Chapter 1

General Introduction

1.1) General Background

Many plants contain volatile oils, either in the bark, flowers, fruit, leaves, roots, plant organs or wood. These oils are called essential oils and consist primarily of monoterpenes and sesquiterpenes. Since the oils are volatile they can be removed from the plant by steam distillation. The oils have a low solubility in water and so are easily recovered using a condensed water trap (1).

The bioactivity of essential oils from Australian native plants has been known since the Aboriginal occupation of Australia first commenced some 40,000 years ago. During this period aborigines in various parts of the country were aware of the medicinal value of these oils. For example, the leaves of eucalypts were steeped in water to make infusions drunk for the treatment of bronchial ailments or the leaves of certain eucalypt species were wrapped around body wounds to promote rapid healing (2).

Bioactivity is a very broad term and it is used to describe those properties of a substance which are related to its interaction with living organisms or biological systems. Many specific forms of bioactivity exist. Antibacterial, antitumor, insecticidal, anticancer and fungicidal activity are all examples of bioactivity (3). In order to determine bioactivity of any substance the type of bioactivity being measured must be clearly defined.

A wide variety of essential oils have been shown to exhibit antimicrobial activity against bacteria, yeast and/or molds. The type of antimicrobial activity shown by essential oils varies from partial or complete inhibition of growth to bactericidal or fungicidal activity (4-39). The spectrum of this antimicrobial activity, is quite broad with some of the more commonly studied essential oils such as tea tree oil showing activity against oral bacteria (40,41), commonly studied pathogens such as *Escherichia coli* and *Staphylococcus aureus* (42), antibiotic resistant microorganisms such as methicillin resistant *S. aureus* (43) and skin microorganisms (44) including...
those causing dermatological problems such as tinea and acne (45,46). Various essential oils are also known to show antimicrobial activity against a number of food spoilage microorganisms (22,23,26,47).

Today, probably the fastest growing essential oil industry in Australia is the tea tree oil industry. The oil is distilled from plantations of the terpinen-4-ol chemical variety of Melaleuca alternifolia. Tea tree oil is becoming increasingly popular as a naturally occurring antimicrobial agent which is used in its natural form or incorporated into an increasing number of health-care products. These products include antiseptic creams, shampoos, conditioners, toothpastes and soaps (5,48). Many essential oils from other Australian natives are also becoming more popular and are now added to many different products for a multitude of reasons. For the essential oil industry to remain competitive in the world market, steps must be taken to understand exactly how essential oils and their components are bioactive. This will provide the industry with the required information to develop better formulations for maximum activity and to guide selective breeding programs aimed at selecting plant chemical types which produce better quality oils.

1.2 Antimicrobial Activity of Terpenes

The antimicrobial properties of essential oils have thus generated interest in determining how their chemical composition relates to their activity. Correlations have been made between the chemical composition of essential oils and their antimicrobial activity in some cases. For example, hierarchical cluster analysis was used to group species of the genus Zieria by the chemical composition of their oils. Minimum Inhibitory Concentrations (MIC) of the oils from each group and their main components was determined allowing chemical composition to be related to antimicrobial activity (20). A similar study also exists on various African essential oils (49). In both cases, it was found that oxygenated terpenoids were the main contributors to antimicrobial activity of the oils, with alcohols being more active than aldehydes and ketones. In comparison to oxygenated monoterpenes, monoterpene hydrocarbons were found to exhibit lower levels of antimicrobial activity.
Links between oxygenated compounds and antimicrobial activity are also seen in many other studies. For example in a study on components of lemon grass oil the oxygenated terpenes tested, α-citral [I], β-citral [II], citronellol [III], citronellal [IV], linalool [V] and geraniol [VI], were found to be active against *E. coli*, *Bacillus subtilis* and *S. aureus* whilst the hydrocarbon terpenes, (+) and (-)-limonene [VII] and myrcene [VIII], showed no activity. However, it was also shown in the same study that myrcene, whilst inactive on its own, did enhance the activity of α- and β-citral against *B. subtilis* (50).

Another example of an active oxygenated terpene is terpinen-4-ol [IX], which has been shown to have bactericidal, fungicidal and insecticidal properties (5, 13,51,52). Tea tree oils with high terpinen-4-ol content have increased antimicrobial activity and this has led to the antimicrobial properties of the oil being attributed mainly to terpinen-4-ol (5,13). More recent research has shown that, of the minor components of tea tree oil, α-terpineol [X] and linalool, have similar antimicrobial
activity to terpinen-4-ol. Although it has been suggested that these compounds contribute to the overall antimicrobial activity of the oil (5), linalool is found in only trace amounts in most commercial tea tree oils and thus its effect on overall activity should be minimal.

1,8-Cineole [XI], a monoterpenic ether, although shown to have little antimicrobial activity in its own right (53-56) was found to have a synergistic effect on microorganisms when present in tea tree oil at levels of greater than 20% while terpinen-4-ol still made up at least 30-40% of the oil (13). The medicinal properties of some eucalyptus species have also been traced to high levels of 1,8-cineole since it has been shown to have the property of reducing the swelling of mucous membranes and of loosening phlegm, thus making breathing easier (55).

Piperitone [XII], a p-methane ketone, has also been shown to have some antimicrobial activity against *Rhodopseudomonas sphaeroides*, *E. coli*, *Proteus vulgaris*, *Micrococcus luteus*, *S. aureus* and *B. subtilis* (54). The bioactivity of this compound also extends to insects. The essential oil of *Zanthoxylum bungeanum*, or the Chinese prickly ash, contains high levels of piperitone and had insect repellent properties. Piperitone when tested on its own had similar feeding deterrent activity (57).

Piperitol [XIII], the alcohol analogue to piperitone, is yet another oxygenated terpene which exhibits bioactivity against bacteria. It has been shown to be active against *Salmonella typhi*. In addition, piperitol has also been linked to the antifungal activity of the essential oil from the bark of *O. usambarensis* (58).
Thymol [XIV] and carvacrol [XV], terpene phenols, have been implicated as the main antimicrobial agents in essential oils from thyme (22) while the hydrocarbons also found in these oils were determined to be relatively inactive. Similarly both of these compounds have been shown to exhibit fungicidal properties while hydrocarbons such as α-pinene [XVI], α-phellandrene [XVII] and (+)-limonene exhibited very little such activity (25).

![Chemical structures](images)

[XIV]  [XV]  [XVI]  [XVII]

Various other oxygenated compounds have also been shown to be active against a multitude of bacteria and fungi; examples are shown in Tables 1.1 and 1.2. In each of these studies, terpenoid hydrocarbons were found to have little or no activity.
### Table 1.1: Oxygenated Terpenoids with Antimicrobial Activity Against Various Bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Terpenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td></td>
</tr>
<tr>
<td>Borneol (7)</td>
<td>Citronellal (7,21,50,59)</td>
</tr>
<tr>
<td>Car-3-en-2-one (20)</td>
<td>Citronellol (7,50)</td>
</tr>
<tr>
<td>Carvacrol (14)</td>
<td>Eugenol (56,59,60,62,63)</td>
</tr>
<tr>
<td>Chrysanthene (20)</td>
<td>Geraniol (7,50,59)</td>
</tr>
<tr>
<td>1,8-Cineole (21,30)</td>
<td>Isoeugenol (7)</td>
</tr>
<tr>
<td>Cinnamic aldehyde (59)</td>
<td>Linalool (7,30,50,59,63)</td>
</tr>
<tr>
<td>Citral (12,21,58,59-61)</td>
<td>Menthol (7,59,62)</td>
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<tr>
<td>Myrtenal (7)</td>
<td></td>
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<tr>
<td>Nerol (7,21)</td>
<td></td>
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<tr>
<td>Terpineol (5,7,20)</td>
<td></td>
</tr>
<tr>
<td>Thymol (14,62)</td>
<td></td>
</tr>
<tr>
<td>Terpinen-4-ol (5,53,55,64)</td>
<td></td>
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<tr>
<td>Verbenone (20)</td>
<td></td>
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<tr>
<td><strong>Salmonella typhimurium</strong></td>
<td></td>
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<tr>
<td>Carvacrol (14,22,55,66)</td>
<td>Geraniol (65,66)</td>
</tr>
<tr>
<td>Citral (65,66)</td>
<td>Linalool (65)</td>
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<td>Citronellal (65)</td>
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<td>Perilla aldehyde (65)</td>
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<tr>
<td>α-Terpineol (22,65)</td>
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<tr>
<td>Thymol (14,22,34,62)</td>
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<tr>
<td><strong>Vibrio vulnificus</strong></td>
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</tr>
<tr>
<td>Carvacrol (65)</td>
<td>Eugenol (65)</td>
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<tr>
<td>Citral (65)</td>
<td>Geraniol (65)</td>
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<td>Citronellal (65)</td>
<td>Linalool (65)</td>
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<tr>
<td>Perilla aldehyde (65)</td>
<td></td>
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<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td></td>
</tr>
<tr>
<td>Carvacrol (22,65)</td>
<td>Geraniol (65)</td>
</tr>
<tr>
<td>Citral (65)</td>
<td>Linalool (65)</td>
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<tr>
<td>Thymol (22)</td>
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<tr>
<td><strong>Bacillus subtilis</strong></td>
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<tr>
<td>Thymol (14)</td>
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<tr>
<td><strong>Micrococcus sp.</strong></td>
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<td>Cinnamic aldehyde (59)</td>
<td>Eugenol (59)</td>
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<tr>
<td>Citral (59)</td>
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<td>Menthol (59)</td>
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<tr>
<td><strong>Enterococcus faecalis</strong></td>
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<td>Cinnamic aldehyde (59)</td>
<td>Eugenol (56,59,60)</td>
</tr>
<tr>
<td>Citral (56,59,60)</td>
<td>Geraniol (59)</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>Car-3-en-2-one (20)</td>
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<td>Carvacrol (14,22,50,65)</td>
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<td>Carvone (7)</td>
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<td>Chrysanthene (20)</td>
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<td>Citronellal (50,65)</td>
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<tr>
<td>Perilla aldehyde (65)</td>
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<tr>
<td>Terpineol-4-ol (5)</td>
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<tr>
<td>Terpineol (5,7,20,22,65)</td>
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<tr>
<td>Verbenone (20)</td>
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<tr>
<td><strong>Mycobacterium smegmatis</strong></td>
<td></td>
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<tr>
<td>Eugenol (62)</td>
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<tr>
<td><strong>Vibrio parahaemolyticus</strong></td>
<td>Menthol (62)</td>
</tr>
<tr>
<td></td>
<td>Thymol (62)</td>
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</table>
Table 1.2: Oxygenated Terpenoids with Antimicrobial Activity Against Various Yeasts and Molds

<table>
<thead>
<tr>
<th>Yeast/Mold</th>
<th>Terpenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizopus species</td>
<td>Carvacrol&lt;sup&gt;(25,67)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspergillus species</td>
<td>Carvacrol&lt;sup&gt;(67)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mucor species</td>
<td>p-Anisaldehyde&lt;sup&gt;(25)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>Citral&lt;sup&gt;(60)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stereum purpureum</td>
<td>Citral&lt;sup&gt;(60)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>p-Anisaldehyde&lt;sup&gt;(25)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>Carvacrol&lt;sup&gt;(18)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Borneol&lt;sup&gt;(7)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Penicillium species</td>
<td>p-Anisaldehyde&lt;sup&gt;(25)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trichophyton species</td>
<td>Cinnamaldehyde&lt;sup&gt;(68,70)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>Cinnamaldehyde&lt;sup&gt;(68,70)&lt;/sup&gt;</td>
</tr>
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</table>

While the focus of this project will be on antimicrobial activity of terpenoids, these compounds do also exhibit other types of bioactivity (57,71-76).

1.2.1 Site of Action of Terpenoids and Essential Oils

Recently investigations have shown that the site of action of terpenoids (77,78) and tea tree oil (79-81) is at the cell membrane. In particular, tea tree oil was found to cause K⁺ leakage in *E. coli* (79), at levels that are known to inhibit or reduce growth of this organism, and to stimulate autolysis (80). Autolysis of stationary and
exponential growth phase cells was indicated by electron micrographs which showed loss of electron dense material, coagulation of cell cytoplasm and formation of extracellular blebs. It has been proposed that the ability of tea tree oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control is the most likely source of its lethal action at minimum inhibitory levels (81).

Carvacrol and thymol have also been shown to act directly against bacterial membranes with both compounds having prominent outer membrane disintegrating properties, as indicated by their enhancing effect on 1-N-phenyl-napththlamine uptake and LPS release as well as sensitization to detergents. These compounds also inhibited bacterial growth at concentrations similar to those required for outer membrane disintegration and increased the permeability of the cytoplasmic membrane to ATP. In contrast, the same study showed that cinnamaldehyde, found to have equal activity to that of thymol and carvacrol, and (+)-carvone, which was less active, exhibited none of these outer membrane disintegrating characteristics nor did they affect levels of intracellular ATP. The mechanism by which carvacrol and thymol attack the outer membrane has yet to be clarified. However, observations that MgCl₂ had no effect on the disintegrating ability of the two compounds suggests a mechanism other than chelation of divalent metal cations (see section 6.1) from the outer membrane exists (78).

In addition to their membrane disintegrating capability terpenes such as limonene, 1,8-cineole and nerilidol are known to increase the permeation of drugs such as oestradiol and 5-fluoracil through human epidermis (82,83) and model systems developed to mimic the stratum corneum (84-86). These three terpenes were also found to affect the structure of both the model matrix and real stratum corneum by affecting the inter and intra arrangements of lipid bilayers (85,87).

β-pinene has also been shown to act at the membrane causing K⁺ and H⁺ leakage in yeast (88) In the same study β-pinene was also found to affect isolated yeast mitochondria by de-energization of the organelle followed by inhibition of respiration. This effect on respiration was attributed to effects on the cytochrome b region of the electron transport chain. Likewise cyclohexane, limonene and β-pinene were found to inhibit respiration and other energy-dependent processes associated with the membrane of the yeast S. cerevisiae (89,90).
The cyclic hydrocarbon tetralin (91) and the hydrocarbon terpenes α-pinene, β-pinene, γ-terpinene and limonene (92) were found to affect structural and functional properties of artificial membranes. These compounds were shown to permeabilize the membranes making them swell and to increase membrane fluidity. This inhibited respiratory enzymes which lead to a partial dissipation of the pH gradient and electrical potential due to the increased permeability to H⁺ ions (91,92). Each of these gradients is crucial to the energy system in a cell.

The effect of several terpenoids on microbial oxygen uptake and oxidative phosphorylation has also been studied (93-95). Most of the terpenoids tested were found to inhibit both processes. In particular the phenolic and non-phenolic alcohols exhibited the strongest inhibitory effects, followed by aldehydes and ketones. The monoterpenic hydrocarbons exhibited very little activity at all. It was suggested that the free OH group possessed by the alcohols may be a key to their activity (93).

Terpenoid compounds also inhibit electron transport, proton translocation, phosphorylation steps and other enzyme dependent reactions in membranes prepared from *Rhodopseudomonas sphaeroides*. Even in lower concentrations, specific terpenoids with functional groups such as phenolics, alcohols or aldehydes interfere with membrane-integrated or associated enzyme proteins (54).

Evidence supports the site of action of terpenoids as being at the membrane, therefore the main focus of this project was on the membrane disrupting nature of the terpenoids and structure/activity relationships. However, in order to do this we need first understand the factors which affect such activity.

### 1.3 Factors Affecting Antimicrobial Activity

Regardless of the mode of action of an antimicrobial compound two major barriers must be overcome. These are, its availability to the cell (i.e. can sufficient amounts of the compound be brought into contact with the cell?) and its ability to reach its site of action (i.e. can the compound pass through the physical barriers presented by the cell?). Since the cell envelope is known to be the site of action of terpenoids and regulates the flow of molecules into and out of the cell it is necessary to include it in determining the factors affecting antimicrobial activity.
1.3.1. Microbial Cell Envelope Structure

1.3.1.1 Bacteria

Eubacteria are unicellular organisms which exist in various general shapes including coccus (spherical), rod (cylindrical), vibrio (cylindrical with a single twist) and spirochete (cork screw-like rods). There are three fundamental structures which exist in almost all bacteria, the cell wall, one or two membranes and the cytoplasm (96). Other structures not strictly associated with the cell membrane also exist and are called surface layers. Various surface layers exist such as capsules, extra-cellular polysaccharides, sheaths and crystalline s-layers. These structures are not as well studied due to their compositional variability but can attribute quite significantly to microbial cell and population survival (97-101). In particular extra-cellular polysaccharides are thought to significantly affect diffusion properties of compounds into and out of bacterial cells (99).

Eubacteria are divided in two major groups based on their cell envelope composition. Gram positive bacteria consist of one membrane, the cytoplasmic membrane, which is surrounded by a thick cell wall. Gram negative bacteria have, in addition to the cytoplasmic membrane and cell wall, an outer membrane that consists largely of phospholipids and lipopolysaccharide (LPS) (Figure 1.1) (77).

