1b, J=5 Hz, J=9 Hz), 3.55 (s, 3H, OCH_3), 1.36 (s, 3H, CH_3), 1.32 (s, 3H, CH_3).

3-(4-Methoxybenzyl)-sn-glycerol. (30)

Crude isopropylidene-sn-glycerol **27** was azeotroped twice with toluene, dissolved in anhyd THF (10 ml) and added dropwise to a suspension of sodium hydride (0.80 g, 80% dispersion in mineral oil, 26 mmol) in 120 ml THF. After stirring for 45 min, 4.07g (3.52 ml, 26 mmol) 4-methoxybenzyl chloride was added in two portions, and stirring continued 16 h. After the careful addition of 10 ml H_2O, the solvent was stripped off and the product was extracted with EtOAc (3 X 100 ml), washed with portions of 0.05N HCl, H_2O, sat sodium bicarbonate solution, and brine. The combined organic layers were dried with $MgSO_4$ and evaporated. The acetonide **29** was immediately hydrolyzed without purification. The product was redissolved in 15 ml MeOH and 10 ml 1N HCl, and stirred for 1h. After extraction with EtOAc and removal of the solvent, the residual oil was purified by flash chromatography using 2% MeOH in $CHCl_3$ as eluant. The product **30** was obtained as a clear oil which solidified on standing in 60% yield from mannitol(3.04 g, 14.3 mmol, mp 40-41°C). TLC (hexane/EtOAc 3:1) R_f: 0.20. ^1H NMR (200 MHz, CDCl3): δ 7.23 (d, 2H, ArH, J= 9 Hz), 6.86 (d,2H, ArH, J= 9 Hz), 4.42 (s, 2H, CH_2Ar), 3.73 (s, 3H, OCH_3), 4.03-3.27 (m, 7H). ^{13}C NMR ($CDCl_3$, 75.4MHz): δ 159.4, 129.7, 129.4, 113.9, 73.2, 71.5, 70.5, 64.1, 55.3. Anal. Calcd for $C_{11}H_{16}O_4$: C, 62.25; H, 7.60. Found: C, 62.05; H, 8.01.

1,2-Dilinoleoyl-3-(4-methoxybenzyl)-sn-glycerol. (31)

The PMB-glycerol **30** (0.257 g, 1.21 mmol) and linoleic acid (0.75 g, 2.7 mmol) were dissolved in 30 ml dry CH_2Cl_2 at 0 °C. A solution of DCC (0.824 g, 4.0 mmol) and DMAP (0.329 g, 2.7 mmol) in 10 ml dry CH_2Cl_2 was added, and the solution allowed to warm to room temperature. After stirring overnight, the dicyclohexylurea was filtered, and the solvent removed. Chromatography using 12% EtOAc in hexanes gave 0.850 g product **31** (1.15 mmol, 95%). TLC (10% EtOAc/hexanes): Rf = 0.34. ^1H NMR (200

MHz, CDCl3): δ 7.22 (d, 2H, ArH, J=8.6 Hz), 6.86 (d, 2H, ArH, J=8.6 Hz), 5.4-5.2 (m, 9H, H_2, 2 H9, 2 H10, 2 H12, 2 H13), 4.46 (d, 2H, ArCH2, J=2Hz), 4.32 (dd, 1H, H_{1a}, J_{ab}=11.8Hz, J_{12}=3.7Hz), 4.17 (dd, 1H, H_{1b}, J_{ab}=11.8Hz, J_{12}=6.5Hz), 3.80 (s, 3H, $ArOCH_3$), 3.54 (d, 2H, H_3, J=5.2Hz), 2.76 (t, 4H, H11, J=6Hz), 2.24 (m, 4H, C_1OCOCH_2, C_2OCOCH_2), 2.03 (q, 8H, H8, H14, J=7Hz), 1.7-1.2 (32H, CH_2), 0.88 (t, 6H, CH_3, J=5Hz).

3-O-Allyl-sn-glycerol. (33)

The procedure for **30** was followed using 22.2 g (168 mmol) glycerol acetonide **27**, sodium hydride (7.4 g, 60% in oil, 185 mmol), Bu_4N I (3.0 g, 8.4 mmol), and allyl bromide (24.4 g, 200 mmol). The acetonide was then hydrolyzed by stirring a solution of the 1,2-isopropylidene-sn-glycerol allyl ether in 100 ml ethanol in the presence of activated Amberlite IR-120 H+ overnight. The product **33** was obtained in 75% overall yield (16.6 g, 126 mmol) after chromatography (EtOAc/hexanes 1:1). 1,2-isopropylidene-3-O-allyl-sn-glycerol: ^{1}H NMR (200 MHz, CDCl3): δ 5.81 (m, 1H, CH=C), 5.35-5.15 (m, 2H, $C=CH_2$), 4.28 (m, 1H, H2), 4.05 (m, 3H, H_{1a}, $CH_2C=C$), 3.75 (q, 1H, H_{1b}), 3.6-3.4 (m, 2H, 2 H3), 1.42 (s, 3H, CH_3), 1.36 (s, 3H, CH_3).

1-Palmitoyl-3-O-allyl-sn-glycerol. (35).

Allyl glycerol **33** (2.00g, 15.1 mmol) and palmitic acid (3.84 g, 15.0 mmol) were dissolved in 150 ml dry CH_2Cl_2 and cooled to 0 °C. A solution of DCC (3.40 g, 16.5 mmol) and DMAP (1.83 g, 15 mmol) in 20 ml dry CH_2Cl_2 was added dropwise over 45 min. The solution was stirred at °C overnight. The precipitated urea was filtered, the solvent removed and the residue chromatographed to give 3.60 g (9.75 mmol, 65%) of the title product **35**, along with **34**. TLC: 15% EtOAc/hexanes, Rf= 0.33. ^{1}H NMR (200 MHz, CDCl3): δ 5.90 (m,1H, CH=C), 5.35-5.15 (m, 2H, $C=CH_2$), 4.15 (m, 2H, 2 H1), 4.05 (m, 3H, H_2, $CH_2C=C$), 3.51 (q, 1H, H_{3a}, J_{ab}= 11Hz, J_{ax}= 3.8Hz), 3.44 (q, 1H, H_{3b}, J_{ab}= 11Hz, J_{bx}= 6.2Hz), 2.36 (t, 2H, CH_2COO, J= 8Hz), 1.8-1.2 (26H, CH_2), 0.86 (t, 3H, CH_3, J=4Hz).

2-Palmitoyl-3-O-allyl-sn-glycerol. (34).

The preparation of **35** also gave 0.83 g (2.25 mmol, 15%) of the isomeric palmitate ester **34**. TLC: 15% EtOAc/hexanes, Rf= 0.22. ^{1}H NMR (200 MHz, CDCl3): δ 5.90 (m,1H, CH=C), 5.35-5.15 (m, 2H, $C=CH_2$), 5.05 (m, 1H, H2), 3.82 (m, 2H, 2 H3), 3.62 (dd, 2H, 2 H3, J=1 Hz, J= 5Hz), 2.37 (t, 2H, CH_2COO, J= 8Hz), 1.8-1.2 (26H, CH_2), 0.86 (t, 3H, CH_3, J=4Hz).

5-(t-Butyldimethylsilyloxy)-stearic acid. (37).

