STRUCTURE-ACTIVITY STUDIES

OF CATIONIC BILAYER STABILIZING INHIBITORS

OF PROTEIN KINASE C

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

September, 1990

MASTER OF SCIENCE (1990)   McMaster University

(Biochemistry)   Hamilton, Ontario

TITLE: Structure Activity Studies of Cationic Bilayer Stabilizing

                 Inhibitors of Protein Kinase C

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NUMBER OF PAGES: xi, 111

ABSTRACT

Several compounds possessing diverse chemical structures have been shown to inhibit the action of protein kinase C (PKC). A general property of some of these compounds is that they inhibit bilayer to hexagonal phase interconversion in phosphatidylethanolamine bilayers. In addition, a large number of PKC inhibitors are positively charged. To clarify the relationship between charge and enzyme inhibition, the effect of the cationic amphiphile sphingosine was studied at different pH's. Inhibition by sphingosine was found to be pH dependent. Above pH 7.75, sphingosine has little or no inhibitory effect. In fact, at pH 8.5 sphingosine slightly enhances enzyme activity above that which occurs when the enzyme is stimulated by diacylglycerol and phosphatidylserine. After correcting for electrostatic repulsion, the intrinsic pK for sphingosine in Triton micelles is 8.5. Inhibition of PKC by sphingosine at physiological pH's therefore correlates with the presence of positive charge. In an attempt to optimize the structural features necessary for inhibition of PKC, a number of compounds which incorporate both positive charge as well as bilayer stabilizing ability were designed. These compounds possess a hydrophobic backbone which does not perturb hydrocarbon packing and have a tertiary or quaternary nitrogen functionality in the head group. All designed amphiphiles inhibit PKC activity; the potency of the amphiphile correlates with the presence of positive charge. Quaternary ammonium bilayer stabilizers are 10 fold

more potent than their tertiary amine precursors, generally inhibiting in the 10 - 60 μM range using the Triton mixed micelle assay. Aside from charge, factors such as the structure of the amine containing head group and its length from the hydrocarbon moiety did not markedly influence inhibitor potency. In contrast, the hydrocarbon backbone did influence potency. Cationic amphiphiles containing a steroid backbone were more potent than their straight chain analogues. These amphiphiles do not appear to alter the partitioning of PKC from the aqueous phase to the membrane surface. A number of bilayer stabilizing compounds possessing carboxylate and sulfate anions in addition to the quaternary nitrogen functionality were also designed. Although inhibitor potency correlated with the amount of charge present on the amphiphile, charge could not account for all the observed effects. Changes in the position of the charged functionalities and hydrocarbon length resulted in marked differences in amphiphile potency. Some inhibited in the submicromolar range. The results of these studies suggest that a combination of positive charge and a bilayer stabilizing structural characterisitic provides a basis for the rational design of PKC inhibitors.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ACS	aqueous counting scintillant
bp	boiling point
BSA	bovine serum albumin
CDI	1,1' carbonyldiimidazole
CPD	cholesterylphosphoryldimethylethanolamine
Cp_{max}	excess specific heat
δ	chemical shift
DAG	*sn*-1,2-diacylglycerol
dansyl PE	dipalmitoyl-N-L-α-phosphatidylethanolamine
$DCI\text{-}NH_4$	direct chemical ionization using ammonia
DEPE	1,2-dielaidoyl-*sn*-glycerol-3-phosphoethanolamine
DLPE	1,2-dilinoleoyl-*sn*-glycerol-3-phosphoethanolamine
DNI	does not inhibit
DSC	differential scanning calorimetry
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGTA	ethyleneglycerol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
EI	electron impact
FAB+	positive ion fast atomic bombardment
^{1}H NMR	proton nuclear magnetic resonance
$H_{\parallel}$	hexagonal phase

HIV	human immunodeficiency virus
Lα	lamellar phase
(M)$^{+\bullet}$	molecular ion
(M+H)$^{+\bullet}$	protonated molecular ion
MDR	multiple drug resistance
mp	melting point
Mr	molecular weight
m/z	charge to mass ratio
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIP$_2$	phosphatidylinositol 4,5 bisphosphate
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PMSF	phenylmethylsulfonylfluoride
PS	phosphatidylserine
TLC	thin layer chromatography
TRIS	tris (hydroxymethyl) aminomethane
UV	ultraviolet
v/v	volume per volume

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1. INTRODUCTION

1.1 General Introduction

Reversible phosphorylation of cellular polypeptides by protein kinases and protein phosphatases has gained significant recognition as a common mechanism for modulating processes of biological importance (Hunter, 1987). With the development of gene cloning and sequencing techniques, the number of known protein kinases has exceeded seventy (Hunter, 1987). Of the second messenger dependent protein kinases, protein kinase C (PKC) has gained considerable attention because it is thought to play a regulatory role in a number of diverse physiological events such as metabolism, exocytosis, and neurotransmission, as well as growth and differentiation (Nishizuka, 1986). PKC has also been implicated in the pathogenesis of certain diseases. In particular, PKC has been suggested to play a critical role in tumour promotion (O'Brian et al., 1988; Weinstein, 1988). Cells constitutively expressing PKC display altered growth and exhibit increased tumorigenicity (Persons et al., 1988). Such cells, when transfected by oncogenes, are more susceptible to transformation (Hsiao et al., 1989). There is considerable evidence that PKC acts as the major receptor for various tumour promoters including phorbol esters, indole alkaloids and certain polyacetates, all of which

markedly enhance the activity of the enzyme at nanomolar concentrations (Nishizuka, 1984; Ashedel, 1985; Jeffrey and Liskamp, 1986). PKC has also been implicated in the P-glycoprotein type multiple drug resistance (MDR) phenotype where cells becomes insensitive to a variety of structurally unrelated cytotoxic agents. Posada and co-workers (1989a,b) reported that certain cell lines exhibiting the MDR phenotype display elevated PKC levels. When stimulated with phorbol esters, P-glycoprotein phosphorylation is enhanced (Hamada et al., 1987), and the MDR phenotype is induced (Fine et al., 1988). Others have implicated the importance of PKC for HIV entry into cells (Fields et al., 1988). As a consequence of PKC's involvement in a number of pleiotrophic biological phenomena there has been an interest in the study of the properties of currently available PKC inhibitors as well as efforts to design novel inhibitors (Hidaka et al., 1987; O'Brian et al., 1988; Weinstein, 1988; Gescher and Dale, 1989; Marasco et al., 1990; O'Brian et al., 1990). Inhibitors are of interest for various reasons. These pharmacological probes can be used to gain information on the mechanism of enzyme action as well as enzyme function in cells. In addition, it has been proposed that specific PKC antagonists could serve as antiproliferative drugs either by inhibiting tumour development (O'Brian et al., 1988; Weinstein, 1988; Gerscher and Dale, 1989), or by reversing the MDR phenotype; once again sensitizing cells to cytotoxic agents (Weisenthal et al., 1987). Furthermore, because the inhibition of PKC blocks HIV entry into CD4+ cells, PKC has been suggested as a potential target for anti HIV therapeutic agents (Fields et al., 1988; Mahoney et al., 1990). Here, a new approach designed to improve the effectiveness of PKC inhibitors is

discussed. In order to appreciate the rationale behind this approach, some biochemical aspects of PKC regulation as well as some of the properties of the well established inhibitors are reviewed in the following paragraphs.

1.2 Biochemical Aspects of Protein Kinase C

Protein kinase C is a serine and threonine kinase that has been shown to be present in a variety of organs and tissues. The enzyme is distributed in the cytosol as well as the plasma membrane of cells, but under physiological conditions it has been shown to be active only when bound to a membrane surface (reviewed by Kikkawa and Nishizuka, 1986a). PKC is dependent on phospholipids for activity. In the presence of calcium, anionic phospholipids such as phosphatidyl-inositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidyl-glycerol (PG), are capable of activating the enzyme (Takai et al., 1979a,b). One of the most potent phospholipid activators is phosphatidylserine (PS). In addition, diacylglycerol (DAG), markedly enhances the activity of the calcium and phospholipid stimulated enzyme (Kishimoto et al., 1980). This interaction is however stereospecific; only DAG's possessing the *sn*-1,2 stereochemistry as opposed to the *sn*-2,3 or *sn*-1,3 configurations will activate the enzyme (reviewed by Rando, 1988). DAG also lowers the calcium requirement of the enzyme to physiological concentrations and thus has been said to act synergistically with calcium (Kishimoto et al., 1980). Phorbol esters can however substitute for DAG

and activate the enzyme for prolonged periods at concentrations far below the effective concentration of DAG (Ashendel, 1985).

PKC is a 77 k Mr single polypeptide possessing a 26 k Mr hydrophobic regulatory domain and a 51 k Mr hydrophilic catalytic domain. It comprises a family of closely related proteins exhibiting distinct properties and uneven tissue distribution. Thus far, seven isozymes have been identified and can be classified into two groups (reviewed by Nishizuka, 1988; Huang, 1989; Kikkawa et al., 1989; Parker et al., 1989). The α, ß (ßI, ßII), and γ isozymes (types III, II, and I respectively) which have been separated by hydroxyapatite chromatography, comprise the first group. The isoforms ßI and ßII are products of the same gene that have undergone alternative mRNA splicings and differ only in their carboxyl terminus domain. The primary structure has been shown to contain four conserved regions (C1 - C4) and five variable regions (V1 - V5). The regulatory domain (V1, C1, V2, C2) comprises the amino terminal end of the protein and is separated from the catalytic domain (C3, V4, C4, V5) by V3. The C1 region contains a tandem repeat of a cysteine rich sequence analogous to a zinc finger DNA binding motif. This region is considered to bind phospholipid, diacylglycerol, and phorbol esters. A stretch of highly basic amino acids also known as the pseudosubstrate sequence, is present in C1 and has been suggested to occupy the catalytic domain and lock the enzyme in an inactive conformation (House and Kemp, 1987). The C2 region has been proposed to be important for binding calcium. The catalytic domain contains a consensus ATP binding sequence conserved in a variety of other kinases; this comprises C3. The function of the fourth conserved region (C4) in

the catalytic domain however remains to be deduced. The δ, ϵ, and ζ isozymes comprise the second group. Although not isolated from tissues, their cDNA's have been expressed in COS 7 cells, their gene products purified and their properties determined (Ono et al., 1988; 1989). Like the other C-kinases, δ, and ϵ are sensitive to the presence of PS and DAG, but unlike the first group, the δ, and ϵ PKC's lack the C2 region and have been shown to be insensitive to calcium (Ono et al., 1988). The ζ isozyme, in addition to the absence of C2, lacks one of the cysteine rich sequences in C1 and is insensitive to DAG and phorbol esters (Ono et al., 1989).

1.3 Enzyme Activation

It is well accepted that as a result of agonist receptor interaction, second messengers such as DAG and inositol trisphosphate (IP_3) are produced which inevitably induce the activation of PKC (reviewed by Berridge, 1984; 1987). The transient production of these second messengers is attributed to the hydrolysis of the minor membrane component phosphatidylinositol 4,5 bisphosphate (PIP_2) by a PIP_2 specific phospholipase C. IP_3 mobilizes calcium from intracellular stores. In the presence of calcium the inactive cytosolic enzyme translocates to the inner membrane surface where it can interact with acidic phospholipids. DAG can then augment the activity of membrane associated PKC. However, PIP_2 hydrolysis yields only a minor amount of DAG. A major source of DAG production is from phosphatidylcholine (PC) hydrolysis either directly mediated by a PC specific

phospholipase, or indirectly by the combined action of phospholipase D and a PA phosphohydrolase (reviewed by Exton, 1990).

The possibility of another enzyme activation mechanism not related to inositide turnover was raised with the observation that arachadonic acid, as well as other unsaturated fatty acids activate neutrophil PKC in a calcium dependent manner (McPhail et al., 1984). Arachadonate can be produced by phospholipase A2 mediated hydrolysis of phospholipids. Only cis unsaturated fatty acids will activate PKC; their potency correlates with an increase in their degree of unsaturation (McPhail et al., 1984). Moreover, Hannson and co-workers (1986) reported that in the presence of calcium, oxygenated metabolites of arachadonic acid such as the eicosanoid lipoxin A stimulate PKC much more than arachadonate. In contrast, others reported that fatty acid activation may occur independently of calcium (Murakami et al., 1986). These observed differences can be explained by differences in PKC sources and the differential sensitivity of the PKC isozymes to arachadonate (Sekiguchi et al., 1987). The α and β isoforms require high arachadonate concentrations in the presence of calcium for full activation, while the γ form is less responsive to arachadonate, but responds in the absence of calcium (Sekiguchi et al., 1987). Whether or not PKC activation by arachadonate occurs *in vivo* still remains speculative (Dreher and Hanley, 1988).

Protein kinase C can also be proteolytically cleaved through the action of a calcium dependent neutral proteinase to produce the 51 k Mr constitutively active protein kinase M (reviewed by Murray et al., 1987). This form has been shown to occur in phorbol ester stimulated platelets and neutrophils. However,

under physiological situations, the irreversible activation of PKC by this mechanism is still speculative.

1.4 Models for Protein Lipid Interactions

The association of PKC with a membrane surface is essential for its activation. However, the molecular mechanism for this association still remains equivocal. In attempting to define the minimal stoichiometry and specificity for PKC - lipid interactions, Bell and co-workers designed the Triton mixed micelle assay which allows for the systematic variation of a number of lipid components (Hannun et al., 1985; 1986a). Based on this study, Bell proposed that through their carboxyl moieties, a minimum of four PS molecules bind calcium and form a complex on the membrane surface. Subsequently, the enzyme binds to this four PS - calcium complex, but still remains inactive until DAG is produced from inositide turnover. DAG is believed to interact with this complex by forming three points of contact: one to calcium and two to PKC. This ionic interaction is thought to be responsible for the activation of the enzyme. Upon DAG removal, PKC may remain inactive on the membrane surface or dissociate from the membrane. However, it has been suggested that factors which influence the activation of PKC are more complex than this PS/DAG/PKC/calcium electrostatic membrane surface interaction. Studies performed in PS/PC vesicles have shown that calcium dependent PKC binding to the membrane does not correlate with activity (Bazzi and Nelsestuen, 1987a). Hydrophobic interactions have also been

shown to be important for the activation of PKC. For instance, highly saturated PS elicits more activation than highly unsaturated PS (Snoek et al., 1988). Moreover, only a slight increase in PKC activation is observed when DAG is added to highly saturated PS, while with unsaturated PS, DAG markedly increases basal PKC activity (Snoek et al., 1988). A two step model for PKC membrane association and activation is now gaining considerable recognition (Bazzi and Nelsestuen, 1988a; Snoek et al, 1988; Newton et al., 1989). Calcium is considered to induce a conformational change in the enzyme. This conformational change has been observed with circular dichroism or by measuring tryptophan fluorescence quenching upon calcium - PKC binding (Lester and Brumfeld, 1990; Huang, 1989). Recently, McLaughlin proposed that the conformational change induced by calcium unmasks the pseudosubstrate region which supposedly locks the enzyme in an inactive conformation. Once free, this highly basic sequence in the enzyme can subsequently bind to anionic PS (Mosior et al., 1990). However, PKC interactions with the membrane also cause a large increase in the membrane surface pressure suggesting that PKC inserts into the bilayer; this event is irreversible (Bazzi and Nelsestuen, 1988a). The second step, activation, had thus been suggested to involve PKC penetration into the bilayer core. Numerous studies lend further support to this idea. Phorbol esters and DAG enhance PKC labelling by a lipid soluble photoreactive probe that will only label proteins penetrating the bilayer (Snoek et al., 1986). Studies using fatty acid labelled probes suggest that PKC penetrates to at least the C16 position of the fatty acid chain (Brumfeld and Lester, 1990). In addition to phorbol esters, high concentrations of calcium can

induce PKC penetration; the activity of the inserted enzyme is no longer calcium dependent (Bazzi and Nelsestuen, 1988b). Although DAG mediates membrane insertion of PKC *in vitro*, it is not as efficient as phorbol ester mediated insertion (Bazzi and Nelsestuen, 1989). However, this insertion is a physiological event. A substantial fraction of cellular PKC is already membrane associated (Kikkawa et al., 1982; Halsey et al., 1987; Yoshida et al., 1988). In this form, the protein cannot be extracted with calcium chelators, salts, or with sonication (Kikkawa et al., 1982; Halsey et al., 1987). Rather, detergents (Halsey et al., 1987), or high pressures (Lester, 1989) are required to release the protein from the membrane. It has been suggested that other factors such as changes in transmembrane potentials or membrane curvature may contribute to DAG's ability to enhance PKC insertion *in vivo* (Bazzi and Nelsestuen, 1989).

1.5   Membrane Biophysical Properties and Protein Kinase C

Just as the activity of a soluble enzyme is dependent on the nature of the surrounding solvent, one would expect that the activity of a membrane associated enzyme would be affected by changes in the properties of its surrounding lipid. There is evidence that the physical state of lipids plays a role in the activation of PKC. Alterations in membrane fluidity and differences in membrane curvature, have been shown to result in differential PKC activation. For instance, large unilamellar vesicles activate PKC to a greater extent than small unilamellar vesicles (Boni and Rando, 1985). Likewise, agents which enhance membrane fluidity

activate PKC (Lester and Baumann, 1990). In the past, many studies have focused on alterations in membrane fluidity as being the only factor modulating the activity of membrane associated enzymes. However, it has been known for some time that membranes, under certain conditions, can adapt different configurations (reviewed by Cullis et al., 1985; 1986). Phospholipids present in biological membranes are commonly arranged as a planar sheet like structure termed a bilayer. Certain phospholipids, in particular the phosphatidylethanolamines, are capable of undergoing a morphological rearrangement to produce an inverted, type II hexagonal phase (H_{II}). Hexagonal phase formation is a consequence of the phospholipid hydrocarbon region occupying a larger volume than its polar head group. As a consequence of an increase in the phospholipid head group spacings, this arrangement is more susceptible to the penetration of water into the bilayer core, leading to the formation of a more stable lipid organization, the hexagonal phase (Figure 1.1). A number of substances which modulate bilayer to hexagonal phase interconversion also alter many membrane associated processes (reviewed by Epand, 1990a). The activity of PKC has been shown to be affected by compounds which alter the bulk biophysical properties of a membrane. Firstly, PE can enhance the ability of PS to activate PKC (Kaibuchi et al., 1981). At the concentrations of calcium and magnesium necessary for enzyme activation, PS/PE mixtures have been shown to undergo hexagonal phase formation (Boni and Rando, 1985). In addition, DAG, the physiological activator of PKC is a potent hexagonal phase promoter (Epand, 1985). This non bilayer phase is dehydrated and may facilitate lipid - PKC interactions, and consequently enzyme activation.
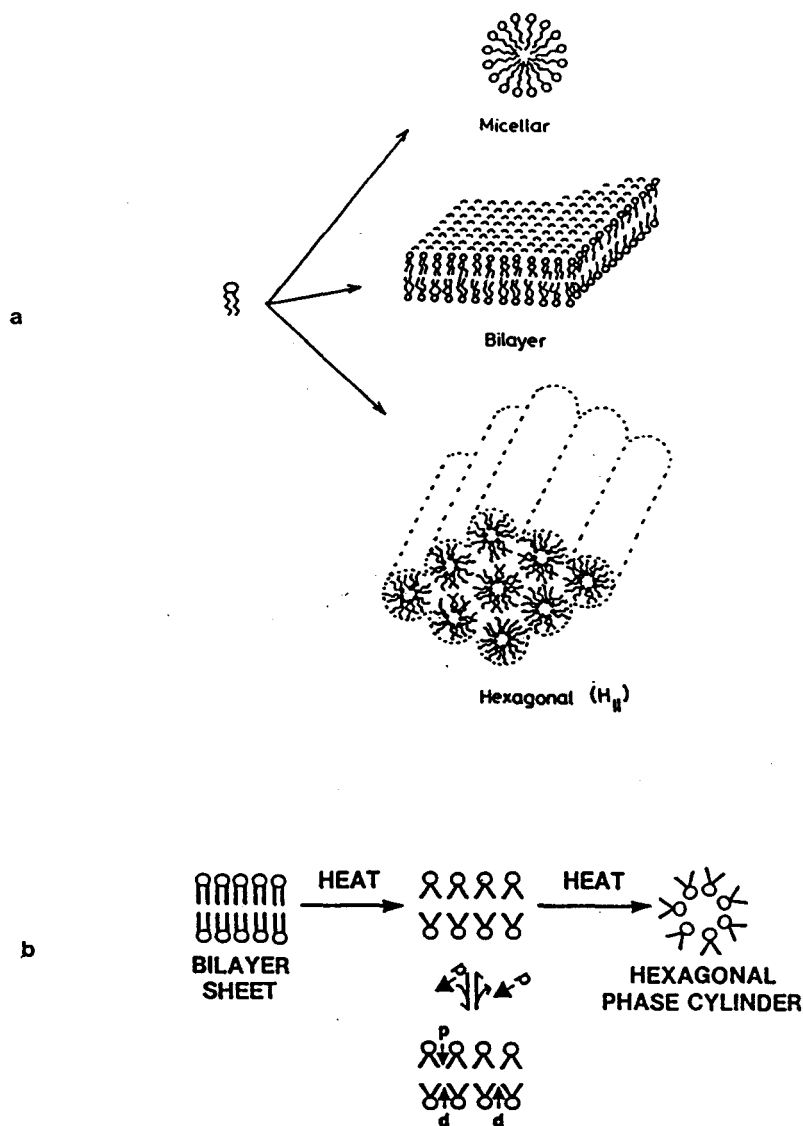
Figure 1.1 *a*: Lipid Phase Preferences. Phospholipids exist in a variety of shapes and can undergo morphological rearrangements. Cylindrical phospholipids favour the bilayer arrangement while inverted cone shaped lipids (lipids with a large head group) favour a micelle structure. Cone shape lipids (lipids with a small head group) favour the formation of an inverted hexagonal array of cylinders. *b*: The hexagonal phase results from an increase in the spacing between the lipid head groups. This unstable situation allows water to penetrate deeper into the exposed bilayer core resulting in hexagonal phase formation. Inverted cone shape molecules (⅄) fill the void spaces between the phospholipid head groups and consequently make hexagonal phase formation less favourable. Figure 1.1*a* was reproduced from Cullis et al., 1985.

However, not all activators of PKC promote hexagonal phase formation and neither are all inhibitors of PKC bilayer stabilizers. If only the uncharged and zwitterionic compounds are considered, then those which activate PKC are hexagonal phase promoters (Epand, 1987). In contrast, uncharged and zwitterionic compounds which are bilayer stabilizers (hexagonal phase inhibitors) have been reported to inhibit PKC (Epand, 1987; Epand et al., 1988; Epand et al., 1989a). The relationship between the ability of a compound to stabilize a membrane and its efficacy in inhibiting PKC is however not quantitative. This is because the hexagonal phase per se is not involved in modulating enzyme activity. Even pure DLPE, a lipid which adapts the H_{II} configuration, is not a potent activating lipid (Boni and Rando, 1985). In addition, hexagonal phases are unlikely to occur in the PS dispersions or Triton mixed micelles which are used to study the effects of activators or inhibitors of PKC. Moreover, these phases are unlikely to occur *in vivo* as this would be disruptive to the membrane and probably result in the breakdown of the cells' permeability barrier (Epand, 1990a). In contrast, amphiphiles which alter bilayer to hexagonal phase interconversion change the surface properties of the membrane and this may affect PKC binding, penetration, and consequently activation. In addition, other factors such as charge play an important role in modulating enzyme function. A number of positively charged compounds have been shown to inhibit polycationic substrate phosphorylation by PKC regardless of a compound's ability to modulate membrane biophysical properties (Epand, 1987).