![Diagram of cell envelope structure](image)

**Figure 1.1** – Schematic representation of the cell envelope of Gram-positive and Gram-negative Bacteria. PP, porin; C, cytoplasmic membarne embedded protein; BP, binding protein; PPS, periplasmic space; A, outer membrane protein; LP, lipoprotein Taken from (77)
1.3.1.1a Cell Wall

The walls of Gram positive bacteria consist of cross-linked or uncross-linked peptidoglycan strands with covalently attached teichoic acids and are generally between 20-50nm in thickness (100). The Gram negative cell wall contains a thinner peptidoglycan layer to which lipoproteins are covalently attached and outside of which lies an LPS layer (77,102).

The cell wall in both Gram positive and negative bacteria performs several important functions. It is involved in retaining cell shape via the rigid peptidoglycan layer and consequently cell integrity against osmotic pressure (100,103). However, one of the most important functions of the cell wall is as an interface with the outside environment. Most bacterial walls, although strongly knitted together by chemical bonds, have some reactive groups available for interaction with extraneous solutes. Usually walls carry an overall net negative charge which make them reactive towards positive ions such as Ca\(^{2+}\) and Mg\(^{2+}\). Gram positive bacteria tend to be more reactive in this manner than Gram negative bacteria. This has been attributed mainly to the abundance of teichoic acids in the Gram positive peptidoglycan layer and the fact the peptidoglycan layer in Gram negative bacteria is hidden behind the outer membrane which has a lower binding capacity (103).

Another important aspect of the cell wall is that the macromolecular component parts are arranged so as to form a physical permeability barrier to external solutes. Permeability of the Gram negative envelope has been extensively studied. The Gram positive cell wall has not been as extensively studied. However given that the Gram positive wall is a thick network of peptidoglycan and associated secondary polymers it is theorized that the sieving threshold would be around the same size as the polymeric interspaces. This has been estimated as anywhere up to 1.75 nm (103).

1.3.1.1b The Cytoplasmic Membrane

Chemically the cytoplasmic membrane is known to consist of phospholipids and protein. The phospholipid molecules of the membrane are arranged in a bilayer with the polar groups directed outwards on both sides with protein molecules either attached to the surface or partially or fully imbedded into the lipid bilayer (see Figure 1.2). The bilayer is considered to be fluid-like in nature and dynamic. This generally accepted model of the cytoplasmic membrane, known as the fluid mosaic model, is
now the most common model used for describing biological membranes (see Figure 1.2) (104,105).

Figure 1.2 – Representation of the Fluid Mosaic Model for the Cytoplasmic Membrane. Modified from (104)

Compared with the cell wall the cytoplasmic membrane is a delicate structure and is also highly metabolically active. It acts as a selective permeability barrier between the cytoplasm and the cell environment as well as providing a stable yet adaptive matrix for membrane associated enzymes (see section 1.3.3.1 for more detail). Various important enzyme systems are situated in the cytoplasmic membrane, including the enzymes associated with the electron transport chain in bacteria and those which are responsible for active transport (96).

The major types of lipids that exist in membranes are; phosphatidylethanolamines, found in both plant and animal membranes but also often the major lipid in microorganism membranes; phosphatidylcholines (lecithins), the most common lipid in animal cell membranes but infrequently found in microorganisms; phosphatidylserines, commonly found in animal, plant and
microorganism membranes but usually at less than 10% and sphingomyelins, which are more commonly found in brain and nerve tissue (106,107) (see Figures 1.3 & 1.4).

Figure 1.3 – Chemical formula for; (a) Phosphatidycholines, (b) Phosphatidylethanolamines, (c) Sphingomyelin and Phosphatidylserines. R₁ – fatty acid acyl chains.

Figure 1.4 – Schematic representation of the amphipathic phosphatidylcholine molecule. S – molecular area at the lipid/water interface. Modified from (109)
Each of these main types of lipids can have various fatty acids attached which form the acyl chains of the phospholipid and affect the physicochemical properties of the lipids. This in turn will affect the physicochemical properties of the membrane. Fatty acid residues commonly associated with membrane lipids are stearic, palmitic, myristic and sometimes oleic (106,108,109).

1.3.1.1c Outer membrane of Gram Negative Bacteria

The outer membrane consists of an outer monolayer of mainly LPS and an inner layer of phospholipids with various proteins attached to or imbedded in the bilayer. LPS itself is an amphiphilic molecule containing three distinct regions; a hydrophobic region (lipid A), a core polysaccharide region and an O-specific side chain (96,102,110).

The lipid A portion of LPS has 5 or 6 fatty acids linked to diglucosamine phosphate. Covalently attached to this is the oligosaccharide core region which is then substituted with O-specific side chains. The O-specific side chains consist of an array of sugars, some of which are unique in nature, and are responsible for specific serological reactions that are often used in identification (96,110,111).

As discussed above the outer surface of bacteria generally carry a net negative charge. This is a result of the net negative charge of LPS. One of the most important features of LPS is that it appears to be anchored in the outer membrane by binding to outer membrane proteins. This is thought to be achieved through hydrophobic interactions with Lipid A and by noncovalent cross-bridging of adjacent LPS molecules with divalent metal cations such as magnesium or calcium (100,110-112).

The combination of negative charge and divalent cation cross bridging of LPS ensures the outer membrane of Gram negative bacteria acts as an effective permeability barrier to many molecules and there are several detailed reviews concerning this feature of the outer membrane as well as greater details of its structure (110-112).

However, while the outer membrane of Gram negative bacteria acts as a protective permeability barrier it must also allow the influx of nutrients and efflux of waste products for the cells to survive (112). It has been suggested that there are three main pathways by which small molecules can enter the cells. The first is the hydrophobic pathway where hydrophobic compounds must diffuse through the LPS
layer of the outer membrane (111). This pathway is considered inefficient in many Gram negative bacteria as seen by resistance of Gram negative bacteria to hydrophobic antibiotics (110) and the fact that hydrophobic compounds of greater than 600 Dalton are very poor at diffusing through the outer membrane (113,114). It has been suggested that this impermeability of the outer membrane to hydrophobic molecules is closely related to the combined effects of divalent metal cation bridging of LPS molecules and high surface negative charge (110,111).

The second pathway is the hydrophilic pathway where hydrophilic compounds pass through the outer membrane via porins (110,111). Porins are outer membrane proteins with a water filled channel that allows nonspecific and spontaneous diffusion of small hydrophilic molecules but excludes hydrophobic molecules (110,114).

Since porins provide water filled channels of defined size the rate of diffusion of molecules through the outer membrane is dependant also on size of the molecule even when the size is well within the exclusion limit (114,115). It has been shown that there is a sharp molecular weight cut off for permeability to porin channels with compounds of less than 500 Dalton being able to almost completely permeate and those greater than 700 Dalton being virtually non-permeating. However, between 500-700 Dalton the diffusion rates can vary up to 100 fold for compounds of similar size (116-118). Although there has been some dispute over how general porins (such as OmpF) regulate uptake across the outer membrane it is currently believed that the negatively charged residues within the porin channels primarily modulate pore selectivity (119,120).

Size exclusion by porins has lead to estimation of the porin channel size. *E. coli* K12 porin Omp F has been estimated to have a channel with a diameter of 1.2nm while another porin, Omp C, of the same organism was estimated to have a 1.1 nm diameter. *P. aeruginosa* PAO1 porin has also been estimated at around 2nm (112) and Omp F from the same organism as 2.2nm (121).

Despite the fact that *P. aeruginosa* porins are generally larger than *E. coli* porins some *P. aeruginosa* strains have demonstrated resistance to a wide range of antibiotics. This resistance has been attributed to the poor permeability of the outer membrane of this organism (122-125) which is a direct result of only 0.2-1% of *P. aeruginosa* porins actually being open (121,126) while wild type *E. coli* is estimated to have the majority if not all of its channels open (115).
The third pathway, called the self-promoting pathway, has been postulated as a mechanism for the uptake of polycationic antibiotics, like polymyxins (127,128). This pathway involves the displacement of divalent cations (in particular Mg$^{2+}$ and Ca$^{2+}$) from LPS by these polycations thus destroying the LPS cross bridging and destabilizing the outer membrane. Because this can result in enhancement of uptake of lysozyme, β-lactams, and hydrophobic fluorescent dyes across the outer membrane it is suggested that such interactions also promote the uptake of the interacting polycationic antibiotic itself (111,129).

1.3.1.2 Yeasts

Yeast, such as *Candida albicans*, have a cell wall outside their plasma membrane (Figure 1.5) which comprises about 30% of the cells dry weight. The wall is composed primarily of polysaccharides consisting of β-glucan, chitin and mannann. The outer layer of mannann forms a hydrophilic barrier to hydrophobic compounds similar to the LPS outer layer of Gram negative bacteria (130).

![Schematic representation of the cell envelope of yeast](image)

**Figure 1.5** – Schematic Representation of the cell envelope of yeast. –P-, phosphodiester linkage; -S-S-, disulphide linkage; C, integral membrane protein.

Modified from Sheperd and Gopal (130)
1.3.2 Bioavailability

Regardless of the pathway by which a compound may enter a cell it must be in a form that is available to the cell in order for it to exhibit antimicrobial activity. It is believed that for compounds of low solubility only those molecules that are dissolved in the aqueous phase are available for intracellular metabolism. Transfer of lipophilic and also hydrophilic substrates proceeds via dissolution in the aqueous phase and subsequent uptake (passively or actively) by the cell (77).

Direct contact between lipophilic compounds and the hydrophobic part of the cell membrane is prevented by the presence of the cell wall and/or the hydrophilic parts of the outer membrane (77). Although this subject has not been well studied for insoluble compounds, the general opinion is that uptake of cyclic hydrocarbons is a passive process. This theory has been supported recently by work on microencapsulation of essential oils in yeast cells where cell viability was found to have no effect on the rate or amount of oil encapsulation (131). An important mechanism in the uptake of lipophilic compounds therefore becomes the partitioning of these molecules into the lipid bilayer of the cytoplasmic membrane (77).

1.3.2.1 Partitioning of solutes into membranes

1.3.2.1a Octanol-Water Partition Coefficients

The Octanol-Water partition coefficient \(K_{ow}\) is the distribution ratio of the equilibrium concentrations of a dissolved substance in a two phase system consisting of the largely immiscible octanol and water (132-134). This ratio can be defined as follows;

\[
\text{Partition Coefficient (}K_{ow}\text{)} = \frac{[X]_{\text{oct}}}{[X]_{w}}
\]

where \([X]_{\text{oct}}\) is the concentration of the substance in the octanol phase and \([X]_{w}\) is the concentration of the substance in the water phase (132,134).

Since the work of Hansch and Fujita (135) the octanol-water system has been widely accepted as being analogous to that of biological membranes (134,136-138).
This has made \( \log K_{ow} \) one of the most commonly reported physical properties of drugs, pesticides and other chemicals (139,140). This is not surprising since in order for compounds to have biological activity, they must be able to traverse or at least partition into biological membranes (139).

Hansch and Fujita (135) suggested that the rate limiting step for the biological action of chemicals could be represented by Figure 1.6.

```
\begin{center}
\begin{tikzpicture}
\t\node[draw] (in) at (2,1) {compound in extracellular phase};
\t\node (site) at (4,1) {site of action in cellular phase};
\t\node (response) at (9,1) {biological response};
\t\node (critical) at (5.5,0) {\text{critical reaction}};
\t\node (t_x) at (5,0.5) {$t_x$};
\t\draw (in) edge (site) (site) edge (response) (response) edge (critical) (critical) edge (t_x) (t_x) edge (response);
\t\node at (2.5,0) {Step I};
\t\node at (6.5,0) {Step II};
\t\node at (8.5,0) {Steps III to \( n \)};
\end{tikzpicture}
\end{center}
```

Figure 1.6 – Pathway required for a chemical to enter a cell and create a biological response. Taken from (135)

Here the first step is pictured as a random walk process in which the molecule in question makes its way from the solution outside the cell to a particular site inside the cell or membrane of the cell (135). This first step is visualised as a relatively slow process, involving the removal of the loosely held water sheath which surrounds organic molecules in aqueous solution (138). The rate of this step is therefore highly dependent on the molecular structure of the compound concerned. Once having arrived at the site of action it may then exert its activity chemically or physically. This action is limited by the available concentration of the compound at the site of action, and hence is directly dependent on the step 1 of this model (135).

Therefore step I is the rate limiting step and can be basically approximated as a simple partitioning process between an aqueous-like phase to an organic-like phase such as the octanol/water system (135). For sparingly soluble compounds such as terpenes Hansch and Fujita’s (135) theory can perhaps be extended because the biological action of a compound is dependent on the partitioning process from water to cell and also dependent on the partitioning process from itself into water. Compounds which are sparingly soluble in water may not be available in high enough
quantities to feed the sites of action with enough compound to exert measurable activity. Also if the rate of partitioning of the compound into water is far slower than that of the partitioning from water to the cell the time required to exert any significant activity may in practical terms be infinitely long.

1.3.2.1b Applications of Partition Coefficients to Biological Activity

Partition coefficients have been used extensively in correlations with biological activity (141) (for review see (138)). For example, they have been used with much success in environmental chemistry in predicting bioconcentration (bioaccumulation). Bioconcentration potential, the ability of a compound to accumulate in an animal from its environment, is critical not only for the animal, but also other members of the food chain. In particular the bioconcentration of compounds from water to fish has been shown to correlate with octanol-water partition coefficients (133,139). For example Chiou et al. (133) found a linear correlation between log $K_{ow}$ and the bioconcentration of several organic compounds including pesticides into rainbow trout. The absorption of chemicals from ground water to soil has also been successfully correlated with partition coefficients (139).

A linear correlation has also been found to exist between log $K_{ow}$ of aliphatic alcohols and their effect on plasma membrane integrity of rat liver epithelial cells. Two types of membrane disruption were measured by monitoring release of lactate dehydrogenase (LDH) from the cells. Mild disruption of the membrane, indicated by a 50% increase in LDH release, and moderate disruption, indicated by release of 50% of the maximal release of LDH, were both found to be linearly correlated to hydrophobicity (142).

It has also been determined that a linear relationship exists between log $K_{ow}$ and the haemolytic activity of a wide range of compounds extending from simple alcohols through to various benzene and naphthalene derivatives. This highlights the broad spanning relevance of this molecular parameter to various types of compounds (143).

Antibacterial activity has also been correlated to partitioning. The antibacterial activity of cephalosporins and penicillins were correlated with partitioning, as measured using reversed-phase thin layer chromatography. It was found that a parabolic relationship existed between lipophilic character and antibacterial activity.
for the cephalosporins against *E. coli* and *S. aureus*. It was suggested that this parabolic relationship occurs because the penetration rate of the compound increases and decreases as its lipophilic character increases progressively and passes through an optimum. This penetration rate is directly linked to activity as the penetration through the cell wall or membrane is a prerequisite for the activity of these compounds. In the case of penicillins against *E. coli*, however, a linear relationship was found, with activity increasing with increased hydrophilicity. It was also noted in this study that those compounds most active against Gram negative organisms were more hydrophilic than those which were most active against Gram positive organisms. This was interpreted on the basis of the different lipid compositions of the cell walls of the Gram positive and Gram negative organisms. The high lipid content of the cell wall of a Gram negative bacterium such as *E. coli* could retain the most lipophilic molecules, which would not reach their site of action. Therefore, only the most hydrophilic compounds could cross the cell wall and exert their toxic effect. In the case of Gram positive bacteria such as *S. aureus* the low lipid content of the cell wall would permit the high activity of more lipophilic compounds provided they can cross the hydrophilic barrier surrounding the cell (144).

Log $K_{ow}$ has also been correlated with progesterone 11α-hydroxylase activity of the fungus *Rhizopus nigricans*. Progesterone 11α-hydroxylase activity was found to be significantly reduced when the organism was grown in various two phase (aqueous-organic) liquid systems. When the organism was grown in the presence of water soluble amounts of the same organic solvents (e.g. cyclohexene) no progesterone 11α-hydroxylase activity was lost. Thus, the effect of the organic medium was found to be directly related to log $K_{ow}$ rather than the water solubility. It has been suggested that this relationship occurs because a critical concentration within the cell membranes is required before progesterone 11α-hydroxylase activity is affected (145). Hence the compounds must not only be available to the cells but also able to interact significantly with the membrane to cause any effect.

Despite the many good correlations between log $K_{ow}$ and various biological activities there is some argument that membranes can not always be appropriately modeled by bulk octanol and water phases. This results from the fact that lipid bilayers have high surface to volume ratios and are, therefore, interfacial phases of matter. In interfacial phases physical properties vary with distance from the interface.
In contrast, in bulk phases, physical properties are uniform throughout. Some studies have shown that in fact the partitioning of compounds such as benzene and hexane into model lipid bilayers is not only dependant on log\(K_{ow}\) but also surface density and chain ordering of the bilayers (146,147).

### 1.3.3 Physicochemical Effects

Although accumulation of lipophilic compounds into membranes via partitioning is important in terms of bioavailability, it is also important because such accumulation can significantly affect the physicochemical properties of membranes and consequently the function of the cell (77,148-150).

The ability of phospholipids to spontaneously form liposomes (i.e. globules containing bilayers of lipid) in water, above their transition temperature, has led to their use as model membranes (108). The physico-chemical properties of membranes are therefore usually described by referring to the properties of such model lipid bilayer systems and the roles of lipids in these bilayers.