To a suspension of methyl 5-oxooctadecanoate (1.00 g, 3.20 mmol) in 10 ml methanol was added excess $NaBH_4$ (0.090 g, 2.3 mmol) in small portions. After the starting material had been consumed the reaction was quenched with 1N HCl. The product was extracted with EtOAc (3 X 50 ml), washed with 0.01 N HCl, water, brine and dried with $MgSO_4$. The crude alcohol (TLC: 5% EtOAc/hexanes, Rf= 0.3) was redissolved in 5 ml dry DMF, to which was added t-butyldimethylchlorosilane (0.75 g, 4.8 mmol) and imidazole (0.40 g, 6.0 mmol). The solution was stirred overnight at room temperature. Water was added (50 ml), and the product extracted with ethyl acetate (3 X 50ml), washed with 0.1 N HCl, water, satd $NaHCO_3$, brine and dried. Chromatography (1% EtOAc/hexanes) gave the methyl ester of **37**. TLC: 5% EtOAc/hexanes, Rf=0.83. The carboxylic acid **37** was obtained by dissolving the crude methyl ester in 30 ml ethanol containing 1.8g (32 mmol) potassium hydroxide, stirring overnight, and extraction of the acidified mixture with EtOAc (3 X 100 ml). Pure product was obtained in 75% yield (1.00 g, 2.4 mmol). ^1H NMR (**37** methyl ester) (200 MHz, $CDCl_3$): δ 3.66 (s, 3H, OCH_3), 3.62 (m, 1H, H5), 2.30 (t, 2H, CH_2COO, J=7Hz), 1.8-1.2 (28H, CH_2), 0.87 (s,12H, CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$).

1,5-Bis-(t-butyldimethylsilyloxy)-octadecane. (38).

This product was isolated in 15% yield (0.25 g, 0.48 mmol) during the preparation of **37**. TLC: 5% EtOAc/hexanes: Rf=0.93. ^1H NMR (200 MHz, $CDCl_3$): δ 3.59 (t, 3H, 2H1, H5, J=6Hz), 1.6-1.2 (30H, CH_2), 0.89 and 0.87 (d,18H, CH_3, 2 $SiC(CH_3)_3$), 0.04 and 0.03 (d, 12H, $Si(CH_3)_2$).

Methyl 13-hydroxystearate. (41).

To a solution of soybean lipoxygenase (Sigma Chemical Co, type I, 30 mg) in 500 ml 0.1M sodium borate buffer (pH 9.0) in a wide mouth flask was added a solution of linoleic acid (0.50 g, 1.79 mmol) in 5 ml ethanol. The solution was stirred in air for 30 min, and solid sodium borohydride (0.25 g) added in small portions. After a further 30 min, the reaction was stopped by adding 1N HCl to pH 4 (much frothing). The mixture of unsaturated alcohols was extracted with ether (3 X 250 ml), dried with $MgSO_4$ and the solvent evaporated. The crude mixture was dissolved in 30 ml absolute ethanol, 0.10 g PtO_2 added, and the solution shaken under 2 atmospheres of H_2 for 1 hour. The platinum catalyst was filtered, and the solvent evaporated. TLC showed two spots (EtOAc/Hexane 1:1, Rf= 0.70, Rf=0.56). The mixture of carboxylic acids was redissolved in ether and treated with diazomethane until the yellow color persisted. The

mixture of hydroxy methyl esters was chromatographed with 10% EtOAc/hexanes and gave 0.376 g **41** (1.20 mmol, 67%) along with **42**. The product was converted to the TBDMS ether and the methyl ester hydrolyzed in the manner described for **37**. ^{1}H NMR (**41** Hydroxy ester)(200 MHz, $CDCl_3$): δ 3.66 (s, 3H, OCH_3), 3.56 (m, 1H, H13), 2.30 (t, 2H, CH_2COO, J=7Hz), 1.8-1.2 (28H, CH_2), 0.87 (t, 3H, CH_3, J=5Hz). (**41** free acid TBDMS ether) (200 MHz, $CDCl_3$): δ 3.60 (m, 1H, H13), 2.34 (t, 2H, CH_2COO, J=7Hz), 1.8-1.2 (28H, CH_2), 0.87 (s, 12H, CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (**41** TBDMS ether) (75.4 MHz, $CDCl_3$): δ 174.4, 72.4, 51.4, 37.1, 34.1, 32.1, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 26.0, 25.4, 25.0, 23.7, 18.2, 14.1. EIMS: m/z= 315 (39%, $M^{+\cdot} - CH_3OCOC_{11}H_{22}$), 173 (100%, $M^{+\cdot} - C_5H_{11}$).

Methyl 9-hydroxystearate. (42).

Isolated from the preparation of **41** in 15-30% yield . ^{1}H NMR (**42** Hydroxy ester)(200 MHz, $CDCl_3$): δ 3.66 (s, 3H, OCH_3), 3.56 (m, 1H, H9), 2.30 (t, 2H, CH_2COO, J=7Hz), 1.8-1.2 (28H, CH_2), 0.87 (t, 3H, CH_3, J=5Hz). (**42** free acid TBDMS ether) (200 MHz, $CDCl_3$): δ 3.60 (m, 1H, H9), 2.34 (t, 2H, CH_2COO, J=7Hz), 1.8-1.2 (28H, CH_2), 0.87 (s, 12H, CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$). ^{13}C NMR (**42** TBDMS ether) (75.4 MHz, $CDCl_3$): δ 174.4, 72.4, 51.5, 37.2, 37.1, 34.1, 32.0, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 26.0, 25.8, 25.4, 25.3, 25.2, 22.7, 18.2, 14.2. EIMS: m/z= 259 (98%, $M^{+\cdot} - C_9H_{19}$), 229 (100%, $M^{+\cdot} - CH_3OCOC_7H_{14}$).

1-Palmitoyl-2-(5-O-TBDMS-stearoyl)-3-O-allyl-sn-glycerol. (43).

A solution of DCC (0.44 g, 2.2 mmol) and DMAP (0.090 g, 1.0 mmol) in 2 ml methylene chloride was added to a solution of acid **37** (0.446 g, 1.08 mmol) and glycerol **35** (0.40 g, 1.08 mmol), and the solution stirred at room temperature. After chromatography, 91% yield (0.75 g, 0.98 mmol) product was obtained. TLC: 10% EtOAc/hexanes, Rf= 0.34. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.85 (m, 1H, CH=C), 5.3-5.1 (m, 3H, H2, $C=CH_2$), 4.32 (dd, 1H, H1a, J_{ab}= 12Hz, J_{ax}= 3.7 Hz), 4.15 (dd, 1H, H1b, J_{ab}= 12Hz, J_{bx}= 6.5Hz), 3.98 (d, 2H, $CH_2C=C$, J= 6Hz), 3.59 (m, 1H, SiOCH), 3.54 (d, 2H, 2 H3, J= 5Hz), 2.29 (m, 4H, 2 CH_2COO), 1.8-1.2 (54H, CH_2), 0.87 (s, 15H, 2 CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$).

1-Palmitoyl-2-(5-O-TBDMS-stearoyl)-3-O-(2-propenyl)-sn-glycerol. (44).

A solution of tris(triphenylphosphine)rhodium^{I} chloride (0.120 g, 0.13 mmol), diazabicyclooctane (0.120 g, 1.1 mmol) and triphenylphosphine (0.085g, 0.33 mmol)

in 10 ml ethanol were heated to reflux for 30 min under an inert atmosphere. A solution of allyl ether **43** in 5 ml THF (1.00g, 1.30 mmol) was then added, and the solution refluxed for one hour. The mixture was redissolved in ether and filtered through a short plug of silica gel to remove the rhodium salts. The isomerization was quantitative, and the product was used in the next step without further purification. TLC: 15% EtOAc/hexanes, Rf= 0.66. ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.22 and 6.15 (m, 1H, OCH=C cis and trans), 5.09 (m, 1H, H2), 5.22 and 4.80 (m, 1H, OC=CH-C), 4.5-3.5 (m, 2 H1, 2 H3, SiOCH), 2.29 (m, 4H, 2 CH_2COO), 1.8-1.2 (57H, OC=C-CH_3, CH_2), 0.87 (s, 15H, 2 CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$).