1.6 Inhibitors of Protein Kinase C

A number of distinct sites within PKC can serve as targets for the design of selective inhibitors. One class of inhibitors including the triphenylethylenes such as tamoxifen (O'Brian et al., 1988), and phenothiazines such as trifluoperazine (Schatzman et al., 1981) interact with the calcium and PS binding sites within the regulatory domain. The amino acridines, another member of this class compete with the DAG and phorbol ester binding sites, although not with the calcium site (Hannun and Bell, 1988). Nucleoside analogues such as sangivamycin (Loomis and Bell, 1987), isoquinolinesulfonamides such as H7 (Hidaka and Hagiwara, 1987), indole carbazoles such as staurosporine (Davis et al., 1989), and flavinoids such as quercetin (Ferriola et al., 1989) comprise another class of compounds which inhibit PKC by competition with the ATP substrate site. Other inhibitors such as the pseudosubstrate octadecapeptide antagonize the enzyme by binding with the protein substrate site (House and Kemp, 1987). A general problem that is associated with the use of some of the well established inhibitors of PKC is their lack of specificity. This has been problematical with substances such as staurosporine or H7 which inhibit tyrosine and other serine kinases (Rüegg and Burgess, 1987). This is not surprising considering that the ATP binding site is highly conserved in all kinases (Hanks et al., 1988). Protein substrate site specific inhibitors, although they are potent and specific, are susceptible to degradation by cellular proteases and have permeability problems making their use in cells difficult (Rüegg and

Burgess, 1987). In contrast, inhibitors which are directed at the regulatory domain of PKC take advantage of the membrane association phenomenon which, unlike other kinases, is required for PKC activation. Although these inhibitors also have a specificity problem, combining features that are targeted to the regulatory domain with characteristics which exploit other sites in PKC could result in a more potent and specific PKC inhibitor. However, before specificity can be introduced, it is necessary to optimize structural features of a molecule to enhance inhibitor potency.

1.7 Objectives

The objective of this study is to attempt to optimize the structural features of a drug which is necessary for inhibiting PKC. Since both uncharged and zwitterionic bilayer stabilizers as well as cationic drugs have been shown to be PKC inhibitors, zwitterionic and cationic bilayer stabilizing amphiphiles will be designed. The design strategy focuses on the use of a steroid backbone which possesses a compact fused ring arrangement. This should allow for facile transfer of the drug from the aqueous phase to the membrane phase, where PKC is said to interact. Furthermore, the fused ring system of the steroid is planar; this avoids introducing hydrocarbon splay and therefore the expansion of the centre of the bilayer. Consequently, hexagonal phase formation is less likely to occur. Combining this feature with a large polar head group possessing amino functionalities will generate a cationic amphiphile with a large ratio of head group

to hydrocarbon volume. This will potentially give the molecule the property of being a good bilayer stabilizer. A tertiary amino group will allow the molecule to be derivatized to produce a zwitterionic sulfobetaine or carboxybetaine. Furthermore, cationic character can be increased further by acylating the tertiary amine with iodomethane. The inhibition of PKC by compounds possessing the steroid moiety as the hydrocarbon backbone will be compared to amphiphilic analogues whose hydrocarbon region is based on a long chain fatty acyl moiety.

2. SYNTHESIS OF CATIONIC BILAYER STABILIZERS

2.1 INTRODUCTION

Amphiphiles can be chemically modified to determine what factors influence PKC activity. To assess the effect of changes in the type and amount of charge, a number of alcohols or primary amines containing additional primary or tertiary amino functionalities can be used to acylate hydrocarbons possessing activated carboxyl groups. Long chain acyl chlorides or carbonyldiimidazole activated steroids have the potential of stabilizing PE bilayers. Moreover, the use of cholesteryl-3ß-carboxylate and cholesteryl hemisuccinate as starting materials allows the location of the charge relative to the steroid moiety to be varied. In this chapter, the synthesis of cationic and zwitterionic amphiphiles will be described.

2.2 REAGENTS AND EQUIPMENT

2.2.1 Reagents

Cholesterol (99+% grade), cholesterol hemisuccinate, 1,1'-carbonyldiimidazole, and trans-tamoxifen were purchased from Sigma Chemical Co.,

St. Louis, MO. Magnesium powder-50 mesh (99+%), thionyl bromide (97%), 1,3-propane sultone (99%), iodomethane (99%), trans-1,2-dichloroethylene (98%), N,N-dimethylaniline (99%), N,N-dimethylethylenediamine (95%), 1,3-bis-dimethylamino-2-propanol (97%), 2-{[2-(dimethylamino)ethyl]methylamino}ethanol (98%), were obtained from Aldrich Chemical Co., Milwauke, WI. Lignoceroyl and behenoyl acid chlorides were purchased from Nu Chek Prep., Elasyian, WI. Methanol, dichloromethane, and acetonitrile were HPLC grade solvents. All other chemicals and solvents, unless specified were reagent grade.

2.2.2   Equipment

Thin layer chromatography (TLC) was performed on Analtech (Newark, DE.) 250 or 1000 micron precoated silica gel GF plates and the chromatograms were visualized with iodine vapour. Preparative TLC was performed on the 1000 micron tapered plates. High pressure liquid chromatography was performed on a Varian Vista 5500 instrument equipped with a UV detector and fitted with a Whatman partisil 10 ODS-3 reverse phase column. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on either a Bruker AC200 MHz or Bruker AM500 MHz spectrometer with all chemical shifts relative to chloroform at δ = 7.24 ppm. Elemental analysis was conducted at Guelph Chemical Laboratories, Guelph, Ont. and mass spectrometry was performed at the McMaster University mass spectrometry facility. The minimum purity of compound **V** and **X** was assessed by NMR and was calculated by assuming that the trace impurity resonance corresponded to a single proton.

2.3 STEROID BASED AMPHIPHILES

2.3.1 Cholesteryl Carboxylate Analogues

A synthetic scheme for cholesteryl carboxylate analogues is shown in Figure 2.1.
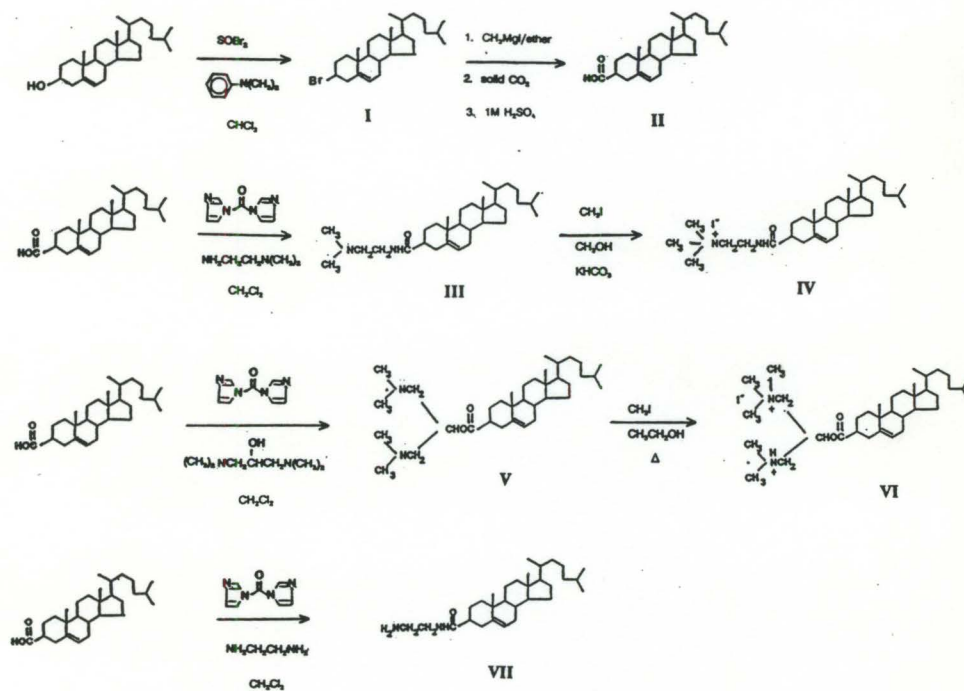
Figure 2.1 Synthetic scheme for cholesteryl carboxylate analogues.

2.3.1.1 Cholesteryl Bromide (I)

Cholesteryl bromide was synthesized from cholesterol by a modified procedure previously described by Roberts and co-workers (1954). Reactions were carried out on a 25 g scale. Cholesterol, (25 g, 64.6 mmol) was dissolved in 10 mL of dimethylaniline (78.9 mmol) and 50 mL of chloroform. While stirring on ice, small quantities of thionyl bromide (6 mL, 77.6 mmol) dissolved in 20 mL of cold chloroform was added slowly over a period of 15 minutes. After the addition of thionyl bromide was complete, the mixture was stirred for an additional 2 hours at room temperature. The resulting solution was poured into 200 mL of ice cold 95% ethanol and left on ice for 1 hour until crystallization was complete. The product was filtered and washed with 25 mL of ice cold 95% ethanol. A small amount of product was recovered from the filtrate with the addition of 75 mL distilled water followed by refrigeration. Finally, the product was recrystallized from 120 mL of acetone giving 21.8 g of cholesteryl bromide (yield, 75%) with a melting point of 93-95 °C (lit 97-98 °C). The identity of the product was confirmed with mass spectrometry (EI) which showed an intense peak with an m/z of 448, corresponding to the molecular ion ($M^{+\bullet}$) of cholesteryl bromide. Also, the bromide molecular weight pattern characteristic of the two different isotopes of bromine (79Br:81Br, 1:1) was observed.

2.3.1.2 Cholest-5-ene-3ß-Carboxylic Acid (II)

The synthesis of cholesteryl-3ß-carboxylate was performed using a Grignard reaction according to the method previously described by Roberts and co-workers (1954). All glassware was oven dried at 110 °C overnight. In a 500 mL three-neck flask set up for reflux, a solution of methyl magnesium iodide was freshly prepared by treating 9 g of oven dried (110°C) magnesium powder in 100 mL anhydrous diethyl ether with 10 mL of methyl iodide. After the vigorous reaction subsided, cholesteryl bromide (25 g, 56 mmol) dissolved in 100 mL of anhydrous diethyl ether was slowly added to the methyl magnesium iodide solution over a three hour period. The solution was refluxed for 36 hours with enough heat required to bring the diethyl ether to a boil. Subsequent to cooling, the Grignard reagent was added to finely ground solid carbon dioxide, and after 1 hour, the complex was hydrolyzed by treatment with ice cold 1 M sulfuric acid. After the steroid was extracted with diethyl ether (3 x 250 mL), the ethereal layer was washed with 10 mM sodium thiosulfate (3 x 50 mL) to remove a persistent orange colour. After removing the water layer, the ether layer was washed with distilled water and filtered to remove an insoluble residue. The ether layer was subsequently dried over anhydrous sodium sulfate and rotary evaporated to give a white-yellow oily suspension. Tituration with pentane yielded 8.6 g of cholesteryl-3ß-carboxylate (yield, 37%) as a fine powder with a melting point of 212-215 °C (lit 218-220 °C). Mass spectrometry (EI) showed an m/z of 414 for the molecular ion ($M^{+ \cdot}$). The product was characterized by proton NMR and the major

assignments are shown in Figure 2.2. The product was lyophilized overnight to give an anhydrous starting material for acylation reactions.
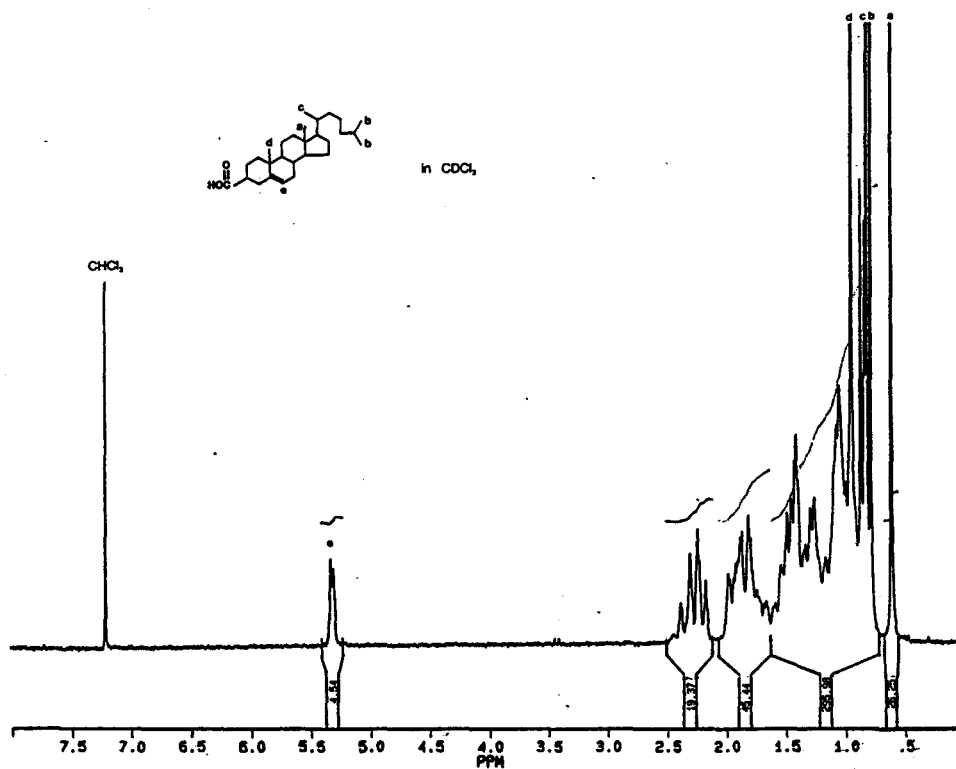
Figure 2.2 200 MHz spectrum of compound **II**, inset top left.

2.3.1.3 Cholesteryl-3ß-Carboxyamidoethylenedimethylamine (III)

The acylation of cholesteryl carboxylate was carried out under a dry argon or nitrogen atmosphere in oven dried glassware. The procedure used was adapted

from one previously described (Morton et al., 1987; Mangroo and Gerber, 1988). Cholesterol carboxylate (2 g, 4.8 mmol) was suspended in 5 mL of dichloromethane (HPLC grade under 4 Å molecular sieves). A 1.5 molar excess of 1,1'-carbonyldiimidazole (CDI, 1.2 g) dissolved in 15 mL dichloromethane was added to the cholesteryl carboxylate suspension in small volumes with intermittent shaking. When the reaction subsided, the solution was stirred overnight. N,N-dimethylethylenediamine (5 mL, 43.2 mmol) was subsequently added and the resulting solution was stirred for 36 hours at room temperature. Dichloromethane was removed by rotary evaporation, after which the reaction was quenched with a small volume of distilled water. The acylated steroid was extracted with diethyl ether (4 x 50 mL). Subsequently, the pooled ether fractions were back extracted with distilled water (3 x 50 mL), dried over anhydrous sodium sulfate, and rotary evaporated under reduced pressure. The residue was then titurated with pentane and the product collected on a sintered glass funnel. A voluminous powder (1.7 g, 73% yield) was obtained and found to be pure by TLC (Rf=0.72) using chloroform:methanol:water (65:25:4; v/v/v) as the developing solvent. The product gave a melting point of 167-169°C. Mass spectrometry (FAB+) showed an intense peak at an m/z of 485 which corresponds to the protonated molecular ion $(M+H)^{+ \cdot}$. The ^{1}H assignments are shown in the NMR spectrum, Figure 2.3.
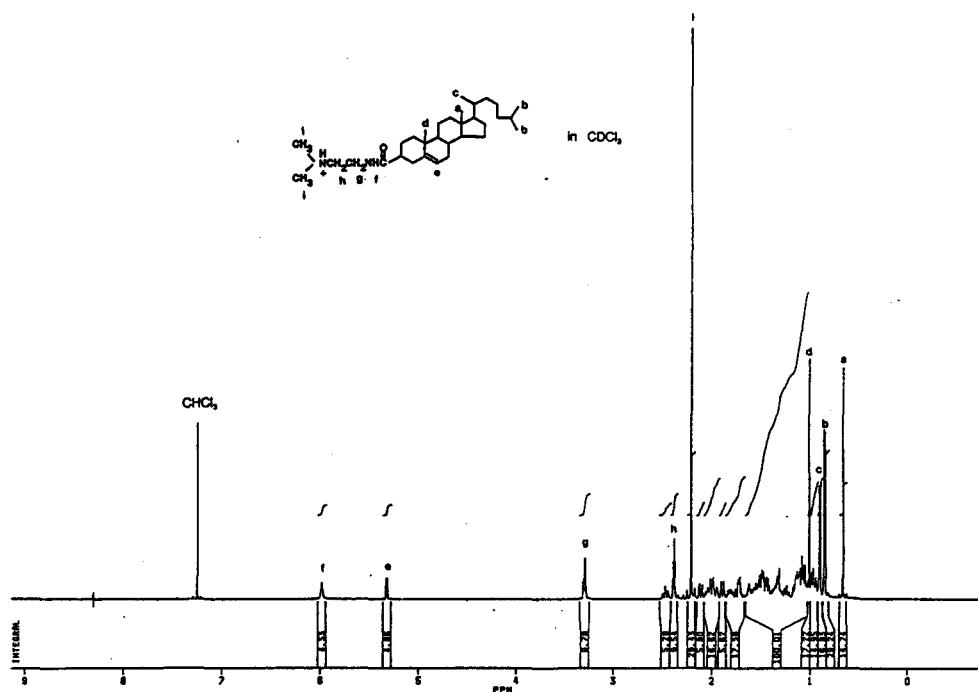
Figure 2.3   500 MHz NMR spectrum of compound III, inset top left.

2.3.1.4   Cholesteryl-3ß-Carboxyamidoethylenetrimethylammonium Iodide (IV)

The quaternization of compound **III** was performed using methyl iodide and potassium bicarbonate by a procedure previously described by Chen and Benoiton (1976). Briefly, 1 g (2.1 mmol) of compound **III** was dissolved in 40 mL of methanol in the presence of 2 g (20 mmol) of potassium bicarbonate and 2 mL (32.1 mmol) of methyl iodide. The reaction was stirred for 24 hours at room temperature.   The solvent was subsequently removed under vaccum and the remaining bicarbonate was neutralized with 1 M HCl until the solution gave a pH

reading of 7. Water was removed by lyophilization and the product was extracted from inorganic salt impurities using a small volume of ice cold methanol. After evaporating the solvent, the product was recrystallized from absolute ethanol and was further purified on a reverse phase column using an acetonitrile/0.1% trifluoroacetic acid gradient (100% to 85% acetonitrile in 60 minutes). The powder was shown to be pure with TLC (Rf=0.10) using chloroform:methanol: water (65:25:4 v/v/v) as the developing solvent. It was shown to melt with decomposition at about 190°C, and had a molecular ion with an m/z of 500 (M$^{+\cdot}$) according to mass spectrometry (FAB+). The NMR spectrum, Figure 2.4, shows the major proton assignments.
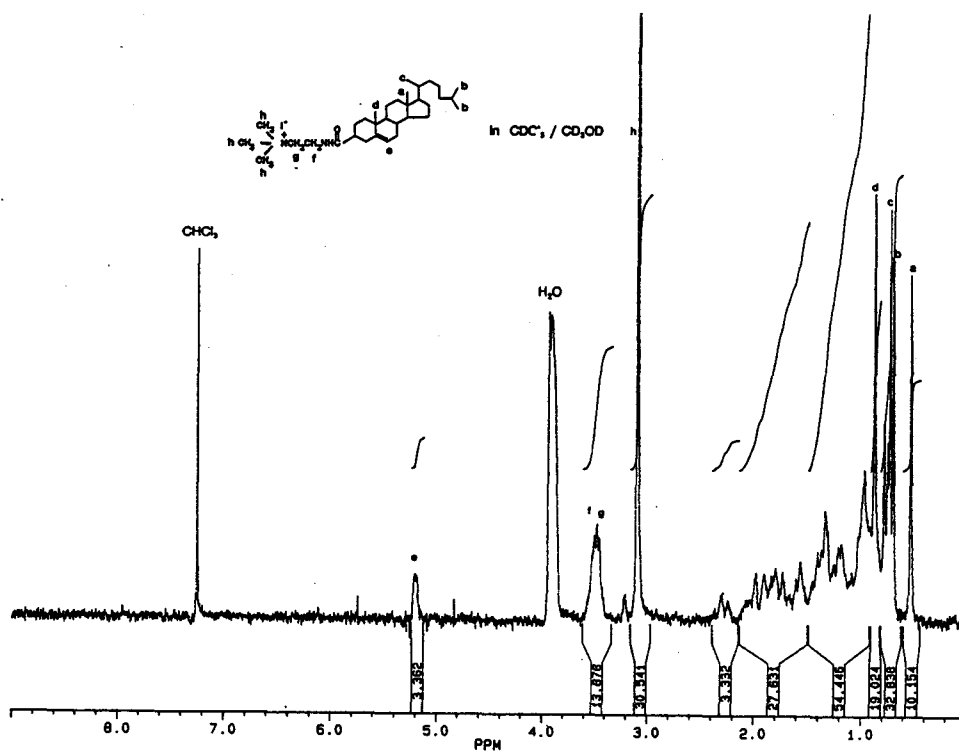
Figure 2.4 200 MHz NMR spectrum of compound IV, inset top left.

2.3.1.5 1,3-Bis-Dimethylamino-2-Propyl-Cholesteryl-3ß-Carboxylate (V)

Acylation was performed using CDI activated cholesteryl-3ß-carboxylate analogous to the method described for compound III, except that 1,3-bis-dimethylamino-2-propanol (8 mL, 47.6 mmol) was the nucleophile. After the addition of the nucleophile, the reaction was stirred at room temperature for 72 hours. The dichloromethane was removed and the remaining oily residue was dissolved in chloroform. Impurities precipitated with a large volume of petroleum ether (bp, 35-60°C). The filtrate was rotary evaporated to dryness, re-dissolved in pentane, and filtered once again. After drying, the pentane soluble material was dried and redissolved in a small volume of diethyl ether and added to a large volume of hot diethyl ether:acetonitrile (30:70, v/v). The product crystallized at -20°C after allowing some of the ether to evaporate. Mass spectroscopy (FAB+) gave an m/z of 543 for the protonated molecular ion $(M+H)^{+\cdot}$. The NMR spectra (Figure 2.5) shows the ^1H assignments. A minor impurity was detected at $\delta = 3.4$ and could not be successfully removed despite numerous crystallization attempts. The estimated purity of the compound from NMR is > 98 %.
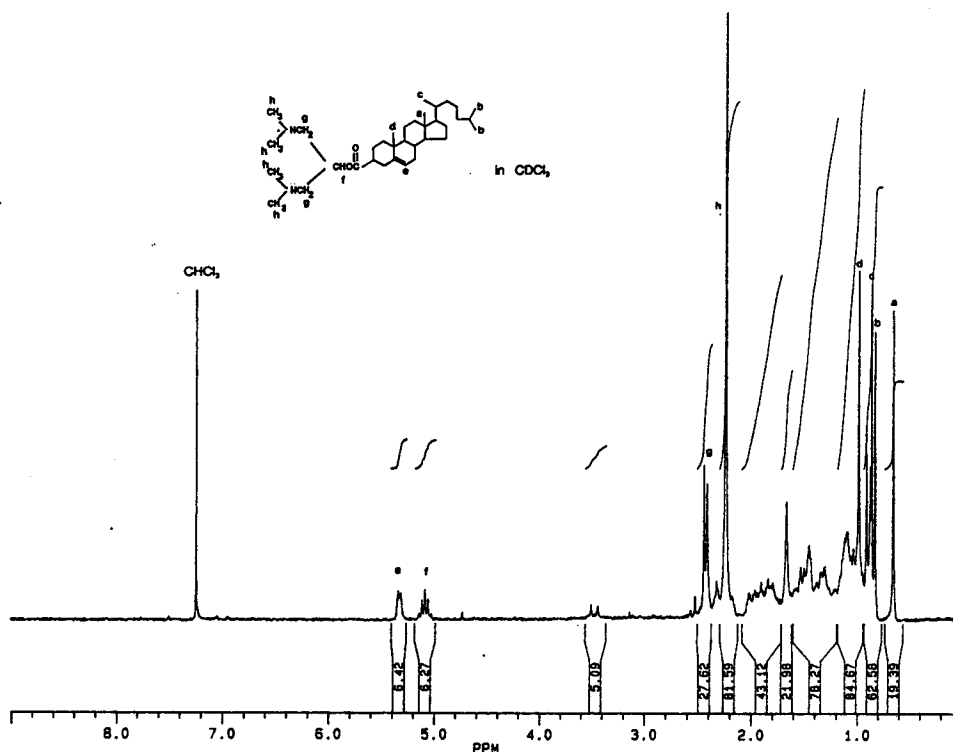
Figure 2.5   200 MHz NMR spectrum of compound V, inset top left.