#### 1.3.3.1 General Overview of the Roles of Lipids in the Fluid Mosaic model of Biological Membranes

Within the terms of the fluid mosaic model for biological membranes the functional roles of lipids fall into categories related to their ability to self-assemble into bilayers on hydration, thus providing a permeability barrier as well as a matrix with which functional membrane proteins can be associated (104,105). Roles of individual lipid components therefore are concerned with establishing the bilayer structure (or order), establishing appropriate permeability characteristics, satisfying insertion and packing requirements around membrane proteins (104,105,151,152), as well as allowing surface association of peripheral proteins via electrostatic interactions. Each of these functional demands for lipids are critical to the function of cells and are linked together in describing the most important, and most difficult to quantitate, physicochemical property of biological membranes, membrane fluidity (148). Influences on membrane fluidity will affect membrane function and therefore the function of the cell.
1.3.3.2 Membrane Fluidity

Membrane fluidity is defined, for normal liquids, as the reciprocal of viscosity, which is an easily measured physical characteristic. However, membranes are anisotropic solutions and require at least two parameters to describe fluidity. Although membrane fluidity is difficult to quantify there are a number of concepts that can be used for discussing the molecular motion in the bilayer and thus act as indicators of effects on membrane functioning. As noted above the most important of the membrane fluidity-related parameters possessed by lipids are order, packing and permeability (148).

1.3.3.2a Membrane Order

The order of a membrane is often described using the order-disorder transition, or gel to liquid-crystalline phase transition of the lipid or lipids which are present in a membrane ($T_C$) (104,153,154).

The order-disorder transition of membrane lipids is a phase transition where the acyl chains of the lipids change from a gel state to a liquid crystal state (see section 5.1.1 for detailed discussion), the nature of which has been deduced and studied through a combination of $^1$H-NMR, $^{13}$C-NMR, $^{31}$P-NMR, X-ray and thermodynamic experiments (106,153,155-166) (see Figure 1.7)

![Diagram of membrane fluidity](image)

$T < T_C$  $\quad \implies \quad \quad \quad \quad T > T_C$

Figure 1.7 – Schematic representation of the reversible gel to liquid-crystalline phase transition ($T_C$) of lipids. $T$; temperature. Modified from (167)
However, effects on $T_C$ transition are most easily measured using Differential Scanning Calorimetry (DSC). This method has been extensively used in the past for measuring this and other thermal transitions in both model and biological membranes (82,163-165,167-187). This technique is based upon the endothermic nature of transitions from a more ordered state, that is the gel state, to a disordered state, that is when the lipids are in the liquid crystalline state. The resulting change in enthalpy from the phase change is measured by gradually increasing the temperature of two cells (one of which contains the sample and the other is a reference) and comparing the heat input required to increase the temperature of both cells equally. The output of such a measurement for pure lipids, such as dipalmitoylphosphatidylcholine (DPPC), is shown in Figure 1.8. The area under the curve is related to the enthalpy of the phase transition, the peak maximum is termed the phase transition temperature (in the case of order disorder the $T_C$), while the front edge of the peak is known as the onset temperature (OT) (104,175,188). The onset temperature is often used in preference to the transition temperature since many lipid-solute or lipid-lipid mixtures have broad and/or asymmetric peaks. This being the case the relative cooperativity of a phase transition, that is the extent of transmission of motional changes from one lipid molecule to the next, can also be evaluated directly from a DSC trace. Since cooperativity is related to the sharpness of the transition peak, the half height width (HHW) is also often determined (188).

![Figure 1.8 - DSC thermogram of DPPC. $T_C$ – transition temperature, OT – Onset temperature, HHW – half height width.](image-url)
The advantage of differential scanning calorimetry is that it provides thermodynamic information without requiring external probes that may perturb the system. Generally, the manipulations involved in DSC do not alter the specimen (104,188). The limitation of this method is that it provides thermodynamic, not mechanistic information about the changes and therefore should be used in conjunction with other techniques (104,170).

In general those compounds which are able to reduce the \( T_C \) of a lipid bilayer are considered to have a disordered effect on the bilayer whilst those which increase the \( T_C \) are considered to increase the order of the bilayer. Either of these two effects can be detrimental to the function and reproducing capabilities of a cell since membrane order can affect permeability, packing and membrane bound protein functioning.

The importance of the \( T_C \) to cell membranes was noted as far back as the late 60's and early 70's during investigations on bacterial (155,167,170,172,189,190) and mammalian membranes (191). Studies on the membrane properties of *Mycoplasma laidlawii* showed that order-disorder transitions not only existed in the membranes of this organism but also could affect physiology and growth. *M. laidlawii* membranes are able to be altered by supplementing the growth medium with the appropriate fatty acids which are then incorporated in the lipid bilayer. Thus *M. laidlawii* membranes that consist of mainly one particular lipid can be formed. By varying the fatty acids added, various membrane types can be formed, each with a different \( T_C \).

Inspection of the morphology of these membranes highlighted the importance of the temperature at which \( T_C \) occurs. It was found that cells such as those grown on oleate, with melting points below the growth temperature of the organism were filamentous, while those grown with a \( T_C \) region spanning the growth temperature of the organism became coccoid. However, cells with transitions above the growth temperature were swollen and eventually lysed. This process was preventable by addition of 0.3 M sucrose although cell division completely ceased. These results suggest that osmotic imbalance occurs when the membrane transition temperature exceeds the growth temperature and that for transport processes to function properly the hydrocarbon chains in the membrane must be in the liquid crystalline state (167).

Since lipid order is related to lipid bilayer stability and permeability it is likely that the osmotic imbalance occurs due to increased permeability of the membrane.
This will increase leakage of small ions which are involved in membrane potential and hence energy of the cells (see section 1.3.3.2c) which probably accounts for cessation of cell division.

Similar results are obtained for *E. coli* when the membrane transition temperature is varied by including 3-decynoyl-N-acetylcystamine (DNAC), an inhibitor of unsaturated fatty acid synthesis. Similar to *M. laidlawii*, *E. coli* growth ceases when the membrane transition in whole cells rises above the growth temperature. In addition, when the $T_C$ is above the growth temperature, permeability of the membrane is increased while enzyme mediated transport across *E. coli* membranes is decreased (170).

Other experiments using mutants of *E. coli* which are unable to synthesise or degrade unsaturated fatty acids (fatty acid auxotrophs) have shown that the changes in $T_C$ of the membrane, by incorporation of different fatty acids in the growth medium, is well correlated with sudden changes in o-nitrophenyl galactoside hydrolysis (192), respiration, membrane transport and cell growth (190,193). Effects on proline transport and succinate dehydrogenase activity have also been related to phase transition of *E. coli* mutant membranes (194).

Visible $T_C$'s have now been observed and studied in other bacteria such as *Acholeplasme laidlawii*, *S. aureus*, *Enterococcus faecalis*, yeasts such as *Saccharomyces cerevisiae*, erythrocyte membranes, and mitochondrial membranes (108,175,193).

1.3.3.2b Membrane Packing (Bilayer Stability)

Membrane packing in lipid bilayers is important to the formation and stability of membranes. It has been shown, through theoretical calculation by Isrealachvili (195), that the packing geometry of lipid molecules determines the packing of the lipids into certain lipid aggregations. From the theoretical model a simplified concept has emerged where each lipid is treated as a building block. Lipids are then simply classified as cones, cylinders or inverted cones depending their packing geometry (see Figure 1.9), which is defined as follows;

\[
\text{Packing Geometry} = \frac{v/l}{a}
\]
where $a$ is the phospholipid headgroup water interfacial area, $l$ is the hydrocarbon chain length, and $v$ the hydrocarbon chain volume.

Those lipids which have a relatively small headgroup area compared to the hydrocarbon area will have a packing parameter of $>1$ and can be visualised as cone shaped lipids. Cone shaped lipids will prefer to form inverted hexagonal II (HII) lipid aggregates. Those lipids which are considered cylindrical (packing parameter 0.5-1) will prefer to form bilayers while those which are considered inverted cones (packing parameter $<0.5$) will prefer to form hexagonal I (HI) aggregations (104,105,148).

<table>
<thead>
<tr>
<th>Molecular shape</th>
<th>Inverted Cone</th>
<th>Cylindrical</th>
<th>Cone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors effecting packing geometry</td>
<td>1) One acyl-chain</td>
<td>1) Smaller headgroup</td>
<td>2) Increased unsaturation</td>
</tr>
<tr>
<td>Packing ($v/l$) geometry ($\alpha$)</td>
<td>$&lt; 0.5$</td>
<td>$0.5 - 1.0$</td>
<td>$&gt; 1.0$</td>
</tr>
<tr>
<td>Configuration of the phospholipids</td>
<td>Hexagonal type I or Micellar</td>
<td>Bilayer</td>
<td>Hexagonal type II</td>
</tr>
</tbody>
</table>

Figure 1.9 – Molecular shape of various phospholipids and their corresponding polymorphic lipid phases. Taken from (148)
In biological membranes the situation is more complex because they consist of a mixture of various lipids and proteins. Other factors such as pH, temperature, presence of divalent metal cations or the ionic strength of the environment will also affect the packing of lipids into certain aggregation structures (148). The lipids must also seal membrane proteins into the bilayer so as to prevent nonspecific leakage and to provide an environment appropriate to a functional protein conformation (104). Such membrane proteins will have varying shapes within the bilayer and hence require cone or inverted cone shaped lipids in the region directly surrounding the protein to achieve this effectively (105).

In addition to simple packing requirements, various microorganisms have been found to utilise non-lamellar lipids, in particular those which have a strong preference for the inverted hexagonal phase (HII). In *A. laidlawii* a proper balance between bilayer and non-bilayer forming lipids is maintained by the microorganisms in response to changes in chemical (196) and environmental conditions (148). In *E. coli* and *Clostridium butyricum* the phase preference of the membrane lipids is regulated by adjustments in the ratio of bilayer to non-bilayer lipids (148). It has also been proposed that local regions of lipids forming transitory non-lamellar phases may be an advantage to some membrane functions such as membrane fusion, cell division and transbilayer movement of lipids and proteins (104,105,148,197,198).

Thus it is crucial to a cell that a balance is kept between those lipids which are bilayer forming and those which are not. Clearly, it is unfavorable for a cell to have too high a proportion of non-bilayer forming lipids in its membrane. Likewise it will also be unfavorable to have existing bilayer forming lipids induced into non-bilayer phases by the partitioning of foreign compounds into the membrane.

One method for assessing bilayer stability is to measure the lamellar-inverted hexagonal phase transitions of lipids (Lα-HIII). For example, egg phosphatidylethanolamine (PE) has a transition from lamellar (Lα) to inverted hexagonal (HIII) phase when the temperature is raised to 30°C (148). The temperature at which this phase transition occurs is termed T_{LH}. As for T_C this can be measured using DSC (199), although ^31_P-NMR is also widely used to monitor T_{LH} and formation of HII phases by lipids (105,197-201).

Those compounds which lower the T_{LH} will have a destabilizing effect on the membrane bilayer structure whilst those which increase it will tend to stabilise the
bilayer. However, increasing or decreasing the $T_{LH}$ may affect the functioning of the membrane. Decreasing the $T_{LH}$ may cause packing problems for proteins and disruption of bilayer integrity, each of which may affect permeability. Raising the $T_{LH}$, although stabilising the membrane, may also prevent or inhibit formation of non bilayer aggregations required as a response to environmental conditions or other functions, such as membrane fusion, as listed above.

X-ray diffraction is another technique that has been used to elucidate changes in bilayer structure and packing arrangements (155,165,166,176,194,199,202-205) and hence detect possible affects of membrane functioning. X-ray diffraction patterns from hydrated lipids will produce repeat periods of certain size which is indicative of bilayer thickness (168,169,202,205). For example, DPPC hydrated to 70% was found to have a repeat period of 65Å (168) whilst hydrated PE has a repeat period of 54Å (203). However, the repeat period for lipids is dependent on the level of hydration of the lipid. DPPC when dry has a repeat period of only 52Å and PE 42Å (203). Therefore hydration levels of lipids must be kept constant if comparative experiments on changes in bilayer thickness are to be made (169,206).

As well as short angle deflections, lipids also have characteristic wide-angle reflection patterns. For example, pure DPPC multilayers in excess water give a sharp 4.2Å wide-angle reflection surrounded by a diffuse broad band (168,169,206). This type of pattern has been interpreted in terms of hydrocarbon chain tilt of about 30° (168,205,206). Effects on this chain tilt may also affect membrane thickness, packing and therefore function.

Electron density profiles (see Figure 1.10) are another output from X-ray diffraction studies which provide some information about bilayer structure. The high density peaks in the profile correspond to the phospholipid headgroup, primarily the high density phosphate group, while the low density troughs in the centre of the bilayers correspond to the lipid terminal methyl groups. The relatively flat medium density region between the head groups and terminal methyl dip is the methylene region of the lipid hydrocarbon chains. The medium density regions at the outside edge of each profile are the fluid spaces between bilayers. Although electron profiles of different lipids will have the same general characteristics, the widths and depths of the different regions of the density profiles will vary according to the chemical composition and physical state of the lipids (169,205,206). Thus, changes in bilayer
thickness or effects on specific regions of the lipids in the bilayer may be viewed in this way. For example the induction of an interdigitated phase is able to be seen using this method (207).

Figure 1.10 – Electron density profiles for pure DPPC (A) and DPPC with excess hexadecane (B), both at 70% water content. Modified from (169)

A more direct method for monitoring effects on bilayer structure and stability has become available with the recent advent of Atomic Force Microscopy (AFM), which has now been applied to many biological and non-biological systems (for a review see (208)). The AFM works by measuring a tiny force (approx. $10^{-9} - 10^{-11}$ N) of contact between the apex of a cantilevered tip and the surface of the sample (209,210). This is achieved with high sensitivity by use of a laser which is able to detect deflection in the cantilevered tip of less than 0.1 nm. Scanning can be done in a dry environment or under water in a fluid chamber (208) (see Figure 1.11). In the conventional contact mode the probe is in continuous contact with the surface during scanning. To reduce the interaction of the AFM tip and the sample, however, Tapping Mode is often used. In this mode, the cantilever is oscillated at a high frequency resulting in intermittent contact between the probe and the sample (211).
Single lipid bilayers or monolayers are able to be formed on surfaces such as mica (called supported lipid bilayers or SPB's) using several different techniques (64,210,213-216) and can be imaged using the AFM (216,217) down to the level of distinguishing individual phospholipid head groups (209,212,218). This, in conjunction with monitoring the thickness of the bilayer (218,219), via use of
naturally occurring holes in the SPB, and visual monitoring of gross physical changes in the bilayer surface, can be used to monitor the effects of compounds on the stability and packing arrangements in the SPB.

Several studies already exist where the effects of compounds such as alkanols (220), cholesterol (219), tris buffer (221) and the antifungal antibiotic filipin (222) on supported lipid bilayers have been determined using AFM (see section 5.2.2.1 for more detailed discussion).

AFM is regarded as a non-intrusive method of monitoring membrane properties and has even been applied to whole cells with varying degrees of success (211,223-226). However, despite the non-intrusive nature of AFM sample destruction or damage is a danger when studying lipid bilayers and either Tapping Mode or low force Contact Mode (i.e. less than 1nN) must be used to reduce tip/sample interaction. Such bilayer damaging and/or destruction has been shown by several workers (210,217,218).

1.3.3.2c Membrane Permeability

The main function of the cell membrane is a permeability barrier, regulating the passage of solutes between the cell and its external environment. The barrier properties of the cytoplasmic membrane is of special importance for the energy transduction of the cell. An intact permeability barrier to small ions such as $K^+$, $Na^+$ and $H^+$, for example, is vital for maintaining electrochemical gradients which give rise to a membrane potential that is used to drive other membrane-mediated transport processes (77,107). An increase in the permeability of the membrane for such ions may lead to a dissipation of the proton motive force, resulting in a less effective energy transduction. Furthermore, an increased permeability of the cell might also affect the internal pH control of the cell or could result in the loss of essential metabolites (77,148). In extreme instances increased permeability of ions into and out of the cell may lead to a large enough imbalance in osmotic pressure to cause cell lysis.

Factors which can affect small metal ion permeability are the lipid composition of the membrane, the stability of the membrane structure and the membrane order (152).
1.3.3.3 Consequences of Membrane Damage and Dysfunction

As well as providing a permeability barrier to the outside environment another of the membrane’s important functions is to provide a matrix for various important membrane bound enzymes. These include ATPases, transport proteins, transerases oxireductases and other enzymes involved in solute transport and electron transport chains (77,148,227). Some phospholipids such as PE have also been shown to regulate the activity of some membrane proteins. For example PE has been shown to have a regulatory effect on a calcium pump protein (151,197).

Studies have shown that the activity of membrane embedded enzymes can be influenced by the physicochemical properties of the membrane. For example compounds which cause thickness changes in the membrane through partitioning may hinder the activity of transmembrane proteins to transport molecules across the lipid bilayer. Membrane expansion may also affect protein-protein interactions in supra molecular protein complexes such as the electron transport chain, in which electron transfer may be slowed when protein complexes become dissociated (77).

In addition, membrane partitioning solvents such as hydrocarbons and alkanols may disrupt orientation of boundary lipids, which are lipids closely associated with membrane proteins and are suspected as being necessary for the correct conformation and orientation of enzymes. Displacement of these boundary lipids by the interaction of hydrophobic solvents with the membrane might result in a lipid/protein mismatch and inactivation of the enzyme. Furthermore, direct interactions of the solvent with hydrophobic parts of the enzyme, which are expected to affect enzyme activity, might also occur (77,148).