1-Palmitoyl-2-(5-O-TBDMS-stearoyl)-sn-glycerol. (45)

Method A: To a solution of vinyl ether **44** (0.255 g, 0.333 mmol) and 0.091 g (0.42 mmol) yellow mercuric oxide in 5 ml acetone at 0 °C was added dropwise a solution of mercuric chloride (0.091g, 0.335 mmol) in 1 ml 1:1 acetone/water. The solution was stirred at °C for 30 min and rt for 30 min. After disappearance of the starting material, the reaction was quenched with an aqueous solution of NaI (10 g/ml). The mixture was extracted with ether (3 X 50 ml), washed with water and brine, and dried with $MgSO_4$. TLC analysis showed a mixture of **45** and **46** which were used immediately in the next reaction.

Method B: To a solution of vinyl ether **44** (0.250 g, 0.326 mmol) in 3.0 ml THF and 0.1 ml water was added dropwise a solution of N-bromosuccinimide (0.060 g, 0.34 mmol) in 2.0 ml THF. The reaction was complete within 5 minutes. The solution was diluted with 50 ml water and extracted with ether (3 X 50 ml), washed with water and brine, and dried with $MgSO_4$. The crude product was used immediately without purification. No trace of isomerization was detected by TLC. TLC: 15% EtOAc/hexanes, Rf= 0.38. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.06 (m, 1H, H2), 4.30 (dd, 1H, H1a, J_{ab}= 12Hz, J_{ax}= 4.4Hz), 4.21 (dd, 1H, H1b, J_{ab}=12Hz, J_{bx}= 5.8Hz), 3.70 (d, 2H, H3, J= 5Hz), 3.57 (m, 1H, SiOCH), 2.31 (m, 4H, 2 CH_2COO), 1.8-1.2 (54H, CH_2), 0.87 (s, 15H, 2 CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$).

1-Palmitoyl-3-(5-O-TBDMS-stearoyl)-sn-glycerol. (46).

From Method A above. TLC: 15% EtOAc/hexanes, Rf= 0.44. ^{1}H NMR (200 MHz, $CDCl_3$): δ 4.3-4.0 (m, 5H, 2 H1, H2, 2 H3), 3.57 (m, 1H, SiOCH), 2,33 (t, 4H, 2 CH_2COO), 1.8-1.2 (54H, CH_2), 0.87 (s, 15H, 2 CH_3, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$).

2-Hydroxyethyl linoleate. (48)

Linoleic acid (1.35 g, 4.8 mmol) was dissolved in 90 ml ethylene glycol, and trimethylsilyl chloride (3.0 ml, 2.6 g, 24 mmol) added. After stirring overnight, water was added, and the product extracted with ethyl acetate (3 X 250 ml), washed with water, satd $NaHCO_3$, brine and dried with $MgSO_4$. After chromatography (25% EtOAc/hexane) 1.45 g product (93%) is isolated. TLC (EtOAc/Hexane 1:1) : Rf = 0.6. ^1H NMR (200 MHz, $CDCl_3$): δ 5.34 (m, 4H, H9, H10, H12, H13), 4.21 (t, 2H, $COOCH_2$, J=5 Hz), 3.82 (t, 2H, CH_2OH, J=5Hz), 2.76 (t, 2H, H11, J=6Hz), 2.34 (t, 2H, CH_2COO, J=8Hz), 2.03 (q, 4H, H8, H14, J=7Hz), 1.7-1.2 (16H, CH2), 0.88 (t, 3H, CH_3, J=5Hz).

(2-Cyanoethyl)-(2-bromoethyl)-(N,N-diisopropylamino)-phosphoramidite. (51).

To a solution of 0.390 ml (1.75 mmol) (2-cyanoethyl)-(N,N-diisopropylamino)-chlorophosphite **50** in 10 ml dry CH_2Cl_2 was added dropwise 0.25 ml (1.78 mmol) triethylamine, followed by 0.125 ml (1.75 mmol) 2-bromoethanol at rt. Stirring was continued for 1 hr, and the solvent pumped off at low temperature. The residue was redissolved in diethyl ether and filtered under an inert atmosphere to remove the amine hydrochloride. Removal of the ether at low temperature gave the crude product as an oil, which was used in the next step immediately.

2-[(2-Bromoethyl)-(2-cyanoethyl)-phospho]-ethyl linoleate. (52).

The alcohol **48** (0.114 g, 0.35 mmol) and phosphoramidite **51** (0.52 mmol prepared fresh) were dissolved in 2.5 ml dry acetonitrile. A solution of tetrazole in acetonitrile (0.060 g, 0.86 mmol in 2.5 ml) was then added. After 5 min, TLC showed the starting material had been converted to the phosphite triester (EtOAc/Hex 1:1, Rf = 0.8). Pyridine was added (0.25 ml) and a solution of iodine in THF/water (2:1, 0.5M) was added dropwise until the yellow color persisted. The product was extracted with EtOAc (3 X 50 ml), washed with 5% $NaHSO_3$, water, 0.01N HCl, brine and dried with $MgSO_4$. The product was purified by flash chromatography (0.157g, 0.30mmol, 86%). TLC: EtOAc/Hex 1:1, Rf = 0.8. ^1H NMR (200 MHz, $CDCl_3$): δ 5.34 (m, 4H, H9, H10, H12, H13), 4.32 [m, 8H, $COOCH_2CH_2OP(O)(OCH_2)(OCH_2)$], 3.72 (t, 2H, CH_2Br, J=6Hz), 2.8 (m, 4H, CH_2CN, H11), 2.34 (t, 2H, CH_2COO, J=8Hz), 2.03 (q, 4H, H8, H14, J=6Hz), 1.7-1.2 (16H, CH2), 0.88 (t, 3H, CH_3 ,J=4Hz). ^{13}C NMR ($CDCl_3$, 75.4MHz): δ 173.4, 130.2, 130.0, 128.0, 127.9, 116.2, 67.5 (J= 5Hz), 66.2 (J=

6Hz), 62.4 (J= 7Hz), 62.1 (J= 5Hz), 42.4, 42.3, 34.0, 31.5, 29.6, 29.3, 29.2, 29.1, 29.0, 28.9, 27.2, 25.6, 24.8, 24.7, 22.5, 19.7, 19.6.

2-(Phosphatidylcholine)-ethyl linoleate. (53)

The phosphate triester **52** (0.132 g, 0.254 mmol) was dissolved in 10 ml dry acetonitrile, and added to 2 ml trimethylamine condensed in a pressure bottle. The container was sealed, and heated to 65 °C for 18 hrs. The excess amine was removed, and the remaining gum was dissolved in 100 ml $CHCl_3$/methanol (2:1), washed with 0.01N HCl, and the solvents evaporated. The residual water was removed by azeotropic distillation of toluene (3 X 25 ml). The residue was chromatographed with $CHCl_3$/methanol/water (65:25:4), to give 0.120 g product (0.245 mmol, 96%). TLC ($CHCl_3$/methanol/water 65:25:4), Rf = 0.25. ^1H NMR (200 MHz, CDCl3): δ 5.36 (m, 4H, H9, H10, H12, H13), 4.4, 4.3, 4.1 (br, 8H, $COOCH_2CH_2$, $POCH_2CH_2N$), 3.45 (s, 9H, $N(CH_3)_3$), 2.78 (t, 2H, H11, J=6Hz), 2.32 (t, 2H, CH_2COO, J=8Hz), 2.05 (q, 4H, H8, H14, J=6Hz), 1.8-1.2 (16H, CH_2), 0.88 (t, 3H, CH3, J=5Hz).