2.3.1.6   **1-Dimethylamino-3-Trimethylammonio-DL-2-Propyl Cholesteryl Carboxylate Iodide Salt (VI)**

The methiodide of compound V was prepared by gently refluxing compound V (0.5 g, 0.9 mmol) and methyl iodide (2 mL, 32.1 mmol) in 20 mL of ethanol for one hour.   After cooling, the precipitate (0.5 g, yield 79%) was recrystallized twice from absolute methanol.   The product melted with decomposition at about 232°C and ran as a single spot on a TLC plate (Rf=0.22) using chloroform:methanol:water (65:25:4, v/v/v) as the developing solvent.   The

product had a molecular ion with an m/z of 557 ($M^{+\bullet}$) with FAB+ mass spectroscopy, consistent with the alkylation of one of the possible two tertiary amine sites. The ^{1}H assignments are given on the NMR spectrum, Figure 2.6.
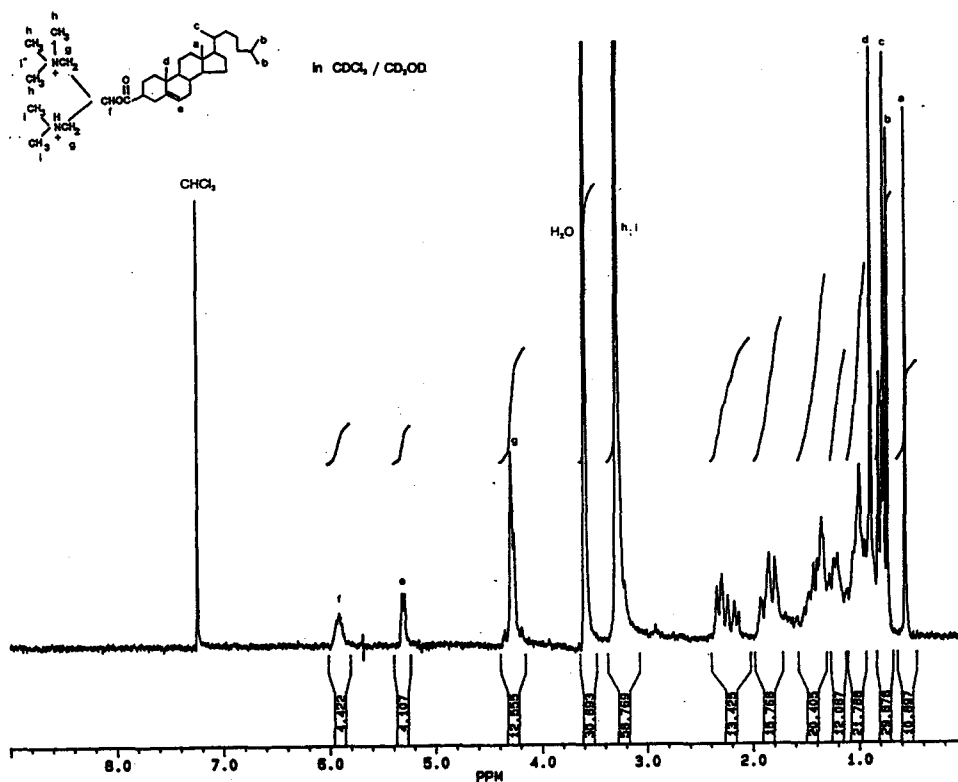
Figure 2.6 200 MHz NMR spectrum of compound VI, inset top left.

2.3.1.7 Cholesteryl-3ß-Carboxyamidoethyleneamine (VII)

To a solution of ethylenediamine (5.11 g, 85 mmol) in 20 mL dichloromethane, a solution of CDI activated cholesteryl carboxylate (0.7 g, 1.7 mmol) in 5 mL of dichloromethane was added dropwise over a 1.5 hour period. When the addition of the activated sterol was complete, the reaction was stirred for 48 hours under nitrogen. After removing the solvent under reduced pressure,

the residue was dissolved in chloroform:methanol (2:1, v/v) and extracted against water (3 x 50 mL). The chloroform phase was subsequently dried with anhydrous sodium sulfate, the solvent removed and the residue purified by preparative TLC using chloroform:methanol:water (65:25:4, v/v/v) as the developing solvent. The band at about Rf = 0.3 was collected, extracted with chloroform:methanol (1:1, v/v) and dried under reduced pressure. The product (0.63 g, yield, 81%) ran as a single spot (Rf = 0.33) and melted with decomposition at about 194°C. Mass spectrometry (FAB+) gave an m/z of 457 for the protonated molecular ion $(M+H)^{+\cdot}$. The NMR spectrum with the major proton assignments is depicted in Figure 2.7.
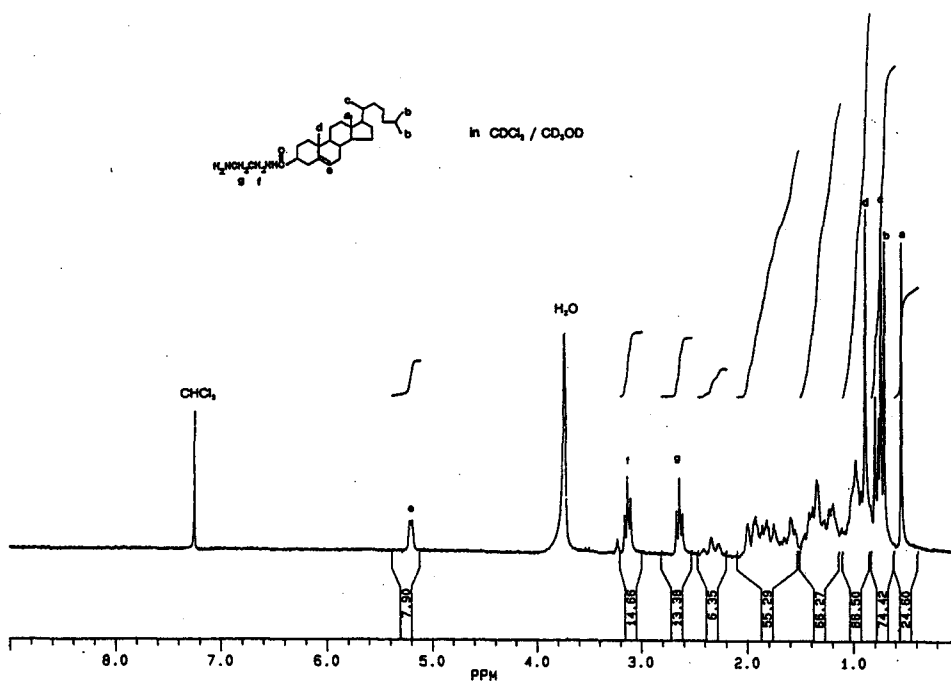
Figure 2.7 200 MHz NMR spectrum of compound VII, inset top left.

2.3.2 Cholesteryl Hemisuccinate Analogues

A scheme describing the various steps is depicted in Figure 2.8.
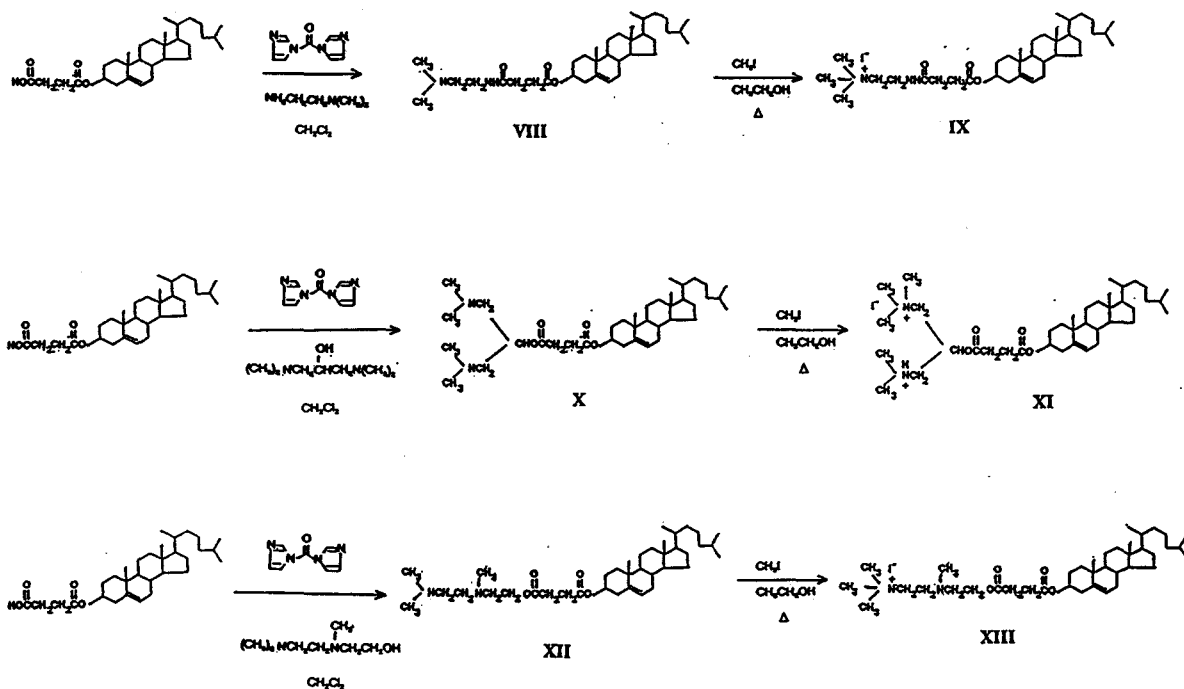
Figure 2.8 Reaction scheme of cholesteryl hemisuccinate analogues.

2.3.2.1 Cholesteryl-3ß-Oxysuccinamidoethylenedimethylamine (VIII)

The synthesis of compound **VIII** first required the acyl imidazolide of cholesteryl hemisuccinate which was prepared by reacting cholesterol hemisuccinate

with N,N-carbonyldiimidazole (CDI) as described for the synthesis of compound III. Briefly, to cholesterol hemisuccinate (2 g, 4.1 mmol) suspended in 5 mL of dichloromethane was added 1.5 equivalents of CDI (1 g) dissolved in 15 mL of dichloromethane. The solution was stirred overnight after which N,N-dimethylethylenediamine (5 mL, 43.2 mmol) was added. Dichloromethane was subsequently removed by rotary evaporation, distilled water was added and the acylated sterol was extracted with diethyl ether (4 x 50 mL). Subsequently, the ether fractions were washed with distilled water (3 x 50 mL) and dried over anhydrous sodium sulfate. The ether was removed by rotary evaporation. The product was washed with 200 mL of pentane, and minor impurities were removed using preparative silica gel TLC. After developing with chloroform:methanol:water (65:25:4 v/v/v) the band present at about an Rf = 0.80 was collected and extracted with chloroform/methanol (2:1 v/v). The residue was purified further using chloroform:ethyl acetate (1:1; v/v) as the second developing solvent. The band at about Rf = 0.2 was extracted with chloroform/methanol (2:1 v/v). The lyophilized product ran as a single spot on TLC with an Rf of 0.75 using chloroform:methanol:water (65:25:4 v/v/v) as the developing solvent and had a melting point of 109-111°C. Mass spectrometry (FAB+) showed an m/z of 557 which would correspond to the protonated molecular ion $(M+H)^{+\cdot}$. The ^{1}H assignments are shown on the NMR spectra, Figure 2.9.
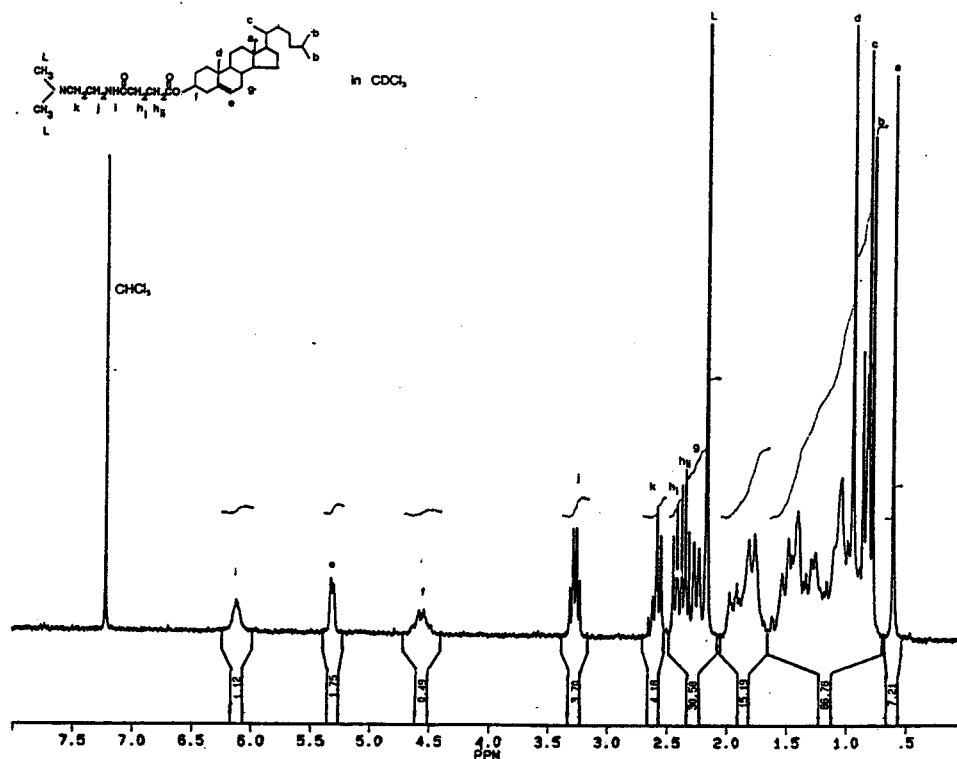
Figure 2.9 200 MHz NMR spectrum of compound **VIII**, inset top left.

2.3.2.2 Cholesteryl-3ß-Oxysuccinamidoethylenetrimethylammonium Iodide

(IX)

The quaternization of compound **VIII** was carried out with methyl iodide in absolute ethanol as described earlier for the synthesis of compound **VI**. Allowing the solution to cool to room temperature afforded 0.5 g (80% yield) of the quaternary ammonium salt. Subsequently, the product was recrystallized from absolute ethanol giving a fine white powder which melted with decomposition at about 196°C. The product ran as a single spot on a TLC plate (Rf = 0.43) using chloroform:methanol:water (65:25:4) as the developing solvent. Mass spectrometry (FAB+) indicated a molecular ion with an m/z of 572 ($M^{+ \cdot}$). NMR gave a

spectrum (Figure 2.10) with the ^{1}H assignments indicated.
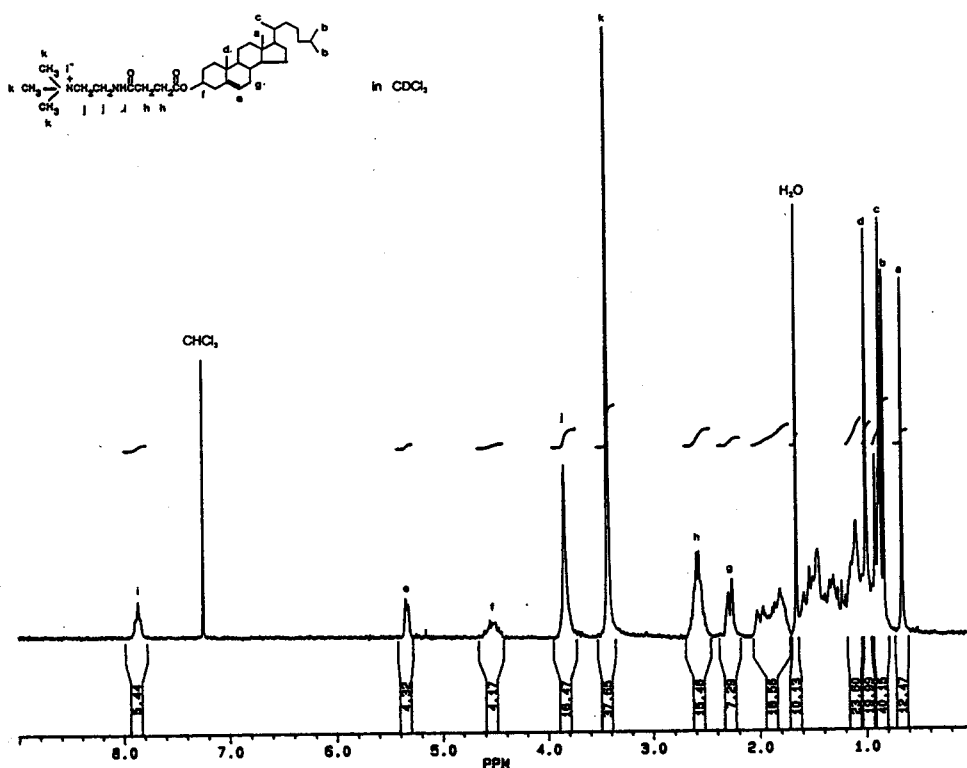
Figure 2.10   200 MHz NMR spectrum of compound IX, inset top left.

2.3.2.3   1,3-Bis-Dimethylamino-2-Propyl-Cholesteryl-3ß-Oxysuccinate (X)

Acylation was performed using CDI activated cholesteryl hemisuccinate according to the procedure described earlier (see 2.3.1.5). After the addition of 1,3-bis-dimethylamino-2-propanol (7 mL, 41.6 mmol), the mixture was stirred for 72 hours, after which the solvent was removed under vaccum. The product, extracted from the residue with diethyl ether (3 x 75 mL), gave an oil following removal of the ether. The addition of pentane precipitated additional impurities; after rotary evaporation, the resulting oil could not be successfully crystallized using

a variety of solvents or by lyophilization. Mass spectrometry (FAB+) indicated a protonated molecular ion with an m/z of 616 (M+H)$^{+\cdot}$. NMR analysis (Figure 2.11) showed an impurity at δ = 3.1 ppm which could not be successfully removed. The estimated purity of the compound is > 98%.
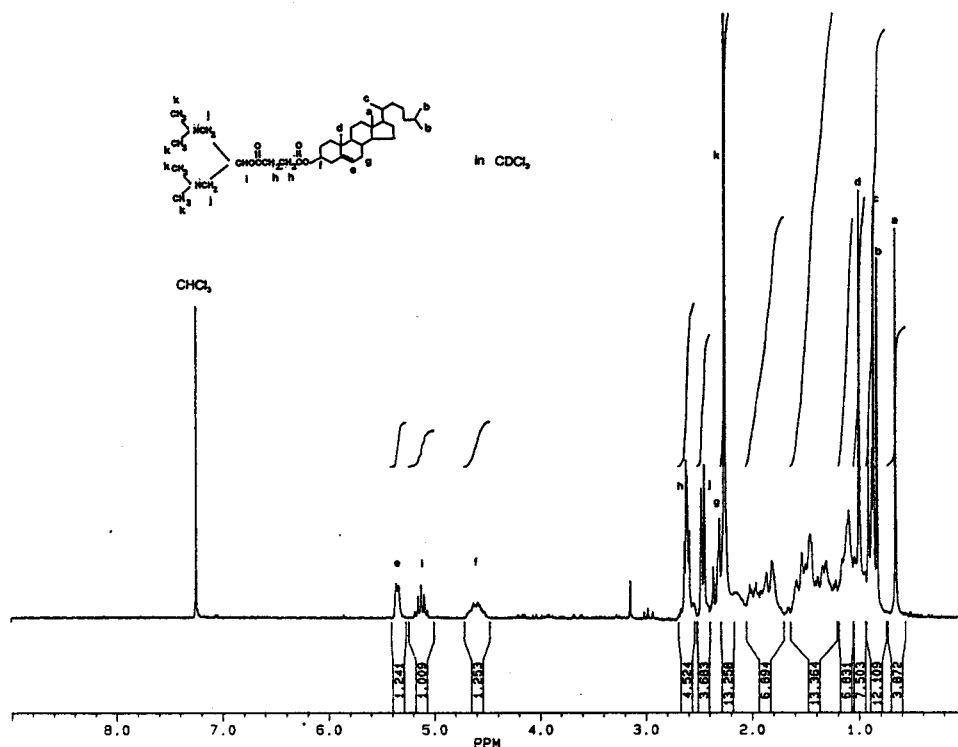
Figure 2.11 200 MHz NMR spectrum of compound X, inset top left.

2.3.2.4   1-Dimethylamino-3-Trimethylammonio-DL-2-Propyl-Cholesteryl-3ß-Oxysuccinate Iodide Salt (XI)

Methylation of compound **X** was performed using the method described in 2.3.1.6. After 1 hour, the solution was cooled and the methiodide recrystallized twice from absolute methanol to give needle shaped crystals which melted with

decomposition at about 222°C. The product ran as a single spot on a TLC plate (Rf = 0.17) using chloroform:methanol:water (65:25:4, v/v/v) as the developing solvent. Mass spectrometry (FAB+) indicated a molecular ion with an m/z of 629 ($M^{+\cdot}$) consistent with the methylation of 1 of a possible 2 tertiary amine sites. The NMR spectrum (Figure 2.12) shows the ^1H assignments.
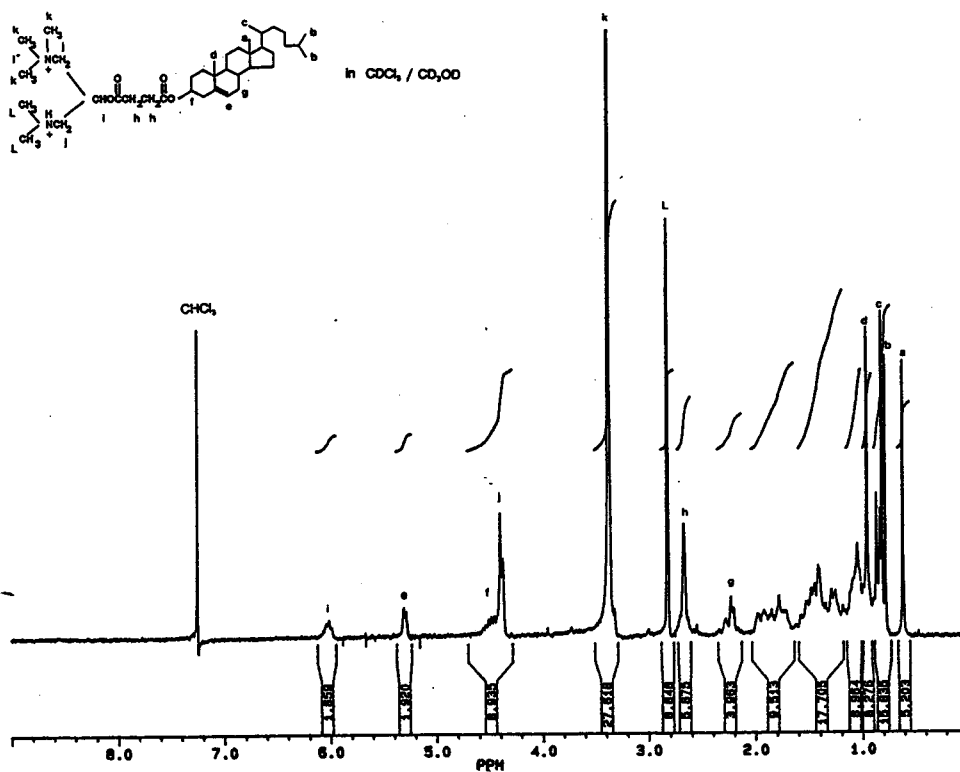
Figure 2.12 200 MHz NMR spectrum of compound **XI**, inset top left.