1.4 Membrane Adaptations

Membrane adaptation mechanisms to toxic solvents fall into two general groups. The first are those adaptations aimed at maintaining a stable functioning bilayer structure. This includes regulating and modifying; the amounts of saturated and unsaturated lipids in the membrane; the types of phospholipid head groups present and; the protein/lipid ratios in the membrane, all of which allow regulation of the T_{LH} and/or T_C. Hence, control of membrane stability and lipid ordering, each of which affect membrane permeability, can be achieved (see Figure 1.12) (77,148).
The second group are those membrane adaptations which are aimed at reducing the actual level of solvent able to partition into the membrane. This can again be achieved by manipulation of the membrane content by the cell such that partitioning into the membrane is no longer as energetically viable (see Figure 1.12) (77).

![Diagram showing ordering of lipid bilayer, physiological adaptations to lipophilic compounds, active excretion, S-layer hydrophobicity, and cell wall hydrophobicity with LPS modification.]

**Figure 1.12** – A summary of physiological adaptation mechanisms that may protect against the toxic effects of lipid soluble solvent molecules.

*(Taken from (77))*

However, for Gram negative organisms there is also the question of the outer membrane which, as described above (see section 1.3.1.1.b), has as part of its structure an LPS layer that has low permeability for hydrophobic compounds. Some strains of the Gram negative bacterium *P. aeruginosa* are noted for their solvent tolerance and coinciding outer membrane impermeability (77). The role of the outer membrane as a solvent tolerance mechanism and factors which affect this mechanism are explored in more detail in chapter 6.
1.5 Objectives

Taking into account the current status of research in the area of antimicrobial activity of essential oils and their major components the terpenoids, the main objectives of this thesis were;

* To further the understanding of the links between molecular properties and structure and the antimicrobial activity of terpenoids.

* To determine the effects of terpenoid compounds on microbial membranes by conducting experiments on both microorganisms and model systems.

* To further the understanding of a general antimicrobial mode of action of terpenoids.
Chapter 2
Materials and Methods

2.1 Chemical Analysis

2.1.1 Terpenoid Standards

The terpenoid standards used in this study and their percentage purity, as determined by Gas chromatography (see section 2.1.1.2), were: (-)-Borneol (96%), (+)-Camphene (96%), (±)-Camphor (98%), Car-2-ene (94%), Car-3-ene (94%), Carvacrol (99%), (-)-Carveol (98%), (R)-(−)-Carvone (99%), 1,8-Cineole (99%), (R)-(−)-Citronellal (95%), β-Citronellol (95%), p-Cymene (>99%), Dihydrocarveol (98%), Dihydrocarvone (99%), Eugenol (97%), (−)-Fenchol (95%), (1R)-(−)-Fenchone (99%), Geraniol (99%), Geranylacetate (98%), α-Ionone (91%), β-Ionone (97%), (±)-Isoborneol (96%), (cis,trans)-Isoeugenol (93%), (+)-Isomenthol (99%), (−)-Limonene (95%), (−)-Limonene (99%), Linalool (96%), Linalool oxide (98%), Linalyl acetate (93%), t-p-Menth-6-ene-2,8-diol (97%), (±)-Menthol (98%), (−)-Menthol (>99%), (−)-Menthone (95%), Mentholacetate (99%), Methyleugenol (99%), (−)-cis-Myrtanyl amine (98%), (1s2s5s)-(−)-Myrtanol (99%), (1R)-(−)-Myrtenal (97%), (1R)-(−)-Myrtanol (94%), Nerol (97%), Neryl acetate (97%), (−)-α-Pinene (97%), (−)-α-Pinene (96%), (s)-(−)-β-Pinene (99%), (+)-Pulegone (85%), α-Terpinene (85%), γ-Terpinene (>99%), Terpinen-4-ol (95%), α-Terpineol (98%), Thymol (>99%), (1s)-(−)-Verbenone (96%), (s)-cis-Verbenol (95%), Limonene oxide (98%) as obtained from Aldrich (Castle Hill, Australia) and 1,4-Cineole (74%), (s)-(−)-Perilla alcohol (95%), (−)-Perilla aldehyde (92%), α-Terpinolene (90%), (α)-(±)-Terpinylicacetate (95%) as obtained from Fluka (Castle Hill, Australia).

In addition Piperitone (92%) was obtained from Harmann and Reimer and Car-3-en-2-one (94%) was extracted and purified from Zieria adenodonta oil as described in section 2.1.1.1.
2.1.1.1 Purification Of Car-3-en-2-one

Car-3-en-2-one was separated from the essential oil of Z. adenodonta using a glass column (3cm x 50cm) which was packed with approximately 70 mL of silica gel 60. The sample was applied to the column neat and fractions were eluted with petroleum ether (200 mL), 99:1 petroleum ether:diethyl ether (100 mL), 9:1 petroleum ether to diethyl ether (100 mL) and finally diethyl ether (100 mL). Fractions were collected in 50 mL aliquots. The composition of each fraction was analysed by GC. Fractions containing car-3-en-2-one were combined and the solvent evaporated using a Buchii Rotovapor-R rotary evaporator with a water bath temperature of between 45°C and 55°C. The resulting car-3-en-2-one (98%) was stored at 4°C until use.

2.1.1.2 Gas Chromatographic (GC) Analysis

All terpenoid purity determinations were carried out using a Hewlett Packard 6890 GC equipped with a Flame Ionisation Detector (FID) and fitted with an SGE BP5 capillary column (50m length, 0.22 mm ID, 1μm film thickness). Chromatograms were acquired under the following conditions; inlet temperature: 240°C, detector temperature: 280°C, temperature program; initial temperature 80°C for 5 minutes then 4°C/min to 180°C then immediately 45°C/min to 280°C and hold for 5 minutes. Carrier gas was hydrogen at 40 cm/sec. The split ratio was 1:10 and injection size was 1 μl. Terpenoid solutions were made up in methanol to between 250-500 ppm.

2.1.2 Water Solubility of Terpenoids

2.1.2.1 Sample Preparation

An excess of the terpenoid in question (usually 100μL) was added to 1 mL of MilliQ water in a 2 mL GC auto-sampler vial which was then sealed. The water/terpenoid mixture was first vortexed for 5 minutes and then sonicated (Bransdon 1200 bath sonicator) for 30 minutes.

After sonication, the mixture was equilibrated in a 25°C water bath for a minimum of 1 hour before centrifuging at 6000 rpm at 25°C for 15 minutes, in order
to break up any possible emulsions that may have formed. During centrifugation the GC auto-sampler vials were inverted so that sampling of the water layer could be performed through the septum of the vial using a gas tight syringe.

Approximately 500 µL of the water layer was withdrawn from the vial in this manner and placed into a fresh auto-sampler vial for direct analysis using GC. These water layer samples were kept at 25°C before sampling using the water jacketed auto-sampler tray of the GC.

For each terpenoid, samples were prepared in triplicate and duplicate injections were made from the water layers sampled from each sample. This was then repeated so that a total of six vials and twelve injections were made per compound for determination of the water solubility.

It should be noted that in the case of the more volatile hydrocarbons the sampled water layer was divided into two parts and placed into separate vials due to significant loss via evaporation between duplicate injections.

2.1.2.2 GC Analysis

All water solubility analyses were carried out using a Hewlett Packard 6890 GC equipped with an FID and fitted with an SGE BPX5 capillary column (50m length, 0.22 mm ID, 1µm film thickness). Chromatograms were acquired under the following conditions; inlet temperature: 280°C, detector temperature: 280°C, temperature program; initial temperature 80°C for 5 minutes then 4°C/min to 180°C then immediately 45°C/min to 300°C and hold for 5 minutes. Carrier gas was hydrogen at 40 cm/sec. The split ratio was 1:5 and injection size was 1 µL.

Quantitation was done using standard curves generated by external standards of each terpenoid. External standard concentrations used to generate standard curves ranged from approximately 10ppm to 2000ppm and were corrected for the purity of the terpenoids used. Compounds with a higher solubility than the upper limit of the standard curve were carefully diluted by known amounts in more water and re-run through the GC for more accurate quantitation.
2.1.3 Octanol/Water Dispersion Coefficient (log $K_{ow}$)

2.1.3.1 Determination of log $K_{ow}$ by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

2.1.3.1a Standards for Determination of log $K_{ow}$

Six compounds (see Table 2.1) of known log $K_{ow}$ and of similar chemical structure to that of the terpenoids were used as standards in determination of log $K_{ow}$ for the non-amine and non-phenolic terpenoids.

<table>
<thead>
<tr>
<th>Standard</th>
<th>log $K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl alcohol</td>
<td>1.10</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1.48</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.03</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.61</td>
</tr>
<tr>
<td>Cyclohexene</td>
<td>2.86</td>
</tr>
<tr>
<td>p-cymene</td>
<td>4.10</td>
</tr>
</tbody>
</table>

$K_{ow}$ values are averages of values taken from (132) and/or (228). Only values determined by the shake-flask method were used to calculate averages.

A further three phenolic compounds (see Table 2.2) were used for amine and phenolic terpenoids. A separate set of standards was required due to the special interaction of amines and phenolic compounds with C18 type columns.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Log $K_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>1.48</td>
</tr>
<tr>
<td>Eugenol</td>
<td>2.99</td>
</tr>
<tr>
<td>Thymol</td>
<td>3.30</td>
</tr>
</tbody>
</table>

$K_{ow}$ values are averages of values taken from (131) and/or (228). Only values determined by the shake-flask method were used to calculate averages.
2.1.3.1b HPLC Analysis

All HPLC analysis of both terpenoids and standard compounds was done using a Hewlett Packard 1090 HPLC fitted with an Alltech Altima C18 column (length : 150mm, internal diameter : 4.6mm, particle size : 5μm), diode array detector and auto-injector. Detection of compounds using the diode array was done at 215nm with 500nm as the reference wavelength. Where compounds were unable to be detected using diode array an Erma ERC-7510 Refractive Index Detector was fitted and used. HPLC conditions for all analyses were as follows; Oven temperature : 37°C, Flow rate : 1 mL/minute, injection volume : 10μL.

All HPLC analysis was done using a range of methanol:water mixtures of between 75 and 30% methanol with the water portion buffered to pH 7.2 using 0.02M 4-morpholinepropanesulfonic acid (MOPS). All analysis at the different methanol:water ratios were done isocratically. All compounds were analysed at a minimum of four different methanol:water ratios, however the lower limit of methanol able to be used was governed by the polarity of the compounds tested. Three injections were made for the terpenoids and five injections for the standards at each methanol:water ratio.

Retention times for all compounds were recorded at each methanol:water ratio along with the hold time of the column as estimated by the retention times of methanol.

2.1.3.1c Calculations

The capacity factors ($k'$) for terpenoids and standards were calculated at each methanol:water ratio from retention times using the formula below;

$$k' = (t_R - t_0)/t_0$$

$t_R$ = sample retention time
$t_0$ = Mobile phase hold time as estimated by retention time of methanol
For the each standard and terpenoid a plot of \( k' \) versus proportion of organic modifier (i.e. methanol) was generated and extrapolated back to 0% modifier to determine the capacity factor of each compound if the eluent were 100% water \( (k_w) \).

In the case of the standards, \( k_w \) was then plotted against the known \( \log K_{ow} \) (Tables 2.1 and 2.2) of the compounds to form a standard curve. Two separate standard curves were plotted, one for non-amine and non-phenolics \( (\log K_{ow} = 1.43 \log k_w - 0.60, r^2 = 0.950, n = 6, \text{ std. err.} = 0.27) \) and the other for amine and phenolic compounds \( (\log K_{ow} = 1.41 \log k_w - 0.15, r^2 = 0.989, n = 3, \text{ std. err.} = 0.148) \). \( k_w \) of the terpenoids was then used to determine their \( \log K_{ow} \) from these standard curves.

2.1.3.2 Determination of \( \log K_{ow} \) by Shake Flask Method

Solutions of known terpenoid concentration were prepared using type I water, pre-saturated with 1-octanol (Aldrich ACS spectroscopic grade) for 24 hours prior to use. For those terpenoids suspected to have \( \log K_{ow} \) values of less than 3, equivalent volumes of the terpenoid and octanol (pre-saturated with type I water for 24 hours before use) were added together. For those terpenoids with a \( \log K_{ow} \) suspected to be greater than 3, a ratio of 1 mL of octanol to 10 mL of water was used. The resulting two phase mixture was repeatedly inverted for one hour using a slow rotating wheel (approx. 1 rev/10 sec) to which the samples were attached.

After mixing, samples were centrifuged for 30 min (6000 rpm) to ensure any possible emulsions were removed. Both fractions of the sample were analyzed using a 6890 Hewlett Packard GC fitted with an SGE BPX5 capillary column (50m length, 0.25 mm ID, 1\( \mu \)m film thickness) and FID. The GC operating parameters for the analysis were as follows: inlet temperature: 240°C, carrier gas: hydrogen at 40 cm/sec, injection size 1\( \mu \)L, detector temperature: 280°C, initial oven temperature 100°C for five minutes, increased at 4°C/min to 160°C then increased at 45°C/min to 250°C and held for 5 min. Injector split ratios were varied according to the sensitivity required. Quantitation was achieved by use of external standards of each of the terpenoids. External standards were run at each of the split ratios and in each of the solvents (i.e. water and octanol) used. Octanol fractions were diluted in methanol before analysis to reduce column overloading by octanol.
2.1.3.3 Calculation of log $K_{ow}$

Compounds were constructed using the molecular modeling program Chemsite version 2.4.2 (Pyramid Learning, USA). These structures were then transferred to the molecular modeling program Molecular Modeling Pro version 2.4 (Chem SW software, USA) where log $K_{ow}$ values were calculated using an atomistic method developed by Ghose and Crippen (229) and a fragment addition method developed by Hansch and Leo (230). These structures were then imported to KOWWIN version 1.54 (Syracuse Research Corporation) where the log $K_{ow}$ values were calculated using an Atom/Fragment Contribution Method as described in Meylan and Howard (231).

2.1.4 Determination of Molecular Parameters of Terpenoids by Molecular Modeling

Compounds were constructed using the molecular modeling program Chemsite version 2.4.2 (Pyramid Learning) and the lowest energy 3-D conformations of the molecules were determined using the energy minimisation function of the program. These structures were then transferred to the molecular modeling program Molecular Modeling Pro version 2.4 (Chem SW software) where they were put through a more rigorous energy minimisation.

After energy minimisation the following molecular parameters were calculated for each molecule; molecular volume ($Å^3$), surface area ($Å^2$), hydrophilic/lipophilic balance (HLB), polarity, hydrogen bonding capacity, percent hydrophilic surface area, hydrogen bond acceptor capacity, hydrogen bond donor capacity.
2.1.5 Effects of Terpenes on the Phase Transition Temperature and Profiles of Dipalmitoyl-D-α-phosphatidylcholine (DPPC)

2.1.5.1 Sample Preparation

The lipids used in these experiments, dipalmitoyl-D-α-phosphatidylcholine (C16:0) (99%) (DPPC) was obtained from Aldrich chemicals and used without further purification.

Terpenoid/DPPC mixtures were prepared, in 1.5 mL Eppendorf tubes, by first mixing approximately 10 mg of lipid, accurately weighed using a five place balance, and terpenoid to give the appropriate mole ratio of lipid to terpene (1:1 mole ratio of lipid:terpene was used for all comparisons of transition temperature deviations between terpenes). Water was then added to the terpenoid/lipid mixtures in a ratio of 2:1 water:l lipid before the whole sample was centrifuged, at 13,000 rpm for 2 minutes, to ensure no lipid powder or terpenoid was left on the sides of the eppendorf tube. Samples were dispersed by heating above the transition temperature of the lipid (41.5°C) and mixing vigorously via vortex, this was repeated at least twice per sample. Pure DPPC, used as standards for determining the phase transition temperatures, was prepared in the same manner as terpenoid/lipid mixtures, but in the absence of the terpenoid.

All samples were transferred to pre-weighed 40 μL aluminium sample pans and sealed ready for analysis using the differential scanning calorimeter. After analysis sample pans were re-weighed to determine the amount of sample present in the pans for enthalpy calculations.

2.1.5.2 Differential Scanning Calorimetry (DSC)

Calorimetry scans were performed using a Perkin-Elmer DSC 7 Differential Scanning Calorimeter (DSC) fitted with a dry box, Intracooler 2 and using nitrogen as purge gas. Aluminium sample pans (40μL) were used (Perkin-Elmer). Scanning conditions for all experiments were; initial temperature : -20°C, final temperature : 80°C, Scan rate : 10°C/minute. Temperature/area calibrations of the DSC 7 were made at the beginning of each sample batch using indium as the calibration standard. All samples were scanned either three or four times. Due to the expense of pure
DPPC most samples were prepared only once. However, several terpenoids were examined in triplicate either on the same day or consecutive days to monitor the reproducibility of the experiment. Phase transition temperatures were found to be reproducible within 1-2% between samples and between days. \( T_C \) was determined as the onset temperature of the first main transition peak.

