STUDY OF THE METABOLISM AND CYTOTOXICITY OF THE ETHER LIPID SRI 62-834

FRANCES ELIZABETH BISHOP

Doctor of Philosophy

UNIVERSITY OF ASTON IN BIRMINGHAM

August 1991

The University of Aston in Birmingham

STUDY OF THE METABOLISM AND CYTOTOXICITY OF THE ETHER LIPID SRI 62-834

by

FRANCES ELIZABETH BISHOP

A thesis submitted for the degree of Doctor of Philosophy

1991

SUMMARY

[2'-(Octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N,N,N*-trimethylammonio]ethyl phosphate (SRI 62-834, SRI) and hexadecylphosphocholine (Miltefosine, Mil) belong to a relatively novel group of antitumour agents, the ether lipids. Some evidence suggests that they possess selective cytotoxicity against tumours, especially certain leukaemias. The work described in this thesis had two aims. The first objective was to clarify the metabolism of SRI and Mil and the second was to compare the cytotoxicities of SRI and Mil with that of their putative metabolites. The toxicity experiments were designed to demonstrate whether metabolism of SRI and Mil was a detoxification process and thus aid with the understanding of their mode of action.

Metabolites of SRI and Mil were postulated from metabolism experiments reported in the literature for other ether and phospholipids. Three potential metabolites of SRI were synthesised. The first was [2'-(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl phosphate which would be the product of SRI metabolism catalysed by phospholipase D. The second metabolite was the alcohol, 2-hydroxymethyl-2-octadecyloxymethyl-tetrahydrofuran, which would result from SRI breakdown, mediated by phospholipase C, or phospholipase D and phosphatase. The third proposed metabolite was the diol, 2,2-bis(hydroxymethyl)tetrahydrofuran, the product of the oxidation of the alcohol by alkylglycerol monooxygenase. The putative metabolites of Mil were hexadecyl phosphate and hexadecanol. The former would be produced via catalysis by phospholipase D and the latter by metabolism mediated by phospholipase C or phospholipase D and phosphatase.

The metabolism of SRI and Mil was elucidated using gas chromatography, UV spectrophotometry and ^{31}P-NMR spectroscopy. Purified phospholipase C and D, post-mitochondrial rat liver fraction and rat liver microsomes were used in these studies. ^{31}P-NMR spectroscopy was the most useful technique for monitoring metabolism by phospholipases *in vitro*. SRI and Mil were metabolised by phospholipase D and subsequently the phosphates were metabolised by phosphatase. Neither SRI nor Mil appeared to be substrates for phospholipase C. Under the conditions used, SRI and Mil did not inhibit the phospholipase C mediated metabolism of platelet activating factor, an important endogenous substrate of this enzyme.

Toxicity of SRI and its proposed metabolites were examined in three cell lines with three different cytotoxicity assays. The toxicities of Mil and its metabolites were compared in the MTT assay. SRI was the most toxic and its metabolism was a detoxification process. Metabolism of Mil gave two products, one was less toxic and one more toxic than the parent molecule. The toxicity profiles of SRI, Mil and their potential metabolites demonstrate the importance of lipophilicity for their activity. The results are consistent with the contention that the membrane is the site of the toxic activity of these ether lipids.

Key words: Cytotoxicity, Metabolism, Miltefosine, SRI 62-834, Synthesis.

This is not the end. It is not even the beginning of the end: But it is perhaps the end of the beginning. Winston Churchill

ACKNOWLEDGEMENTS

I would first like to thank my supervisors, Professor Andreas Gescher and Dr. Sally Freeman for discussions, inspiration and constructive criticism. I am grateful to Dr. Caroline Dive especially for her advice during the preparation of my cytotoxicity chapter.

I wish to thank the MRC for funding my research and enabling me to attend the AACR conference in Houston and also the CRC for their contribution to my work.

I would also like to take this opportunity to thank the technicians in the department who have given me practical assistance, especially Donna Phipps who helped me set up my MTT assay. I wish to acknowledge Dr. Mike Perry for his initial involvement in running ^{31}P-NMR spectra and Graham Smith for his assistance with presentation of the NMR data. Thanks also to Dr. Peter Lambert and Dr. Brian Denny for their time spent demonstrating the molecular modelling techniques. I am thankful to my friends in the department for making it such an enjoyable place to work.

I wish to express my gratitude to my fiance Dr. Andrew Malyan for his encouragement, support and especially for his knowledge of the application of the Macintosh computer. Finally my thanks go to my parents and God for their love and for being there when I needed them.

CONTENTS

ABBREVIATIONS

AA	: Arachidonic acid
AA-PC	: Arachidonyl phosphatidylcholine
ADP	: Adenosine diphosphate
AGMO	: Alkylglycerol monooxygenase
ATP	: Adenosine triphosphate
B cereus	: *Bacillus cereus*
BnP	: Benzylphosphonate
brs	: Broad singlet
brt	: Broad triplet
$[Ca^{2+}]_i$	: Intracellular calcium concentration
$CDCl_3$	: Deuterated chloroform
$CHCl_3$	: Chloroform
CH_3OH	: Methanol
CoA	: Coenzyme A
C perf	: *Clostridium perfringens*
Cyt P450	: Cytochrome P450
DIBAH	: Diisobutyl aluminium hydride
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribose nucleic acid
D_2O	: Deuterated water
EDTA	: Ethylene diamine tetraacetic acid
ET-18-OCH_3	: 1-O-Octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine
Et_3N	: Triethylamine
FA	: Fatty acid
FCS	: Foetal calf serum
Fig.	: Figure
GC	: Gas chromatography
H_2O	: Water
H_2SO_4	: Sulphuric acid
HCl	: Hydrochloric acid
HPLC	: High performance liquid chromatography
IC_{50}	: Concentration required to produce 50% toxicity
$InsP_3$	: Inositol(1,4,5)triphosphate
J	: Coupling constant
LDH	: Lactate dehydrogenase
lit	: Literature
LPC	: Lyso-phosphatidylcholine
M^+	: Molecular ion
MES	: 2[*N*-Morpholino]ethane sulfonic acid
MHz	: Megahertz
Mil	: Miltefosine
MNDO	: Modified neglect of diatomic differential overlap
$MPtH_4$	: 6-Methyl-tetrahydropterine
$MPtH_2$	: 6-Methyl-dihydropterine
MS	: Mass spectrometry
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.
n	: Number of experiments

NADH	: Nicotinamide adenine dinucleotide (reduced form)
NADPH	: Nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	: Nuclear magnetic resonance
PAF	: Platelet activating factor
PBS	: Phosphate buffered saline
PC	: Phosphatidylcholine
PCl_3	: Phosphorus trichloride
PtdIns	: Phosphatidylinositol
PtdInsP	: Phosphatidylinositol phosphate
$PtdInsP_2$	: Phosphatidylinositol(4,5)biphosphate
Pyr	: Pyruvate
S9	: Post mitochondrial supernatant liver fraction
SD	: Standard deviation
SE	: Standard error
Strep	: *Streptomyces chromofuscus*
SRI	: [2'-(Octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N*,*N*,*N*-trimethylammonio]ethyl phosphate, SRI 62-834
TEAB	: Triethylammonium bicarbonate
THF	: Tetrahydrofuran
TLC	: Thin layer chromatography
TPA	: 12-O-Tetradecanoyl phorbol 13-acetate
tris	: Tris[hydroxymethyl]amino methane
u	: Units

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1. Cancer and its treatment at present

Within the European Community there are over three quarters of a million deaths due to cancer each year [McVie, 1989b]. The number of cases is slowly increasing even with the improving knowledge of this disease. The incidence of neoplasia in a population is related to both its size and age structure. Cancer is mainly a disease of the old, therefore the total number of cases rises as the number of elderly in the population increases.

Fewer novel chemical substances are finding their way into cancer clinics as potential therapeutic agents. Most of the chemicals used were first introduced in the 1950's and 60's and few agents have been found satisfactory to replace them [McVie, 1989a]. Anticancer drugs have generally been developed by modification of existing active agents to reduce their toxicity. Most approaches to cancer chemotherapy have centred around the idea that cytotoxic compounds can be used to eradicate proliferating neoplastic cells. Cytotoxicity induced by drugs is thought to be due to genetic damage, and DNA has served as a primary focus for drug development [Tritton and Hickman, 1990].

Cancer is associated with aberrant control of cellular differentiation and proliferation. DNA controls cellular function and although it has been the main site for chemotherapeutic attack, it may not be the most appropriate target [Hickman, 1988]. Present therapy has many limitations, the main one being its lack of toxic selectivity. It is very difficult to kill cancer cells without harming normal cells. Most drugs used in chemotherapy target rapidly dividing cells so can kill some tumour cells. They also tend to be toxic to cells such as those in the haemopoetic system and gastrointestinal mucosa, which also have a rapid turnover. Such compounds are not successful in treating tumours with low growth potential and many have been shown to be mutagenic. Difficulties also arise i) in the treatment of solid tumours with a low blood supply, ii) due to tumour heterogeneity and iii) where resistance of cells to treatment develops. For selective treatment and improved efficiency, new targets for chemotherapy need to be explored [Hickman, 1988].

A more appropriate target for chemotherapy could be control of differentiation and proliferation. Many of the factors involved in these processes are located in or associated with the membrane, such as protein kinase C [Kikkawa et al, 1989]. Therefore, the membrane has been considered as a primary target for more recently developed

chemotherapeutic agents. Disruption of the membrane could affect cell surface transduction and growth control [Tritton and Hickman, 1990].

Rational drug development has seen the introduction of biological response modifiers and agents that upset the pathways that cells use to control normal growth [Tritton and Hickman, 1990]. There are many problems associated with biological response modifiers which include lack of selectivity for induction of the immune response. This deficiency can lead to severe immune reactions, toxicity and these agents have not shown much success in the treatment of cancer [Talmage and Clark, 1987]. Ether lipids were developed as biological response modifiers for cancer chemotherapy. Ether lipids are structurally related to endogenous platelet activating factor (PAF) (Fig. 1a, p15), which has been implicated in immune reactions [Snyder et al, 1989]. Ether lipids, like PAF can activate macrophages [Berdel et al, 1980]. They also appear to be directly toxic to cells and this action may be mediated via membrane damage or an effect on signalling [Zheng et al, 1990]. A hallmark of cancer is the inappropriate expression of oncogenes. Many oncogene products have a role in signal transduction, hence alteration of signalling might allow selective disruption of neoplastic cell growth [Tritton and Hickman, 1990].

2. Ether lipids

This thesis is concerned with the investigation of a number of compounds which belong to a relatively novel class of anticancer agents, the ether lipids. In 1970, Brohult described a reduced mortality rate in cancer patients given glycerol ethers from greenland shark liver oil [Brohult et al, 1970]. Since this time interest in ether lipids has grown, especially because they are a group of compounds which appear to have no effect on DNA. They have been well tolerated in preliminary trials, at doses calculated from the concentrations that produced an anticancer response in rats [Berdel et al, 1981]. They show antitumour activity both *in vitro* and *in vivo* in rodents. Such compounds appear to be able to activate macrophages and have a directly toxic affect on tumour cells [Berdel et al, 1981]. Ether lipids are thought to produce their toxicity via attack on the membrane and have been shown to inhibit several important enzymes in the cell signal transduction process, as discussed later in this chapter (section 2.4.2, p19) . Ether lipids seem to be selectively toxic towards cancer cells, and they have been used to purge bone marrow of leukaemic cells [Glasser et al, 1984]. One of the first ether lipids tested was ET-18-OCH_3 (Fig.1b, p15) and newly developed ether lipids are compared with this compound for activity [Houlihan et al, 1987; Unger et al, 1987].

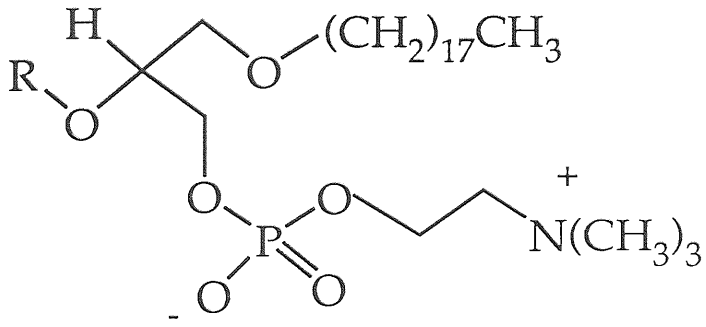
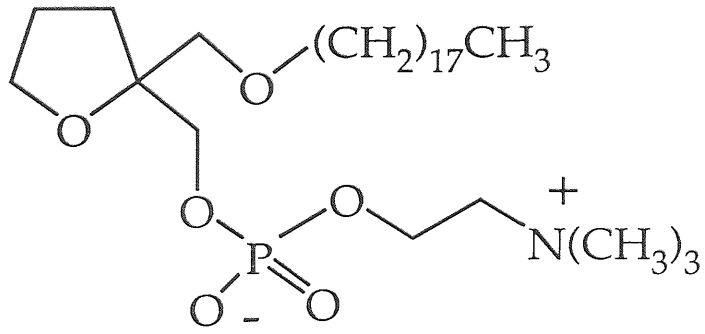


Fig. 1 Chemical Structure of a) PAF (R = acetyl), and
b) ET-18-OCH_3 (R = methyl)

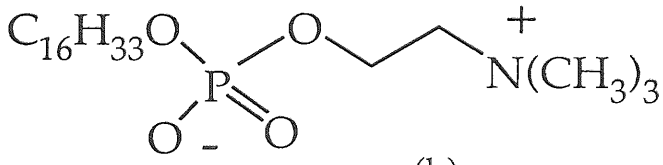
2.1. Cytotoxic activity of the ether lipids

The major topic of the research discussed here is the ether lipid SRI 62-834 (SRI, 2'[(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N,N,N*-trimethylammonio]ethyl phosphate) (Fig. 2a, p16). SRI is structurally related to ET-18-OCH_3 (Fig. 1b) [Houlihan et al, 1987]. Another agent, on which work is described here, is Miltefosine (Mil, hexadecylphosphocholine) which is an ether lipid analogue. Mil has antitumour activity [Unger et al, 1989] and is an alkylphosphatidylcholine (Fig. 2b, p16), with the alkyl group directly attached to the phosphatidylcholine moiety. In this thesis Mil will be referred to as an ether lipid, which is strictly speaking incorrect.

SRI and Mil have shown antitumour activity *in vivo* and *in vitro* in rodents [Houlihan et al, 1987; Unger et al, 1989]. SRI has activity against HL60 leukaemia cells and WEHI-3B leukaemia cells [Lazenby et al, 1990; Bazill and Dexter, 1989] and it is slightly less active against EMT6 mouse mammary tumour cells [Dive et al, 1991]. The K562 leukaemia cell line is more resistant towards SRI than HL60 cells [Lazenby et al, 1990]. Mil shows a similar pattern of cytotoxicity, with HL60 cells more sensitive than Raji or K562 leukaemia cells [Unger et al, 1987]. Mil also shows activity against mammary carcinoma induced in rats by dimethyl benzanthracene and methyl nitrosourea [Unger et al, 1989; Muschiol et al, 1987] and when applied topically to skin tumours [Unger et al, 1989].



(a)



(b)

Fig. 2 Chemical structures of (a) SRI and (b) Mil.

2.2. Toxicity of ether lipids

Ether lipids exhibit low systemic toxicity in rodents. Mil and ET-18-OCH_3 are antineoplastic at non-toxic doses in rats [Scherf et al, 1987]. At high doses Mil produces toxicity in the liver, kidney and spleen, when given to rats [Muschiol et al, 1987]. The dose limiting toxicity in breast cancer patients who received Mil was nausea [Unger et al, 1989]. Ether lipids do not seem to affect the bone marrow and they have been used to purge bone marrow of leukaemic cells, prior to its re-introduction into the patient [Glasser et al, 1984]. Treatment with ether lipids left most of the normal stem cells but eliminated the leukaemic blasts.

2.3. Structure activity relationship

Ether lipids are structurally related to PAF (Fig. 1a, p15). PAF has many physiological functions, both beneficial and detrimental. It is involved in the immune system and released in immediate hypersensitivity type reactions linked to allergic responses. PAF causes platelet and neutrophil aggregation, bronchoconstriction in asthma and decreases blood pressure in toxic shock [Snyder et al, 1989]. PAF has also been implicated in prolonging inflammation and ulceration associated with inflammatory bowel disease [Wallace et al,

1989]. It is also necessary for fertilisation and parturition [Snyder et al, 1989]. PAF also possesses cytotoxic activity, the naturally occurring isomer L-PAF has an IC_{50} of 59μM in HL60 cells [Dive et al, 1991] and D-PAF is about four and a half times more toxic than the L form [Hoffman et al, 1984]. Ether lipids possess some of the properties of PAF in that they can modulate the immune system and activate macrophages [Berdel et al, 1980] but they are generally more cytotoxic.

The antineoplastic activity of ether lipids requires certain molecular characteristics. They need an alkyl moiety in position 1 of the glycerol backbone, a metabolically stable group in postion 2 and phosphatidylcholine in position 3 [Hoffman et al, 1984]. Reducing the length of the alkyl chain in positon 1 below C_{16} leads to a dramatic loss of activity, as does introduction of unsaturation into the molecular skeleton [Bonjouklian et al, 1986]. Substitution of sulphur instead of the ether oxygen in position 1 marginally increases the lipophilicity of the molecule. This property may increase the ease with which it inserts into the membrane so slightly increasing its cytotoxicity. Ether lipids have been referred to as alkyl lysophospholipids in the literature due to the presence of a small substituent in position 2. A small group occurs in lysophospholipids such as lyso-PAF, following the metabolism of the long chain fatty acid (Fig. 4, p24) which is present in naturally occurring phospholipids. Mil was designed to possess the minimal structural requirements of ether lipids for antineoplastic activity [Unger et al, 1989]. Mil does not have the glycerol backbone and ether linked long chain alcohol so is not strictly an ether lipid and it does not appear as cytotoxic as the others [Uberall et al, 1991].

2.4. Mode of action of ether lipids

Ether lipids appear to act on tumours by indirect and direct attack. Their indirect toxicity is mediated by generation of cytotoxic cells. Ether lipids are able to activate macrophages, which can then attack the tumour more vigorously. These compounds have also been shown to activate mouse peritoneal macrophages [Yamamoto and Ngwenya, 1987]. They also activate bone marrow macrophages, which can then inhibit the development of metastases of 3-Lewis lung tumour in mice [Berdel et al, 1980].

There are various theories as to how ether lipids produce their direct toxicity in cells. Ether lipids are not genotoxic and it is generally thought that they act at the membranes of cells to initiate their toxicity [Berdel et al, 1981]. There is a pattern of events which leads to cell death. The first detectable biological event, at 4h, is a decrease in both cellular uptake and incorporation of [^{3}H] thymidine into DNA. This is followed by a release of lactate dehydrogenase (LDH) around 8h and then cell numbers start to decline after about 12h incubation with ether lipid [Hoffman et al, 1984]. One of the first morphological changes

that occurs in cells is the formation of blebbing and ruffling on the cell surface. The bleb formation may be related to changes in calcium levels that occur in the presence of ether lipid, as discussed later in this chapter (section 2.4.2., p19). Holes develop in the membrane. Large holes probably occur where the membrane over the top of large vacuoles, which develop in response to ether lipid, ruptures. Small holes also appear in the membrane and are not related to vacuole formation. At high concentrations of ether lipids cells swell and rupture very readily [Noseda et al, 1989].

2.4.1. Cytotoxic selectivity

Certain cell lines (eg HL60) are more sensitive to the toxicity of ether lipids, than others (eg K562). Ether lipids also appear more toxic to certain tumours than to normal cells (Glasser et al, 1984). There are three main theories as to the reason for the difference in sensitivity. The first associates differences in sensitivity with metabolism, the second implicates a variation in binding sites and the third suggests differential uptake mechanisms.

Originally it was suggested that the selective toxicity of ether lipids arose because of their selective accumulation in tumour cells, due to a low level of metabolism. Initial studies showed that tumour cells had a lower level of the alkyl cleavage (or alkylglycerol monooxygenase enzyme, AGMO) than normal cells. AGMO is required for the cleavage of the alkylether group [Soodsma et al, 1970]. Tumour cells would then accumulate the ether lipid which in turn could interfere with many cellular processes involving phospholipids, such as membrane permeability [Hoffman et al, 1986]. However, more recently it has been shown that many ether lipids such as ET-18-OCH_3 and PAF itself, are not substrates for AGMO and so differences in enzyme level could not explain selective toxicity. HL60 cells are more sensitive to ether lipids than K562 cells but these two cell lines have very similar levels of AGMO [Hoffman et al, 1986]. Similarly AGMO activity could not explain the differences in the sensitivities of HL60 and L1210 cells toward ET-18-OCH_3. Their levels of specific cleavage enzyme activity were also very similar [Unger et al, 1987]. It was suggested that differences in phospholipase activity or uptake of compound may explain selective toxicity, if the resulting glycerol metabolite was the active compound. However, other research has shown that the glycerol metabolite of 1-alkyl-2-O-methyl-phospho-choline generated by phospholipase C was not toxic. Hence differential metabolism by phospholipase C could not explain differences in sensitivity [Vallari et al, 1988].

It has been shown that cellular concentration of ether lipid after equilibrium binding is 3 - 4 times lower in rMethA sarcoma cells resistant to ether lipids compared to ether lipid sensitive MethA sarcoma cells [Storch and Munder, 1987]. ET-18-OCH_3 was thought to be stored in discrete cellular structures and ether lipid binding sites were suggested to be

responsible for ether lipid-induced cell lysis. Clustering of these sites or increase in their number could explain the difference in sensitivity of these cell lines. Another possibility was that ether lipids were transported into the cells by a specific membrane protein and differences in sensitivity were due to changes in the number of binding sites for this protein.

More recent research suggests that uptake of ether lipids could be responsible for the differences in the sensitivity of cells to these compounds [Hoffman et al, 1986]. Labelled ether lipid was shown to accumulate at the periphery of HL60 cells, whereas it was more uniformly distributed in polymorphonuclear neutrophils and K562 cells. PAF at non toxic doses was also shown to be evenly distibuted throughout cells. Ether lipids appear to accumulate at the surface membrane of sensitive cells, where they might elicit their toxic action. Enrichment of the membrane with exogenous ether lipids could inhibit vital cell functions such as those catalysed by the lipid metabolising enzymes involved in membrane assembly [Hoffman et al, 1986].

SRI has been shown to be more than twenty-five times as toxic to murine WEHI myeloid leukaemic as to normal murine myeloid cells in the presence of serum, and one hundred times more toxic in the absence of serum [Bazill and Dexter, 1989]. The toxic selectivity of SRI was thought to be related to its cellular uptake. The rate of uptake of SRI by WEHI-3B was six times greater than that by bone marrow cells. WEHI-3B cells accumulated more ether lipid than the bone marrow cells and they were more sensitive to SRI [Bazill and Dexter, 1989].

2.4.2. The effect of ether lipids on cell membrane and signalling pathways

An early phenomenon detected after exposure to alkylmethoxy-glycero-phosphocholine was the impairment of membrane transport of small molecules in sensitive cells. Changes in membrane transport could be part of the toxic action of ether lipids. A decrease in choline uptake has been detected in sensitive HL60 cells treated with ether lipid. This was not observed in resistant differentiated HL60 cells. Choline is important for membrane integrity and its loss could lead to membrane perturbation [Vallari et al, 1988].

Ether lipids have also been shown to have an inhibitory effect at other sites of membrane transport including the Na,K-ATPase involved in ion transport [Zheng et al, 1990]. This inhibition may be by direct or indirect interaction with sodium binding sites on the enzyme located at the intracellular surface of the plasma membrane. Ether lipids probably interfere with boundary phospholipid to increase the local fluidity and/or decrease the interaction of acidic boundary phospholipids with the Na,K-ATPase. The inhibition is competitive with

respect to Na^+ and non competitive with K^+. Inhibition of this enzyme could greatly affect the ionic balance within the cell and disrupt many other enzymes and functions.

Membrane transport can be interrupted by changes in the membrane. Such changes can follow disruption of phospholipid metabolism, as phospholipids are the main component of membranes. Ether lipids are antimetabolites in 3-sn-phosphatidylcholine synthesis and tumour cell death can be correlated with disturbance of this metabolic route. Decreases in phosphatidylcholine synthesis and increases in its degradation are observed during ether lipid incubation [Modolell et al, 1979].

Sensitive tumour cells accumulate ether lipids in their membrane. Their presence has been shown to increase the structural order of the membrane and reduce its fluidity [van Blitterswijk et al, 1987]. Change in fluidity was shown to prevent the tumour cells invading normal tissue. This inhibition was demonstrated by prevention of invasion of tumour cells into embryonic chick heart. However, ether lipid incorporation and the resultant reduction in fluidity did not strictly correlate with the inhibition of cell invasion.

Ether lipids have been shown to interfere with tumour invasiveness at concentrations that permit growth of the tumour cells. ET-18-OCH_3 inhibits invasion but still allows microtubule assembly. It was previously thought that microtubule damage was essential for inhibition of invasion. Prevention of spread of tumour cells was thought to be due to the increase in membrane fluidity seen with ether lipids [Storme et al, 1985]. Alterations in membrane fluidity can affect many metabolic processes associated with the membrane, such as phospholipid turnover, phosphatidylcholine synthesis and protein kinase C activity. Ether lipids partition into phospholipid domains which then change their physical properties [Noseda et al, 1988].

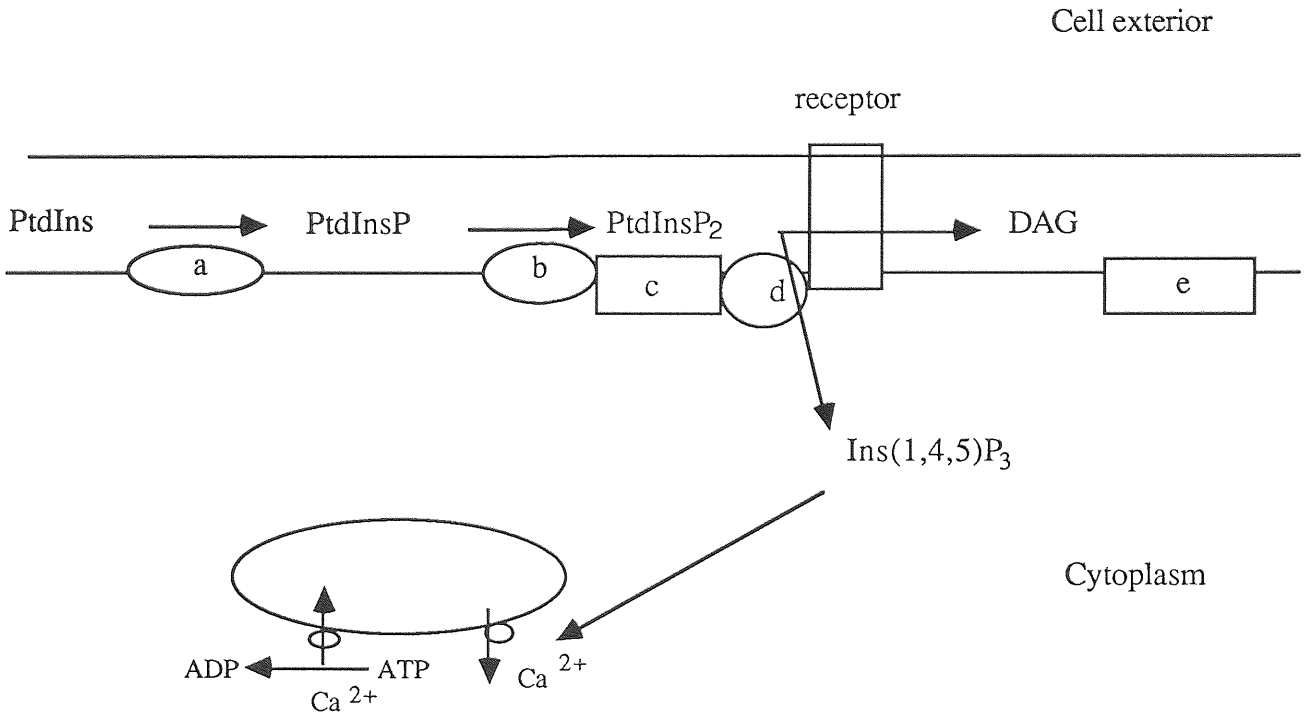
Ether lipids have been shown to inhibit several enzymes involved in cellular functions. They inhibit phospholipid sensitive Ca^{2+} dependent protein kinases, which are the predominant phosphorylating system in leukaemic cells [Helfman et al, 1983]. Many ether lipids have also been shown to inhibit protein kinase C. This enzyme is essential for growth factor signal transduction and cellular proliferation. It exerts both positive forward control as well as negative feedback control over various steps of the cell signalling process [Kikkawa et al, 1989]. The thioether BM41.440 and ET-18-OCH_3 competitively inhibit protein kinase C with respect to phosphatidylserine and also inhibit 12-O-tetradecanoyl phorbol 13-acetate (TPA) activated protein kinase C. They are specific for this kinase as they do not inhibit myosin light chain or cAMP-dependent protein kinases [Shoji et al,

1988]. Inhibition of protein kinase C and Na,K-ATPase could act synergistically to produce ether lipid cytotoxicity [Zheng et al, 1990].

Mil also competitively inhibits protein kinase C both in cell free extracts and intact cells [Uberall et al, 1991]. SRI was shown to inhibit this kinase only at concentrations 100 fold greater than its cytotoxic dose suggesting that protein kinase C was not involved in its mode of action [Houlihan et al, 1987]. However, more recently SRI was shown to cause a release of Ca^{2+} at cytotoxic levels in HL60 cells. This Ca^{2+} release was inhibited by TPA in a concentration and time dependent manner and suggests an involvement for protein kinase C in SRI toxicity [Lazenby et al, 1990].

Ether lipids could also inhibit cell signalling by interaction at different stages in the signalling pathway, other than at protein kinase C (Fig. 3, p22). Ether lipids have been shown to inhibit inositol phosphate-mediated $[Ca^{2+}]_i$ signalling and this could inhibit the actions of growth factors and mitogens and so contribute to their mode of action [Seewald et al, 1991]. Inositol(1,4,5)triphosphate ($InsP_3$) is a second messenger that releases Ca^{2+} from the endoplasmic reticulum. $InsP_3$ is formed by the ligand activated phospholipase C which catalyses the hydrolysis of membrane phosphatidylinositol-4,5-biphosphate ($PtdInsP_2$). ET-18-OCH_3 has been shown to inhibit Ca^{2+} uptake and $InsP_3$ induced Ca^{2+} release. In Swiss 3T3 fibroblasts, ET-18-OCH_3 inhibited the inositol formation stimulated by platelet-derived growth factor and its associated rise in $[Ca^{2+}]_i$. Ether lipids are general inhibitors of inositol formation induced by growth factor stimulation. They are also selective inhibitors of the increases in $[Ca^{2+}]_i$ stimulated by inositol phosphate. Ether lipids may inhibit the action of phospholipase C in cell signalling by depletion of $PtdInsP_2$ or by increasing the breakdown of the triphosphate messenger [Seewald et al, 1990]. SRI causes a rise in $[Ca^{2+}]_i$ at cytotoxic levels. This rise involves an initial release from intracellular stores and subsequent opening of a verapamil / prenylamine insensitive membrane channel in HL60 cells. SRI may inhibit protein kinase C [Lazenby et al, 1990], alternatively the release of Ca^{2+} may be via $InsP_3$ [Seewald et al, 1990].

Ca^{2+} has a pivotal role in regulating cytoskeletal structure and function. Changes in extramitochondrial Ca^{2+} have been associated with bleb formation in hepatocytes. These morphological alterations are thought to be due to the effect of Ca^{2+} on the cytoskeleton [Jewell et al, 1982]. The increases in $[Ca^{2+}]_i$ caused by ether lipids may also affect the cytoskeleton and help to explain bleb formation associated with ether lipid treatment of tumour cells [Noseda et al, 1989].



a) Phosphatidylinositol kinase
b) Phosphatidylinositol phosphate kinase
c) Phospholipase C (inositol specific)
d) G protein
e) Protein kinase C

Fig. 3 The phosphoinositol cell signalling pathway.

[Whitman and Cantley, 1988]

Phosphorylated phosphoinositides were shown to accumulate in cells treated with Mil [Uberall et al, 1991]. This phenomenon indicates that treatment associated depression of $InsP_3$ generation was not caused by an inhibiton of phosphoinositide kinases. There was no increase in the rate of triphosphate turnover and formation of $PtdInsP_2$ was not inhibited. The depression of triphosphate generation was probably related to inhibiton of phosphoinositidase C either by competition for the catalytic site or by an indirect action of perturbation of the phospholipid environment. The antitumour activity of Mil and ether lipids may be related, in part, to the inhibition of mitogenic signal transduction and of second messenger generation [Uberall et al, 1991].

There are suggestions that, due to their structural similarity to PAF, ether lipids act at the PAF receptor. WEHI-3B cells have been protected from the toxic activity of ET-18-OCH_3 and SRI by incubation with the PAF antagonist L-652,731 [Bazill and Dexter, 1989] and SRI has also been shown to inhibit PAF binding to human platelet receptors [Houlihan et al, 1987]. The PAF receptor is stereospecific, accepting only L-PAF as a ligand, whereas D-PAF is inactive at PAF receptors. However, D-PAF is more cytotoxic than L-PAF, which suggests that cytotoxic action is mediated via a different mechanism [Hoffman et al, 1984]. More recently treatment with PAF antagonist WEB 2086 did not modulate the cytotoxicity of PAF, SRI or ET-18-OCH_3, supporting a mode of action for ether lipids that does not involve receptors [Workman et al, 1991].

SRI caused a rapid dose dependent rise in membrane permeability and a decrease in cell size [Dive et al, 1991]. Antagonist WEB 2086 and low concentrations of PAF had no affect on SRI induced membrane permeability and neither could mediate the toxicity of SRI. These results also support the theory that ether lipids do not act through PAF receptors and that SRI has a membrane damaging role. SRI caused elevation of $[Ca^{2+}]_i$ at concentrations which caused a modest change in membrane permeability. Ether lipids may initially undergo an interaction with the cell membrane which leads to a rise in $[Ca^{2+}]_i$ prior to more extensive modification of membrane structure and function [Dive et al, 1991].

3. Metabolism

SRI is structurally related to PAF (Figures 1a, p15 and 2a, p16) and it may undergo similar enzymatic breakdown. The main route of PAF metabolism is via the deacylation-acylation pathway (Fig. 4, p24) [Kramer et al, 1984] which involves inactivation of the PAF molecule by acetyl hydrolase and transacylase. However, ether lipids cannot enter this cycle because a lysophospholipase cannot cleave the ether bond and acylation is blocked in most of these compounds by a stable substituent in position 2 of the glycerol backbone [Storch and Munder, 1987].

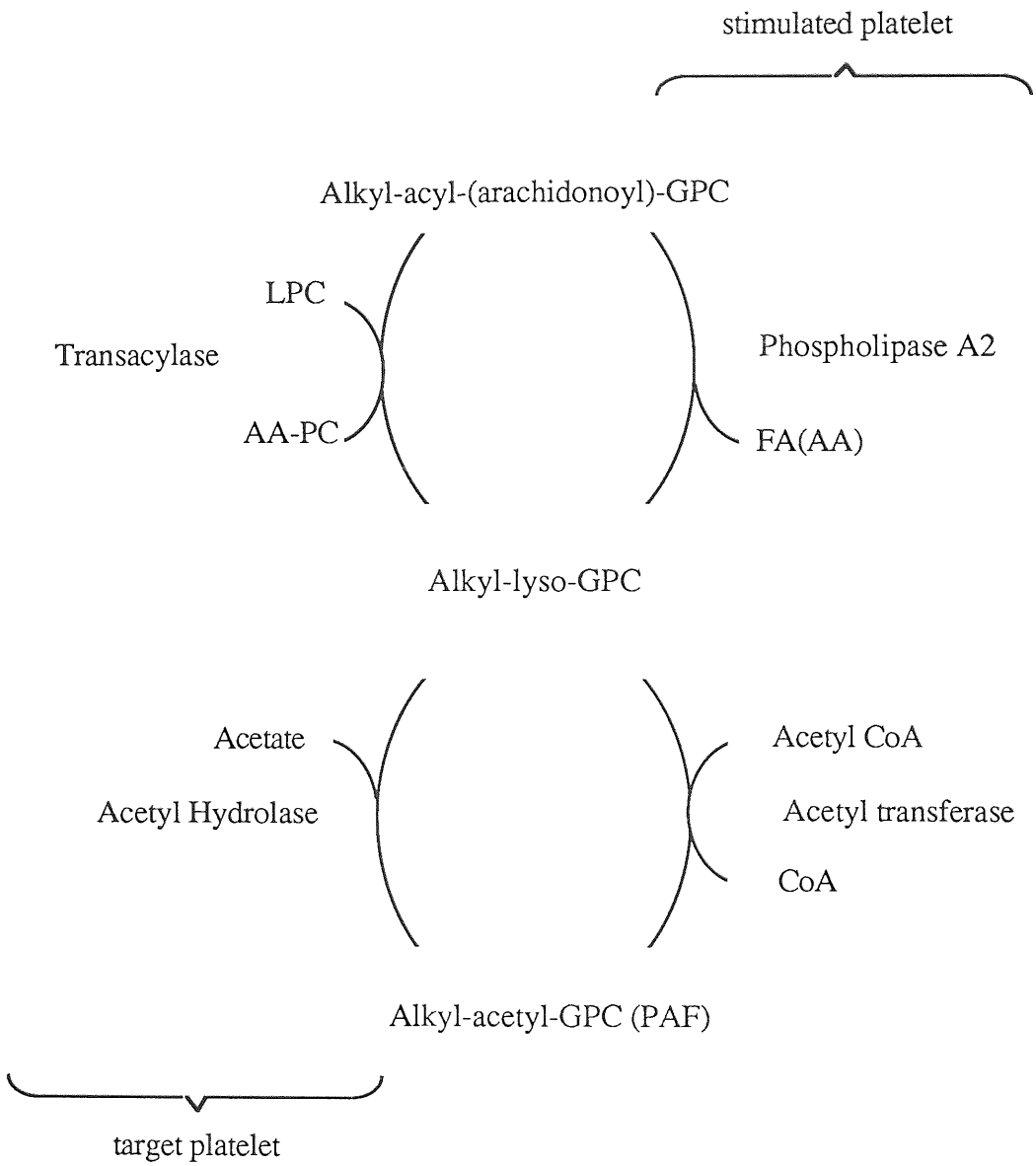


Fig. 4 Proposed pathway for the metabolism of PAF in platelets.

Phospholipids are susceptible to metabolic attack by phospholipases [Rossiter and Strickland, 1960; Kates, 1960]. Fig. 5 (p25) shows the sites at which phospholipases A, B, C and D catalyse metabolism. Phospholipases A and B hydrolyse fatty esters rather than ether linkages and would be unable to metabolise SRI. PAF is metabolised by phospholipase C to yield the alkylglycerol and phosphatidylcholine (Fig. 6, p26) [Wilcox

et al, 1987] and it can be metabolised by phospholipase D to the alkylglycerol phosphate and choline (Fig. 6). The alkylglycerol phosphate can be further metabolised by phosphatase to yield the alkylglycerol and inorganic phosphate (Fig. 6) [Qian et al, 1989]. ET-18-OCH_3 has been shown to be metabolised by phospholipases C and D [Wilcox et al, 1987; Fleer et al, 1986]. It can thus be postulated that SRI would be metabolised as shown in Fig. 7 (p27). Mil is also a phosphatidylcholine and may be susceptible to metabolic attack by phospholipases C and D [Unger et al, 1989; Fleer et al, 1986] and its possible enzymic degradation is shown in Fig. 8 (p28). The susceptibility of SRI and Mil to metabolism by phospholipase C and D is one question which is addressed in this thesis.

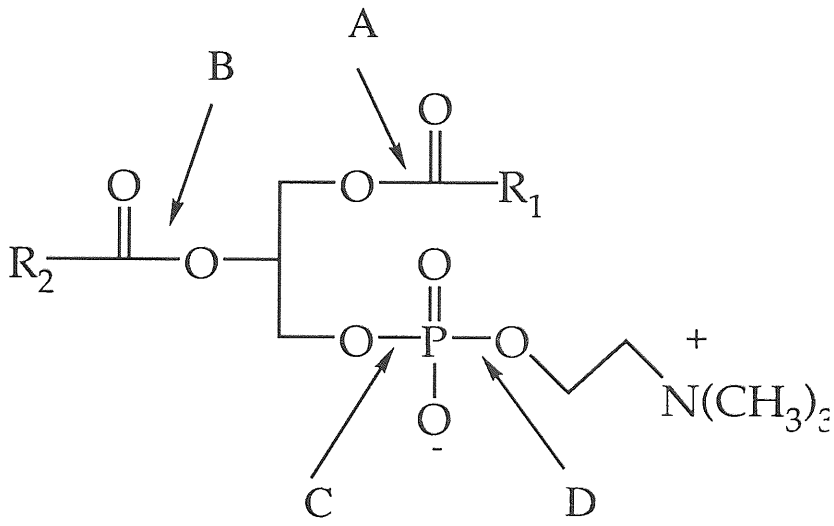


Fig. 5 Sites of action of the phospholipase enzymes.

There are two other sites in the SRI molecule which would be postulated to be liable to enzymic attack. The first is the alkyl chain substituent on carbon 2 of the tetrahydrofuran (THF) ring. The second site of attack would be the THF ring itself. PAF undergoes sequential metabolism by the following enzymes [Okayasu et al, 1986]. Firstly a cytosolic acetyl hydrolase removes the acetate from position 2 of the glycerol backbone. Second, a lysosomal phospholipase C cleaves the phosphatidylcholine from lyso-PAF to form the alkylglycerol. The third step involves microsomal AGMO which removes the alkyl residue from alkylglycerol, and lyso-PAF, to form the fatty aldehyde [Okayasu et al, 1986]. The aldehyde is then oxidised to the acid or reduced to the alcohol. SRI may require a similar sequential metabolism before it is metabolised by AGMO especially as ether linkages are generally metabolically unreactive [Tietz et al, 1964].

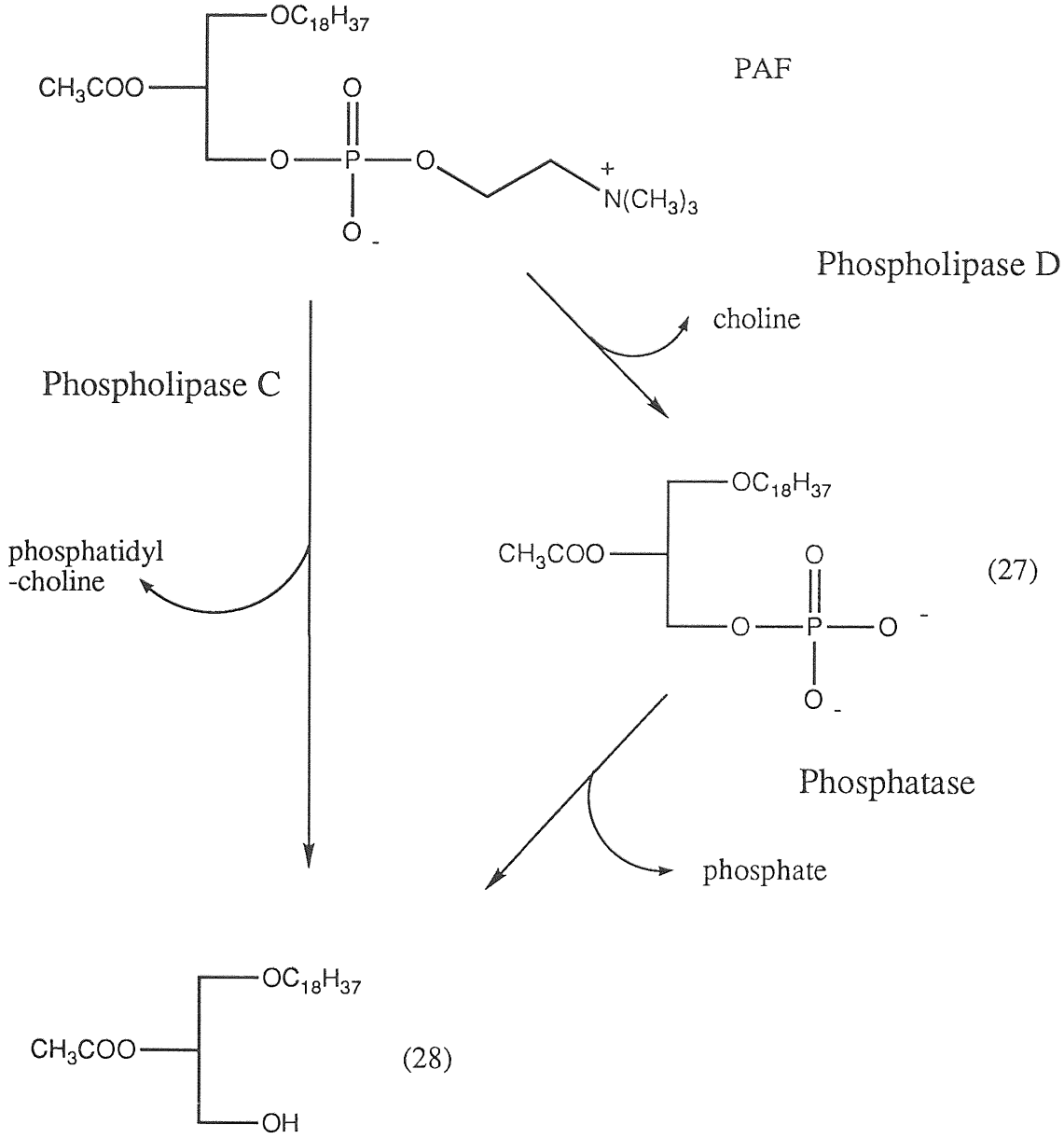


Fig. 6 Routes of the metabolism of PAF by phospholipases

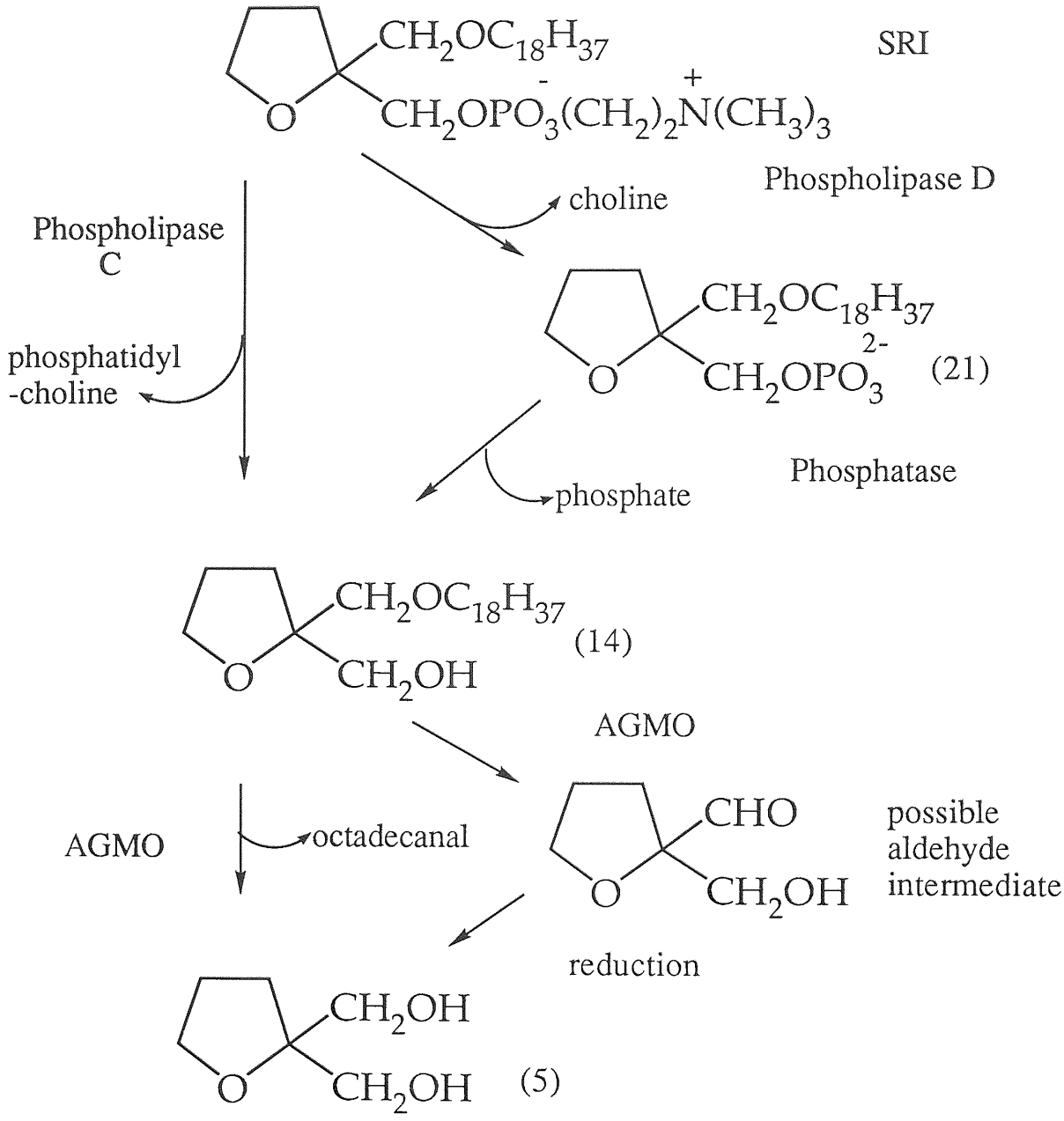


Fig. 7 Possible routes for the metabolism of SRI.