1-Palmitoyl-2-(5-O-TBDMS-stearoyl)-sn-glycero-3-[(2-bromoethyl)-(2-cyanoethyl)-phosphate]. (54).

A solution of 0.47 mmol 1,2-diacylglycerol **45** (prepared immediately prior to use by method B from 0.359 g vinyl ether **44**) in 5 ml CH_3CN was treated with 1.5 mmol **51** (also freshly prepared), followed by a solution of 0.175 g tetrazole (2.5 mmol) in 5 ml CH_3CN. Once the alcohol was consumed, 1.0 ml pyridine was added, followed by a solution of iodine in THF/water 4:1 (0.26 g I_2/ml) until the color of iodine persisted. The solution was extracted with EtOAc (3 X75 ml), washed with 5% $NaHSO_3$, 0.01N HCl, water, sat $NaHCO_3$, brine and dried with $MgSO_4$. TLC: 50% EtOAc/hexanes, Rf= 0.48. ^1H NMR (200 MHz, CDCl3): δ 5.24 (m, 1H, H2), 4.4-4.0 (m, 8H, 2H1, 2H3, OCH_2CH_2CN, OCH_2CH_2Br), 3,70 (t, 2H, CH_2Br, J= 6Hz), 3.62 (m, 1H, SiOCH), 2.76 (t, 2H, CH_2CN, J= 6Hz), 2.31 (q, 4H, CH_2COO), 1.8-1.1 (54H, CH_2), 0.86 (s, 15H, $2CH_3$, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$. ^{13}C NMR ($CDCl_3$, 75.4MHz): δ 173.2, 172.6, 116.1, 71.9, 69.2 (J= 7Hz), 67.3 (J= 5Hz), 66.1 (J= 5Hz), 62.2 (J= 5Hz), 61.4, 37.1, 36.3, 34.2, 34.0, 31.9, 29.9, 29.7, 29.5, 29.4, 29.3, 29.1, 25.9, 25.3, 24.8, 22.7, 22.4, 20.6, 19.7, 19.6, 18.1, 14.1.

1-Palmitoyl-2-[(2-bromoethyl)-(2-cyanoethyl)-phospho]-sn-glycero-3-(5-O-TBDMS-stearate). (54b)

Isolated from the reaction of mixtures of **45** and **46** with **51** under the same conditions as above. TLC: 50% EtOAc/hexanes, Rf= 0.60. ^1H NMR (200 MHz, CDCl3): δ 4.77 (m,

1H, H2), 4.4-4.1 (m, 8H, 2H1, 2H3, OCH_2CH_2CN, OCH_2CH_2Br), 3,70 (t, 2H, CH_2Br, J= 6Hz), 3.62 (m, 1H, SiOCH), 2.77 (t, 2H, CH_2CN, J= 6Hz), 2.33 (t, 4H, CH_2COO), 1.8-1.1 (54H, CH_2), 0.86 (s, 15H, $2CH_3$, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$. ^{13}C NMR ($CDCl_3$, 75.4MHz): δ 173.1, 173.0, 116.1, 74.7 (J= 6Hz), 71.9, 67.6 (J= 6Hz), 62.5 (J= 4Hz), 62.2 (J= 5Hz), 42.3, 42.2, 37.0, 36.3, 36.2, 34.1, 34.0, 31.9, 29.8, 29.7, 29.5, 29.3, 29.1, 29.0, 25.9, 25.8, 25.3, 24.8, 22.7, 20.6, 19.6, 19.5, 18.1, 14.1.

1-Palmitoyl-2-(5-O-TBDMS-stearoyl)-sn-glycero-3-phosphocholine, (55).

A solution of phosphate triester **54** (0.440 g, 0.465 mmol) and NaI (0.20 g, 1.3 mmol) in 15 ml CH_3CN was added to ≈ 5 ml condensed trimethylamine in a pressure vessel. The solution was heated in a sealed tube overnight. The trimethylamine was distilled off and the residue dissolved in $CHCl_3$/methanol 2:1, washed with 0.1 N HCl and methanol/water 2:1. The solvent was evaporated and the residue dried by azeotroping with toluene. The product was purified by flash chromatography with $CHCl_3$/methanol/water 65:25:4 to give 0.37 g **55** (0.43 mmol, 92%). ^1H NMR (200 MHz, 2:1 $CD_3OD/CDCl_3$): δ 5.1 (br s, 1H, H2), 4.3 and 4.0 (2 dd, 2H, 2 H1), 4.15 (br s, 2H, $POCH_2CH_2N$), 3.85 (t, 2H, 2 H3), 3.5 (m, 3H, SiOCH, CH_2N), 3.1 (s, 9H, $N(CH_3)_3$), 2.2 (q, 4H, 2 CH_2COO), 1.6-1.1 (54H, CH_2), 0.76 (s, 15H, 2 CH3, $SiC(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 75.4MHz): δ 173.5, 172.9, 71.9, 70.5, 70.4 (J=6Hz), 66.3 (J= 4Hz), 63.3 (J= 5Hz), 63.0, 59.3 (J= 4Hz), 54.3, 37.1, 36.4, 34.4, 34.1, 31.9, 30.0, 29.9, 29.7, 29.6, 29.3, 29.2, 26.1, 25.9, 25.3, 24.8, 22.7, 20.8, 18.1, 14.1.

1-Palmitoyl-2-(5-hydroxystearoyl)-sn-glycero-3-phosphocholine, (56).

The silyl ether **55** (0.37 g, 0.43 mmol) was left at room temperature in the solid state for 21 days. A characteristic earthy smell of silanol developed. The product was purified by flash chromatography and was found to have desilylated spontaneously to give 0.100 g (0.116 mmol, 25%) hydroxy phosphatidylcholine **56**. ^1H NMR (200 MHz, 2:1 $CD_3OD/CDCl_3$): δ 5.2 (br s, 1H, H2), 4.4-4.0 (br m, 4H, 2 H1, $POCH_2CH_2N$) 3.9 (br m, 2H, 2 H3), 3.6 (br m, 2H, CH_2N), 3.55 (br m, 1H, HOCH), 3.15 (s, 9H, $N(CH_3)_3$), 2.2 (br m, 4H, 2 CH_2COO), 1.6-1.1 (54H, CH_2), 0.8 (br t, 6H, 2 CH_3). ^{13}C NMR (75.4 MHz, $CDCl_3/CD_3OD$ 2:1): δ 173.7, 173.4, 70.4 (J= 7Hz), 66.2 (J=

4Hz), 63.8 (J= 5Hz), 62.4, 59.0 (J= 5Hz), 54.2, 37.2, 37.1, 36.4, 34.1, 33.9, 31.8, 29.7, 29.6, 29.4, 29.3, 29.2, 29.0, 25.7, 24.7, 22.6, 21.1, 14.0.

3-Benzyl-sn-glycerol. (61).

This product was made by following the method for **33**, using benzyl bromide instead of allyl bromide.

1,2-Dilinoleoyl-3-benzyl-sn-glycerol. (62).