2.3.2.5   2-{[2-(dimethylamino)ethyl]methylamino}ethyl-Cholesteryl-3ß-Oxysuccinate (XII)

The synthesis of compound **XII** was analogous to the method described for the acylation of compound **VIII** except that 2-{[2-(dimethylamino)ethyl]-

methylamino}ethanol (7 mL, 42.0 mmol) was the amino alcohol used as the

nucleophile.   After extraction with diethyl ether, the product was lyophilized dry

and further purified by preparative TLC using chloroform:methanol:water (65:25:4;

v/v/v).   After the band present at Rf = 0.80 was collected and extracted with

chloroform:methanol, (2:1, v/v), the residue was purified further using

chloroform:ethyl acetate (1:1; v/v) as the second TLC developing solvent.   The

band which were present at about Rf = 0.2 was collected and the silica was

extracted with chloroform:methanol (2:1, v/v).   The product, which ran as a single

spot on a TLC plate (Rf = 0.72) using chloroform:methanol: water (65:25:4 v/v/v)

as the developing solvent gave a melting point of 50-52°C.   Mass spectrometry

(FAB+) showed a protonated molecular ion with an m/z of 615 $(M+H)^{+ \cdot}$.   The

^{1}H assignments are shown in the NMR spectrum (Figure 2.13).
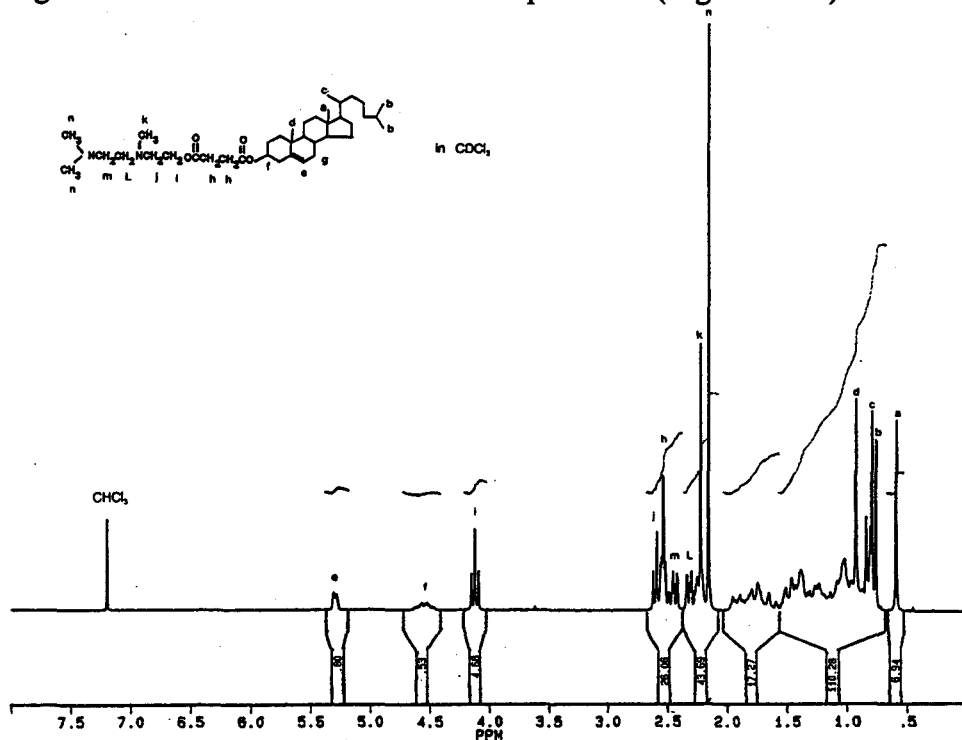
Figure 2.13   200 MHz NMR spectrum of compound XII, inset top left.

2.3.2.6   2-{[2-Trimethylammonio)ethyl]methylamino}ethyl-Cholesteryl-3ß-Oxysuccinate Iodine Salt (XIII)

The acylation of compound **XII** (0.5 g, 0.8 mmol) was carried out under reflux conditions with methyl iodide in absolute ethanol as described in 2.3.1.6. The precipitate was recrystallized twice from absolute methanol and stained as a single spot on a TLC plate (Rf = 0.22) using chloroform:methanol:water (65:25:4; v/v/v) as the developing solvent. The crystals melted with decomposition at about 172°C. Mass spectrometry (FAB+) gave an m/z of 629 for the molecular ion (M$^{+\cdot}$) consistent with the methylation of only one of the possible two tertiary amine sites. The NMR spectrum shown in Figure 2.14 shows the ^{1}H assignments.
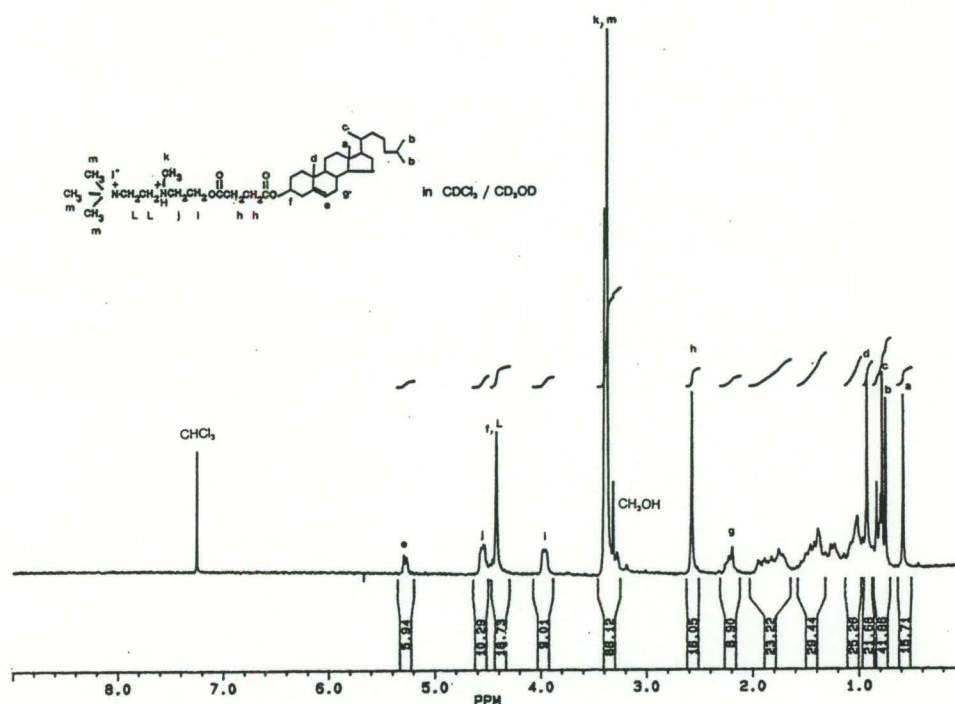
Figure 2.14   200 MHz NMR spectrum of compound **XIII**, inset top left.

2.3.3 Sulfopropylation and Carboxyethylation of Steroid Derivatives

A synthetic scheme for the synthesis of zwitterionic steroids is shown in Figure 2.15.
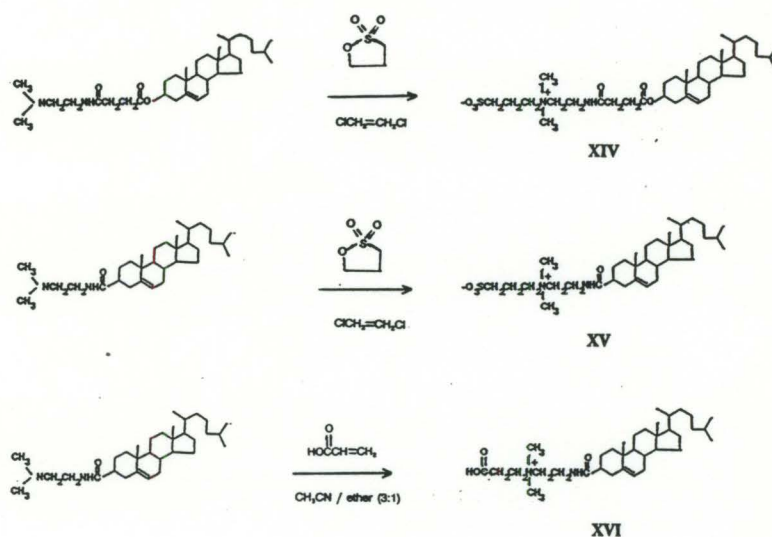
Figure 2.15 Synthetic scheme for zwitterionic steroids.

2.3.3.1   Sulfopropylation of Steroid Derivatives

2.3.3.1.1 3-[(2-Cholesteryl-3ß-Oxysuccinylamidoethylenedimethylammonio)]-1-Propane Sulfonate (XIV)

The sulfopropylation of compound **III** was carried out with modification to a procedure previously described by Linfield and co-workers (1963).

Cholesteryl-3ß-oxysuccinylamidoethylenedimethylamine (1 g, 1.8 mmol) was dissolved in 10 mL of ethylene dichloride in a reflux apparatus. A solution of propane sultone (0.45 g, 3.7 mmol) in 5 mL ethylene dichloride was added in small quantities. The entire 5 mL of sultone solution was added within a 0.5 hour period. Afterwards, the solution was refluxed for approximately 2 hours at 50 °C. When the reaction was complete (judged by cloudiness or a significant amount of precipitate in the reaction vessel), the product was then poured into 300 mL of acetone where further precipitation occurred. The product was collected by vacuum filtration using a sintered glass funnel, and was recrystallized from a large volume of isopropyl alcohol. Prior to recrystallization, 1 g of sulfobetaine, yield, 82 % was obtained. Mass spectrometry (FAB+) showed the protonated molecular ion with an m/z of 679 $(M+H)^{+\cdot}$. Elemental analysis gave C, 66.22%; H, 9.99%; N, 4.12%; S, 4.92% (theoretical, C, 67.21%; H, 9.80%; N, 4.13%; S, 4.72%). The product ran as a single spot on a TLC plate (Rf = 0.26) and melted with decomposition at about 212°C. The NMR spectrum is depicted in Figure 2.16 and shows the ^{1}H assignments.
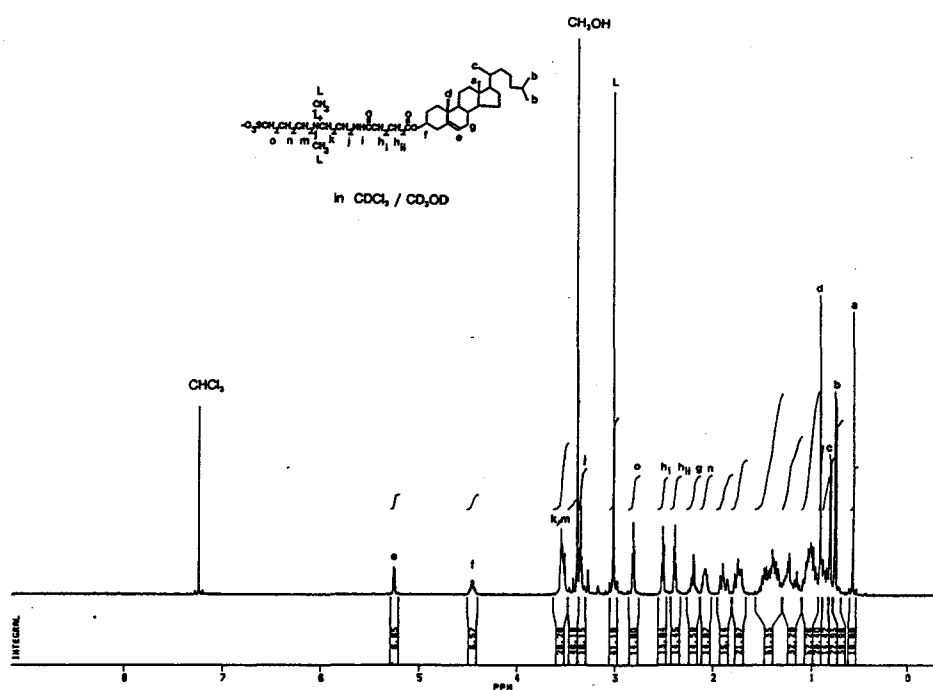
Figure 2.16 500 MHz NMR spectrum of compound **XIV**, inset top left.

2.3.3.1.2 3-[(2-Cholesteryl-3ß-Carboxyamidoethyl)dimethylammonio]-1-Propane Sulfonate (XV)

The sulfopropylation of compound **III** (1 g, 2.1 mmol) was performed in the manner described for the previous synthesis. The product (1 g, 78% yield) could not be successfully recrystallized. Mass spectroscopy (FAB+) indicated a protonated molecular ion with an m/z of 607 $(M+H)^{+\bullet}$, and elemental analysis gave C, 69.70%; H, 10.52%; N, 4.81%; S, 5.50% (theoretical, C, 69.25%; H, 10.32%; N, 4.62%; S, 5.27%). The product ran as a single spot on a TLC plate (Rf = 0.29) using chloroform:methanol:water (65:25:4 v/v/v). The sulfobetaine was

found to melt with decomposition at about 249°C. The NMR spectrum (not shown) displayed broad resonances in a variety of solvents. This was attributed to micelle formation.

2.3.3.2 Carboxyethylation of Steroid Derivatives

2.3.3.2.1 2-[(2-Cholesteryl-3ß-Carboxyamidoethyl)dimethylammonio]-1-Ethane Carboxylate (XVI)

The carboxyethylation of compound **III** was performed according to the procedure described by Ernst and Miller (1982). Cholesteryl-3ß-carboxyamidoethylenedimethylamine (0.53 g, 1.1 mmol) was dissolved in 10 mL of a mixture of diethylether:acetonitrile (3:1, v/v). A solution of acrylic acid (0.258 mL, 3.8 mmol) in 2 mL of diethyl ether:acetonitrile (3:1, v/v) was added at room temperature. The solution was stirred under nitrogen at room temperature for three days. A fine white powder precipitated. The product was filtered on a sintered glass funnel and washed with 3 x 2 mL of diethyl ether:acetonitrile (3:1, v/v) followed by 3 x 2 mL ether. The powder was dried under high vaccum, and gave 0.34 g of carboxybetaine, (yield, 56%), which melted with decomposition at 190°C. The compound ran as a single spot (Rf = 0.16) on silica gel TLC using chloroform:methanol:water (65:25:4, v/v/v) as the developing solvent. Mass spectromety analysis indicated a molecular ion with an m/z of 557 ($M^{+•}$). NMR spectrometry gave a spectrum (Figure 2.17) which shows the major ^1H assignments.
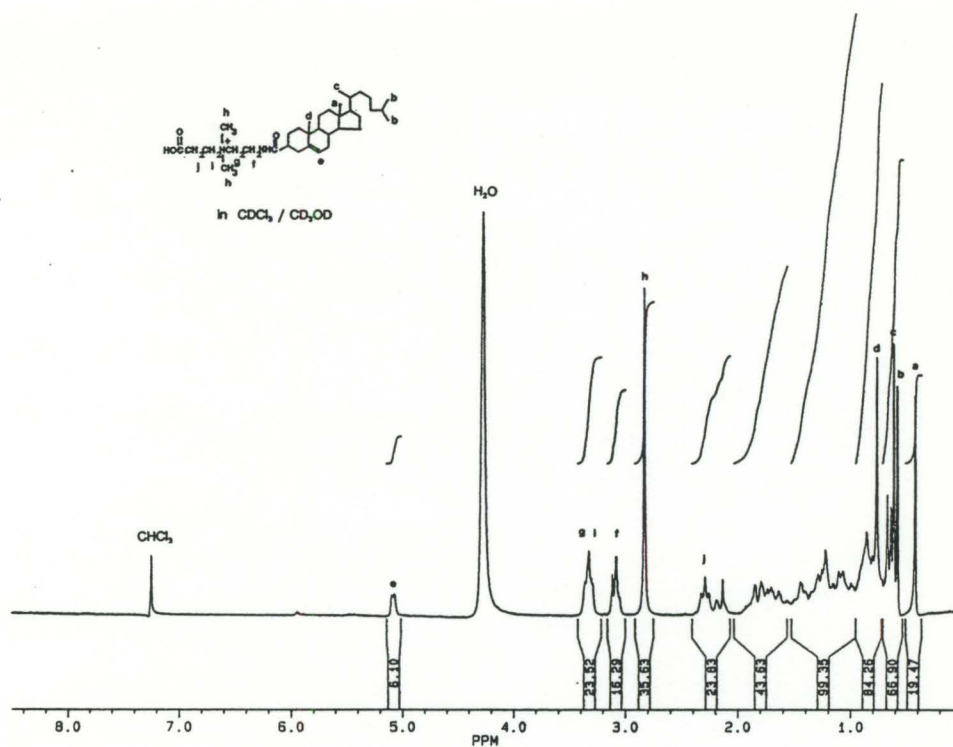
Figure 2.17 200 MHz NMR spectrum of compound **XVI**, inset top left.

2.4 Straight Chain Amphiphiles

Reactions for the synthesis of long chain bases are shown in Figure 2.18.
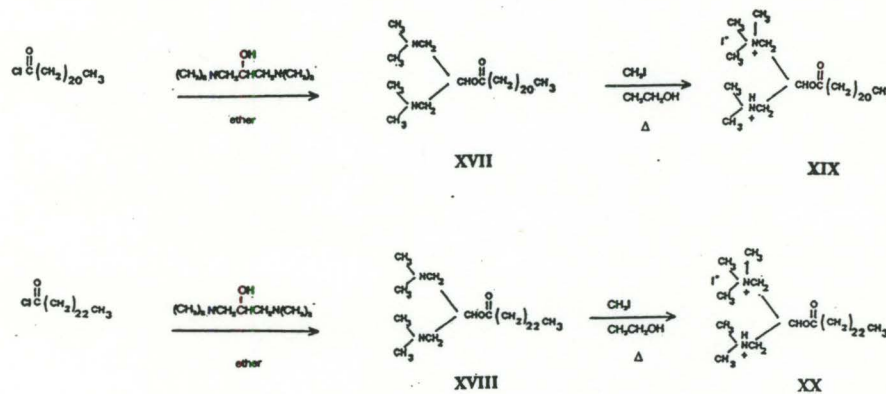
Figure 2.18 Reaction scheme for the synthesis of long chain bases.

2.4.1 1,3-Bis-Dimethylamino-2-Propyl Behenate (<u>XVII</u>)

The behenoyl ester of 1,3-bis-dimethylamino-2-propanol were prepared by dissolving behenoyl acid chloride (1 g, 2.8 mmol) in 50 mL of diethyl ether. The solution was slightly warmed and 1,3-bis-dimethylamino-2-propanol (2.5 mL, 15.3 mmol) was added. Almost immediately, a precipitate was formed. The reaction was gently refluxed for an additional hour, after which the solution was filtered. The ether phase which contained the product was back extracted three times with water to remove the excess unreacted base, after which it was dried with anhydrous sodium sulfate and rotary evaporated to dryness. The compound was subsequently lyophilized overnight. The ester was then crystallized by first dissolving it in a small volume of chloroform and adding this to a large volume of acetone. The homogeneous solution was allowed to cool at -20°C for 6 hours. White needle shaped crystals were obtained. The compound was found to be pure on a TLC plate using chloroform:methanol:water (65:25:4; v/v/v), Rf = 0.89, and chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v/v), Rf = 0.30, as the developing solvents. Mass spectrometry analysis (DCI-NH$_4$) showed the protonated molecular ion with an m/z of 469.6 (M+H)$^{+\bullet}$ for 1,3-bis-dimethylamino-2-propyl behenate. A melting point of 38-40°C was obtained. The NMR spectrum showing the ^{1}H assignments is depicted in Figure 2.19.
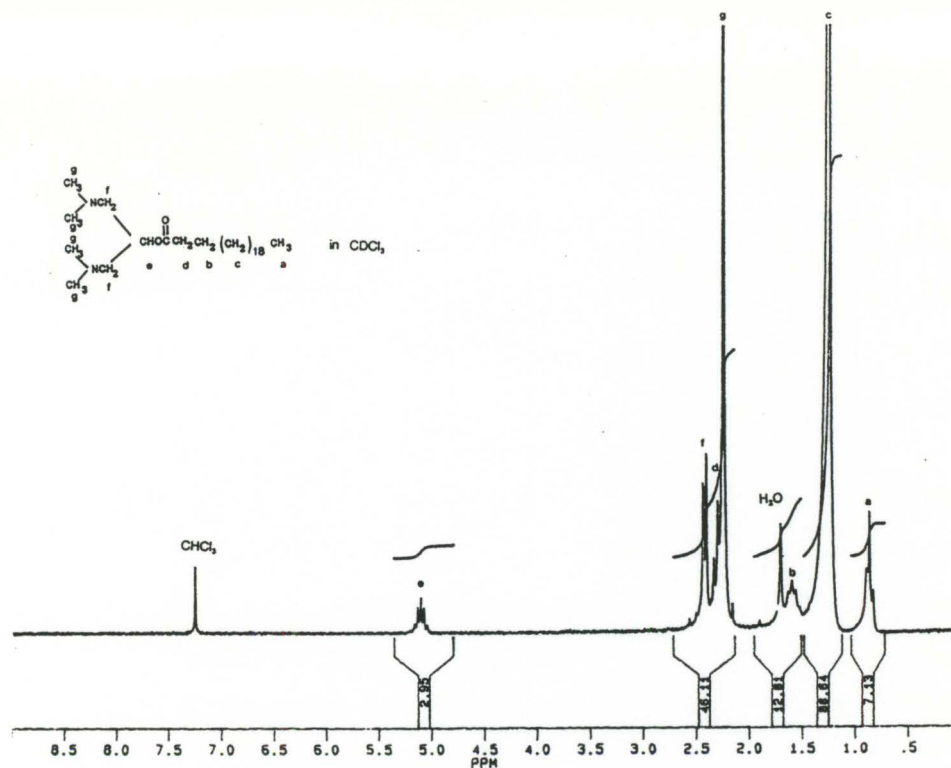
Figure 2.19 200 MHz NMR spectrum of compound **XVII**, inset top left.

2.4.2 1,3-Bis-Dimethylamino-2-Propyl Lignocerate (XVIII)

Lignoceroyl acid chloride (1 g, 2.6 mmol) was acylated with 1,3-bis-dimethylamino-2-propanol (2.5 mL, 15.3 mmol) as described for the preparation of compound **XV**. Crystallization from acetone afforded white needle shaped crystals which melted at 44-46°C. The product ran as a single spot and gave the same Rf's on a TLC plate as 1,3-bis-dimethylamino-2-propyl behenate. Mass spectroscopy analysis (DCI-NH4) showed the protonated molecular ion with an m/z of 497.5 $(M+H)^{+\bullet}$. The NMR spectra was similar to that obtained for compound **XVII**.