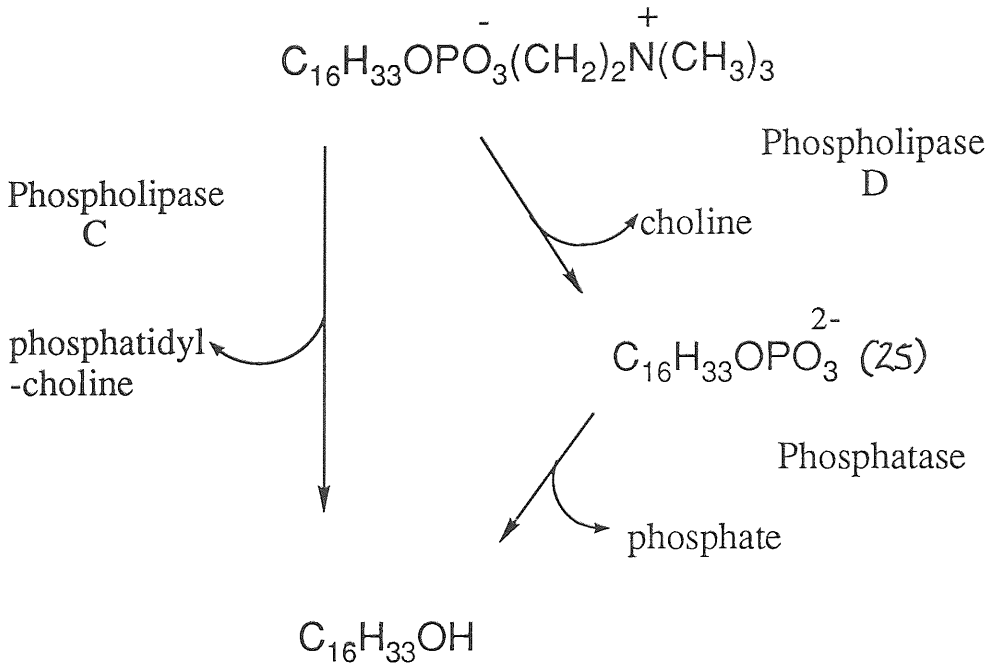
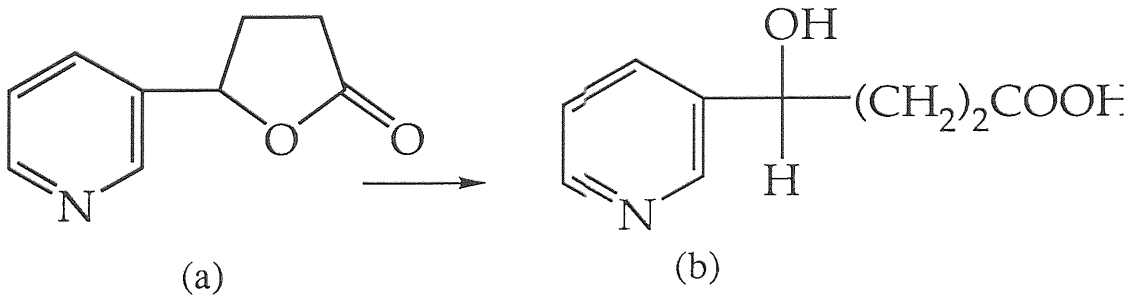


Fig. 8 Proposed routes for the metabolism of Mil

The THF ring is found in many xenobiotics and a general route of metabolism is via oxidative ring opening. 5-(3'-Pyridyl)tetrahydrofuran-2-one is a metabolite of nicotine and is further metabolised to 4-(3'-pyridyl)-4-hydroxybutanoic acid (Fig. 9, p29) [Bowman, 1968]. Similarly studies on tetrahydrofurfurylmercaptan also demonstrate oxidative cleavage of the THF ring to valeric acid (Fig. 10, p29) [Fujita and Suzuoki, 1973]. The ring is first hydroxylated at the 5-position in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. The product is further oxidised to the ring opened acid by cytosolic enzymes. This ring oxidation is also seen in the metabolism of the diuretic, Mefruside, *N*-(4'-chloro-3'-sulphamoylbenzene-sulphonyl)-*N*-methyl-2-amino-methyl-2-methyl tetrahydrofuran [Fujita and Suzuoki, 1973]. The ring opening in the examples given would probably proceed by simple hydrolysis. This reaction is related to the fact that the metabolic intermediates are lactones, rather than the THF ring itself being susceptible to hydrolysis. SRI would have to be oxidised before it would undergo ring opening. The opened ring could then be cleaved after or prior to alkyl chain metabolism.

Metabolism of the alkyl chain of SRI may occur via catalysis by AGMO as this is the only known mechanism for the degradation of the alkylether bond and it may have an important role in regulating the ether lipid content of cells [Rock et al, 1976]. Metabolism by AGMO requires molecular oxygen, reduced pteridine and pteridine reductase. The product of the

initial enzyme transformation is thought to be a hemiacetal which breaks down spontaneously to yield the long chain aldehyde and free glycerol (Fig. 11, p30). The aldehyde can then be oxidised to the acid or reduced to the alcohol [Tietz et al, 1964].



(a) (b)

(a) 5-(3'-pyridyl)tetrahydrofuran-2-one
(b) 4-(3'-pyridyl)-4-hydroxybutanoic acid

Fig. 9 Intermediates in the metabolism of nicotine.

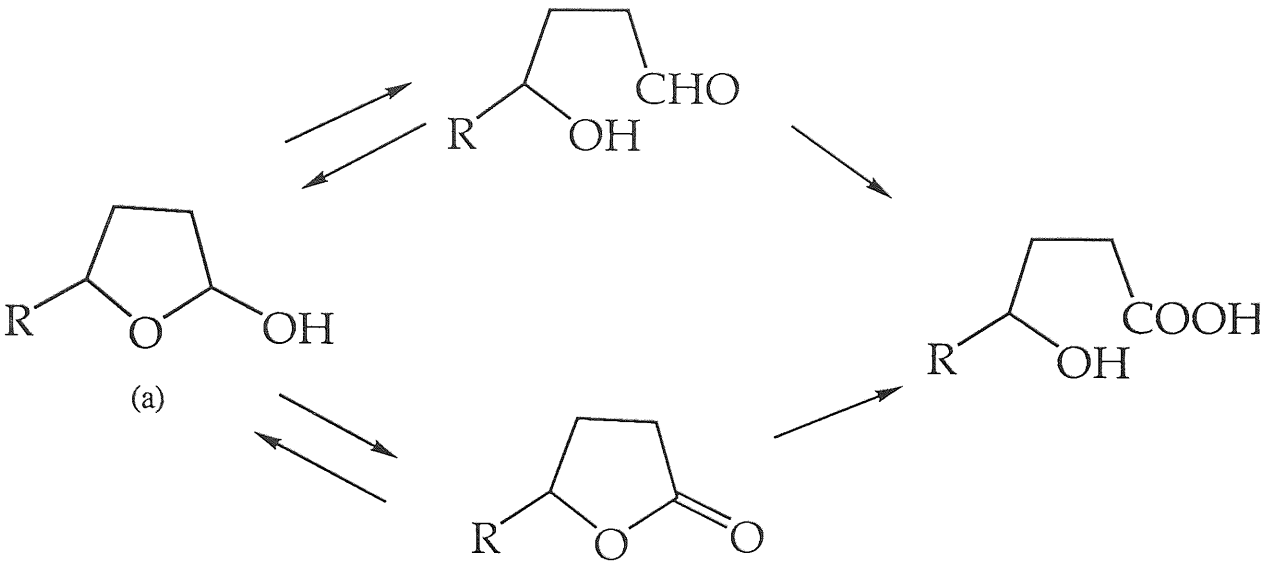


FIG. 10 Possible intermediates in the oxidation and ring opening of tetrahydrofuran ring of tetrahydrofurfuryl mercaptan (a), ($R=CH_3SCH_2$).

The cytosol of cells contains a heat-sensitive non-dialysable soluble factor which is also necessary for maximum activity of AGMO. This stimulatory component was found to be catalase which was thought to protect the enzyme from inactivation by hydrogen peroxide. Catalase also retards the non-enzymatic oxidation of the pteridine co-factor, NAD^+ [Rock et al, 1976]. The reduced pteridine participates as a co-substrate and reduces one atom of dioxygen to water whilst the other hydroxylates the substrate [Soodsma et al, 1972].

AGMO cleaves alkyl chains which have greater than six carbons. In di-O-alkylglycerols it only cleaves the long alkyl chain if the other chain is less than five carbon atoms long. ET-18-OCH_3 only becomes a substrate for AGMO on cleavage of the phosphatidylcholine moiety, and PAF is only a substrate once the acetate group is cleaved [Koetting et al, 1987b]. SRI may require metabolism of the phosphatidylcholine group or the THF ring prior to its metabolism by AGMO.

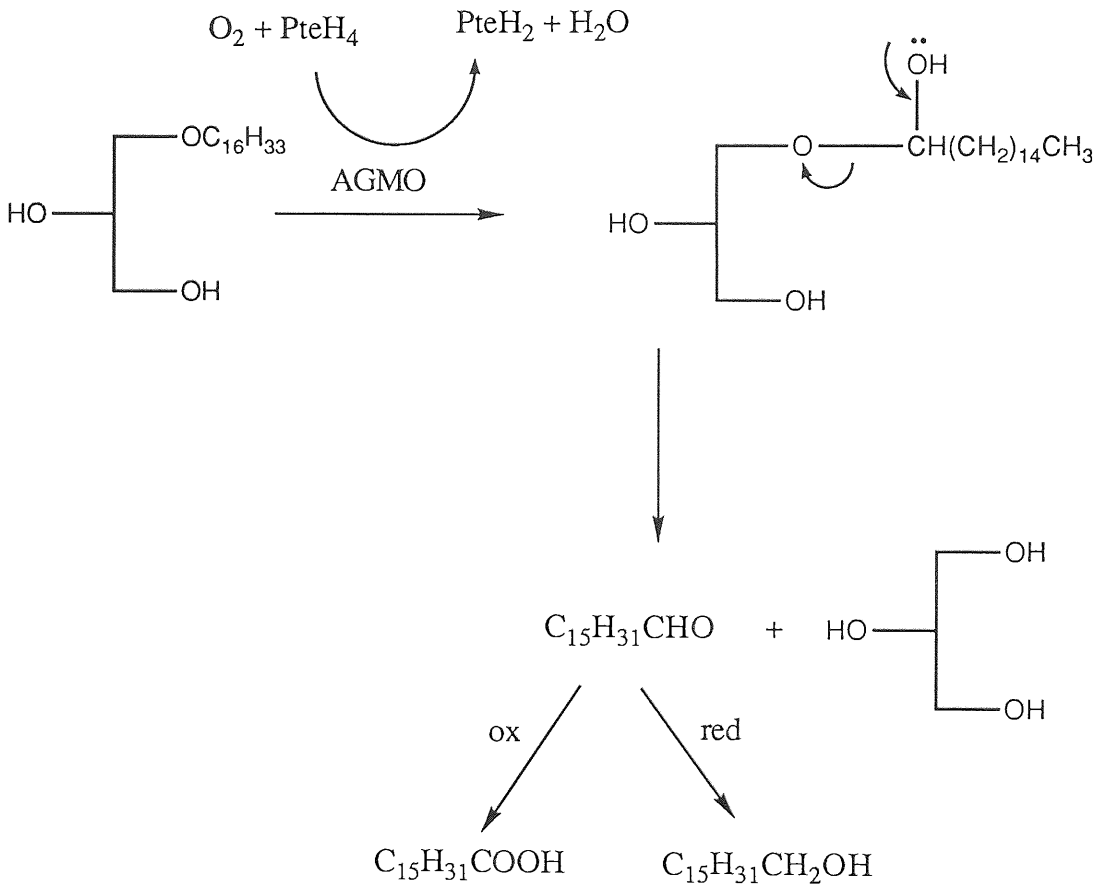


Fig. 11 Cleavage of the alkyl group from 1-hexadecylglycerol by alkylglycerol monooxygenase.

4. ^{31}P-NMR spectroscopy

^{31}P-NMR spectroscopy has been used to study metabolism *in vitro* [Hachisuka et al, 1990; Boyd et al, 1986]. Hence it was considered for use in the study of metabolism of the phosphatidylcholine group of SRI and Mil. ^{1}H-NMR spectroscopy would be more sensitive than ^{31}P-NMR spectroscopy for studying metabolism. However, the spectrum from ^{1}H-NMR spectroscopy would have more peaks and would be more difficult to interpret. The spectra obtained from ^{31}P-NMR spectroscopy are easier to understand. Identification of substrate and metabolite peaks is less complex with no interfering signals from the buffering system.

^{31}P-NMR spectroscopy in the clinical situation is still an experimental procedure. It may be of importance for the study of tumours, in diagnosis, monitoring of drug therapy and analysis of tumour resistance to drugs. An increase in phospholipid turnover may accompany tumourogenesis. This feature could be a distinction from normal tissue and be of use in diagnosis and size determination [Daly and Cohen, 1989]. Myo-inositol-1,2-(cyclic)phosphate has been detected by ^{31}P-NMR spectroscopy in Morris rat hepatomas but not in normal rat liver [Graham et al, 1987]. This phosphate appeared to be less active in releasing Ca^{2+} than the normal non-cyclic inositol phosphate that is produced by phospholipase metabolism of $InsP_3$. The high concentrations of the cyclic phosphate indicated a metabolic defect in the phosphatidylinositol metabolism in the fast growing Morris hepatoma. Its detection may be of use in diagnosis of hepatoma [Graham et al, 1987]. Adenosine triphosphate (ATP) studies may be used to judge the biochemical status of tumours during treatment and could be used to monitor phosphorus containing drug levels. Differences in phospholipid turnover may be associated with tumour resistance and could be detected by this technique [Daly and Cohen, 1989].

^{31}P-NMR spectroscopy has been used to study the effects of ether lipids on human leukaemic cell lines [Long et al, 1983]. ATP: ADP ratios were monitored to determine the mitochondrial capacity of cells. This ratio did not change on ether lipid treatment, indicating that these compounds do not act through mitochondria. The use of both ^{31}P-NMR and ^{1}H-NMR spectroscopy demonstrated that ether lipids could affect membrane integrity.

^{31}P-NMR spectroscopy has more recently been used to study tissue metabolism both *in vitro, ex vivo* and *in vivo*. Degradation of phospholipids is induced by organ ischaemia and is responsible for irreversible organ injury [Hachisuka et al, 1990]. Previously *in vivo* analysis of perchloric acid extracts of livers, preserved for transplantation, were studied for high energy phosphates. This method was used to monitor the metabolic state and hence viability of the tissue. ^{31}P-NMR spectroscopy has been found to be a more sensitive

technique for detecting alterations in phospholipids in tissue extracts and provided more detailed information about the phospho mono and diesters, than older methods such as thin layer chromatographic (TLC) determination. Ischaemia was detected as a depletion of cellular phospholipids which closely correlated with organ viability. In well preserved livers, phosphatidylcholine and phosphatidylethanolamine levels did not change, indicating absence of phospholipid anabolism and membrane catabolism. ^{31}P-NMR spectroscopy was able to detect subcellular alterations in liver preservation and estimate cell viability, prior to the appearance of morphological changes [Hachisuka et al, 1990].

Tissue metabolism has also been studied by ^{31}P-NMR spectroscopy in an *ex vivo* isolated Morris hepatoma (7777) [Graham et al, 1991]. The tumour was implanted in the inguinal region of Buffalo rats, such that it developed a blood supply that was independent of the surrounding tissue. ^{31}P-NMR spectroscopy was used to monitor ATP and other phosphorus containing metabolites, and showed the *ex vivo* model to be a reasonable representation of the *in vivo* situation. Use of an *ex vivo* model could remove many of the host influences. Phosphorus signals originated from the entire tumour and were not affected by signals from the surrounding normal tissue. This model in conjunction with ^{31}P-NMR spectroscopy could enable study of tumour metabolism, physiology and biochemistry, with greater relevance to the *in vivo* situation [Graham et al, 1991].

^{31}P-NMR spectroscopy has also been used to study metabolism of compounds in solution, in cell culture and *in vivo*. Cyclophosphamide is known to be metabolised to a toxic aziridinium ion *in vivo* (Fig. 12). ^{31}P-NMR spectroscopy has been used to study the affects of pH control on the rate of intramolecular cyclisation of cyclophosphamide to the aziridinium ion and hydrolysis of this reactive alkylating agent. Ion formation was found to be pH dependent and that the rate of its formation decreased in acid. Some tumours may be acidic in nature and this would slow cyclisation and cyclophosphamide would be less effective in these tumours [Engle et al, 1979]. ^{31}P-NMR spectroscopy has also been used to monitor the formation of the toxic aldophosphamide from cyclophosphamide (Fig. 12) in U937 lymphoma cells. The cells were entrapped in low gelling agarose threads and analysis was carried out on the perfusate [Boyd et al, 1986]. Mafosfomide is structurally related to cyclophosphamide with $-SCH_2CH_2SO_3^-$ substituted in position 4 of this molecule. Its metabolism to the 4-ketocyclophosphamide by P388 mice leukaemic cells has also been studied by ^{31}P-NMR spectroscopy [Sonowat et al, 1990].

^{31}P-NMR has been used to study choline kinase, choline phosphate cytidyl transferase, choline phosphotransferase and phospholipase C activity both *in vitro* and *in vivo* [Edwards and Hands, 1976]. 2-Hydroxyethyltrimethyl phosphonium chloride was used as an

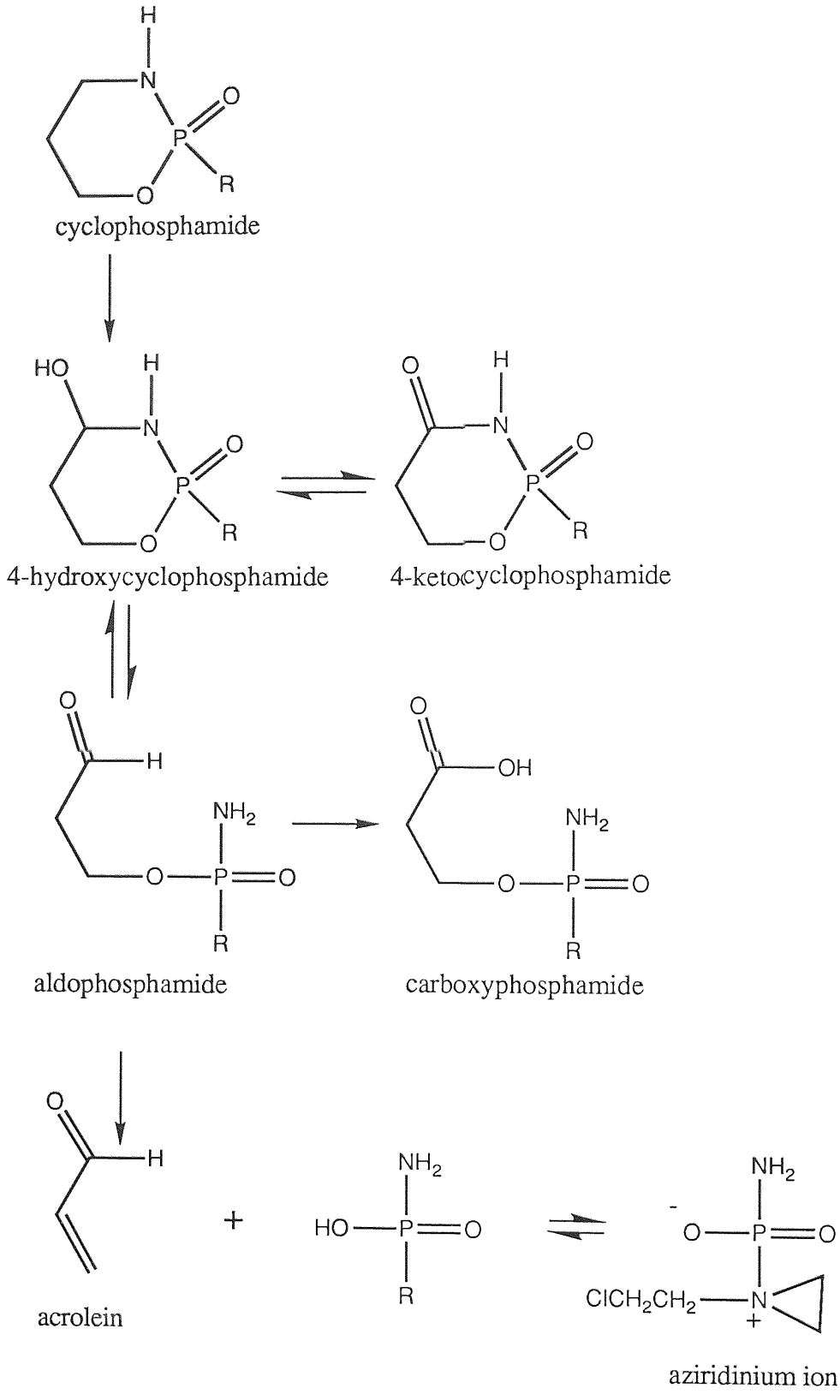


Fig. 12 Metabolism of cyclophosphamide, where R = $N(CH_2CH_2Cl)_2$.

analogue of choline for these studies. In this molecule the nitrogen of choline is replaced by phosphorus to give the compound $HOCH_2CH_2P^+(CH_3)_3$. Metabolism of this compound and phosphocholine-substituted choline analogues such as CDP-phosphocholine was followed on incubation with the enzymes or on extraction following incubation with crude tissue homogenates. This phosphonium was also administered to rats and the phosphorus containing compounds were monitored to study the sites of its incorporation into phospholipids. The choline analogue was used for metabolism studies as it closely resembles choline and it was metabolised and utilised in the same way. The presence of the phosphorus in phosphocholine enabled experiments to be monitored by ^{31}P-NMR spectroscopy [Edwards and Hands, 1976].

5. Objectives of this project.

One of the main objectives of this project was to study the metabolism of the ether lipids SRI and Mil, to contribute to the body of knowledge on xenobiotic metabolism. SRI and Mil are phosphorus containing compounds, so the hypothesis was tested that ^{31}P-NMR spectroscopy could be used to study the metabolism of these compound by purified enzymes and liver homogenate *in vitro*. Putative metabolites of SRI and Mil were synthesised as reference compounds for analytical detection. The hypothesis that SRI could undergo metabolism by AGMO was also explored. SRI metabolism was studied in liver homogenate and analysed by gas chromatography (GC) and an NADH coupled UV assay. SRI and Mil are both undergoing clinical trial and a study of their metabolism may eventually help in the monitoring of blood levels of these ether lipids and their metabolites. An understanding of the route of excretion can aid in the identification of possible sites of toxicity.

Uberall et al [1991] postulated that Mil produces its cytotoxicity by inhibiting inositol-specific phospholipase C, so interfering with cell signal transduction. The hypothesis was tested that SRI and Mil could inhibit phosphatidylcholine specific phospholipase C. PAF is known to be metabolised by phospholipase C [Wilcox et al, 1987]. To check for inhibition, SRI and Mil were incubated with phospholipase C and then PAF was added to observe if its rate of metabolism was decreased. These experiments were carried out using ^{31}P-NMR spectroscopy.

Metabolism can both increase and decrease the toxicity of compounds, it can also pharmacologically activate or deactivate agents. Ether lipids are thought to exert their cytotoxic action via the cell membrane (chapter 1, section 2.4, p17). Metabolites of SRI and Mil were envisaged on the basis of metabolism experiments on other ether lipids, phospholipids or PAF as reported in the literature. The hypothesis that metabolism is a

detoxification process was tested. It has been postulated that metabolism of ether lipids would reduce toxicity if their mode of action was via attack of the membrane. Metabolism usually decreases the lipid solubility of a compound and would thus reduce its ability to penetrate membranes. Three assays were used to assess the cytotoxic potential of SRI and its proposed metabolites. The assays used were the study of growth characteristics, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay and release into the cellular medium of LDH (chapter 4, section 1.3., p104). These three assays detect toxicity by measuring different parameters. Variations in the sensitivity of these tests were thought to give information on the site of toxicity of the ether lipids (chapter 4, section 1.3., p104). The MTT assay was used to compare the toxicity of Mil and its potential metabolites. The toxicity of SRI, Mil and their postulated metabolites was compared in two tumour cell lines originating from different tissues (A549 from human lung and JB1 from rat liver) to observe if there was a difference in tumour sensitivity. Differences may be expected as the JB1 cell line was shown by Manson et al [1981] to have an ability to metabolise certain compounds, though its ability to metabolise phospholipids was not tested (chapter 4, section 1.2.2., p103). The metabolic ability of JB1 cells may be greater than that of the A549 cells. Comparison of compound toxicity was also made between a tumour cell line (JB1) and an untransformed cell line (BL8) from the same tissue, rat liver, to find out if tumours were more sensitive to ether lipids than "normal" cells. If SRI were cytotoxic to BL8 cells, toxicity could be suspected to occur *in vivo*. During clinical trials liver function could then be monitored to identify early signs of damage. SRI and Mil are zwitterionic and have the potential to bind to serum proteins. The hypothesis was tested that the presence of serum in cell culture reduces the toxicity of these compounds. Finally the hypothesis was tested that SRI and Mil are of comparable toxicity to ET-18-OCH_3, the ether lipid which is most often used in research. This theory was examined by comparing the IC_{50} values measured for SRI and Mil with those reported in the literature for ET-18-OCH_3.

CHAPTER 2

CHEMISTRY

1. RESULTS AND DISCUSSION.

Synthesis of SRI and its metabolites.

Fig. 13 outlines the basic steps that had to be accomplished in the synthesis of SRI and its putative metabolites, diol (**5**), alcohol (**14**) and phosphate (**21**).

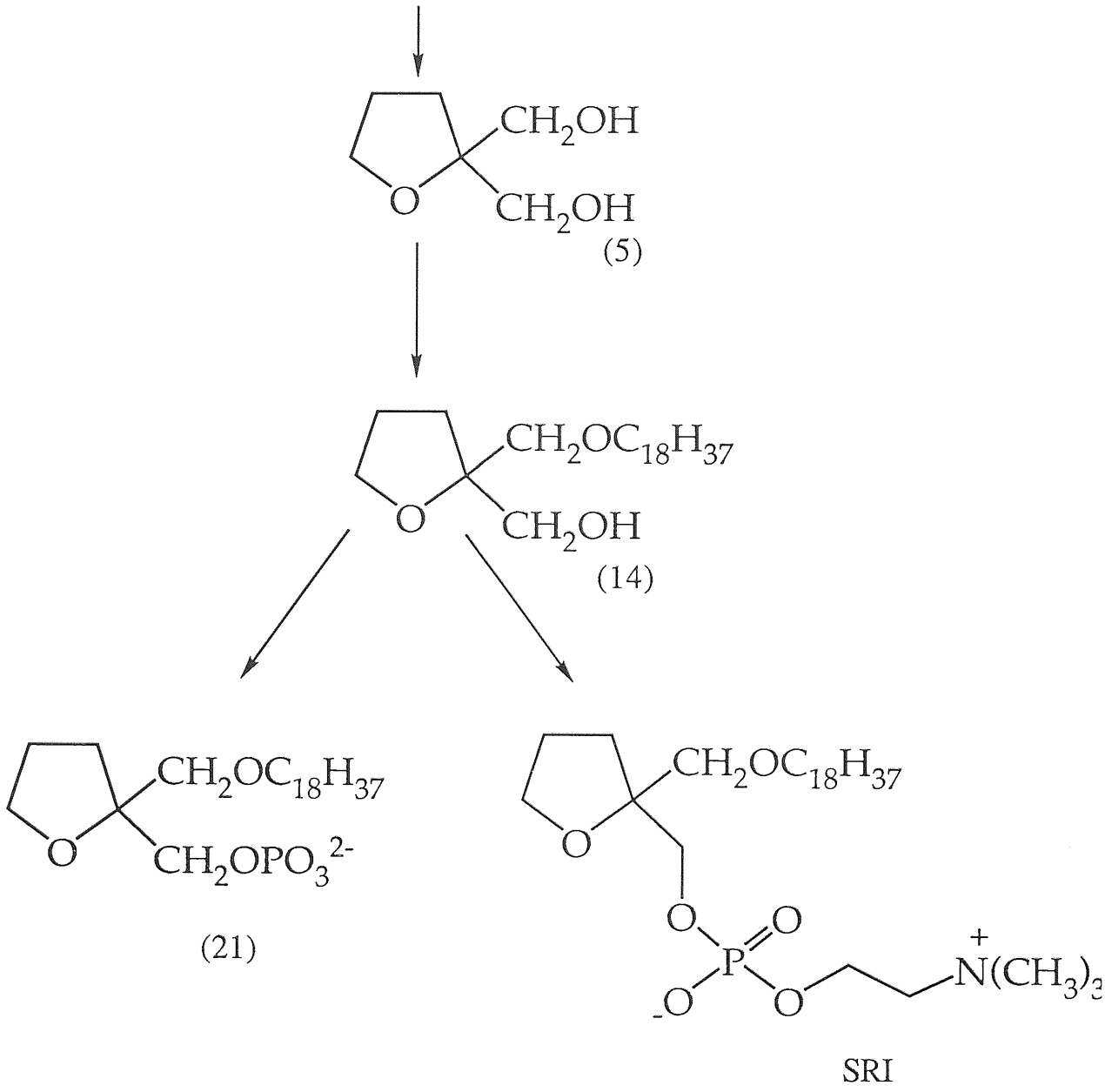


Fig. 13 Scheme for the synthesis of SRI and its putative metabolites.

1.1. Synthesis of SRI

The chemical synthesis of this compound can be considered in three stages,

1) Preparation of 2,2-bis(hydroxymethyl)tetrahydrofuran [diol (**5**)]

2) Synthesis of 2-hydroxymethyl-2-octadecyloxymethyltetrahydrofuran [alcohol (**14**)].

3) Addition of phosphatidylcholine group to (**14**) to give SRI

1.1.1. Preparation of 2,2-bis(hydroxymethyl)tetrahydrofuran (5)

The first method considered for the synthesis of the diol (**5**) was that described by Houlihan et al [1987]. The initial stage of this method involved production of methyl tetrahydro-2-furoate (**3**) shown in Fig. 14. Step one was hydrogenation of 2-furoic acid (**1**) in the presence of a palladium catalyst, to give tetrahydro-2-furoic acid (**2**). A 100% yield was obtained when (**1**) was boiled with activated charcoal in water, filtered and recrystallised prior to hydrogenation. This purification step reduced the chance of "poisoning" the catalyst. Step two was esterification of (**2**) with HCl / methanol and it also proceeded giving a good yield (74%) of ester (**3**). Hydrogenation of (**1**) in the presence of methanol and an acid catalyst was attempted to combine the two steps. However, although this method gave ester (**3**), the yield was small and the product was difficult to purify. Therefore, the two step process was generally used.

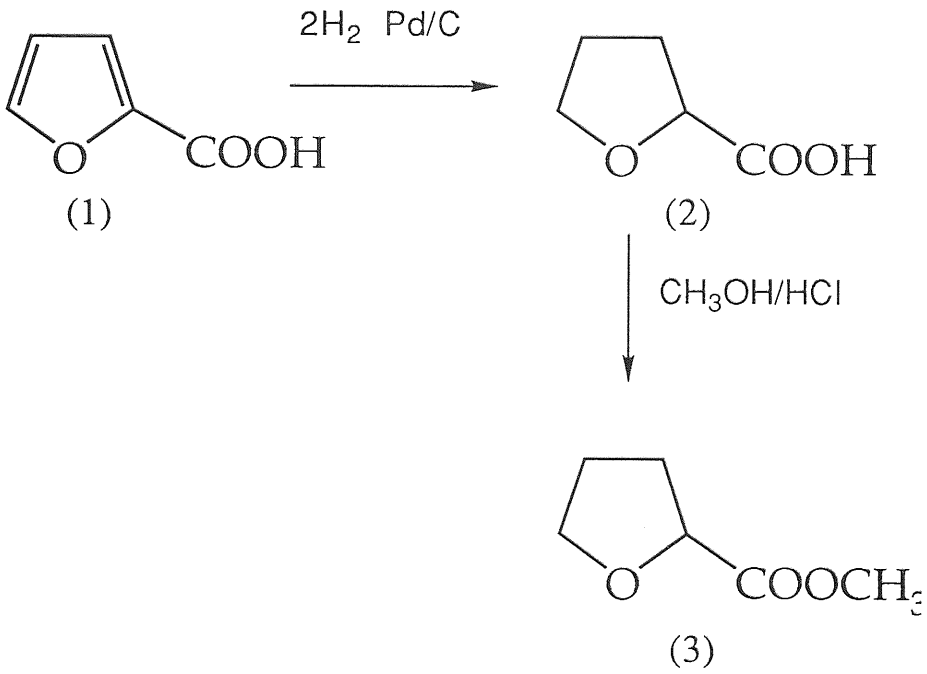


Fig. 14 Synthesis of methyl tetrahydro-2-furoate

The next stage in the synthesis of 2,2-bis(hydroxymethyl)tetrahydrofuran (**5**) was the conversion of ester (**3**) to diol (**5**). The reaction proceeded via the intermediate,

tetrahydrofuran-2-carboxaldehyde (**4**) (Fig. 15, p38). The two step reaction was performed without isolation of (**4**). Diisobutyl aluminium hydride (DIBAH) was used to reduce ester (**3**) to aldehyde (**4**) at temperatures of < -60°C under argon to protect from oxygen and moisture. The mixture was then rapidly transferred by cannula under argon into a solution of sodium hydroxide in aqueous formaldehyde. This reaction gave a very poor yield (100mg, <0.5%) of diol (**5**) together with a number of unidentified by-products. A second problem was that, occasionally after the addition of DIBAH, a large amount of solid precipitated out, which was presumably a complex of (**4**). This precipitate made the transfer of the mixture into the formaldehyde solution impossible.

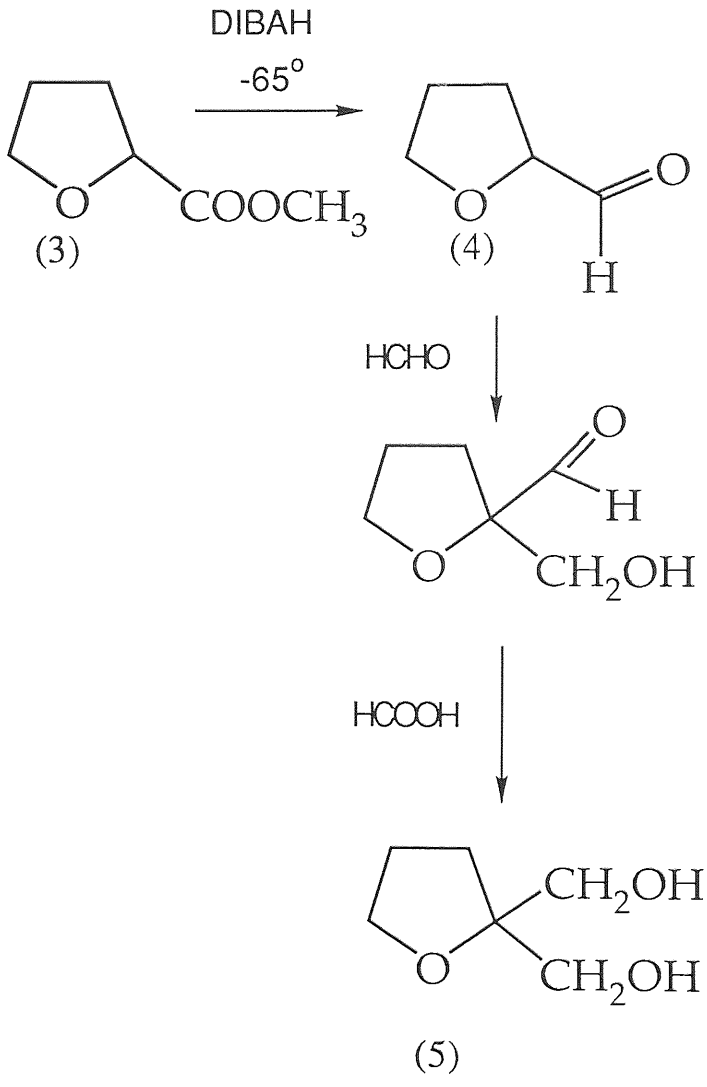


Fig. 15 Synthesis of 2,2-bis(hydroxymethyl)tetrahydrofuran.

The poor yield led to the exploration of alternative methods for the synthesis of aldehyde (**4**) avoiding DIBAH. In the synthesis of diol (**5**) from ester (**3**), via aldehyde (**4**), (Fig. 15) the two steps were carried out progressively. It was difficult to judge whether the overall

reaction had failed at the first or second step of the DIBAH reaction. It was decided that the formation of (**5**) should be divided into the synthesis of aldehyde (**4**) and then the production of diol (**5**).

The first method to be discussed for the synthesis of aldehyde (**4**) used tetrahydrofurfuryl-amine (**6**) [Belanger and Williams, 1983] (Fig. 16). Synthesis of tetrahydrofuran-2-carboxaldehyde oxime (**7**) from amine (**6**) gave a mixture of 30% syn and 70% anti geometrical isomers in good yield (52%). ^{1}H-NMR spectrum showed two set of doublets for the vinyl protons, one at 7.4 (anti) and the other at 6.8ppm (syn). The anti isomer occurred in larger quantities due to the steric hindrance between the THF ring and the -OH group in the syn isomer. The cleavage of the oxime group to yield aldehyde (**4**) was attempted by two methods. In the first, titanium trichloride was used. However, this reagent reacted violently with the water used as a solvent and only low yields (25%) of (**4**) were obtained. Another method for cleaving the oxime group, using sodium bisulphite, was unsucessful [Pines et al, 1966].

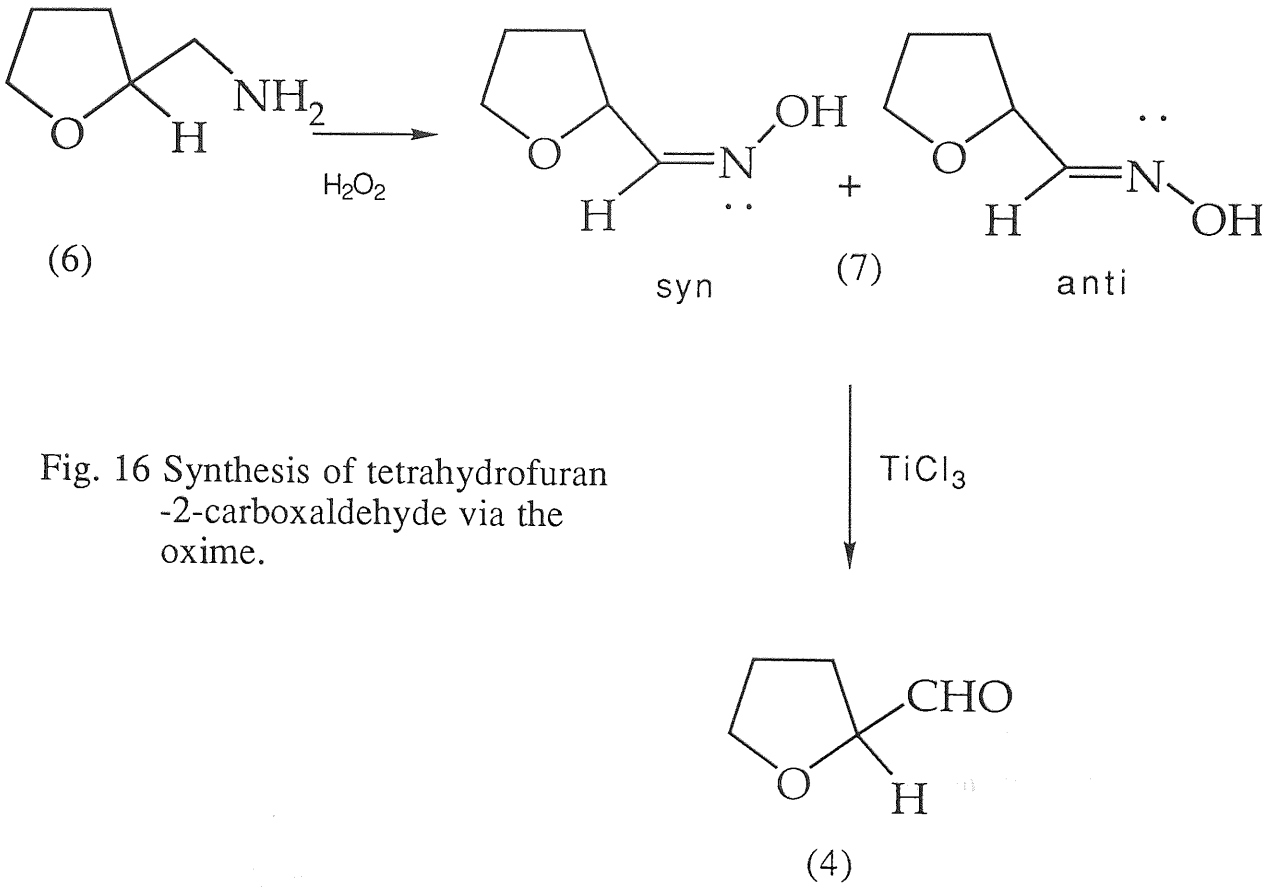


Fig. 16 Synthesis of tetrahydrofuran -2-carboxaldehyde via the oxime.

The best yield of aldehyde (**4**) was obtained by hydrogenation of 2-furaldehyde (**8**). Although the product was not pure, large amounts (20 - 25g) of (**8**) could be reacted at one

time. The mixture was then used in its impure state in the synthesis of diol (**5**) by substituting it in the reaction with formaldehyde (Fig. 15, p38), to give 5 - 10g of diol (**5**). This method, however, was inconvenient for the preparation of aldehyde (**4**) as it was not very stable and could not be stored for long periods of time. The method for the isolation of aldehyde (**4**) from the hydrogenation mixture also involved several steps which could increase its degradation. Aldehyde (**4**) formed by hydrogenation had to be used almost immediately for the synthesis of diol (**5**). A more convenient method for the preparation of aldehyde (**4**) was developed.

A different method for the preparation of aldehyde (**4**) involved the use of protecting groups for the aldehyde functionality of 2-furaldehyde (**8**) during hydrogenation. These groups could then be cleaved to regenerate the aldehyde group at a suitable time. The first protecting group used was an acetal. Furaldehyde diethyl acetal (**9**) was produced by stirring aldehyde (**8**) with ethanol and HCl for 24h, then distilling [Adkins et al, 1931] (Fig. 17, p41). Acetal (**9**) was readily hydrogenated to give 2-tetrahydrofuraldehyde diethyl acetal (**10**) (Fig. 17). A method for deprotection using silica was attempted [Huet et al, 1978] as diethyl acetal (**10**) had showed signs of decomposition on the acidic sites of silica. This deacetalization required the addition of an acid. Oxalic acid did not cause any cleavage of the acetal group of (**10**), however, sulphuric acid gave the desired product in a 60% yield. The mixture of aldehyde (**4**) and acetal (**10**) could also be substituted in the formaldehyde reaction (Fig. 15, p38) (section 2.3.6.1b, p59) and diol (**5**) purified by distillation. The main problem with this method was that only small amounts (1-2g) of acetal (**10**) were deprotected at one time as this required large amounts of silica.

Amberlite IR-120 cationic exchange resin has been used to deacetalize the dibutyl acetal of aldehyde (**4**) [Defaye, 1968]. This resin produced complete deprotection of the aldehyde group but leached out a large number of impurities. Amberlite deprotection required 30g of resin to deacetalize 1.5g of acetal (**10**), so again this method was only suitable for preparation of small quantities of aldehyde. Use of other cationic resins did not cause any deprotection.

The second protecting group for the aldehyde was diacetate. Furfural diacetate (**11**) was prepared in a 60% yield from the reaction of aldehyde (**8**) with acetic anhydride (Fig. 18, p42) [Bertz, 1963]. Hydrogenation of diacetate (**11**) was catalysed by palladium on carbon and gave tetrahydrofurfural diacetate (**12**) (Fig. 18) in a 90% yield. The method for deprotection with NaOH, previously used for other diacetates [Amouroux et al, 1981] initially proved unsuccessful. NaOH could cleave the acetate groups of (**11**) so

modifications were made to the method and diacetate (**12**) was successfully deprotected in quantitative yields, to give aldehyde (**4**).

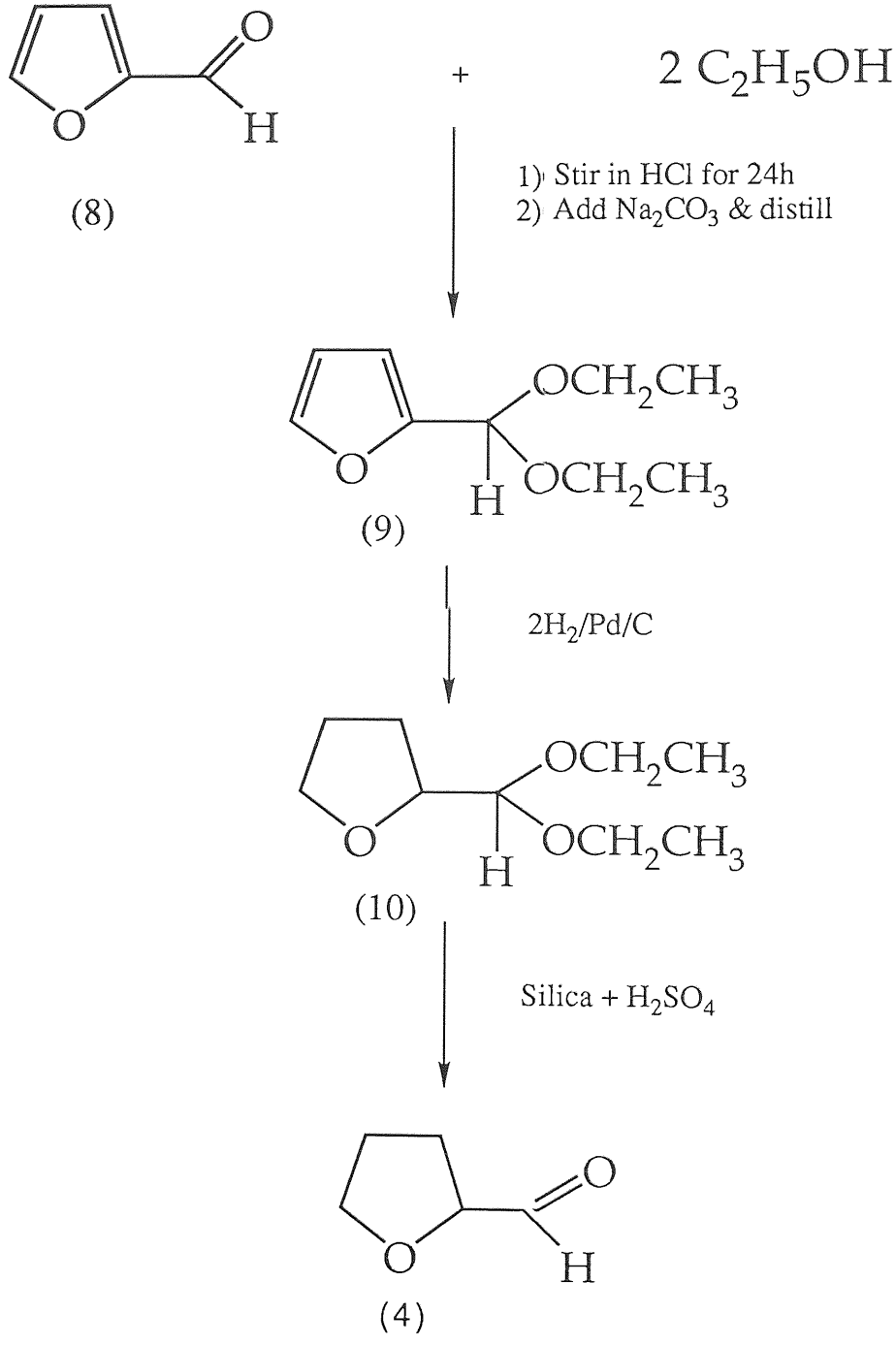


Fig. 17 Synthesis of the aldehyde (**4**) via the diethyl acetal (**10**).

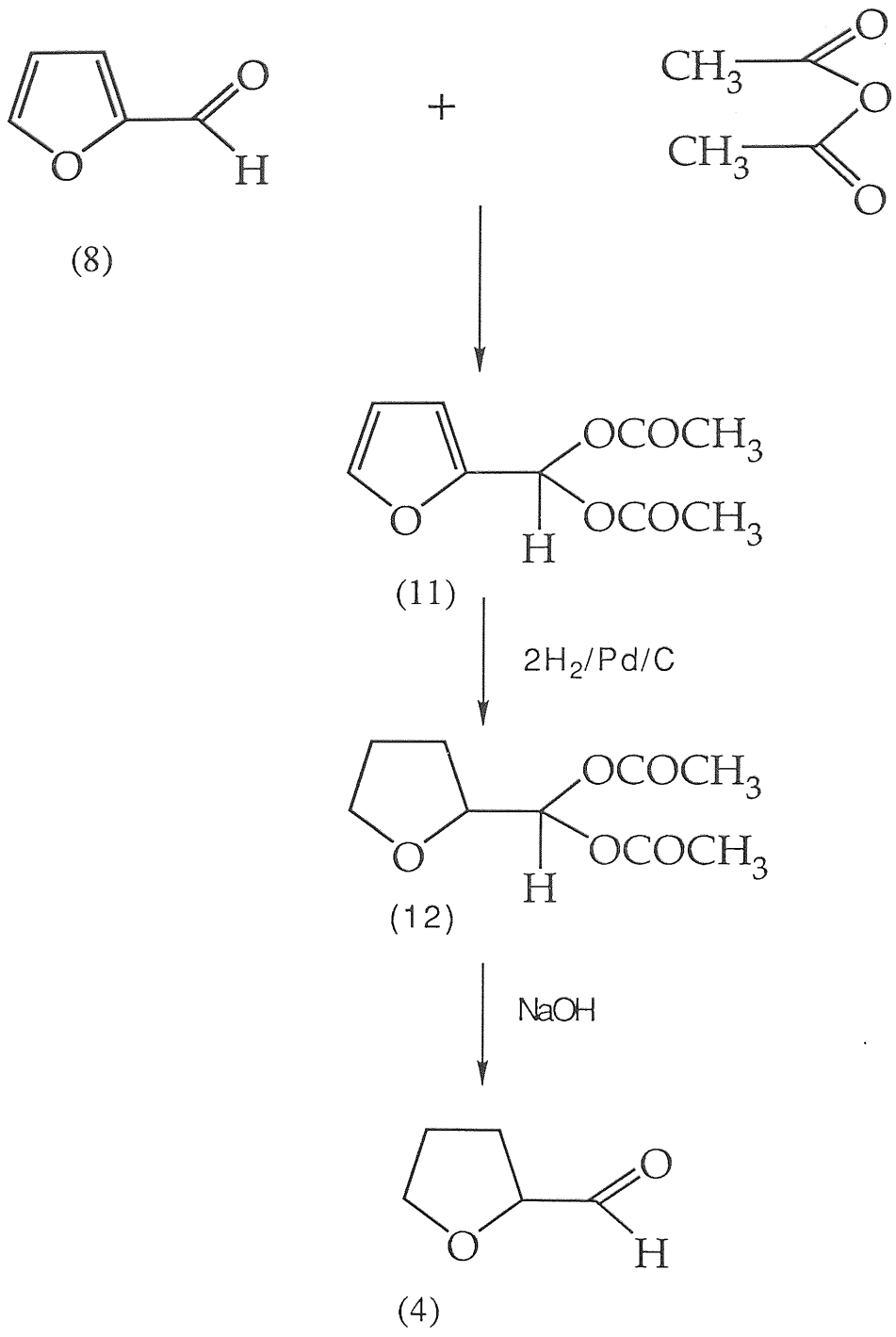


Fig. 18 Synthesis of the aldehyde (**4**) via the diacetate (**12**).

The best method for the preparation of aldehyde (**4**) was by deprotection of diacetate (**12**). Although hydrogenation of (**8**) could yield larger quantities of (**4**), the deprotection route was more convenient, enabling storage of diacetate (**12**) and its deprotection to give aldehyde (**4**) as required.

There were two methods which could be used to synthesise diol (**5**) from aldehyde (**4**). In the first, (**4**) was heated with base and formaldehyde. The synthesis relied on the Cannizzaro reaction to convert formaldehyde to formic acid which would then reduce aldehyde (**4**) to the alcohol (Figures 19 and 20) [Eftax and Dunlop, 1961]. Aldehyde (**4**) was not very stable and readily polymerised, especially at raised temperatures [Minne and Adkins, 1933], so the yield of diol (**5**) from this reaction was low (8%). The method of warming aldehyde (**4**) with formaldehyde also used n-butanol to extract diol (**5**). This solvent was difficult to completely remove by distillation.

Fig. 19 The Cannizzaro reaction for formaldehyde.

The second approach used for the preparation of diol (**5**) from aldehyde (**4**) (Fig. 20) was based on that of Houlihan et al [1987]. Aldehyde (**4**) was treated with base to form the anion which was then reacted with formaldehyde to yield the aldehyde intermediate (Fig. 20). Formic acid was then added to reduce the remaining aldehyde to the diol (**5**). This method was more successful with yields of 40%.