The same procedure as for **31**was followed. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.3 (5H, ArH), 5.5-5.2 (m, 9H, H2, 4 HC=CH), 4.53 (s, 2H, CH_2Ar), 4.33 (dd, 1H, H1a, J_{ab}= 12Hz, J_{ax}= 3.8Hz), 4.18 (dd, 1H, H1b, J_{ab}= 12Hz, J_{bx}= 6.4Hz), 3.58 (d, 2H, 2 H3, J= 5Hz), 2.76 (t, 4H, 2 C=C-CH_2-C=C, J= 6Hz), 2.29 (q, 4H, 2 CH_2COO, J= 8Hz), 2.04 (q, 8H, 2 CH_2C=C, 2 C=CCH_2), 1.8-1.2 (32H, CH_2), 0.88 (t, 6H, 2 CH3, J= 5Hz).

13-Tetradecyn-1-ol. (63a)

A suspension of lithium wire (1.05 g, 150 mmoles) in 75 ml 1,3-diaminopropane (distilled under nitrogen from barium oxide and stored over 4 Å molecular sieve) was heated at 70°C until the blue color discharged (2 h). The reaction mixture was cooled to rt and potassium tert-butoxide (11.2 g, 100 mmoles) was added. The resultant mixture was stirred for 30 min and then 3-tetradecyn-1-ol (5.25 g, 25 mmoles, solubilized in 5 ml diaminopropane) was injected *via* syringe. After 45 minutes, the mixture was carefully hydrolysed with ice-cold H_2O, acidified with an aqueous solution of 10% HCl and extracted with hexane (3X200 ml). The organic phase was washed with sat aqueous sodium bicarbonate and brine, dried over $MgSO_4$ and evaporated. The crude product was chromatographed on a silica gel column (CH_2Cl_2, then hexane/ EtOAc 8:2) and recrystallized at -18°C (hexane) to yield the product (4.472 g; 75%, mp 37-40°C). TLC (hexane/ EtOAc 8:2): Rf:0.28. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.36 (t, 2H, CH_2OH, J=7Hz), 2.20 (dt, 2H, C≡CCH_2, J=7Hz, J=4Hz), 1.95 (t, 1H, HC≡C-, J=4Hz), 1.6-1.2 (m, 20H, CH_2). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 84.8, 68.0, 63.0, 32.8, 29.6, 29.6, 29.4, 29.1, 28.7, 28.5, 25.7, 18.4. Anal. Calcd for $C_{14}H_{26}O$: C, 79.9; H, 12.5. Found: C, 79.7; H, 12.7.

15-Hexadecyn-1-ol. (63b).

The procedure for **63a** was followed, using 7-hexadecyn-1-ol as starting material. The product was converted directly to **64b** by oxidation with Jones reagent as described below.

13-Tetradecyn-1-oic acid . (64a).

To a solution of 13-tetradecyn-1-ol **63a** (3.0 g, 14.3 mmoles) in 150 ml acetone was added dropwise a solution of Jones reagent until the characteristic orange color of the reagent persisted. Isopropanol was added to neutralize the excess reagent. The chromium salts were filtered, the acetone evaporated and the residue dissolved in EtOAc , washed three times with 0.01N HCl, dried over $MgSO_4$ and evaporated. The crude product was recrystallized in hexane at -18°C to yield 4a (2.614 g, 82%). TLC (hexane/EtOAc 1:1), R_f=0.5. mp 45-47°C. ^{1}NMR (200MHz, $CDCl_3$): δ 2.35 (t, 2H, CH_2CO, J=7Hz), 2.20 (dt, 2H, $C{\equiv}CCH_2$, J=7Hz and J=4Hz), 1.95 (t, 1H, $HC{\equiv}C$, J=4Hz), 1.7-1.3 (m, 4H, CH_2CH_2COO and $CH_2CH_2C{\equiv}C$), 1.25 (s, 14H, CH_2). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 179.6, 84.8, 68.0, 33.9, 29.5, 29.4, 29.3, 29.2, 29.1, 28.7, 28.5, 24.7, 18.4.

15-Hexadecyn-1-oic acid. (64b).

The procedure for **64a** was followed. ^{1}H NMR (200MHz, $CDCl_3$): δ 2.35 (t, 2H, CH_2CO, J=7Hz), 2.20 (dt, 2H, $C{\equiv}CCH_2$, J=7Hz and J=4Hz), 1.95 (t, 1H, $HC{\equiv}C$, J=4Hz), 1.7-1.3 (m, 4H, CH_2CH_2COO and $CH_2CH_2C{\equiv}C$), 1.25 (s, 18H, CH_2). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 180.0, 84.8, 68.0, 34.0, 29.6, 29.5, 29.4, 29.2, 29.1, 29.0, 28.7, 28.5, 24.7, 18.4.

1,2-Di-(13-tetradecyn-1-oyl)-3-(4-methoxybenzyl)-sn-glycerol. (65).

DCC(0.94g, 4.6 mmol) and DMAP (0.52g, 4.3 mmol) in 5 ml dry CH_2Cl_2 were added to a solution of PMB glycerol**30** (0.40g, 1.9 mmol) and 13-tetradecynoic acid (0.965g, 4.3 mmol) in 15 ml CH_2Cl_2 at rt in an inert atmosphere. After 2 h the urea precipitate was filtered, the solvent evaporated, and the residue purified by flash chromatography to give 0.917g (1.47mmol, 77%) **65** as a waxy solid. TLC (hexane/EtOAc 9:1) R_f:0.34. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.22 (d,2H, ArH, J=8.6Hz), 6.86 (d,2H, ArH, J=8.6Hz), 5.21 (m, 1H, H_2, J_{23}=5.1Hz, J_{1a2}=3.8Hz, J_{1b2}=6.4Hz), 4.46 (ABq,2H, ArCH2), 4.31 (q, 1H, H_{1a}, J_{ab}=11.9Hz, J_{1a2}=3.8Hz), 4.17 (q, 1H, H1b, Jab=11.9Hz, J1b2=6.4Hz), 3.78 (s, 3H, $ArOCH_3$), 3.54 (d, 2H, H_3, J_{23}=5.1Hz), 2.29 (m, 4H, C_1OCOCH_2, C_2OCOCH_2), 2.17 (dt, 4H, $CH_2C{\equiv}CH$, ^{3}J=6.8Hz, ^{4}J=2.7Hz), 1.93 (t, 2H, $CH_2C{\equiv}CH$, ^{4}J=2.7Hz), 1.8-1.2 (36H, CH_2). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 173.4, 173.1, 159.3, 129.7, 129.3, 113.8, 84.8, 72.9, 70.0, 68.0, 67.9, 62.7, 55.2, 34.3, 34.1, 29.6, 29.5, 29.4, 29.3, 29.1, 28.9, 28.7, 28.6, 28.5, 24.9, 24.8, 18.4. Anal. Calcd for $C_{39}H_{60}O_6$: C, 74.96; H, 9.68. Found: C, 74.82; H9.96.

MS (CI): m/z 642 ($M+NH_4^+$, 100%), 625 ($M+H^+$, 42%). HRMS (CI): calcd for $C_{39}H_{61}O_6$: 625.44681; Found: 625.44651.

1,2-(13,15-octacosadiyn-1,28-dioyl)-3-(4-methoxybenzyl)-sn-glycerol. PMB-Macrocycle. (66).