2.4.3   1-Dimethylamino-3-Trimethylammonio-DL-2-Propyl Behenate Iodide Salt (<u>XVIX</u>)

The quaternary ammonium analogue of compound <u>XVII</u> (0.5 g, 1.1 mmol) was produced by acylating with methyl iodide (2 mL, 32.1 mmol) in 10 mL of absolute ethanol as described for the synthesis of compound <u>VII</u> except that the reaction was allowed to continue for 8 hours. After 4 hours, an additional aliquot of methyl iodide (2 mL, 32.1 mmol) was added to the reaction mixture. The product crystallized out after cooling and was recrystallized from absolute ethanol. The methiodide ester, dissolved in a small volume of chloroform with a trace of methanol, was added to a large volume of acetone. Cooling this homogeneous solution to -20°C afforded a fine powder which ran as a single spot (Rf = 0.21) on a TLC plate using chloroform:methanol:water (65:25:4, v/v/v) as the developing solvent. The methiodide melted with decomposition at about 170°C. Mass spectrometry (FAB+) gave a molecular ion peak at an m/z of 484 ($M^{+\cdot}$), consistent with the methylation of only one of the possible two tertiary amine sites. This was also confirmed by the integral of the quaternary ammonium peak in the [^{1}H] NMR spectrum which corresponded to only 15 protons. The NMR spectrum with the ^{1}H assignments is depicted in Figure 2.20.
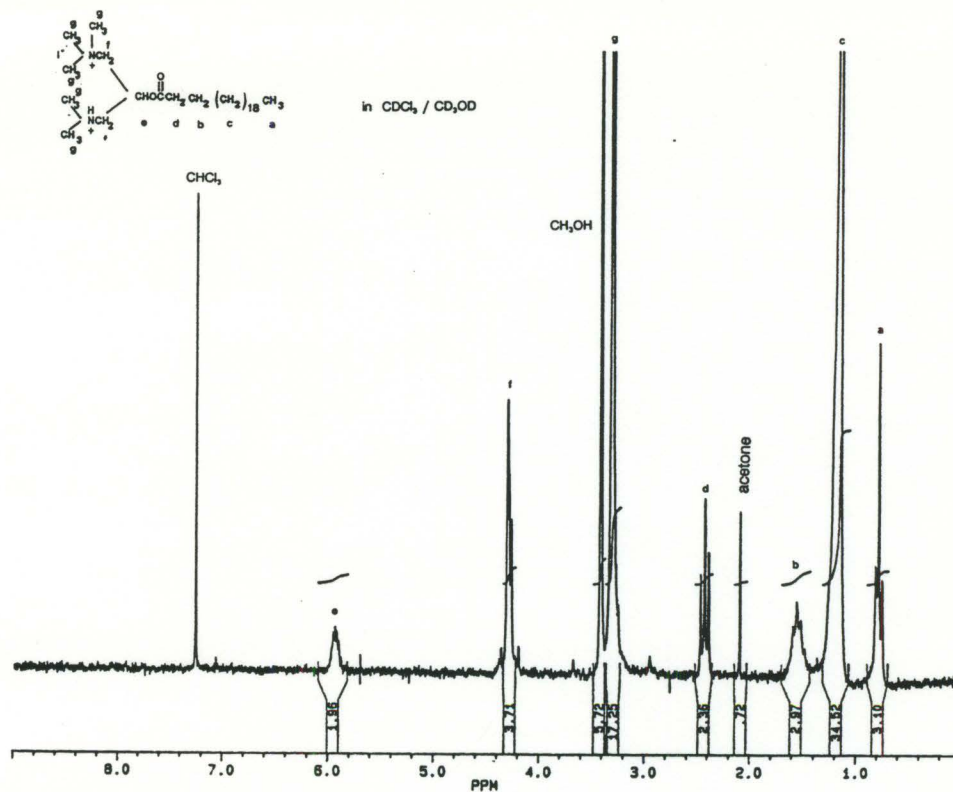
Figure 2.20   200 MHz NMR spectrum of compound **XIX**, inset top left.

2.4.4   1-Dimethylamino-3-Trimethylammonio-DL-2-Propyl Lignocerate Iodide Salt (XX)

The quaternary ammonium analogue of compound **XVIII** was produced as described of the synthesis of compound **XIX**. The powder obtained from crystallization in acetone gave a molecular ion with an m/z of 512 (M$^{+\bullet}$), and ran as a single spot on a TLC plate (Rf = 0.21) using chloroform:methanol:water (65:25:4; v/v/v) as the developing solvent. The compound melted with decomposition at about 170°C. The NMR spectrum was similar to that shown in Figure 2.20.

2.5 Tamoxifen Analogues

The synthesis of tamoxifen trimethyl amine is shown in Figure 2.21.
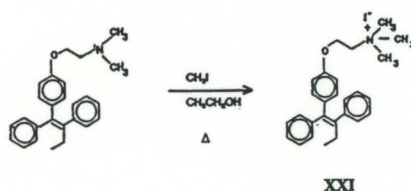
Figure 2.21 Reaction for the synthesis of tamoxifen trimethyl ammonium iodide.

2.5.1 Tamoxifen Trimethylammonium Iodide Salt (XXI)

The quaternization of trans-tamoxifen was performed according to the method described for the methylation of compound **IX** (see 2.3.2.2). Tamoxifen (0.5 g, 1.3 mmol) dissolved in absolute ethanol (10 mL) was refluxed for 0.5 hours with methyl iodide (2 mL, 32.1 mmol). The product crystallized out after cooling and ran as a single spot on a TLC plate (Rf = 0.46 vs Rf = 0.86 for trans tamoxifen). The product was further characterized with mass spectrometry (FAB+) which showed a molecular ion at an m/z of 386 ($M^{+\bullet}$). The NMR spectrum, Figure 2.22, depicts the proton assignments.

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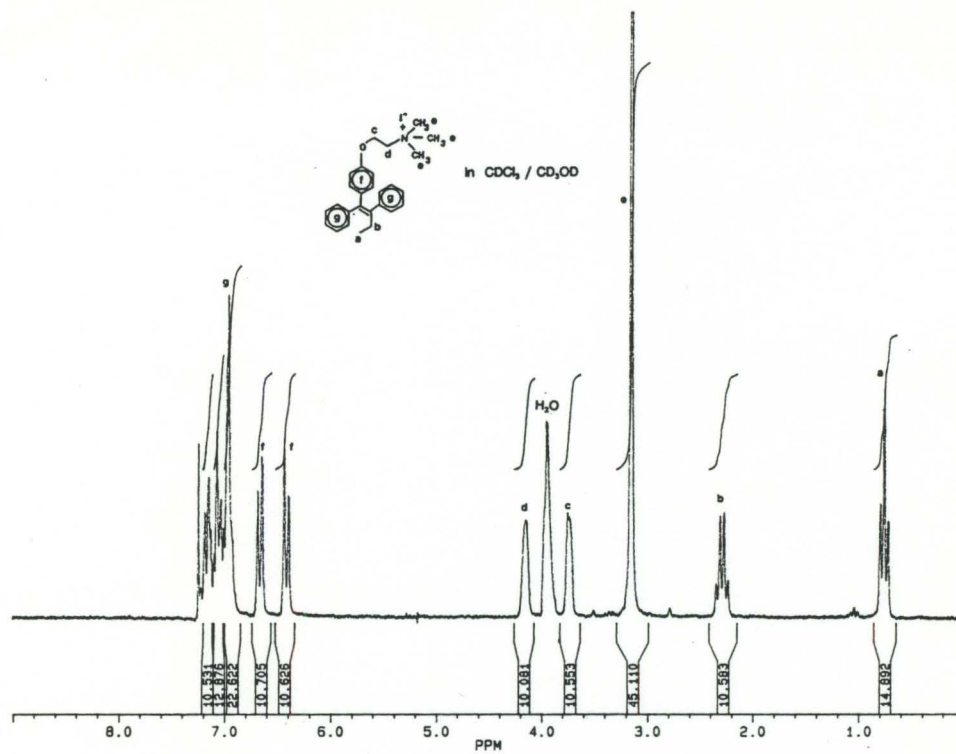
Figure 2.22 200 MHz NMR spectrum of compound **XXI**, inset top left.

3. THE EFFECT OF CATIONIC AND ZWITTERIONIC COMPOUNDS ON MEMBRANE BIOPHYSICAL PROPERTIES

3.1 INTRODUCTION

The ability of amphiphiles to modulate bilayer to hexagonal phase interconversion in model systems can be measured using a variety of techniques. Differential scanning calorimetry is a powerful, non-perturbing technique that can be used to determine the thermodynamic parameters associated with thermally induced lipid phase transitions (reviewed by McElhaney, 1986). The simplest model system to use for determining the structural properties of phospholipids is a dispersion of lipid in aqueous buffer. A lipid dispersion which can undergo a thermotropic transition is heated simultaneously with buffer. The buffer, which does not undergo a transition acts as a reference. When there is no thermal transition in the sample, the temperature of both the sample and reference cell remains equal and increases linearly with time. However, as the lipid undergoes a thermally induced transition, some of the heat supplied to the sample cell is absorbed by the lipid; the temperature differential between the sample and reference cells is detected and the instrument supplies more heat to the sample

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cell in order to maintain equal temperatures in both cells. A trace of the excess heat as a function of temperature is recorded; the transition appears as a vertical deflection from the baseline. An upward deflection indicates an endothermic event; its magnitude is proportional to the amount of excess specific heat absorbed. The lipid phase transition temperature is the temperature observed when the excess specific heat absorbed by the lipid reaches a maximum.

Phosphatidylethanolamines which are capable of undergoing bilayer to hexagonal phase interconversion are markedly sensitive to the presence of certain additives (Epand, 1985). Hydrophobic compounds generally promote hexagonal phase formation while amphiphilic compounds with a large ratio of head group to hydrocarbon volume inhibit its formation (Cullis et al., 1985; 1986). In this chapter, the bilayer stabilizing ability of the amphiphiles synthesized as described in chapter 2 will be determined using differential scanning calorimetry.

3.2 MATERIALS AND METHODS

3.2.1 Materials

1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) was purchased from Avanti Polar Lipids, Pelham AL. DEPE exhibited a sharp gel to liquid crystalline transition indicating a high degree of purity. Tamoxifen was obtained from Sigma Chemical Co., St. Louis MO and was shown to be chromatographically pure with TLC using chloroform:methanol:water (65:25:4) as the developing solvent.

3.2.2 Methods

3.2.2.1 Differential Scanning Calorimetry

DEPE (10 mg) and different concentrations of additive were dissolved together in a solution of chloroform:methanol, (2:1, v/v). The solvent was evaporated under a stream of nitrogen leaving a lipid film deposited on the walls of a glass test tube. Traces of remaining solvent were removed into a liquid nitrogen trap under high vacuum for 2 hours at 40°C. The lipid film was hydrated with 2 mL of 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, 0.02 mg/mL NaN_3, pH 7.4, warmed to about 45°C and vortexed vigorously for 30 seconds to suspend the lipid. Both buffer and suspended lipid solution were degassed for 20 minutes prior to loading into the reference and sample cell respectively of an MC-2 high sensitivity differential scanning calorimeter. A scan rate of 45 K/hour was employed. The enthalpy of the bilayer to hexagonal transition temperature was determined by fitting the hexagonal phase transition trace to a single Van't Hoff component using Microcal Co. software. The T_{max} of this fit was used as the value for the $L\alpha \rightarrow H_{\parallel}$ transition temperature. In cases where the transition was sharp and symmetrical, the fit T_{max} was the same as the temperature where the excess heat capacity is maximum (Cp_{max}). For the broader, less cooperative transitions, the fit T_{max} occasionally deviated (<0.2°C) from the temperature observed at Cp_{max}.

3.3 RESULTS

3.3.1 Amphiphiles in DEPE Bilayers

A number of cationic compounds have been designed to inhibit hexagonal phase formation. A convenient lipid to study the effects of additives on lamellar to hexagonal phase interconversion is DEPE. Generally, the concentration of additive was varied from 0 to 14 mole percent of the lipid. A series of DSC thermograms of DEPE with varying amounts of a cationic steroid is depicted in Figure 3.1. Pure DEPE undergoes a bilayer to hexagonal phase transition at approximately 65.6 °C. As the concentration of the drug is increased, the temperature at which the lipid undergoes this transition also increases. The change in bilayer to hexagonal transition temperature is plotted as a function of mole fraction of additive in Figure 3.2. The slope of this linear plot is a useful parameter describing the effect of an additive on the stability of the membrane. Hexagonal phase promoters (membrane destabilizers) give rise to negative slopes while bilayer stabilizers (inhibitors of the hexagonal phase) display positive slopes. The larger the magnitude of the slope, the more potent the additive is at either stabilizing or destabilizing the membrane. All zwitterionic and cationic steroid derivatives are bilayer stabilizers (Table 3.1, 3.2). The bilayer stabilizing effect of the cationic steroids is markedly increased when the positive charge is fixed on the molecule by quaternization. However, what is particularly interesting, is the effect of the tertiary amine straight chain amphiphiles on bilayer stability (Figure 3.2,

Table 3.3). These compounds are potent hexagonal phase promoters despite their large head group. Tamoxifen, a commonly used antineoplastic drug is also a potent hexagonal phase promoter (Table 3.5). However, after placing a permanent positive charge on these drugs bilayer stabilizing ability is markedly enhanced, particularly for the straight chain amphiphiles (Figure 3.2, Table 3.3, Table 3.5).
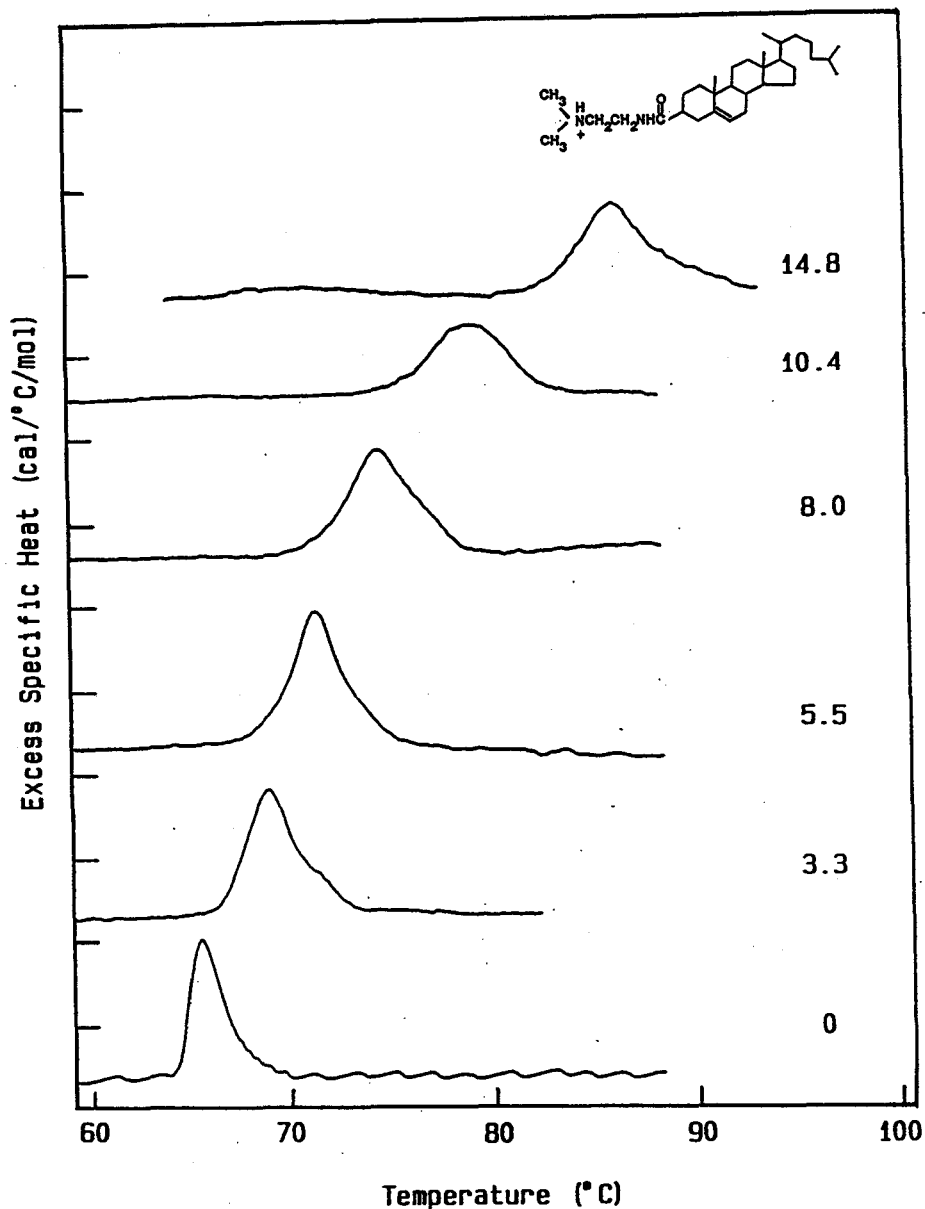
Figure 3.1   Differential scanning calorimetry (DSC) traces of the bilayer to hexagonal phase transition of DEPE in the presence of increasing concentrations of cholesteryl-3ß-carboxyamidoethylenedimethylamine (inset, top right) hydrated with 20 mM PIPES, 1 mM EDTA, and 150 mM NaCl containing 0.02 mg/mL NaN$_3$, pH 7.40.   The lipid concentration was 5 mg/mL.   The concentration of additive as a mole percent of DEPE is given to the right of the DSC trace.   The traces for various experiments were offset for clarity.   Each division on the ordinate axis represents 100 cal/°C/mol.
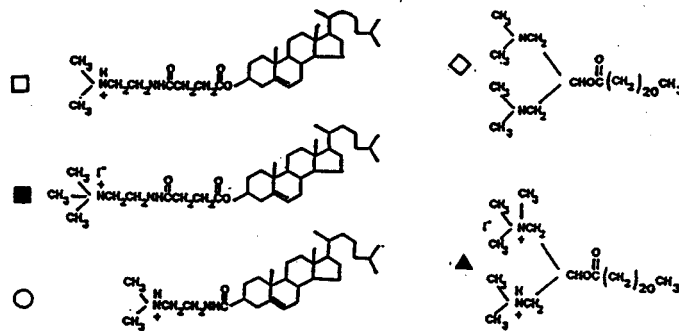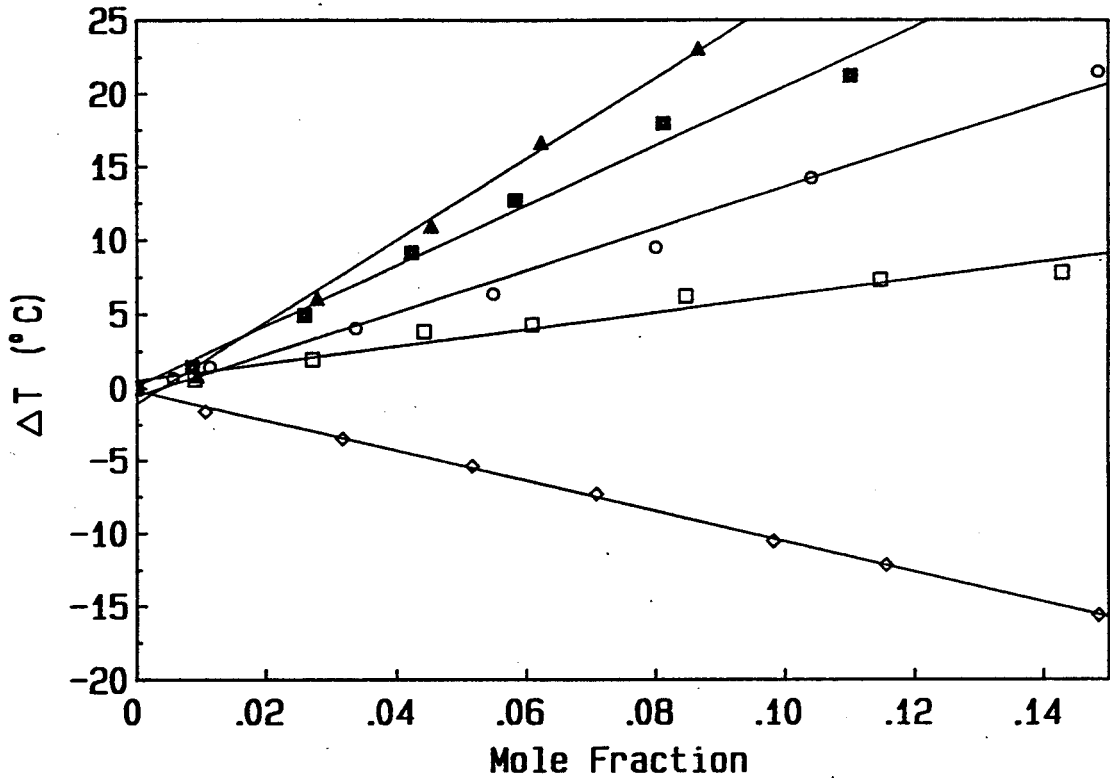
Figure 3.2 The shift in the bilayer to hexagonal phase transition temperature of DEPE as a function of different concentrations of additives. ΔT is the change in transition temperature from pure DEPE. The lipid (5 mg/mL) and additive were hydrated with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, and 0.02 mg/mL NaN$_3$, pH 7.40. The structures of the compounds giving the above correlations are shown.

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Table 3.1   Effect of Cationic Steroids on the $L\alpha \rightarrow H_{||}$ Phase Transition Temperature of DEPE.
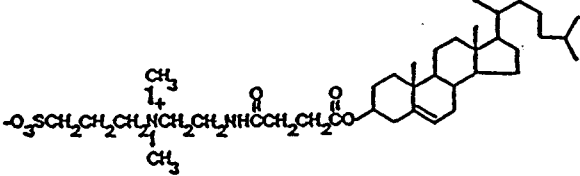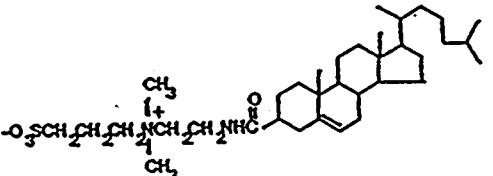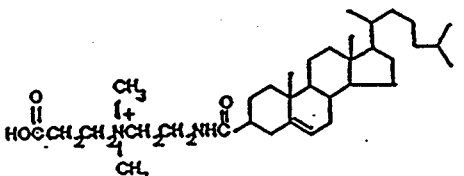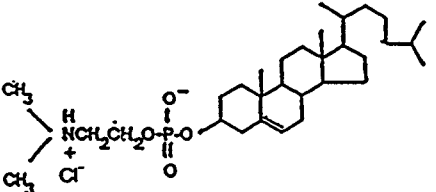
Amphiphile		Slope^a(K/mole fraction)
VIII		57 ± 5
IX		203 ± 10
III		147 ± 7
IV		159 ± 24
XII		136 ± 4
XIII		190 ± 7

^{a}The slope was obtained from a plot of bilayer to hexagonal phase transition temperature vs the mol fraction of additive (see Figure 3.1, Figure 3.2).

Table 3.1   continued
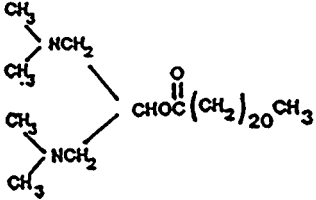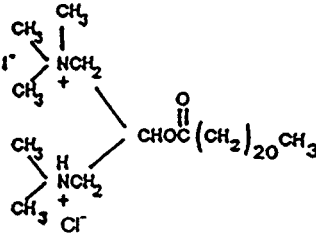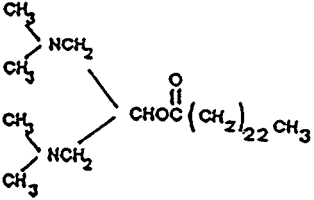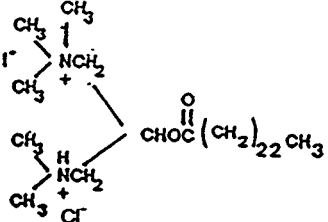
Amphiphile	Slope^a (K/mole fraction)
X	126 ± 4
XI	244 ± 9
V	52 ± 5
VI	239 ± 7
VII	44 ± 6

Table 3.2   Effect of Zwitterionic Steroids on the Lα → H$_{\parallel}$ Phase Transition Temperature of DEPE.