2.1.6 Monitoring Effects of Terpenes on the Stability and Packing of DPPC Supported Lipid Bilayers

2.1.6.1 Bilayer Preparation

A lipid/water mixture (ca. 0.5 mg/mL) was prepared and sonicated at its phase transition temperature until transparent (a transparent mixture indicating formation of lipid micelles between 20-50nm in diameter). Freshly cleaved mica was placed into the micelle solution and heated to between 41-48°C for 30 minutes. Mica was then removed, washed and stored at room temperature under water until analysis (typically no more than 24 hours after initial preparation).

2.1.6.2 Bilayer Stability and Lipid Packing - Atomic Force Microscopy (AFM)

Prepared bilayers were imaged under water using a Nanoscope III Atomic Force Microscope (Digital Instruments, Santa Barbara) in Tapping Mode with a low force constant tip (100 \( \mu \text{m} \), narrow legs, 0.38 kN/m, silicon nitride) and fitted with a fluid cell. Tapping Mode was used for the large area scans to reduce the forces exerted by the tip on these fragile bilayers. However, even in Tapping Mode initial images showed some damage. Therefore, at the beginning of each experiment the force exerted by the tip was minimised using force-distance curves. This was able to effectively prevent tip damage to the bilayer as indicated by 10 repeat scans showing no visible changes in the bilayer surface. Consecutive images of the same area (without removing the tip from the surface) of bilayer were then collected before, during and after addition of increasing concentrations of terpene.

High resolution scans (imaging of lipid headgroups) were collected using the same systems as above, but with the tip in Contact Mode and the microscope placed on a vibration isolation platform to reduce vibrational interference.
All images were flattened before being analysed. The high resolution (lipid headgroup) images were Fourier Transformed to reduce noise. These were the only manipulations performed on the data before analysis. The data analysis software provided on the Nanoscope was used for all the results presented in this thesis.

2.1.7 Calcium Complexing

Terpene solutions (30-50 ppm) were prepared to the desired concentrations using MilliQ water. These solutions (3 ml) were added to an equivalent amount calcium chloride in 20 mL headspace vials such that the final terpene/Ca$^{2+}$ ratio was approximately 10:1. The vials were sealed, vortexed for 1 minute then left for 24 hours before reading. Calcium complexing of terpenes was measured using a combination Calcium ion selective/reference electrode connected to a pH/mV meter (Activon Scientific, Gladesville, Australia). Standard curves for the electrode were constructed using calcium chloride solutions ranging in concentration from 1-5000 ppm (ie. 0.0001-0.05 M). Results were calculated as the number of moles of Ca$^{2+}$ complexed per molecule of terpene.

2.2 Microbiological Analysis

2.2.1 Stock Cultures

The sources and strain numbers of all of the organisms used in this study are shown in Table 2.3.

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>SOURCE &amp; STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive Bacteria</strong>:</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>NCTC 8325</td>
</tr>
<tr>
<td><strong>Gram negative Bacteria</strong>:</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>AG 100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>NCTC 6749</td>
</tr>
<tr>
<td><strong>Yeast</strong>:</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>KEM H5</td>
</tr>
</tbody>
</table>

Table 2.3: Stock Cultures - Sources and Strain Numbers
2.2.2 Maintenance Of Stock Cultures

Bacterial stock cultures were maintained on Nutrient Agar (Oxoid, Australia) slopes at 20°C and were subcultured monthly. The yeast stock culture was maintained on Malt Extract Agar (Oxoid, Australia) slopes at 20°C and was subcultured monthly. The incubation period for all stock cultures before storage was 24 hours at 37°C. Purity of bacterial working cultures was assessed on a regular basis by viewing Gram stain results and cell morphology using an BH2 Olympus microscope.

2.2.3 Inoculum Preparation

All bacteria were subcultured from stock cultures into 10 mL of Isosensitest Broth (ISB) (Oxoid, Australia), Malt Extract Broth (Oxoid, Australia) for *C. albicans*, and incubated at 37°C for 24 hours. For MIC and kill rate assays, this broth was then subcultured into a fresh 10 mL broth and incubated for a further 18 hours under the same conditions. For potassium leakage experiments, this broth was subcultured into a fresh 250 mL broth and incubated at 37°C for 18 hours on a shaking incubator (80 rpm). The cell density of each resulting 18 hour broth culture was determined using a Pharmacia Novaspec II spectrophotometer, at 420 nm for bacterial cultures and 600 nm for *C. albicans*. The cultures were then diluted to give a cell density of 1x10^6 cfu/ml for MIC tests (see section 2.2.4) and 1x10^7 cfu/ml for kill rate assays (see section 2.2.5). For potassium leakage experiments, cultures were pelleted by centrifuging at 4000 rpm for 15 minutes. Cell pellets were then re-suspended in 100 mM phosphate buffer (pH 7.1), pelleted and re-suspended again to give a final cell concentration of 4 x 10^9 cfu/mL.

2.2.4 Minimum Inhibitory Concentration (MIC) Agar Dilution Method

Terpenoids were added aseptically to 15 mL of sterile molten Isosensitest Agar (Oxoid, Australia), containing 0.5% v/v Tween 20, at the appropriate volumes to produce final concentrations of terpenoid of 2%, 1%, 0.7, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.05% and 0.01% v/v. Where the volume of terpenoid available was limited the 2% concentrations were omitted. 2% was the upper limit of the agar dilution method
as above this level 0.5% Tween 20 did not adequately disperse the terpenoids throughout the growth medium.

The agar/terpenoid mixtures were vortexed for 15 seconds and immediately poured into petri dishes. The plates were left to set for 30 minutes and then inoculated by pipetting 10 µL from a prepared inoculum (see section 2.2.3) of the desired organism onto the plates. The plates were left to stand for 20 minutes then incubated for 24 hours at 37°C. After incubation the plates were examined for the presence or absence of growth and results were recorded.

Two controls were included with each batch of tests. The first was a negative control involving the presence of the test material, but not the organism to check for contamination of the test material. The second was a positive control involving the presence of the microorganisms, but absence of the test material. All MIC tests were carried out triplicate and repeated on consecutive days.

From the recorded results for each compound, the MIC was determined using the following key and set of rules in order to reduce the subjectivity in reading the results.

**2.2.4.1 Key**

-  = growth
-  = no visible growth
+- = less than 20 isolated colonies or barely visible growth, much less growth than positive control

**2.2.4.2 Rules**

1) Level at which the MIC occurs is the concentration of terpenoid where 5 out of 6 plates show no visible growth. Where intermediate results occur proceed to rule two.

2) Two types of intermediate result are possible;

   a) Positive intermediate – where two or more plates show +− type growth while any other plates show visible growth. In this case the MIC is considered
the next highest concentration level where rule 1 is true. For example if a positive intermediate result is found at the 0.3% v/v level and the criteria for rule 1 are met at the 0.4% v/v level (see Table 2.4) then the MIC is considered to be 0.4% v/v.

<table>
<thead>
<tr>
<th>Test Level (% v/v)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>0.2</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.4 – Example of a Positive Intermediate Result in the Agar Dilution MIC Assay

b) Negative Intermediate – where two or more results show + type growth while all remaining plates show no visible growth. In this case the MIC is considered in between the level where the negative intermediate result occurs and the next highest concentration level where rule 1 is true. For example if a negative intermediate result occurs at the 0.3% v/v level and the criteria for rule 1 are met at the 0.4% v/v level (see Table 2.5) then the MIC is considered to be between 0.3% v/v and 0.4% v/v and is reported as 0.35%.

<table>
<thead>
<tr>
<th>Test Level (% v/v)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>0.2</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.5 – Example of a Negative Intermediate Result in the Agar Dilution MIC Assay

2.2.5 Kill Rate Assay

Aliquots (20 mL) of $1 \times 10^7$ cfu/mL *P. aeruginosa* or *E. coli* cultures were added to sterile 100 mL conical flasks containing sterile magnetic fleas. Cultures not treated with Polymyxin B Nona-peptide (PMBN) were stirred at high speed and aliquots of the cultures equivalent to the amount of monoterpenoid to be used were
removed. To the control flask an equivalent aliquot of sterile ISB was added to the culture whilst to the other flasks the same amount of monoterpenoid was added.

Cultures treated with PMBN (10 μg/mL) were first stirred at half the speed used during the kill rate for 10 minutes after the PMBN was added. The stirring speed was then increased to that used for the non treated kill rate experiments and the procedure outlined above was repeated. Note that although stirring speeds were not measured, they were kept consistent throughout all experiments.

Replicate samples (1 mL) were removed from the cultures immediately after the addition of the monoterpenoid and subsequently at 5, 15, 30, 60 and 120 minutes. Samples were placed immediately into a pre-warmed (37°C) neutralising broth consisting of Tryptone Soy Broth (Oxoid, Australia) (30 g/L), Neutralised Liver Digest (30 g/L) and lecithin (10 g/L). This was left to stand for 10 minutes and placed into a water bath at 10-12°C until plated out.

The samples in the neutralising broths were diluted in 0.1% peptone and poured plated using Tryptone Soy Agar (Oxoid, Australia). Plates were incubated at 37°C for 72 hours before counting.

2.2.6 Potassium Leakage of *E. coli* Cells when Exposed to Terpenoids

Potassium leakage from *E. coli* cells was measured using a combination potassium ion selective/reference electrode connected to a pH/mV meter (Activon Scientific, Gladesville, Australia). Standard curves and electrode slope checks were done using various dilutions of 1000 ppm KCl solution. Terpenes were added directly to 1 mL of 100 mM phosphate buffer (pH 7.1) at levels where they were soluble in the final 2 mL sample volume (700-2000 ppm depending on terpene). Terpene/phosphate buffer mixtures were stirred at moderate speed for 5 minutes before 1 mL of the $4 \times 10^5$ cfu/mL *E. coli* cell cultures were added to give a final cell concentration of $2 \times 10^5$ cfu/mL.

Free potassium was then immediately measured using the ion selective electrode and monitored at 5, 10, 15, 20, 30 and 60 minutes after addition of the cells. For each experiment terpene treatments were carried out in duplicate along with an untreated cell culture as a control. Experiments were repeated three times and on different days for each of the terpenes.
2.3 Statistical Analysis

The statistical package used for all data analysis for this study was the Statsoft software package Statistica version 4.5 (Statsoft Inc).

2.3.1 Cluster Analysis

MIC’s (see section 2.2.4) were calculated in parts per million (ppm) and then converted to maximum non-inhibitory concentrations (MnIC). The MnIC was defined as the maximum concentration tested at which growth still occurred. This was done to avoid problems with MIC values that were greater than the highest levels able to be tested. Multivariate statistical analysis was then performed on the MnIC data.

The first multivariate analysis used was hierarchical cluster analysis which was used to group the terpenoids via the level and specificity of their activity against the four test organisms. The number and members of each of the groups was then confirmed using K-means cluster analysis. K-means cluster analysis was then used to generate a plot of means for each group to highlight the differences in specificity and levels of activity of the compounds in each group.

2.3.2 Discriminant Analysis (DA)

The molecular parameters (see section 2.1.4) of each molecule along with solubilities and log $k_{ow}$ values, as determined by measurement, were used in the following discriminant analysis. Each terpenoid was placed into one of five groups based on prior classification using hierarchical cluster analysis derived from biological activity data (i.e. MIC). Discriminant Analysis (DA) was then performed on the molecular parameter data to determine which, if any, of these parameters most contribute to the formation of these groups. All DA was done using the forward stepwise technique with F to enter set at 2.07 and F to remove set at 1.07, as suggested by Afifi and Clark (232), to give p values of 0.15 and 0.30 respectively. Where outliers occurred in any of the molecular parameters they were substituted by means. Due to its limited size, group III was omitted from all DA. More specific differences between individual groups of compounds were then determined by performing DA on pairs of groups in order to determine factors which affected specificity of compounds against a
particular organism(s). Group III was again omitted due to its limited size and unusual organism specificity.
Chapter 3
Antimicrobial Activity Patterns of Terpenoids

3.1. Introduction

There are two general types of microbial assay techniques that are employed in testing of antimicrobial activity of antibiotics and disinfectants. The first, known as diffusion techniques, include those methods which do not require a homogeneous dispersion of the test compound in water. The second, known as dilution techniques, includes those techniques which do require a homogeneous dispersion of the test material in water (233). However these methods have been developed for water soluble antimicrobial agents and require modification for use with insoluble agents such as essential oils and their components.

3.1.1 Diffusion Techniques

Disc- and well-diffusion tests, otherwise known as the Zone of Inhibition (ZOI) method, have been the method of choice of many workers for the comparison of the antimicrobial activity of essential oils (9,10,19,53,234). The principle of this method is that the test sample will diffuse out into the agar medium, producing a concentration gradient. If the antimicrobial is effective against the test microorganism, a zone of inhibition will form around the well or disc (13,235,236). The size of the zone provides some indication of the relative activity of the substance, however, a number of factors including the volume and type of medium, the concentration and age of inoculum, the incubation conditions and the size, charge and conformation of the active ingredient(s) will all affect the result (235,237). This method is ideal for assessing water soluble antibiotics and disinfectants. Most essential oils are however predominantly water insoluble with some components, namely oxygenated monoterpenes, being sparingly soluble in water (see Results, Table 3.1). Consequently the agar diffusion tests are not true indicators of the antimicrobial activity of an essential oil but rather its diffusion capacity in agar. Thus
although this technique has been standardised for antibiotic testing to give reproducible results, which correlate with Minimum Inhibitory Concentration values, it suffers from inconsistent results and a lack of correlation with MIC values when the method is applied to the testing of water-insoluble essential oils and their components (5,7,11,233). One particular example was noted when comparing the results of two recent studies by Tantawy et al. (238) and Cosentino et al. (22). Tantawy et al. (238) when using a disc diffusion method, determined that α-pinene and Taxodium distichum oil (87% α-pinene) were active against P. aeruginosa 27853 while Cosentino et al. (22) using a broth microdilution method determined that α-pinene was inactive against the same strain of P. aeruginosa. This type of discrepancy is not an isolated incident and is compounded by the subjective nature of deciding what size of inhibition zones indicate activity. This can potentially lead to misleading interpretation of data (see section 3.2 for further discussion).

The reason for these problems is the poor water solubility of many of the terpenoids, which make up the essential oils, thus limiting their diffusion through the agar. For example in an oil such as tea tree oil only the more water soluble components, such as terpinen-4-ol, 1,8-cineole and α-terpineol, diffuse into the agar from the disc. However, the hydrocarbon components either remain on the disc or evaporate (13,235). Consequently, the contribution of these components to the activity of the oil cannot be assessed as only the activity of the more water soluble oil components is being measured. This may result in an incorrect estimation of the oil’s activity. Whilst the method is easy to perform and requires only small volumes of the agent, its use as a screening tool continues, but it is not appropriate where more quantitative measurements are required, or where the antimicrobial activity of oils of different composition are being compared.

Since reservoir techniques rely heavily on a component’s ability to diffuse in agar, an alternative method has been developed where the reservoir, in this case a paper disc, is placed in the lid of the petri dish which is incubated inverted (233,239). The theory behind this method is that since the oils are volatile then their vapors will still inhibit growth of the test organisms. This method has shown some discrepancies with the standard disc diffusion technique in that some organisms are sensitive in one test but not the other (233). Activity of compounds is also reliant on their volatility.
3.1.2 Dilution Techniques

The Minimum Inhibitory Concentration (MIC) technique is a dilution method. MIC measurements have been used extensively to quantify the antimicrobial activity of essential oils and a number of variations of the method have been published (5,11,13,240-244). The principle of the assay is that the test organism is added to a series of dilutions of the agent prepared in either a solid or liquid nutrient medium, and presence or absence of growth is determined after a period of incubation. The MIC is recorded as the lowest concentration of the agent which inhibits growth.

MIC methods can be carried out using solid or liquid growth media. These two techniques are called the agar dilution and broth dilution methods respectively. The agar dilution method generally consists of different amounts of test material being added to molten agar to give several concentrations of test material in the plates onto which organisms are either streaked or pipetted (13,236). The problem with this method is again the insolubility of the oils in water. This causes the oils to separate from the agar after it has been poured and before it sets. This may result in erroneous results since the oil is no longer homogeneously distributed throughout the growth medium (11).

Broth methods, especially those carried out in microtitre trays, have the advantage over agar methods of lower workloads for a larger number of replicates and the use of small volumes of the test substance and growth medium. In broth methods, however, turbidity of the oil-water emulsion can interfere with the reading of the endpoint, particularly in microtitre assays. In one study, where the susceptibility of three bacteria to *M. alternifolia* oil using broth and agar dilution methods was compared, in some cases it was almost impossible to differentiate between growth and the turbidity caused by addition of the oil (12).

For this reason, indicators such as fluorescein diacetate (245), p-iodonitrotetrazolium violet (240), triphenyl tetrazolium chloride (TTC) (246) and resazurin (247) have been used. However, problems can occur when using some indicators. For example Carson and Riley (45) report that color changes of TTC do not correlate exactly with MIC. Agar dilution methods overcome these turbidity and indicator problems associated with broth methods and can be modified to suit any organism by changing the growth medium and incubation conditions.
Whether broth or agar methods are chosen it is important to obtain uniform oil
dispersion throughout the growth medium to ensure prolonged contact with the test
organism. However, since most essential oils are water insoluble, obtaining a uniform
oil dispersion throughout the growth medium becomes a significant problem.