A solution of bis-acetylenic diacylglycerol **65** (0.55g, 0.88 mmol) in 10 ml freshly distilled pyridine was added over a period of 4 h via syringe pump to a gently refluxing solution of $Cu(OAc)_2 \cdot H_2O$ in 60 ml pyridine. Heating was continued one hour after the addition was complete, at which time most of the pyridine was distilled *in vacuo* at rt. The residue was diluted with H_2O (50 ml), acidified to pH 2 with 1N HCl, extracted with EtOAc (3X100 ml). The organic layers were washed with successive portions of 0.05N HCl, sat sodium bicarbonate and brine, and the combined organic phases were dried with $MgSO_4$ and evaporated. After flash chromatography (hexanes/EtOAc 9:1) 0.30g (0.48 mmol, 54%) cyclic product was obtained. TLC (hexane/EtOAc 9:1) R_f: 0 29. ^1H NMR (200 MHz, $CDCl_3$): δ 7.22 (d, 2H, ArH, J=8.7Hz), 6.86 (d, 2h, ArH, J=8.7Hz), 5.21 (m, 1H, H_2, J_{12}= 3.7, 6.6Hz, J_{23}=5.3Hz), 4.46 (s, 2H, $ArCH_2$), 4.32 (dd, 1H, H_{1a}, J_{ab}=11.8Hz, J_{12}=3.7 Hz), 4.16 (dd, 1H, H_{1b}, J_{ab}=11.8Hz, J_{12}=6 6Hz), 3 79 (s, 3H, OCH_3), 3.54 (d, 2H, H_3, J_{23}=5.3Hz), 2.4-2 1 (m, 8H, C_1OCOCH_2, C_2OCOCH_2, $CH_2C{\equiv}C\text{-}C{\equiv}C\text{-}CH_2$), 1.8-1.2 (m, 36H, CH_2). ^{13}C NMR (75.4 MHz, $CDCL_3$). δ 173.3, 173.0, 159.2, 129.7, 129.2, 113.8, 72.9, 70.0, 67.9, 65.5, 62.8, 55.2, 34.3, 34.1, 29.3, 29.2, 29.1, 29.0, 28.8, 28.7, 28.3, 28.2, 27.8, 25.8, 25.7, 19.0. MS (CI): m/z 640 ($M+NH_4^+$, 100%), 623 ($M+H^+$, 69%). HRMS (CI)· calcd for $C_{39}H_{59}O_6$: 623.43116. Found: 623.43089

1,2-(13,15-octacosadiyn-1,28-dioyl)-sn-glycerol. Macrocycle glycerol. (67).

Dimethylboron bromide (0.068ml, 0.7 mmol) was added to a solution of PMB ether **66** (0.30g, 0.48 mmol) in 5 ml anhyd CH_2Cl_2 at -78°C. After 5 minutes, the solution was quenched with 100 ml diethyl ether and washed with distilled H_2O until neutral. The organic phase was dried with $MgSO_4$, the solvent removed *in vacuo*, and the product used immediately in the next step. TLC (hexane/EtOAc 3:1) R_f: 0.28.

1,2-(13,15-octacosadiyn-1,28-dioyl)-sn-glycero-3-[(2-bromoethyl)-(2-cyanoethyl)-phosphate]. Macrocycle phosphate triester. (68).

To a solution of diacylglycerol **67** (0.48 mmol) and dialkyl phosphoramidite **51** (1.0 mmol) in 5 ml anhyd acetonitrile at rt was added a solution of tetrazole (0.105g, 1.5

mmol) in 5 ml acetonitrile. After 15 min, TLC showed complete disappearance of the starting material. Aqueous hydrogen peroxide (0.2ml 30% H_2O_2, 6.5 mmol) was then added, and stirring continued for 1 h. After completion of the oxidation,100 ml H_2O was added, and the solution was extracted with CH_2Cl_2 (2X200 ml), washed with 0.05N HCl, sat sodium bicarbonate solution, brine, and the organic layer dried with $MgSO_4$. Evaporation of the solvent and flash chomatography of the residue using EtOAc/hexane 3:1 gave 0.288g (0.39 mmol, 81%) phosphate triester. TLC (hexane/EtOAc 1:1) R_f:0.14. ^1H NMR (200 MHz, $CDCl_3$): δ 5.28 (m, 1H, H_2), 4.5-4.0 (m, 8H, $2H_1$, $2H_3$, $POCH_2CH_2Br$, $POCH_2CH_2CN$), 3.55 (t, 2H, CH_2Br, J=6.0 Hz), 2.78 (t, 2H, CH_2CN, J=6.0 Hz), 2.4-2.2 (m, 8H, C_1OCOCH_2, C_2OCOCH_2, $CH_2C{\equiv}C\text{-}C{\equiv}C\text{-}CH_2$), 1.7-1.2 (m, 36H, CH_2). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 173.2, 172.8, 116.2, 69.2 ($^3J_{PC}$=8Hz), 67.4 ($^2J_{PC}$=6Hz), 66.1 ($^2J_{PC}$=6Hz), 65.5, 62.3 ($^2J_{PC}$=5Hz), 61.6, 34.2, 34.0, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 28.8, 28.4, 28.3, 27.9, 24.8, 19.7 ($^3J_{PC}$=7Hz), 19.1. FABMS (NBA): m/z 745 ($M+H^+$, 3.6%), 743 ($M+H^+$, 4.0%), 486 (100%). Anal. Calcd for $C_{36}H_{57}NO_8PBr$: C, 58.22; H, 7.74; N, 1.89; P, 4.17. Found: C, 57.68; H, 7.75; N, 2.00; P, 4.24.

1,2-(13,15-Octacosadiyn-1,28-dioyl)-sn-glycero-3-phosphatidylcholine. Macrocycle Phosphatidylcholine. (69).

A solution of phosphate triester **68** (288 mg, 0.39 mmol) in 5 ml dry acetonitrile was added to 10 ml anhyd trimethylamine at -78°C in a pressure bottle, followed by a solution of sodium iodide (0.080g, 0.53 mmol) in 5 ml acetonitrile. The bottle was sealed and the solution stirred at 65°C overnight. The excess trimethylamine was distilled off, the solution was acidified to pH 2 with 1N HCl, and extracted with 2:1 $CHCl_3$/MeOH (3X200 ml). The combined organic layers were washed once with pH 2 MeOH/H_2O 1:1 (200ml), evaporated, and the H_2O removed from the residue by azeotropic distillation with toluene under reduced pressure. The product was purified by flash chromatography with $CHCl_3$/MeOH/H_2O 65:25:4, giving 0.221g (0.33 mmol, 85%) of the title product. The overall yield from 15 was 69%. TLC ($CHCl_3$/MeOH/H_2O 65:25:4) R_f:0.5. ^1H NMR (200 MHz, $CDCl_3$): δ 5.22 (m, 1H, H_2), 4.5-4.2 (br m, 3H, H_{1a}, $POCH_2$), 4.12 (br dd, 1H, H_{1b}), 3.93 (br t, 2H, H_3), 3.80 (br s, 2H, CH_2N), 3.35 (s, 9H, $N(CH_3)_3$), 2.4-2.2 (m, 8H, C_1OCOCH_2, C_2OCOCH_2, $CH_2C{\equiv}C\text{-}C{\equiv}C\text{-}CH_2$), 1.7-1.2 (m, 36H, CH_2). ^{13}C NMR (75.4 MHz, $CDCL_3$): δ 173.5, 173.2, 70.5 ($^3J_{PC}$=8Hz), 66.3, 65.6, 63.4 ($^2J_{PC}$=5Hz), 63.0, 59.3 ($^2J_{PC}$=4Hz), 54.4, 34.4, 34.2, 29.5, 29.3, 29.1, 29.0, 28.9, 28.8, 28.7, 28.3, 28.2, 27.9, 27.8, 24.9, 24.8, 19.1, 19.0. FABMS (NBA): m/z 669 ($M+H^+$, 35.6%), 184 (100%). Anal.