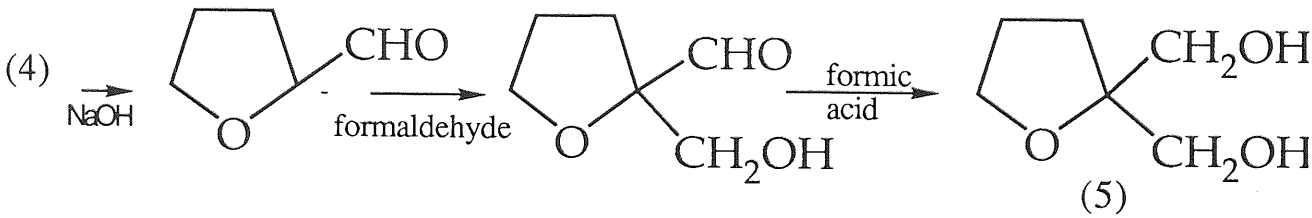


Fig. 20 Synthesis of diol (**5**) from aldehyde (**4**).

1.1.2. Synthesis of 2-hydroxymethyl-2-octadecyloxymethyltetrahydrofuran (14)

The next stage in the synthesis of SRI, involved the nucleophilic substitution of n-octadecyl -bromide with one alcohol group of diol (**5**) (Fig. 21, p44). Only 0.3 equivalents of n-octadecylbromide was used in the reaction to reduce the probability of diol (**5**) reacting with two equivalents of n-octadecylbromide. The reaction gave reasonable amounts (3g) of

2-hydroxymethyl-2-octadecyloxymethyltetrahydrofuran (**14**) in 62% yield based on n-octadecylbromide. Alcohol (**14**) was purified by flash column chromatography [Still et al, 1978] using ethyl acetate - hexane (1: 2 v/v).

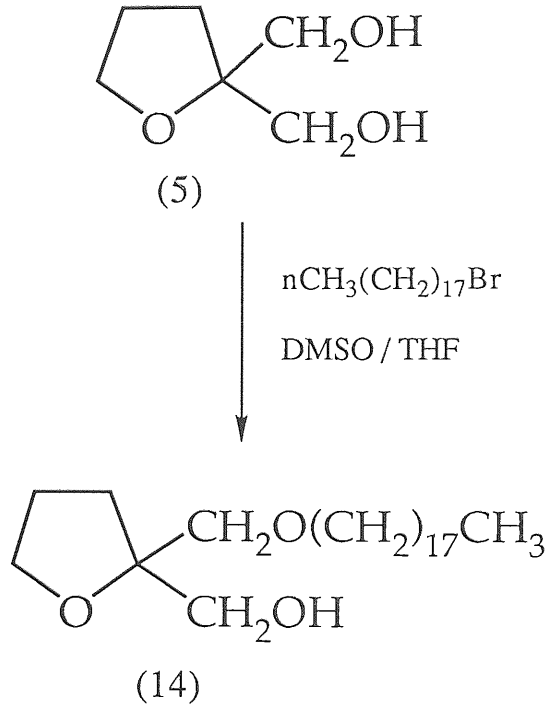


Fig. 21 Synthesis of 2-hydroxymethyl-2-octadecyloxymethyl -tetrahydrofuran.

1.1.3. Addition of phosphatidylcholine group to alcohol (14) to give SRI

The phosphatidylcholine group could be added via the reaction of alcohol (**14**) with 2-chloro-2-oxo-1,2,3-dioxophospholane (**16**). This method was based on that reported by Houlihan et al [1987] (Fig. 22, p45). Phospholane (**16**) was readily prepared in an 80% yield from the reaction of 2-chloro-1,2,3-dioxaphospholane (**15**) with oxygen (Fig. 23, p46) [Edmundson, 1962]. Phospholane (**15**) hydrolysed on contact with moisture so the oxygen had to be dried by passing it through silica, prior to its entry into the reaction vessel.

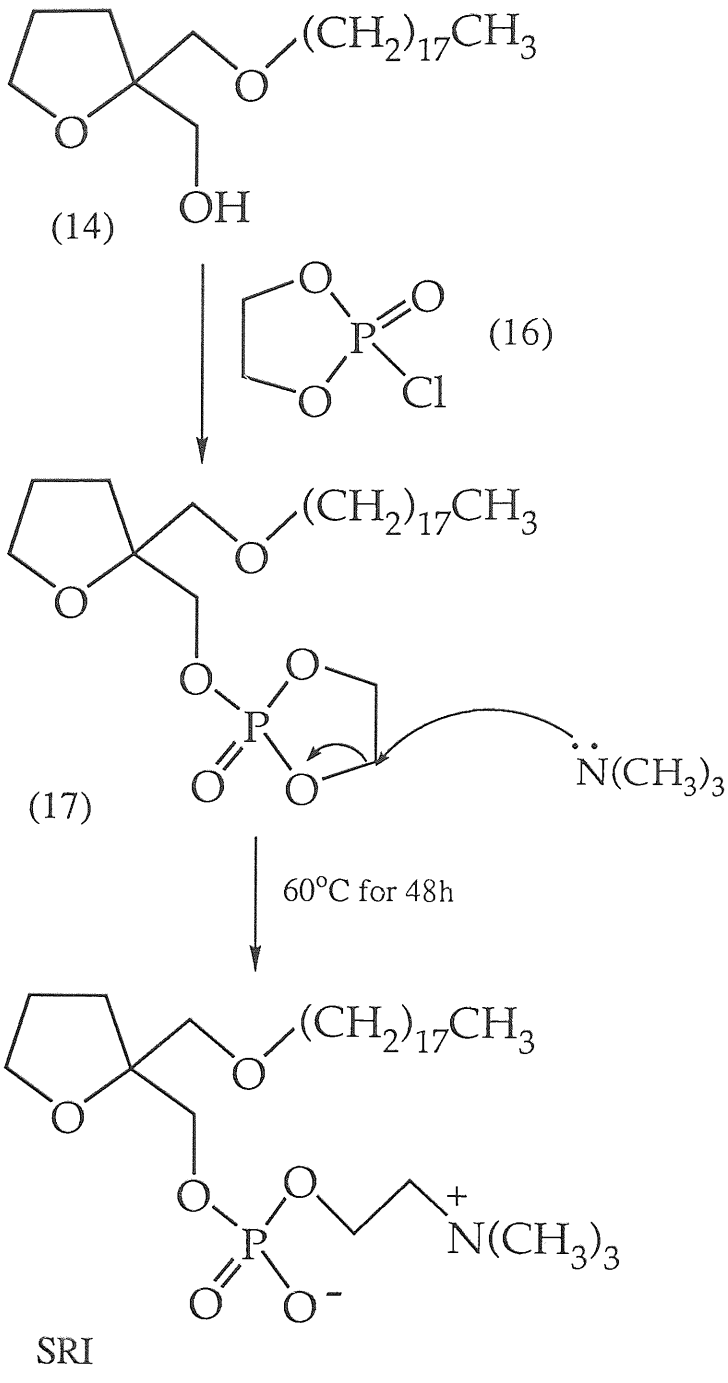
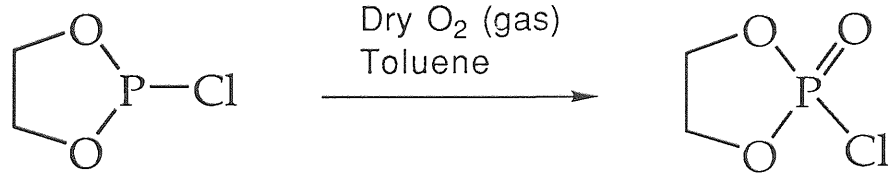


Fig. 22 Final steps in the synthesis of SRI.

The crude phospholane derivative (**17**) was prepared by the reaction of alcohol (**14**) with phospholane (**16**) (Fig. 22, p45) and was not isolated. Crude intermediate (**17**) was sealed in a pressure bottle with trimethylamine for completion of the synthesis of SRI (Fig. 22). Purification was carried out using flash column chromatography which gave a large amount of unchanged starting material and a small amount of white solid. This solid had an R_f and NMR spectral properties similar to those of the authentic sample of SRI.



(15) (16)

Fig. 23 Oxidation of 2-chloro-1,2,3-dioxaphospholane.

A similar route was also used unsuccessfully in the attempt to synthesise [2'-(hydroxy-methyl)tetrahydrofuran-2'-yl]methyl 2-[*N*,*N*,*N*-trimethylammonio]ethyl phosphate (**18**) (Fig. 24, p47). A possible reason for the failure of this reaction was the use of methanol to improve the solubility of diol (**5**) in toluene. Methanol is a better nucleophile than diol (**5**) and would more readily attack phospholane (**16**). This reaction would increase impurities and reduce the yield of phosphate (**18**). Methanol was used in this synthesis as diol (**5**) was hydrophilic and very difficult to dissolve in organic solvents. Due to the low yields of phosphate (**18**) and SRI, different methods were attempted for attaching a phosphatidylcholine group to alcohol (**14**).

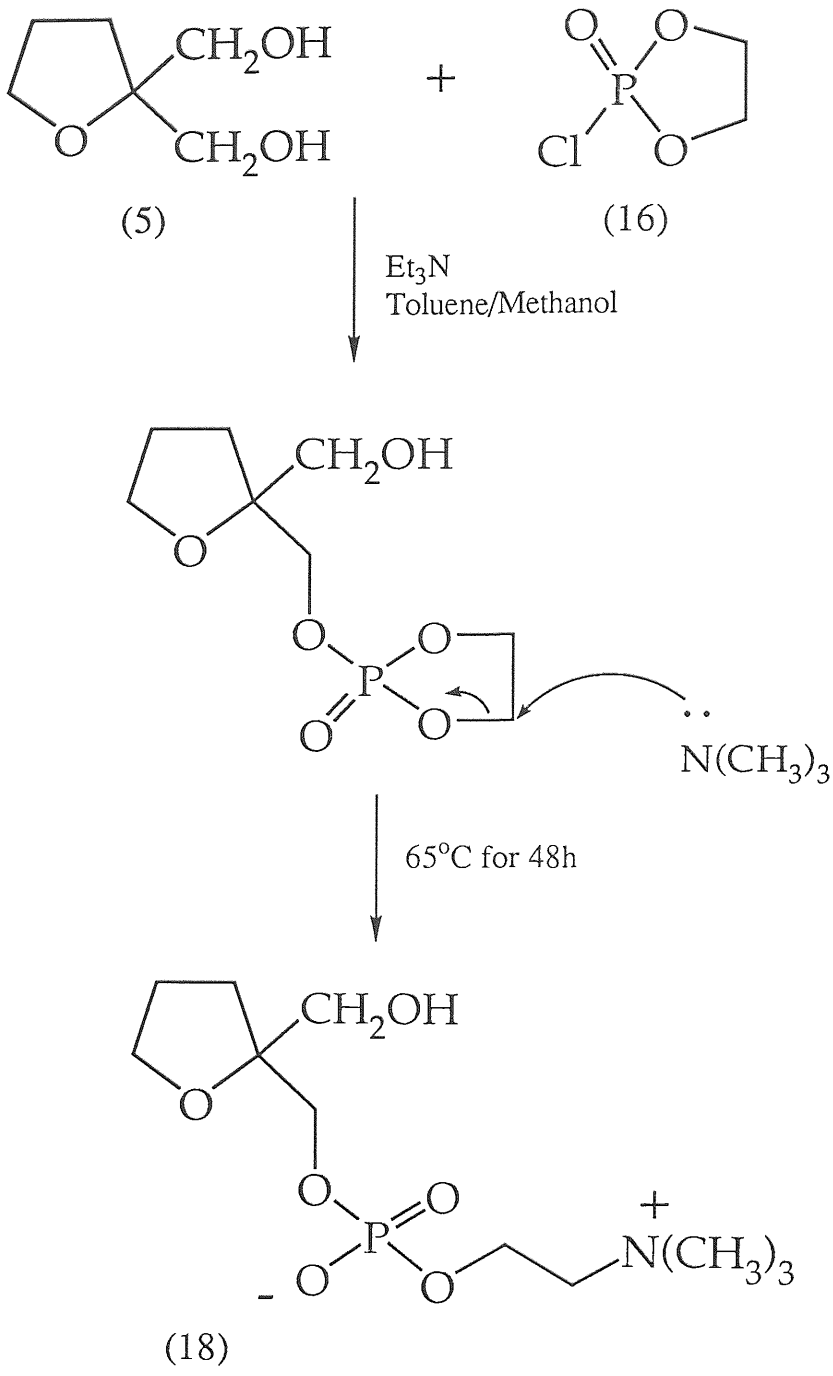


Fig. 24 Synthesis of [2'-(hydroxymethyl)tetrahydrofuran-2'-yl] -methyl 2-[N,N,N-trimethylammonio]ethyl phosphate.

Synthesis of [2'-(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N*-methylammonio] -ethyl phosphate (**20**) was attempted using both methods described by Euerby et al [1987] (Figures 25 and 26). Each route would produce the same intermediate (**19**), which could be hydrolysed with acid to (**20**) and then, theoretically, methylated to SRI (Fig. 25).

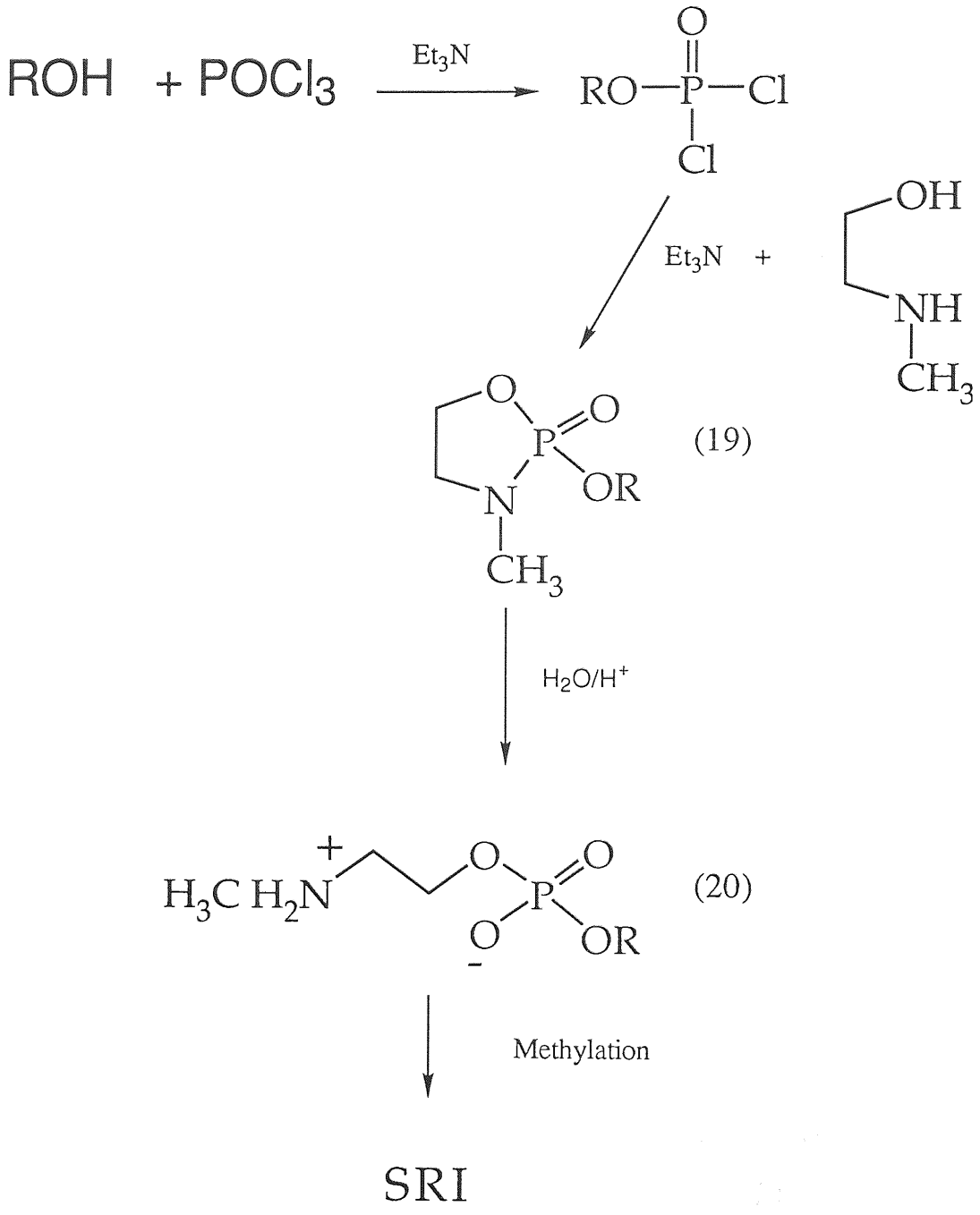


Fig. 25 Alternative route for synthesising SRI (Euerby et al, 1987). ROH = (**14**)

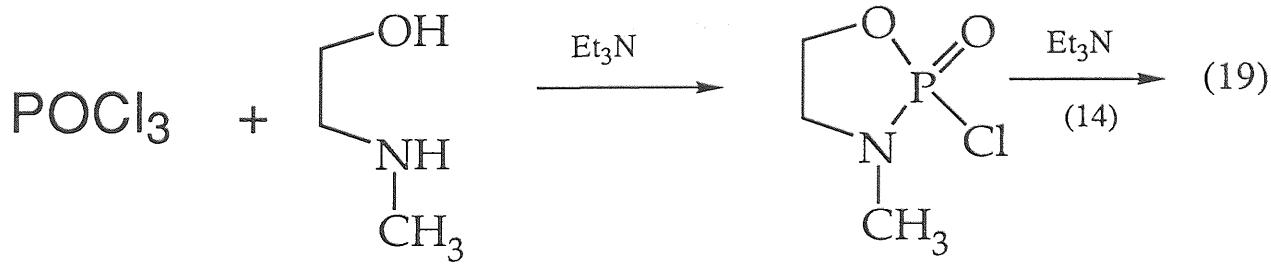


Fig. 26 Second route for synthesising intermediate (**19**) (Euerby et al, 1987).

Neither of the methods reported by Euerby et al [1987] were successful for the synthesis of (**19**), and methylation of (**20**) was considered very difficult [McGuigan, personal communication]. A third method to prepare (**20**) via intermediate (**19**), based on the work of Anson and McGuigan [1989] (Fig. 27) was not attempted as it also required methylation of the NH_2 group.

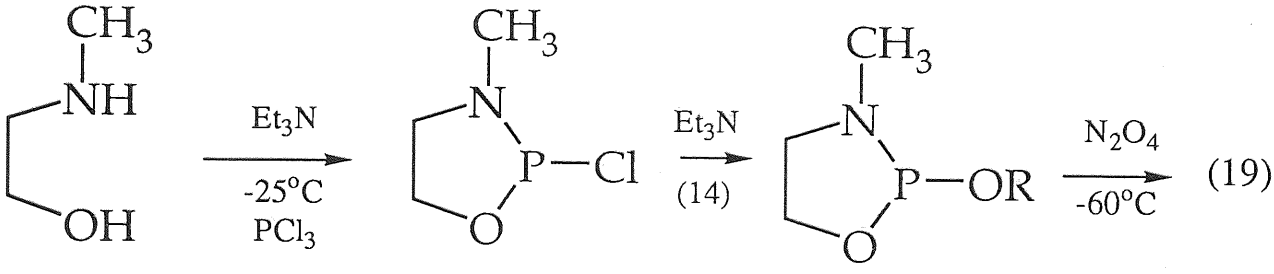


Fig. 27 Third route for preparing intermediate (**19**) (Anson and McGuigan, 1989).

1.1.4. Development of a HPLC method for analysis of SRI

The method used in the Aston Molecules Standard Operating Procedure for SRI [Baer, 1988] was first considered for HPLC analysis of samples of SRI. This compound does not have a chromophore, therefore it cannot be easily detected by UV spectrophotometry. Post column derivatisation was considered so that SRI could be detected by fluorescence spectroscopy. 8-Anilino-1-naphthalene sulphonic acid (ANS) was used but would only detect the parent compound and not its metabolites. The basis for the use of ANS was provided by Schaefer and Rohdewald [1989] who employed this reagent for detecting ET-18-OCH_3 in blood using TLC. ANS has to be bound to both the phosphorus and the alkyl chain to fluoresce [Muesing and Nishida, 1971]. SRI emerged from the reverse phase column in a broad band over several fractions. Altering the ratio of phosphate buffer to methanol, changing the pH and use of ion pairing agents such as triethylamine and hexanesulphonic acid did not improve the peak shape of the HPLC trace. A normal phase column with lichrosorb was then used for separation. A variety of mobile phases were tried and the most successful mixture was chloroform - methanol - water (12: 6: 0.5) with which SRI appeared in fractions 6 - 8 (1ml each) at a reasonable retention time (6 - 8min).

1.2. Synthesis of the phosphate metabolites of SRI and Mil

The two compounds which would be of most use for ^{31}P-NMR metabolism studies were dihydrogen hexadecyl phosphate (**25**) and dihydrogen [2'-(octadecyloxymethyl)-tetrahydrofuran-2'-yl]methyl phosphate (**21**). These compounds would be the proposed metabolites of Mil and SRI, respectively, after their incubation with phospholipase D.

1.2.1 Use of phosphorus oxychloride

The synthesis of the monosodium and disodium salts of phosphate (**21**) were attempted by the reaction of phosphorus oxychloride with alcohol (**14**) (Fig. 28, p51), based on the method of Eibl & Blume [1979]. After 2h, TLC indicated 100% disappearance of (**14**) and production of the dichloride. The dichloride was hydrolysed with aqueous sodium acetate using the method of Jahnig et al [1979] to prepare the monosodium salt. TLC and NMR data showed the presence of the required compound but also a large number of impurities. The monosodium salt of (**21**) was then converted to the disodium salt by using cation-exchange chromatography. The impure disodium phosphate (**21**) was run on an anion exchange column and the fractions analysed for the presence of phosphate. Many of the fractions gave a positive response but when they were concentrated under vacuum and analysed using ^{31}P-NMR spectroscopy, phosphorus was not detected. A different method was considered for the synthesis of (**21**) as the free acid.

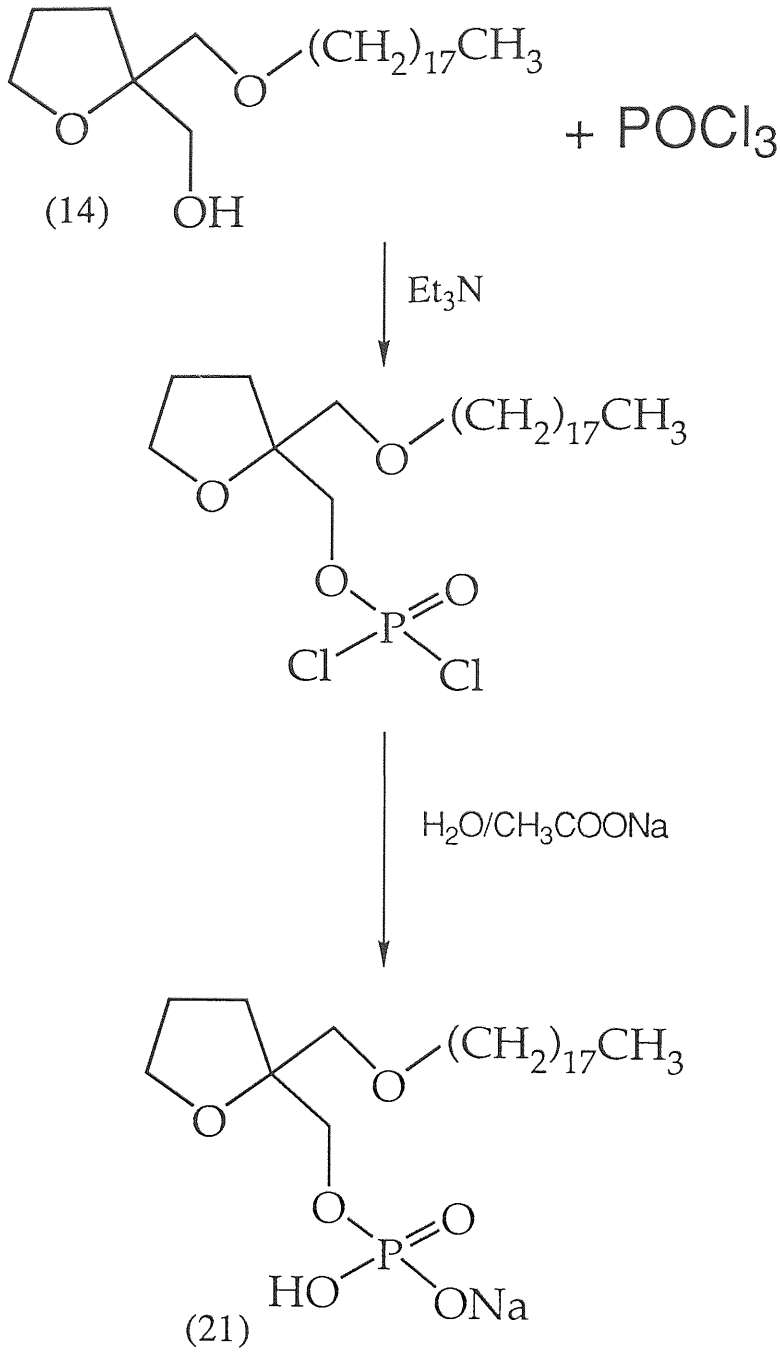


Fig. 28 Synthesis of hydrogen sodium [2'-(octadecyloxymethyl) -tetrahydrofuran-2'-yl]methyl phosphate

1.2.2. Di-*tert*-butyl *N,N*-diethylphosphoramidite as phosphorylating agent

Since traditional methods of phosphorylation with phosphorus oxychloride were unsuccessful, a new method described by Perich and Johns [1988], was employed. This method, involving a reactive PIII phosphorylating agent, was used to prepare phosphates (**25**) and (**21**).

The first step was the synthesis of *N,N*-diethylphosphoramidous dichloride (**22**) from diethylamine and phosphorus trichloride (Fig. 29, p53). This product was formed in a yield of 69% and was then treated with *tert*-butanol to give di-*tert*-butyl *N,N*-diethylphosphoramidite (**23**) in a 40% yield (Fig. 29). Product (**23**) could be stored for several weeks in a fridge under argon. Success of the phosphorylation of *tert*-butanol was shown by the change in the ^{31}P-NMR chemical shift from 162.3 to 133.4ppm.

The phosphorylating agent (**23**) was then reacted with either hexadecanol or alcohol (**14**). The phosphorus was then oxidised to the P(V) form with m-chloroperoxybenzoic acid (MCPBA) (Fig. 30, p54) giving triester (**24**) or (**26**) respectively. The published method used the crude phosphate triester in the deprotection step but it was decided that the crude ester (**24**) or (**26**) should be purified by flash column chromatography. Formation of triester (**24**) or (**26**) was confirmed using ^{31}P-NMR spectroscopy [δ_p -9.65 for (**24**) and -10.35ppm for (**26**)] and mass spectrometry to identify (**26**).

Initial attempts to remove the *tert*-butyl protecting groups by the method of Perich and Johns [1988] using 1M HCl / dioxane were disappointing. For successful deprotection, phosphate triester (**24**) or (**26**) had to be treated with 6M HCl / dioxane and stirred rapidly for 48hrs. The sample was then concentrated under vacuum to remove the volatile butene by-product. The solid was then treated with a mixture of chloroform and water (1: 1 v/v) and phosphates (**25**) and (**21**) dissolved in the chloroform layer. To purify both phosphates the chloroform layers were treated with aqueous NaOH (10M), which gave the sodium salt of the phosphates. The sodium salt of (**25**) extracted into the aqueous layer. The sodium salt of (**21**) remained in the chloroform but many of its impurities were removed by extraction. Both phosphates were acidified and extracted into chloroform and analysed by NMR and mass spectrometry.

The sodium salt of (**25**) was used in tissue culture and metabolism experiments as it was soluble in the phosphate buffered saline (PBS). Phosphate (**21**) was used as the acid form as it could be dissolved in small quantities of DMSO and then diluted with culture medium.

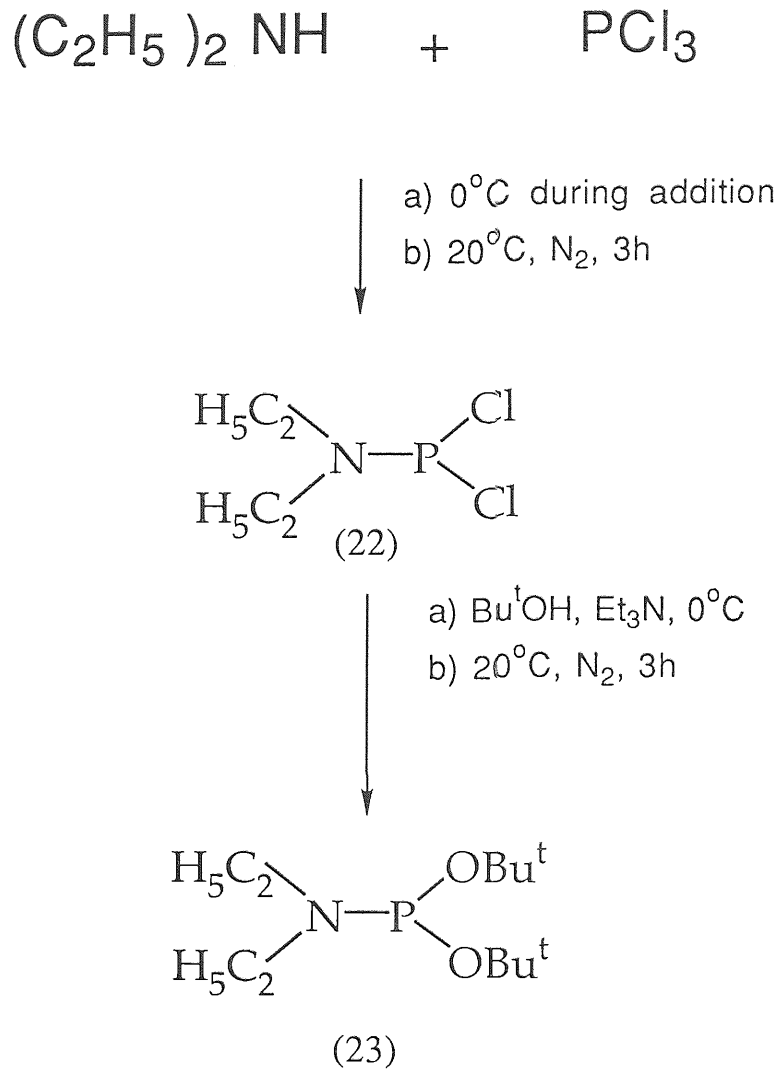
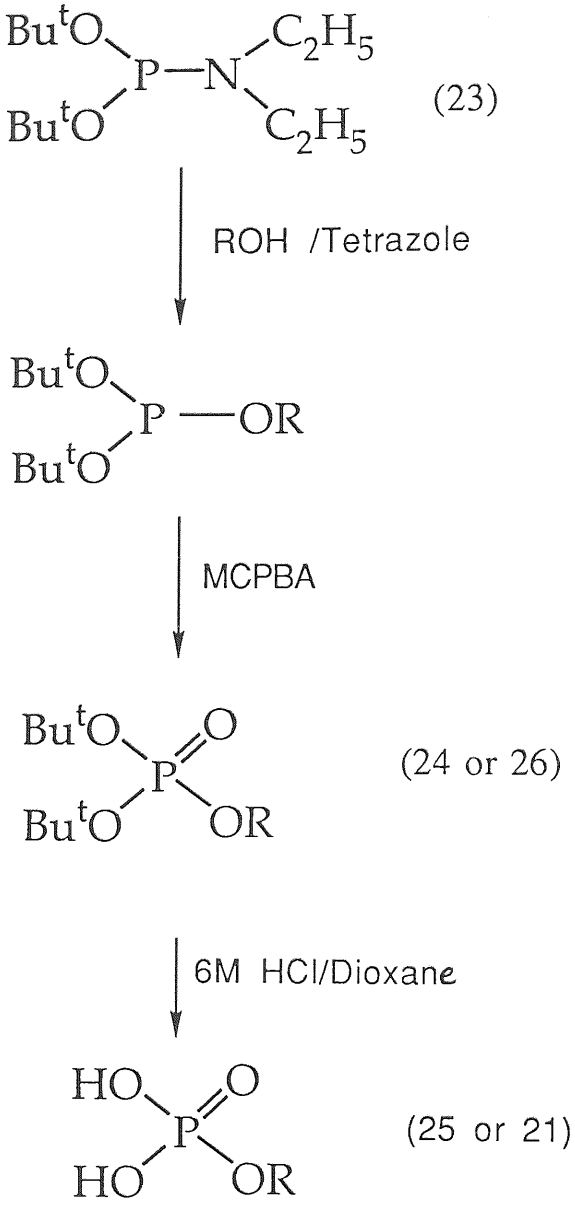


Fig. 29 Synthesis of di-*tert*-butyl N,N-diethylphosphoramidite



ROH = $C_{16}H_{33}OH$ - Product is hexadecyl phosphate (25)

ROH = (14) Product is dihydrogen [2'(octadecyloxy-methyl)tetrahydrofuran-2'-yl]methyl phosphate (21)

Fig. 30 Synthesis of alkyl phosphates

2. EXPERIMENTAL

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM360A 60MHz continuous wave spectrometer, and Bruker FT AC 250 or 300MHz spectrometers. ^{1}H-NMR data are reported δ (ppm) downfield from tetramethylsilane. Assignment of protons in the furan and tetrahydrofuran ring are based on the ring numbering system shown in Fig. 31. ^{31}P-NMR data are reported δ_p (ppm) downfield of 85% phosphoric acid. Infra red data were recorded on a Perkin-Elmer 1310 IR spectrophotometer. Ultra violet data were recorded on a Pye-Unicam SP 8000 UV spectrophotometer. Mass Spectral analysis was performed at the SERC Mass Spectrometry Service Centre, Chemistry Department, Universiy College Swansea, West Glamorgan, South Wales. Melting points were determined using a Gallenkamp melting point apparatus. Chemicals were obtained from Aldrich Chemical Co, Dorset, UK unless otherwise stated. Mil was provided by Dr P. Hilgard, Asta Pharma, Bielefeld, Germany. A small amount of SRI was supplied by Dr W. J. Houlihan, Sandoz Research, New Jersey. Solvents were of reagent grade and dried prior to use when required. Reaction progress and the purity of the final products were determined on E Merck Silica Gel TLC plates. Silica deacetalization of 2-furaldehyde diethyl acetal were carried out using E Merck Silica Gel 40 (70 - 230 Mesh ASTM). Flash column chromatography was carried out using Colpak Sorbsil C60 Silica Gel (40 - 60 μm) [Still et al, 1978].

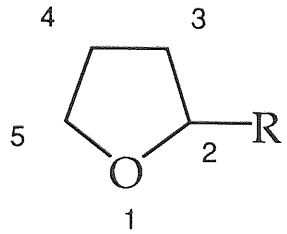


Fig. 31 Carbon numbering system used for interpretation of NMR data.

Methanol and ethanol were dried by refluxing with magnesium to form magnesium methoxide or ethoxide. Any water present then hydrolysed the complex to give magnesium hydroxide and released the methanol / ethanol which was then isolated by distillation [Vogel, 1981]. THF was dried by refluxing over sodium wire and using benzophenone to indicate when it was dry for distillation. Dimethyl sulphoxide (DMSO) was dried by distilling and discarding the first 20% [Burfield and Smithers, 1978]. Toluene was dried by refluxing over sodium for 2h then distilling. Acetonitrile was dried by refluxing over calcium hydride for 1h then distilling. Dry diethylamine was obtained by distillation following reflux over sodium hydroxide.

2.1. TLC Systems for analysis of phosphates

2.1.1. Propan-2-ol - aqueous ammonia - water (7: 1: 2)

inorganic phosphate	R_f 0.0
R O PO_3^{2-}	R_f 0.25
$(RO)_2$ POO^-	R_f 0.7
$(RO)_3$ P O	R_f 0.9 - 1

2.1.2. Butanol - glacial acetic acid - water (5: 3: 2)

R O PO_3^{2-}	R_f 0.5
inorganic phosphate	R_f 0.3

2.2. Spray reagents for TLC

2.2.1. Malachite green spray reagent for detecting phosphates

For detection, organic phospholipids had to be hydrolysed to inorganic phosphate using perchloric acid and then reacted with the spray reagent to give a coloured reaction [Vaskovsky and Latyshev, 1975]. A solution of sodium molybdate (5g) in 2M hydrochloric acid (150ml) was added to a solution of malachite green (1g) in 2M hydrochloric acid and mixed thoroughly. The solution was diluted to 500ml with water and after standing for 2h it was filtered through filter paper. The spray remained stable for several months when stored in an amber glass bottle, in the dark, at room temperature.

2.2.2. Anisaldehyde spray reagent

Ethanol (93ml), glacial acetic acid (1ml), *p*-anisaldehyde (2.5ml) and concentrated sulphuric acid (3.5ml) were mixed together. Test compounds were developed on glass backed TLC plates and allowed to dry. The plates were then sprayed with anisaldehyde reagent and heated at 110°C for 5 - 10min.

2.3. Two component phosphate spray

Spray A: Water (90ml) and concentrated sulphuric acid (10ml) were added to ammonium molybdate (1g). The mixture was stirred until the compound dissolved.

Spray B: Concentrated sulphuric acid (10ml) was added to stannous chloride (10g) and the mixture heated until it dissolved. The solution was then cooled and added to water (90ml).

2.3. Synthesis of SRI

2.3.1. Preparation of 2,2-bis(hydroxymethyl)tetrahydrofuran (5)

2.3.1.1. Methyl tetrahydro-2-furoate (3)

Ester (3) was prepared using the literature procedure of Houlihan et al [1987]. A hot aqueous solution of 2-furoic acid (1) was purified by stirring with charcoal. The charcoal was removed by filtration and on cooling the filtrate crystallised to give acid (1). A suspension of (1) (34.9g, 0.31mol), 95% ethanol (125ml) and 10% palladium on activated charcoal (1.6g) was placed in a steel pressure bottle and affixed to a Parr hydrogenation apparatus. The suspension was maintained at 60psi and room temperature for 8h, with constant agitation. The mixture was filtered through celite, under vacuum, to remove the catalyst. Concentration *in vacuo* gave 36g (0.31mol, 100%) tetrahydro-2-furoic acid (2), R_f (CH_3OH) 0.6, loss of UV absorption compared to (1), which had a maximum absorption at 225nm; ^{1}H-NMR (60MHz, $CDCl_3$) δ 12.1 (1H, s, -COO<u>H</u>), 4.3 - 4.0 (1H, m, -C(2)<u>H</u>), 3.9 - 3.3 (2H, m, -C(5)<u>H_2</u>), 2.3 - 1.4 (4H, m, -C(3)<u>H_2</u>, and -C(4)<u>H_2</u>); ν_{max} (Nujol): 3000 (OH), 1720 (C=O) cm^{-1}, no C=C when compared to (1).

The carboxylic acid (2) (36g, 0.31mol) was added to a saturated solution of hydrogen chloride in dry methanol (200ml). The reaction mixture was stirred for 5h at room temperature then concentrated under vacuum. The residue was dissolved in dichloromethane (150ml) and washed with 10% sodium bicarbonate solution (2 x 150ml) to extract any acid still present. The organic layer was then washed with water (2 x 150ml) and dried over sodium sulphate. The extract was concentrated under vacuum to yield 30g (0.23mol, 74%) of methyl tetrahydro-2-furoate (3), R_f (CH_3OH) 0.8; ^{1}H-NMR δ (60MHz, $CDCl_3$) 4.4 - 4.1 (1H, m, -C(2)<u>H</u>), 4 - 3.6 (2H, m, -C(5)<u>H_2</u>), 3.5 (3H, s, -OC<u>H_3</u>), 2.3 - 1.6 (4H, m, -C(3)<u>H_2</u>, and -C(4)<u>H_2</u>); ν_{max} (thin film) 1750cm^{-1} (C=O).

A method was tried to combine the two stages of this reaction into one process. 2-Furoic acid (1) (35g, 0.31mol), dry methanol (120ml), 10% palladium on carbon catalyst (1.6g) and a catalytic amount of concentrated hydrochloric acid (0.5ml) was placed in a pressure bottle. This steel vessel was fixed to a Parr hydrogenator and agitated for 16h at room temperature and 60psi of hydrogen. The mixture was filtered through celite and concentrated under vacuum. TLC and NMR indicated that there was a mixture of (1), (2), and the methyl esters of both acids.

2.3.1.2. 2,2-Bis(hydroxymethyl)tetrahydrofuran (5)

Using a previously published method [Houlihan et al, 1987] a solution of ester (3) (21g, 0.16mol) in anhydrous tetrahydrofuran (THF) (180ml) was stirred under argon. The mixture was cooled to an internal temperature of -65°C using a dry ice / isopropanol bath.

Diisobutyl aluminium hydride (DIBAH, 25% solution in toluene, 102g, 0.17mol) was added dropwise to the mixture at a rate which did not allow the internal temperature to rise above -60°C. The mixture was stirred for 1.5h at -60°C, then anhydrous methanol (23ml) was added slowly so that the temperature did not rise above -50°C. This solution was allowed to warm slowly to 5°C. It was then transferred into a solution of 37% aqueous formaldehyde (226ml, 3.9mol), NaOH (52g, 1.3mol), and water (170ml) using a wide bore double ended needle to avoid contact with oxygen or moisture. The mixture was stirred for 15min while maintaining the temperature at 10°C by using an ice bath. An aqueous solution of 98% formic acid (6.8ml) was added to the reaction, stirred for 10min and then the mixture was concentrated under vacuum. The residue was treated with dichloromethane (90ml) and stirred for 15min. The organic layer was decanted and the slurry extracted further with dichloromethane (3 x 100ml). The four organic extracts were combined and concentrated. The crude 2,2-bis(hydroxymethyl)tetrahydrofuran (**5**) was distilled to give a very low yield (0.1g, 0.7mmol, 0.5%) of a colourless oil which gradually solidified at room temperature to give white crystals, R_f (CH_3OH) 0.2, bp 102 - 108°C (0.3mmHg) (lit bp 110°C (0.47mmHg) [Eftax and Dunlop, 1961]), mp 51°C (lit mp 51-53°C [Eftax and Dunlop, 1961]); ^{1}H-NMR (60MHz, $CDCl_3$) δ 4.6 - 4.4 (2H, m, -$CH_2O\underline{H})_2$) 4.0 - 3.7 (2H, m, -C(5)$\underline{H}_2$), 3.5 (4H, s, (-$C\underline{H}_2OH)_2$), 2.3 - 1.7 (4H, m, -C(3)$\underline{H}_2$, -C(4)$\underline{H}_2$); ν_{max} (thin film) 3400cm^{-1} (OH).

2.3.1.3. Tetrahydrofuran-2-carboxaldehyde oxime (7)

The procedure was based on the method used for the synthesis of single isomers of oxime (**7**) [Belanger and Williams, 1983]. Hydrogen peroxide (30%, 12ml, 125mmol) was added dropwise over 3h to a solution of tetrahydrofurfurylamine (**6**) (5.05g, 50mmol) and sodium tungstate (500mg) in water (50ml). The solution was maintained at 10°C by use of an ice bath. After stirring for a further 30min, sodium chloride was added to saturate the solution, which was then extracted with ethyl acetate (5 x 100ml). The combined organic extracts were dried over sodium sulphate and concentrated under vacuum. Tetrahydrofuran-2-carboxaldehyde oxime (**7**) was purified by distillation under vacuum. Oxime (**7**) was made up of both anti and syn geometric isomers (Fig. 16, p39). Yield 3g, (26mmol, 52%), bp 100°C (0.08mmHg); ^{1}H-NMR (60MHz, $CDCl_3$) δ 9.2 (1H, s, -N$\underline{O}$H) 7.4 (70%, anti), 6.8 (30% syn) (1H, both d, J_{HH} = 7Hz, -$\underline{C}$H=NOH), 4.6 - 4.2 (1H, m, -C(2)$\underline{H}$), 4.1 - 3.5 (2H, m, -C(5)$\underline{H}_2$), 2.6 - 1.6 (4H, m, -C(3)$\underline{H}_2$, and -C(4)$\underline{H}_2$); ν_{max} (thin film) 2995 (OH), 1660 (C=N) cm^{-1}.

2.3.1.4. Cleavage of the oxime group, using titanium trichloride, to give tetrahydrofuran-2-carboxaldehyde (4)

Oxime cleavage was attempted using the method of Belanger and Williams [1983]. Oxime (7) (2.3g, 20mmol) was added to a stirred solution of citric acid monohydrate (6.1g, 30mmol) and concentrated ammonium hydroxide (2ml) in water (40ml), under argon. The mixture was placed in an ice bath and titanium trichloride (7.4g, 48mmol) was transferred under argon, into this mixture. Care was taken to add the trichloride in small portions as it reacted violently with aqueous solutions. The mixture was stirred for another 2h, then sodium chloride (6g) was added. The solution was then extracted with dichloromethane (5 x 100ml) and the organic phases combined and dried over sodium sulphate. The dichloromethane was removed by evaporation to yield crude tetrahydrofuran-2-carboxaldehyde (4) (0.5g, 5mmol, 25%), R_f (CH_3OH) 0.8; ^{1}H-NMR (60MHz $CDCl_3$) δ 9.6 (1H, s, -C<u>H</u>O), 4.5 - 4.1 (1H, m, -C(2)<u>H</u>), 4.0 - 3.6 (2H, m, -C(5)<u>H_2</u>), 2.5 - 1.6 (4H, m, -C(3)<u>H_2</u>, and -C(4)<u>H_2</u>); ν_{max} (thin layer) 1750cm^{-1} (C=O).

2.3.1.5. Cleavage of the oxime group, using sodium bisulphite, to give tetrahydrofuran-2-carboxaldehyde (4)

The oxime (7) (2.3g, 20mmol) was dissolved in aqueous ethanol (50%, 100ml) and heated under reflux with sodium bisulphite (2.7g, 70mmol) [Pines et al, 1966]. TLC R_f (toluene) 0.3 showed that heating under reflux for 48h did not cleave the oxime group.

2.3.1.6. Synthesis of 2,2-bis(hydroxymethyl)tetrahydrofuran (5) from tetrahydrofuran-2-carboxaldehyde (4)

a) Method 1

This method was based on that used by Eftax and Dunlop [1961] to prepare diol (5).The aldehyde (4) (1g, 10mmol) was added to a stirred solution of aqueous formaldehyde (37%, 1.5ml, 25mmol). NaOH (500mg, 11.5mmol) in water (5ml) was added dropwise over a period of 10min, while maintaining the internal temperature at 50°C with an ice bath. The internal temperature started to decline after 10min and heat was applied (55 - 60°C) for 90min. On cooling, the aqueous phase was extracted with n-butanol (5 x 10ml). The n-butanol was removed by evaporation to yield a very small amount of crude diol (5) which was then distilled (0.1g, 0.76mmol, 7.6%). The colourless oil had the same properties as described earlier [section 2.3.1.2, p57, in the synthesis of the diol (5) from the ester (3)].

b) Method 2

This second method was based on that previously described for production of diol (5) from ester (3) [Houlihan et al, 1987] (p57). A solution of the aldehyde (4) (10g, 0.1mol) in methanol (10ml) was added to a stirred solution of aqueous formaldehyde (37%, 113ml,

2mol), and sodium hydroxide (26g, 0.65mol), in water (85ml). The mixture was stirred in an ice bath for 15min, formic acid (3.4ml) was then added and the solution stirred for another 15min. The suspension was concentrated under vacuum and the residue treated with dichloromethane (60ml) and left to stir for 15min. The dichloromethane was decanted and the slurry extracted further with solvent (3 x 50ml). The organic layers were combined and concentrated under vacuum. The oil obtained was distilled to yield pure diol (**5**) (5g, 0.04mol, 40%) bp 112°C (0.5mmHg), ^{1}H-NMR data was as described in section 2.3.1.2.

2.3.1.7. Preparation of aldehyde (4) by hydrogenation of 2-furaldehyde (8)

Hydrogenation of 2-furaldehyde (**8**) (25g, 0.26mol) was performed in the same way as that of acid (**1**) in the preparation of methyl tetrahydro-2-furoate (**3**) (section 2.3.1.1., p57). A mixture of aldehyde (**4**) and unidentified impurities was obtained. This mixture was used to produce diol (**5**) by reaction with formaldehyde and NaOH (as described in the synthesis of (**5**) section 2.3.1.6b., p59). It was then purified by distillation. The aldehyde (**4**) formed was impure and readily degraded and it therefore had to be used rapidly to prepare diol (**5**).

2.3.1.8. 2-Furaldehyde diethyl acetal (9)

This acetal was prepared using the published method of Adkins et al [1931]. Freshly distilled aldehyde (**8**) (9.6g, 0.1mol) was mixed with dry ethanol (28g, 0.6mol) and a catalytic amount of concentrated hydrochloric acid. The mixture was allowed to stand for 24h after which time aqueous sodium bicarbonate (0.1g in 1ml) was added to neutralise the acid. The mixture was then fractionated through a Widner column at a rate of not more than 2ml/min. The yield of 2-furaldehyde diethyl acetal (**9**) was 3.9g (0.2mol, 19%), bp 110°C (60mmHg) (lit bp 77 - 79°C (16mmHg) [Adkins et al, 1931]); ^{1}H-NMR (60MHz $CDCl_3$) δ 7.35 (1H, s, -C(5)H), 6.35 (2H, s, -C(3)H, and -C(4)H), 5.2 (1H, s, -CH$(OCH_2CH_3)_2$), 3.6 (4H, q, J_{HH} = 8Hz, -$(OCH_2CH_3)_2$), 1.2 (6H, t, J_{HH} = 8Hz, -$(OCH_2CH_3)_2$); ν_{max} (thin film) 1080 (C-O), 715 (C=CH) cm^{-1}.

2.3.1.9. Tetrahydro-2-furaldehyde diethyl acetal (10)

Diethyl acetal (**9**) (15.6g, 0.09mol) was reacted with hydrogen in the same way as acid (**1**) (in section 2.3.1.1., p57) to yield tetrahydro-2-furaldehyde diethyl acetal (**10**). Yield 15.9g (0.09mol, 100%); ^{1}H-NMR (60MHz $CDCl_3$) δ 4.4 (1H, d, J_{HH} = 6Hz, -CH(OCH_2CH_3), 4.2 - 3.4 (7H, m, -C(2)H, -C(5)H_2, and -$(OCH_2CH_3)_2$), 2.2 - 1.7 (4H, m, -C(3)H_2, and -C(4)H_2), 1.22 (3H, t, J_{HH} = 7Hz, -$(OCH_2CH_3)_2$); ν_{max} (thin film) 1080 cm^{-1} (C-O) and no C=C in the 715cm^{-1} region that was observed with (**9**).

2.3.1.10. Preparation of aldehyde (4) by deprotection of diethyl acetal (10)

The diethyl acetal (**10**) showed tailing on silica TLC due to decomposition. This indicated that it might be possible to deprotect it using the literature method for the deacetalization of acetals [Huet et al, 1978]. The method was successful for acetal (**9**) giving aldehyde (**8**), so it was then tried on acetal (**10**).

A 10% solution of oxalic acid in water (11 drops) was added to a continuously stirred suspension of silica gel (3g) in dichloromethane (30ml). The water phase disappeared after 2 - 3 min due to its adsorption on to the silica gel surface. Acetal (**10**) (2g, 6mmol) was added to this reaction mixture and the slurry stirred for 4h, during which time further solvent was added to prevent the silica drying. Sodium bicarbonate (0.1g) was added to the slurry to neutralise the oxalic acid and the mixture was stirred for a further 5min. The mixture was then filtered through a sintered glass funnel, under vacuum, and the silica washed with dichloromethane (3 x 50ml). The filtrate was concentrated under vacuum but ^{1}H-NMR spectral analysis showed that hydrolysis of the acetal group had not occurred. On stirring the slurry for 48h only starting material (**10**) was isolated.