In the absence of a solubilising or emulsifying agent, the oil separates from the
agar or broth and is visible as a layer on the surface of the test medium. Therefore
many different solvents and dispersing agents have been tested for use in such
methods to overcome this (233). An ideal emulsifier would have no effect on the test
organism, would not interact chemically with the constituents of the oil and would act
neither synergistically nor antagonistically with the oil (11).

Whilst cationic emulsifiers have a slight bactericidal activity (11) non-ionic
emulsifiers, such as Tween 20 and Tween 80, are relatively inactive when tested
alone and have been used with success (11,20,240,245). One notable exception to this
is Mycobacterium where Tween 80 has been reported to be bactericidal to this
organism. Therefore care must be taken to avoid use of Tween 80 with
Mycobacterium (248).

In addition to taking care with choice of emulsifiers, researchers need to be
aware that increasing the concentration of Tween from 0.1% to 5% has been shown to
increase MIC values, that is decrease antimicrobial activity, in some cases (247,249).
A possible explanation is that the active components of the oil are partitioned out of
the aqueous phase as they become solubilised within micelles of the surfactant (250).
As a result they do not come into contact with the microorganism (251). Other studies
suggest that low concentrations of surface-active agents in the mixture may actually
enhance the activity of the antimicrobial through causing changes to the permeability
of the cell membrane of the microorganism (252).

Difficulties with the use of emulsifiers has seen an attempt to avoid their
incorporation by the use of agar solutions to disperse the oils (249). The results
revealed that when a 0.2% agar solution was used as dispersing agent when mixing
oils into media for MIC tests much lower MIC values were seen than when
emulsifiers or solvents were used.

Regardless of the emulsifier used MIC values are also affected by the method
of preparation and the concentration of the inoculum, the test medium, the contact
time and the method of determining the end-point. Due to different interpretations of
these variables, it is difficult to directly compare the results of various authors, and there is a strong need for a reliable standardized procedure for the testing of oils which is applicable to a wide range of microorganisms. The outline of such a method has been suggested and was used in this project (241).

3.2 Results and Discussion

The MIC's of 60 terpenoids (Table 3.1) were determined in order to assess their relative antimicrobial activities and to act as a base on which to formulate structure/antimicrobial activity relationships of the terpenoids through subsequent experiments (note: water solubility of the terpenoids is presented in Table 3.1 for the readers convenience and will be discussed in chapter 4).

The plate MIC method used in our experiments has been shown to give reproducible results up to a level of 2% v/v of test components (241). Therefore the data presented here is the largest internally consistent source of MIC results, for terpenoids, presented to date. Such an accumulation of data is essential if any structure/activity relationships of the terpenoids is to be made.

Hierarchical cluster analysis (Figure 3.1) of the activity data showed that five groups exist for the 60 terpenoids based on the level and specificity of their activity against *P. aeruginosa, E. coli, S. aureus* and *C. albicans*. K-means cluster analysis was used to confirm these groupings and to generate a plot of means (Figure 3.2) which shows these differences graphically. It can be seen from Figure 3.2 that the terpene activity groups vary from inactive against all four organisms (Group IV) to active against all four organisms (Group I).
Table 3.1: Minimum Inhibitory Concentrations (ppm) against P. aeruginosa, E. coli, S. aureus and C. albicans and Water Solubility (ppm) of Terpenoids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>C. albicans</th>
<th>Solubility a (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-(+)-Citronellal</td>
<td>&gt;16700</td>
<td>&gt;16700</td>
<td>2100</td>
<td>850</td>
<td>33.7 ± 0.3</td>
</tr>
<tr>
<td>β-Citronellol</td>
<td>&gt;16800</td>
<td>&gt;16800</td>
<td>860</td>
<td>430</td>
<td>224 ± 6</td>
</tr>
<tr>
<td>Geraniol</td>
<td>&gt;17400</td>
<td>1800</td>
<td>890</td>
<td>440</td>
<td>520 ± 24</td>
</tr>
<tr>
<td>Geranylacetate</td>
<td>&gt;18000</td>
<td>&gt;18000</td>
<td>&gt;18000</td>
<td>&gt;18000</td>
<td>17.1 ± 1.8</td>
</tr>
<tr>
<td>Linalool</td>
<td>&gt;17100</td>
<td>1700</td>
<td>1700</td>
<td>1300</td>
<td>754 ± 12</td>
</tr>
<tr>
<td>Linalool Oxide</td>
<td>1900</td>
<td>9400</td>
<td>&gt;18500</td>
<td>5500</td>
<td>36580 ± 270</td>
</tr>
<tr>
<td>Linalyl acetate</td>
<td>&gt;19300</td>
<td>&gt;19300</td>
<td>&gt;19300</td>
<td>&gt;19300</td>
<td>2.9 ± 0.09</td>
</tr>
<tr>
<td>Nerol</td>
<td>&gt;17200</td>
<td>880</td>
<td>880</td>
<td>880</td>
<td>629 ± 15</td>
</tr>
<tr>
<td>Neryl acetate</td>
<td>&gt;17800</td>
<td>&gt;17800</td>
<td>&gt;17800</td>
<td>&gt;17800</td>
<td>19.5 ± 0.7</td>
</tr>
</tbody>
</table>

3.1.1. Bicyclic

| (1s,2s,5s)-(-)-Myrtanol | 9600 | 1900 | 970 | 970 | 524 ± 7 |
| (-)-cis-Myrtanyl amine | 910 | 460 | 900 | 460 | 1436 ± 35 |
| (1R,3R)-Myrtenal | 9800 | 3000 | 2000 | 990 | 394 ± 23 |
| (1R,3S)-Myrtenol | >18700 | 950 | 950 | 950 | 880 ± 41 |
| (-)-β-Pinene | >16800 | >16600 | >16800 | >16800 | 1.9 ± 0.3 |
| (+)-β-Pinene | >16800 | >16600 | >16800 | 1680 | 0.57 ± 0.09 |
| (-)-γ-Pinene | >16800 | >16600 | >12700 | 3400 | 0.9 ± 0.15 |
| (s)-cis-Verbenol | 3900 | 2000 | 2000 | 1000 | 738 ± 56 |
| (1s)-(-)-Verbenone | >16200 | 3900 | 2400 | 2400 | 7917 ± 135 |

p-Menthane

| Carveol | 3900 | 980 | 980 | 490 | 830 ± 10 |
| (-)-Carveol | 4500 | 1500 | 3000 | 1100 | 1601 ± 7 |
| (R)-(-)-Carvone | 3800 | 1900 | 560 | 960 | 916 ± 20 |
| 1,4-Cineole | >17500 | >17500 | >17500 | >17500 | 599 ± 49 |
| 1,8-Cineole | >18100 | 9100 | >18100 | >18100 | 907 ± 60 |
| p-Cymene | >16900 | >16900 | >16900 | >16900 | 6.2 ± 0.35 |
| DiHydrocarveol | 2800 | 930 | 930 | 690 | 1058 ± 11 |
| DiHydrocarveone | 28200 | 9300 | 9300 | 2000 | 263 ± 31 |
| (+)-Isomenthol | 9600 | 9600 | 4400 | 2000 | 263 ± 31 |
| (+)-Limonene | >16500 | >16500 | >16500 | >16500 | 10 ± 0.11 |
| (-)-Limonene | >16500 | >16500 | >16500 | >16500 | 10 ± 0.11 |
| Limonene oxide | >16200 | 1900 | >18200 | 4200 | 349 ± 11 |
| p-Menth-8-ene-2,8-diol | 9500 | 9500 | 9500 | 9500 | 16220 ± 270 |
| (-)-Menthol | >18200 | >19200 | 1000 | 500 | 297 ± 8 |
| (-)-Menthone | >17500 | >17500 | >17500 | 4000 | 236 ± 5 |
| (s)-Limonene | >19100 | >19100 | 1000 | 500 | 244 ± 5 |
| Mentholacetate | >18100 | >18100 | >18100 | >18100 | 17 ± 3.5 |
| (-)-Perilla aldehyde | 9100 | 970 | 1900 | 480 | 349 ± 12 |
| Piperitone | 9100 | 1600 | 1800 | 1400 | 1676 ± 19 |
| (+)-Pulegone | >18400 | >2800 | >16900 | >18900 | 27.9 ± 1.5 |
| (α,β)-Terpinylacetate | 2900 | 960 | 960 | 480 | 758 ± 9 |
| (α,β)-Perilla alcohol | >16400 | >16400 | >16400 | >16400 | 8.2 ± 0.16 |
| α-Terpine | >16600 | >16600 | >16600 | >16600 | 10.3 ± 0.24 |
| γ-Terpinene | >16300 | >1900 | >16900 | >16900 | 1827 ± 47 |
| α-Terpinolene | >16900 | >16900 | >16900 | >16900 | 4.3 ± 0.1 |
| Terpinen-4-ol | 6200 | 1900 | 1900 | 930 | 1491 ± 28 |
| Thymol | 2000 | 1000 | 1000 | 500 | 846 ± 9 |

Carneos

| Car-2-ene | >16900 | >16900 | >16900 | >16900 | 1.3 ± 0.11 |
| Car-3-ene | >16800 | >16800 | >16800 | >16800 | 1.2 ± 0.22 |
| Car-3-en-2-one | 9800 | 2500 | 2000 | 1500 | 370 ± 11 |

2.1.1. Bicyclics

| (-)-Bornol | >19000 | >19000 | 1500 | 1000 | 405 ± 9 |
| (+)-Camphene | 9600 | 9600 | 9600 | 9600 | 0.8 ± 0.05 |
| (1)-Camphor | 9500 | 4500 | 3000 | 2000 | 1040 ± 25 |
| (+)-Fenchol | 7100 | 2000 | 2000 | 1000 | 840 ± 49 |
| (1R,1S)-Fenchone | >18500 | 4700 | >18600 | 4200 | 1134 ± 12 |
| (+)-Isoborneol | 9600 | 9800 | 2000 | 1000 | 330 ± 14 |

Aromatic-Ethers

| Eugenol | 10600 | 2100 | 2100 | 800 | 2406 ± 91 |
| (cis,trans)-Isoeugenol | >21300 | 1100 | 1100 | 810 | 1086 ± 23 |
| Methyleneugenol | >20300 | >20300 | >10300 | 1600 | 531 ± 16 |

Isoterpines

| α-limonone | >18200 | 2800 | >18200 | 5400 | 66 ± 3.5 |
| β-limonone | >18500 | 2800 | >18500 | 3800 | 58 ± 2.2 |

a Solubility quoted with 95% confidence interval
Figure 3.1 – Hierarchical clustering of 60 terpenoids based on Minimum inhibitory concentrations against *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albicans*
Figure 3.2 - Activity patterns of terpenoid groups represented by a plot of mean Maximum-non-Inhibitory Concentrations (ppm) for each group against *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albicans*.
Analysis of the activity of the compounds comprising the five clusters (Figure 3.1) showed a similar trend of activity to that established by Knobloch et al. (6) (see chapter 4 for further detail), that is;

<table>
<thead>
<tr>
<th>Acetates</th>
<th>Ethers</th>
<th>Ketones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocarbons</td>
<td>Oxides</td>
<td>Aldehydes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcohols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenols</td>
</tr>
</tbody>
</table>

This is illustrated by the fact that the terpenoids which showed antimicrobial activity (Groups I, II and V) tended to be alcohols, ketones and aldehydes while those terpenoids which were inactive (Group IV) were mainly hydrocarbons and acetates. Group III on the other hand was of intermediate activity and contained mainly oxides and ethers. Beyond generalised functional group trends, no other immediately obvious structural trends were found for the terpenoids.

It can be seen from the Figures 3.1 and 3.2 that *P. aeruginosa* is resistant to the greatest number of terpenoids (i.e. all those compounds excluding those in Group I), followed by *E. coli*, *S. aureus* with *C. albicans* being resistant to the least number. This is important to note since the resistance of microorganisms to the terpenoids is equally as important in the determination of their antimicrobial action as is determining their susceptibility. Hence, the resistance of *P. aeruginosa* and *E. coli* to compounds, which are found to be active against both *S. aureus* and *C. albicans*, was also investigated in order to determine the method of resistance of these organisms to the action of terpenoids (see Chapter 6).

While the results of this experiment have shown similar structure/activity trends to previous work (6) and similar MIC values to other studies (7,14,18,22,41,62,65), some data published in the literature is in disagreement with these results. The disparity between results presented here and other work seems to generally occur where investigators are using the disc diffusion assay. In each of the examples discussed the contradictory results are able to be attributed to problems with this method and its use for quantitation purposes. The discrepancies between results seem to be mainly false positives and involve terpene hydrocarbons. As has already
been discussed the disc diffusion technique is not suitable for insoluble compounds, a problem of this technique which can potentially result in misleading data.

For example, it has been suggested that \( \alpha \)-pinene [I] is active against a variety of organisms including *C. albicans*, *E. coli* and *P. aeruginosa* (238). While the strains of these organisms are different to those used in this study, it is clear from Table 3.1 that \( \alpha \)-pinene is inactive against *P. aeruginosa* and *E. coli* but does show some activity against *C. albicans*. This disparity between results is most likely a product of the disc diffusion method employed. In fact \( \alpha \)-pinene, being a hydrocarbon, should not disperse significantly across the agar on its own (13). The diffusion zones produced therefore were most probably a factor of the solvent used to dissolve the terpenes for inoculation onto the filter disc. Further evidence that the disc diffusion assay gave false positive results is seen when comparing the results of (238) to that of (22) who used an MIC method to test the activity of a small group of terpenoids. Comparison of the two studies showed that the same strains of *E. coli* and *P. aeruginosa* were examined in both cases. Where the disc diffusion assay showed activity of \( \alpha \)-pinene against both organisms the MIC method showed them to be inactive. In addition, other MIC values reported by (22), for terpenoids such as carvacrol, thymol and linalool, were similar to the values presented in this chapter, despite the use of different strains.

![Chemical Structures]

\([I] \quad [II]\)

A similar disparity involving \( \alpha \)-pinene is also found in a study on the relative antimicrobial activities of its (-)- and (+)- enantiomers [II] (253). This study again used a disc diffusion technique for assessing antimicrobial activity. The authors in this study suggest that both enantiomers show activity against *E. coli* and *S. aureus* and a
wide variety of other organisms and that the (-)- enantiomer is more active than the (+)- enantiomer. The suggestion that one enantiomer is more potent than another is also debatable as three problems exist with the method used in this study. Firstly, the study does not report standard deviation for the zone measurements and thus it is difficult to ascertain the significance of the differences in activity especially since in many cases the difference between zone size was minimal. Second, and most significant was the use of a 25 °C incubation temperature for organisms that should be grown at 37°C. This may affect the organisms' susceptibility to the compound and result in larger than normal inhibition zones. Finally, no indication is given as to whether the well size was incorporated into the zone measurement. If it was incorporated this would reduce many of the results and it could be debated whether they were significantly active or not.

A final example involving false positive results shows the problems with using such data for quantitative analysis for comparing structure/function. In this case, the contradiction between disc diffusion data and the MIC data presented here is concerning the activity of the enantiomers of limonene. In a study on the activities of these two compounds (254) it was suggested that while both compounds possess significant antimicrobial activity the (+)- enantiomer was more potent than the (-)-enantiomer. However, our experiments show (Table 3.1) that neither of the enantiomers are active nor is there any apparent difference in their relative activities. In support of the MIC data presented here is the fact that very little or no difference was found between these two enantiomers in terms of their molecular properties (see chapter 4) and/or their effects on the order of model membranes (see chapter 5). The evidence presented in subsequent chapters will also show that terpenoids do not seem to have specific sites of action such that one enantiomer is preferred over the other.

While the majority of discrepancies between the results presented here and other data involves false positive results, there are also instances where false negative results arise from the disc diffusion assay. These are also attributable to problems with the disc diffusion method. For example, in two separate studies, it has been reported that the oxygenated terpenes borneol (255) and camphor (255,256) are inactive against almost all of the organisms tested against. This disparity with MIC values reported here can be easily explained by the fact that both borneol and camphor are solid at room and incubation temperatures. Therefore once the solvent that was used
to dispense the terpenoids evaporates only solid terpenoid will remain. It is no wonder that no migration of these two compounds was observed. This indicates very clearly the care which must be taken in assessing antimicrobial activity of insoluble compounds otherwise potentially useful antimicrobial agents may be missed due to false negative results.

The examples of the technical problems of the disc diffusion assay, when applied to insoluble compounds, presented in this discussion represent only a few of the common types of discrepancies between results in the literature. Various other examples can also be found in reviews by Hulin et al. (235) and Markham (48).

3.3 Conclusion

MIC values were determined for the 60 terpenoids against *S. aureus*, *E. coli*, *C. albicans* and *P. aeruginosa*. The results have shown that five groups of compounds exist based on their activity patterns against these organisms. In general this data was consistent with the functional group versus antimicrobial activity trend established by Knobloch (6). However, functional group and simple structural similarities between terpenoids did not explain completely the separation of the terpenes into the five separate groups. Molecular properties affecting the classification of the terpenoids into the five groups is explored in chapter 4.

Activity patterns also showed that *P. aeruginosa* was resistant to the largest numbers of terpenoids followed by *E. coli*. The mechanism of resistance of these organisms is investigated in chapter 6.