Amphiphile	Slope[a] (K/mole fraction)
XIV	208 ± 7
XV	65 ± 39
XVI	189 ± 8
	236 ± 13[b]
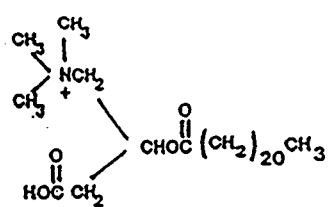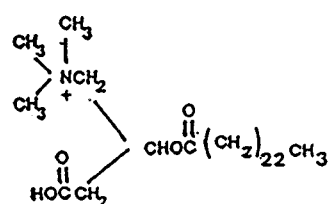
[a]The slope was obtained from a plot of bilayer to hexagonal phase transition temperature vs the mol fraction of additive (see Figure 3.1, Figure 3.2). [b]Epand et al., 1989a.

Table 3.3   Effect of Cationic Straight Chain Amphiphiles on the $L\alpha \rightarrow H_{\parallel}$ Phase Transition Temperature of DEPE.

Amphiphile	Slope[a] (K/mole fraction)
XVII	-104 ± 1
XIX	277 ± 10
XVIII	-110 ± 5
XX	246 ± 9
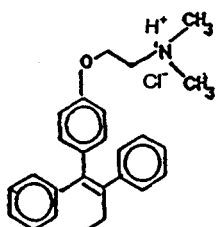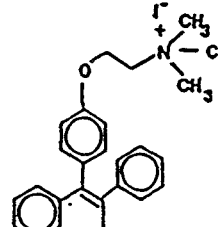
[a]The slope was obtained from a plot of bilayer to hexagonal phase transition temperature vs the mol fraction of additive (see Figure 3.1, Figure 3.2).

Table 3.4   Effect of Zwitterionic Straight Chain Amphiphiles on the L$\alpha \rightarrow$ H$_{\parallel}$ Phase Transition Temperature of DEPE.

Amphiphile	Slope[a] (K/mole fraction)
(structure: CH_3, CH_3, CH_3 groups on N^+CH_2; $CHOC(=O)(CH_2)_{20}CH_3$; $HOC(=O)CH_2$)	62 ± 17
(structure: CH_3, CH_3, CH_3 groups on N^+CH_2; $CHOC(=O)(CH_2)_{22}CH_3$; $HOC(=O)CH_2$)	100 ± 18

[a]The slope was obtained from a plot of bilayer to hexagonal phase transition temperature vs the mol fraction of additive.
Data was obtained from Epand et al., 1989b.

Table 3.5   Effect of Tamoxifen and Tamoxifen Trimethylammonium Iodide on the Lα → H$_{\parallel}$ Phase Transition Temperature of DEPE.

Amphiphile	Slope^a (K/mole fraction)
	-146 ± 8
XXI	83 ± 14

^{a}The slope was obtained from a plot of bilayer to hexagonal phase transition temperature vs the mol fraction of additive (see Figure 3.1, Figure 3.2).

3.4 DISCUSSION

The phase preference of lipids has been shown to be modulated by a number of factors such as hydrocarbon unsaturation, head group size, charge and hydration (Cullis et al., 1986). The molecular shape concept which considers a variety of these forces is a simple qualitative way to explain the effects observed for the various amphiphiles synthesized. The ability of an amphiphile to modulate bilayer to hexagonal phase equilibria can be predicted based on the shape parameter S, where $S = v/al$ (Israelachvili, 1980; Cullis et al., 1986). Here, v is the volume of the amphiphile, a is the head group surface area at the lipid - water interface, and l is the length of the extended hydrocarbon region. Values of $S > 1$ describes a molecules' preference to form the hexagonal phase, while values of $S < 1$ suggests that the molecule prefers to adapt the bilayer configuration. The steroid amines have a large head group cross sectional area (ie. a large value for a; $S < 1$) and will stabilize the bilayer. These results can also be explained by considering hydrogen bonding. A loss of hydrogen bonding decreases the tendency of PE to adopt a H_{II} configuration (Boggs, 1987; Epand, 1990b). The tertiary amine head group has the potential to form only one hydrogen bond. However, hydrogen bond formation with tertiary amines is difficult because of the steric hinderance around the nitrogen atom. As a consequence, the PE - PE hydrogen bonded lattice is disrupted around the amphiphile. Water fills the hydrogen bonding requirement; this results in a larger amphiphile head group volume, and

consequently an increase in the hexagonal phase transition temperature. Quaternization of the tertiary amine head group results in a number of changes. Quaternization gives the molecule the property of being more polar; it will be more hydrated and consequently a will be larger. Moreover, quaternization completely eliminates the molecules' ability to hydrogen bond subsequently decreasing hexagonal phase tendency. The ability of tamoxifen to promote $H_{\parallel}$ formation is largely due to its large bulky hydrocarbon volume (ie, a large value for v, $S > 1$). This could induce hydrocarbon splay in the centre of the bilayer and facilitate $H_{\parallel}$ phase formation. After quaternization, the molecule's head group volume and polarity are increased and subsequently it cannot partition as deeply into the bilayer, thus minimizing hydrocarbon splay. The shape parameter is however not a defined measurable quantity, and can change depending on the environment of the lipid. Gruner (1985) proposed the more quantitative curvature parameter which predicts a ligands' phase preference. The curvature parameter describes the ability of a lipid monolayer to bend from a flat planar surface with an intrinsic or relaxed radius of curvature. A small intrinsic radius of curvature favours hexagonal phase formation, while a large intrinsic radius of curvature is suggestive of a molecules' preference to form the bilayer configuration. The formation of the hexagonal phase is described as a competition between the intrinsic radius of curvature and hydrocarbon packing constraints which occurs in the hexagonal phase. In order for hexagonal phase formation to be favourable, void spaces between the hexagonal phase cylinders must be filled with hydrocarbon. The ability of 1,3-bis-dimethylamino-2-propyl analogues of the C22 and C24 straight

chains to promote hexagonal phase formation can be more easily explained by this concept. The C22 and C24 chains are much longer than the steroid hydrocarbon region, and most importantly, longer than the acyl chains of DEPE. Furthermore, the protonation state of the molecule could affect its position in the bilayer. An unprotonated molecule may penetrate deeper into the bilayer, increasing the hydrocarbon length. Consequently, the hydrocarbon region of these molecules can intercalate into the void spaces between the hexagonal phase cylinders, reducing the hydrocarbon packing constraints. This makes the formation of the hexagonal phase less unfavourable. Quaternization of the molecule would not allow the molecule to partition as deeply into the membrane, thus increasing hydrocarbon packing constraints. The large amount of positive charge would increase electrostatic repulsion in the hexagonal phase making it less favourable. Alternatively, quaternization which increase the head group size and hydration, also increases the intrinsic radius of curvature making hexagonal phase formation less favourable.

4. PROTEIN KINASE C ACTIVITY STUDIES

4.1 INTRODUCTION

Since the recognition that PKC is a major regulatory enzyme controlling numerous cellular processes, different classes of compounds have been reported to inhibit the enzyme. Many of the well established inhibitors of PKC have been assayed using either sonicated PS dispersions or Triton mixed micelles. The use of the former has received criticism for a number of reasons. The enzyme does not encounter a pure PS bilayer under physiological conditions. Moreover, vesicles formed from sonicated dispersions of PS are not structurally uniform (Hannun et al., 1985). Furthermore, pure PS does not form stable bilayers under the conditions used to assay PKC; defects formed on the vesicle surface may enhance enzyme activity and affect reproducibility (Boni and Rando, 1985). On the other hand, the use of the Triton mixed micelle assay allows for the variation of the lipid composition to give a more defined surface to present lipid co-factors necessary for enzyme activity (Hannun et al., 1985; Hannun et al., 1986a).

A drug design strategy was developed to incorporate both bilayer stabilizing ability and positive charge in attempt to optimize the features necessary to inhibit the enzyme. Moreover, cationic compounds such as sphingosine can inhibit PKC regardless of their ability to affect $L\alpha \rightarrow H_{\parallel}$ interconversion (Epand,

1987). In this chapter, the Triton mixed micelle assay is used to determine the importance of the protonation state of sphingosine for the inhibition of PKC. The Triton mixed micelle assay is also used to assess the efficacy of cationic bilayer stabilizing compounds at inhibiting PKC. The potency of such compounds is correlated with respect to their effect on bilayer stability and on their protonation state in the Triton mixed micelle.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Bovine brain phosphatidylserine was obtained from Avanti Polar Lipids, Pelham AL. Dipalmitoyl-N-dansyl-L-α-phosphatidylethanolamine (dansyl PE) was from Molecular Probes, Oregon. *sn*-1,2- diolein was purchased from Nu Chek Prep. Inc. Elysian, MN. Phenylmethylsulfonyl fluoride (PMSF), histone III-S, D-sphingosine, trans tamoxifen and polylysine agarose were obtained from Sigma Chemical Co., St. Louis, MI. DEAE Sepharose fast flow, Phenyl Sepharose CL-4B, and Sephacryl S-200HR were obtained from Pharmacia-LKB. Leupeptin was from IAF Biochem. Int., Montreal, Quebec and γ-^{32}P-adenosine 5'-triphosphate was from NEN, Quebec. Behenoyl and lignoceroyl carnitines were synthesized by Kelli Robinson as previously described (Epand et al., 1990b).

4.2.2 Methods

4.2.2.1 Isolation of Protein Kinase C

Protein kinase C was purified to near homogeneity by a modification to a procedure previously described by Huang and co-workers (1986). As rapidly as possible, brains from 25 Sprague-Dawley rats (150 - 200 g) were removed, washed with 100 mL of 20 mM TRIS, 1 mM EDTA, 1 mM EGTA, pH 7.5, and homogenized in 150 mL of ice cold 20 mM TRIS, 10 mM EGTA, 2 mM EDTA, 10 mM DTT, 0.25 M sucrose, 2 mM PMSF and 100 μg/mL leupeptin, pH 7.5. The homogenate was immediately centrifuged at 100,000 g for 40 minutes at 4°C in a Beckman Ti 50.2 rotor. The supernatant was applied to a 2.5 x 20 cm column of DEAE Sepharose (fast flow) containing 60 mL of resin equilibrated with 20 mM TRIS, 1 mM EDTA, 1 mM DTT, pH 7.5 (buffer A). The column was washed with 300 mL of buffer A and an additional 200 mL of buffer A containing 0.03 M KCl. Protein kinase C was eluted with a 500 mL continuous KCl gradient (0.03 - 0.3 M KCl). Fractions of 5 mL volumes were collected. Fractions showing calcium and phospholipid dependence were pooled; the salt concentration was adjusted to 1.5 M KCl with the appropriate quantity of solid KCl. The crude sample containing 1.5 M KCl was stirred for 15 minutes and subsequently loaded onto a 1 x 10 cm column containing 9 mL Phenyl Sepharose equilibrated with 1.5 M KCl in 20 mM TRIS, 0.5 mM EGTA, 1 mM DTT, pH 7.5 (buffer B). The column was washed with 90 mL of buffer B containing 1.5 M KCl. PKC was

eluted with a 100 mL continuous KCl gradient (1.5 - 0 M KCl). Fractions of 3 mL volumes were collected. The column was washed with an additional 50 mL of buffer B. Most of the enzyme activity eluted during this stage. Fractions showing calcium and phospholipid dependence were pooled and concentrated to 4 mL using an Amicon ultrafiltration cell fitted with a YM-10 filter. The concentrated sample was loaded onto a 2.5 x 100 cm column containing 400 mL of Sephacryl S-200 HR beads equilibrated with buffer B containing 10% glycerol (buffer C). Fractions of 3 mL volumes were collected. About 150 mL of buffer was run through; PKC eluted very close to the column void volume. The fractions showing calcium and phospholipid dependence were pooled and loaded onto a 0.5 x 5 cm column containing 2.5 mL polylysine agarose equilibrated with buffer C. PKC was eluted with a 40 mL continuous KCl gradient (0 - 0.8 M KCl). Fractions of 1 mL volumes were collected. The first few active fractions were contaminated. The uncontaminated fractions were pooled, concentrated, and diluted with buffer C to remove the high salt content. After reconcentrating, the sample was divided into working portions, frozen in liquid nitrogen and stored at - 80°C. Full activity was regained after rapid thawing. Trace impurities (116 k, 66 k, and 50 k Mr) could still be detected when the gel was silver stained heavily. The enzyme gave a specific activity of 200 nmoles phosphate incorporated per minute per milligram of protein when assayed for histone phosphorylation using the Triton mixed micelle assay with 6.5 mole % phosphatidylserine, 2.5 mole % DAG and 100 μM calcium present. Specific activities ranging from 30 nmoles/min/mg (Hannun et al., 1985) to 600 nmoles/min/mg (Hannun and Bell, 1988; Loomis and Bell, 1988) have been

observed for PKC using the Triton mixed micelle assay under the same conditions.

4.2.2.2   Mixed Micelle Assay of Protein Kinase C

The Triton X-100 assay previously described by Bell and coworkers was used to measure enzyme activity (Hannun et al., 1985). Phosphatidylserine and 1,2-diolein with and without additive were dissolved in a solution of chloroform/methanol (2:1, v/v). Solvent was evaporated with a stream of nitrogen and last traces removed using a vacuum desiccator at 40°C. The lipid films were then solubilized by the addition of 3% Triton X-100, vortexed vigorously for 30 sec and then incubated at 30°C for 10 minutes to allow for equilibration. A 25 μL aliquot of this solution was used in a final assay volume of 250 μL, containing 20 mM TRIS-HCl, pH 7.5, 10 mM $MgCl_2$, 200 μg/mL histone III-S, 100 μM $CaCl_2$, 10 μM [γ-^{32}P] adenosine 5' triphosphate, 2.75 mM Triton X-100, with 300 μM (6.5 mole percent) phosphatidylserine and 107 μM (2.5 mole percent) 1,2-diolein. For controls, 25 μL of 20 mM EGTA replaced the $CaCl_2$. To initiate the reaction, 150 ng of protein was added. After briefly mixing, the tubes were incubated for 10 minutes at 30°C. The reaction was terminated by adding 1 mL of cold 0.5 mg/mL BSA and 1 mL of cold 25% trichloroacetic acid. This mixture was passed through a GF/C Whatman filter and washed five times with 2 mL of 25% trichloroacetic acid. After drying, the filters were counted with 6 mL ACS scintillation fluid.

4.2.2.3 Vesicle Assay for Protein Kinase C

Lipid films containing PS, 1,2 diolein, in the presence or absence of additive were prepared as described for the mixed micelle assay. The films were subsequently suspended in water and sonicated under argon for 10 minutes. Each assay tube contained 10 μM [γ-^{32}P] ATP, 10 mM MgCl$_2$, 200 μg/mL PS with or without additive. The reaction was initiated with the addition of PKC (150 ng). After 10 minutes, 100 μL of BSA (1 mg/mL) followed by 1 mL of 25% trichloroacetic acid were added. After cooling on ice for 10 minutes, each assay tube was filtered through a GF/C Whatman filter and washed five times with 2 mL of 25% trichloroacetic acid. The filters were dried and counted with 6 mL of ACS scintillation fluid.

4.2.2.4 Estimation of the Intrinsic pK of Cationic Amphiphiles in Triton Micelles

The intrinsic pK of sphingosine, tamoxifen, behenoyl ester, and cholesteryl-3ß-carboxyamidoethylenedimethylamine were estimated using [^{1}H] NMR. NMR spectra were recorded on a Bruker-AM500 spectrometer. A sample for NMR was prepared by taking a 10% Triton solution made up in 5% D$_2$O, 95% deionized distilled water, and vortexing vigorously with the appropriate quantity of drug to give a 10 mg/mL solution. The resulting suspensions required brief sonication. Both sphingosine and 1,3-dimethylamino-2-propyl behenate gave clear, homogeneous solution after sonication, while tamoxifen and cholesteryl-3ß-

carboxyamidoethylenedimethylamine remained cloudy until the pH was brought below 5.5. Drug containing micelles were titrated with 10 or 100 mM NaOH or 10 or 100 mM HCl, taking a pH reading before and after the NMR spectra were recorded. In an independent experiment the same result for sphingosine was confirmed by potentiometric titration after correcting for the amount of base required to alter the pH of a solution of the detergent alone.

4.2.2.5 Protein Kinase C Binding to Phospholipid Vesicles

The binding of PKC to phospholipid vesicles in the presence or absence of a cationic amphiphile was measured using fluorescence energy transfer according to the procedure previously described by Bazzi and Nelsestuen (1987a). Phosphatidylserine was mixed with dansyl PE to give a 1:10 weight percent ratio of dansyl PE to PS. As a representative of the cationic amphiphiles, compound XI (see 2.3.2.4) was the drug under investigation. The drug concentrations, where present, were 8.8, 26.5, and 48.5 mole percent of the PS concentration. After drying the lipid solution under a stream of nitrogen, last traces of chloroform/methanol were removed from the lipid film under high vacuum. The lipid film was suspended in 20 mM TRIS, 100 mM NaCl, pH 7.5, vortexed vigorously and sonicated using a bath type sonicator until the solution was homogeneous. Sonication was performed under argon for approximately 10 minutes. The resulting solution was passed through a 1.5 x 20 cm Sephadex G-50 column equilibrated with the same buffer as above to separate the vesicles from

any unincorporated probe. Phospholipid vesicles eluted at the column void volume. Fluorescence energy transfer experiments were performed using an MPF-44 Perkin Elmer spectrofluorimeter with an excitation wavelength of 284 nm and an emission wavelength of 500 nm. The vesicles were generally diluted 100 fold in the fluorometer cuvette. PKC (18 μg) and subsequently $CaCl_2$ (final concentration, 200 μM) were added to the cuvette. The temperature was maintained at 25°C. Changes in the fluorescence intensity in the presence of PKC and calcium before and after the addition of EGTA were recorded.

4.3 RESULTS

4.3.1 The Importance of Positive Charge on Sphingosine

4.3.1.1 pH Effects on the Inhibition of Protein Kinase C by Sphingosine

The role of the 2-amino group of sphingosine on the *in vitro* inhibition of PKC was investigated by comparing PKC activity in the presence and absence of sphingosine over the pH range of 7 to 8.5. The pH range for optimal enzyme activity in the absence of sphingosine is between 7.5 and 8.0, agreeing with previous reports (Kikkawa and Nishizuka, 1986b). When sphingosine is present in Triton micelles, inhibition of PKC occurs in a pH dependent manner (Figure 4.1). Between the pH's of 7 and 7.5, inhibition appears to remain constant; the curves of the inhibited and uninhibited enzyme are roughly parallel. However, at pH's
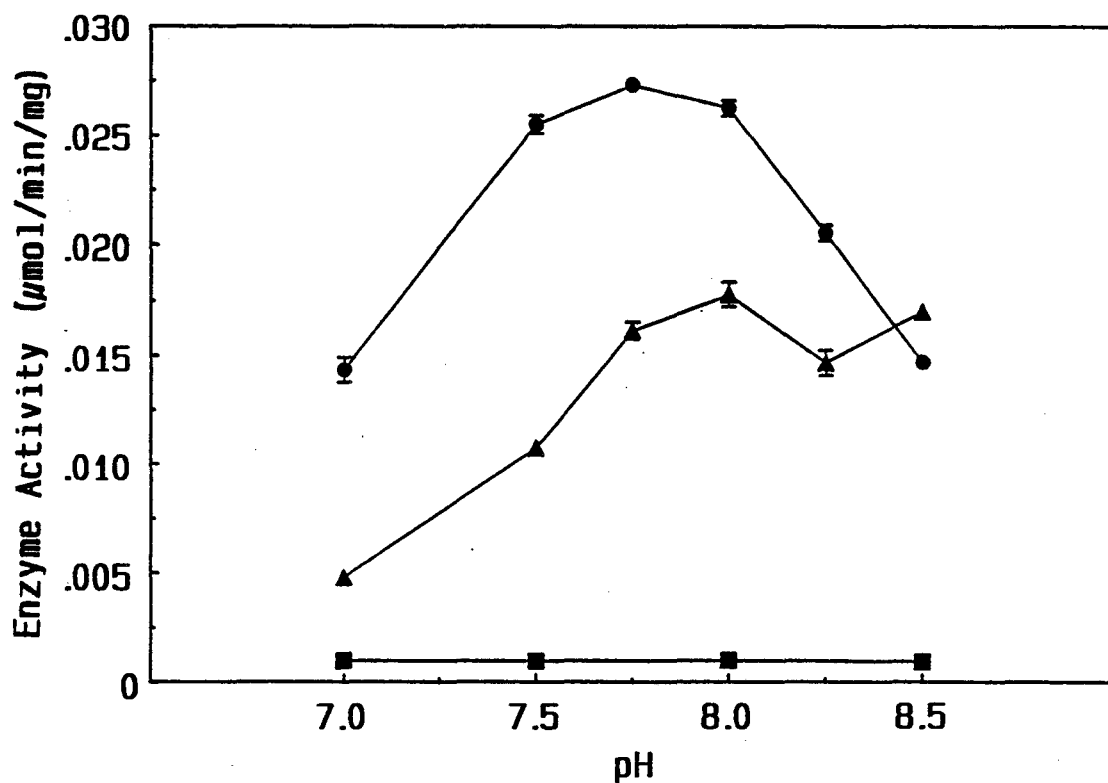
Figure 4.1 The pH dependence of calcium, phosphatidylserine and diacylglycerol stimulated PKC activity assayed with Triton X-100 micelles in the absence of sphingosine (●) and in the presence of 160 μM sphingosine (▲). Control in Triton X-100 micelles with EGTA and with no other additives (■). Reproduced from Bottega et al., 1989.

above 7.75, sphingosine is not as potent an inhibitor; both curves begin to converge. At pH 8.5, sphingosine actually activates the enzyme to a small extent. Unfortunately, this study could not be extended to lower or higher pH's because of loss of buffering capacity or loss of enzyme activity respectively. The decreasing inhibition with increasing pH suggested an effect resulting from a change in the state of ionization of sphingosine.