The use of 15% sulphuric acid (11 drops) instead of oxalic acid produced some deacetalization with a maximum yield of 1.2g (60%). Stirring the slurry for up to 5 days did not improve the yield and the aldehyde (**4**) began to polymerise.

Amberlite IR-120, a cationic exchange resin has been used to catalyse the hydrolysis of the dibutyl acetal of aldehyde (**4**) (Defaye, 1968). The amberlite was prepared by first washing it in a column with 6 volumes of 6M hydrochloric acid. The resin was then thoroughly washed with distilled water to remove excess acid (the pH of the washings were tested until they were neutral). To complete the preparation of the amberlite it was washed with 6 column volumes of acetone - water (16: 1 v/v). Acetal (**10**) (2g) was mixed with acetone (80ml) and water (5ml) then added to the amberlite. The mixture was stirred for 20h at 50°C. The amberlite was removed by filtration, and the filtrate was concentrated under vacuum. The aqueous residue was extracted with dichloromethane (4 x 20ml) and the organic layer was dried over sodium sulphate and evaporated to give aldehyde (**4**) (NMR data as before, p59) in 40% yield. The sample did not contain any starting material (**10**) but the amberlite leached a large number of impurities into the sample. Many of these impurities were removed when crude (**4**) was used to prepare diol (**5**). This product was then purified by distillation. Other cationic resins (such as IR-50 and Dowex-50) were used instead of IR-120 but they did not produce any cleavage of the acetal group.

In an attempt to clean up the amberlite [Cleland unpublished], potassium hydroxide pellets were added to a suspension of amberlite in water, so that the supernatant was still at least 1M. Liquid bromine (2ml for 100ml suspension) was pipetted in, then stirred to give a yellow supernatant. The amberlite was filtered on a Buchner funnel and washed thoroughly with water and then with 6M hydrochloric acid. It was then packed into a column and prepared as before. This cleaning process greatly reduced the impurities that leached from the amberlite but did not completely abolish them.

2.3.1.11. Furfural diacetate (11)

Acetic anhydride (10.2g, 0.1mol) and concentrated sulphuric acid (0.01ml) were mixed in a Claisen flask and the mixture cooled to 10°C in an ice bath [Bertz, 1963]. Over a period of 10min freshly distilled 2-furaldehyde (**8**) (9.6g, 0.1mol) was added with constant stirring. The solution was allowed to warm up spontaneously (a temperature of 35°C was reached in 5min). Once the internal temperature had dropped back to room temperature (approximately 20 - 30min) anhydrous sodium acetate (0.04g) was added. The mixture was then distilled under low vacuum (≈20mmHg) to remove aldehyde (**8**) and unreacted acetic anhydride. The vacuum was increased to distill the furfural diacetate (**11**) which was a white crystalline solid. In the synthesis of diacetate (**11**) it was important to ensure that the sulphuric acid catalyst was completely neutralised by the sodium acetate. This prevented the breakdown of the product by acid during distillation. Diacetate (**11**) solidified in the condenser, but could be melted again by passing a current of warm air over the condenser. Fractional distillation was required because if the collection of (**11**) was started too early the crystals began to darken due to contamination with aldehyde (**8**), as the latter is light sensitive. Bp 90 - 104°C at 3mmHg. (lit bp 140 - 142°C (20mmHg)[Bertz, 1963]), mp 45°C; ^{1}H-NMR (60MHz $CDCl_3$) δ 7.7 (1H, s, -C<u>H</u>$(OCOCH_3)_2$), 7.4 (1H, s, -C(5)<u>H</u>), 6.7 - 6.2 (2H, m, -C(3)<u>H</u>, and -C(4)<u>H</u>), 2.2 (6H, s, -$(OCOC\underline{H}_3)_2$); ν_{max} (Nujol) 1750 (C=O), 750 (C=<u>CH</u>) cm^{-1}.

2.3.1.12. Tetrahydrofurfural diacetate (12)

A pressure bottle containing furfural diacetate (**11**) (18g, 0.09mol) dissolved in absolute ethanol (60ml) and 10% palladium on carbon catalyst (0.3g) was attached to a Parr hydrogenator. The mixture was maintained at 100psi of hydrogen for 6h, with agitation. Filtration through celite and concentration under vacuum gave tetrahydrofurfural diacetate (**12**) as a yellow oil (16.4g, 0.08mol, 90%), R_f (ethyl acetate) 0.5; ^{1}H-NMR (60MHz $CHCl_3$) δ 6.65 (1H, d, J_{HH} = 6Hz, -C<u>H</u>$(OCOCH_3)_2$), 4.2 - 3.3 (3H, m, -C(2)<u>H</u>, and -C(5)$\underline{H}_2$), 2.2 (6H, s, (-$OCOC\underline{H}_3)_2$), 2.0 - 1.8 (4H, m, -C(3)$\underline{H}_2$, and -C(4)$\underline{H}_2$); ν_{max} (thin film) 1750cm^{-1}(C=O) and no C=<u>CH</u> as seen in (**11**).

2.3.1.13. Deprotection of diacetate (12) to give aldehyde (4)

Initially the method recommended by Amouroux et al [1981] was used for deprotection of the acetate groups. A solution of diacetate (**12**) (30g, 0.15mol) and NaOH (12g, 0.21mol) in water (60ml) was cooled to 0°C and stirred for 1h. The unreacted starting material was extracted with diethyl ether (100ml), then the mixture was extracted with dichloromethane (3 x 100ml). The dichloromethane layers were combined, washed with a saturated solution of aqueous sodium chloride (100ml) and concentrated. ^{1}H-NMR spectroscopy showed that the starting material (**12**) was present in the ether layer and that the aldehyde (**4**) was not present in the dichloromethane extract. Cleavage of the acetate groups had not occurred.

Aqueous NaOH could be used to deprotect the unsaturated diacetate (**11**) releasing aldehyde (**8**), under similar conditions used by Amouroux et al [1981]. Therefore, further attempts were made to cleave the acetate from (**12**). Diacetate (**12**) (1g, 5mmol) was stirred rapidly and a solution of NaOH (0.4g, 7mmol) in water (2ml) was slowly added dropwise to ensure complete mixing and good contact between the two phases. Deprotection was monitored using TLC and occurred within 1h. The aldehyde (**4**) was extracted using dichloromethane (4 x 5ml). Concentration of the organic layers under vacuum gave a yellow oil which was confirmed to be aldehyde (**4**) by ^{1}H-NMR spectroscopy. Yield of aldehyde (**4**) was 0.5g (5mmol, 100%), R_f (ethyl acetate) 0.2; ^{1}H-NMR as described earlier (section 2.3.1.4., p59). This method could be used very successfully to deprotect up to 10g of diacetate. The yield of (**4**) decreased above 3g as it was more difficult to ensure complete mixing of the two phases. Aldehyde (**4**) was then immediately used in method 2 (described in section 2.3.1.6b., p59) to prepare diol (**5**).

2.3.2. 2-Hydroxymethyl-2-octadecyloxymethyltetrahydrofuran (14)

Powdered potassium hydroxide (2.1g, 38mmol) was added to a stirred solution of diol (**5**) (5g, 38mmol) in DMSO (25ml) and THF (25ml) at room temperature. A solution of n-octadecylbromide (4.1g 12.5mmol) in THF (15ml) was added dropwise to this mixture and stirred for 16h before concentrating *in vacuo*. The residue was treated with water (50ml) and then extracted with diethyl ether (3 x 50ml). The ether layers were combined, washed with a saturated aqueous sodium chloride solution (100ml), dried over sodium sulphate and concentrated. The residue was purified by flash column chromatography (ethyl acetate - hexane, 1: 2) giving a cream semi-solid of 2-hydroxymethyl-2-octadecyl-oxymethytetrahydrofuran (**14**) (3g, 7.8mmol, 62%), R_f (ethyl acetate - hexane, 1: 2) 0.4; ^{1}H-NMR (300MHz $CDCl_3$) δ 3.84 (2H, t, J_{HH} = 6.5Hz, -OC$\underline{H}_2$(CH$_2$)$_{16}$CH$_3$), 3.58 (1H, dd (second order), J_{gem} = 11.2, $J_{HHtrans}$ = 6.5Hz, -C(5)$\underline{H}$a), 3.50 (1H, dd (second order), J_{gem} = 11.2, $J_{HHtrans}$ = 6.0Hz, -C(5)$\underline{H}$b), 3.47 - 3.38 (2H, m, -CH$_2$OH), 3.435 (1H, d, J_{gem} = 9.5Hz, -$\underline{H}$CHOC$_{18}$H$_{37}$), 3.335 (1H, d, J_{gem} = 9.5Hz, -HC$\underline{H}$OC$_{18}$H$_{37}$),

1.98 - 1.82 (2H, m, -C(4)$\underline{H_2}$), 1.82 - 1.7 (2H, m, -$OCH_2C\underline{H_2}(CH_2)_{15}CH_3$), 1.54 (2H, tt, $J_{HH} = J_{HH} = 6.5$Hz, -C(3)$\underline{H_2}$), 1.38 - 0.96 (30H, brs, -$OCH_2CH_2(C\underline{H_2})_{15}CH_3$), 0.86 (3H, t, $J_{HH} = 6.5$Hz, -$OCH_2CH_2(CH_2)_{15}C\underline{H_3}$); ν_{max} (Nujol) 3130 (OH), 1050 (C-O) cm^{-1}.

2.3.3. Addition of phosphatidylcholine group to alcohol (14) to give SRI

2.3.3.1. 2-Chloro-2-oxo-1,2,3-dioxophospholane (16)

Oxygen, dried by passing through silica, was bubbled into a solution of 2-chloro-1,2,3-dioxaphosphalane (**15**) (25g, 0.2mol) in toluene (50ml) over approximately 8h, until the exothermic reaction was complete (Edmundson, 1962). The toluene was evaporated and 2-chloro-2-oxo-1,2,3-dioxophospholane (**16**) was distilled to yield a colourless oil, (20g, 0.14mol, 70%), bp 79°C (0.4mmHg) (lit bp 90°C (0.8mmHg) [Edmundson, 1962]); ^{1}H-NMR (60MHz $CDCl_3$) δ 4.9 - 4.3 (4H, m, -$C\underline{H_2}C\underline{H_2}$-); ^{31}P-NMR (121.5MHz $CDCl_3$) δ_p 22.4 (s, ^{1}H decoupled) (m, ^{1}H coupled). [(**15**) ^{31}P-NMR (121.5MHz $CDCl_3$) δ_p 167.23 (s, ^{1}H decoupled).]

2.3.3.2. 2-{Hydroxy[tetrahydro-2-(octadecyloxy)methylfuran-2-yl-methoxyl]-phosphinyloxy}-*N,N,N*-trimethylethaniminium hydroxide inner salt (SRI 62-834; SRI)

A more systematic name for this compound is:
[2'-(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N,N,N*-trimethylammonio]-ethyl phosphate.

The final step in the synthesis of SRI followed the method of Houlihan et al [1987]. 4-Dimethylaminopyridine (86mg, 0.7mmol) and phospholane (**16**) (1.4g, 9.8mmol) were added to a solution of alcohol (**14**) (2.9g, 7.6mmol) in anhydrous toluene (50ml). The reaction mixture was maintained at room temperature, under a nitrogen atmosphere, and stirred for 16h. The mixture was concentrated *in vacuo* and the residue treated with anhydrous acetonitrile (40ml). The mixture was transferred to a pressure bottle, equipped with a magnetic stirrer, and cooled with the aid of a dry ice / isopropanol bath. Trimethylamine gas was generated by adding NaOH (1M) dropwise to trimethylamine hydrochloride crystals (a gas cylinder was later obtained). The gas was dried by passing it through NaOH pellets and then bubbled into the acetonitrile. After 30min, once the solution was saturated with trimethylamine, the bottle was rapidly capped, warmed to 60 - 65°C and left to stir for 48h. Acetonitrile was evaporated under vacuum and the dark yellow solid remaining purified by flash column chromatography with $CHCl_3$ - CH_3OH - H_2O (10: 5: 1). SRI was isolated in a low yield (0.03g, 0.08mmol, <1%), R_f ($CHCl_3$-CH_3OH-H_2O, 10: 5: 1) 0.3; ^{1}H-NMR (300MHz $CDCl_3$) δ 4.17 (2H, brs, -$POC\underline{H_2}CH_2N(CH_3)_3$),

3.86 (4H, bs, -C(5)$\underline{H}_2$, and -OC$\underline{H}_2$(CH_2)$_{16}$$CH_3$), 3.71 (2H, brs, -C$\underline{H}_2$N), 3.55 - 3.50 (4H, m, -C(2)(C$\underline{H}_2$O-)$_2$), 3.39 (9H, s -N(C$\underline{H}_3$)$_3$), 1.91 (4H, m, -C(3)$\underline{H}_2$, and -C(4)$\underline{H}_2$), 1.55 (2H, brs, -OCH$_2$C$\underline{H}_2$(CH_2)$_{15}$$CH_3$), 1.26 (30H, brs, -OCH$_2CH_2$(C$\underline{H}_2$)$_{15}$$CH_3$), 0.88 (3H, brt, -OCH$_2CH_2$($CH_2$)$_{15}C\underline{H}_3$); ^{31}P-NMR (121.5MHz $CDCl_3$) δ_p 2.07 (s, ^{1}H decoupled)(m, ^{1}H coupled).

2.3.3.3. HPLC analysis of SRI

Initially, for HPLC analysis of SRI a Waters Radial PAK Cyanol 20 x 0.8cm column was used. The mobile phase was methanol - phosphate buffer (65: 35), passing at a flow rate of 1.5ml/min. The buffer was 0.02M sodium dihydrogen phosphate in water, pH adjusted to 7.4 with NaOH (1M). SRI in water (10mM, 25µl) was injected onto the column [Baer J, Aston Molecules Standard Operating Procedure, 1988]. SRI eluted as a broad peak over ten fractions. Altering pH, methanol concentration or adding ion pairing agents eg triethylamine and hexanesulphonic acid did not improve the peak shape.

SRI was detected in fractions 6 - 8 when a normal phase lichrosorb column was used, with a mobile phase of chloroform - methanol - water (12: 6: 0.5).

Detection of SRI in HPLC fractions.

Fractions (1.5ml) were collected for post column derivatisation and they were initially concentrated under vacuum. However, it was found that the methanol did not interfere with the results so its evaporation was unnecessary. To each fraction, a solution of freshly prepared 10mM ANS (1ml) was added and the mixture shaken. Aliquots (1ml) were placed in a fluorimeter in a cuvette of path length 1cm, and fluorescence read with excitation wavelength 430nm, emission wavelength 495nm. The results were compared with a standard curve produced using 0 - 400µl of 10mM SRI (in water) and made up to 1.5ml with methanol - phosphate buffer (65: 35) and adding ANS (1ml) (Fig. 32, p66).

2.3.3.4. Detection of SRI on TLC plates using spray reagents

a) Detection of SRI using the malachite spray reagent

This detection method was based on that used by [Vaskovsky and Latyshev, 1975]. SRI was developed on glass backed TLC plates using the mobile phase described earlier (section 2.3.3.2., p64). The plates were dried, sprayed thoroughly with perchloric acid (57% - 72%) and then placed on a hot plate at 250 - 300°C. Once the perchloric acid had reacted and the excess evaporated, the plates were allowed to cool then sprayed with malachite green spray (section 2.2.1., p56). According to the published method the phosphorus containing compounds should appear as green spots on an orange background.

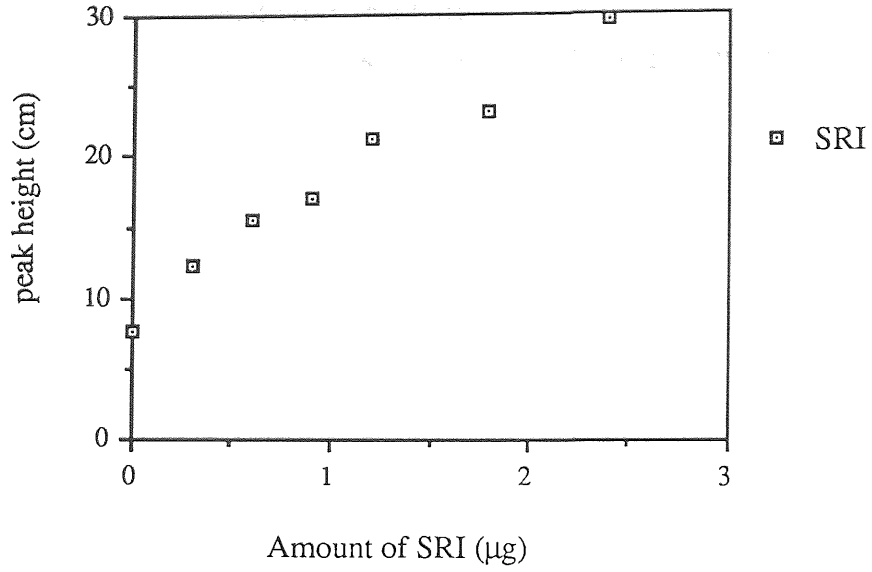


Fig. 32 Standard curve for HPLC determination of SRI

For SRI the reverse pattern was seen, orange spots on a dark green background. The spots were not clearly distinguishable and altering the acid concentration did not improve the results.

b) Anisaldehyde spray reagent and detection of SRI

SRI was developed on glass backed plates as described earlier and allowed to dry. The plates were sprayed with anisaldehyde reagent (section 2.2.2., p56) and heated at 110°C for 5 - 10min. SRI gave purple spots on a white background. These spots were more clearly distinguished than those produced by the malachite spray, but this spray is not selective for phosphorus.

c) Two component phosphate spray

Details for the preparation of these sprays is given in section 2.2.3. (p56). The compound under test eg SRI or phosphate (**21**) was developed on glass or aluminium backed plates using a suitable mobile phase. The plates were then sprayed with A and heated on a hot plate at 200 - 300°C, until dry. The plates were allowed to cool then sprayed with B. If spots did not appear the plates were gently warmed. Phosphorus containing compounds showed up as yellow spots on a blue background and were easily seen. (Trimethylamine, often used in reactions described here, was observed as a dark blue spot.)

2.3.3.5. [2'-(Hydroxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N*,*N*,*N*-trimethylammonio]ethyl phosphate (18)

The synthesis of phosphate (**18**) was attempted using the method described by Houlihan et al [1987] for the addition of the phosphatidylcholine group to (**14**). A solution of diol (**5**) (0.51g, 3.8mmol) in dry toluene (17ml) and methanol [2ml, to increase the solubility of (**5**)] was stirred under argon. Triethylamine (0.6ml, 4.4mmol), 4-dimethylaminopyridine (43mg) and phospholane (**16**) (0.5g, 3.8mmol) were added and the mixture stirred for 16h at room temperature, then concentrated under vacuum. The residue was resuspended in dry acetonitrile (20ml) and transferred to a pressure bottle. The mixture was cooled in dry ice / isopropanol and trimethylamine gas was bubbled through to saturation. The bottle was rapidly capped and allowed to warm to room temperature prior to heating to 60 - 65°C for 48h in an oil bath. The solution was then cooled to room temperature and concentrated under vacuum. R_f (Propanol mixture) 0.1, R_f (Butanol mixture) 0.3. NMR and TLC data indicated an impure product which was too difficult to separate.

2.3.3.6. [2'-(Octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl 2-[*N*-methyl-ammonio]ethyl phosphate (20)

Synthesis was based on method B used by Euerby et al [1987] (Fig. 20). A solution of alcohol (**14**) (4.4g, 11.35mmol) and triethylamine (1.3g, 12.93mmol) in THF (25ml) was added dropwise, over a period of 30min, to a stirred solution of phosphorus oxychloride (1.8g, 11.8mmol) in THF (25ml) at 0°C. The reaction was allowed to warm to room temperature and stirred overnight. TLC in ethyl acetate was used to confirm the disappearance of (**14**). To this mixture, a solution of 2-(*N*-methylamino)ethanol (0.9g, 12.3mmol) and triethylamine (2.9g, 28.4mmol) in THF were added dropwise over a period of 30min, at 0°C. The reaction was allowed to warm to room temperature and stirred for a further 5h. Filtration removed the precipitated triethylammonium hydrochloride. The intermediate 1,3,2-oxazaphospholidin-2-one (**19**) formed was not isolated. The mixture was treated with acetic acid (20% in THF) and stirred overnight, to hydrolyse the P-N bond. The reaction mixture was concentrated under vacuum and the components separated by flash column chromatography with $CHCl_3$ - CH_3OH - H_2O (10: 5: 1). Anisaldehyde spray was used to identify the relevant spots on TLC and the fractions combined. R_f 0.4; ^{1}H-NMR (250MHz $CDCl_3$) δ 4.3 - 4.2 (2H, m, -POC$\underline{H}_2$CH$_2$N(CH$_3$)H$_2$), 3.79 - 3.60 (8H, m, -C$\underline{H}_2$OPO-, -CH$_2$OC$\underline{H}_2$CH$_2$(CH$_2$)$_{15}$CH$_3$ -C$\underline{H}_2$OCH$_2$CH$_2$(CH$_2$)$_{15}$CH$_3$, and -C(5)$\underline{H}_2$), 3.3 (2H, brs, -C$\underline{H}_2$N(CH$_3$)H$_2$), 3.18 (3H, brs, -N(C$\underline{H}_3$)H$_2$), 1.8 (4H, m, -C(3)$\underline{H}_2$, and -C(4)$\underline{H}_2$), 1.5 (2H, m, OCH$_2$C$\underline{H}_2$(CH$_2$)$_{15}$CH$_3$), 1.19 (30H, brs, -OCH$_2$CH$_2$(C$\underline{H}_2$)$_{15}$CH$_3$), 0.84 - 0.79 (3H, brt, -OCH$_2$CH$_2$(CH$_2$)$_{15}$C$\underline{H}_3$); ^{31}P-NMR (121.5MHz $CDCl_3$) δ_p -1.35 (s, ^{1}H decoupled)(m, ^{1}H coupled).

2.4. Synthesis of phosphate metabolites of SRI and Mil

2.4.1. Synthesis using phosphorus oxychloride

2.4.1.1. Hydrogen sodium [2'-(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl phosphate (21)

The synthesis was based on the method of Eibl and Blume [1979]. Freshly distilled phosphorus oxychloride (1.8g 11.8mmol) was dissolved in THF (20ml) and stirred in an ice bath. A solution of triethylamine (2g, 20mmol) in THF (20ml) was added to this solution. Alcohol (**14**) (3.84g, 10mmol) was dissolved in THF (60ml) and added dropwise to the stirred mixture which was then allowed to warm to room temperature and left for a further 60min. Phosphorylation was followed by TLC (ethyl acetate - hexane, 2: 1). Alcohol (**14**), R_f 0.4 completely reacted giving a product with R_f 0. Filtration under vacuum removed the triethylammonium hydrochloride precipitate which was washed with THF and the filtrates combined.

Hydrolysis of the dichloride to the phosphate was achieved using the method of Jahnig et al [1979]. Sodium acetate, 2M in water (50ml) was added to the filtrate and stirred at room temperature for 2h. TLC ($CHCl_3$) was used to follow the hydrolysis. THF was removed by evaporation and the remaining aqueous solution was treated with $CHCl_3$-CH_3OH (20: 12) (the methanol was used to facilitate phase separation). The aqueous layer was further extracted with chloroform (3 x 30ml), the organic phases were combined and concentrated. Crude product (21) was purified by flash column chromatography eluting with $CHCl_3$ - CH_3OH - H_2O (10: 5: 1) R_f 0.5, R_f [Propanol mixture] 0.3, R_f [Butanol mixture] 0.6.

The mono sodium salt of phosphate (**21**) was then converted to the disodium salt by passing it through a Dowex 50 cation exchange column and eluting with water. R_f ($CHCl_3$) 0.4. This was then purified by flash column chromatography by eluting with $CHCl_3$ - CH_3OH - H_2O (12: 6: 0.5) R_f 0.4, R_f [Propanol mixture] 0.4; ^{1}H-NMR (250MHz $CDCl_3$) δ 3.38 - 3.33 (8H, m, -C(5)$\underline{H}_2$ -C$\underline{H}_2$OCH$_2$CH$_2$(CH$_2$)$_{15}$CH$_3$, and -OC$\underline{H}_2$CH$_2$(CH$_2$)$_{15}$CH$_3$, -C$\underline{H}_2$OP-), 1.70 - 1.69 (4H, m, -C(3)$\underline{H}_2$, -C(4)$\underline{H}_2$), 1.38 (2H, brs, -OCH$_2$C$\underline{H}_2$(CH$_2$)$_{15}$CH$_3$), 1.15 - 1.08 (30H, brs, -OCH$_2$CH$_2$(C$\underline{H}_2$)$_{15}$CH$_3$), 0.70 - 0.65 (3H, brt, -OCH$_2$CH$_2$(CH$_2$)$_{15}$C$\underline{H}_3$); ^{31}P-NMR (121.5MHz $CDCl_3$) δ_p 1.35 (s, ^{1}H decoupled)

Ion exchange chromatography was then attempted to further purify the disodium salt of (**21**). The buffer used was triethylammonium bicarbonate (TEAB).

2.4.1.1.1 Ion exchange chromatography

a) Preparation of TEAB buffer (1M)

Triethylamine (348.5ml, 2.5mol) was made up to 2.25litres with distilled water. Carbon dioxide (gas) from solid dry ice was bubbled through the buffer, maintained on ice. The gas was passed through until the pH had fallen to 7 - 7.5 (overnight). The volume was made up to 2.5litres, and the pH rechecked. TEAB buffer (1M) was diluted with water to concentrations of 500 and 50mM, checking pH and adjusting to pH 7 - 7.5.

b) Preparation of the resin and the column

The amount of resin required for the experiment had to be calculated. This was achieved using the following formula, moles of charge on 0.75g (**21**) were 0.75/509 x 2, (where 509 is the molecular weight) = 3mmol. The resin capacity was 1.33mmol of charge/ml of resin hence a minimum of 2.3ml of resin should be used. An excess was desirable and the amount of resin used was 23ml. The resin (AG1-X8) was defined by taking 50ml (wet volume) and mixing it with TEAB buffer (1M, 100ml). The slurry was swirled and left to stand for 30min. The buffer was decanted and the process repeated to remove any fine particles which could cause the solvent to pass only slowly through the column. The resin was then suspended in TEAB buffer (1M, 100ml) and poured into a column (20 - 30ml) which was then eluted first with 10 column volumes of TEAB buffer (1M) and then with the same volume of the diluted TEAB buffer (50mM).

The sample of disodium (**21**) (0.75g) was dissolved in water (200ml) so that its conductivity was less than that of the TEAB buffer (50mM). It was then eluted on to the column which was washed with water and a little TEAB buffer (50mM). The washings were kept and later analysed for the presence of disodium (**21**) in case it had passed through the column.

c) Column elution.

A linear concentration gradient was required and 1litre of buffer was passed through [50mM (500ml), 500mM (500ml)]. Initially 50mM buffer was eluted through the column which was gradually mixed with 500mM TEAB buffer until 100% 500mM eluted through. A fraction collector was used to collect the fractions (200 drops).

d) Analysis of the fractions for phosphate

A spot of each fraction was placed on a glass backed TLC plate and sprayed with perchloric acid and the malachite green spray. A large number of positives were observed. It was then decided to use a quantitative phosphate assay to examine every third fraction. Aliquots (100 - 1000µl) of standard 0.1µmol/ml KH_2PO_4 were placed in tubes. Water (200 and

400µl) was added to two separate tubes to act as blanks. From every third fraction, 25µl was taken and placed in a separate tube. All the samples were placed in an oven at 97°C and evaporated to dryness (4h). Perchloric acid (150µl) was then added to each tube which was then covered in foil and heated at 140°C for 12h. The samples were allowed to cool to room temperature and the assay mixture (1ml) was added. [The assay mixture contained ammonium molybdate (72.5mg), ascorbic acid (290mg) and water (to 25ml)]. Following addition, each tube was immediately mixed by vortex and then all samples were heated at 100°C for 5min. The tubes and rack were cooled in ice and their optical densities measured at 660nm using a Cecil CE 594 double beam UV spectrophotometer. Standards were used in preparing a calibration curve for comparison with unknown fractions.

Groups of fractions were positive with the assay and they were combined and concentrated. Isopropanol was then added to form an azeotropic mixture with TEAB buffer and evaporated. This was repeated three times, 1M NaOH (2 x 100µl) was added to reduce the acidity of the samples, due to the bicarbonate. Although the fractions gave positive responses for phosphate, no phosphorus signals were observed by ^{31}P-NMR spectroscopy. Disodium (**21**) was not found in the column eluent or in combined samples

2.4.2. Di-*tert*-butyl *N,N*-diethylphosphoramidite as phosphorylating agent

2.4.2.1. *N,N*-Diethylphosphoramidous dichloride (22)

The method used was based on that of Perich and Johns [1988]. Dry diethylamine (20.7ml, 14.6g, 0.2mol) was added dropwise to a vigorously stirred solution of freshly distilled phosphorus trichloride (13.7g, 0.1mol) in anhydrous ether (60ml), under nitrogen. During the addition (1.5h) the temperature was maintained below 0°C by use of a dry ice / isopropanol bath. The mixture was allowed to warm to 20°C and stirred for 3h. The diethylammonium hydrochloride was filtered by suction under nitrogen and the precipitate washed with dry ether (4 x 20ml). Filtrates were combined and concentrated under vacuum. The crude liquid was distilled giving dichloride (**22**) (12g, 0.07mol, 70%), bp 61 - 62°C (9mmHg) (lit bp 72 - 75°C (14mmHg) [Perich and Johns, 1988]); ^{1}H-NMR (250MHz $CDCl_3$) δ 3.42 - 3.28 (4H, m, $-N(C\underline{H}_2CH_3)_2$), 1.26 - 1.15 (6H, t, J_{HH} = 7.5Hz, $-N(CH_2C\underline{H}_3)_2$); ^{31}P-NMR (101.3Hz, $CDCl_3$) δ_p 162.34 (s, ^{1}H decoupled).

2.4.2.2. Di-*tert*-butyl *N,N*-diethylphosphoramidite (23)

Synthesis of (**23**) was based on the method of Perich and Johns [1988]. A solution of *tert*-butanol (2.96g, 40mmol) and triethylamine (4.44g, 44mmol) in dry ether (14ml) was slowly added to dichloride (22) (3.46g, 20mmol) in dry ether (6ml) under nitrogen. Addition rate was such that the reaction temperature was kept below 0°C by use of a dry ice / isopropanol bath. The mixture was allowed to warm to 20°C and stirred for a further 3h.

A solution of 5% aqueous sodium bicarbonate (70ml) was added and the emulsion transferred to a separating funnel. The aqueous layer was discarded and the ether layer washed with more 5% sodium bicarbonate solution (2 x 6ml) and saturated aqueous sodium chloride (6ml) then dried over sodium sulphate and filtered. The filtrate was concentrated under vacuum and the crude residue distilled to give ester (**23**) (2g, 8mmol, 40%); bp 74 - 76°C (6mmHg); ^{1}H-NMR (250MHz $CDCl_3$) δ 3.1 - 2.9 (4H, m, $-N(C\underline{H}_2CH_3)_2$) 1.33 (18H, s, $-C(C\underline{H}_3)_3$), 1.08 - 1.06 (6H, t, J_{HH} = 5Hz, $-N(CH_2C\underline{H}_3)_2$); ^{31}P-NMR (101.3MHz $CDCl_3$) δ_p 133.39 (s, ^{1}H decoupled).

4.2.3. Hexadecyl di-*tert*-butyl phosphate (24)

1H-Tetrazole (0.76g, 9mmol) was added as one portion to a stirred solution of hexadecanol (0.8g, 3.3mmol) and ester (**23**) (0.75g, 3mmol) in dry THF (3ml), at room temperature. After 5min the mixture was cooled to -40°C using a dry ice / isopropanol bath and a solution of 50% MCPBA (1.38g, 4mmol) in dichloromethane (4ml) was added. Rate of addition did not allow the internal temperature to rise above 0°C. The mixture was then allowed to warm to room temperature and stirred for 5min. An aqueous solution of 10% sodium bisulphite (10ml) was added and the solution stirred for 10min. The mixture was transferred to a separating funnel and the flask rinsed with ether (10ml). The aqueous layer was discarded and the ethereal phase washed with 10% aqueous sodium bisulphite (2 x 20ml), 5% aqueous sodium bicarbonate (2 x 20ml), dried over sodium sulphate, and filtered. The filtrate was concentrated and purified by flash column chromatography to give the ester (**24**) (1.0g, 2.2mmol, 73%), R_f (ethyl acetate - hexane, 1: 1) 0.5; ^{1}H-NMR (250MHz $CDCl_3$) δ 3.95 - 3.90 (2H, s, $-OC\underline{H}_2CH_2(CH_2)_{13}CH_3$), 1.8 - 1.7 (2H, m, $-OCH_2C\underline{H}_2(CH_2)_{13}CH_3$), 1.49 (18H, s, $-C(C\underline{H}_3)_3$), 1.27 (26H, s, $-OCH_2CH_2(C\underline{H}_2)_{13}CH_3$), 0.89 (3H, brt, $-OCH_2CH_2(CH_2)_{13}C\underline{H}_3$); ^{31}P-NMR (101.3MHz $CDCl_3$) δ_p -9.65 (s, ^{1}H decoupled) (t, J_{PH} = 6.6Hz, ^{1}H coupled).

2.4.2.4. Hexadecyl phosphate (25)

The di-*tert*-butyl ester (**24**) (1g, 2.2mmol) was stirred in a solution of 6M HCl (2ml) and dioxane (2ml) at room temperature for 48h. The solvent was evaporated, the solid resuspended in water (2ml) and hexadecyl phosphate (**25**) extracted into ether. Purification was achieved by extracting (**25**) as the sodium salt into NaOH (10M, 4ml). The aqueous layer was concentrated, resuspended in water (1ml), acidified to pH1 with concentrated hydrochloric acid and back extracted into chloroform. The chloroform was evaporated under vacuum to yield 0.4g of phosphate (**25**) as a colourless waxy solid (1.2mmol, 55%); ^{1}H-NMR (250MHz $CDCl_3$) δ 5.4 - 5.2 (2H, brs, $-P(O\underline{H})_2$), 4.0 - 3.8 (2H, brs, $-OC\underline{H}_2CH_2(CH_2)_{13}CH_3$), 1.8 - 1.7 (2H, m, $-OCH_2C\underline{H}_2(CH_2)_{13}CH_3$) 1.25 (26H, s, $-OCH_2CH_2(C\underline{H}_2)_{13}CH_3$), 0.9 (3H, brt, $-OCH_2CH_2(CH_2)_{13}C\underline{H}_3$); ^{31}P-NMR (101.3MHz

$CDCl_3$) δ_p 1.15 (s, ^1H decoupled) 1.2 (t, J_{PH} = 5.5Hz, ^1H coupled); m/z (CI, ammonia) 453 (44%) 340 (M + NH_4, 100), 323 (M + H, 55). Observed accurate CI m/z on (M + NH_4) gives 340.2617, $C_{16}H_{35}O_4P.NH_4$ requires 340.2617.

2.4.2.5. [2'-(Octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl di-*tert*-butyl phosphate (26)

1H Tetrazole (0.38g, 4.5mmol) was added to a stirred solution of alcohol (**14**) (0.65g, 1.7mmol) and phosphoramidite (**23**) (0.38g, 1.5mmol) in dry THF (3ml) and maintained at room temperature for 15min. The mixture was then treated with 50% MCPBA (0.7g, 2mmol), extracted, washed, concentrated then purified by flash column chromatography (as described for ester (**24**) above). Di-*tert*-butyl phosphate (**26**) was a yellow oil (0.70g, 1.2mmol, 80%); R_f (ethyl acetate - hexane, 1: 1) 0.7; ^1H-NMR (250MHz $CDCl_3$) δ 3.88 - 3.82 (4H, m, $(-C\underline{H}_2O)_2$), 3.45 - 3.39 (4H, m, $-OC\underline{H}_2CH_2(CH_2)_{15}CH_3$ and $-C(5)\underline{H}_2$), 2.02 - 1.83 (4H, m, $-C(3)\underline{H}_2$, and $-C(4)\underline{H}_2$), 1.80 - 1.76 (2H, m, $-OCH_2C\underline{H}_2(CH_2)_{15}CH_3$), 1.50 - 1.45 (18H, t, J_{HH} = 6.8Hz, $(-C(C\underline{H}_3)_3)_2$), 1.22 (30H, s, $-OCH_2CH_2(C\underline{H}_2)_{15}CH_3$), 0.88 - 0.82 (3H, t, J_{HH} = 7.5Hz, $-OCH_2CH_2(CH_2)_{15}C\underline{H}_3$); ^{31}P-NMR (101.3MHz $CDCl_3$) δ_p -10.35 (s, ^1H decoupled) (m, ^1H coupled); m/z (CI, ammonia). 577.5 (M + H, 15%), 521.4 (M + H - butene, 10%), 465.4 (M + H - 2(butene), 100) 96.9 (25). Observed accurate CI m/z on (M + H) gives 577.4597. $C_{32}H_{66}O_6P$ requires 577.4597.

2.4.2.6. Dihydrogen [2'-(octadecyloxymethyl)tetrahydrofuran-2'-yl]methyl phosphate (21)

A solution of the di-*tert*-butyl ester (**26**) (0.5g, 1.1mmol) in 6M hydrochloric acid (1ml) and dioxane (1ml) was stirred for 48h at room temperature. The solvent was evaporated and water (1ml) and chloroform (1ml) were added. Phosphate (**21**) was extracted into the chloroform layer which was decanted and the water layer extracted with more chloroform (1ml). The organic layers were combined and washed first with 10M NaOH (2ml), then concentrated hydrochloric acid (checking the pH of the emulsion formed). Chloroform was removed by evaporation to yield phosphate (**21**) as a colourless sticky solid (300mg, 0.5mmol, 45%); ^1H-NMR (250MHz $CDCl_3$), δ 4.00 (1H, brs, $-PO\underline{H}$), 3.90 (1H, brs, $-PO\underline{H}$), 3.50 - 3.30 (8H, m, $(-C\underline{H}_2O-)_2$, $-OC\underline{H}_2CH_2(CH_2)_{15}CH_3$, and $-C(5)\underline{H}_2$), 2.00 - 1.80 (4H, brs, $-C(3)\underline{H}_2$, and $-C(4)\underline{H}_2$), 1.60 - 1.45 (2H, brs, $-OCH_2C\underline{H}_2(CH_2)_{15}CH_3$), 1.34 - 1.14 (30H, brs, $-OCH_2CH_2(C\underline{H}_2)_{15}CH_3$), 0.88 - 0.82 (3H, t, J_{HH} = 6.5Hz, $-OCH_2CH_2(CH_2)_{15}C\underline{H}_3$); ^{31}P-NMR (101.3MHz $CDCl_3$) δ_p -0.17 (s, ^1H decoupled); m/z (CI, ammonia). 465 (M + H, 10%), 403 (M - PO_3 + NH_4, 5%), 385 (M - PO_3, 30%), 97 (100). Observed accurate CI m/z on (M = H) gives 465.3345. $C_{24}H_{50}O_6P$ requires 465.3345.

CHAPTER 3

METABOLISM

1. INTRODUCTION

There are three main sites in the SRI molecule at which metabolic enzymes can attack, the alkyl chain, the phosphatidylcholine group, and the furan ring (see chapter 1, section 3, p23). This chapter is concerned with the hypotheses that a) ^{31}P-NMR spectroscopy can be used to study the metabolism of SRI and Mil mediated by phospholipases and b) SRI can undergo enzymic attack at sites other than the phosphatidylcholine moiety.

Metabolism of the alkyl chain was studied using rat and mouse liver post mitochondrial supernatant and microsomes, with analysis of the metabolites by GC. Oxidation of NADH associated with AGMO activity was monitored by UV spectrophotometry at 340nm.

Metabolism of the phosphatidylcholine side chain by phospholipase enzymes was observed using the liver fractions described above and purified phospholipase C and D. The methods used for metabolite detection were GC and ^{31}P-NMR spectroscopy.

2. METHODS

2.1. Materials

Chemicals and enzymes were obtained from Sigma Chemical, Poole, Dorset. PAF was obtained from Cascade Biochemicals, White Knights Campus, Reading University, UK. Buffers were prepared as described in appendix 1.

2.2. Preparation of liver post mitochondrial supernatant fraction

Male BALB/c mice (weight 20g) or Wistar rats (weight 100g) obtained from Banting and Kingman, The Field Station, Grimston, Aldbrough, Hull, were killed by cervical dislocation. All equipment and the buffer were maintained on ice. The liver was carefully but rapidly removed and the gall bladder was discarded from the mouse liver. Liver tissue was then washed in ice cold tris[hydroxymethyl]amino methane (Tris) buffer (0.05M) with sucrose (0.25M) pH 7.6, weighed and roughly chopped using scissors. Four times its weight of buffer was then added before further mincing. The crude homogenate was then placed in a Potter-Elvenhjem homogeniser and its teflon pestle attached to a Camlab homogeniser (model S63C TRi-R 1). The pestle was spun slowly and guided up and down in the tube until the liver was completely homogenised. The mixture was transferred to Beckman polycarbonate centrifuge tubes and spun in a Beckman 48 - 60M ultracentrifuge at 9000g for 22min at 4^{o}C. The supernatant was decanted and used either to prepare

microsomes or for metabolic studies. The post mitochondrial supernatant fraction prepared will be referred to as S9.

Rat liver S9 was also used to study metabolism in conjunction with ^{31}P-NMR spectroscopy. For these experiments liver S9 was prepared as described earlier except that Sprague-Dawley rats were used. Pepstatin and phenylmethylsulphonyl fluoride (protease inhibitors) were added in acetone (500µl) just prior to homogenisation [Scopes, 1982].

2.3. Preparation of microsomes

The liver S9 was transferred into clean centrifuge tubes and spun at 105000g for 1h, to separate microsomes from soluble cell fraction. The supernatant was discarded and microsomal pellet resuspended in Tris buffer, pH 7.6, by gentle hand homogenisation with a teflon pestle. The microsomal suspension was again spun at 105000g for 1h to remove any haemoglobin. Supernatant was discarded and the microsomes resuspended in buffer to give approximately 20mg protein/ml.

2.4. Lowry Protein assay

The method used was based on that of Lowry et al [1951]. From a stock solution of bovine serum albumin (50mg in 50ml, 0.5M NaOH), standard protein concentrations ranging from 0 to 200µg protein/ml were prepared. The microsomal suspension was diluted 20 or 40 fold with water. The Lowry reagent (appendix 1, p161) was prepared and aliquots (2.5ml) placed in test tubes. Standard protein solution or diluted microsomal sample (500µl) was mixed with reagent and the resultant solution allowed to stand for 10min. Folin Ciocalteaus phenol reagent (2M) was diluted with an equal volume of water and an aliquot (250µl) added to each sample. The samples were then mixed using a vortex and allowed to stand for 30min. Sample absorbance was read at 750nm, on a Cecil CE594 double beam UV spectrophotometer. A sample prepared as above but without protein was used as a blank.

A calibration curve was produced using absorbance recorded for standard protein samples. This curve was used to calculate protein content of the microsomal sample.

2.5. Incubations of SRI and 1-hexadecylglycerol with liver S9 or microsomes and analysis by GC

Aliquots (2ml) of liver S9 or microsomal suspension were placed in open vials. SRI was added (50µl of a 40mM solution in Tris buffer pH 7.6) and incubated at 37°C in a water bath, with gentle agitation. After 2h, the incubations were treated with ethyl acetate (2ml) to extract the metabolites. Samples were mixed in a vortex and ethyl acetate decanted into

Gyrovap glass tubes. A further 1ml of ethyl acetate was added to each sample, shaken and on separation, decanted. Ethyl acetate extracts were combined and evaporated to dryness using a Gyrovap, V. A. Howe & Co Ltd, London. The solid remaining was resuspended in ethyl acetate (100µl) and analysed by GC.

1-Hexadecylglycerol has been shown to be a substrate for AGMO [Koetting et al, 1987a] and was used in experiments as a control. A second method of sample extraction was developed using methanol to stop metabolism and deproteinise the samples. Methanol was used here because glycerol, a metabolite of 1-hexadecylglycerol [Tietz et al, 1964], could not be extracted readily into ethyl acetate [Matarase 1983, Cronholm et al 1984]. SRI and 1-hexadecylglycerol were incubated with liver S9 or microsomes as above. After 2h, methanol (2ml) was added and the samples were centrifuged, to pellet microsomal material. The supernatant was decanted, concentrated in the Gyrovap and the solid remaining was resuspended in methanol (100µl). The samples were analysed using GC.

2.6. Efficiency of extraction

Alcohol (**14**) and diol (**5**) (50µl of 40mM solutions, in ethyl acetate) were added to microsomes (2ml). Ethyl acetate was then immediately added to extract these compounds and prepare them for GC analysis, as described in section 2.5. above. The results were compared with a control sample in which ethyl acetate (2ml) was added to alcohol (**14**) and diol (**5**) (50µl of 40mM solutions, in ethyl acetate) and then treated as above.

The efficiency of the methanol extraction was also tested. Alcohol (**14**), diol (**5**), glycerol and hexadecanol (50µl of 40mM methanolic solutions) were added to microsomes (2ml) and extracted with methanol as described in section 2.5. The extracts were prepared for GC analysis and compared with a control, as above.

Efficiency of extraction by ethyl acetate was calculated to be 82% for alcohol (**14**), 12 - 15% for diol (**5**), 72% for hexadecanol and 2% for glycerol. Methanol was more effective for extracting glycerol, with efficiencies of 70% for alcohol (**14**), 80% for diol (**5**), 65% for hexadecanol and 85% for glycerol.

2.7. Conditions for GC analysis

Gas pressures were hydrogen - 20psi, air - 20psi, nitrogen - 1psi. Flame ionisation was used to detect compounds. The column was 1.5m in length, had an internal diameter of 4mm and was packed with 3% OV-17 on Chrom W-AWDMCS. Injector temperature was 300°C and the running conditions using method A were an initial column temperature of 170°C for 5min followed by a 6°C rise per min to 270°C and remaining at this for 30min.

Method B started at 150°C then followed the same temperature programming. This second method was used when metabolism of 1-hexadecylglycerol was studied.

The retention times of chemicals by GC are shown in Table 1 (p77). The retention times of peaks extracted from liver S9 by ethyl acetate and methanol are shown in Tables 2 (p78) and 3 (p79) respectively. This demonstrates that although there were several impurities seen in the extracts the potential metabolites could still be distinguished. The minimum levels of detection for alcohol (**14**), diol (**5**) and 1-hexadecylglycerol were 38.4µg in 1ml, 38.4ng injected onto the column in a 1ml injection volume; 132.0µg in 1ml, 132ng injected onto the column; and 6.3mg in 1ml, 6.3µg injected onto the column, respectively.

2.8. Study of AGMO activity

The method was based on that of Koetting et al [1987a] and the assay system was developed on the basis of the reaction shown in Fig. 33 (p83). AGMO uses 6-methyl-tetrahydropterine ($MPtH_4$) as a cofactor, which is converted to a quinoid 6-methyl-dihydropterine (q-$MPtH_2$) which can tautomerise in a side reaction to its 7,8 isomer. The quinoid tautomer is regenerated to $MPtH_4$ by dihydropteridine reductase, using $NADH/H^+$ as co-factor. The decrease in NADH absorption can be followed spectrophotometrically at 340nm.

The following constituents were placed in sample and reference cuvettes, in a temperature controlled (37°C) cell holder of a Cecil CE 594 double beam UV spectrophotometer, microsomes (50µl), 20µl NADH solution (10µmol/ml), 2.5µg dihydropterine reductase (5µl) and 500nmol of SRI or 1-hexadecylglycerol (292µg or 158µg in 50µl Tris buffer, respectively). Tris buffer (0.1M, pH 8.8) was added to make the solutions up to 1ml. This assay was also performed with a larger volume using 3ml cuvettes. Sample and reference cuvettes were adjusted to give an absorption of 0 at 340nm and the optical stability checked for 30s. The cuvettes should balance, i.e. autoxidation of NADH in each sample, step 2 in Fig. 33 (p83), should be equal. The reaction to be investigated was initiated by addition of 10µl $MPtH_4$ (0.2µM) to the sample cuvette. Absorbance was then followed for 2 - 3min.

A control reaction was set up with an identical incubation mixture but without the substrate. This was necessary to eliminate the contribution of autoxidation of $MPtH_4$ and tautomerisation, steps 3 and 1 in Fig. 33 (p83).

Standards	Temp °C	Retention Time /min	
		A	B
Glycerol	150		1.5
Diol (5)	156	6.3	8.6
Acetate (12)	193	12.2	14.5
Hexadecanol	230	16.1	18.4
Hexadecylglycerol	270	28.5	30.8
Octadecanol	270	31.9	33.9
Alcohol (14)	270	41.3	43.6

Table 1 Retention times and temperatures of possible metabolite standards (A and B refer to the GC conditions described in section 2.7.).

Peak assignment	Temp °C	Retention Times /min
		Method (A)
peak 1	187.8	11.3
" 2	232.8	18.8
" 3	239.4	19.9
" 4	270	29.1
" 5	270	38.4
" 6	270	45.9

Table 2 Retention times and temperatures for ethyl acetate extraction of rat liver S9 fraction.

Peak assignment	Temp °C	Retention Time /min
		Method (B)
Peak 1	150	2.6
Peak 2	150	4.3
Peak 3	162.6	7.1
Peak 4	179.4	9.9
Peak 5	203.4	13.9
Peak 6	270	26.6

Table 3 Retention times and temperatures of methanol extract of rat liver S9.

2.9. Incubation of SRI with phospholipases

The incubation method was developed from that of Wilcox et al [1987]. A 20mM solution of SRI (11.7mg in 1ml PBS) was prepared and aliquots (50µl) were taken and added to PBS (450µl) in sealable vials. Phospholipase C (*Clostridium perfringens* (*C perf*), Type XIV, 12.5units, in 500µl PBS) or D (*Streptomyces chromofuscus* (*Strep*), Type VI, 1250 units in 500µl PBS) were added to the vials to give a final concentration of SRI as 1mM.

The samples were maintained in a water bath at 37°C. With gentle agitation, under a nitrogen atmosphere, the incubations were left for 1h. The reactions were terminated by extraction with ethyl acetate (2 x 2ml) and extracts prepared for GC analysis as described in section 2.5.

2.10. Metabolism studies using ^{31}P-NMR spectroscopy

Studies were performed using a Bruker FT AC 250MHz NMR spectrometer. ^{31}P-NMR spectra were recorded at 121.5MHz and are reported δ_p ppm downfield of 85% phosphoric acid. SRI, Mil or PAF (10µmol) were dissolved in 900µl Tris buffer (0.1M) or 2[*N*-morpholino]ethane sulfonic acid (MES) buffer (0.1M). Tris buffer of pH 7.3 was used to study metabolism by phospholipase C from *C perf*, Type XIV and *Bacillus cereus* (*B cereus*), Type III and pH 8.0 for that of phospholipase D from *Strep*, Type VI. MES of pH 5.6 was used for experiments with phospholipase D from peanut, Type II and pH 6.0 with phospholipase C from *B cereus*, Type XIII. D_2O (100µl), was added to each sample to be the spectrometer lock. Solutions were filtered through a small cotton wool bung into the NMR tubes, and a spectrum recorded. The enzymes were prepared in optimum pH buffers eg Phospholipase D (*Strep*) 2500 units was made up in Tris buffer pH 8.0 (1ml). An NMR spectrum was recorded immediately after the addition of 25 units of the enzyme (10µl). The NMR tubes were then incubated in a water bath at 37°C and they were analysed at regular intervals by ^{31}P-NMR spectroscopy.