As already discussed, the disc diffusion method, while appropriate for screening for antimicrobial activity should not be used for quantitative comparisons when dealing with water insoluble compounds. The continuing use of diffusion methods therefore needs to be addressed and conclusions from such data need to be limited, unless presented with more quantitative data such as MIC tests. It should also be noted that although the data presented here contradicts some data already present in the literature, it is only found to do so for those studies which have used diffusion assays. However, at the same time our MIC data is consistent with a number of more substantial studies which have used various MIC techniques.
Chapter 4
The Role of Structure and Molecular Properties of Terpenoids in Determining their Antimicrobial Activity

4.1 Introduction

Knobloch et al. (6,54), surveyed the antimicrobial activity of a large number of terpenes and concluded, that the general order of activity for terpenoids is;

Alcohols, phenols > ketones, aldehydes > oxides, ethers > hydrocarbons

While various studies support this (20,25,49), it should be noted that this order of activity is only considered a general rule and compounds which do not comply with it can be found in various studies on antimicrobial activity. For example, some studies show citral (18,56,59,60,65) and cinnamic aldehyde (59) to be of similar or greater antimicrobial activity as terpene alcohols. Conversely menthol has been reported as having less antimicrobial activity than ketone and aldehyde terpenoids (8,59).

Whilst the level of antimicrobial activity have been determined for many terpenoids (see Tables 1.1 and 1.2) and most adhere to the activity trend suggested by Knobloch et al. (6,54), actual structure/activity relationships of these compounds are not well understood. For example it is known that carbonylation of terpenoids increases their bacteriostatic activity, though not necessarily their bactericidal activity, but with no real understanding as to why (257).

A limited attempt has been made to correlate various chemical properties of some terpenoids and their antimicrobial activity. For example it was found that the antifungal activity of cinnamaldehyde, perilla aldehyde, citral and citronellal was directly related to their electron acceptor properties, as defined by the energy values
of their lowest empty molecular orbitals, with the better electron acceptors being more active than poor electron acceptors (70).

It has also been reported that terpenoids have different antiseptic potency depending on their solubility in water and it has been suggested that the inactivity of the monoterpane hydrocarbons is largely due to such insolubility. However, there are some anomalies with oxygenated compounds such as thymol, carvacrol, eugenol and cinnamaldehyde which are of low water solubility but highly antiseptic (54). It is therefore clear that neither water solubility nor functional group are the only factors involved in determining the level of antimicrobial activity.

Despite the fact that essential oils and their terpenoid components are weakly to moderately soluble in water they are readily dissolved in lipids. Their solubility in the phospholipid bilayer of biological membranes appears to be of importance. Recently investigations have shown that the site of action of terpenoids (77,78) and tea tree oil (79-81) is at the cell membrane. However, the effectiveness of a particular component in this respect, again depends heavily on its water solubility and its ability to penetrate the different cell walls associated with Gram-positive and Gram-negative organisms. Thus particular components of essential oils may have different antibacterial activity on Gram positive and Gram negative bacteria.

In this set of experiments ten molecular properties of the terpenoids have been examined in order to further understand the links between structure and molecular properties of terpenoids to their antimicrobial activity. The results presented in this chapter show the influence of their molecular parameters on antimicrobial activity against representative Gram positive and Gram negative bacteria and yeast.

4.2 Results and Discussion

As discussed in chapter 3, hierarchical cluster analysis (Figure 3.1) showed that five groups exist for the 60 terpenoids based on the level and specificity of their activity (Table 3.1) against *P. aeruginosa*, *E. coli*, *S. aureus* and *C. albicans*. K-means cluster analysis was used to confirm these groupings and to generate a plot of means (Figure 3.2) which shows graphically these differences.

The following molecular properties were then calculated or measured for each of the terpenoids; water solubility, hydrogen bonding capacity, hydrogen bond donor
capacity, hydrogen bond acceptor capacity, polarity, hydrophilic/lipophilic balance, log $K_{ow}$, molecular volume, molecular surface area and percentage hydrophilic area. Discriminant analysis (DA) was then used to ascertain which molecular property or combination of molecular properties (Table 4.1) of the terpenoids were associated with these different activity patterns and hence associated with activity against specific organisms (Group III was omitted from all DA analysis due to its limited size). Values of the molecular properties for each of the individual terpenoids can be found in Appendix I except for water solubility which is presented in Table 3.1 and log $K_{ow}$ values which are presented in Table 7.1.

<table>
<thead>
<tr>
<th>Groups and Group Pairings</th>
<th>Activity Difference</th>
<th>Discriminating Variables$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I versus II</td>
<td><em>P. aeruginosa</em></td>
<td>H-bond donor capacity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Surface area</td>
</tr>
<tr>
<td>II versus V</td>
<td><em>E. coli</em></td>
<td>H-bond acceptor capacity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-bonding capacity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Molecular volume</td>
</tr>
<tr>
<td>V versus IV</td>
<td><em>S. aureus</em> and <em>C. albicans</em></td>
<td>H-bond acceptor capacity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-bonding capacity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polarity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% Hydrophilic area</td>
</tr>
<tr>
<td>I versus IV</td>
<td>Activity/No activity</td>
<td>Water solubility</td>
</tr>
<tr>
<td>Groups I, II, IV and V</td>
<td>All four organisms</td>
<td>log $K_{ow}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-bonding capacity$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water solubility</td>
</tr>
</tbody>
</table>

$^a$ Discriminating variables are listed in descending order of largest contributor to smallest contributor in the discriminating function.

$^b$ H-bonding capacity was the largest contributor to the first discriminating function (root 1) while water solubility was the largest contributor to the second discriminating function (root2).

### 4.2.1 Group Characteristics

DA of groups I, II, IV and V was carried out in order to determine which of the molecular properties were the main contributors to the discrimination between all of the four groups (Table 4.1) and to determine how well the variables were able to discriminate thus giving some indication of their overall importance. The DA showed
that there were two discriminating functions (roots). However, of the ten variables only hydrogen bonding capacity and water solubility were found to participate in the discriminating functions, with H-bonding being the larger contributor to the discrimination in the first root and water solubility in the second root. The first root was found to discriminate groups IV from groups I, II and V. The second root was found to discriminate Group V from groups I, II. This discrimination however was not as clearly defined as that for the first root. The discrimination can be seen visually in a scatter plot of the two roots against one another (Figure 4.1).

Specifically, those compounds in groups IV tended to have a lower H-bonding capacity and be less soluble than those compounds found in the other three groups (Table 4.2). To a lesser extent compounds in Group V tended to be less soluble than those compounds in Groups I and II. Group V compounds were also found to have a lower H-bonding capacity than Group I compounds and a lower H-bonding donor capacity than Group II compounds.

<table>
<thead>
<tr>
<th>Molecular Properties</th>
<th>Group I</th>
<th>II</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-bonding capacity</td>
<td>10.47</td>
<td>8.88</td>
<td>4.68</td>
<td>9.86</td>
</tr>
<tr>
<td>H-bond acceptor capacity</td>
<td>0.37</td>
<td>0.20</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>H-bond donor capacity</td>
<td>0.24</td>
<td>0.15</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Water solubility</td>
<td>1084</td>
<td>1528</td>
<td>149</td>
<td>291</td>
</tr>
<tr>
<td>Molecular volume</td>
<td>97.6</td>
<td>97.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface area</td>
<td>12.68</td>
<td>13.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLB</td>
<td>2.56</td>
<td>3.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polarity</td>
<td></td>
<td></td>
<td>2.94</td>
<td>4.01</td>
</tr>
<tr>
<td>log K_{ow}</td>
<td>3.05</td>
<td>3.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Hydrophilic Area</td>
<td></td>
<td></td>
<td>6.88</td>
<td>12.91</td>
</tr>
</tbody>
</table>

Table 4.2: Means of discriminating molecular properties for each terpenoid group.
Figure 4.1 - Scatter plot of the first (root 1) and second (root 2) discriminating functions of the discriminant analysis of Groups I, II, IV and V. Group III was omitted from DA due to its limited size.
DA of groups I and IV was used to determine the molecular parameters affecting activity/inactivity. It was found that the largest contributor to the discrimination was water solubility followed by H-bonding capacity and log $K_{ow}$ (Table 4.1). Specifically, compounds which tended to show activity against all of the test organisms (Group I), predominantly unsaturated alcohols, tended to have a higher solubility and H-bonding capacity but a lower log $K_{ow}$ than those compounds (Group IV) which are inactive (Table 4.2). Group IV was found to contain mainly monoterpene hydrocarbons and terpene acetates. The structural variety found within this group does not appear to influence activity and it seems likely that it is their low water solubility and low hydrogen bonding capacity that result in their lack of activity. This is consistent with the work of Knobloch et al. (6,54). It should be noted that even though the compounds were dispersed evenly through the growth medium by use of Tween 20 this does not ensure they are able to traverse the small water layer that is associated with the outside of the organisms. It should be noted that although log $K_{ow}$ was not the main discriminating parameter between activity/inactivity it does not preclude its importance in this respect. However, in the case of the terpenoids their relatively low water solubility is more likely the limiting factor in their bioavailability. As water solubility increases, as with some antibiotics, log $K_{ow}$ will become the limiting factor and hence more important.

On initial inspection, compounds from groups I and II appeared to be very similar in both structure and molecular properties, however DA highlighted the importance of subtle differences. DA of groups I and II showed that in general compounds active against $P. aeruginosa$ (Group I) tended to have a greater capacity to donate a hydrogen bond when H-bonding but also tend to be of smaller surface area and have a lower HLB than those compounds of group II.

When comparing compounds in Groups II and V, whose activities differed mainly against $E. coli$ (Figure 3.2), there was again no clear trend in structural characteristics. However DA of Groups II and V showed that, generally, compounds of smaller volume and with greater capacity to accept a H-bond when H-bonding but having an overall lower H-bonding capacity, tended to be active against $E. coli$. Those compounds without this combination of properties tended to be inactive (Table 4.2). Molecular volume and hydrogen bond acceptance capacity were the two largest contributors in the discrimination (Table 4.1).
Therefore, against Gram negative bacteria it would seem that smaller molecules which are able to donate or accept lone pair electrons in a hydrogen bond situation may be better able to inhibit the growth of these organisms. This may be related to the outer membrane of these bacteria. It is already known that hydrophobic molecules are forced to diffuse through the impermeable LPS layer whilst small hydrophilic compounds are able pass more readily through the outer membrane via porins. Porins are also able to exclude hydrophilic compounds by size (114). Thus smaller terpenoids which are able to form hydrogen bonds and therefore significantly interact with water may be better able to traverse these pathways.

None of the terpenoids tested was active against only *S. aureus* or *C. albicans* thus no inferences could be made about molecular requirements for specific activity against either of these organisms. However DA of groups V and IV showed the molecular properties (Table 4.1) which were associated with activity against both of these organisms. Specifically, compounds which tended to show activity against these organisms (Group V) tended to have a higher H-bonding capacity (largest contributing variable), H-bond acceptance capacity, percent hydrophilic area, and polarity than those compounds which are inactive (Table 4.2).

As with the Gram negative organisms it is observed that H-bonding factors are associated with differences in activity patterns. However, for *S. aureus* and *C. albicans* there were no size parameters as discriminating properties, indicating that size may be more important for Gram negative organisms.

It can be seen clearly throughout each set of DA results that H-bonding parameters are important in defining the antimicrobial activity patterns of the terpenoids and that water solubility is also associated with terpenes being capable of exhibiting activity. However, it is important to note that H-bonding parameters and water solubility did not discriminate all four groups from one another. Thus although these two molecular properties are associated with the activity patterns of the terpenoids they do not account for all the trends in activity. This suggests that either; a) not all of the molecular properties which have a crucial effect on antimicrobial activity of the terpenoids have been measured or b) molecular properties of the terpenoids can account for only part of the antimicrobial action and are unable to predict completely specific terpenoid/microorganisms interactions. Thus for a more
detailed examination of the antimicrobial action of terpenoids further work is required to determine specific compound organism interactions.

4.2.2 Structure/Activity Relationships

Several different structural types of terpenoids were investigated in this study. The various structure/activity relationships found to exist within these structural types are discussed below. Hydrocarbons and acetates will not be discussed since they were found to be inactive in all cases.

4.2.2.1 Acyclic Terpenoids

Geraniol [I] and nerol [II] (geometric isomers) showed only slight differences in their activities against the four test organisms. Thus geometric isomerism does not seem to have a large effect on antimicrobial activity in this case.

Geraniol and nerol (primary alcohols) and linalool [III] (a tertiary alcohol) also showed little difference in activity patterns. Therefore, the position of the functional group on the carbon skeleton of these acyclic terpenoids does not seem to have a large effect on activity. This indicates that the presence of the alcohol functional group rather than its location along the carbon chain is more crucial for activity against E. coli, S. aureus and C. albicans. However this may not be the case for all acyclic monoterpenoid alcohols as illustrated by the fact that citronellol [IV] was inactive against E. coli whereas geraniol, nerol and linalool were active. The only structural difference between citronellol and geraniol or nerol is the absence of a double bond. This slight structural difference is however significant enough to affect important molecular parameters such as hydrogen bonding capacity and hence activity against E. coli.

Citronellal [V], the corresponding aldehyde to citronellol, was also inactive against E. coli and had a lower solubility than the less saturated geraniol, nerol and linalool. When comparing alcohol to aldehyde citronellol showed greater activity against S. aureus than citronellal. Knobloch et al. (6,54) also showed that alcohols were generally more active than aldehydes against a number of organisms including S. aureus.
4.2.2.2 [3.1.1.] Bicyclic Terpenoids

The majority of oxygenated compounds of this structural type were Group II compounds (i.e. not active against *P. aeruginosa*). Only verbenol and myrtanylamine were active against *P. aeruginosa* (see Figure 3.1).

Comparison of 3.1.1. bicyclics found in Group II showed that myrtenal [VI] was slightly less active than its corresponding alcohol, myrtenol [VII], against *E. coli* and *S. aureus* (Table 3.1). However, this functional group difference did not cause large enough changes in important molecular properties, such as hydrogen bonding parameters or molecular size, to produce significant activity towards *P. aeruginosa*.

As for the acyclic terpenoids, the level of saturation of the molecule was found to be important for the 3.1.1. bicyclics. When comparing myrtenol and myrtanol [VIII] it was found that the less saturated and more soluble myrtenol showed slightly greater activity against *E. coli* but similar activity against the other organisms. Thus the degree of saturation does affect activity against *E. coli* and is reflected in slight changes in molecular properties, which tend towards those required for activity against *P. aeruginosa*.

The Group I compound verbenol shows that the position of the alcohol functional group on the 3.1.1. bicyclics does have an effect on the activity against *P. aeruginosa*. Myrtenol and myrtanol, each having terminal alcohol functional groups, were not as effective against this organism. Verbenone, the corresponding ketone to verbenol, was not active against *P. aeruginosa*, showing that the ketone group is not as effective against *P. aeruginosa* as an alcohol functional group.
Myrtanylamine was found to be active against all of the test organisms yet has a terminal functional group rather than a ring substitution. Terpenoid amines may therefore be more effective antimicrobial agents against *P. aeruginosa* especially if their structures correspond to those oxygenated terpenoids already known to show activity against this organism. However further research is required to confirm this observation.

### 4.2.2.3 p-Menthane Terpenoids

p-Menthane compounds found to be active against all four organisms (Group I) were alcohols or phenols with each of the alcohols having at least one double bond present on the molecule. The only non-alcohol active against all four organisms was carvone [IX] which is even less saturated than the alcohols.

The effect that subtle differences in structure have on molecular properties and hence activity of p-menthanes is apparent when comparing carvone and dihydrocarvone [X], with the more saturated dihydrocarvone being less soluble, inactive against *P. aeruginosa* and less active against *E. coli* (Table 3.1). Naigre *et al.* (257) reported a similar result for *P. aeruginosa* CIP A 22 but a reverse effect on *E. coli* CIP 54 127 which shows that the importance of subtle structural changes may be strain dependent.

In contrast to this, carveol [XI] and dihydrocarveol [XII] showed no difference in activity. This shows that although important, increased saturation of the p-menthane terpenoids does not always equate to molecular property changes that can affect activity against *E. coli* or *P. aeruginosa*. 

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![Chemical structures](image)
Another example where slight structural changes in the p-menthanes results in significant changes in activity patterns can be seen when comparing terpinen-4-ol [XIII] and α-terpineol [XIV], whose only structural difference is the position of the alcohol functional group. However, this slight difference was found to affect molecular properties of the molecule, such as hydrogen bonding capacity, and hence the activity of the molecules against *P. aeruginosa* with terpinen-4-ol being active and α-terpineol inactive.

The susceptibility of *P. aeruginosa* to changes in the molecular properties of the p-menthan terpenoids was also seen when comparing perilla alcohol and perilla aldehyde with the alcohol being active and the aldehyde being inactive.