4.3.1.2   The Protonation State of Sphingosine in Triton Micelles

To assess the state of protonation of sphingosine in Triton micelles, a titration measuring the changes in the NMR spectra as a function of pH was performed. The chemical shift of the CH proton adjacent to the amino group is sensitive to the protonation state of the amino group and was displaced from 4.24 to 3.88 ppm between the pH's of 4.38 and 8.95 (data not shown). A complete titration curve for sphingosine could not be generated at higher pH's because the CH proton signal of interest merged with a resonance generated by the detergent. Nevertheless, at extremely high pH's, the chemical shift was not displaced beyond this resonance frequency to a lower chemical shift allowing for an approximation of the upper limit of the titration curve. In an independent experiment, a potentiometric titration gave a curve that was superimposable with that which was obtained by NMR (Figure 4.2). From the titration curve, the apparent pK of sphingosine in Triton micelles is 7.7. This is in contrast to a recent study which reported the pK to be 6.7 (Merrill et al., 1989). This may be due to the use of

a 20 fold greater concentration of sphingosine and Triton X-100. Although the sphingosine to Triton ratio is the same in both studies, more acid and base is required for titrating the 20 fold greater quantity of sphingosine present in these micelles. Consequently, the ionic strength of the solution would be increased. This would suppress electrostatic repulsion and as a result, raise the apparent pK to higher values probably contributing to the observed discrepancy. Regardless of the precise details of the ionization properties of sphingosine, it is clear that inhibition of protein kinase C by sphingosine correlates with its state of protonation. However, an unusual feature of this titration curve is that unlike the titration curves of small, soluble molecules, the titration curve obtained for sphingosine is broad suggesting that there are electrostatic interactions among adjacent sphingosine molecules within the detergent micelle. Figure 4.3 shows the data for the titration curve of sphingosine plotted to take into account electrostatic repulsion. In cases where electrostatic interactions do not occur, $pH-log[\alpha/(1-\alpha)]$ is independent of pH (Tanford, 1961). However, this is not the case for sphingosine in Triton micelles, suggesting that the apparent pK is affected by electrostatic repulsion in the micelle. When the net charge of sphingosine is zero (i.e. when the degree of dissociation, α is 1), the intrinsic pK of the drug in the micelle can be obtained from the value of the ordinate. Thus, the intrinsic pK for sphingosine in Triton micelles corrected for electrostatic repulsion is 8.5.
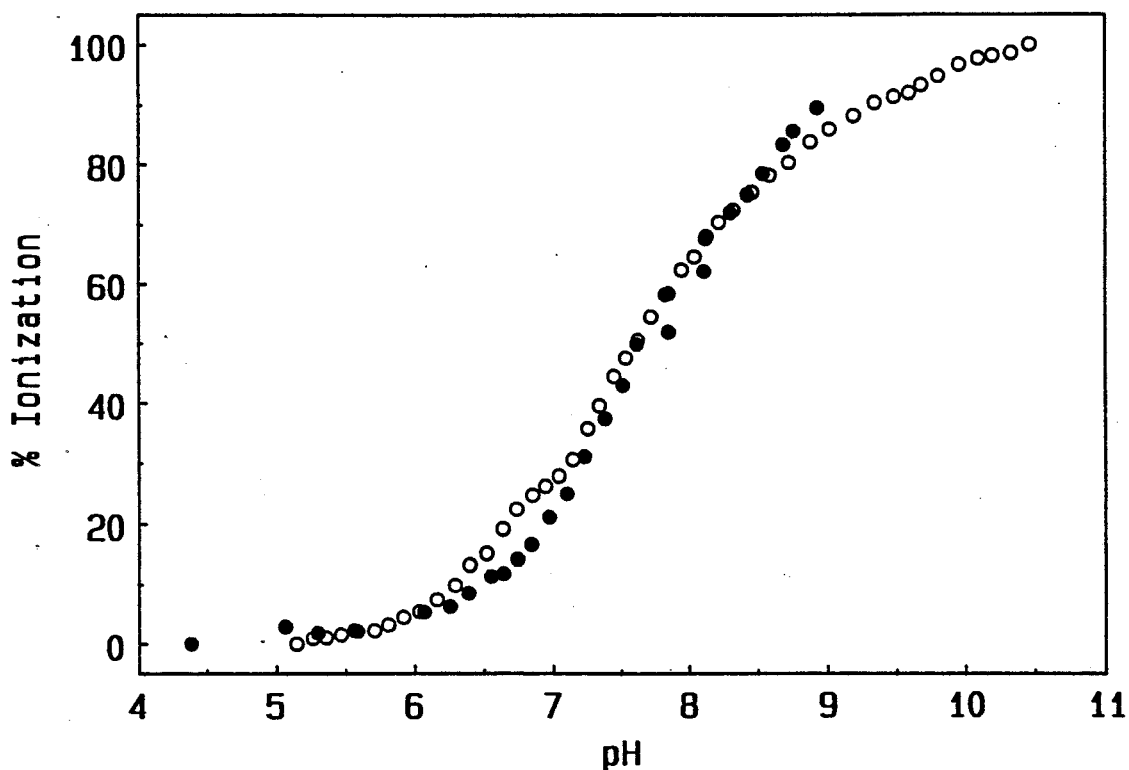
Figure 4.2   pH titration curves of sphingosine in Triton-X100 measured potentiometrically (O) and with [¹H] NMR (●) as described under methods. The sphingosine concentration was 10 mg/mL in a 10% solution of Triton X-100 made up in 95% H_2O, 5% D_2O. D_2O served as a lock signal for the magnetic field throughout the experiment. The deviation observed between pH 6.4-7.4 for the potentiometric titration curve is an artifact. Reproduced from Bottega et al., 1989.
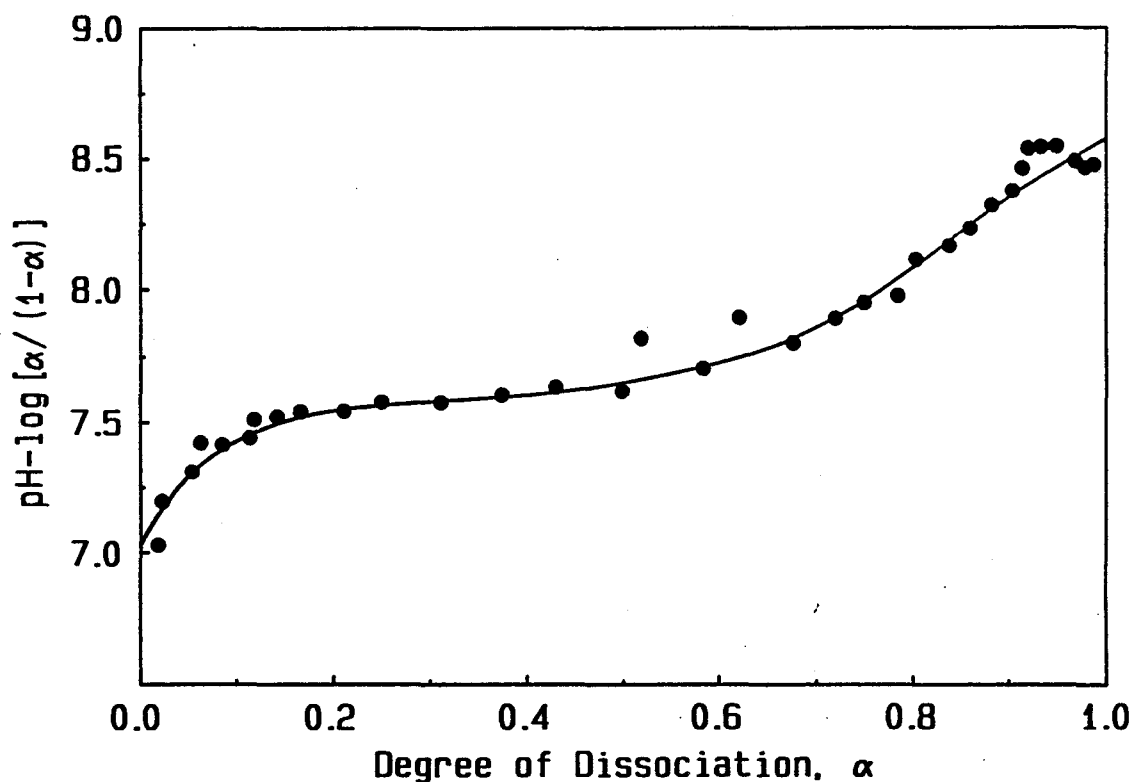
Figure 4.3   Dependence of pH-log[α/1-α] on the degree of dissociation of sphingosine (α). The data used are from Figure 4.2. Because the NMR titration method does not require correction for a blank, the data from NMR is considered more accurate and is used to generate this analysis. However, above pH 9, the potentiometric titration curve was used to complete this plot. Reproduced from Bottega et al., 1989.

4.3.2 Inhibition of Protein Kinase C by Charged Amphiphiles

Drugs containing a steroid or straight chain hydrocarbon backbone were synthesized to determine whether or not the nature of the hydrocarbon moiety alters the ability of a compound to inhibit PKC. The head group structure was also varied to ascertain whether or not the amount of charge or location of the charge (spatially close or distant from the membrane surface) enhances the capability of an amphiphile to inhibit PKC activity. Drug potency was determined by measuring the concentration giving 50% inhibition of the enzyme. This is expressed as an IC_{50}. The IC_{50} is presented in Tables 4.1 - 4.5 for all the amphiphiles studied.

4.3.2.1 Steroid Based Cationic Amphiphiles

The cationic steroids possessing a tertiary amine head group inhibited PKC with an IC_{50} in the range of 150 - 650 μM (Table 4.1). However, there was a marked effect upon their quaternization. Introducing a fixed positive charge on the steroid resulted in inhibitors which were generally 10 fold more potent than their precursor tertiary amine counterparts. The dose response curves for a steroid containing a tertiary amine head group and its corresponding quaternary amine counterpart (Figure 4.4) show that upon quaternization the enzyme inhibition curve is markedly shifted to lower amphiphile concentrations. The quaternary amine steroids completely eliminated calcium, PS and diolein induced stimulation of the
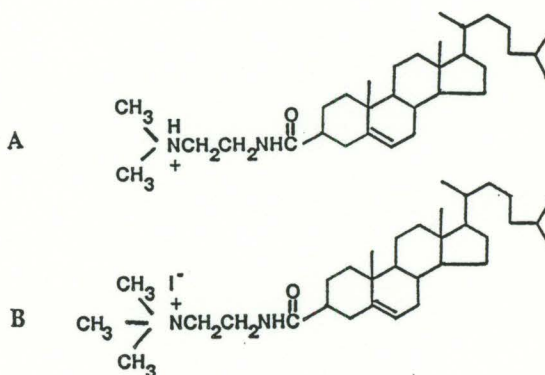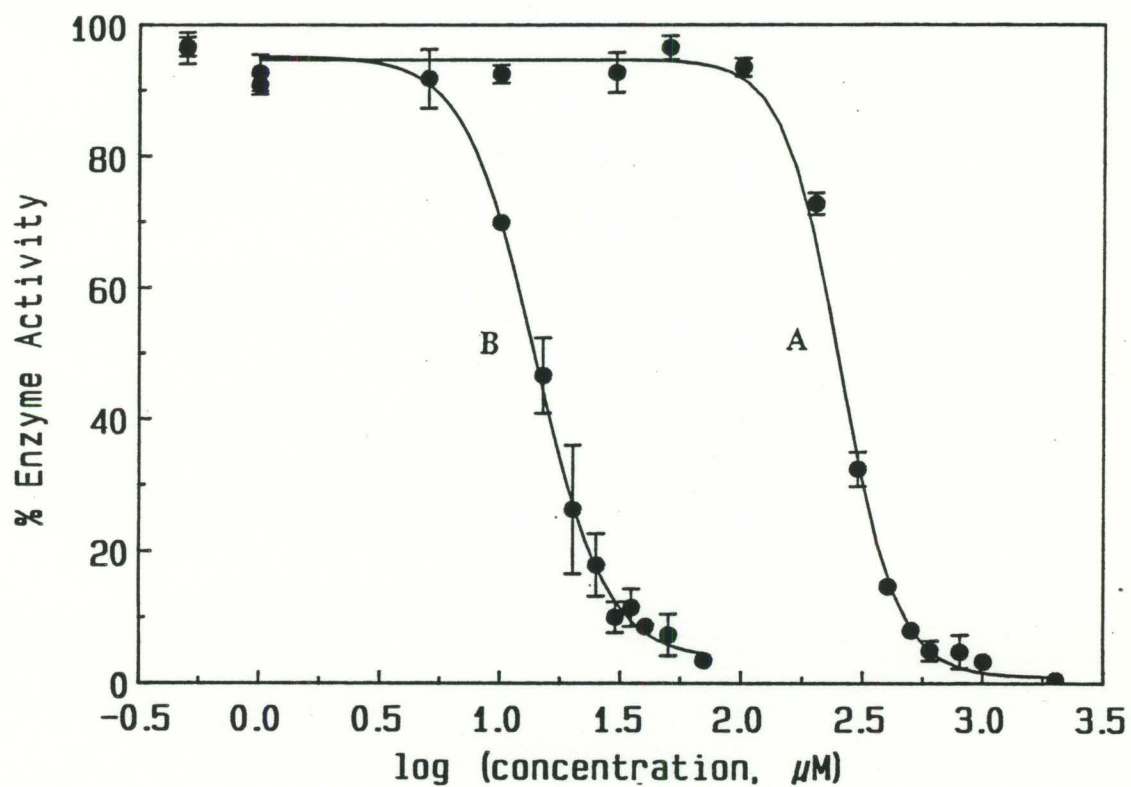
Figure 4.4 The effect of a cationic tertiary amine steroid (A) and its quaternary ammonium counterpart (B) on the inhibition of calcium, phosphatidylserine and diolein stimulated PKC assayed using Triton X-100 mixed micelles as described under methods.

Table 4.1   Potency of Cationic Bilayer Stabilizing Steroids on the Inhibition of Calcium, Phosphatidylserine and Diacylglycerol Stimulated Protein Kinase C. Enzyme activity was measured using the Triton mixed micelle assay as described under methods.
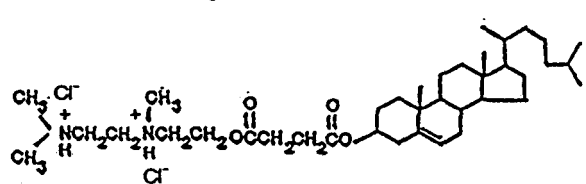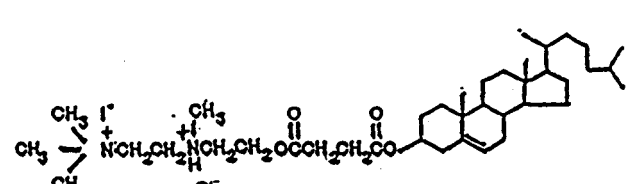
Amphiphile		IC50 (µM)
VIII		191
IX		59
III		258
IV		12
XII		164
XIII		20

Table 4.1 continued
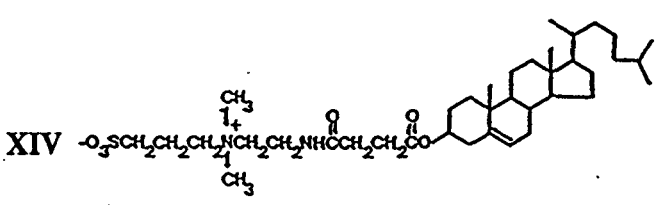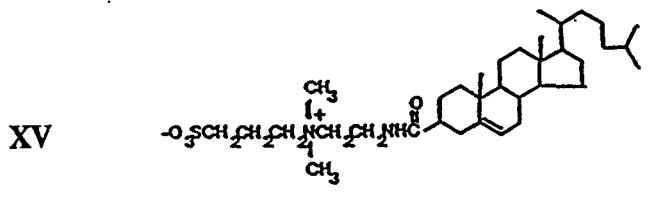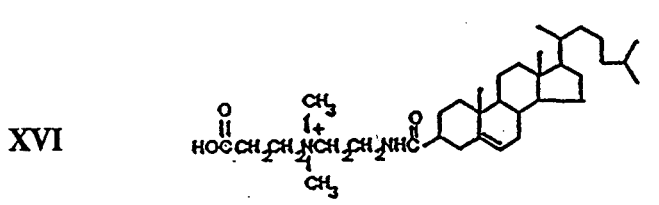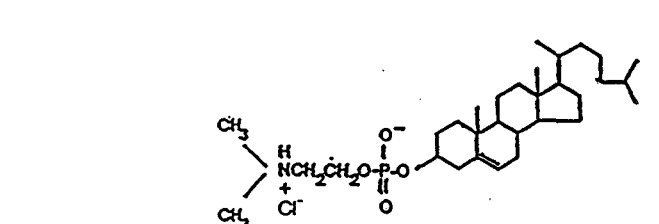
Amphiphile	IC50 (μM)
X	408
XI	15
V	643
VI	11
VII	246

enzyme, with 50% inhibition occurring in the 11 - 59 μM range (Table 4.1). Apart from quaternization, the size and structure of the amine moiety did not appear to contribute significantly to the inhibition of the enzyme.

4.3.2.2 Steroid Based Zwitterionic Amphiphiles

Cholesterylphosphoryldimethylethanolamine (CPD), a zwitterionic steroid bilayer stabilizer, has previously been reported to be a potent inhibitor of PKC giving an IC_{50} of 29 μM (Epand et al., 1989). This inhibitor contains a phosphoryl anion and a tertiary amine functionality. Additional zwitterionic steroids were synthesized to determine whether or not enzyme activity is affected by the nature of the charged moieties. Sulfobetaines which contain a sulfate group or carboxybetaines which possess a carboxylate anion are zwitterionic molecules whose anionic site is separated from a quaternary ammonium group by a short hydrocarbon spacer. They also differ from CPD in that the charged functionalities are reversed. For instance, CPD possesses a negative charge close to the steroid *A* ring while the sulfobetaines and carboxybetaines possess the positive charge closer to the steroid *A* ring. The sulfopropylation of two steroid tertiary amines gave sulfobetaines with 50% inhibition occurring in the 400 - 700 μM range (Table 4.2). Cholesteryl-3ß-carboxyamidoethylene sulfobetaine inhibited with an IC_{50} of 625 μM. However, if the sulfate moiety is replaced by a carboxyl group, the carboxybetaine of cholesteryl-3ß-carboxyamidoethylenedimethylamine was 3 fold more potent at inhibiting the enzyme than its sulfated analogue with an IC_{50} of 200 μM. It was still significantly less potent than CPD.

Table 4.2   Potency of Zwitterionic Bilayer Stabilizing Steroids on the Inhibition of Calcium, Phosphatidylserine and Diacylglycerol Stimulated Protein Kinase C. Enzyme activity was measured using the Triton mixed micelle assay as described under methods.
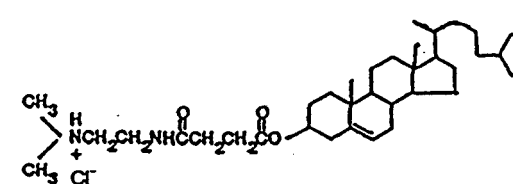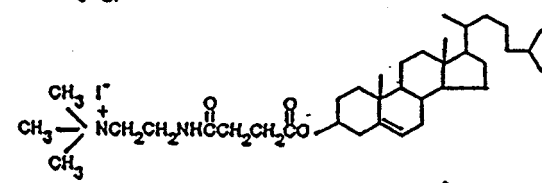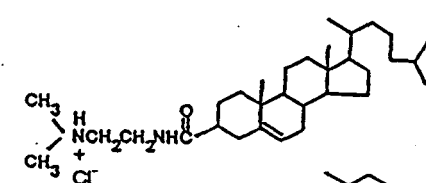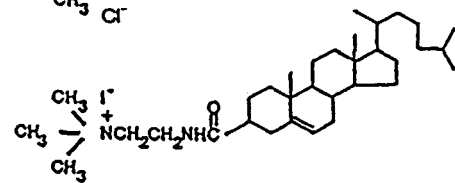
Amphiphile	IC50 (μM)
XIV	447
XV	625
XVI	200
	29 [a]

[a]Epand et al., 1989a.

4.3.2.3 Zwitterionic Straight Chain Amphiphiles

One of the well established zwitterionic amphiphilic inhibitors of PKC is palmitoyl carnitine. A study with acyl carnitines comprised of different hydrocarbon chain lengths has also been performed (Wise and Kuo, 1983). However, the longest chain analyzed was the C18 analogue. Recently, carnitine analogues containing either a C22 or C24 saturated chain (behenoyl and lignoceroyl carnitine respectively) have been synthesized (Epand et al., 1989b). Behenoyl and lignoceroyl carnitines inhibit PKC with 50% inhibition occurring at 0.2 and 0.6 μM respectively (Table 4.3). The vesicle assay gave comparable potencies, with half maximal inhibition occurring at submicromolar concentrations.

4.3.2.4 Cationic Straight Chain Amphiphiles

In order to assess whether or not the C22 or C24 hydrocarbon backbone contributed to the potency of the C22 or C24 carnitine analogues, straight chain hydrocarbon derivatives were designed to contain two tertiary amine sites. These compounds are analogous to 1,3-dimethylamino-2-propyl cholesteryl-3ß-carboxylate except that the steroid backbone has been substituted by a straight chain hydrocarbon. It was striking that when the steroid backbone is replaced with a straight chain hydrocarbon, the tertiary amine does not inhibit PKC at concentration up to 1 mM. Moreover, these compounds are hexagonal phase promoters (Table 3.3). Their lack of potency at inhibiting PKC, and their tendency

to promote hexagonal phase formation suggests that they are uncharged in the Triton micelle. Based on their ability to promote hexagonal phase formation, it would be expected that these compounds are activators of PKC. This was shown to be negligible in the absence and presence of DAG. However, quaternization of these compounds generated PKC inhibitors with half maximal inhibition occurring at concentrations that were comparable to the quaternized cationic steroids (Table 4.4). However, these quaternary ammonium compounds were still 10 fold less potent that the C22 and C24 carnitines, suggesting that the presence of the carboxylate moiety increases the potency of the C22 and C24 acyl carnitines (Table 4.3).

4.3.2.5 Tamoxifen Analogues

As seen with sphingosine, not all cationic inhibitors of PKC are bilayer stabilizers. Another cationic hexagonal phase promoter is tamoxifen. Tamoxifen antagonized PKC with half maximal inhibition occurring at concentrations comparable to the tertiary amine steroids (Table 4.5). Quaternizing tamoxifen however resulted in a bilayer stabilizing inhibitor that is 10 fold more potent that tamoxifen itself. Its potency was comparable to the quaternized steroid derivatives (Table 4.1).

Table 4.3   Potency of Zwitterionic Straight Chain Amphiphiles on the Inhibition of Calcium, Phosphatidylserine and Diacylglycerol Stimulated Protein Kinase C.   Enzyme activity was measured using the Triton mixed micelle assay as described under methods.

Amphiphile	IC50 (μM)
	0.2
	0.6

CH_3, CH_3, NCH$_2$, CH_3^+, CHOC$(CH_2)_{20}CH_3$, O, HOC CH$_2$

CH_3, CH_3, NCH$_2$, CH_3^+, CHOC$(CH_2)_{22}CH_3$, O, HOC CH$_2$
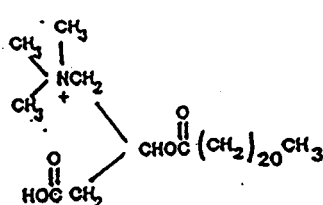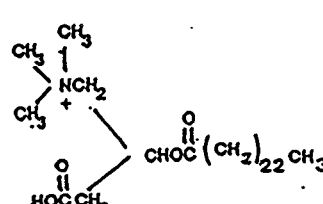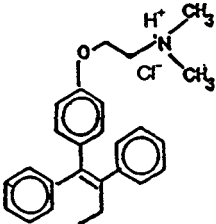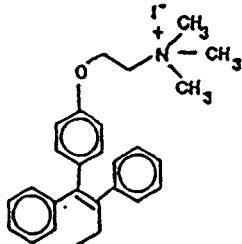
Table 4.4   Potency of Cationic Straight Chain Amphiphiles on the Inhibition of Calcium, Phosphatidylserine and Diacylglycerol Stimulated Protein Kinase C.   Enzyme activity was measured using the Triton mixed micelle assay as described under methods.

	Amphiphile	IC50 (µM)
XVII		DNI
XIX		26
XVIII		DNI
XX		40

DNI - does not inhibit

Table 4.5   Potency of Tamoxifen and Tamoxifen Trimethylammmonium Iodide on the Inhibition of Calcium, Phosphatidylserine and Diacylglycerol Stimulated Protein Kinase C. Enzyme activity was measured using the Triton mixed micelle assay as described under methods.