Time course studies on reactions catalysed by phospholipase C (*C perf*) and D (from *Strep* or peanuts) were performed on samples of SRI prepared as above, except that a solution of benzylphosphonate (Bn<u>P</u>) (5µmol, 0.9mg) in the D_2O (100µl) was added as a standard. Initially inorganic phosphate was used as an internal reference but it had the same chemical shift as the phosphate metabolites generated by phospholipase D. The phosphate internal standard also had a very broad peak shape which would have made its quantitative measurement difficult. The temperature of the NMR probe was warmed to 37°C and spectra were run constantly at the beginning of the reaction. The speed of metabolism dictated the frequency for recording subsequent spectra.

The S9 from rat liver was also used to study metabolism with analysis by ^{31}P-NMR spectroscopy. PAF, SRI and Mil (10μmoles) were dissolved in 500μl Tris buffer pH 7.3 and D_2O (100μl), an NMR spectrum was run prior to the introduction of liver S9 (400μl). Another NMR spectrum was recorded. Samples were incubated in a water bath at 37°C and spectra taken at regular intervals.

In all samples containing phospholipase D, metabolism was stopped by the addition of two drops of 10M NaOH. This alkali was used to sharpen the peaks in the ^{31}P-NMR spectrum. In the case of PAF the base also catalysed the hydrolysis of the 2-acetoxy ester group. This was confirmed by adding two drops of 10M NaOH to a sample of PAF (10μmol) in Tris buffer pH 8.0 (900μl) and D_2O (100μl) and following the reaction by ^{31}P-NMR spectroscopy which gave peaks at δ_p -0.07ppm (PAF) and 0.39ppm. ^{1}H-NMR spectroscopy was used to detect the production of acetate, δ_p 1.95ppm (confirmed by addition of standard, acetic acid). Products of metabolism were identified by adding reference compounds, eg phosphatidylcholine chloride calcium salt. Alkaline phosphatase was added to confirm the presence of organic phosphate monoesters as these were cleaved to yield inorganic phosphate.

Metabolites formed on addition of phospholipase D to SRI and Mil were isolated by acidifying the sample, extracting with $CDCl_3$ (1ml), and then back extracting into 10M NaOH (1ml). The presence of phosphatase activity in samples of phospholipase D was confirmed by incubating 25 units of enzyme in Tris buffer pH 8 (1ml) with the disodium salt of *p*-nitrophenyl phosphate (10mg), which produced a bright yellow colour on cleavage of the phosphate for *p*-nitrophenolate.

Neither Mil nor SRI were metabolised by phospholipase C. Experiments were set up to measure the ability of these two ether lipids to inhibit phospholipase C. SRI and Mil (10μmoles) were dissolved in MES (500μl) pH 6 and D_2O (100μl) and their ^{31}P-NMR spectra were run. The samples were then incubated for 30min at 37°C with phospholipase C, Type XIII, isolated from *B cereus* (12.5units). ^{31}P-NMR spectra were recorded after the incubation period and immediately after the addition of PAF (10μmoles in 400μl of MES pH 6). The rate of PAF metabolism was compared with a control sample, omitting the ether lipid. For this sample ^{31}P-NMR spectra were run prior to and immediately after the addition of phospholipase C (12.5units).

Phospholipase C (*B cereus*) produced very rapid metabolism of PAF. Phospholipase C, Type XIV from *C perf* (12.5 units), was also used as a catalyst. The experiments were repeated in the same way as above but the buffer used was Tris pH 7.3.

3. RESULTS

3.1. Metabolism by AGMO

SRI was incubated for up to 2h with S9 or microsomes isolated from the livers of rats or mice and the mixture was extracted with ethyl acetate. The extracts were examined for metabolites by GC analysis. Table 1 (p77) shows the retention times of important standard compounds by GC analysis. Table 2 (p78) shows the main peaks obtained from an ethyl acetate extract of an incubate of SRI with liver S9. Retention times were not identical to those of putative reference metabolites of SRI. SRI was not apparently metabolised by AGMO.

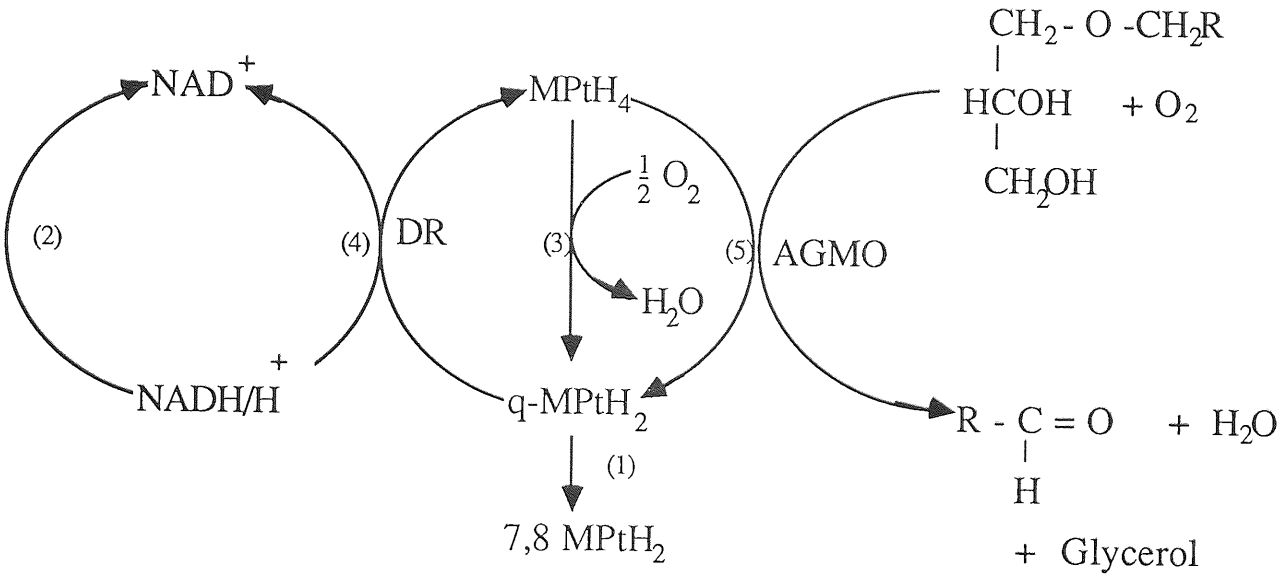
It has been shown for many glycerophospholipids that inorder to be a substrate for AGMO they have to undergo cleavage of the phosphatidylcholine moiety [Koetting at al, 1987b]. Initially studies were intended to demonstrate generation of alcohol (**14**) from SRI. Alcohol (**14**) may be metabolised to diol (**5**) and octadecanal, by AGMO. The efficiency of extraction with ethyl acetate was 11 - 15% for (**5**), but 82% for alcohol (**14**). Incubations of SRI with liver S9 or microsomes over 2h did not show production of alcohol (**14**) or diol (**5**). As metabolism of SRI was not observed it was necessary to have a positive control substance to confirm the presence of AGMO activity in liver fractions.

To that end 1-hexadecylglycerol was incubated with rat liver microsomes, as it has been shown to be a substrate for AGMO [Koetting et al, 1987a]. Glycerol, the product of 1-hexadecylglycerol metabolism mediated by AGMO [Tietz et al, 1964], could not be extracted efficiently into ethyl acetate. It was shown to be effectively extracted into methanol. However, to separate glycerol from methanol by GC the initial column temperature had to be reduced to 150°C. Methanol extraction of microsomes showed many background peaks. They had different retention times compared to those of the proposed metabolites of 1-hexadecylglycerol (Tables 1, p77 and 3, p79). One metabolite of 1-hexadecylglycerol co-eluted with glycerol, though this was difficult to verify due to its proximity to the solvent peak. Hexadecanol was not detected. A similar experiment with SRI, using the methanol extraction method, did not detect any metabolism.

The optimum pH for the activity of AGMO is 9 [Soodsma et al, 1972], however the microsomal incubations with SRI and 1-hexadecylglycerol were performed at pH 7.6. This difference in pH may have been sufficient to slow the metabolism by AGMO to an undetectable level.

Since metabolism of SRI or 1-hexadecylglycerol was not observed using GC analysis, a more sensitive assay was used to study AGMO metabolism. The method used is based on

the reaction shown in Fig. 33. The AGMO-catalysed reaction is coupled to oxidation of NADH to NAD^+ and observed as a decline in UV absorption at 340nm. Even though this assay was claimed to be rapid, sensitive and simple [Koetting et al, 1987a], I was unable to detect metabolism of SRI or 1-hexadecylglycerol. Modifications made to the assay such as increasing microsomal content, addition of dihydropteridine reductase and use of the more soluble 1-hexadecyl glycero-3-phosphocholine as a control, did not lead to detectable metabolism by AGMO. It has also been suggested that AGMO requires the presence of soluble fraction from preparation of liver homogenate for its activity, due to co-operation with a second enzyme, catalase [Soodsma et al, 1972]. However, metabolism of SRI was not detected in liver S9.



DR - Dihydropteridine Reductase
AGMO - Alkyl glycerol monooxygenase
$MPtH_4$ - 6 - methyltetrahydropterine

Fig. 33 Reactions in the coupled AGMO assay

Among the reasons which may explain this failure to observe metabolism are the lack of sensitivity of the method and the short period (2 - 3min) for which the reaction was monitored.

3.2. Metabolism by phospholipase C

3.2.1. PAF

PAF, a natural substrate for phospholipase C [Okayasu et al, 1986] was used as a positive control in ^{31}P-NMR spectroscopy studies on metabolism, as shown in Fig. 34 (p85). PAF [δ_p 0.13 (s, ^{1}H decoupled)(pent, ^{1}H coupled, J_{PH} = 7Hz)] was rapidly metabolised by phospholipase C from both *C perf* and *B cereus*. Complete metabolism occurred within 5min of adding *B cereus* and within 30min for *C perf*. The metabolite had a chemical shift of 3.86ppm which corresponded to that of phosphatidylcholine [δ_p 3.86 (s, ^{1}H decoupled)(t, ^{1}H coupled J_{PH} = 5Hz)], and when an authentic reference compound was added to the PAF metabolism sample this peak grew relative to the PAF peak. The metabolite was not inorganic phosphate (δ_p 1.79ppm). The PAF metabolite was incubated with alkaline phosphatase and the product (δ_p 2.28ppm) corresponded to inorganic phosphate, confirmed by addition of authentic reference compound. The change in the chemical shift of inorganic phosphate was due to a slight change in pH. In conclusion, the metabolism of PAF could be monitored by ^{31}P-NMR spectroscopy.

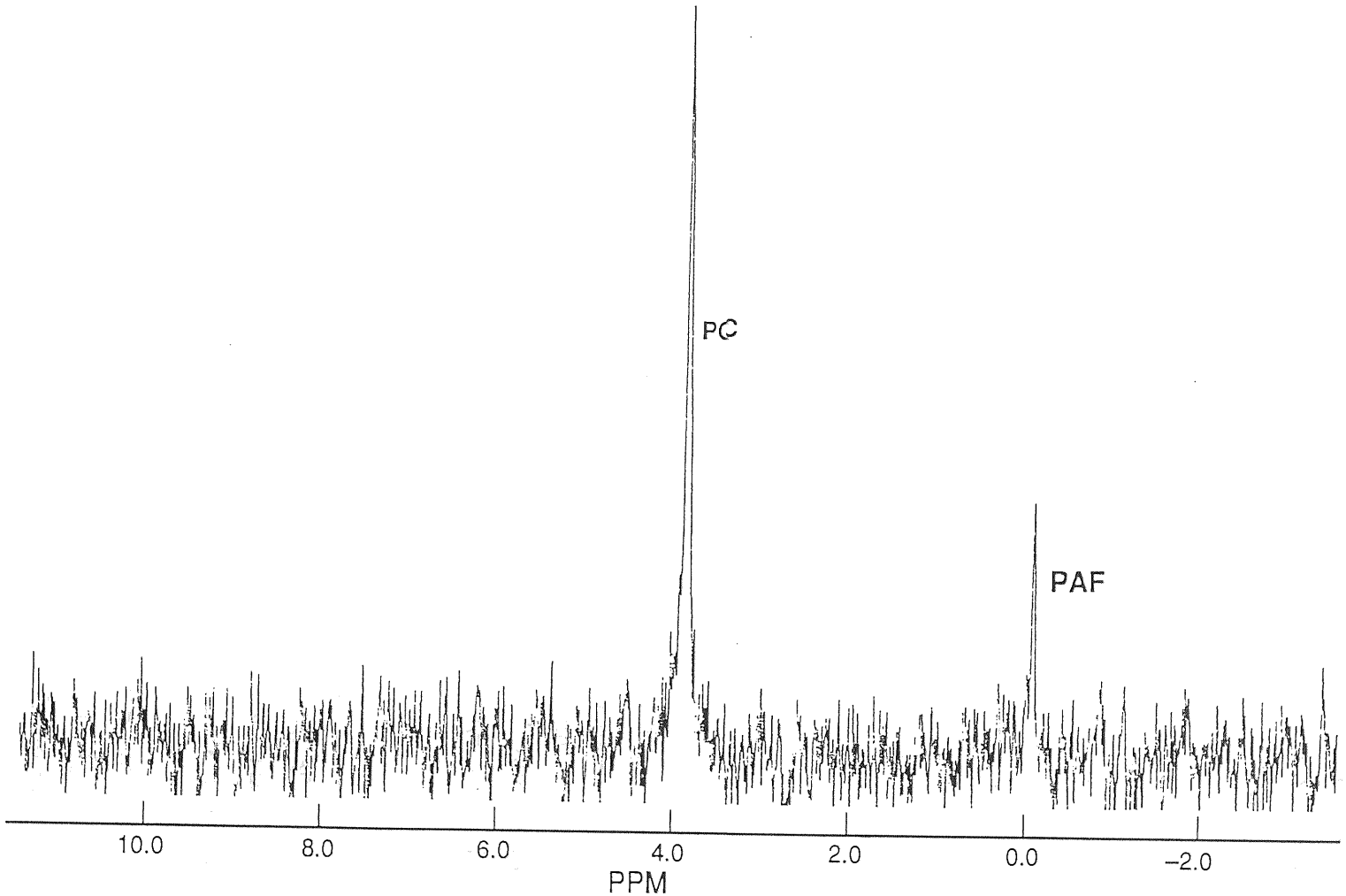
3.2.2. SRI and Mil

SRI was incubated with phospholipase C from both *C perf* and *B cereus*. Expected metabolites of this enzyme are alcohol (**14**) and phosphatidylcholine. However, GC analysis of extracts did not show any formation of alcohol (**14**). The ^{31}P-NMR spectra of incubates of SRI with phospholipase C did not have any additional peaks, when compared to the spectrum obtained prior to addition of enzyme. Metabolism was not evident even after 48h incubation with either phospholipase C enzyme. Similarly in the case of incubates of Mil with phospholipase C, the ^{31}P-NMR spectrum did not change during a 48h incubation period. Unlike PAF, SRI and Mil do not appear to be a substrates for phospholipase C.

Figure 34

^{31}P-NMR spectrum of the metabolism of PAF by phospholipase C.

(T = 10 min) (PC = phosphatidyl choline)



3.3. Metabolism by Phospholipase D

3.3.1. PAF

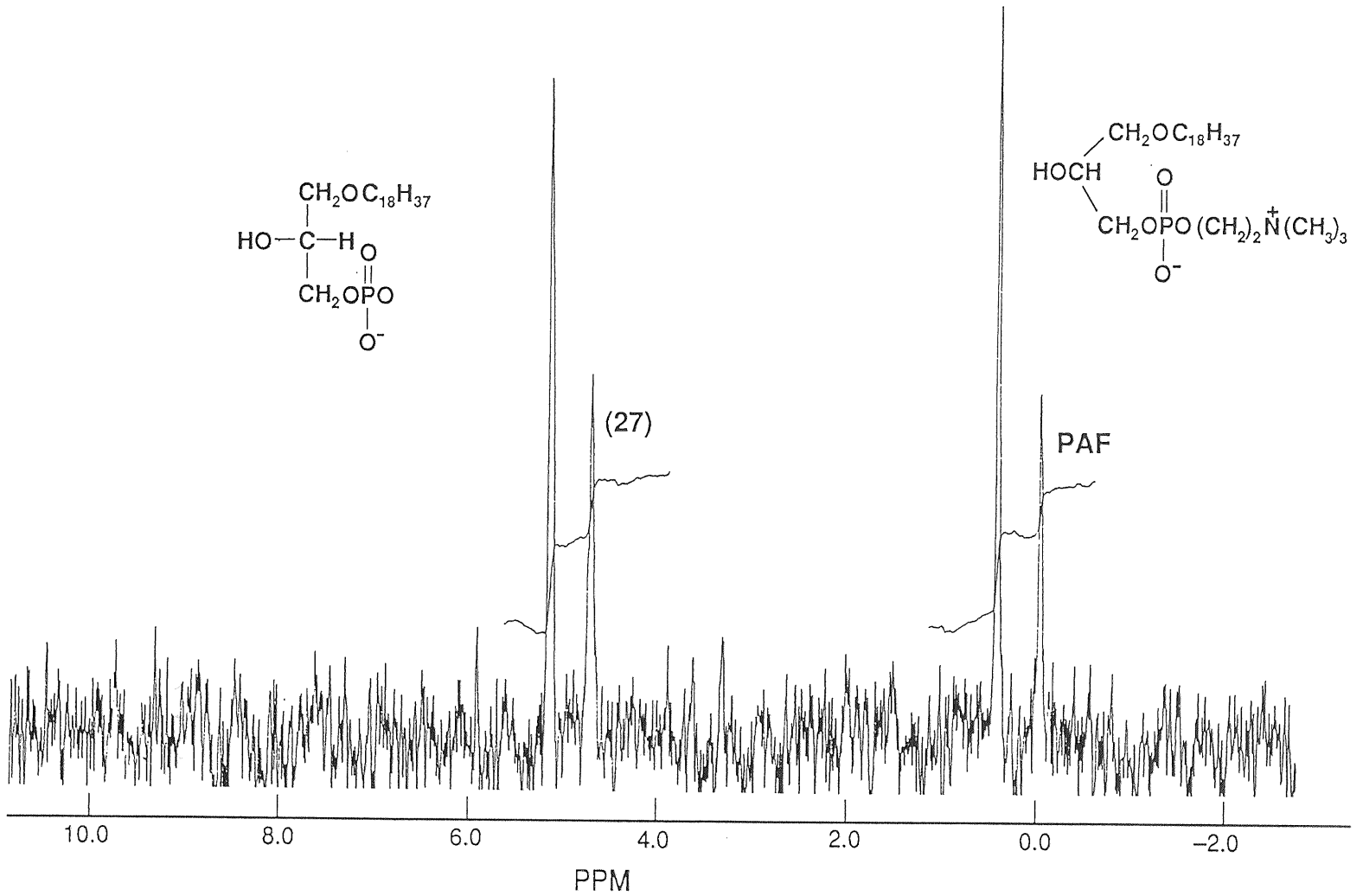
PAF, a natural substrate for phospholipase D [Qian et al, 1989] was metabolised more slowly by phospholipase D than C. PAF [δ_p -0.13ppm, (s, ^1H decoupled)(pentet, ^1H coupled, J_{PH} = 7Hz)] was first incubated with phospholipase D (*Strep*) whose optimum pH is 8. Around 50% metabolism had occurred at 24h, giving a broad peak at 3.7 - 2.5ppm. After the addition of a few drops of NaOH (10M), four singlets emerged at δ_p 5.17, 4.73, 0.45 and 0.00ppm (Fig. 35, p87). With time, the peaks at 4.73 and 0.00ppm disappeared. The peak at 4.73ppm (s, ^1H decoupled)(t, ^1H coupled, J_{PH} = 5Hz) was neither inorganic phosphate [δ_p 5.91ppm (s,^1H decoupled)(s, ^1H coupled)] nor phosphatidylcholine [δ_p 4.07ppm (s, ^1H decoupled) (t, ^1H coupled, J_{PH} = 5Hz)]. The presence of the triplet in the ^1H-coupled spectrum provided evidence that the metabolite was the phosphate of PAF (**27**), obtained by cleavage of choline. The peak at 0.00ppm was due to PAF. The peak at 0.45ppm was probably lyso-PAF, formed by hydrolytic cleavage of the ester group in position 2, catalysed by the addition of alkali. The peak at 5.17ppm was the phosphate metabolite (**27**) with the 2-acetyl group removed by hydrolysis. Fig. 36 (p88) shows the reactions involved in producing the three compounds as a result of metabolism and chemical hydrolysis of PAF.

To confirm the chemical hydrolysis, NaOH (10M) was added to a buffered solution, pH 8, of PAF (δ_p -0.07ppm) which gave a peak at δ_p 0.39ppm (Fig. 37, p89). After 2h all the PAF had reacted and the peak at 0.39ppm showed the same coupling pattern in the ^1H coupled spectra as PAF, showing that phosphorus remained part of a phosphatidylcholine group. The ^1H-NMR spectrum of this sample included a peak at δ_H 1.9ppm which corresponded to the methyl group of sodium acetate. These results confirm that sodium hydroxide catalyses ester hydrolysis (Fig. 36, p88).

PAF (δ_p -0.13ppm) was also incubated with phospholipase D from peanuts, which has a pH optimum of 5.6. Metabolism was observed within 5min. After 24h, 50% of the substrate had been metabolised. A broad peak developed at δ_p 0.81ppm, but on addition of NaOH it split into three peaks, one at 6.14ppm and the other two very close together at 5.10ppm. These latter two peaks merged into one (δ_p 5.14ppm) with time and resulted from carboxyl ester hydrolysis, as described above. The peak at δ_p 6.14ppm corresponded to inorganic phosphate. The second peak was not due to phosphatidylcholine (δ_p 4.12ppm) so it was thought to be the phosphate of lyso-PAF. It had a shift similar to that of the metabolite produced by phospholipase D (*Strep*). The production of inorganic phosphate was unexpected, therefore both phospholipase D enzymes were tested for the presence of phosphatase activity by incubating them with *p*-nitrophenyl phosphate.

Figure 35

^{31}P-NMR spectrum of the metabolism of PAF by phospholipase D, following the addition of NaOH, (T = 24h)



A small degree of phosphatase activity was identified in the *Strep* enzyme, but far greater activity in that isolated from peanuts. This difference aids in explaining why inorganic phosphate was formed with phospholipase D from peanuts.

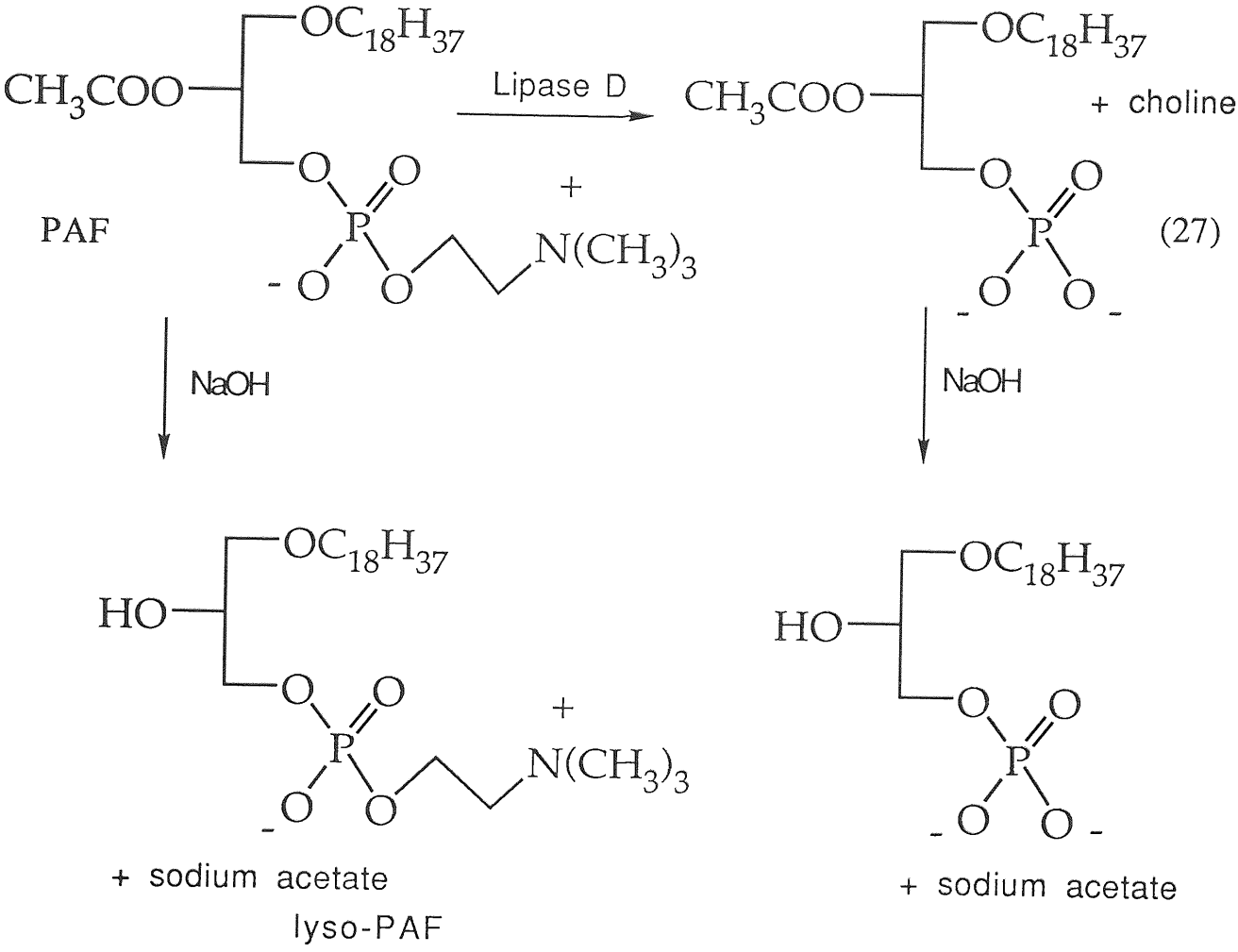
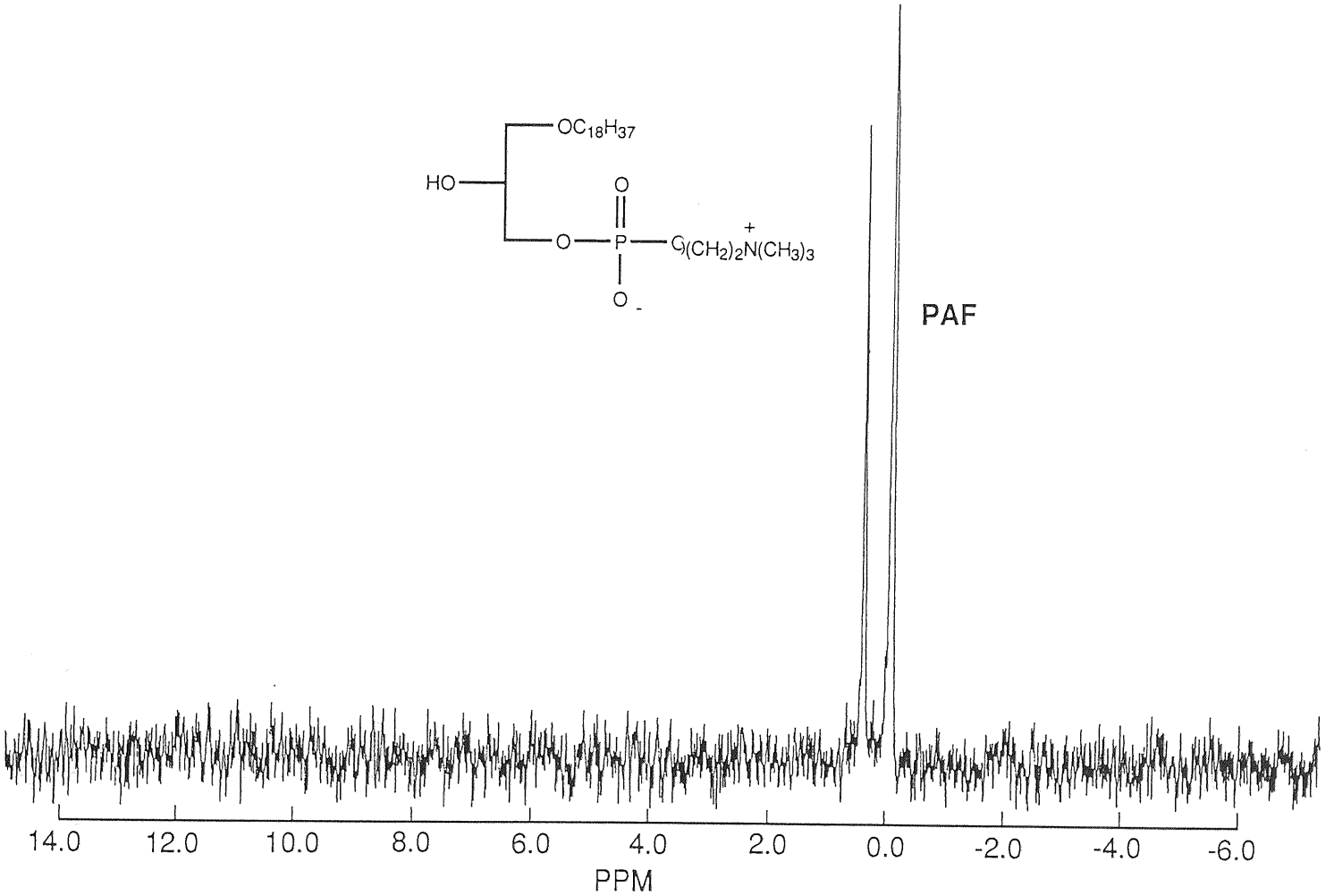


Fig. 36 Metabolism of PAF and the effect of adding sodium hydroxide.

Figure 37

^{31}P-NMR spectrum demonstrating the affect of adding NaOH to a solution of PAF in D_2O (T = 10 min)



3.3.2. SRI

SRI was incubated with phospholipase D. Upon metabolism SRI would be expected to yield phosphate (**21**). However, the presence of phosphatase enzyme could convert this product to alcohol (**14**) and inorganic phosphate. Phosphate (**21**) was too polar to be eluted from the GC column but its metabolite, alcohol (**14**) could be detected if present. Extracts of SRI incubated with phospholipase D (*Strep*) gave a peak with a retention time similar to that of alcohol (**14**). However, an extract of the enzyme solution also showed a small amount of material at this retention time. This result suggests that SRI might be a substrate for phospholipase D. Evidence for the formation of phosphate (**21**) and inorganic phosphate was pursued by ^{31}P-NMR spectroscopy.

In ^{31}P-NMR spectroscopy studies, SRI was first incubated with phospholipase D isolated from *Strep*. Trisodium inorganic phosphate was initially used as an internal standard but at pH 8 it showed the same chemical shift as the SRI metabolite, phosphate (**21**). Addition of NaOH could separate the inorganic phosphate reference (δ_p 7.76ppm) from SRI phosphate metabolite (δ_p 6.73ppm), but it also stopped the reaction. At 24h approximately 75% metabolism had occurred. A further problem of using trisodium inorganic phosphate as internal standard was that inorganic phosphate may be a product of metabolism. To be able to follow the time course of the metabolism of SRI, BnP was used as internal standard. Its chemical shift of 18.79ppm was significantly different from that of either SRI or any putative metabolites. The C-P bond in BnP is very resistant to chemical or enzymic cleavage and it is very unlikely to be metabolised by phospholipase D.

In incubates of SRI with phospholipase D the appearance of a broad metabolite peak (δ_p 6.00 - 5.40ppm) occurred within 10min of adding enzyme. The reaction was monitored over 21h. Peak areas of SRI and BnP were calculated and the ratio used to monitor the rate of SRI metabolism (Fig. 38, p91). Ratio of metabolite / internal standard was too difficult to measure due to the very broad nature of the metabolite peak. Since only a further 10% metabolism had occurred between 2h and 21h the reaction was stopped after 21h by addition of NaOH (Fig. 39, p92). Approximately 70% of SRI had been metabolised. A metabolite peak occurred at δ_p 5.08ppm and did not correspond to inorganic phosphate (δ_p 6.15ppm) or phosphatidylcholine (δ_p 4.74ppm). The metabolite was most likely the SRI molecule without the choline group. This was confirmed in three ways: i) Incubation with alkaline phosphatase gave inorganic phosphate (δ_p 6.15ppm).ii) Isolation of the metabolite by the addition of hydrochloric acid and then extraction into chloroform, and comparison with the phosphate standard (**21**) using TLC and NMR. iii) Synthesis of authentic SRI phosphate (**21**), which had a similar shift to the metabolite and produced inorganic phosphate (δ_p 6.15ppm) on incubation with alkaline phosphatase.

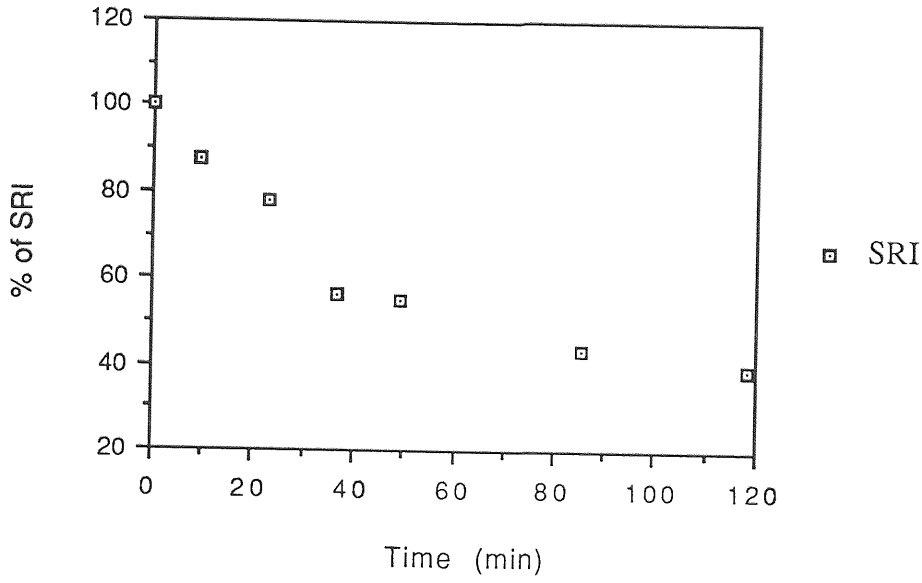
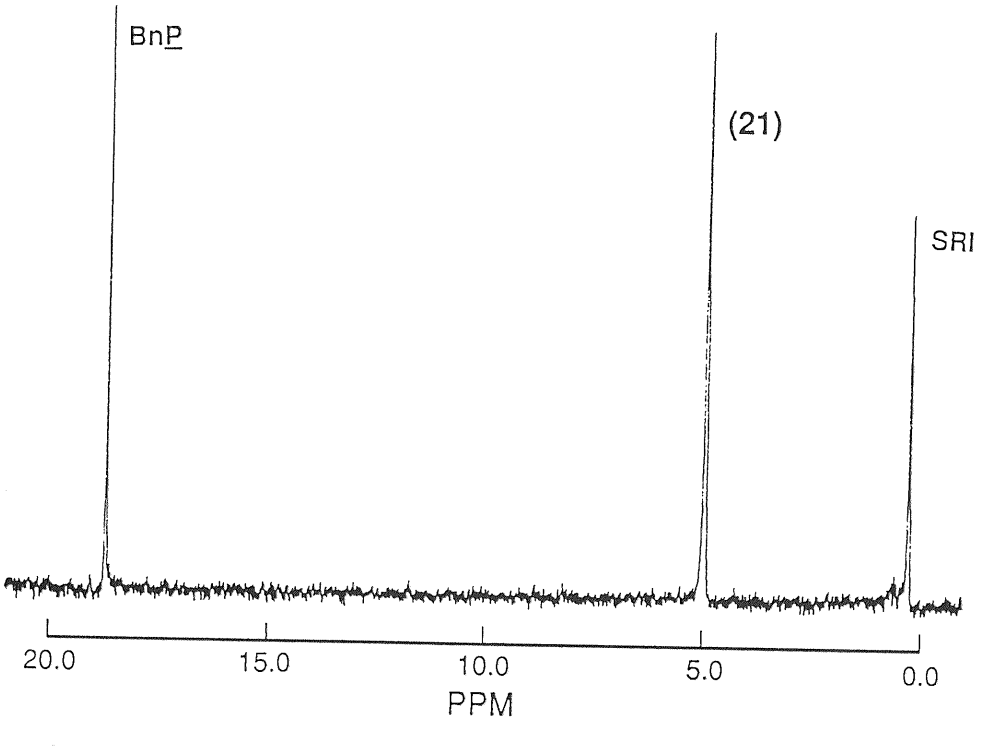


Fig. 38 SRI metabolism catalysed by Phospholipase D (*Strep*)

SRI was also incubated with phospholipase D isolated from peanuts. After 24h only 30% of the SRI (δ_p 0.27ppm) had been metabolised to a singlet at δ_p 1.01ppm. On addition of NaOH this peak split into two, the major one δ_p 6.02ppm (25%) corresponded to inorganic phosphate, probably due to the high level of phosphatase activity in this enzyme. The minor peak at δ_p 4.87ppm (5%) was for phosphate (**21**).

In a control experiment SRI was incubated with heat inactivated phospholipase D or Tris buffer pH 8. Metabolite peaks did not appear even after 48h incubation. This result indicates that the formation of phosphate (**21**) was due to enzymic activity and not chemical degradation.

Figure 39
^{31}P-NMR spectrum of the metabolism of SRI by phospholipase D, after the addition of NaOH. (T = 48 h)



3.3.3. Mil

Mil [δ_p 0.20ppm (s, ^1H decoupled)(pentet, ^1H coupled J_{PH} = 6Hz)] was first incubated with phospholipase D isolated from *Strep*. The pattern of metabolism was similar to that of SRI. A broad metabolite peak (δ_p 2.6-2.1ppm) developed which on addition of NaOH sharpened to a peak at δ_p 4.67ppm (40%) and a very small peak at 6.18ppm (10%) (Fig. 40, p94). The metabolism of Mil was slower than that of SRI and only 50% was metabolised within 24h. In a coupled spectrum the peak at 4.67ppm became a triplet (J_{PH} = 5.5Hz). It was shown to be phosphate (**25**), the monoester of Mil, without the choline moiety. The small peak at δ_p 6.18ppm corresponded to inorganic phosphate, generated by phosphatase activity present in this enzyme preparation.

Mil was also incubated with phospholipase D isolated from peanuts. Mil (δ_p 0.13ppm) was metabolised to a peak at δ_p 0.95ppm (90% conversion) which was split into two peaks on addition of NaOH. In the ^1H-coupled spectrum the singlet peak at 5.83ppm (60%) responded at the frequency of inorganic phosphate (δ_p 5.83ppm). In contrast the peak at 4.67ppm (30%), a triplet (J_{PH} = 5.5Hz), corresponded to standard (**25**), the phosphate of Mil.

Mil was incubated with heat inactivated phospholipase D and Tris buffer pH 8, to indicate that the production of phosphate (**25**) and inorganic phosphate were due to enzymes and not chemical breakdown.

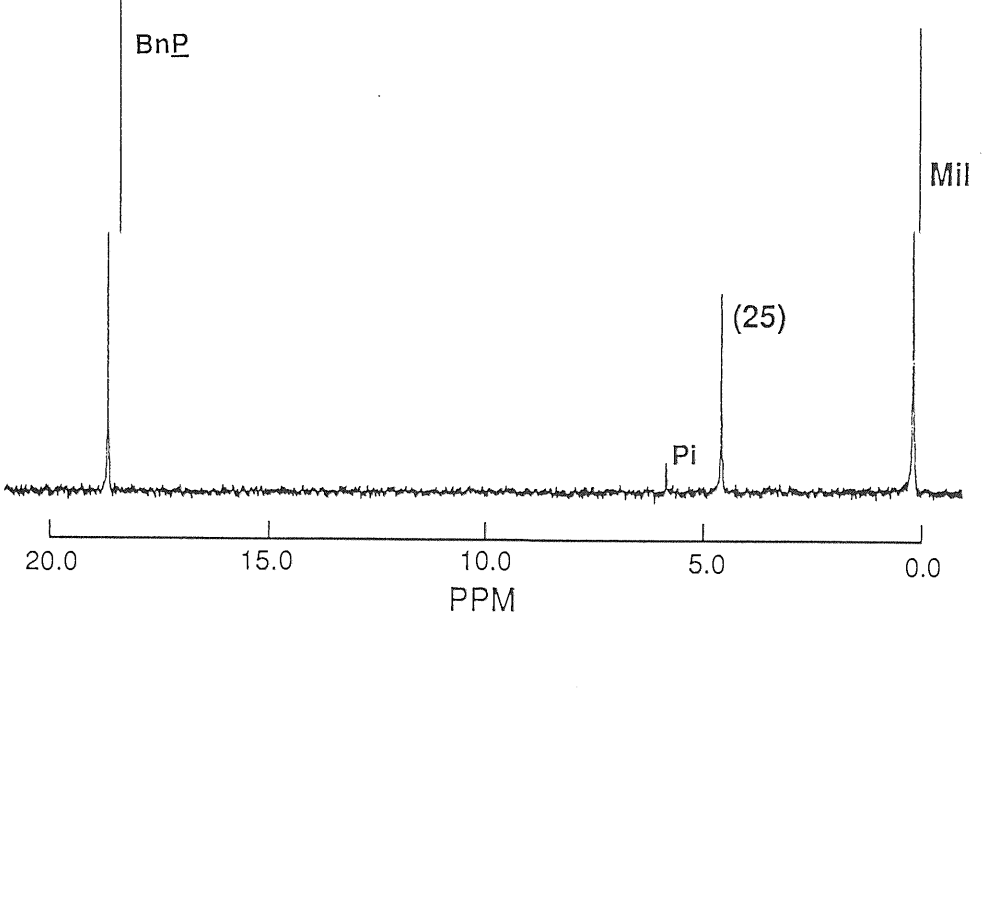
3.4. ^{31}P-NMR studies of metabolism by rat liver S9

3.4.1. PAF

Incubation of rat liver S9 alone gave weak peaks at δ_p 2.77, 3.93 and 4.71ppm. After incubation for 24h these peaks had merged into one at 2.61ppm. On addition of NaOH this peak sharpened and shifted to δ_p 7.66ppm, corresponding to inorganic phosphate. PAF (δ_p -0.11ppm) was incubated with liver S9 for 72h, after which time NaOH was added and left for 30min to allow hydrolysis of the carboxy ester. The ^{31}P-NMR spectrum showed that approximately 50% of PAF had been metabolised. A peak at δ_p 5.95ppm (34%) corresponded to inorganic phosphate, which had increased in size when compared to the inorganic phosphate peak in the liver S9 control incubate. The smallest peak at δ_p 5.60ppm (7%) was thought to be the phosphate of lyso-PAF. The peak at δ_p 4.09ppm (8%) corresponded to phosphatidylcholine and the largest peak at δ_p 0.37ppm was probably lyso-PAF. The results suggest metabolism of PAF mediated by both phospholipase C and D and by phosphatase.

Figure 40

^{31}P-NMR spectrum of the metabolism of Mil by phospholipase D, after the addition of NaOH. (T = 24h)



3.4.2. SRI

SRI (δ_p 0.01ppm) was incubated with rat liver S9 for 72h and a broad peak (δ_p1.95 - 0.9ppm) emerged. About 30% of SRI was metabolised. The broad peak sharpened on addition of NaOH to give two peaks at δ_p 5.84ppm (24%) and 3.90ppm (6%). The former was inorganic phosphate and although present in the control liver S9, had increased in size. The small peak at δ_p 3.90ppm corresponded to phosphate (**21**), SRI with the choline group cleaved. These results suggest that metabolism of SRI by phospholipase D gives phosphate (**21**) with subsequent metabolism of (**21**) by phosphatase to inorganic phosphate and alcohol (**14**). Again metabolism by phospholipase C was not observed, in agreement with the results obtained with purified enzymes.

3.4.3. Mil

Mil (δ_p 0.07ppm) was metabolised to a broad peak at δ_p 3.0 - 2.4ppm, with approximately 25% disappearance of substrate. This peak, on addition of NaOH sharpened into two peaks δ_p 5.88ppm (20%) and 4.05ppm (5%) which were identified as inorganic phosphate and phosphate (**25**), respectively. As with SRI, Mil seems to be metabolised by phospholipase D to give (**25**), which is then metabolised by phosphatase to give inorganic phosphate. Again metabolism by phospholipase C was not observed.

3.5. The effects of SRI and Mil on phospholipase C catalysed metabolism of PAF

Mil and ET-18-OCH_3 are thought to inhibit inositol specific phospholipase C [Uberall et al, 1991; Seewald et al, 1990]. Neither Mil nor SRI were metabolised by phospholipase C. So experiments were designed to test the hypothesis that these two ether lipids could inhibit the activity of phospholipase C.

SRI (δ_p 0.16ppm) and Mil (δ_p 0.26ppm) were incubated at pH6 with phospholipase C from *B cereus* for 30min. There was no change in their chemical shifts and other peaks for metabolites were not present. PAF at pH 6 gave a peak at δ_p 0.03ppm. On addition of PAF to the SRI / enzyme and the Mil / enzyme mixtures a peak manifested at δ_p 2.90 - 2.20ppm. This peak corresponded to phosphatidylcholine, and PAF was completely metabolised within 5min. This rate was comparable to that observed in the control where PAF was incubated with phospholipase C (*B cereus*).

SRI (δ_p 0.16ppm) and Mil (δ_p 0.28ppm) were also incubated with phospholipase C from *C perf*, at pH 7.3. After 30min incubation, the ^{31}P-NMR spectrum showed no change in the shift for SRI or Mil and other peaks did not appear. PAF (δ_p 0.00ppm) was added to this mixture. A peak at δ_p 3.93ppm developed in the SRI incubate and at 3.83ppm in the Mil sample, which corresponded to phosphatidylcholine. PAF was more slowly

metabolised to phosphatidylcholine by phospholipase C from *C perf* and complete metabolism took 2h. This rate was very similar to that observed for the PAF control.

The results suggest that SRI and Mil did not affect the rate of metabolism of PAF by phospholipase C from either *B cereus* or *C perf*. Neither ether lipid seemed to inhibit phospholipase C, under the conditions used for this experiment.

4. DISCUSSION

Metabolism of SRI by liver microsomes or S9 was not detected by GC analysis or by the AGMO assay. The lack of detection of metabolism may be due to the low sensitivity of the methods or to the fact that SRI was not a good substrate for AGMO. Failure to show metabolism with the control 1-hexadecylglycerol may be related to its very poor water solubility. The detection sensitivity for 1-hexadecylglycerol was also lower than that for alcohol (**14**). Metabolism of 1-O-hexadecylglycero-3-phosphocholine was not detected in the AGMO assay. This compound had improved water solubility but its metabolism is thought to be slower than that of 1-hexadecylglycerol [Koetting et al, 1987a]. Cleavage of the phosphatidylcholine moiety of this compound would have to occur prior to metabolism by AGMO. The time for observing its metabolism in this assay was probably too short to discern significant changes. The assays used may not be sensitive enough to detect the low level of metabolism.

Metabolism of ether lipids appears to involve a series of enzyme catalysed steps. AGMO mediated metabolism seems to occur at a late stage in the metabolism cascade. ET-18-OCH_3 is not a substrate for AGMO. ET-18-OCH_3 and SRI are structurally related (Figures 1b, p15 and 2a, p16). They both possess an octadecyl chain linked via an ether linkage and could undergo a similar metabolic process, though they differ in their substituent in position 2. ET-18-OCH_3 becomes a good substrate for AGMO once the phosphatidylcholine moiety has been cleaved [Koetting et al, 1987b]. SRI is also structurally related to PAF, and PAF only becomes a good substrate for AGMO once the acetyl group at position 2 is cleaved [Koetting et al, 1987b; Lee et al, 1981]. A substrate for AGMO requires one sterically unhindered position in the glycerol derivative. It is possible that SRI would not be a good substrate for AGMO and that it may require metabolism by phospholipase C, or phospholipase D and phosphatase before it could be metabolised by AGMO. Hence the time for studying metabolism in the AGMO assay would be too short to detect changes in NADH. The bulky THF group in position 2 of the "glycerol backbone" of SRI and alcohol (**14**) may also interfere with enzyme binding and inhibit metabolism.

There were two main problems encountered in studying metabolism of SRI by AGMO. The first relates to the fact that catalysis by AGMO is probably not the first step in the breakdown of SRI. SRI is only slowly metabolised by phospholipase D, which probably has to occur prior to AGMO metabolism. Hence the time for incubation of SRI with liver S9 or microsomes was probably insufficient to produce enough of diol (**5**), the AGMO metabolite, to be detected by GC. The second problem was that there were a possible six metabolites which could be produced from AGMO catalysed metabolism of alcohol (**14**). Only diol (**5**) and octadecanol were used as standards for GC analysis. Potential THF ring metabolites would be tetrahydrofurfuryl alcohol with either CH_2OH [diol(**5**)] or CHO substituted in position 2 (Fig. 7, p27). The aldehyde group could be further oxidised to a carboxylic acid or reduced to diol (**5**). The alkyl chain in the SRI molecule could be biotransformed to octadecanol or octadecanal, and the octadecanal could yield octadecanoic acid or octadecanol. Products of metabolism of alcohol (**14**) by AGMO were expected to be diol (**5**) and octadecanal. This suggestion is based on the production of glycerol and hexadecanal from 1-hexadecylglycerol by AGMO (Fig. 11, p30) [Tietz et al, 1964]. Octadecanol was measured rather than octadecanal, the expected second metabolite, since the latter would probably be reduced octadecanol (Fig. 11). Presence of octadecanol and absence of diol (**5**) could have indicated if metabolism of (**14**) went via the tetrahydrofurfuryl aldehyde and octadecanol rather than diol (**5**) and octadecanal. The tetrahydrofurfuryl aldehyde metabolite was not prepared and so could not be looked for in the GC analysis of incubates of SRI.

The parent ether lipid SRI did not appear to be metabolised by AGMO. SRI is probably not a substrate for this enzyme but if it had undergone alkylglycerol monooxygenation, phosphate (**18**) would have been the product. The synthesis of this phosphate (**18**) was very difficult and a pure sample was not isolated (chapter 2, section 2.3.3.5., p67). Hence a standard could not be used for GC analysis.

^{31}P-NMR spectroscopy did not detect any AGMO-mediated metabolism of SRI. There are three possible explanations, the first is that SRI is not a substrate for AGMO. The second is that AGMO metabolism occurs after cleavage of the phosphatidylcholine chain, as discussed earlier. ^{31}P-NMR spectroscopy would only be able to detect changes close to the phosphorus. The third possible explanation is that if SRI was metabolised by AGMO to phosphate (**18**), cleavage of the alkyl group would have a minimal effect on the chemical shift in ^{31}P-NMR spectroscopy.

GC analysis of extracts of incubates of SRI with phospholipase C suggest that SRI was at best only a very poor substrate for this enzyme as alcohol (**14**), the putative metabolite, was

not detected. There was an indication that SRI was a poor substrate for phospholipase D, when the extract was analysed by GC.