Calcd for $C_{36}H_{62}NO_8P$: C, 64.74; H, 9.36; N, 2.10; P, 4.64. Found: C, 62.80; H, 9.47; N, 2.18; P, 5.41.

1-Palmitoyl-2-(15-hexadecyn-1-oyl)-3-(4-methoxybenzyl)-sn-glycerol. (71).

A solution of DCC (0.250g, 1.2 mmol) and DMAP (0.060g, 0.5 mmol) in 5ml dry CH_2Cl_2 was added to a solution of 15-hexadecynoic acid (0.255g, 1.0 mmol) and **70** (0.410g, 0.91mmol) in 5 ml CH_2Cl_2 at rt. Stirring was continued 5h until the starting material had disappeared. The solution was filtered and the solvent removed. Flash chromatography of the residue (hexane/EtOAc 9:1) gave 0.617g (99%) of the product as a waxy solid. TLC (hexane/EtOAc 3:1) R_f:0.78. ^1H NMR (200 MHz, $CDCl_3$): δ 7.22 (d, 2H, ArH, J=8.6 Hz), 6.86 (d, 2H, ArH, J=8.6 Hz), 5.21 (ddd, 1H, H_2, J_{23}=5.2Hz, J_{12}=3.7 Hz, J_{12}=6.5Hz), 4.46 (ABq, 2H, ArCH2), 4.32 (dd, 1H, H_{1a}, J_{ab}=11.8Hz, J_{12}=3.7Hz), 4.17 (dd, 1H, H_{1b}, J_{ab}=11.8Hz, J_{12}=6 5Hz), 3 80 (s, 3H, $ArOCH_3$), 3.54 (d, 2H, H_3, J=5.2Hz), 2.24 (m, 4H, C_1OCOCH_2, C_2OCOCH_2), 2.17 (dt, 2H, $CH_2C{\equiv}CH$, J=2.7, 7.0Hz), 1.93 (t, 1H, $CH_2C{\equiv}CH$, J=2.7Hz), 1.8-1.4 (m, 6H, $C_1OCOCH_2CH_2$, $C_2OCOCH_2CH_2$, $CH_2CH_2C{\equiv}C$), 1.25 (s, 42H, CH_2), 0.87 (t, 3H, CH_3, J=6.7Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 173.4, 173.1, 159.2, 129.7, 129.2, 113.8, 72.9, 70.0, 68.0, 67.8, 62.6, 55.2, 34.3, 34.1, 31.9, 29.7, 29.6, 29.4, 29.3, 29.2, 29.1, 28.7, 28.4, 24.9, 23.8, 22.6, 18.3, 14.1. FABMS (NBA): m/z 685 ($M+H^+$, 4.9%), 548 (50%), 313 (48%), 121(100%). Anal. Calcd for $C_{43}H_{72}O_6$: C, 75.17; H, 10.86. Found: C,74.95; H, 10.87.

2,2'-(15,17-Dotriacontadiyn-1,32-dioyl)-bis-(1-palmitoyl-3-(4-methoxybenzyl)-sn-glycerol). PMB-Bolaform. (72).

Protected acetylenic diacylglycerol **71** (0.446g, 0.65 mmol) and $Cu(OAc)_2{\cdot}H_2O$ (1.30g, 6.5 mmol) were dissolved in 5 ml freshly distilled pyridine under argon. The blue solution was warmed to 80°C in an oil bath for 2 h. The now green solution was acidified to pH 2 with 1N HCl. The aqueous layer was extracted with EtOAc (3X100 ml), washed with 1% HCl, sat sodium bicarbonate solution, brine, dried with $MgSO_4$ and evaporated. Flash chromatography using 10% EtOAc in hexanes as eluant gave 0.297g (0.22 mmol, 68%) product as a white waxy solid. TLC (hexane/EtOAc 3:1) R_f: 0.66. ^1H NMR (200 MHz, $CDCl_3$): δ 7.22 (d, 2H, ArH, J=8.6Hz), 6.85 (d, 2H, ArH, J=8.6Hz), 5.21 (m, 2H, H_2, J_{23}= 5.2Hz, J_{ax}= 3.7Hz, J_{bx}=6.6Hz), 4.45 (s, 4H, ArCH2), 4.31 (dd, 2H, H_{1a}, J_{ab}=11.8Hz, J_{ax}=3.7Hz), 4.16 (dd, 2H, H_{1b}, J_{ab}=11.8Hz, J_{bx}=6.6Hz), 3.79 (s, 6H, $ArOCH_3$), 3.54 (d, 4H, H_3, J_{23}= 5.2Hz),

2.4-2.1 (m, 12H, C_1OCOCH_2, C_2OCOCH_2, $CH_2C{\equiv}C$), 1.8-1.4 (m, 12H, $C_1OCOCH_2CH_2$, $C_2OCOCH_2CH_2$, $CH_2CH_2C{\equiv}C$), 1.24 (s, 96H, CH_2), 0.87 (t, 6H, CH3, J=6.7Hz). ^{13}C NMR (75.4 MHz, $CDCL_3$): δ 173.3, 173.0, 159.2, 129.7, 129.2, 113.7, 72.9, 69.4, 67.8, 65.2, 62.6, 55.2, 34.3, 34.2, 31.9, 39.6, 29.4, 29.3, 29.2, 29.1, 28.8, 28.3, 24.9, 24.8, 22.6, 19.2, 14.1. FABMS (NBA): m/z 1369 ($M+H^+$,0.6%), 1367 (0.7%), 1230 (0.9%), 1110 (2.3%), 313 (11.4%).

2,2'-(15,17-Dotriacontadiyn-1,32-dioyl)-bis-(1-palmitoyl-sn-glycerol). Bolaform alcohol. (73)

A solution of bis-PMB ether **72** (0.567g, 0.414 mmol) in 5 ml dry CH_2Cl_2 was cooled to -78°C under Ar. Dimethylboron bromide (0.150 ml, 1.5 mmol) was added neat, and the suspension of bolaform was warmed to rt. As soon as the solution became clear (5 min) it was quenched with 50 ml diethyl ether. The ether layer was washed with distilled H_2O until the washings were neutral (4X50 ml). After drying with $MgSO_4$ and evaporation under reduced pressure, the crude bis-alcohol **73** was used immediately without purification in the phosphitylation reaction. TLC (hexane/EtOAc 1:1) R_f:0.55.

2,2'-(15,17-Dotriacontadiyn-1,32-dioyl)-bis-{1-palmitoyl-sn-glycero-3-[(2-bromoethyl)-(2-cyanoethyl)-phosphate]}. Bolaform bis(phosphate triester). (74).