Amphiphile   IC50 (µM)

194

XXI

19

4.3.3   Protonation Studies of Tertiary Amine Amphiphiles in Triton Micelles

In order to correlate the potencies of the inhibitors with the charge of the amphiphile in the Triton micelle, the protonation state of amphiphiles containing a tertiary amine head group was studied with [¹H] NMR spectroscopy. Amphiphiles of interest included 1,3-bis-dimethylamino-2-propyl behenate, tamoxifen, and cholesteryl-3ß-carboxyamidoethylenedimethylamine. The chemical shift of the methyl substituents present on a tertiary amine nitrogen is sensitive to the protonation state of the neighbouring nitrogen atom. When the amino group is deprotonated, these protons give a characteristic chemical shift at about 2.2 ppm. Protonation at lower pH's leads to deshielding of the methyl protons and therefore, a downfield shift to approximately 2.9 to 3.1 ppm. The deshielding effect is larger for 1,3-bis-dimethylamino-2-propyl behenate because of two neighbouring amine groups resulting in chemical shifts closer to 3.1 ppm. Conveniently, there is an absence of proton resonances in the region of 1.8 to 3.2 ppm of the Triton micelle spectrum. The shift of the methyl protons for 1,3-bis-dimethylamino-2-propyl behenate in Triton micelles is shown in Figure 4.5. The change in ionization state of the drug with respect to pH is depicted in Figure 4.6. What is striking is that at the pH of the enzyme assay, (pH 7.5), the compound is for the most part deprotonated. The apparent pK of 1,3-bis-dimethylamino-2-propyl behenate in Triton micelles is 5.5. Apart from electrostatic repulsion, the broad titration curve can be attributed to the presence of two tertiary amine sites on this molecule as opposed to one. If one amino functionality is protonated, it will be more difficult to protonate the second one. On the other hand, tamoxifen and cholesteryl-3ß-

pH

2.12

2.545

4.225

4.82

5.52

6.58

7.12

7.525

8.335

11.1

TRITON-XIOO

3.0 PPM
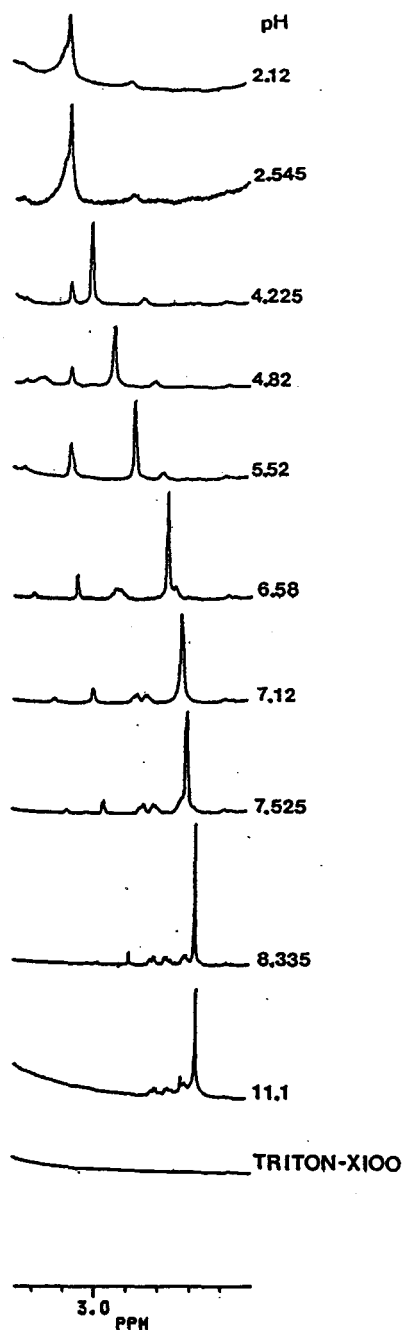
Figure 4.5 The pH dependence of the chemical shift of the tertiary amine methyl protons of 1,3-bis-dimethylamino-2-propyl behenate (10 mg/mL) in 10% Triton X-100 made up in 95% H_2O, 5% D_2O. D_2O served as a lock signal for the magnetic field throughout the experiment. The spectra were recorded at 500 MHz using presaturation of the water signal. The bottom spectrum depicts the absence of ^{1}H resonances of Triton X-100 between 2.0 and 3.4 ppm. The addition of drug accounts for the numerous resonances in the remaining spectra. The most intense peak was assigned to the tertiary amine methyl protons of 1,3-bis-dimethylamino-2-propyl behenate, and was used to generate the titration curve.
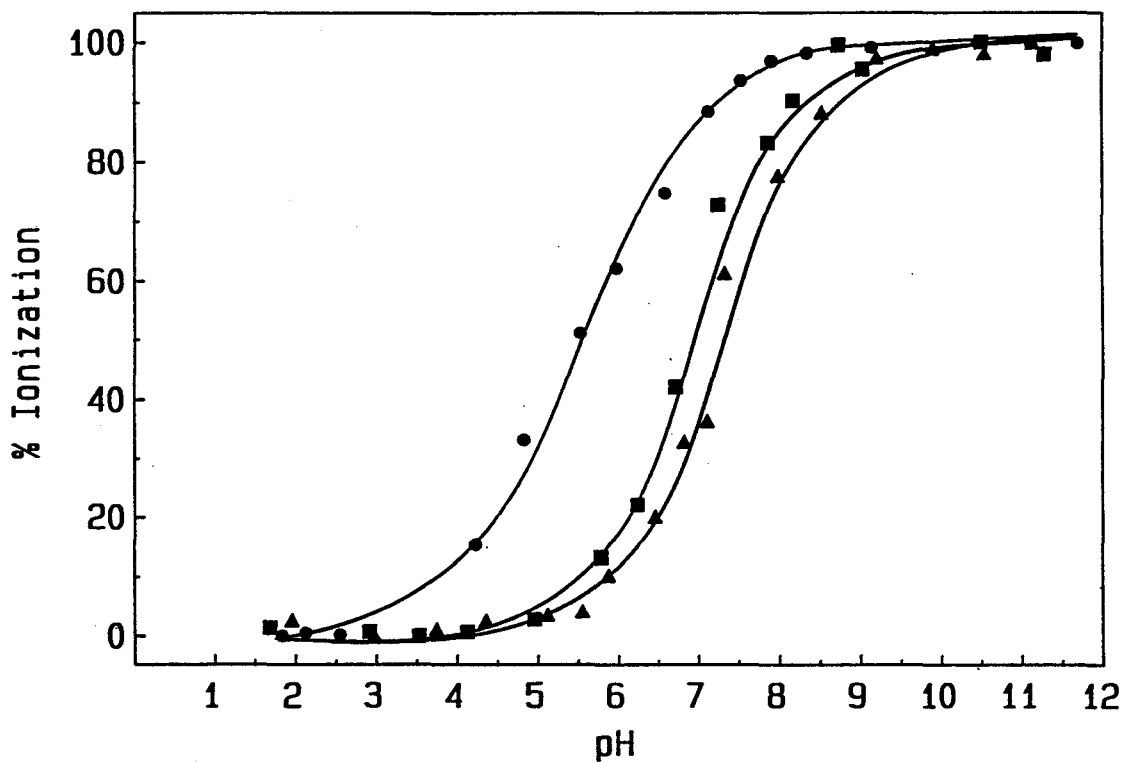
Figure 4.6 pH titration curves of 1,3-bis-dimethylamino-2-propyl behenate (●), tamoxifen (■) and cholesteryl-3ß-carboxyamidoethylenedimethylamine (▲) in Triton X-100 measured with ¹H NMR spectroscopy. The drug concentration (10 mg/mL) in a solution of Triton X-100 made up in 95% H_2O, 5% D_2O. D_2O served as a lock signal for the magnetic field throughout the experiment.

carboxyamidoethylenedimethylamine still bear a significant amount of charge at pH 7.5 (Figure 4.6). The apparent pK's of these drugs are 6.9 and 7.2 respectively.

4.3.4   Binding of Protein Kinase C to Phospholipid Vesicles

Fluorescence energy transfer from the tryptophan moieties of PKC to the dansyl moiety of dansyl PE was used to determine whether or not cationic bilayer stabilizers inhibit PKC binding to the membrane surface. Energy transfer is dependent on the Förster distance of the donor-acceptor pair and thus will only occur when the tryptophan residues of the protein are present at the membrane surface in close proximity to the dansyl moiety (Devaux and Seigneuret, 1985). In the absence of added calcium, there was a slight increase in the fluorescence intensity with the addition of PKC; this may be attributed to a minor calcium contamination in the buffers. However, the addition of PKC and calcium to labelled vesicles resulted in a 50% increase in the fluorescence intensity (data not shown). The presence of 8.8, 26.5 or 48.5 mole percent of cationic amphiphile (compound XI, see 2.3.2.4) relative to PS did not affect this increase in fluorescence suggesting that the drug was not affecting PKC binding to the membrane surface. The addition of EGTA resulted in the dissociation of PKC from the membrane surface. However, the increase in fluorescence intensity did not decrease below 10%, suggesting that some of the kinase still remained irreversibly bound to the membrane.

4.4 DISCUSSION

4.4.1 Structure Activity Studies

Although many compounds have been developed to inhibit PKC, only a few structure activity studies have been performed to determine the important features that are necessary for optimal enzyme inhibition. A large number of cationic substances have been shown to antagonize PKC. Furthermore, Epand (1987) has demonstrated that uncharged or zwitterionic PKC antagonists inhibit the formation of the hexagonal phase in phosphatidylethanolamine bilayers. A series of bilayer stabilizing amphiphiles were designed to incorporate positive charge in an attempt to optimize the structural features of a molecule necessary for the inhibition of PKC. These contained either a steroid or straight chain hydrocarbon with variations in the structure of the charged polar group. It is clear that a substantial amount of cationic character is required for the inhibition of PKC. Quaternary ammonium steroids contain a permanent positive charge and were found to be the most potent inhibitors of the kinase. The tertiary amines, including the steroids and tamoxifen gave half maximal inhibition of the enzyme at concentrations 10 fold higher (150 - 650 μM) than the corresponding quaternary amines (Table 4.1). The observed potencies correlate with their protonation state in the Triton micelle. At the pH of the assay, tamoxifen and cholesteryl-3ß-carboxyamidoethylenedimethylamine are 73 % and 62.5 % dissociated respectively. Although they are not completely protonated, they are more protonated than 1,3-

bis-dimethylamino-2-propyl behenate, which at pH 7.5 is 93 % dissociated. This latter substance did not inhibit PKC up to 1 mM. However, when a permanent positive charge is fixed on 1,3-bis-dimethylamino-2-propyl behenate, its potency is equivalent to that of quaternized tamoxifen (tamoxifen trimethylamine, Table 4.5) and the quaternary ammonium steroids (Table 4.1). Perhaps the most convincing evidence that the amount of positive charge on a substance dictates how it will affect PKC comes from studies with sphingosine. The decreasing inhibition with increasing pH suggested an effect resulting from a change in the ionization state of sphingosine either in the Triton mixed micelle or when bound to PKC. At pH 7.5, sphingosine is only 47 % ionized and is consequently more charged than the tertiary amine steroids or tamoxifen. Its potency (IC_{50} = 100 μM; Hannun et al., 1986a) corresponds well with respect to the less charged tertiary amine steroids and tamoxifen and the more charged quaternary ammonium compounds. However, at pH 8.5 where about 78 % of the positive charge is removed, sphingosine activates the enzyme to a small extent, which was not observed for the hexagonal phase promoting 1,3-bis-dimethylamino-2-propyl derivatives of the C22 and C24 straight chains in the presence or absence of DAG.

The results of other studies have noted the necessity of a large hydrophobic region and an amino moiety implicating the importance of positive charge for the inhibition of PKC. Since there are differences in the PS vesicle and the Triton mixed micelle assay, it is difficult to compare absolute values for the drug potencies. For instance, O'Brian and co-workers (1988) reported that tamoxifen antagonized PKC with half maximal inhibition occurring at 100 μM.

The results of this study find tamoxifen inhibiting PKC with an IC_{50} of 194 μM. These types of discrepancies in the potencies assessed using different assays have been previously observed (Epand et al., 1989a) and they probably arise largely from different extents of inhibitor dilution into vesicles or micelles used for monitoring PKC activity. Nevertheless, the relative effects on PKC by slight changes in drug structure can be correlated with positive charge. Through an inductive effect from the attached methyl substitutions, a tertiary amine nitrogen would be expected to protonate easier and consequently have more positive charge than a primary or secondary amine. However, the reverse is the case. The steric crowding around the nitrogen atom would make the protonation of tertiary amines more difficult (or deprotonation easier). Consequently, tertiary amines have a lower pK than primary or secondary amines. This effect would be even greater in the membrane. Since tertiary amines are more hydrophobic, they would partition deeper into the membrane and this would make deprotonation more favourable. Tamoxifen, a tertiary amine, is 10 fold less potent than N-des methyl tamoxifen, a drug with one less methyl substituent on the nitrogen atom (O'Brian et al., 1988). Moreover, amino acridines possessing primary amine substitutions are more potent than those possessing tertiary amine substitutions at the same site (Hannun and Bell, 1988). In addition, Merrill and co-workers (1989) reported that N-methyl sphingosine (IC_{50} = 4 mole %) is more potent than N,N-dimethyl sphingosine (IC_{50} = 10 mole %). It is likely that N,N-dimethyl sphingosine is almost completely deprotonated in the Triton micelle as was observed for 1,3-bis-dimethylamino-2-propyl behenate. Furthermore, N-acetyl sphingosine, an analogue

of sphingosine without a positive charge does not alter the activity of isolated PKC nor the effect of this enzyme in human platelets (Hannun et al., 1987). In fact, N-methyl-N-isopropyl sphingosine, an analogue with an increased head group hydrophobicity activated PKC (Merrill et al., 1989). The lack of overall positive charge on a substance may explain why the steroid sulfobetaines are significantly less potent than the carboxybetaine or their tertiary amine precursors. A sulfate moiety ionizes to a greater extent than a carboxylate, and subsequently, the overall positive charge of a sulfobetaine is expected to be less positive than a carboxybetaine. In contrast, CPD, has a potency comparable to the quaternized steroids (Epand et al., 1989a). Although possessing a tertiary amine functionality, it is unlikely to be as charged as the quaternized steroids and consequently inhibition by CPD cannot be explained simply by charge effects. The same is true for the C22 and C24 acyl carnitines. Lignoceroyl and behenoyl carnitines contain a quaternary nitrogen functionality as well as a carboxylate anion as does cholesteryl-3ß-carboxyamidoethylene carboxybetaine. The carboxybetaine is 1000 fold less potent than the C22 and C24 carnitines suggesting that the spacial location of the charged groups $((CH_3)_3N+)$ and (CO_2-) may be important for inhibitor potency. However, the C22 and C24 acyl carnitines are greater than 1000 fold more potent than the C20, C18, C16, and C14 acyl carnitines when assayed using the mixed micelle assay (Alan Stafford, personal communication), suggesting that the long chain hydrocarbon moiety (> C20) also contributes to the observed effect. There is also a difference between the potencies of 1,3-bis-dimethylamino-2-propyl analogues of the C22 and C24 straight chains and of the steroids. The

steroid analogue antagonizes PKC with half maximal inhibition occurring at 643 μM while the straight chains with the same head group abolishes any inhibition of PKC at this concentration. These differences may reflect the pK of the drug in a membrane environment. A steroid moiety may not partition as deeply into the bilayer core as would a C22 or C24 chain and subsequently the pK may not be perturbed as markedly as that observed with a straight chain. The fact that the C22 or C24 tertiary amines induce $H_{\parallel}$ phase formation suggest they partition deeply into the membrane, this event may be attributed to their lack of charge.

4.4.2 Mechanism of Inhibition

The mechanism by which bilayer stabilizing amphiphiles inhibit PKC is far from being completely determined and is not likely to be identical for all amphiphiles. The major goal of this study was structure-activity relationships in an attempt to optimize the features necessary to evaluate enzyme inhibition. The cationic amphiphiles were designed to alter the bulk biophysical properties of a membrane. There is already evidence that agents which affect PKC activity also affect lipid polymorphism. DAG, the physiological activator of PKC potentiates hexagonal phase formation (Epand, 1985) and has been shown to increase the spacing between phospholipid head groups (Das and Rand, 1986). This could enhance lipid protein interactions (Epand and Lester, 1990). However, not all hexagonal phase promoters activate the enzyme. Sphingosine, a promoter of the $L\alpha \rightarrow H_{\parallel}$ phase inhibits the enzyme. This is however attributed to its charge; when

the charge is completely removed, sphingosine slightly activates the enzyme as would be predicted on the basis of alterations in the lipid polymorphism. All the synthesized amphiphiles which inhibit PKC also inhibit the formation of the hexagonal phase. However, hexagonal phase formation per se is unlikely to be involved in the inhibition process. There is a lack of correlation with regards to the ability of these substances to stabilize a PE bilayer and their ability to inhibit PKC. For instance, cholesteryl-3ß-oxysuccinylamidoethyldimethylamine (compound **XIV**) is a potent bilayer stabilizer (slope = 208 ± 7 K/mole fraction), but not a potent inhibitor of PKC (IC_{50}=447 μM), while cholesteryl-ß-carboxyamidoethyl-enetrimethyl ammonium iodide (compound **IV**), a less potent bilayer stabilizer (slope = 159 ± 24 K/mole fraction), is a more potent PKC inhibitor (IC_{50}=12 μM). In addition to specific interactions between amphiphile and PKC, an alternative explanation based on membrane physical properties is that substances which inhibit hexagonal phase formation also make the membrane surface more hydrophilic and this could antagonize PKC binding. However, an amphiphile concentration as high as 48.5 mole percent relative to PS did not affect PKC binding to the membrane surface when monitored using fluorescence energy transfer. Although the effects of only one cationic drug was assessed for its ability to alter PKC binding to phospholipid vesicles, other bilayer stabilizers such as palmitoyl carnitine (Nakadate and Blumberg, 1987) and CPD (Epand et al, 1989a) also did not alter PKC binding. This suggests that bilayer stabilizers do not appear to antagonize PKC by altering its ability to partition into membranes. However, previous studies have shown that binding does not always correlate with activity

(Bazzi and Nelsestuen, 1987a). The activation process has been suggested to involve PKC insertion into the membrane (Bazzi and Nelsestuen, 1988a,b). It is possible that these compounds antagonize the extent to which PKC penetrates bilayers or affect the conformation it attains in the membrane therefore inhibiting PKC activation.

The other possibility is that the drug interacts directly with the enzyme. One of the general properties of cationic drugs that inhibit polycationic substrate phosphorylation by PKC is that they compete with phosphatidylserine for PKC binding. This has been shown to be the case for polymyxin B (Mazzei et al., 1982), mellitin (Katoh et al., 1982) amino acridines (Hannun and Bell, 1988), tamoxifen and rhodamine 6G (O'Brian et al., 1988), N-myristyl-Lys-Arg-Thr-Leu-Arg (O'Brian et al., 1990) and the recently synthesized quaternary ammonium alkylglycerols (Marasco et al., 1990). Preliminary results suggest that cationic bilayer stabilizing amphiphiles also compete with the PS binding site (Alan Stafford, personal communication), and may therefore antagonize the phospholipid requirement of the enzyme. The other possibility is that the observed effects are the result of a cationic drug - substrate interaction. Cationic peptides, such as histone, are commonly used as PKC substrates because they can spontaneously associate with phospholipid vesicles (Bazzi and Nelsestuen, 1987c). Recently, it was proposed that electrostatic interactions between the cationic substrate and anionic membrane surface are important for presenting the substrate to PKC (Bazzi and Nelsestuen, 1987c; Newton and Koshland, 1990). Increasing the negative charge on the membrane surface by increasing the anionic phospholipid concentration

would facilitate the binding of a cationic substrate and act as a matrix to bring both the substrate and the kinase together (Newton and Koshland, 1990). This type of interaction was prevented by increasing the salt concentration of the assay (Newton and Koshland, 1990). Consequently, in the presence of cationic amphiphiles positively charged substrates may not be allowed to approach the vesicle surface either because of a charge neutralization of PS or through electrostatic repulsion of the substrate which can occur with sphingosine (Bazzi and Nelsestuen, 1987b). If this is the case, then the cationic substrates used *in vitro* may not be a good representative of *in vivo* substrates which may not be cationic, and consequently, these amphiphiles may not function with physiological substrates. However, a charge neutralization of PS is unlikely because PS which is present at 300 μM, is in large excess over the quaternary ammonium analogues which antagonize PKC with half maximal inhibition occurring at about 15 μM. Therefore, histone repulsion is also unlikely. Since the enzyme is present at a concentration of approximately 8 nM, there is likely enough free PS available to fill the activation requirement of the kinase, or enough negative charge on the membrane surface to allow the substrate to bind. Recently, O'Brian and Ward (1990) reported that in the presence of calcium and phospholipid, autophosphorylated PKC has an ATPase activity and this could be used as a probe for the allosteric regulation of the enzyme. Cationic amphiphiles such as mellitin, polymyxin B, and N-des methyl tamoxifen inhibited this ATPase activity with a potency comparable to their IC_{50} with histone phosphorylation (O'Brain and Ward, 1990) suggesting that cationic drugs function directly on the protein and the

observed effects are not artifacts of substrate - drug interactions. Moreover, polymyxin B has been shown to inhibit the phosphorylation of physiological substrates such as liver cytosolic proteins and to a lesser extent liver particulate proteins (Pugazhenthi et al., 1990). Consequently, cationic drugs can also function in physiological situations implying that the observed inhibition is not the consequence of a substrate effect. Aside from the phosphatidylserine site, cationic amphiphiles may interact electrostatically with an anionic site such as an aspartate or glutamate residue on the protein. A possible site may include the calcium site. For example, sphingosine was reported to compete with calcium binding to PKC (Hannun et al., 1986b). Another anionic site of interaction could be the peptide substrate binding site. An anionic site within the phosphate acceptor substrate site has been implicated with the observation that the highly basic pseudosubstrate region of PKC occupies the active site when the kinase is not stimulated (House and Kemp, 1987). Furthermore, the hydroxylated amino acid which is phosphorylated by PKC is dependent on basic amino acids which surround it (Ferrari et al., 1985; Kishimoto et al., 1985; Woodgett et al., 1986; Kitamura et al., 1989). Consequently, these amphiphiles may interact electrostatically with anionic residues within the catalytic domain and impede a substrate interaction. Interestingly, the bilayer stabilizer CPD which competed with the PS binding site also interacted with the catalytic domain of trypsinized PKC (Epand et al., 1989a). However, there is no kinetic evidence to support a competition of cationic drugs with the histone phosphoacceptor site.

5. SUMMARY AND FUTURE STUDIES

The inhibition of PKC correlates with the presence of positive charge. This is supported by a number of observations. The quaternization of a tertiary amine head group markedly enhanced inhibitor potency. In addition, the removal of the positive charge present on sphingosine by an increase in pH abolished inhibition. Moreover, the lack of inhibition with the C22 and C24 tertiary amines correlated with their lack of positive charge. Furthermore, inhibitor potency decreased with a decrease in overall positive charge resulting from sulfopropylation or carboxyethylation. However, from the structure activity studies, changes in the position of the charged functionalities and hydrocarbon length also resulted in marked differences in inhibitor potency. To more clearly assess the role of head group structure, carboxyethylbetaines of C22 and C24 hydrocarbon chains could be synthesized and compared with the potencies of the C22 and C24 carnitines. In addition, the studies on the role of the hydrocarbon structure and length could be completed by comparing the potencies of the C22 and C24 acyl carnitines with the potency of a carnitine - steroid ester. Collectively, these results raise a number of questions. Which isoform(s) of PKC do these cationic bilayer stabilizers inhibit? What enzyme site(s) do they interact with? Do they all work in a similar fashion or are there differences in their mechanism of action? These questions could be answered with an extensive kinetic study on a number of amphiphiles. A direct

effect on the enzyme could be assessed more clearly by studying the effects of cationic bilayer stabilizers on the ATPase reaction of autophosphorylated PKC (O'Brian and Ward, 1990). Furthermore, although these drugs do not appear to alter the binding of PKC to the membrane surface, they may affect the extent to which PKC penetrates the lipid bilayer. The penetration of PKC in the presence or absence of amphiphile can be assessed by monitoring tryptophan fluorescence quenching by doxylstearates. Doxylstearates can be obtained with a nitroxide free radical at various positions (C5, C12, and C16) along the stearate backbone. Maximum quenching occurs when the tryptophan residues of the protein are in very close proximity (5-7 Å) to the nitroxide spin label (Devaux and Seigneuret, 1985). Thus, the depth to which PKC penetrates into the bilayer in the presence of drug can be compared to its position in the bilayer in the absence of drug. Although many questions remain to be answered, it is clear that a combination of positive charge and bilayer stabilizing ability provides a basis for the rational design of new PKC inhibitors.

Cationic compounds were used in this study as inhibitors of PKC. They could also be used as probes to study membrane fusion phenomena. Since they are capable of stabilizing the bilayer, they would be expected to inhibit membrane fusion. Furthermore, these cationic amphiphiles could be used to enhance transfection. Preliminary results have shown that those containing tertiary amine functionalities are more potent at enhancing transfection than lipofectin (Dr L. Huang, unpublished results).

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