As shown using ^{31}P-NMR spectroscopy, SRI and Mil were not metabolised by phospholipase C. For a compound to be a substrate for phospholipase C the polar trimethylammonio head and the fatty acid groups are essential for substrate recognition. The enzyme favours substituents with carbon chains of 12 - 16 in length to produce a hydrophobic binding site [Bomalaski and Clark, 1987]. Both SRI and Mil have at least a C_{16} chain length but they have ether rather than ester linkages. Substitution of ester linkages with ether moieties yield very poor substrates. The carbonyl group and its immediate environment are important for phospholipid interaction with phospholipase C [El-Sayed et al, 1985]. This fact helps to explain why SRI and Mil are not metabolised by this enzyme. Mil also has its long alkyl chain attached directly to the phosphatidylcholine group and it is possible that the lack of glycerol character could reduce its binding to the enzyme.

SRI and Mil were shown to be metabolised by phospholipase D by the use of ^{31}P-NMR spectroscopy. Metabolism of SRI and Mil did not go to completion. Addition of extra enzyme could not catalyse complete breakdown of these ether lipids. It was apparent that the inhibition of metabolism was not due to the enzyme simply being inactivated. SRI has a chiral centre and the sample used was a racemic mixture of both enantiomers. Phospholipase C has a preference for one stereoisomer of phosphatidylcholines [Reidy and Snyder, 1987]. Phospholipase D is thought to be stereospecific for the same enantiomer of phospholipids as phospholipase C [Jiang et al, 1984]. Therefore, metabolism of the less favoured isomer of SRI would occur more slowly, or not at all. Another possibility for incomplete metabolism of SRI, which would also apply to Mil is that of product inhibition by either choline or the phosphate metabolites (**21**) and (**25**) respectively.

When PAF was incubated with rat liver S9, the metabolites produced suggested that it was metabolised by both phospholipase C, D and by phosphatase. This fact supports the findings for the incubation of PAF with pure enzymes. Peaks corresponding to phosphates (**21**) and (**25**) were present in incubates of rat liver S9 with SRI and Mil, respectively. The presence of these metabolites suggest choline cleavage by phospholipase D. The inorganic phosphate peak present in these incubates also increased in size relative to the control. This increase is consistent with the proposal that inorganic phosphate is a metabolite of these ether lipids. In theory, the formation of inorganic phosphate could be due to metabolism of the ether lipid by phospholipase C to give phosphatidylcholine and its subsequent metabolism by phosphatase. Inorganic phosphate could also be produced following

metabolism by phospholipase D to the ether lipid phosphate and then phosphate cleavage by phosphatase. SRI and Mil did not appear to be substrates for phospholipase C and phosphatidylcholine was not detected. It would seem that metabolism of the phosphates (**21**) and (**25**) by phosphatase was responsible for the production of inorganic phosphate. This last step was very rapid and hence inorganic phosphate was the main metabolite of the breakdown of SRI and Mil by rat liver S9.

Previous experiments involving incubation of Mil with phospholipase C have identified phosphatidylcholine as a metabolite [Breiser et al, 1987] consistent with the premise that Mil is a substrate of phospholipase C. These experiments used Mil with the choline moiety labelled with tritium to detect metabolism in whole cells. Detection by radioactivity is more sensitive than ^{31}P-NMR spectroscopy. If Mil is only a very poor substrate for phospholipase C then ^{31}P-NMR spectroscopy may not have detected the small amount of phosphatidylcholine produced. Another possibility for the production of phosphatidylcholine would be via labelled choline transfer, catalysed by phospholipase D within cells [Pritchard and Vance, 1981]. Such a transfer would not occur in cell free solution.

ET-18-OCH_3 and Mil have been postulated to inhibit inositol specific phospholipase C [Seewald et al, 1990; Uberall et al, 1991]. The experimental results suggest that SRI and Mil were not substrates for phosphatidylcholine specific phospholipase C. Thus the hypothesis was considered that these ether lipids may inhibit this enzyme. PAF is a substrate for phospholipase C, as discussed earlier in chapter 3 (in section 3.2.1., p83), but neither SRI nor Mil seemed to inhibit or alter its rate of metabolism. Inhibition of phospholipase C by these ether lipids was not apparent, under the condition used.

A possible explanation for the lack of inhibition of PAF metabolism by SRI and Mil could be that PAF may have a greater binding affinity for phospholipase C than SRI or Mil. Such a hypothesis could be tested by using lower concentrations of PAF in further experiments. However, such experiments could not be performed using ^{31}P-NMR spectroscopy due to its lack of sensitivity. Mil inhibits inositol specific phospholipase C [Uberall et al, 1991] but it need not necessarily inhibit the phosphatidylcholine specific phospholipase C used in these studies. The phospholipases have different substrate specificities and they have been isolated from different sources. Uberall et al [1991] used phospholipase C from fibroblasts whereas phospholipase C was isolated from bacteria for the experiments reported here. These variations could affect enzyme activity. Ether lipids have been shown to alter membrane fluidity [Noseda et al, 1989] and this change could alter phospholipase C

activity [Uberall et al, 1991]. It is possible that Mil and SRI do not inhibit the phospholipase C in cell free solutions due to their lack of interaction with cell membrane.

In summary ^{31}P-NMR spectroscopy can be used to study the metabolism of SRI and Mil by phospholipases. Figures 41 and 42 show the metabolic studies performed with SRI and Mil, respectively, demonstrating their routes of metabolism. Both SRI and Mil were metabolised by phospholipase D, from *Strep* and peanuts, and then subsequently by phosphatase. Neither SRI nor Mil appeared to be substrates for phospholipase C, from *C perf* or *B cereus*. They did not inhibit the activity of this enzyme toward PAF metabolism, under the conditions used. SRI possesses an ether-linked octadecyl chain in position 2, but metabolism by AGMO was not detected by GC or spectrophotometric analysis. The THF ring of SRI may interfere with the binding of AGMO to the octadecyl chain and so inhibit its cleavage.

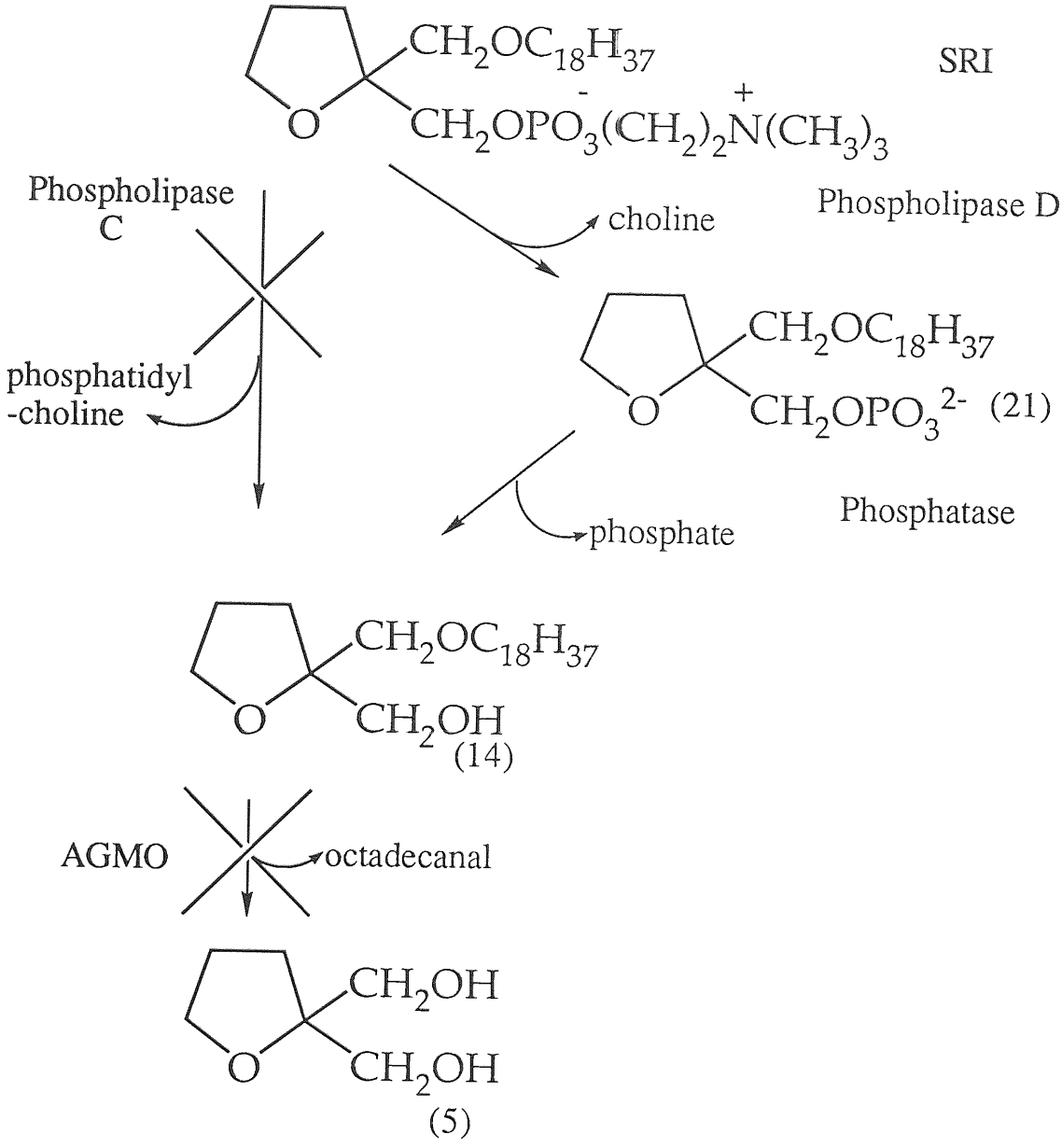


Fig. 41 Route of the metabolism of SRI.

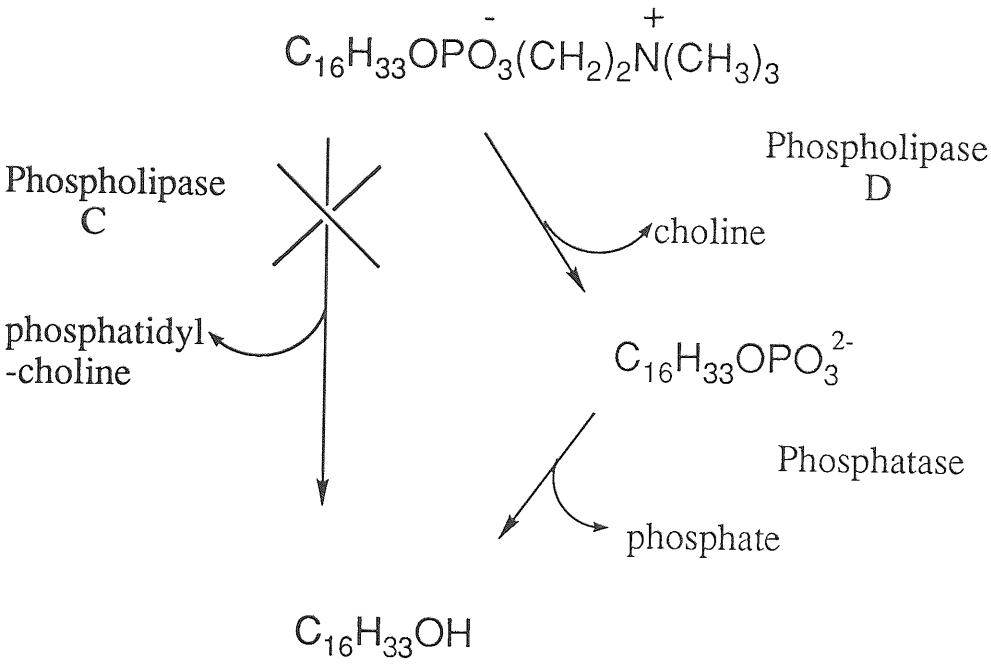


Fig. 42 Route of the metabolism of Mil

CHAPTER 4

CYTOTOXICITY TESTING

1. INTRODUCTION

1.1. Objective

The hypothesis was tested that metabolism of SRI and Mil is a detoxification process. The cytotoxicity of SRI, Mil and their putative metabolites was compared in three cell lines. Potential metabolites were postulated on the basis of literature work on PAF, ether lipids and phospholipids (chapter 1, section 3, p23) and of results described in chapter 3. In addition the hypothesis was tested that lipophilicity is important for the toxicity of SRI, Mil and their metabolites. Metabolism generally increases water solubility. Therefore a decrease in cytotoxicity might be expected. Such information may contribute to the body of knowledge concerning the mechanism of ether lipid cytotoxicity.

The cell lines used were two malignant lines, derived from lung and liver, and one line initiated from normal liver. These cell lines were thought to allow comparison of cytotoxicity toward malignant and normal cells from the same tissue (liver) and towards malignant cells of two completely different tissues of origin. The lung cells were derived from human tissue, whereas the liver lines were from rats, this difference in source might also allow a preliminary species comparison to be made. Three different assays were used to compare the cytotoxicity of SRI and its metabolites to aid in understanding its mode of action. Finally the hypothesis was tested that the cytotoxicity of SRI and Mil is affected by the presence of serum.

1.2. Description of cell lines

1.2.1. A549 cells

A549 cells were derived from a human alveolar cell carcinoma and grown in culture as monolayers [Girard et al, 1973]. They have a human karyotype and possess multilamellar cytoplasmic inclusion bodies typical of those found in type II alveolar epithelial cells of lungs. They are differentiated in as much that they produce and secrete pulmonary surfactant [Lieber et al, 1976]. The rationale for choosing this cell line was the fact that lung cancer is at present very difficult to treat with chemotherapy. Hence, it is important to screen for *in situ* novel agents which might have antitumour activity at this site.

1.2.2. JB1 and BL8 cells

JB1 is a monolayer epithelial cell line derived from a hepatoma induced in rats by aflatoxin B_1 [Judah et al, 1977]. Malignant transformation has permitted these cells to replicate, with

some retention of their differentiated structure. Normal hepatocytes can not generally maintain their specialization in culture [Manson et al, 1981]. Transformation does not increase the growth rate of JB1 cells, when compared to the BL8 non-transformed rat liver cell line. BL8 cells are also a monolayer epithelial cell line but with a relatively undifferentiated ultrastructure. The BL8 cell line was initially obtained by collagenase perfusion of rat liver [Judah et al, 1977; Manson et al, 1981]. JB1 cells resemble BL8 cells in that they both exhibit contact inhibition in culture, but differ in that only JB1 cells can be grown *in vivo* in nude mice. BL8 hepatocytes differ quite markedly from primary hepatocytes both in appearance and biologically. BL8 cells are less differentiated than normal hepatocytes and they do not have bile canaliculi. BL8 cells do however form tight gap junctions with neighbouring cells.

JB1 cells have been shown to have certain metabolic capabilities but not others. JB1 cells have higher levels of γ-glutamyl transferase and show a greater sensitivity to γ-glutamyl-*p*-phenylene diamine mustard trifluoroacetate than BL8 cells [Manson et al, 1981]. JB1 cells exhibit a higher degree of activation of this mustard than BL8 cells, as the former could more easily hydrolyse the γ-glutamyl derivative to the free mustard. There was no difference in sensitivity between JB1 and BL8 cells to the parent phenylene diamine mustard, confirming that the JB1 cell sensitivity to the γ-glutamyl mustard is related to its level of γ–glutamyl transferase. At least five Cytochrome P450 isoenzymes have been reported to activate alflatoxin B_1, including Cyt P450 $3A_4$ and $1A_2$ [Crespi et al, 1991]. Cytochrome P450 is rapidly lost during culturing of hepatocyte cell lines but BL8 cells retain some metabolic capacity for activation of this compound [Manson et al, 1981]. In contrast, JB1 cells do not appear to have any ability to activate aflatoxin B_1. In the work described here the JB1 cell line was used in cytotoxicity studies to be compared with A549 cells which were derived from a different tissue and species. Similarly to lung cancer, liver cancer is almost untreatable with chemotherapy, so it is important to screen for compounds with anti-hepatoma activity. The BL8 cell line was used to represent the response of "normal" liver cells to SRI, Mil and their potential metabolites. Extrapolation of toxicity results from cultured BL8 hepatocytes to the normal primary hepatocytes has to be done with caution.

1.3. Cytotoxicity assays

Three assays were used to measure the cytotoxicity of SRI and its putative metabolites. These were the measurement of cell growth characteristics, the MTT assay and the LDH assay. Use of three different assays was intended to aid in the elucidation of the mode of action of SRI. Only the MTT assay was used for comparison of the toxicities of Mil and its metabolites.

1.3.1. Assessment of cell growth characteristics

In this assay the number of treated and control cells are counted over a period of days and the results compared. The observations give an indication of the effect of the test compound on the balance between cell proliferation and cell death. Compounds which affect DNA can cause cytostasis before signs of toxicity are seen. Protein kinase C is one of the many enzymes involved in the control of cell proliferation [Kikkawa et al, 1989]. Mil and SRI have been shown to inhibit protein kinase C [Uberall et al, 1991; Thompson and Hickman, 1988], so they might be expected to modulate cell growth.

Ether lipids have been reported to cause cells to swell and membrane blebs to be formed [Noseda et al, 1989]. The advantage of this assay was that use of a haemocytometer to count cells allowed observation of changes in cell morphology. A disadvantage of this assay is that it cannot rapidly detect acute toxicity. This limitation is due to the fact that at short treatment intervals the control cell number is low, as cells have not had time to proliferate.

1.3.2. MTT assay

The MTT assay is based on a reaction thought to be catalysed by dehydrogenases present in viable but not dead cells. The enzymes convert the yellow water soluble MTT salt to a purple insoluble formazan product [Mosman, 1983; Twentyman and Luscombe, 1987]. The formazan is soluble in DMSO and the resulting optical density recorded is proportional to cell number. This assay is based on a biochemical interpretation of cell viability as, formazan is produced by mitochondrial activity in living cells. Only metabolically active cells reduce MTT to formazan. Dead cells do not interfere with MTT absorbance since cell debris is not stained by formazan [Keepers et al, 1991]. It is a versatile assay and can be automated to allow the rapid testing of a large number of samples, eg nine concentrations of compound can be tested on one 96 well plate. This technique has been used by the National Cancer Institute in screening anticancer agents. The assay shows good correlation between absorbance and cell number [Mosman, 1983].

However, when this method is used several disadvantages have to be considered. The optical density of formazan depends both on the MTT concentration and incubation time. Optimum conditions have to be identified for each cell line used [Keepers et al, 1991]. MTT is toxic, so an incubation time point has to be selected which gives maximal formazan formation with minimal MTT toxicity. Other disadvantages of the MTT assay include the fact that serum interferes with optical density of the formazan solution. Therefore, medium should be removed, as much as is possible and cells should be rinsed. DMSO catalyses the production of formazan from MTT. Plates cannot be stored and have to be read swiftly

following the addition of DMSO [Twentyman and Luscombe, 1987]. The MTT assay does not give linear results with increasing cell number, especially at high cell densities. Hence, at the end of the cell dosing period, when MTT is added, it is important for the cells to be subconfluent [Keepers et al, 1991]

1.3.3. LDH assay

This assay measures the leakage of LDH from cells damaged by a toxic insult. LDH is a cytoplasmic enzyme and when cell membranes are sufficiently damaged it leaks out into the extracellular environment and can be detected. The amount of LDH present in cell culture medium can be used as an indicator of cell death. LDH has been measured in body fluids such as serum and cerebrospinal fluid for diagnosis and prognosis of myocardial infarction, hepatitis, leukaemia and stroke [Cadaud et al, 1958]. The method used here relies on the fact that LDH catalyses the following reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \text{----------> Lactate} + NAD^+$$

LDH activity is determined by the rate of oxidation of NADH. The conversion of NADH to NAD^+ is accompanied by a decrease in absorption, when the UV spectrum is observed at 340nm. Results of the LDH assay are expressed per cell number. This fact is important as maximal LDH release has to be calculated in cells after treatment with triton. Cells in control incubates continue to grow, whilst treated cells are either completely inhibited or grow more slowly, if a test agent is toxic. If cell number was not taken into account, LDH release from control incubates would be unduly high and that from treated cells proportionally small, underestimating toxicity. The sensitivity of the LDH assay in detecting toxicity is not high. Sensitivity is poor because LDH is a relatively large protein, made up of four subunits (molecular weight 135 - 140 kDa), which will only be released when there is severe damage to the cell membrane. However, the LDH assay could still be suitable for measuring cytotoxicity due to membrane active agents such as ether lipids. The assay is coupled to the conversion of NADH to NAD^+. There are endogenous serum enzymes present which utilise NADH. Hence the rate of NAD^+ production must be monitored soon after the addition of test sample, to ensure conditions in the cuvette remain optimum for the assay. A sample of medium taken from control cells must also be analysed for LDH activity to provide the background level of NADH oxidation.

An advantage of this assay over the MTT assay is that samples can be stored at 4°C, overnight with minimal loss of LDH activity [Leathwood and Plummer, 1969].

The comparison of the results obtained with the three assays in A549, JB1 and BL8 cells was thought to shed light on the role of metabolism of SRI and its mechanism of action.

2. METHODS

2.1. Materials

Chemicals were obtained from Sigma Chemicals, unless otherwise stated. Tissue culture medium was from Gibco Ltd, Paisley, Renfrewshire, Scotland and tissue culture flasks and plates were from Nunc, Longbridgeway, Uxbridge, Middlesex. Samples were prepared using a Whirlimixer from Fisons, Loughborough and cell samples were centrifuged using a Labofuge 6000, Heraeus, London. Compounds (**5**), (**14**), (**21**) and (**25**) were synthesised as described in chapter 2 and stock solutions were made up as described in appendix 2. Diol (**5**) has two CH_2OH groups attached to the 2 position of a THF ring, it is the proposed product of the metabolism of alcohol (**14**) by AGMO. Alcohol (**14**) is the potential compound formed from phospholipase catalysed breakdown of SRI. Alcohol (**14**) has a $CH_2OC_{18}H_{37}$ and a CH_2OH group attached to the 2 position of the THF ring. Phosphates (**21**) and (**25**) are the putative metabolites of phospholipase D mediated metabolism of SRI and Mil respectively. The structures of (**21**) and (**25**) are the same as their parent molecules but the choline group has been cleaved.

2.2. Maintaining cells in culture

2.2.1. A549 cells

A549 cells were obtained from the American Type Culture Collection, Rockville, Maryland, USA. They were maintained in Hams F12 nutrient medium supplemented with 10% foetal calf serum (FCS), penicillin (1000u/ml), streptomycin (10pg/ml) and 2mM glutamine. The medium was replenished every 2 - 3 days and the cells passaged every 5 - 7 days, once they had reached confluency. Under these conditions the cells had a doubling time of 24h. Cells could be stored under liquid nitrogen. They were frozen and thawed as described in appendix 3.

2.2.2. JB1 and BL8 cells

JB1 and BL8 cells were kindly provided by Dr R. F. Legg, MRC Toxicology unit, MRC Laboratories, Carshalton, Surrey. They were seeded at 2 x 10^5 cells in 100ml flasks in 15 - 20ml medium. The medium used was Williams E, supplemented with 5% FCS, 2mM glutamine and 50mg/ml gentamycin. The cells were replenished every 2 - 3 days then gased for 40s with 5% carbon dioxide / 95% air and passaged every 7 days, once confluent. JB1 cells had a doubling time of 22h and that for BL8 was 17h. Incubation was at 37°C. Extra cells were maintained in liquid nitrogen and thawed as required (appendix 3).

2.3. Removal of cells from monolayers

2.3.1. A549 cells

The medium was aspirated and replaced with sufficient trypsin / versene stock solution to cover the cells (2ml to each 100ml flask). Cells were incubated for 5 - 10min at 37°C until they began to detach from the plastic. An equal volume of medium was added to inactivate the trypsin. The cell suspension was diluted with medium, either for counting with a Coulter counter or haemocytometer, or for passaging.

2.3.2. JB1 and BL8 cells.

Medium was aspirated and the cells rinsed twice with trypsin in PBS (appendix 3) (5ml). The trypsin was removed and the flask incubated at 37°C until the cells had begun to detach, about 5 - 10min in the case of JB1 and 10 - 15 min for BL8 cells. Cells were then resuspended in medium, about 18 - 25ml, at a low enough density to allow the cells to be counted.

2.4. Counting Cells.

A model ZM Coulter Counter (Coulter Electronics Ltd, Luton, Bedford) was used to count A549 cells. Cells were treated with trypsin, as above, an aliquot (200µl) taken and diluted to 10ml with Isoton (Coulter Electronics Ltd). The instrument settings were current 130; attenuation 16; lower threshold (T_L) 12 and upper threshold (T_U) 99.9. Cell samples were counted twice and an average calculated for number of cells/ml. A549 cells were counted using a haemocytometer, after treatment with ether lipids, to observe any associated change in morphology. JB1 and BL8 cells were also counted using a haemocytometer, which enabled these cells to be viewed.

2.5. Measurement of cell growth characteristics

2.5.1. A549 cells

Cells ($1 \times 10^{5)}$ were added to each well of six well plates, each containing medium (3ml). The cells were counted every other day following detachment with trypsin.

To assess the effect of compounds on cell growth, cells were set up as above and treated with either

i) several concentrations of SRI (100, 50, 10, 5, 1, 0.5, 0.1µM), alcohol (**14**) or diol (**5**) (100, 10, 1µM) and the cells were counted after 72h.

or

ii) The cells were treated with SRI, alcohol (**14**), diol (**5**) or Mil at a concentration of 100, 10 or 1µM and then counted at 12, 24, 48 and 72h.

In each case, wells were treated in triplicate and controls were treated with the appropriate test solvent. Cell numbers were represented as 100 - (number of treated cells expressed as a percentage of control).

2.5.2. JB1 and BL8 cells

Cells were removed from the flasks using trypsin and counted. Cells (1×10^5) were added to medium (10ml) in 50ml flasks. The cells from two flasks were counted each day. Average cell number was plotted against time.

To observe the effect of SRI, alcohol (**14**) and diol (**5**) on cell growth, six well plates were used. Cells (1×10^5) were added to each well containing medium (3ml). Triplicate wells were set up per concentration, per time point and the cells allowed to adhere for 4h. Test compound (10µl) was then added so as to give a final concentration of 100, 10 or 1µM. Solvent (10µl) was added to the three control wells per time point of 12, 24, 48 and 72h. Cell numbers were represented as 100-(number of treated cells expressed as a percentage of controls).

2.6. MTT Assay

2.6.1. Optimisation of conditions for the MTT assay

The method used for this assay was based on those described by Mosman [1983] and by Twentyman and Luscombe [1987]. Various factors in this assay had to be modified prior to its use in measuring cytotoxicity. Optimisation of experimental conditions was performed for each cell line. The number of cells used at the start of the assay depended on their growth rate. The initial number of cells used had to give sufficient cells at the end of the treatment period to cover 80% of the well surface. The treatment period of 72h was based on the results of previous experiment and ensured sufficient time for alcohol (**14**) to produce its activity. Unlike the LDH and cell growth assays only one treatment time period was used for the MTT assay. With this assay the optimum initial number of cells would have to be calculated for each time point. The MTT concentration and its time for incubation with the cells was identified to give an adequate colour development for measurement, with minimal MTT toxicity.

Two main problems had to be overcome prior to using the MTT assay.

i) The presence of medium remaining after aspiration could affect the optical density of the formazan solution. Twentyman and Luscombe [1987] showed that medium (10µl) increases optical density with smaller rises in optical density on addition of increasing volumes of up to 40µl of medium. On further addition of medium above 40µl the optical density starts to decline.

ii) A choice of solvents was available for dissolving formazan and the best one had to be selected. A mixture of hydrochloric acid (0.04M) and isopropanol had previously been used as the solvent for dissolving the formazan product [Mosman, 1983]. However, it has been shown that DMSO is a better solvent, solubilising the crystals more rapidly and being less affected by the presence of medium [Twentyman and Luscombe, 1987]. Unfortunately it has also been shown that "when DMSO is added to a small volume of medium containing unconverted MTT (even in the absence of cells) production of formazan proceeds to a significant extent over the next few hours" [Twentyman and Luscombe, 1987].

To overcome the problem of cell culture medium, the medium was removed carefully and washed off where possible. To remove medium from the A549 cell wells, a PBS rinse was added. However, PBS dislodged some of the cells and increased the variability of the results. Rinsing with PBS could not be used in assays with JB1 or BL8 cells, as it resulted in detachment of too many cells and made comparison of the test and control results difficult. The increased ease of detachment may have been due to MTT toxicity since the cells were not as readily dislodged in its absence. With careful aspiration of medium its affects could be minimised, especially by using four repeat wells and a paired set of control wells. DMSO was used as the solvent for dissolving formazan and the plates read as swiftly as possible to minimse the DMSO catalysed production of formazan. A medium blank was also included in the experiment to account for any formazan production in the absence of cells.

When the JB1 cells were grown in the absence of serum they had a slower growth rate (Fig. 43b, p114) than when grown in the presence of serum. They produced less formazan during 5h incubation with MTT. Nevertheless the formazan produced under these conditions was sufficient to assay and the absence of serum reduced interference from medium. To improve formazan yield in cells grown without serum, initial cell numbers were twice those used in the assays for cells grown in the presence of serum.

For reasons unknown, BL8 cells were more sensitive than JB1 cells to changes in their environment. These cells required their humidity to be maintained by regularly topping up the water held in their incubator. Plates had to be placed in single layers to ensure correct conditions for cells to grow at the outer edges of the plates. BL8 cells exhibited increased variability compared to JB1 cells, when assessed in the MTT assay.

Exposure of cells to test compound was designed such that each row of 4 treated wells was accompanied by 4 control wells. The optical density of the controls was considered to be 100%, following subtraction of the background reading. Using these modifications, the

problems associated with the MTT assay using A549, JB1 and BL8 cells were reduced and variability minimised.

2.6.2. MTT assay procedure

A549 cells were detached using trypsin, counted and resuspended at 6 x 10^3/ml. Aliquots (200µl) were added to individual wells of a 96 well plate. The first row of eight wells was left empty and the second received medium only to act as a blank. Cells were allowed 1h to adhere, prior to treatment. Test compound (20µl) was added to the wells to give a final concentration range of 0.1 - 200µM. For each concentration there were four replicate wells and a further four wells received solvent alone, to act as controls. The plates were then incubated at 37°C for 72h.

A stock solution of MTT (5mg/ml) was prepared and passed through a 0.2µm filter, to remove any undissolved impurities and to sterilise the solution. To each well an aliquot (20µl) of this solution was added and the plates incubated for a further 6h. Medium was carefully aspirated to reduce disruption of the formazan product in the cells. PBS (200µl) was added to each well and then aspirated. DMSO (200µl) was then introduced and the plates agitated for 5min on a Titertek plate shaker. The optical density was read in a Titertek multiscan spectrophotometer at 550nm.

Results from replicate wells were averaged and the mean blank value was subtracted from this average. Test results were expressed as a percentage of the controls and plotted against log concentration to calculate the IC_{50} value.

The MTT assay was also carried out as above, using JB1 and BL8 cells. However, there were a few changes made to the assay technique.

i) Cells were initially suspended at 9 x 10^3/ml.

ii) Incubation with MTT was for 5h, which was optimal for these cell lines.

iii) Following the incubation period with MTT, the cells were not rinsed with PBS but the medium was aspirated and the cells treated directly with DMSO.

JB1 cells were used to study the affect of serum on the toxicity of SRI, Mil and their putative metabolites. Cells were detached with trypsin, centrifuged at 1500rpm for 5min, then resuspended in serum-free Williams E medium. The MTT assay was then performed as described above using 1.8 x 10^4 cells/ml.

2.7. Lactate dehydrogenase (LDH) assay

The method used for this assay was based on those of Leathwood and Plummer [1969] and Bergmeyer and Bernt [1974]. Cells were suspended and counted. A cell sample (1×10^5) was added to the wells of six well plates containing 3ml medium. Wells were set up in triplicate per concentration of test compound and per time point. Cells were allowed to adhere for 4h, then the medium was aspirated and replaced with appropriate medium containing 1% FCS. Serum concentration had to be reduced as the endogenous NADH oxidising enzymes present in serum interfered significantly with the assay, when present in higher concentrations.

Aliquots (10µl) of SRI, alcohol (**14**) or diol (**5**) were added to the wells to give a final concentration of 100, 10 or 1µM. Three wells per time point were treated with either PBS or DMSO (10µl), the same solvent used in the test wells. After incubation with test compound (1 - 72h) medium was aspirated, pipetted into tubes and stored at 4°C until analysis. Maximum LDH release was calculated by treating control wells with triton. To each well 20% v/v triton (160µl) was added to give a final concentration of 1% v/v, and left for several minutes. Medium was then collected and analysed for LDH along with the other samples. Prior to analysis, all sample tubes were centrifuged for 5min at 1500rpm to pellet any cell debris and intact cells present.

PBS (2.4ml), NADH stock solution (100µl, to give a final concentration of 0.12mM) and sample (400µl) were placed in a cuvette with 1cm path length. The reference cuvette contained the same as the test cuvette but the appropriate fresh medium with 1% FCS (400µl) was added instead of sample medium. The UV spectrophotometer was set at 340nm and the cuvette maintained at 37°C.

To initiate the reaction, pyruvate stock solution (100µl, to give a final concentration of 1mM) was added and the reaction followed using a UV spectrophotometer. The LDH activity was calculated by measuring the gradients of the reactions, averages were recorded for each concentration and time point. The mean control value was subtracted from all the calculations and the results expressed as a % of LDH activity released by triton (1% v/v) per 10^5 cells.

2.8. Statistical Analysis.

Standard deviation (SD) was calculated using the Student t Test. Standard error (SE) was then calculated by using the SD value in the equation SE = 2SD/square route of n. The number of experiments is given by n and SE is attained within 95% confidence limits.

3. RESULTS

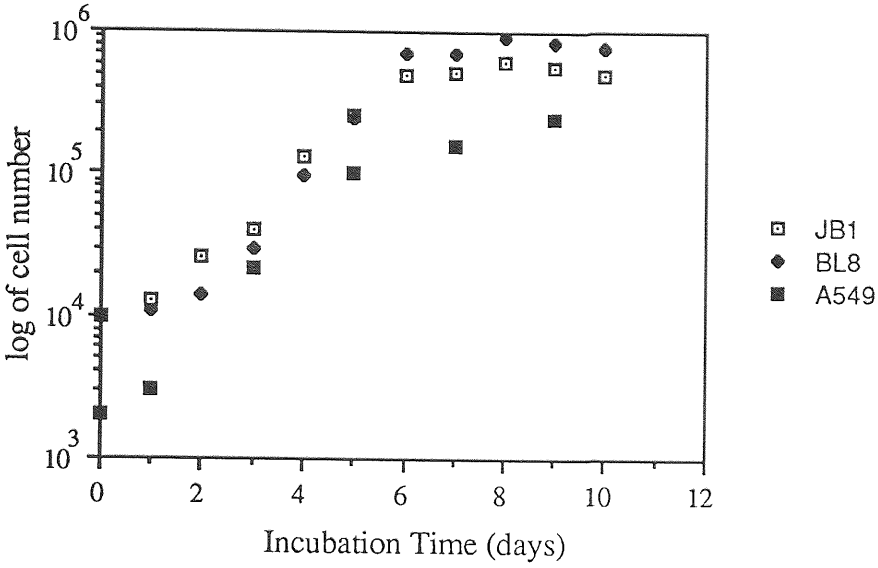
3.1. Growth Characteristics

BL8 and JB1 cells divided slightly more rapidly than A549 cells (Fig. 43a, p114). BL8 were smaller than JB1 cells and had the greatest cell density at confluency. JB1 cells continued to grow in the absence of serum, but at a much diminished rate compared to their growth in the presence of serum (Fig.43b, p114). All three cell lines grew readily in 1% FCS for up to 72h.

Fig. 44a (p115) shows the effects of SRI, alcohol (**14**) and diol (**5**), on the growth of A549 cells, observed after 72h incubation. SRI was the most toxic compound (IC_{50} = 0.1μM) followed by alcohol (**14**) (IC_{50} = 60μM). Diol (**5**) up to 1mM had no significant effect on the growth of A549 cells. Mil was slightly more toxic than alcohol (**14**) with an IC_{50} of 32μM (Fig.44b, p115), but it was far less toxic than SRI. Mil at 100μM produced 100% inhibition of growth of A549 cells after 24h, but 10μM had little effect even after 72h (Fig. 44b, p115).

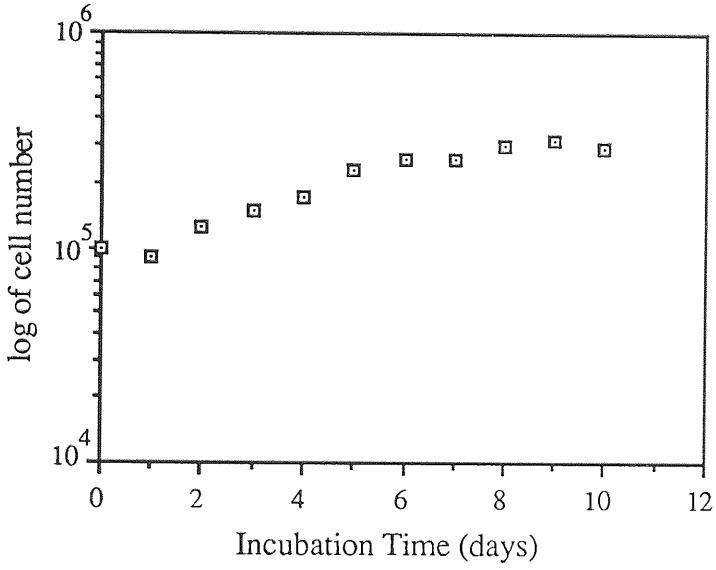
Dependence of growth inhibition on time was studied in A549, JB1 and BL8 cells. Results obtained for JB1 cells are shown in Fig. 45 (p116). SRI produced 100% toxicity within 15min at 100μM and within 24h at 10μM (Fig. 45a, p116). Alcohol (**14**) (100μM) required 72h for 100% inhibition of cell growth, lower concentrations had no effect (Fig. 45b, p116). Diol (**5**) had no significant effect on growth (Fig. 45c, p116). Similar results were seen in all three cell lines (Tables 4 - 6, p117 - 119).

Cells treated with SRI (10μM) were seen to swell prior to death. Swelling occurred very rapidly with SRI (100μM) and occasionally they were observed to "burst". Cells treated with alcohol (**14**) for 72h also became rounded and swollen. When cells burst, little debris remained and nuclei did not initially remain attached to the flasks as was the case after triton treatment



(n = 2, A549 SE = +/- 0.8)
(BL8 SE = +/- 3.3; JB1 SE = +/- 2.5)

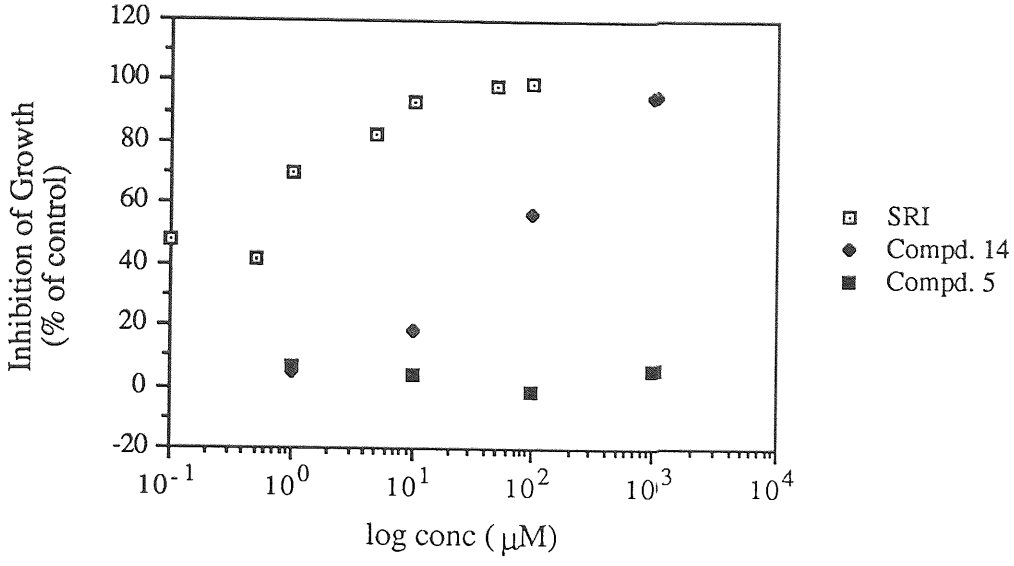
(a)



(n = 2, SE = +/- 0.3)

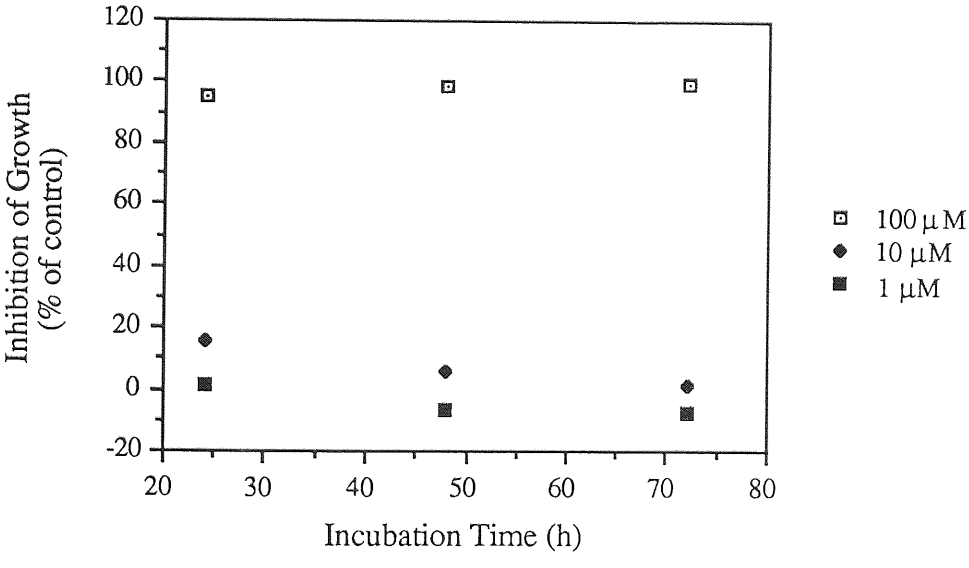
(b)

Fig.43 Growth curves: (a) A549, BL8 and JB1 cells
(b) JB1 cells in the absence of serum.



(n= 4 - 8, SE = +/- 0.9 - 14.8)

Fig. 44(a) The effect of SRI, alcohol (**14**) and diol (**5**) on the growth of A549 cells after incubation for 72h.



(n = 2, SE = +/- 1.0 - 13.6))

Fig. 44(b) Time course of effects of Mil on the growth of A549 cells.

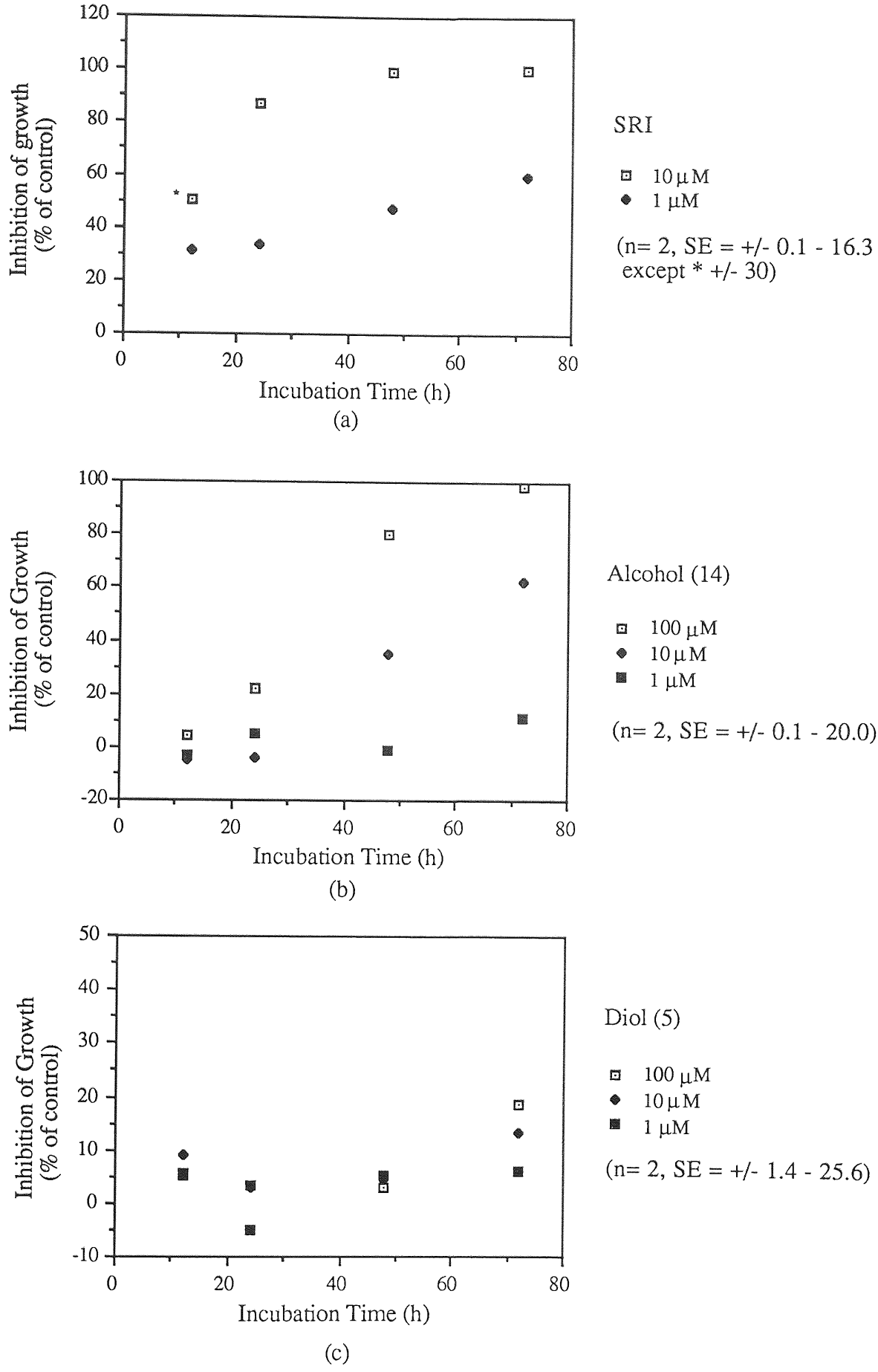


Fig. 45 Time dependence of inhibition of growth in JB1 cells induced by (a) SRI, (b) alcohol (**14**) and (c) diol (**5**).

SRI conc (μM)	Cell Type	Growth Inhibition (%) 12h	24h	48h	72h
10	A549	42.8+	85.2	98.3	99.0
	BL8	56.7*	88.0	99.6	99.8
	JB1	50.7**	86.9	99.4	99.8
1	A549	8.6	10.7	31.7	41.5
	BL8	7.5	19.9	50.0	39.5
	JB1	31.2	33.5	47.4	59.9

(n = 2)

(A549 SE = +/- 0.3 - 15.6 except + +/-24.1)
(BL8 SE = +/- 0 - 12.9 except * +/-22.8)
(JB1 SE = +/- 0.1 - 16.3 except ** +/-30)

Table 4 Comparison of the growth inhibiton caused by SRI (10 and 1 μM) in three cell lines.

Alcohol (14) conc. (μM)	Cell Type	Growth Inhibition (%) 12h	24h	48h	72h
100	A549	24.5	36.7	79.7	96.1
	BL8	13.0	28.1	90.9	99.2
	JB1	4.5	22.3	80.0	98.5
10	A549	13.4	-3.4*	11.6	8.7
	BL8	8.2	3.4	15.1	31.4
	JB1	-4.7	-3.7	34.9	62.8
1	A549	11.7	11.0	18.6	20.0
	BL8	18.6	5.2	25.0	12.9
	JB1	-2.9	5.3	-0.6	11.6

(n = 2)
(A549 SE = +/- 0.1 - 19.8 except * +/- 23.5)
(BL8 SE = +/- 0.3 - 24.3)
(JB1 SE = +/- 0.1 - 20.0)

Table 5 Comparison of effects on growth of alcohol (**14**) in three cell lines.

Diol (5) conc. (μM)	Cell Type	Growth Inhibition (%) 12h	24h	48h	72h
100	A549	-3.0	4.8	7.6	9.3
	BL8	-0.1	4.4	7.2	6.0
	JB1	5.7	3.2	3.1	18.7
10	A549	-3.7	-4.5	0.0	-0.5
	BL8	3.1	-0.1	5.9	-4.1
	JB1	9.2	3.1	4.7	13.3
1	A549	5.9	10.4	11.3	-1.0
	BL8	9.4	6.0	4.1	-1.6
	JB1	5.2	-5.2	5.4	6.1

(n = 2)

(A549 SE = +/- 1.0 - 13.6)
(BL8 SE = +/- 1.6 - 17.5)
(JB1 SE = +/- 1.4 - 25.6)

Table 6 Comparison of growth inhibitory effects of diol (**5**) in three cell lines.

3.2. Cytotoxicity as indicated by the MTT Assay

The results from the growth curve analysis suggest that all three cell lines respond in a similar manner to the ether lipids and their potential metabolites. SRI was more toxic than Mil, alcohol (**14**) or diol (**5**). In addition, using the MTT assay, all three cell lines exhibited closely similar trends (Table 7, p123). Therefore, only the results obtained with JB1 cells will be discussed in detail. Drug induced effects on the viability of JB1 cells are shown in Fig. 46 (p121). SRI was the most toxic of the compounds tested with an IC_{50} of 6 - 9μM. Compounds (**14**), (**21**) and (**5**), the proposed metabolites of SRI, had lower IC_{50} values: 40 - 70μM, >100μM and >200μM, respectively. Water soluble phosphate (**21**) showed a greater degree of toxicity than the hydrophilic diol (**5**) (Fig. 46 c and d, p121), but their IC_{50} values were not calculated. Similar trends were observed in BL8 and A549 cells (Table 7, p123).

Mil exhibited an IC_{50} of 50μM in BL8 cells and of 37μM in JB1 cells. The toxicity of the proposed metabolites of Mil, ie hexadecanol and phosphate (**25**), were examined only in JB1 cells (Fig. 47, p122). Mil was more toxic than phosphate (**25**), which showed an IC_{50} of 140μM, but was slightly less toxic than hexadecanol, which exhibited an IC_{50} of 28μM.

The IC_{50} value for SRI in JB1 cells decreased from 9μM in the presence of serum to 2μM in its absence (Table 8, p123). Similarly the toxicity of Mil increased on incubation in the absence of serum (IC_{50} = 20μM). The toxicity of alcohol (**14**) also increased on removal of serum, with its IC_{50} decreasing from 45 to 20μM. The presence or absence of serum did not affect the toxicity of diol (**5**).