The crude bolaform diol **73** (0.414 mmol) was dissolved in 10 ml 1:1 acetonitrile/ THF, and a solution of freshly prepared phosphoramidite **51** (1.75 mmol) in 5 ml THF was added. Solid tetrazole (0.14g, 2.0 mmol) was then added, and the solution stirred for 1 h until the starting material had disappeared. The solution was treated with 0.25 ml 30% H_2O_2 (8.2 mmol) and stirring continued for a further 1 h. When the oxidation was judged complete by TLC, 100 ml H_2O was added, and the solution was extracted with CH_2Cl_2 (2X200 ml), washed with 0.05N HCl, sat sodium bicarbonate solution, brine, and the organic layer dried with $MgSO_4$. Evaporation of the solvent and flash chromatography of the residue using EtOAc/hexane 3:1 gave 0.432g (2.7 mmol, 65%) of the bis phosphate triester. TLC (EtOAc) R_f: 0.60. ^1H NMR (200 MHz, $CDCl_3$): δ 5.25 (m, 2H, H_2), 4.5-4.1 (m, 16H, $POCH_2CH_2Br$, $POCH_2CH_2CN$, H_3, H_1), 3.55 (t, 4H, CH_2Br), 2.87 (t, 4H, CH_2CN), 2.4-2.2 (m, 12H, CH_2COO, $CH_2C{\equiv}C$), 1.7-1.5 (m, 12H, 2 $C_1OCOCH_2CH_2$, 2 $C_2OCOCH_2CH_2$, 2 $CH_2CH_2C{\equiv}C$), 1.30 (s, 96H, CH_2), 0.85 (t, 6H, CH_3, J=6.6Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 173.2, 172.8, 116.2, 69.2 ($^3J_{PC}$=7Hz), 67.4 ($^2J_{PC}$=6Hz), 66.1 ($^2J_{PC}$=6Hz), 65.2, 62.3 ($^2J_{PC}$=5Hz), 61.4,

34.1, 34.0, 31.9, 30.4, 30.3, 29.6, 29.5, 29.4, 29.3, 29.1, 28.9, 28.6, 28.5, 28.4, 24.8, 22.7, 22.5, 22.4, 19.7 ($^4J_{PC}$=7Hz), 19.2, 14.1.

2,2'-(15,17-Dotriacontadiyn-1,32-dioyl)-bis-(1-palmitoyl-sn-glycero-3-phosphatidylcholine). Bolaform-PC. (75).

Trimethylamine (10 ml) was condensed in a pressure vessel at -78°C, and a solution of phosphate triester **74** (0.364g, 0.225 mmol) in 5 ml toluene was added, followed by sodium iodide (0.041g, 0.27 mmol) in 6 ml dry acetonitrile. The bottle was sealed and the solution stirred at 65°C overnight. The excess trimethylamine was distilled off, the solution was acidified to pH 2 with 1N HCl, and extracted with 2:1 $CHCl_3$/MeOH (3X200 ml). The combined organic layers were washed once with pH 2 MeOH/H_2O 1:1 (200ml), evaporated, and the H_2O removed from the residue by azeotropic distillation with toluene under reduced pressure. The product was purified by flash chromatography with $CHCl_3$/MeOH/H_2O 65:25:4, then 65:35:6, yielding 0.185g (0.127 mmol, 56%) bolaform as a tan solid. Overall yield from **72** was 36%. TLC ($CHCl_3$/MeOH/H_2O 65:25:4) R_f:0.08. ^{1}H NMR (200 MHz, $CDCl_3$/CD_3OD 2:1): δ 5.00 (m, 2H, H_2), 4.20 (dd, 2H, H_{1a}), 4.02 (m, 4H, $POCH_2CH_2N$), 3.90 (dd, 2H, H_{1b}), 3.78 (t, 4H, H_3), 3.38 (t, 4H, $POCH_2CH_2N$), 3.00 (s, 18H, $N(CH_3)_3$), 2.2-1.9 (m, 12H, C_1OCOCH_2, C_2OCOCH_2, $CH_2C{\equiv}C$), 1.5-1.2 (m, 12H, $C_1OCOCH_2CH_2$, $C_2OCOCH_2CH_2$, $CH_2CH_2C{\equiv}C$), 1.05 (s, 96H, CH_2), 0.68 (t, 6H, CH3). ^{13}C NMR (75.4 MHz, $CDCl_3$/CD_3OD 2:1): δ 173.6, 173.2, 70.0 ($^3J_{PC}$=9Hz), 66.0 ($^3J_{PC}$=6Hz), 65.2, 63.2 ($^2J_{PC}$=4Hz), 62.3, 58.7 ($^2J_{PC}$=5Hz), 53.6, 33.8, 33.7, 31.5, 29.2, 29.1, 28.9, 28.8, 28.7, 28.4, 28.2, 28.1, 28.0, 27.9, 27.8, 24.5, 24.4, 22.2, 18.7, 13.5. Anal. Calcd for $C_{80}H_{150}N_2O_{16}P_2$: C, 65.90; H, 10.37; N, 1.92; P, 4.25. Found: C, 58.28; H, 10.33; N, 2.18; P, 4.23.

1,2-(Octacosane-1,28-dioyl)-sn-glycero-3-phosphatidylcholine. Saturated Macrocycle Phosphatidylcholine. (76).

The macrocyclic diyne PC **70** (0.0469 g, 0.0702 mmol) was dissolved in 10 ml methanol to which was added 30 mg 10% Pd on charcoal. The solution was shaken under 2 atmospheres of hydrogen for 2 hours. The solution was filtered on a short plug of silica gel and eluted with $CHCl_3$/methanol/water 65/25/4. Evaporation and removal of the water by azeotropic removal under reduced pressure gave the saturated product in 97% yield (0.0460 g, 0.068 mmol). ^{1}H NMR (200 MHz, $CDCl_3$/CD_3OD 2:1): δ 5.0 (m, 1H, H2), 4.2-3.9 (br m, 4H, 2 H1, OCH_2CH_2N), 3.8 (br t, 2H, 2 H1), 3.4 (br s, 2H, CH_2N), 3.0 (s, 9H, $N(CH_3)_3$), 2.1 (br m, 4H, 2 CH_2COO), 1.6-1.0 (48H, CH_2).

^{13}C NMR (75.4 MHz, $CDCl_3/CD_3OD$ 2:1): δ 173.6, 173.3, 70.0 (J= 8Hz), 66.0 (J= 4Hz), 63.2 (J= 5Hz), 62.4, 58.6 (J= 5Hz), 53.7, 33.9, 33.7, 29.3, 29.0, 28.9, 28.8, 28.7, 28.6, 28.5, 28.4, 28.3, 28.1, 28.0, 27.9, 27.8, 24.6, 24.5.

2,2'-(Dotriacontane-1,32-dioyl)-bis-(1-palmitoyl-sn-glycero-3-phosphatidylcholine). Saturated Bolaform-PC. (77).

A solution of diacetylenic bolaform PC **75** (0.0490 g, 0.0336 mmol) was dissolved in 10 ml methanol. Platinum oxide (PtO_2, 35 mg) was added and the solution shaken under 3 atmospheres of H_2 for 18 hours. The solution was then filtered through a short plug of silica and eluted with $CHCl_3$/methanol/water 65:35:6. The solvents were evaporated, and the product was precipitated from $CHCl_3$/methanol (minimal volume) with acetone in 85% yield (0.0420 g, 0.0286 mmol). ^1H NMR (200 MHz, $CDCl_3/CD_3OD$ 2:1): δ 5.0 (m, 2H, H2), 4.2-3.9 (br m, 8H, H1a, H1b, $POCH_2CH_2N$), 3.8 (br t, 4H, H1), 3.4 (br s, 4H, CH_2N), 3.0 (s, 18H, $N(CH_3)_3$), 2.1 (br m, 8H, 2 CH_2COO), 1.6-1.0 (108H, CH_2), 0.7 (br t, 6H, CH_3). ^{13}C NMR (75.4 MHz, $CDCl_3/CD_3OD$ 2:1): δ 173.6, 173.2, 70.0(J= 8Hz), 66.0, 63.2 (J= 5Hz), 62.3, 58.7 (J= 5Hz), 53.7, 33.8, 33.7, 32.2, 31.5, 29.3, 29.1, 28.9, 28.7, 24.5, 24.4, 22.2, 13.5.