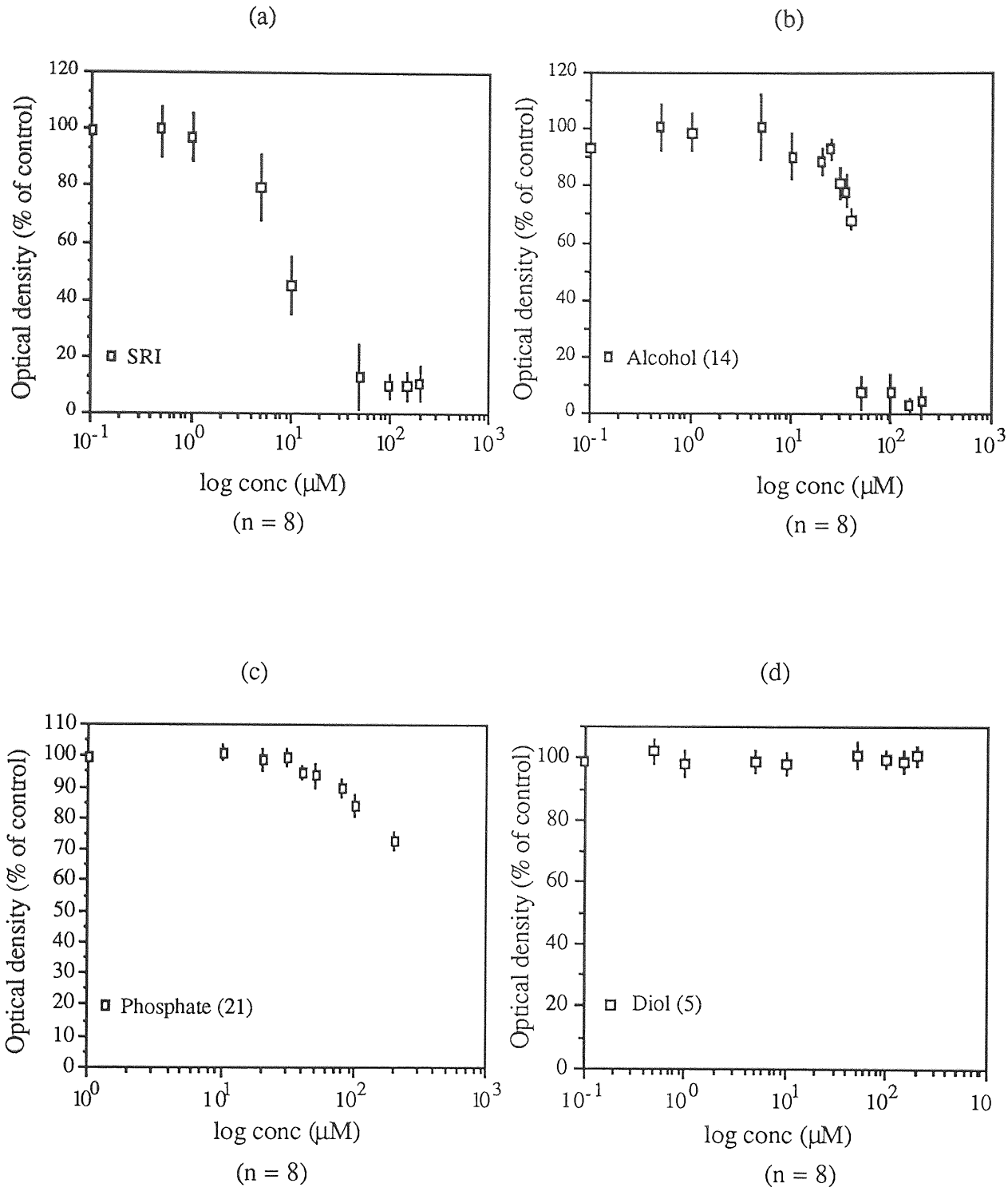
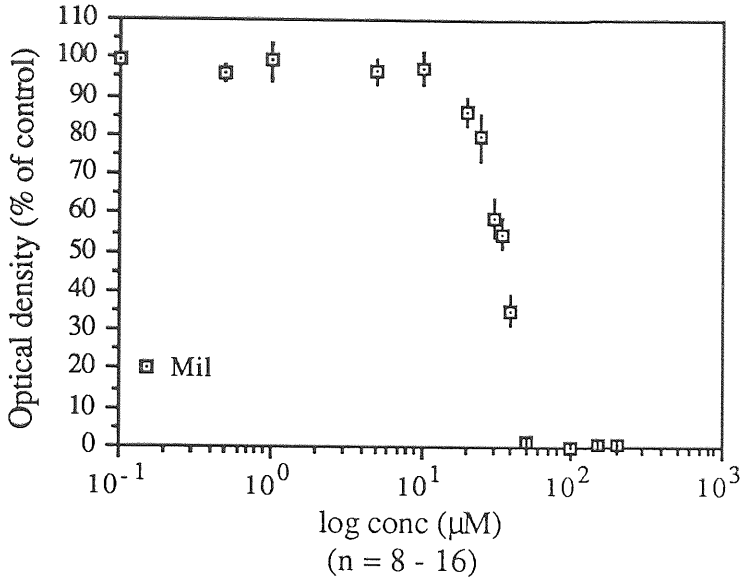
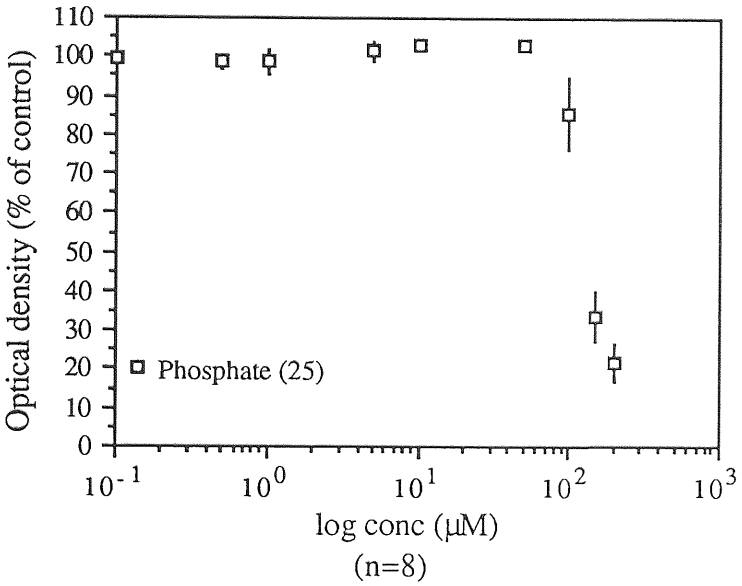


Fig. 46 Cytotoxicity of SRI and its putative metabolites in JB1 cells measured using the MTT assay at 72h





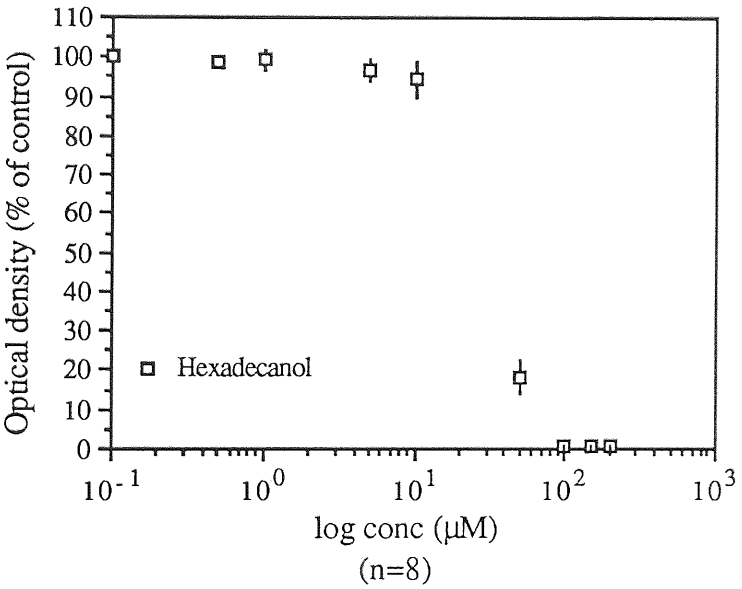


Fig. 47 Cytotoxicity of Mil and its putative metabolites in JB1 cells measured by the MTT assay at 72h.

Compound	IC_{50} (μM) A549	n	BL8	n	JB1	(n=8)
SRI	6	(6)	7	(7)	9	
(14)	70	(8-16)	40	(6)	45	
(5)	>200	(4)	>200	(8)	>200	
Mil			50	(8)	37	

(BL8 SE = +/- 0.1 - 12. A549 SE = + / - 1 - 26)

Table 7 Comparison of IC_{50} values calculated from results of MTT assay (Number of experiments in brackets).

Compound	IC_{50}(μM) + Serum	(n=8)	- Serum	(n=8)
SRI	9		2	
(14)	45		20	
(5)	> 200		> 200	
Mil	37		20	

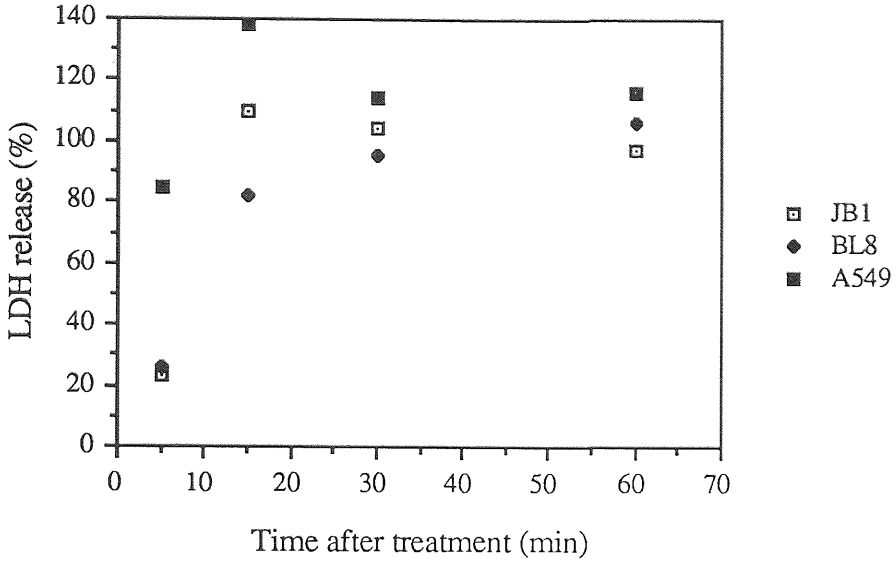
(+ serum SE = +/- 2.2 - 11.2, - serum SE = +/- 0.6 - 9.2)

Table 8 Comparison of IC_{50} values of compounds in JB1 cells in the presence and absence of serum, measured by MTT assay.

3.3. Cytotoxicity as indicated by the LDH assay

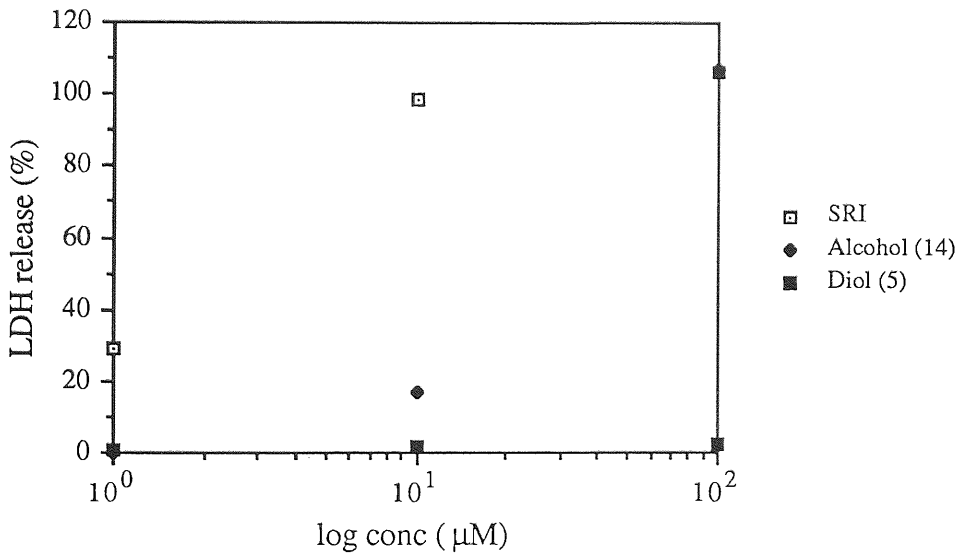
The LDH assay was used to observe the time course of the development of toxicity of SRI and its putative metabolites, alcohol (**14**) and diol (**5**). The method allowed detection of toxicity earlier or at similar times compared to the other assays used. Unlike the MTT assay, assessment of viability using LDH release allows easy monitoring of the kinetics of cell death. The effect of SRI, alcohol (**14**) and diol (**5**) on the viability of A549, JB1 and BL8 cells was tested at periods between 1 and 72h.

The three cell lines tested produced a similar pattern of LDH release in response to compound treatment. SRI (100μM) produced 100% release of LDH within 15min (Fig. 48, p125). The IC_{50} value for SRI in JB1 cells at 72h was 2μM, for alcohol (**14**) it was 24μM and for diol (**5**) >100μM (Fig. 49, p125). Table 9 (p128) shows a comparison of the IC_{50} values calculated for SRI and its proposed metabolites in three cell lines. Within 24h SRI (10μM) produced 100% LDH release, and it still had an effect at 1μM (Fig. 50, p126). Alcohol (**14**) did not act as rapidly as SRI and it was not as toxic. Incubation with alcohol (**14**) for 72h was required to produce 100% LDH release and 10μM had little effect (Fig. 51, p127). Diol (**5**) produced very little LDH release even after 72h incubation, so only the results for JB1 cells are shown (Fig. 52, p128).



(JB1 n=3, SE=+/- 2.2-17.6, BL8 n=3, SE=+/- 6-9.1)
(A549 n=4, SE=+/- 11.7-18.8)

Fig. 48 Time course of cytotoxicity of SRI (100 μM) in JB1, BL8 and A549 cells, expressed as a percentage of LDH released after addition of triton.



(n = 3, SE = +/- 0.2 - 21.4)

Fig. 49 Cytotoxicity of SRI and its putative metabolites in JB1 cells after 72h incubation, expressed as percentage LDH released after triton addition.

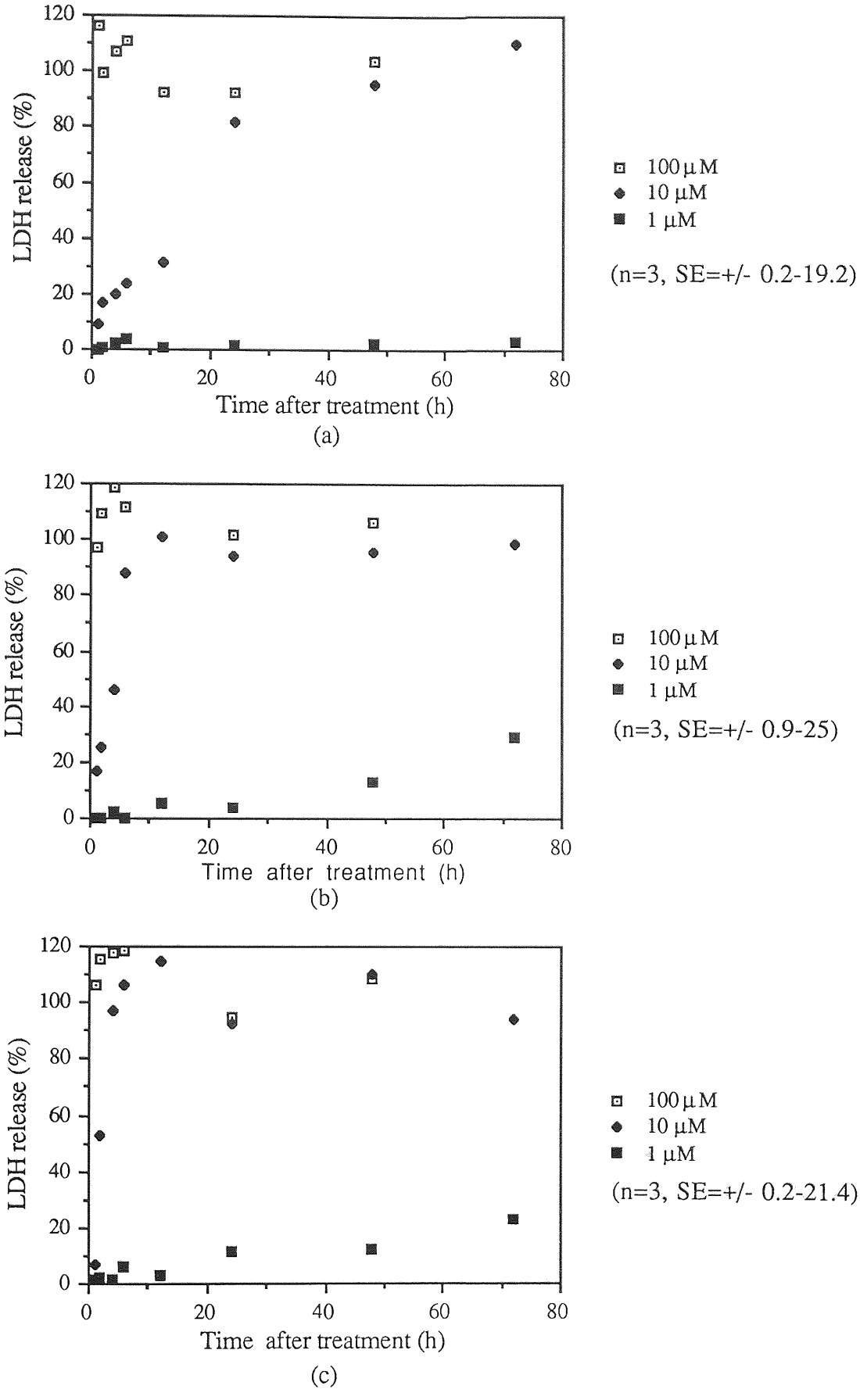


Fig. 50 Time dependent cytotoxicity of SRI in (a) A549 cells (b) JB1 cells and (c) BL8 cells, expressed as percentage LDH released after addition of triton.

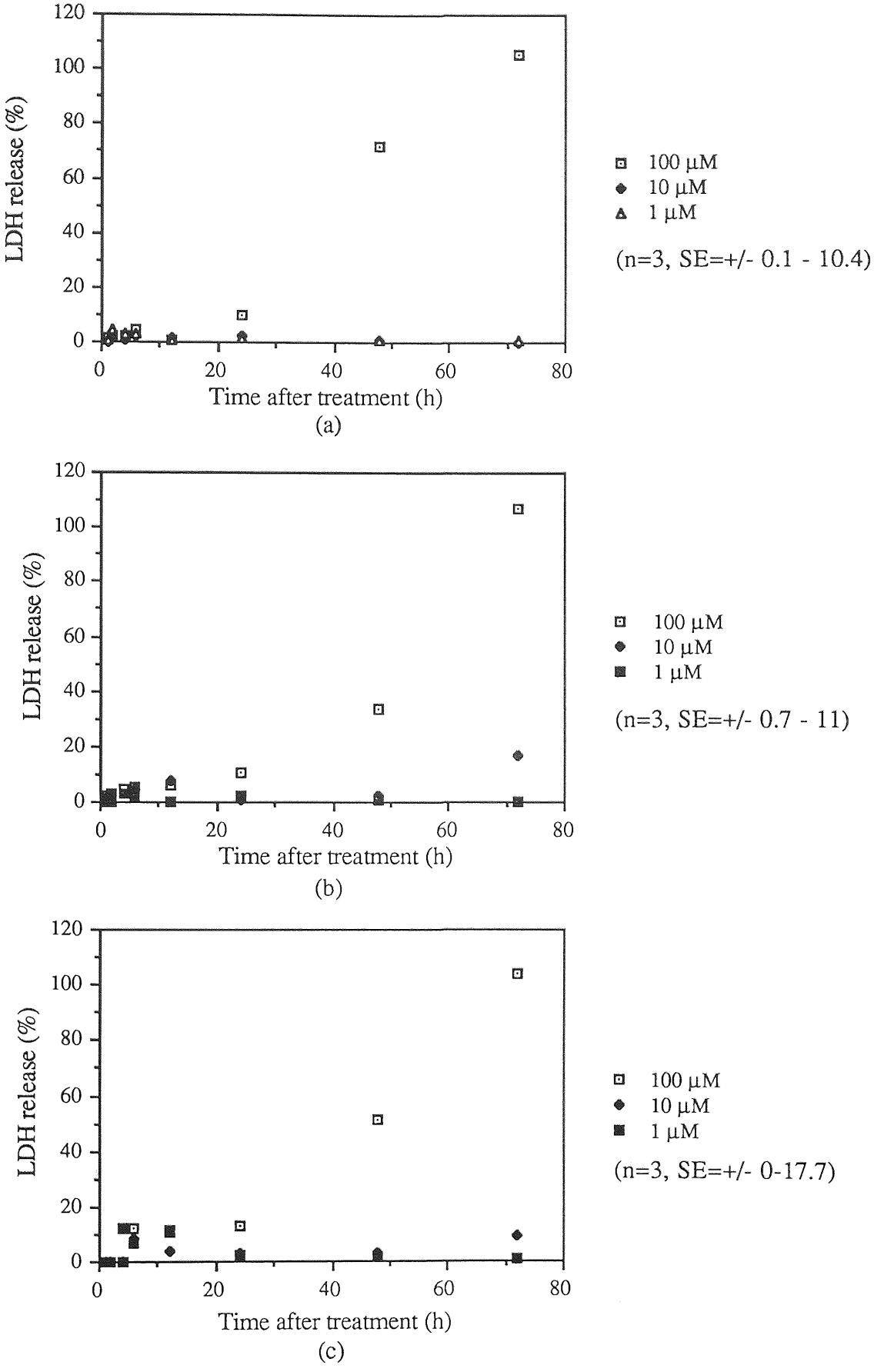


Fig. 51 Time dependent cytotoxicity of alcohol (**14**) in (a) A549 cells (b) JB1 cells and (c) BL8 cells, expressed as a percentage LDH released after the addition of triton.

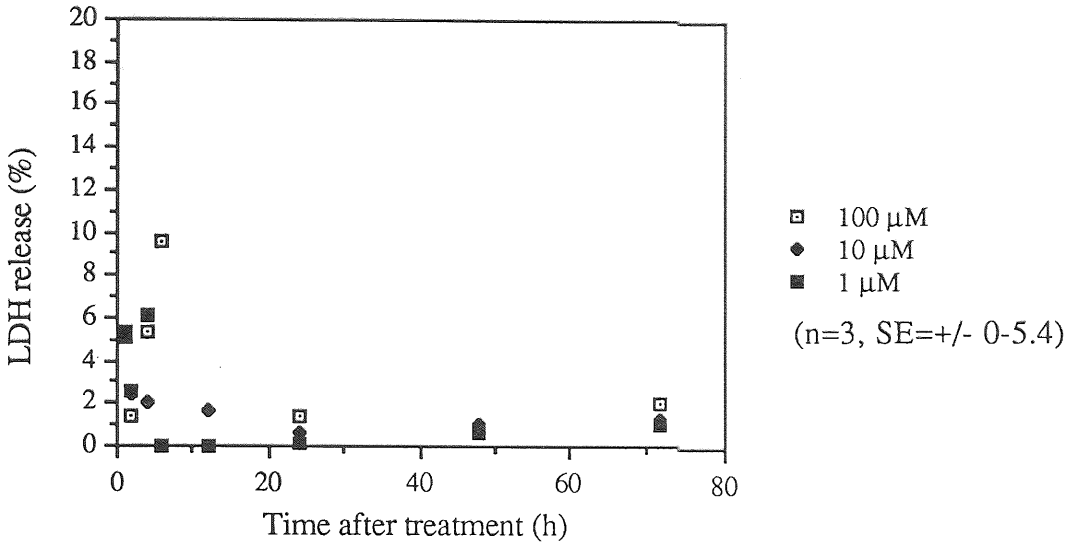


Fig. 52 The effects of diol (5) on LDH release in JB1 cells, expressed as a percentage of LDH released after addition of triton.

	IC_{50}(µM)		
Compound	A549	BL8	JB1
SRI	2.8	2.5	2.0
(14)	29.0	27.0	24.0
(5)	>200	>200	>200

(JB1 n=3, SE = +/- 0.7 - 25.5. BL8 n = 3, SE = +/- 0.2 - 21.4)

(A549 n = 3, SE = +/- 0.5 - 19.2)

Table 9 Comparison of IC_{50} values determined by the LDH assay after 72h incubation with compound.

4. DISCUSSION

The aim of the tissue culture experiments described here was to compare the toxicity of SRI and Mil with that of their putative metabolites. Three cell lines were used to observe if there was any difference in the response to ether lipids of i) two malignant cell lines derived from different tissues (lung and liver) and ii) transformed and non-transformed cells isolated from the same tissue (liver). The experiments presented in this chapter were designed to explore the hypotheses that metabolism of SRI and Mil is a detoxification process and that lipophilicity is important for cytotoxicity. The use of three different cytotoxicity assays was aimed at confirming the identification of the membrane as the site of toxic attack of parent compounds SRI and Mil.

The three cell lines used responded in a similar way to SRI, its proposed metabolites (Table 10, p130) and to Mil. This fact is consistent with the notion that these lines do not metabolise SRI extensively. If the liver-derived cells were able to metabolise SRI, JB1 and BL8 cells would have a different toxicity profile when compared with A549 cells. A decrease in toxicity would occur as metabolite (**14**) is less toxic than SRI. SRI was not selectively toxic towards liver tumour cells as it was practically equitoxic to BL8 cells derived from normal liver. However, it is conceivable that *in vivo,* SRI would not be as toxic to liver cells as it was to BL8 cells *in vitro*. Primary hepatocytes are very different in their ability to metabolise compounds compared to cultured BL8 cells. SRI could undergo metabolism by hepatocytes which would not occur in the BL8 cells which have lost most of their metabolic capacity. Mil at high concentrations has been shown to cause liver toxicity in rats [Muschiol et al, 1987]. The demonstration of toxicity of SRI towards BL8 cells may mean that it will also be hepatotoxic *in vivo*. The results presented here suggest that during clinical trials with SRI and Mil, patient liver function should be monitored to detect early signs of hepatotoxicity.

The three cytotoxicity assays, growth curves, MTT and LDH produced similar results for SRI and its potential metabolites (Table 10, p130). LDH appeared more sensitive than the cell growth assay to SRI toxicity at high concentrations and at time points prior to 24h. This sensitivity was probably related to the fact that the growth inhibition results were dependent on control cell number, and prior to 24h cell number did not increase greatly. The rapid release of LDH by high concentrations of SRI (100μM) is consistent with a "detergent-like", ie biophysical, mode of action on the plasma membrane. At lower concentrations of SRI, LDH was released more slowly and this feature suggests that a more "biochemical" effect might be occurring. Similarly, alcohol (**14**) did not appear to act like a detergent. LDH is a large enzyme which is presumably only released as a consequence of gross membrane damage. The MTT assay detects more subtle interference

with cell biochemistry. The similarity in the results for the LDH and MTT assays is consistent with the plasma membrane being the site of toxicity for SRI.

Compound	Method	IC_{50} (μM) A549	BL8	JB1
SRI	Growth inhibition	0.1	1.5	0.6
	MTT assay	6.0	7.0	9.0
	LDH assay	2.8	2.5	2.0
(14)	Growth inhibition	60.0	28.0	55.5
	MTT assay	70.0	40.0	45.0
	LDH assay	29.0	27.0	24.0

Table 10 Comparison of IC_{50} values calculated at 72h for three cell lines by three different assays.

The results from the three cell lines and three assays show that SRI was the most potently cytotoxic of the compounds tested. In Table 10, the IC_{50} values for SRI and alcohol (**14**) are compared in three cell lines as measured by the different assay systems, after 72h incubation. Alcohol (**14**) also showed some toxicity but it was consistently less potent than SRI and required a longer incubation period to elicit its activity. The IC_{50} for alcohol (**14**) was similar to that of PAF in HL60 cells (59μM) found by Dive et al [1991]. The consequence of the direct cytotoxicity of either agent *in vivo* is presumably different. PAF undergoes rapid inactivation by metabolism, which is unlikely to occur with alcohol (**14**). There was a degree of variation between assays and cell types but the results showed similar trends. This fact seems to indicate very little difference in cell sensitivity to the

compounds tested. Diol (**5**) was relatively non-toxic up to 1mM in the three cell lines and assays tested and hence its results are not given in Table 10. Since diol (**5**) was not toxic to any of the cell lines, metabolism of SRI appears to be a detoxification process. It must be noted that diol (**5**) was not detected during metabolism studies, chapter 3.

Treatment with SRI, alcohol (**14**) and Mil caused changes in cell morphology, cells became swollen and rounded. Change in cell size and membrane permeability to fluorescent dyes has previously been demonstrated by flow cytometry [Dive et al, 1991]. Growth curves indicated that SRI was more toxic than Mil, and that Mil (100μM) produced its toxicity more slowly than SRI (100μM). The differences in the toxicity of SRI and Mil and the variation in time it took for toxicities to emerge suggest that they may act on different sites in the membrane or have different permeability characteristics.

The MTT assay was used to compare the toxicities of SRI and Mil with that of their metabolites (Figures 46, p121 and 47, p122 respectively). The IC_{50} value calculated for Mil was similar to that observed in NIH3T3 cells (65μM) by Uberall et al [1991], but higher than that seen by Unger et al [1987] in HL60 cells (12.5μM), and U937 cells (7.35μM). The IC_{50} was similar to that of 36.8μM found in ether lipid-resistant K562 cells by Unger et al [1987]. The metabolism of Mil to its phosphate (**25**) seems to be a detoxification route. However, the metabolite of phosphate (**25**), hexadecanol, produced by phosphatase-catalysed metabolism was as toxic as the parent compound. Therefore, only one of the studied metabolism routes of Mil leads to detoxification.

The three cell lines and three cytotoxicity assays produced similar results. Mil appeared as toxic to JB1 cells as A549 and BL8 cells. So the toxicity of Mil and its potential metabolites were only measured in the MTT assay. The MTT assay was chosen as it allowed comparison of a large number of drug concentrations on one plate, and plates were read automatically. For similar reasons the MTT assay was used to compare effects of serum on the toxicity of SRI, Mil, alcohol (**14**) and diol (**5**) (Table 8, p123). Absence of serum from the MTT assay reduced variability of the results.

The IC_{50} value calculated in the MTT assay for SRI in the presence of serum (p123) was similar to the ID_{50} (8μM) calculated for HL60 cells by Lazenby et al [1990]. The IC_{50} value for SRI in JB1 cells, in the absence of serum, was similar to that found for SRI in HL60 cells (2.5μM) [Dive et al, 1991]. This decrease in IC_{50} value was probably due to an increased availability of free SRI in the absence of serum, caused by decreased serum protein binding. Alcohol (**14**), the metabolite of SRI, may also bind to protein, as its IC_{50} value decreased from 45 to 20μM on exclusion of serum (see Table 8, p123). Alcohol (**14**)

was far less toxic than SRI in the presence and absence of serum. Toxicity of diol (**5**), the hydrophilic putative metabolite of SRI, was unaffected by the presence of serum. Toxicity of Mil increased in the absence of serum which suggests that it could bind to serum proteins. These results are consistent with those reported by Uberall et al [1991].

The LDH assay produced similar IC_{50} values as the other assays for SRI, alcohol (**14**) and diol (**5**) at 72h. This assay was also used to monitor emergence of toxicity of these compounds with time. Diol (**5**) was not toxic at the concentrations tested for up to 72h. SRI (100μM) produced 100% toxicity within 15min, but at 10μM, 100% toxicity required 12 - 24h incubation. Alcohol (**14**) at 100μM required 72h incubation for toxicity and at 10μM it exhibited little effect. The speed with which SRI (100μM) produced its toxicity in cells was consistent with the membrane as a target for toxicity and suggests a detergent-like activity. Similarity in the structures of PAF, SRI and known detergents suggests that they may have a detergent-like action. PAF has been reported to act as a general membrane perturbant at concentrations above 4μM, causing substantial disturbance of the lipid bilayer [Dive et al, 1991]. At low concentrations of ether lipids their biochemical effects may predominate, but at higher exposure levels a more severe detergent-like interaction may occur. Modulation of SRI activity by a PAF antagonist [Bazill and Dexter, 1989] and inhibition of SRI toxicity by phorbol esters [Thompson and Hickman, 1988] suggest the potential for either receptor mediated or other specific cell signalling effects, rather than the more general detergent-like perturbation [Dive et al, 1991].

The mode of action of antineoplastic agents of the ether lipid type is still subject to much discussion. Several hypotheses have been extended to address the mechanism of action of this novel group of agents. Ether lipids have been postulated to produce their toxic activity by action on the membrane (chapter 1, section 2.4.2. p19). Unger et al [1987] suggested that once they have accessed the membrane, ether lipids are activated by cleavage of the phosphatidylcholine moiety. In contrast, Vallari et al [1988] proposed that the toxicity of ether lipids is not related to the production of the long chain alcohol by metabolism. The fact that Mil and its alcohol metabolite, hexadecanol, showed comparable cytotoxicity could be consistent with the ideas of Unger et al [1987]. It is conceivable that a difference in the rate of penetration into the membrane masks the actual toxic potential. Mil might be taken up into cells much more rapidly than hexadecanol. So if hexadecanol was more inherently cytotoxic than Mil, its slower access to the site of action might render this increased cytotoxicity less apparent.

In the case of SRI the proposal of Unger et al [1987] does not appear to be applicable as the parent ether lipid was markedly more toxic than its metabolite alcohol (**14**). It is however

possible that the presence of the bulky THF moiety in the structure of alcohol (**14**) could greatly impede its uptake into cells. SRI was more toxic than Mil and this may be related to their structural differences. These compounds may act at different sites within the membrane, which could also help to explain differences in toxicity profiles of their potential metabolites. SRI more closely resembles the "optimal structure" for cytotoxic activity as described in chapter 1 (section 2.3., p16). Mil does not possess a glycerol backbone, which has been described as essential for cytotoxic activity [Hoffman et al, 1984].

Cellular uptake of ether lipids is thought to be an important factor in their cytotoxicity [Hoffman et al, 1986]. ET-18-OCH_3 was shown to accumulate at the membranes of sensitive HL60 cells. In K562 cells which are resistant to ether lipid cytotoxicity, ET-18-OCH_3 was more evenly distributed throughout the cell. Ether lipids appear to be preferentially sequestered in the plasma membrane of sensitive cells. Once within the membrane ether lipids could inhibit a range of vital cell functions such as the lipid metabolising enzymes involved in assembly of membranes. They could also inhibit Ca^{2+} phospholipid dependent protein kinases or inhibit transport of required nutrients [Hoffman et al, 1986]. To inhibit protein kinase C [Shoji et al, 1988] or intracellular $[Ca^{2+}]_i$ signalling [Seewald et al, 1990] ether lipids would have to interact with membrane components. Ether lipids may inhibit cell signalling by interaction with phosphatidylinositol specific phospholipase C [Seewald et al, 1990; Uberall et al, 1991]. Ether lipids have been shown to interact with sodium binding sites of Na-K-ATPase, located at the intracellular surface of the membrane and this could be another site for their cytotoxic action [Zheng et al, 1990]. Ether lipids increase the fluidity of the plasma membrane [Noseda et al, 1988]. They partition into the phospholipid domain of the membrane and change its physical properties. The increase in fluidity can affect many membrane associated enzymes and seems to be responsible for the antiinvasive effects of ether lipids [Noseda et al, 1988; Storme et al, 1985]. Ether lipids could either affect signalling enzymes directly or they could alter membrane properties and so inhibit these enzymes indirectly.

The toxicity profiles of SRI, Mil and their putative metabolites support the hypothesis that lipophilicity is an important determinant in their cytotoxic activity. This hypothesis is consistent with the membrane as the site of their toxic activity. SRI and Mil are zwitterionic and they can both penetrate the membrane to initiate their toxic action. SRI was more toxic than Mil which is probably related to the fact that Mil does not have a glycerol like structure. Phosphates (**21**) and (**25**) are dianionic and hydrophilic, they would be unlikely to enter the membrane as readily as SRI and Mil. The phosphates were only slightly toxic (Figures 46b, p121 and 47b, p122 respectively). Similarly diol (**5**) is very hydrophilic and not very toxic. Alcohol (**14**) and hexadecanol are lipophilic so can penetrate the membrane,

but without the phosphatidylcholine moiety they would perhaps interact to a lesser extent with charged phospholipids of the membrane. These alcohols may be less readily taken up into the cell to act on cell signalling enzymes or they may alter membrane structure to a smaller extent.

In summary, metabolism of SRI is a detoxification process. Enzymic breakdown initially detoxifies Mil but then produces a metabolite which was as toxic as the parent ether lipid. The toxicity profiles of SRI, Mil and their metabolites demonstrate the importance of lipophilicity for cytotoxic activity. This feature is in accordance with the membrane being the site of toxic attack. At lower ether lipid concentrations, where rapid cytolysis is not seen, the detoxification of ether lipids via metabolism may be an important factor to be considered in the clinical use of these agents.

CHAPTER 5

MOLECULAR MODELLING

1. INTRODUCTION

Many compounds which are structurally related to PAF, differ in their susceptibility to metabolism catalysed by phospholipase C. Molecular modelling was used to compare the charges on the atoms at the potential site of enzyme attack, in a group of such compounds. The hypothesis was examined that a difference in metabolism could be explained by a variance in charge at the metabolic site of the compound. The compounds studied were 1-octadecyl-2-acetyl-glycero-3-phosphocholine (PAF), 1,2-dipalmitoyl-glycero-3-phospho - choline (I), 1-palmitoyl-glycero-3-phosphocholine (II), 1-octadecyl-2-methyl-glycero-3-phosphocholine (III), 1-hexadecyl-glycero-3-phosphocholine (IV), 1,2-dihexadecyl-glycero-3-phosphocholine (V), Mil and SRI. Mil does not have a glycerol backbone like PAF but has an alkyl chain attached to phosphatidylcholine moiety. PAF, (I) and (II) are readily metabolised by phospholipase C, (III) and (IV) are only slowly metabolised [Unger and Eibl, 1986] and (V) [Unger and Eibl, 1986], Mil and SRI (chapter 3) do not appear to be metabolised by this enzyme.

2. METHODS

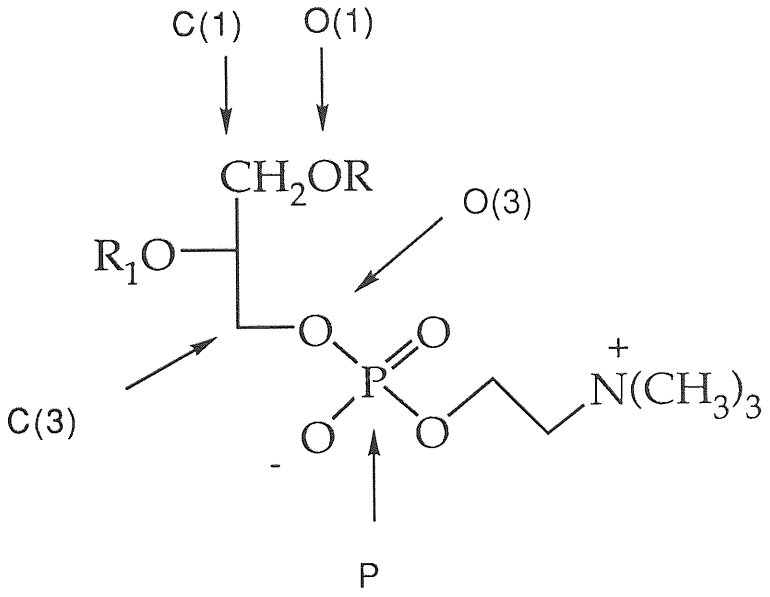
Calculations and modelling were performed with the aid of Dr B. Denny and Dr P. Lambert, Pharmaceutical Sciences Institute, Aston University, Birmingham, UK.

The atomic coordinates of the phosphatidylcholine fragment were obtained from the Cambridge Crystallographic Database, Daresbury Laboratories, SERC, Daresbury. The fragment was taken from the cytidine 5'-diphosphate choline monohydrate (4262 CDPCHM) crystal structure [Nakamachi et al, 1975]. Other fragments were obtained and modified as required using the structure building facility of the Chem-X molecular modelling package [Davies, 1986]. The long alkyl chains had to be confined to three carbons in length, to simplify calculations.

The geometry of the generated structures were optimised using the molecular mechanics routines available in Chem-X, followed by semi-empirical quantum mechanics routines MNDO and PM3 within MOPAC [Stewart, 1983]. The PM3 routine was chosen specifically for phosphorus containing compounds. The Chem-X modelling software was run on a Vax 8650 computer and structures were displayed on a Sigmex 5688 terminal. Chem-X was also used to superimpose the optimised structures of SRI and Mil on PAF, to demonstrate the similarities between their structures.

3. RESULTS

Fig. 53 shows the general structure of the compounds tested and the number of the atoms compared for charge differences. Figures 2a and 2b (p 16) show the structures of SRI and Mil, respectively. Table 11 shows the charges on specific atoms calculated by the MNDO method. Table 12 shows the same results calculated by the PM3 method. Plates 1 and 2 show the structures of PAF and SRI. Plates 3 and 4 show the superimposing of the structures of SRI and Mil on that of PAF (respectively).



	R	R_1
PAF	Octadecyl	Acetyl
I	Palmitoyl	Palmitoyl
II	Palmitoyl	--
III	Octadecyl	Methyl
IV	Hexadecyl	--
V	Hexadecyl	Hexadecyl

Fig. 53 Compounds compared in the molecular modelling study, with the atoms labelled, whose charges were calculated.

Compound	Calculated Charge				
	O(3)	C(3)	P	C(1)	O(1)
PAF	-0.504	0.169	1.366	0.113	-0.323
I	-0.504	0.169	1.366	0.113	-0.323
II	-0.509	0.152	1.367	0.100	-0.320
III	-0.500	0.170	1.370	0.110	-0.330
IV	-0.546	0.200	1.348	0.117	-0.335
V	-0.500	0.151	1.365	0.102	-0.315
Mil	-0.533	0.211	1.343	0.022	
SRI	-0.503	0.151	1.365	0.110	-0.321

Table 11 Charge calculations made using the MNDO routine, within MOPAC.

Compound	Calculated Charge				
	O(3)	C(3)	P	C(1)	O(1)
PAF	-0.667	0.135	2.098	-0.010	-0.249
I	-0.657	0.142	2.093	0.041	-0.267
II	-0.665	0.103	2.101	0.026	-0.324
III	-0.660	0.110	2.090	0.007	-0.270
IV	-0.673	0.106	2.102	0.047	-0.366
V	-0.651	0.118	2.092	0.034	-0.279
Mil	-0.655	0.142	2.088	-0.133	
SRI	-0.652	0.140	2.092	0.072	-0.282

Table 12 Charge Calculations made using the PM3 routine within MOPAC.

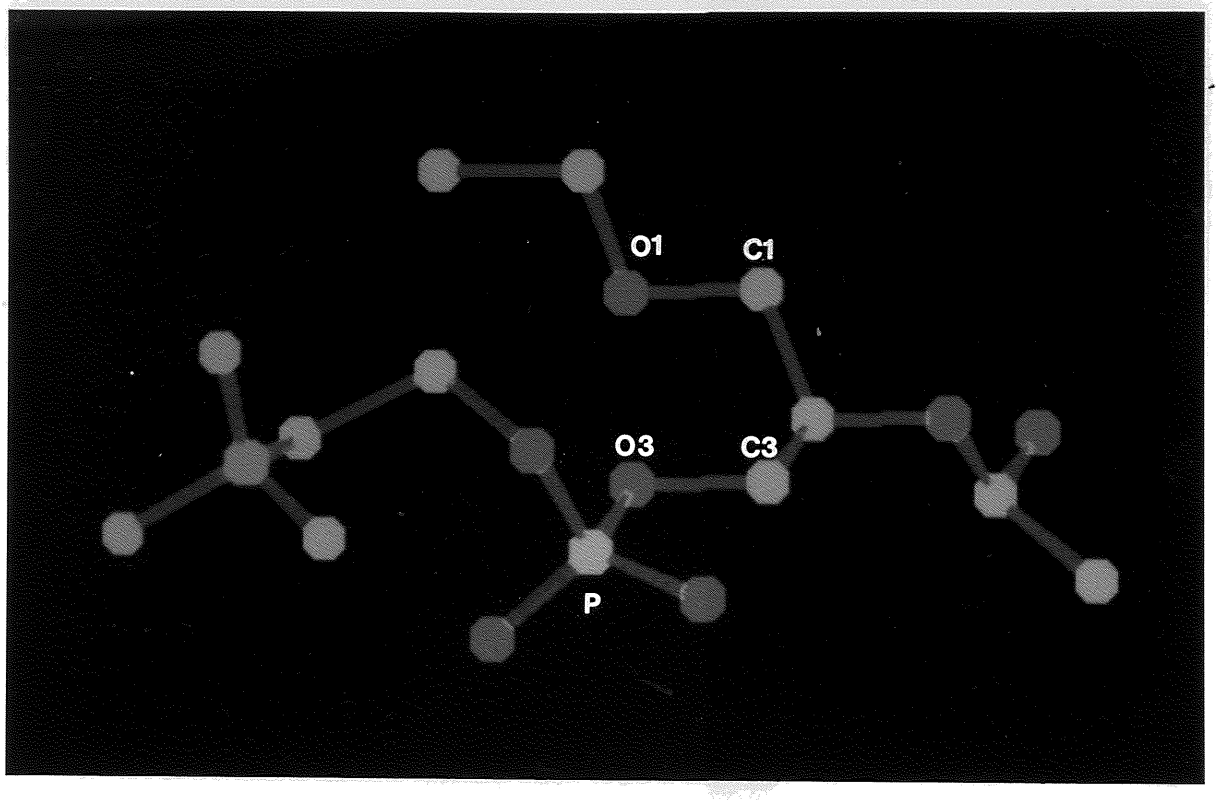


Plate 1. Structure of PAF

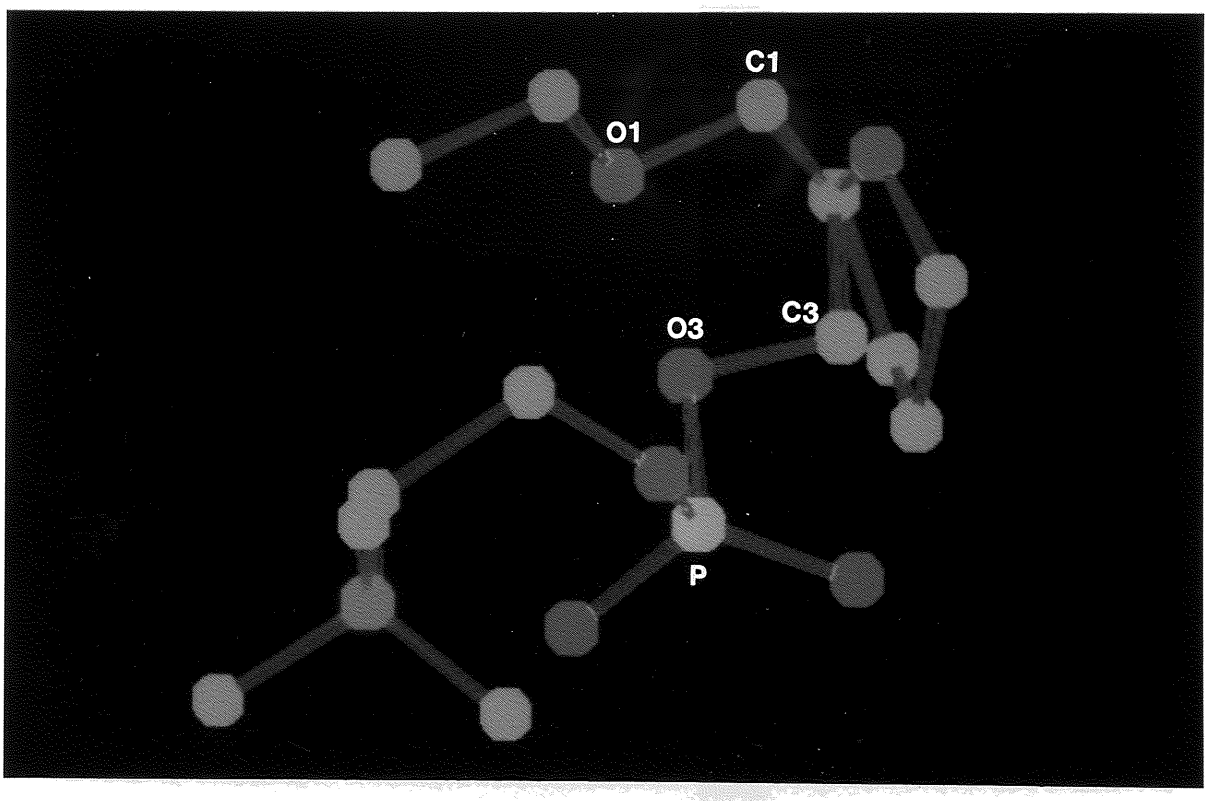


Plate 2. Structure of SRI

(Alkyl chains are shortened to three carbon atoms in length for ease of calculation. Carbon atoms are represented in light grey, nitrogen atoms are in blue, oxygen atoms in red and phosphorus atoms in yellow)

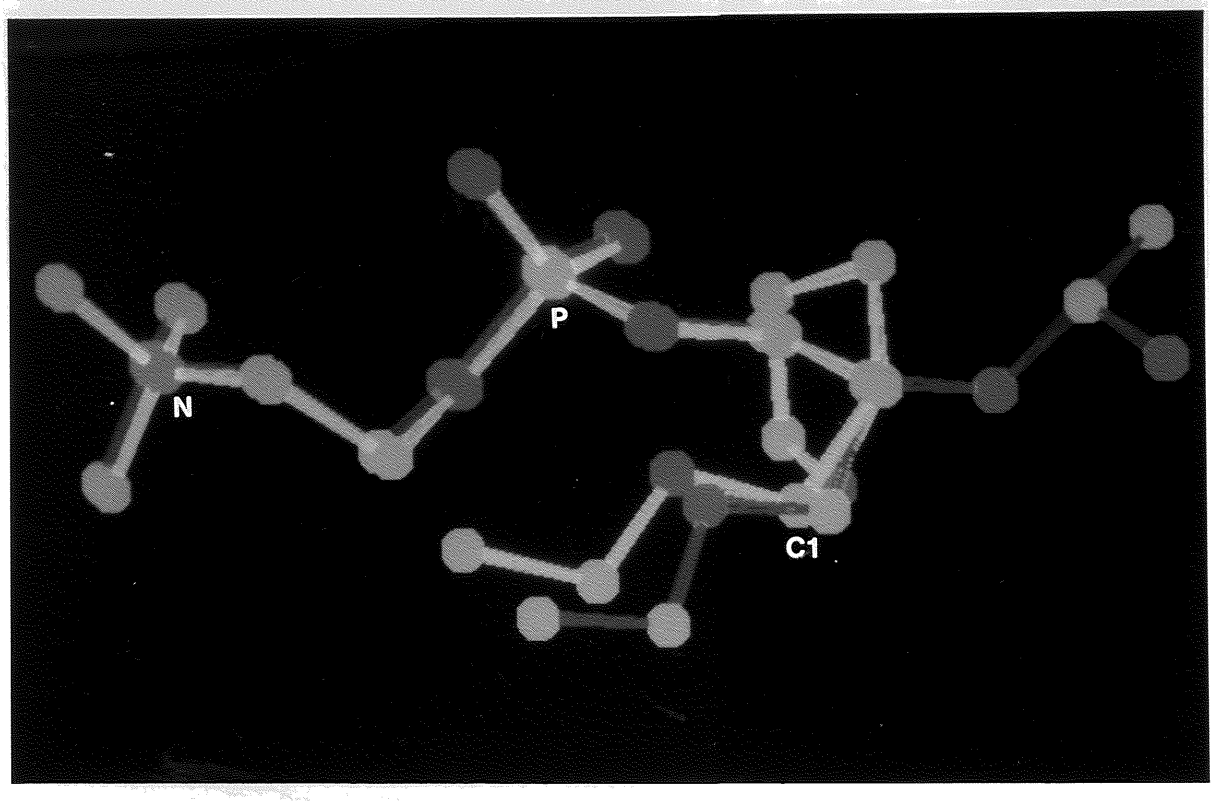


Plate 3. Structure of SRI superimposed on PAF

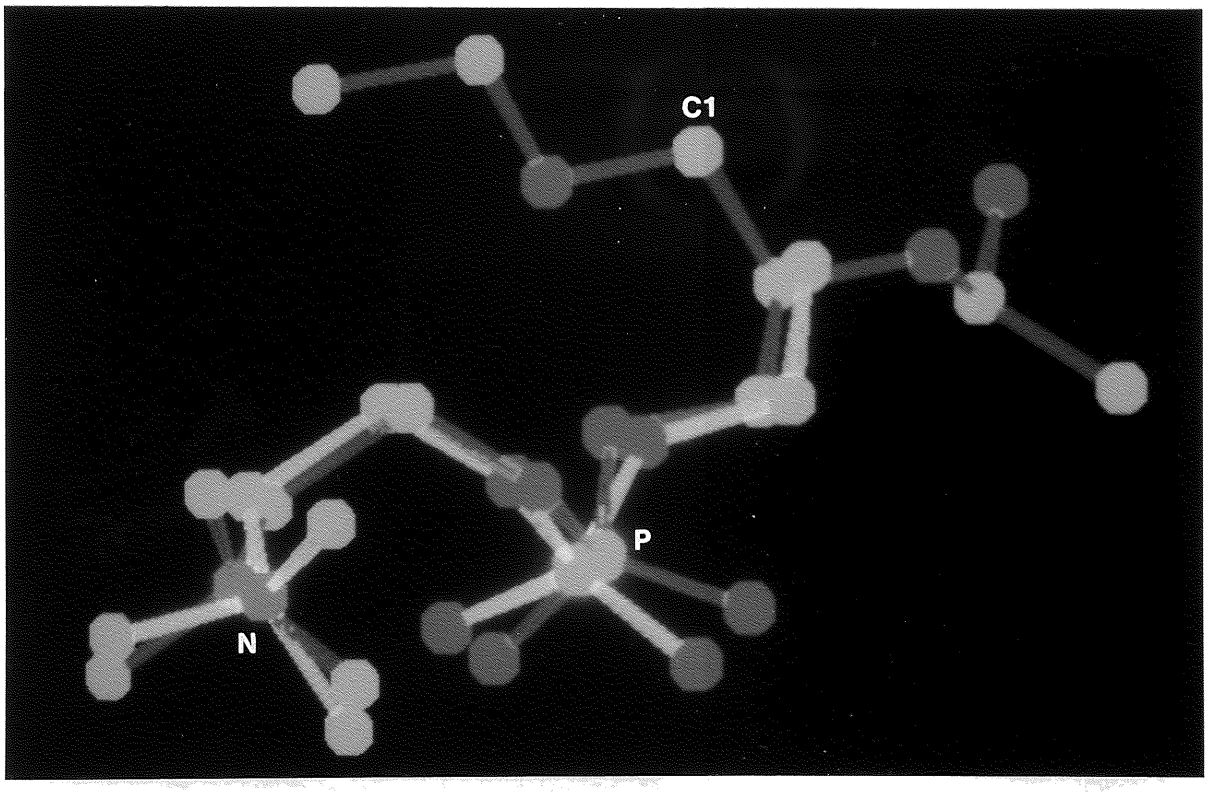


Plate 4. Structure of Mil superimposed on PAF

(Alkyl chains are represented as three carbon atoms in length. PAF is represented with green bonds, SRI and Mil have yellow bond. Carbon atoms are light grey, nitrogen atoms are dark blue, oxygen atoms, red, and phosphorus atoms are light blue.)

4. DISCUSSION

SRI, Mil and each of the compounds shown in Fig. 53 exhibited variation in their calculated charges on the studied atoms. However, there did not appear to be a trend which could explain the differences between the ability of phospholipase C to metabolise these compounds. Rate of metabolism did not appear to be related to the charge on phosphorus or on the atoms around the proposed site of enzyme attack.

The charges calculated for Mil were significantly different to those calculated for the other molecules. Mil, unlike the other compounds studied, has its alkyl chain directly attached to the phosphatidylcholine moiety rather than via a glycerol backbone. The lack of glycerol character probably affects binding and may explain the lack of metabolism of Mil mediated by phospholipase C. All the other compounds compared had an alkyl chain longer than C16, which would be required for hydrophobic binding together with a polar head group [Bomalski and Clark, 1987]. Differences in metabolism seem to be related to the substituent in position 2 of glycerol. Metabolism can occur if there is an ester or a hydroxy group in this position. A long alkyl ester can be cleaved by phospholipase A_2 prior to metabolism by phospholipase C [El-Sayed et al, 1985]. The environment around position 2 is important for binding. The difference between the rapidly and slowly metabolised compounds is the substituent in position 1 of the glycerol backbone. It has been shown that a compound with an ester substituent in position 1 is more rapidly metabolised than one with an ether substituent in this position [El-Sayed et al, 1985].

All the compounds studied have very flexible structures. The side chains of SRI can rotate reasonably freely. Similarly the side chains of PAF rotate readily around the carbon in position 2. Mil is a single chain and also has a large degree of flexibility. The optimised structures of SRI and Mil could be superimposed on that of PAF with a great degree of similarity. This fact suggests that SRI and Mil could occupy the enzyme active site in a similar way to PAF but due to the difference at position 2, could not complete binding with phospholipase C and so be metabolised.

CHAPTER 6

GENERAL DISCUSSION

The major objectives of this thesis were to study the metabolism of the ether lipid SRI and to characterise the cytotoxicity of its metabolites. Such studies may allow a better insight into the mechanism by which ether lipids exert their antitumour activity. Cytotoxicity testing could add to the existing mechanistic knowledge about SRI and might allow the identification of tumours which are susceptible to its affects. A better understanding of ether lipid metabolism and properties of their metabolites could be useful in the design of clinical trials and may increase awareness of their potential side effects. Metabolism of Mil, an antineoplastic ether lipid analogue, was also monitored to allow comparison with SRI. The toxicity of Mil and its putative metabolites was elucidated to support the conclusions drawn from experiments with SRI. In order to test the hypotheses discussed in chapter 1 (section 5, p34), SRI, its proposed metabolites and hexadecyl phosphate (**25**), a potential metabolite of Mil, were synthesised (chapter 2).

The original goals of the research were i) to clarify the metabolic pathways of SRI and ii) to indicate whether metabolism of SRI is a detoxification process. It was considered that the elucidation of the toxicity profiles of SRI, Mil and their proposed metabolites would either support or refute the hypothesis that the membrane is the site of ether lipid activity. These original aims have been partially fulfilled:

i) Metabolism of SRI by phospholipase D was clarified and the potential of liver cytosol to catalyse SRI metabolism was also demonstrated. Metabolism of SRI by AGMO was not detected. SRI may not be a substrate for the AGMO system but, as discussed in chapter 3 (p97) its primary metabolite, alcohol (**14**), may be. However the assay systems used did not detect any metabolism of alcohol (**14**). The reasons for the apparent absence of metabolism could be associated with several factors, as discussed in chapter 3. ^{31}P-NMR spectroscopy was used to show that SRI, Mil and PAF, all of which are structurally related (Figures 2a, 2b, p16 and 1a, p15), were metabolised by phospholipase D. Their phosphate products were subsequently metabolised by phosphatase on incubation with the isolated enzymes or with rat liver S9 (chapter 3). PAF was rapidly metabolised by phospholipase C, but SRI and Mil did not appear to be substrates for this enzyme. Ostensibly neither ether lipid inhibited this enzyme. Inhibition of inositol-specific phospholipase C has previously been reported for Mil [Uberall et al, 1991]. The lack of inhibition observed here may be due to variation in the enzyme source or the substrate specificity between the experiments reported by Uberall et al [1991] and those described in chapter 3. Another

difference between the experiments reported here and those previously outlined is the fact that the former were performed in cell free solutions and the latter in cell culture. It is possible that ether lipids could inhibit phospholipase C by altering its association with the cell membrane.

SRI, Mil, PAF, ether and ester lipids differ in their susceptibility to metabolism catalysed by phospholipase C. The hypothesis was tested that these differences were related to subtle variations in the charges on the atoms in these molecules. Using molecular graphics (chapter 5, p135) atomic charges on PAF, SRI, Mil and five other phospholipid molecules which undergo varying degrees of metabolism by phospholipase C, were calculated. There did not appear to be a trend in differences in charge, on the atoms studied, which would explain the variation in their susceptibility toward metabolism by this phospholipase. It is possible that the great difference in structure of the SRI and Mil molecules, compared to PAF, determine their liability to metabolism by phospholipase C. SRI has a bulky THF group in position 2 of the "glycerol backbone" and this is likely to interfere with its binding to the enzyme site. Mil does not have a glycerol-like structure and this feature may give it only poor binding to the catalytic site on the enzyme. Molecular modelling was used to demonstrate that the structures of SRI and Mil could be superimposed onto that of PAF. It is possible that SRI and Mil can enter the active site of phospholipase C but cannot bind properly to the enzyme active site in order to undergo metabolism.

In view of the work discussed here one could ask the question of how useful ^{31}P-NMR spectroscopy would be in the analysis of ether lipids and their metabolites in clinical trials. ^{31}P-NMR spectroscopy has the advantage over ^{1}H-NMR spectroscopy, *in vitro*, in that its spectra show fewer peaks, with one peak per substrate or metabolite. ^{1}H-NMR spectra of many compounds in aqueous solution are complex, with signals arising from the solvent and buffer as well as the compound under study. However, ^{31}P-NMR spectroscopy also has several disadvantages. Firstly ^{31}P-NMR spectroscopy is not very sensitive. For a convenient analysis time, it requires compounds to be present in concentrations of around 10mM. The lack of sensitivity rules out the use of this technique for measuring ether lipids or their metabolites in the body fluids of patients. A second disadvantage of this technique is that only metabolism at or close to the phosphorus is monitored. Hence neither AGMO activity nor metabolic cleavage of the THF ring within SRI are likely to be detected. A third disadvantage is that whilst it is a useful *in vitro* technique, in cells, body fluids or in tissues, spectra would be extremely difficult to interpret with the requirement to distinguish between drug and its metabolites, and naturally occurring phosphates eg ATP and ADP.

Discussions at the 1991 annual BACR meeting in Manchester, with clinicians who have been involved with clinical trials confirmed that SRI is very difficult to detect. The analytical determination of ether lipids at the low levels found in the body fluids is generally considered to be an obstacle and the presence of endogenous phospholipid interfere with most detection systems [Schaefer and Rohdewald, 1989]. SRI and its putative metabolites do not possess a chromophore group so UV detection cannot be used. As discussed in chapter 2, (section 2.3.3.3. p64) SRI can be isolated using HPLC and analysed by post column derivatisation with the fluorescent agent ANS. This agent could not be used to derivatise the metabolites of SRI and so could not be employed in metabolism studies. There is also the problem that endogenous phospholipids will give positive responses in the presence of ANS. ANS has been used to monitor ET-18-OCH_3 in blood [Schaefer and Rohdewald, 1989] and this technique could be used to follow the kinetics of SRI metabolism or excretion by monitoring its loss from the blood. HPLC with refractive index detection has been suggested for SRI [Baer, 1988] and for lipids in general [Pei et al, 1975]. Good separation of fatty acids and their esters can be attained, but the detection system is not selective for phospholipids.

Most recently a combination of capillary GC or LC and electron capture mass spectroscopy (MS) has been used for quantitative determination of PAF [Balazy et al, 1991]. This technique was also able to measure PAF metabolites and alkyl ether phosphatidylcholines in extracts obtained from rabbit leukocytes. The phosphatidylcholine moiety had to be cleaved prior to derivatisation and GC analysis because it is an impedement to vaporisation of the lipid. Cleavage and derivatisation were performed with pentafluorobenzoyl chloride at raised temperatures. With subsequent analysis by GC or LC and MS this technique was shown to be sensitive and reproducible for monitoring phosphatidylcholines. However, GC-MS could detect lower concentrations of PAF than LC-MS. It was suggested by Balazy et al [1991] that this method could be used to study low levels of PAF and its metabolites present in biological tissues and fluids. Since SRI is a phosphatidylcholine it is possible that GC-MS may be useful in determining its concentration in serum taken from patients during clinical trials. In previous GC methods, phosphatidylcholine was cleaved using phospholipase C [Santouchi et al, 1981]. Such a system would not be suitable for analysis of SRI since as described in chapter 3, this ether lipid does not appear to be a substrate for this phospholipase. The method of Balazy et al [1991] used a single step for cleavage and derivatisation which could be a problem for analysing SRI and its metabolites as alcohol (**14**), phosphate (**21**) and SRI, on cleavage of the phosphate or phosphatidylcholine moiety, are likely to produce the same derivative. Perhaps SRI and its metabolites would first need to be separated prior to the reaction with pentafluorobenzoyl chloride and injection onto the GC column. SRI and its metabolites would have to be well

separated from endogenous phospholipids normally present in body fluids to prevent their interference with SRI determination.

TLC could be used for the qualitative determination of SRI and its metabolites providing a good detection system could be found. Sprays sensitive to phosphate were unable to identify SRI clearly from other products on TLC (chapter 2, p65). Spraying the TLC plate with concentrated sulphuric acid and then charring them has been used for identification of lipids, however it is not selective for phospholipids. Lipids can be detected by placing TLC plates in iodine tanks but again this technique is not selective. Whichever method is chosen for SRI analysis in the clinic, it is unlikely to be a simple one step process.

ii) The second aim of the project was to evaluate whether metabolism of SRI and Mil is a detoxification processes. Biotransformation of SRI was shown to lead to biologically inactive products, whereas Mil was only partially detoxified by metabolism. The toxicity profiles of SRI, Mil and their potential metabolites demonstrate the importance of lipid solubility for their activity and this factor is consistent with the theory that the membrane is the site of their toxic action.

SRI was more toxic than its proposed metabolites, phosphate (**21**), alcohol (**14**) or diol (**5**). These results support the hypothesis that metabolism is a detoxification process (chapter 4). Metabolism of Mil included both a detoxification step and a toxification step. Phosphate (**25**), the metabolite formed from phospholipase D catalysed breakdown of Mil, showed minimal toxicity. Hexadecanol, the product of the metabolism of phosphate (**25**) modulated by phosphatase, was as toxic as Mil. Three cytotoxicity assays were used to measure the activity of SRI and its postulated metabolites. These assays were measurement of growth characteristics, the MTT assay and the LDH assay. The assays produced similar results so it was not possible to interpret differences with the aim of characterising the site of toxic activity (see chapter 4). However, the toxicity profiles of SRI, Mil and their putative metabolites were consistent with the idea that lipophilicity is an important determinant of the cytotoxicity of these agents. SRI and Mil are zwitterionic and they can penetrate the membrane to initiate their toxic action. Phosphates (**21**) and (**25**) are dianionic and hydrophilic, and they are less likely to enter the membrane as readily as SRI and Mil. The phosphates were only slightly toxic (Figures 46c, p121 and 47b, p122). Similarly diol (**5**) is hydrophilic and not very toxic. Alcohol (**14**) and hexadecanol are more lipophilic and can therefore more readily penetrate the membrane than phosphates (**21**) and (**25**). The absence of the phosphatidylcholine moiety in these alcohols may mean that perhaps they would interact to a lesser extent with charged phospholipids of the membrane than the parent

molecules. These alcohols may be taken up into the cell less readily than their parent ether lipids and hence have a reduced toxic affect.

SRI is more toxic than Mil and this fact may be related to their structural differences. These compounds may act at different sites within the membrane, which could also help to explain the variation in the toxicity profiles of their proposed metabolites. SRI more closely resembles the optimal structure for cytotoxic activity as discussed in chapter 1 (section 2.3., p16). Mil does not possess a glycerol backbone which has been described as essential for cytotoxic activity [Hoffman et al, 1984].

SRI and Mil both showed increased toxicity toward cells when they were incubated in the absence of serum (Table 8, p123). This result is consistent with the notion that SRI and Mil can be bound to serum proteins and that the free drug mediates toxicity. Compounds which have a high binding affinity for serum proteins and a low rate of dissociation from these binding sites have a low elimination rate [Bowman and Rand, 1980]. Such compounds can accumulate in the body and increase the risk of side effects. Hence care has to be taken to monitor patient blood levels during clinical trials. Also patient with low serum protein levels, eg hypoalbuminaemia during liver dysfunction, may require a lower dosing regimen as they could have higher levels of the unbound active drug and so be more susceptible to side effects, which has been observed with morphine and diazepam [Bowman and Rand, 1980]. Clinicians also need to be aware of the possibility of interaction with other protein bound drugs.

Comparison of the toxicity of SRI, Mil and their potential metabolites in the two tumour cell lines and the non-transformed cell line indicate very little difference in the sensitivity of these cells to ether lipids (Table 10, p130). It appeared that neither SRI nor Mil were selective for these cell models. Previous work on ether lipids has suggested that they exhibit a toxic selectivity for tumours especially certain leukaemias (Glasser et al, 1984). This has also been demonstrated with SRI (Bazill and Dexter, 1989). One interpretation for the lack of selectivity described in chapter 4 could be that neither JB1 nor BL8 cells have the capacity to metabolise SRI or Mil. Even though SRI and Mil do not seem to be selective *in vitro* in the cell lines tested here, they may be more selective *in vivo*. Phospholipid ethers have been shown to accumulate in certain tumours and this fact has been used in tumour imaging using radiolabelled ether lipids [Plotzke et al, 1991]. It is possible that accumulation in tumours could reduce the concentration of ether lipids in the blood. This feature may in turn reduce the availability of ether lipids to other tissues and so lower their toxicity to normal tissues. Nevertheless, during clinical trials of Mil and SRI liver function

should be monitored, as it has been shown that rats given high doses of Mil suffered from liver toxicity [Muschiol et al, 1987]

SRI and Mil are at present undergoing clinical trial. The trials conducted so far have failed to detect significant antineoplastic activity of these agents against human malignancies. Ether lipids do not seem to be excessively toxic but they also may not be efficacious *in vivo*. Ether lipids have been used for purging bone marrow of leukaemic cells [Glasser et al, 1984]. SRI and Mil may have a clinical application in the treatment of leukaemic bone marrow, as SRI has demonstrated selective toxicity towards leukaemia cells compared to normal bone marrow cells [Bazill and Dexter, 1989]. Mil has also shown some activity when applied topically to breast cancer patients [Unger et al, 1989]. It is still conceivable that ether lipids may have a place in combination therapy to enhance the activity of traditional anticancer agents, without contributing to toxicity.

REFERENCES

Adkins H.; Semb J. and Bolander L. M. Some relationships of the ratio of reactants to the extent of conversion of benzaldehyde and furfuraldehyde to their acetals. *J. Am. Chem. Soc.* 53: 1853 - 1858; 1931.

Amouroux R.; Chastrette F. and Chastrette M. Synthese de polyethers polycycliques. 1. Stereoselectivite de l'heterocyclisation d'alcools tetrahydrofurfuryliques γ,δ-ethyleniques par les sels mercuriques. *J. Het. Chem.* 18: 565 - 569; 1981.

Andreesen R.; Modolell M.; Oepke G. H. F. and Munder P. G. Temperature dependence of leukemic cell destruction by alkyl-lysophospholipids (NSC 324368). *Exp. Hematol* 11: 564 - 570; 1983.

Anson M.S. and McGuigan C. The synthesis by phosphoramidite methodology of novel phospholipids related to ethylenediamine.
J. Chem. Soc. Perkin. Trans I: 715 - 720; 1989.

Baer J. Standard Operating Procedure. SRI 62-834 monograph. Aston Molecules, Aston University 1988.

Balazy M.; Braquet P. and Bazan N. G. Determination of platelet-activating factor and alkylether phospholipids by gas chromatography-mass spectrometry via direct derivatisation. *Anal. Chem.* 196: 1 - 10; 1991.

Bazill G. W. and Dexter T. M. An antagonist to platelet activating factor counteracts the tumouricidal action of alkyl lysophospholipids. *Biochem. Pharmacol* 38: 374 - 377; 1989.

Belanger P. C. and Williams H. W. R. Syntheses of optically active 2-tetrahydrofuran derivatives. *Can. J. Chem.* 61: 1383 - 1386; 1983.

Berdel W. E.; Bausert W. R. E.; Fink U.; Rastetter J. and Munder P. G. Anti-tumour action of alkyl-lysophospholipids (Review). *Anticancer Res.* 1: 345 - 352; 1981.

Berdel W. E.; Bausert W. R.; Weltzein H. U.; Modolell M. L.; Widman K. H. and Munder P. G. The influence of alkyl-lysophospholipids and lysophospholipid-activated macrophages on the development of metastasis of 3-Lewis lung carcinoma. *Eur. J. Cancer* 16: 1199 - 1204; 1980.

Bergmeyer H. U. and Bernt E. Lactate dehydrogenase. In Bergmeyer H. U. (ed) *Methods of Enzymatic Analysis Vol* II: pp 574 - 579. New York. Academic Press. 1974.

Bertz R. T. Furfural diacetate (2-furanmethanediol, diacetate). *Org. Syn.: Coll. Vol* IV: 489 - 490; 1963.

Bomalski J. S. and Clark M. A. The effect of sn-2 fatty acid substitution on phospholipase C enzyme activities. *Biochem. J.* 244: 497 - 502; 1987.

Bonjouklian R.; Phillips M. L.; Kuhler K. M.; Grindey G. B.; Poore G. A.; Schultz R. M. and Altom M. G. Studies of antitumour activity of (2-alkoxyalkyl) and (2-alkoxyalkenyl) phosphocholines. *J. Med. Chem.* 29: 2472 - 2477; 1986.

Bowman E. R. Studies on the excretion of 5-(3-pyridyl)tetrahydrofuranone-2 and its intermediary role in the metabolism of nicotine. *Virginia J. Sci.* 19: 115 - 121: 1968.

Bowman W. C. and Rand M. J. Absorption, distribution, excretion and metabolism of drugs: Biopharmaceutics and pharmacokinetics. In Bowman W. C. and Rand M. J. (ed) *Textbook of Pharmacology 2nd edition* pp40.23 - 40.24. Oxford. Blackwell Scientific Publications. 1980.

Boyd V. L.; Robbins J. D.; Egan W. and Ludeman S. M. ^{31}P Nuclear magnetic resonance spectroscopic observation of the intracellular transformations of oncostatic cyclophosphamide metabolites. *J. Med. Chem.* 29: 1206 - 1210; 1986.

Breiser A.; Kim D.-J.; Fleer E. A. M.; Damenz W.; Drube A.; Berger M.; Nagel G. A.; Eibl H. and Unger C. Distibution and metabolism of hexadecylphosphocholine in mice. *Lipids* 22: 925 - 926; 1987.

Brohult A.; Brohult J. and Brohult S. Biochemical effects of alkoxyglycerols and their use in cancer therapy. *Acta Chem. Scand.* 24: 730 - 734; 1970.

Burfield D. R.; Smithers R. H. Desiccant efficiency in solvent drying. 3. Dipolar aprotic solvents. *J. Org. Chem.* 43: 3966 - 3968; 1978.

Cadaud P. G.; Wroblewski F. and Ruggerio V. Colorimetric measurement of lactic dehydrogenase activity of body fluids. *Am. J. Clin. Pathol.* 30: 234 - 236; 1958.

Coe D.; Flitsch S. L.; Hilpert H.; Liebster M.; Roberts S. M.; Turner N. J. An improved method for the synthesis of highly substituted alkyl phosphates including nucleoside 5'-phosphates. *Chem. Ind.* 724 - 725; 1989.

Crespi C. L.; Penman B. W.; Steimel D. T.; Gelboin H. V. and Gonzalez F. J. The development of a human cell line stably expressing human $Cyp3A_4$: Role in the metabolic activation of Aflatoxin B_1 and comparison to $Cyp1A_2$ and $Cyp2A_3$. *Carcinogenesis* 12: 355 - 359; 1991.

Cronholm T.; Curstedt T. Heterogeneity of sn-glycero-3-phosphate pool in isolated hepatocytes, demonstrated by the use of deuterated glycerols and ethanol. *Biochem. J.* 224: 731 - 739; 1984.

Daly P. F. and Cohen J. S. Magnetic resonance spectoscopy of tumours and potential *in vivo* clinical applications: A review. *Cancer Res.* 49: 770 - 779; 1989.

Davies K. Chem-X, developed and distributed by Chemical Design Ltd., Oxford, UK 1986.

Defaye J. Elimination de di-O-p-toluene-sulfonates vicinaux secondaires, en serie tetrahydrofurannique, par l'iodure de sodium. Acces aux derives optiquement actifs de l'aldehyde tetrahydrofurfurylique (Second Partie). *Bull. Soc. Chim. Franc.* 5: 2099 - 2102; 1968.

Dive C.; Watson J. V. and Workman P. Multiparametric flow cytometry of the modulation of tumor cell membrane permeability by developmental antitumor ether lipid SRI 62-834 in EMT6 mouse mammary tumor and HL60 human promyelocytic leukemia cells. *Cancer Res.* 51: 799 - 806; 1991.

Edmundson R.S. Oxidation of cyclic phosphorochloridites. *Chem. Ind.* 1828 - 1829; 1962.

Edwards R. G. and Hands A. R. The metabolism of the phosphonium analogue of choline *in vitro* and *in vivo*, and its detection in phospholipids by ^{31}P-NMR. *Biochim. Biophys. Acta* 431: 303 - 316; 1976.

Eftax D. S. P. and Dunlop A. P. New diols in the furan and pyran series. *J. Org. Chem.* 26: 2106 - 2107; 1961.

Eibl H. and Blume A. The influence of charge on phosphatidic acid bilayer membranes. *Biochim. et Biophys. Acta* 553: 476 - 488; 1979.

El-Sayed M. Y.; DeBose C. D.; Coury L. A. and Roberts M. F. Sensitivity of phospholipase C (Bacillus cereus) activity to phosphatidylcholine structural modifications. *Biochim. Biophys. Acta* 837: 325 - 335; 1985.

Engle T. W.; Zon G. and Egan W. ^{31}P-NMR investigation of phosphoramide mustard: Evaluation of pH control over the rate of intramolecular cyclization to aziridium ion and hydrolysis of this reactive alkylator. *J. Med. Chem.* 22: 897 - 899; 1979.

Euerby M. R.; Partridge L. Z.; Learmonth M. P.; Ball H. L. and Giddons W. A. Chemistry and biochemistry of helminths. Part 1. The use of 1,3,2-oxazaphospholidin-2-ones in the synthesis of alkoxy- and aryloxy-phosphorylethanolamine derivatives. *J. Chem. Res. (S)* 74 - 75; 1987. *J. Chem. Res. (M)* 0815 - 0830; 1987.

Fleer E. A. M.; Kim D.-J.; Unger C. and Eibl H. 1-Octadecyl-2-O-methyl-rac-glycero-3-phospho[3H-methyl]choline: Chemical preparation and metabolism in leukemic Raji cells. In Muccino R. R. (ed) Synthesis and applications of isotopically labelled compounds. *Proc. of the 2nd Int. Symp.* Kansas City, MO pp473 - 478. Amsterdam. Elsevier Science Publishers. 1986.

Fujita T. and Suzuoki Z. Enzymic studies on the metabolism of the tetrahydrofurfuryl mercaptan moiety of thiamine tetrahydrofurfuryl disulfide. III Oxidative cleavage of the tetrahydrofuran moiety. *J. Biochem.* 74: 733 - 738; 1973.

Girard D. J.; Aaronson S.A.; Todaro G. J.; Arnstein P.; Kersey J. H.; Dosik H. and Parks W. P. *In vitro* cultivation of human tumors: Establishment of cell lines derived from a series of solid tumors. *J. Nat. Cancer. Inst.* 51: 1417 - 1423; 1973.

Glasser L.; Somberg L. B. and Vogler W. R. Purging murine leukemic marrow with alkyl-lysophospholipids. *Blood* 64: 1288 - 1291; 1984.

Graham R. A.; Meyer R. A.; Szwergold B. S. and Brown T. R. Observation of myo-inositol 1,2-(cyclic)-phosphate in Morris hepatoma by ^{31}P-NMR. *J. Biol. Chem.* 262: 35 - 37; 1987.

Graham R. A.; Brown T. R. and Meyer R. A. An *ex vivo* model for the study of tumor metabolism by nuclear magnetic resonance: Characterization of the phosphorus-31 spectrum of the isolated perfused Morris hepatoma 7777. *Cancer Res.* 51: 841 - 849; 1991.

Hachisuka T.; Nakayama S.; Tomita T. and Takagi H. ^{31}P Nuclear magnetic resonance study of phospholipid metabolites in hypothermic-preserved liver. *Transplant. Proc.* 22: 485 - 487; 1990.

Helfman D. M.; Barnes K. C.; Kinkade J. M.; Vogler W. R.; Shoji M. and Kuo J. F. Phospholipid-sensitive Ca^{2+}-dependent protein phosphorylation system in various types of leukaemic cells from human patients and in human leukaemic cell lines HL60 and K562 and its inhibition by alkyl lysophospholipids. *Cancer Res.* 43: 2955 - 2961; 1983.

Hickman J. A. Membrane targets in cancer chemotherapy. A report of a one day symposium of the EORTC-PAM group meeting held at the Centre Antoine Lacassagne, Nice, France, 4th December 1987. *Eur. J. Cancer Clin. Oncol.* 24: 1385 - 1389; 1988.

Hoffman D. R.; Hajdu J. and Snyder F. Cytotoxicity of platelet activating factor and related alkyl phospholipid analogs in human leukemia cells, polymorphonuclear neutrophils, and skin fibroblasts. *Blood* 63: 545 - 552; 1984.

Hoffman D. R.; Hoffman L. M. and Snyder F. Cytotoxicity and metabolism of alkyl phospholipid analogues in neoplastic cells. *Cancer Res.* 46 : 5803 - 5809; 1986.

Houlihan W. J.; Lee M. L.; Munder P. G.; Nemecek G. M.; Handley D. A.; Winslow C. M.; Happy J. and Jaeggi C. Antitumour activity of SRI 62-834, a cyclic ether analog of ET-18-OCH_3. *Lipids* 22: 884 - 890; 1987.

Huet F.; Lechevallier A.; Pellet M.; Conia J. M. Wet Silica Gel; a convenient reagent for deacetalization. *Synthesis* 63 - 65; 1978.

Jahnig F.; Harlos K.; Vogel H. and Eibl H. Electrostatic interactions at charged lipid membranes. Electrostatically induced tilt. *Biochem.* 18: 1459 - 1468; 1979.

Jewell S. A.; Bellomo G.; Thor M.; Orrenius S and Smith M. T. Bled formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. *Science* 217: 1257 - 1259; 1982.

Jiang R.-T.; Shyy Y.-J. and Tsai M.-D. Phospholipids chiral at phosphorus. Absolute configuration of chiral thiophospholipids and stereospecificity of phospholipase D. *Biochem.* 23: 1661 - 1667; 1984.

Judah D. J.; Legg R. F. and Neal G. E. Development of resistance to cytotoxicity during aflatoxin carcinogenesis. *Nature* 265: 343 - 345; 1977.

Kates M. Lipolytic enzymes. In Bloch K. (ed) *Lipide Metabolism* pp165 - 185. London. J. Wiley and Sons Inc. 1960.

Keepers Y. P.; Pizao P. E.; Peters G. J.; van Ark-Otte J; Winograd B. and Pinedo H. M. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *Eur. J. Cancer* 27: 897 - 900; 1991.

Kikkawa U.; Kishimoto A. and Nishizuka Y. The protein kinase C family: Heterogeneity and its implications. *Ann. Rev. Biochem.* 58: 31 - 44; 1989.

Koetting J.; Unger C. and Eibl H. A contiuous assay for O-alkylglycerol monooxygenase (E.C. 1.14.16.5). *Lipids* 22: 824 - 830: 1987a.

Koetting J.; Unger C. and Eibl H. Substrate specificity of O-alkylglycerol monooxygenase (E. C. 1.14.16.5), solubilized from rat liver microsomes. *Lipids* 22: 831 - 835; 1987b.

Kramer R. M.; Patton G. M.; Pritzker C. R. and Deykin D. Metabolism of platelet activating factor in human platelets. Transacylase-mediated synthesis of 1-O-alkyl-2-arachidonyl-sn-glycero-3-phosphocholine. *J. Biol. Chem.* 259: 13316 - 13320; 1984.

Lazenby C. M.; Thompson M. G. and Hickman J. A. Elevation of leukemic cell intracellular calcium by the ether lipid SRI 62-834. *Cancer Res.* 50: 3327 - 3330; 1990.

Leathwood P. D. and Plummer D. T. Enzymes in rat urine I. A metabolism cage for the complete separation of urine and faeces. *Enzymologia* 37: 240 - 250; 1969.

Lee T.-C.; Blant M. L.; Fitzgerald V. and Snyder F. Substrate specificity in the biocleavage of the O-alkyl bond: 1-Alkyl-2-acetyl-sn-glycero-3-phosphocholine (a hypotensive and platelet activating lipid) and its metabolites. *Arch. Biochem. Biophys.* 208: 353 - 357; 1981.

Lieber M.; Smith B.; Szakal A.; Nelson-Rees W. and Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* 17: 62 - 70; 1976.

Long R.C.; Small W. C.; Brynes R. K.; Tidwell T.; Goldstein J. H. and Vogler W. R. Effects of alkyl-lysophospholipids on human leukemic cell lines measured by nuclear magnetic resonance. *Cancer Res.* 43: 770 - 775; 1983.

Lowry O. H.; Rosebrough N. J.; Farr A. L. and Randall R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265 - 275; 1951.

Manson M. M.; Legg R. F.; Watson J. V.; Green J. A. and Neal G.E. An examination of the relative resistances to aflatoxin B_1 and susceptibility to γ-glutamyl *p*-phenylene diamine mustard of γ-glutamyl transferase negative and positive cell lines. *Carcinogenesis* 2: 661 - 670; 1981.

Matarese R.M. Determination of glycerol in biological samples. *J. Chromatog.* 273: 398 - 405; 1983.

McVie J. G. (ed) The Cancer Research Campaign. In *European Cancer News* 2 (5): 1; 1989a.

McVie J. G. (ed) Cancer in the European Community. In *European Cancer News* 2 (8): 1 - 2; 1989b.

Minne N. and Adkins H. Structure of reactants and the extent of acetal formation. *J. Am. Chem. Soc.* 55: 299 - 309; 1933.

Modolell M.; Andreeson R.; Pahlke W.; Brugger U. and Munder P. G. Disturbance of phospholipid metabolism during the selective destruction of tumor cells induced by alkyl-lysophospholipids. *Cancer Res.* 39: 4681 - 4686; 1979.

Morris-Natshke S.; Surles J. R.; Daniel L. W.; Berens M. E.; Modest E. J. and Piantadosi C. Synthesis of sulfur analogues of alkyl lysophospholipid and neoplastic cell growth inhibitory properties. *J. Med. Chem.* 29: 2114 - 2117; 1986.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Applications to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65: 55 - 63; 1983.

Muesing R.A. and Nishida T. Disruption of low and high density human plasma lipoproteins and phospholipid dispersions by 1-Anilinonaphthalene-8-sulfonate. *Biochem.* 10: 2952 - 2962; 1971.

Muschiol C.; Berger M.; Schuler B.; Scherf H. R.; Garzon F. T.; Zeller W. J.; Unger C.; Eibl H. J. and Schmahl D. Alkyl phosphocholines: Toxicity and anticancer properties. *Lipids* 22: 930 - 934; 1987.

Nakamachi H.; Kamiya K.; Wada Y.; Fujii S.; Matsukura Y.; Nakamura H. and Nishikawa M. Cytidine 5'-diphosphate choline monohydrate. *J. Takeda Res. Lab.* 34: 358; 1975.

Noseda A.; Godwin P. L. and Modest E. J. Effects of antineoplastic ether lipids on model and biological membranes. *Biochim. Biophys. Acta* 945: 92 - 100; 1988.

Noseda A.; White J. G.; Godwin P. L.; Jerome W. G. and Modest E. J. Membrane damage in leukemic cells induced by ether and ester lipids: An electron microscopic study. *Exp. Mol. Pathol.* 50: 69 - 83; 1989.

Okayasu T.; Hoshii K.; Seyama K.; Ishibashi T. and Imai Y. Metabolism of platelet activating factor in primary cultured adult rat hepatocytes by a new pathway involving phospholipase C and alkyl monooxygenase. *Biochim. Biophys. Acta* 876: 58 - 64; 1986.

Pei P. T.-S.; Henly R. S. and Ramachandran S. New application of high pressure reversed-phase liquid chromatography in lipids. *Lipids* 10: 152 - 156; 1975.

Perich J. W. and Johns R. B. Di-*tert*-butyl *N,N*-diethylphosphoramidite. A new phosphitylating agent for the efficient phosphorylation of alcohols. *Synthesis* 142 - 144; 1988.

Pines S. H.; Chemerda J. M. and Kozlowski M. A. Cleavage of oximes with bisulfite. A general procedure. *J. Org. Chem.* 31: 3446-3447; 1966.

Plotzke K. P.; Ruyan M.; Skinner R. W. S.; Gross M. D.; Wahl R. and Counsell R. E. Tumor retension and metabolism of radioiodinated phospholipid ethers. *Proceedings of the American Association for Cancer Research* 32: 401; 1991.

Pritchard P. H. and Vance D. E. Choline metabolism and phosphatidylcholine biosynthesis in cultured rat hepatocytes. *Biochem. J.* 196: 261 - 267; 1981.

Qian C.; Lee T.-C. and Snyder F. Metabolism of platelet activating factor (PAF) and related ether lipids by neonatal rat myocytes. *J. Lipid. Med.* 1: 113 - 123; 1989.

Reidy G. P. and Snyder W. R. The stereoselectivity of Clostridium perfringens phospholipase C: Hydrolysis of thiophosphate analogs of phosphatidylcholine. *Arch. Biochem. Biophys.* 258: 504 - 509; 1987.

Rock C. O.; Baker R. C.; Fitzgerald V. and Snyder F. Stimulation of the microsomal alkylglycerol monooxygenase by catalase. *Biochim. Biophys. Acta* 450: 469 - 473; 1976.

Rossiter R. J. and Strickland K. P. The metabolism and function of phosphatides. In Bloch K. (ed.) *Lipide Metabolism* pp69 - 127. London. J. Wiley and Sons Inc. 1960.

Santouchi K.; Pinckard R. N. and Hanahan J. Influence of alkyl ether chain length of acetyl glyceryl ether phosphorylcholine and its ethanolamine analog on biological activity toward rabbit platelets. *Arch. Biochem. Biophys.* 211: 683 - 688; 1981.

Schaefer H.G.; Rohdewald P. Determination of the alkyl lysophospholipid derivative ET-18-OCH_3, a new antineoplastic drug, in plasma. *Clin. Chem.* 35: 821 - 823; 1989.

Scherf H. R.; Schuler B.; Berger M. R. and Schmahl D. Therapeutic activity of ET-18-OCH_3 and hexadecylphosphocholine against mammary tumors in BD-V1 rats. *Lipids* 22: 927 - 929; 1987.

Scopes R. Maintenance of active enzymes. In Scopes R. (ed) *Protein Purification: Principles and Practice* pp194 - 200. New York. Springer-Verlag. 1982.

Seewald M. J.; Olsen R. A.; Sehgal I.; Melder D. C.; Modest E. J. and Powis G. Inhibition of growth factor-dependent inositol phosphate Ca^{2+} signaling by antitumour ether lipid analogues. *Cancer Res.* 50: 4458 - 4463; 1990.

Shoji M.; Raynor R. L.; Berdel W. E.; Vogler W. R. and Kuo J. F. Effects of thioether phospholipid BM 41.440 on protein kinase C and phorbol ester-induced differentiation of human leukaemia HL60 and KG-1 cells. *Cancer Res.* 48: 6669 - 6673; 1988.

Snyder F. Biochemistry of platelet-activating factor: A unique class of biologically active phospholipids (42839). *Proc. Soc. Exp. Biol. Med.* 190: 125 - 135; 1989.

Snyder W. R. Bacillus cereus phospholipase C: carboxylic acid ester specificity and stereoselectivity. *Biochim. Biophys. Acta* 920: 155 - 160; 1987.

Sonowat H. M.; Leibfritz D.; Engel J. and Hilgard P. Biotransformation of mafosfamide in P388 mice leukemia cells: Intracellular ^{31}P-NMR studies. *Biochim. Biophys Acta* 1052: 36 - 41; 1990.

Soodsma J. F.; Piantadosi C. and Snyder F. The biocleavage of alkyl glyceryl ethers in Morris hepatomas and other transplantable neoplasms. *Cancer Res.* 30: 309 - 311; 1970.

Soodsma J. F.; Piantadosi C. and Snyder F. Partial characterization of the alkylglycerol cleavage enzyme system of rat liver. *J. Biol. Chem.* 247: 3923 - 3929; 1972.

Stewart J. J. P. QCPE Bulletin 3, 43. Quantum chemistry program exchange. Indiana University Chemistry Department, Indiana, 1983.

Still W. C.; Kahn M. and Mitra A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43: 2923 - 2925; 1978.
Storch J. and Munder P. G. Increased membrane permeability for an antitumoral alkyl lysophospholipid in sensitive tumor cells. *Lipids* 22: 813 - 819; 1987.

Storme G. A.; Berdel W. E.; van Blitterswijk W. J.; Bruyneel E. A.; De Bruyne G. K. and Mareel M. M. Antiinvasive effect of racemic 1-O-octadecyl-2-O-methylglycero-3-phosphocholine on MO_4 mouse fibrosarcoma cells *in vitro*. *Cancer Res.* 45: 351 - 357; 1985.

Talmadge J. E. and Clark J. Biological response modifiers: Preclinical and clinical results. In Pinedo H. M.; Longo D. L. and Chabner B. A. (eds). *Cancer chemotherapy and biological response modifiers, annual 9*. pp454 - 472 . Amsterdam. Elsevier. 1987.

Thompson M. G. and Hickman J. A. Elevation of HL-60 cell intracellular calcium by the cytotoxic ether lipid SRI 62-834 and antagonism by 12-O-tetradecanoylphorbol 13-acetate. *Biochem. Soc. Trans.* 16: 278; 1988.

Tietz A.; Lindberg M. and Kennedy E. P. A new pteridine-requiring enzyme system for the oxidation of glyceryl ethers. *J. Biol. Chem.* 239: 4081 - 4090; 1964.

Tritton T. R. and Hickman J. A. How to kill cancer cells: Membranes and cell signalling as targets in cancer chemotherapy. *Cancer Cells* 2: 95 - 105; 1990.

Twentyman P. R. and Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br. J. Cancer.* 56: 279 - 285; 1987.

Uberall F.; Oberhuber H.; Maly K.; Zaknun J.; Demuth L. and Grunicke H. H. Hexadecylphosphocholine inhibits inositol phosphate formation and protein kinase C activity. *Cancer Res.* 51: 807 - 812; 1991.

Unger C.; Damenz W.; Fleer E. A. M.; Kim D. J.; Breiser A.; Hilgard P.; Engel J.; Nagel G. and Eibl H. Hexadecylphosphocholine, a new ether lipid analogue. Studies on the antineoplastic activity *in vitro* and *in vivo*. *Acta Oncologica* 28 : 213 - 218; 1989.

Unger C. and Eibl H. Phospholipide als antitumomittel - zytotoxische prinzipien als ergebnis von strukturvariation. In Nagel G. A. (ed) *Mamarkarzinone* pp115 - 123. Berlin. Springer-Verlag. 1986.

Unger C.; Eibl H.; Kim D. J.; Fleer E. A.; Koetting J.; Bartsch H. H.; Nagel G. A. and Pfizenmaler K. Sensitivity of leukemia cell lines to cytotoxic alkyl-lysophospholipids in relation to O-alkyl cleavage enzyme activities. *J. N. C. I.* 78: 219 - 222; 1987.

Unger C.; Eibl H.; vonHeyden H.-W. and Nagel G. A. new assay for the O-alkyl cleavage enzyme with alkyl-lysophospholipids as substrates. *Cancer Res.* 45: 616 - 618; 1985.

Vallari D. S.; Smith Z. L. and Snyder F. HL60 cells become resistant towards antitumour ether-linked phospholipids following differentiation into a granulocytic form. *Biochem. Biophys. Res. Comm.* 156: 1 - 8; 1988.

van Blitterswijk W.; Hilkman H.; Storme G. A. Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* 22: 820 - 823; 1987.

Vaskovsky V. E. and Latyshev N. A. Modified Jungnickel's reagent for detecting phospholipids and other phosphorus compounds on thin-layer chromatograms. *J Chromatog.* 115: 246 -249; 1975.

Vogel A. Experimental techniques. In *Vogel's Textbook of Practical Organic Chemistry 4th edition* ppI23 - I24. Harlo, Essex. Longman Group Ltd. 1978.

Wallace J. L.; Braquet P.; Ibbotson G.C.; MacNaughton W. K. and Cirino G. Assessment of the role of platelet-activating factor in an animal model of inflammatory bowel disease. *J. Lipid Mediators* 1: 13 - 23; 1989.

Whitman M and Cantley L. Phosphoinositide metabolism and the control of cell proliferation. *Biochem. Biophys. Acta* 948: 327 - 344; 1988.

Wilcox R. W.; Wykle R. L.; Schmitt J. D. and Daniel L. W. The degradation of platelet-activating factor and related lipids: Susceptibility to phospholipases C and D. *Lipids* 22: 800 - 807; 1987.

Workman P.; Donaldson J. and Lohmeyer M. Platelet-activating factor (PAF) antagonist WEB 2086 does not modulate the cytotoxicity of PAF or antitumour alkyllysophospholipids ET-18-OMethyl and SRI 62-834 in HL-60 promyelocytic leukaemia cells. *Biochem. Pharmacol.* 41: 319 - 332; 1991.

Yamamoto N. and Ngwenya B. Z. Activation of mouse peritoneal macrophages by lysophospholipids and their ether derivatives of neutral lipids and phospholipids. *Cancer Res.* 47: 2008 - 2013; 1987.

Zheng B.; Oishi K.; Shoji M.; Eibl H.; Berdel W. E.; Hajdu J.; Vogler W. R. and Kuo J. F. Inhibition of protein kinase C, (sodium plus potassium)-activated adenosine triphosphatase, and sodium pump by synthetic phospholipid analogues. *Cancer Res.* 50: 3025 - 3031; 1990.

PUBLICATIONS

Bishop F.; Dive C.; Freeman S. and Gescher A. Metabolism of antineoplastic ether-lipids SRI 62-834 (I) and Miltefosine (V) and cytotoxicity of putative metabolites of I. *Br. J. Cancer* 63: 34; 1991.

Bishop F.; Dive C.; Freeman S. and Gescher A. Comparative cytotoxicity of ether lipid SRI 62-834 (SRI) with two putative metabolites. *Proceedings of the American Association for Cancer Research* 32: 401: 1991.

APPENDICES

APPENDIX 1

Stock solutions and buffers used in the metabolism studies

Tris buffers

Tris buffer (0.05M) pH 7.6, with sucrose (0.25M) was prepared by dissolving sucrose (42.8g) in water (450ml) and then adding Tris (3.03g). The pH was adjusted to 7.6 with concentrated hydrochloric acid. Volume was made up to 500ml with water and the pH confirmed to be 7.6.

Tris buffers (0.1M, 1.21g in 100ml distilled water) of pH 7.3 and 7.6 were prepared in the same way but without the addition of sucrose.

Tris buffer (0.1M) of pH 8.0 and 8.8 were also prepared, adjusting the pH with NaOH (10M).

MES buffers

MES (1.95g) was dissolved in 90ml water and the pH adjusted to 5.6 with hydrochloric acid. Water was added to give a total volume of 100ml and the pH confirmed to be 5.6.

MES buffer, pH 6.0 was also prepared.

Lowry reagent

Copper sulphate solution, 1% w/v (2ml), potassium sodium tartrate, 2% w/v (2ml) and sodium carbonate, 2% w/v (200ml) were added together and mixed thoroughly.

APPENDIX 2

Stock Solutions used in tissue culture and cytotoxicity assays

Versene

PBS tablets (20), EDTA (742mg) and phenol red (100mg) were dissolved in distilled water (150ml) and the pH adjusted to 7.4 using NaOH (1M). Distilled water was then added to 200ml and the solution autoclaved.

Trypsin / Versene for A549 cells

Versene stock solution (15ml), trypsin / EDTA (10x) (15ml) and distilled water (120ml) were added together under sterile conditions.

Trypsin for JB1 and BL8 cells

Trypsin / EDTA (x10) (10ml) was made up to 100ml with sterile PBS.

NADH 3.5mM

NADH (9.92mg) was dissolved in distilled water (4ml). This solution was prepared just prior to use and stored on ice.

Pyruvate 32mM

Sodium pyruvate (14.08mg) was dissolved in distilled water (4ml). This solution was prepared freshly on the day of usage and stored on ice.

Triton 20% v/v

Triton (x100) (20.0ml) was made up to 100.0ml with distilled water. The preparation required warming to ensure complete mixing of the triton.

SRI (100mM)

SRI (58.6mg) was dissolved in sterile PBS (1ml), it was only slowly soluble required shaking and occasionally, warming. This solution was then diluted with sterile PBS as required.

Alcohol (14) (100mM)

Compound (**14**) (38.4mg) was dissolved in DMSO (1ml). This compound was poorly soluble and required mixing with a vortex and warming to dissolve. The solution then had to be diluted or used immediately before it formed a gel. Solutions were diluted with DMSO for the LDH assay. For MTT assay the stock solution was diluted to 20mM with

DMSO and then further diluted with cell culture medium to reduce the percentage of DMSO added to the wells.

Diol (5) (100mM)
Compound (**5**) (13.2mg) was dissolved in PBS (1ml). It was readily soluble and further dilutions were prepared with PBS as required.

Phosphate (21) (2mM)
Compound (**21**) (9.3mg) was dissolved in DMSO (1ml) then made up to 10ml with tissue culture medium. Further dilutions were also prepared with medium.

Mil (2mM)
Mil (8.2mg) was dissolved readily in sterile PBS (10ml). This solution was further diluted with PBS as required.

Phosphate (25) (2mM)
Hexadecyl phosphate (**25**) (7.3mg) was dissolved in sterile PBS (10ml). PBS was used to dilute this solution to the concentrations required for the MTT cytotoxicity assay.

Hexadecanol (2mM)
Hexadecanol (4.9mg) was dissolved in DMSO (1ml) and then diluted to 10.0ml with cell culture medium. Further dilutions of this solution were made with medium.

MTT solution (5mg/ml)
MTT (50mg) was made up to 10.0ml with sterile PBS. The solution was then passed through a 0.2μm filter.

APPENDIX 3

Freezing and thawing cells

1. A549 cells

The cells were detached from the flask using trypsin, counted and then pelleted at 1500rpm for 5min, using a Labofuge 6000 bench top centrifuge. Cells were then resuspended at 1 x 10^6 cells/ml, in fresh medium supplemented with 10% DMSO. Aliquots (1ml) were pipetted into freezing vials and maintained at -80°C in the gas above liquid nitrogen for 4h prior to immersion in the liquid phase.

To recover frozen cells, they were rapidly thawed then an aliquot (500µl) was added dropwise into a flask of fresh culture medium at 37°C (20ml). The cells were allowed to adhere for 6h before medium was aspirated and fresh DMSO free medium added.

2. JB1 and BL8 cells

JB1 and BL8 cells were frozen and thawed as described above for A549 cells, but Williams E medium was used rather than Hams F12.