### 4.2.2.4 Carenes, Aromatic ethers, 2.1.1. Bicyclic’s and Ionones

Inadequate numbers of the remaining four structural types prevented any meaningful examination of structure activity relationships within these groups apart from the fact that carenes and 2.1.1. bicyclic hydrocarbons were inactive against all four organisms. Methylation of the alcohol functional group in eugenol to produce methyleugenol was also found to render the molecule inactive against the Gram negative bacteria showing once again the importance of this functional group in activity. This effect has also been noted by Knobloch *et al.* (6,54).
4.3 Conclusion

In this set of experiments it was found that ketones, aldehydes and alcohols showed antimicrobial activity but with differing specificity and levels of activity. Specificity and level of activity were not always defined by the functional group present but were associated with hydrogen bonding parameters in all cases and for Gram negative organisms a combination of hydrogen bonding parameters and molecular size parameters. In general a smaller surface area or molecular volume was associated with those compounds active against the Gram negative organisms. This may relate to the ability of these compounds to penetrate the relatively impermeable outer membrane of these organisms (see Chapter 6). The importance of subtle structural differences of the p-menthane terpenoids on molecular properties and activity, especially against *P. aeruginosa* has also been demonstrated.

It was also shown that terpene acetates and hydrocarbons tend to be relatively inactive regardless of their structural type and this inactivity appears to be closely related to their limited hydrogen bond capacity, water solubility and to a lesser extent log $K_{ow}$. All of these factors are expected to affect bioavailability of the terpenoids however water solubility seems to be the limiting factor in the expression of activity versus inactivity.
Chapter 5
Effects of Terpenes on Lipid Bilayer Properties

Incorporation of a solute in a bilayer perturbs lipid-lipid and lipid-water as well as lipid-protein interactions and consequently affects the functioning of the membrane. This can be the result of effects of the solute on organisational and motional characteristic of the lipids in the bilayer, which will in turn affect permeability. Solute incorporation in to the membrane can also induce the formation of non-bilayer lipid aggregates (77,148,149).

5.1 Introduction Part A : Effects on Lipid Order

The importance of lipid ordering on membrane fluidity and membrane function has been outlined in the introduction. One of the most commonly studied properties used to represent lipid (or membrane) ordering is the gel to liquid-crystal phase transition ($T_C$) which can be easily measured using differential scanning calorimetry (148,167-171,258,259).

5.1.1 Nature of the Order/Disorder (gel/Liquid-crystal) Phase Transition for Lipids

Lipids can undergo one or more phase transitions as the temperature is increased toward their melting point. This is known as mesomorphism. Lipid bilayers formed in association with water also exhibit mesomorphism. The transition change common in most lipid bilayers is the gel to liquid crystal transition. This phase transition of lipids in water can be explained by looking at their phase diagrams.

For example, the phase diagram of the 1,2-diacyl-L-phosphatidylcholine-water system is shown in Figure 5.1. On addition of water the transition temperature, $T$, of the phospholipid is lowered to a limiting value, $T_1^-$. This transition temperature is the
minimum temperature required for water to penetrate between the layers of the lipid molecules (175,193,260).

Within the lipid mole fraction range where the temperature of the phase transition is still decreasing toward $T_i^*$ (1.0 ≥ mole fraction of lipid > 0.8) no transition is observed for the melting of ice or freezing of water on the Differential Scanning Calorimeter (DSC). When the water content is greater than 20% (lipid mole fraction < 0.8) the lipid endothermic transition temperature remains constant and a peak at 0°C, due to melting of water, appears on the DSC scan. As the concentration of water in the mixture is increased further so does the size of this peak (193). Some of the water added to the phospholipid therefore appears to be ‘bound’ to the lipid. The ‘bound’ water may have a considerable relevance to interactions of anesthetics, drugs and ions within biological membranes (108). The composition of the system at maximum hydration is 40 wt% water. Addition of more than 50 wt% water gives rise to a two phase system consisting of fragments of the lamellar phase at maximum hydration dispersed in excess water (175,193,260).

![Phase diagram](image)

**Figure 5.1 – Phase diagram of the 1,2-dipalmytoyl-L-phosphatidylcholine-water system.** (Taken from 193).
Above the $T_1^*$ line the phosphatidylcholine-water system exists in a mesomorphic (liquid crystal) lamellar phase, in which the hydrocarbon chains are in a liquid like state (175,193,260). When the phosphatidylcholine-water system is cooled below the $T_1^*$ line, the hydrocarbon chains adopt an ordered packing and are in a crystalline form. The structure of this phase is lamellar, with the hydrocarbons packed in an hexagonal subcell with the chain axes inclined at 58° to the lipid-water interface (175,193). Hence the $T_1^*$ becomes the gel-liquid crystal phase transition ($T_C$) provided the proportion of water added to the system is above the point where $T_1^*$ becomes limiting.

The phase diagrams of different chain length lecithin-water systems are essentially equivalent and are disposed along the temperature axis according to the melting temperature of the hydrocarbon chains. The gel-liquid crystal phase transition temperatures of several lipids are given in Table 5.1 (157).

**Table 5.1 – Gel to Liquid-Crystalline Phase Transition Temperatures for the 1,2-diacyl-L-phosphatidylcholines.** Modified from (157)

<table>
<thead>
<tr>
<th>Acyl Chain Length</th>
<th>Transition Temperature $T_C$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behenoyl ($C_{23}$)</td>
<td>75</td>
</tr>
<tr>
<td>Stearoyl ($C_{18}$)</td>
<td>58</td>
</tr>
<tr>
<td>Palmitoyl ($C_{16}$)</td>
<td>41</td>
</tr>
<tr>
<td>Myristoyl ($C_{14}$)</td>
<td>23</td>
</tr>
<tr>
<td>Oleoyl ($C_{18}$)</td>
<td>-22</td>
</tr>
</tbody>
</table>

For some phosphatidylcholines a small pre-transition peak associated with the gel-liquid crystal peak is also observed when water content in the lipid-water mixture is above the point where $T_1^*$ is at its limiting value. The temperature interval between this peak and the main endothermic peak increases as the chain length of the lecithin becomes shorter. This pre-transition peak (34.5°C for DPPC) has been shown to be associated with a change of polar group organization which accompanies a changes of acyl chain orientation with respect to the plane of the lamellae (193).

For example, below the phase transition, both DMPC and DPPC exhibit temperature dependent structural changes associated with the thermal pre-transition. At low temperatures below the pre-transition a one dimensional lamellar lattice is observed ($L_\beta$). The hydrocarbon chains are fully extended and tilted with respect to the plane of the bilayer (Figure 5.2). With increasing temperature the angle of tilt of
the hydrocarbon chains decreases, reaching a minimum of 30° at the pre-transition. During the pre-transition a structural transformation from a one to two-dimensional monoclinic lattice occurs where the lipid lamellae are distorted by a periodic undulation or ripple. In this ripple phase \((P_{\beta})\) the hydrocarbon chains are tilted with respect to the bilayer at minimum tilt angle. At the chain melting transition the hydrocarbon chains of the phospholipid melt assuming a liquid-crystal conformation and the lattice reverts to a one dimensional lamellar \((L_{\alpha})\) \((259,260)\).

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Figure 5.2 – Structural models associated with the thermal transitions in synthetic lecithins. Modified from \((259)\).
5.1.2 Effects of Membrane Partitioning Compounds on $T_c$

5.1.2.1 Effects of Bilayer Partitioning Compounds on Size of Lipid Cooperative Units

Small molecules which partition into a lipid bilayer and are able to associate either within or between the acyl chains of the lipid(s) will be disruptive and thus be expected to influence the mode of packing of the acyl chains and hence the order-disorder transition. In a bilayer, in gel phase the motion in one fatty acid chain is to some extent transmitted to the chains next to it and so on to form a cooperative unit (indicated by thickness of the DSC peak). Thus the conformational changes can be transmitted over a substantial portion of the cooperative unit. Foreign molecules however would be expected to block this transmitting system, so that the cooperative motion was damped out, ensuring that changes in the bilayer remain relatively local instead of involving the cooperative unit as a whole. Thus, the foreign molecules may modify the phase transition profile of the lipid as well as the temperature at which it occurs. Indeed they do so and the effect is dependent both on the structure and the concentration of the additive (180,181,261).

5.1.2.2 Position of solvent partitioning into membranes

The overall effects which a lipophilic compound has on the integrity of a membrane also depends on the site of accumulation in the membrane. A solute near the phospholipid headgroup area will have a different effect on the membrane compared to a solute partitioning deeply in the lipid acyl chains. Depending on the hydrophobicity of the solute it will accumulate more or less deeply into the bilayer (77,148,149). The position of solvents in membranes has been determined using a variety of techniques, including X-ray diffraction (169,206,262), fluorescence spectroscopy (92), and $^{31}$P nuclear magnetic resonance spectroscopy (NMR), $^2$H NMR (262-265) and $^{13}$C NMR (266).

Effects on the transition profiles can also provide information about the regions at which compounds are interacting with lipid bilayers (261).
5.1.2.2a Relationship between Phase Transition Profiles and Solvent Positioning

Jain and Wu (261) noted, during a survey of the effects of ninety lipid soluble compounds on the order-disorder phase transition of DPPC, that the type of transition profile obtained was dependent on the nature of the compound added, whereas the extent of the effect was concentration dependent. These profiles were then used to classify the types of effects of molecules on lipid bilayers.

Five different phase transition profiles were found to exist. The first class of transition profile, referred to as type $C\downarrow$, is where the added compound induces a transition peak whose shape (height, area ($\Delta H$) and half-height width) are almost identical to that of the pure lipid transition peak, but whose $T_C$ is decreased as a function of concentration (see Figure 5.3). The sharp transition profile shifted along the temperature axis, as seen in type C profiles, would imply that the size of the cooperative unit is unchanged, but the packing of these units can be perturbed at lower temperatures (261).

The second transition profile, referred to as type $A\downarrow$, is split into two subtypes. $A\downarrow$ is where the added compound induces a transition peak which is broad compared to that of the pure lipid transition peak, but whose $T_C$ is decreased as a function of concentration (see Figure 5.4) and whose area remains constant. $A\uparrow$ type profiles differ from $A\downarrow$ only in the fact that the $T_C$ is increased as a function of concentration. The broad transition in type A profiles implies that the cooperative unit undergoing transition is small. The shape of type A profiles is also consistent with the fact that the bilayer may consist of a range of phases which differ only slightly in their packing characteristics.

The third transition profile, referred to as $B\downarrow$, is also split into two subtypes. $B\downarrow$ profiles are characterised by a shoulder towards the lower temperature on the main peak which has a half-height width greater than the parent peak. With increasing additive concentration only the area and height of the main peak decreases, with the half-height width staying the same. Both the half-height width and the area of the new peak (shoulder) increase with increasing additive concentration. However the total area of the entire profile remains unchanged. The temperature at the beginning of the overall transition changes drastically at low additive concentration but it changes only
slightly at higher levels. B† type profiles are the same but with the shoulder peak appearing at a temperature higher than the parent peak (see Figure 5.5).

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The fourth set, referred to as $D^\uparrow$, is really one of two subsets of B and represents one of two extreme cases of B type profiles. In $D^\uparrow$ profiles a new peak is formed at a higher temperature. As the concentration of the additive is increased the new peak increases in size as the parent peak decreases. This differs from $B^\uparrow$ profiles in that the new peak is very similar to the original peak. The appearance of a new peak in type D and B implies that a new phase exists in the bilayer that coexist with the unaffected pure lipid phase. The properties of the new phase are indicated by the shape and position of the new peak. A broad peak or shoulder as seen in type B profiles indicates that the new phase has smaller cooperative units, whereas the narrow peak as seen in type D profiles implies that the new phase has cooperative units of comparable size to that of the pure lipid.

The fifth set, referred to as E, is the second subset of the B type profiles. In this case the area of the main peak decreases but no new peak appears in the profile. The lack of any new peak appearing in type E profiles indicates that the new phase formed has no phase transition and is most likely in liquid phase.

The types of compounds which modify the phase transition profile also provide information about their orientation within the bilayer. The molecules which induce type A profiles were found to be long molecules with a one polar end (e.g. C5 and higher alkanols and alkanoic acids). In contrast, the compounds which induced type C profiles were small with weak dipolar character. Compounds which induced type B profiles were relatively large disk-shaped, asymmetric and reasonably polar. The solutes which induced type D profiles were almost exclusively ionic or have an ionic end. Thus if the different classes of compounds are arranged in order of increasing polarity, the order is:

$$\text{Type C} \rightarrow \text{Type A} \rightarrow \text{Type B} \rightarrow \text{Type D}$$

This means that type C compounds would be localised farthest away from the interface and the type D compounds would be localised closest to or on the interface (261). The localisation of Type E compounds is unable to be deduced from DSC data only.

Using this argument and the fact that the order parameter for each of the methylene groups of DPPC is known, the suggested positioning of compounds in a
lipid bilayer, based on their effects on phase transition profiles is; Type D compounds localised at the phosphorylcholine, Type B compounds localised at the glycerol backbone, Type A compounds localised around C1-C8 methylene groups of the lipid acyl chains and Type C compounds localised in the area in the C9-C16 methylene group region of the lipid acyl chains or between the terminal methylene groups of both sides of the bilayer (261).

Thus from the type of transition profile one can not only determine whether a particular compound would “fluidize” (disorder) or “solidify” (order) the bilayer, but a modified profile also provides the information about the thermodynamic properties of the new phase. Such data also indicates whether the whole bilayer undergoes a change or only part of it does.

5.1.2.3 Compounds Which Affect the $T_C$ and Transition Profiles

Numerous studies have shown that interactions of a variety of compounds with the lipid bilayers affect their gel to liquid-crystalline transition temperatures and in some cases the pre-transition. The position of these molecules within the lipid bilayer and the relationship to effects on $T_C$ have also been investigated.

5.1.2.3a Alkanols

It is now well accepted that alkanols, such as octanol, are orientated with their hydroxyl group anchored to the phospholipid polar headgroup, near the lipid water interface, and their aliphatic chains intercalated between the acyl chains of the phospholipids (148,149,262,263,267). This has been shown using both $^2$H NMR (262,263,267), $^{13}$C NMR (266) and X-ray diffraction experiments (262).

In addition to $^2$H NMR, $^{31}$P NMR experiments have shown that n-alkanols tend to cause a large disorder in the glycerol backbone which decreases as alkanol chain length increases. This results because the n-alkanols when anchored to the headgroup of the lipid leave gaps below them in between the lipid acyl chains. These gaps are energetically non-viable and must then be filled by the surrounding chains hence increasing chain disorder. As the alkanol chain length increases the gaps between the acyl chain become less significant and thus acyl chain order decreases back towards its original value (148,149).
Since n-alkanols accumulate in the membrane with their hydroxyl moiety near the phospholipid headgroup and their hydrocarbon chain aligned between the phospholipid acyl chains, the effect of these alkanols on $T_C$ are related to the phospholipid acyl chain length. The closer the alkanol chain length is to the phospholipid acyl chain length the stronger is the interaction between the two, which results in a stabilization of the gel state and hence an increase in the $T_C$ (148,149,180,268). For example, it has been shown that n-alkanols with a chain length of greater than 10 carbon atoms increase the $T_C$ of DPPC, whereas those with a chain length of C6-C10 lower the $T_C$, that is have a disordering effect on the lipid bilayer (148,149). This trend has also been noted for n-monocarboxylic acids (180).

The tendency of short chain alkanols to accumulate in a biological membrane is not very high. Therefore, quite high concentrations of these alcohols have to be used to detect an effect on the $T_C$ (186,269,270). Jain and Wu (261) determined the overall alkanol concentration which was required to cause a decrease in the transition temperature by a certain amount. As can be seen in Table 5.2 much higher concentrations of the short chain alcohols are required to affect the $T_C$ than the long chain alkanols. This can be explained by considering that alkanols partition in the bilayer with their hydroxyl moiety near the phospholipid headgroup. Short chain alkanols will thus not interfere strongly with the lipid acyl chains and will therefore have a less pronounced effect on the $T_C$ than the longer chain alkanols (148).

However, at higher concentrations, ethanol has been noted to have a biphasic effect on the $T_C$ of lipid bilayers, such as DPPC (186,261,269,271-275) and DSPC (271,275), although not PE (272,274). In particular for DPPC it has been found that as the ethanol concentration is raised from 40 mg/mL up through to 60 mg/mL a break occurs in the $T_C$ at 50 mg/mL (Figure 5.7). The linear decrease in the $T_C$ at lower concentration is explained by more ethanol partitioning into the liquid-crystalline phase than the gel phase (207,269,272). The break, at 50 mg/mL ethanol for DPPC, is caused by the onset of the interdigitated phase (see section 5.2.1.1), as shown by electron density profiles using X-ray diffraction (see section 1.3.3.2b). The induction of this phase causes the break in the $T_C$ curve since the interdigitated phase has twice as many hydrocarbon chains per lipid headgroup at the interface (and therefore more ethanol binding sites) than the usual lamellar bilayer. Consequently the partition coefficient becomes greater on the gel phase than the liquid-crystalline phase and
therefore causes $T_C$ to increase with increasing concentration until a concentration is reached where the interdigitated phase becomes saturated (207,272).

Table 5.2 – Membrane concentration of alkanol which causes a constant shift in transition temperature in DPPC lipid bilayers (75 mM). $HHW_{100}$ is the concentration at which the half-height width of the obtained DSC profile is shifted 100% along the temperature axis. Modified from (148).

<table>
<thead>
<tr>
<th>Alkanol</th>
<th>$HHW_{100}$ (Total amount of alkanol (mM) added)</th>
<th>Membrane concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>1400</td>
<td>54</td>
</tr>
<tr>
<td>Ethanol</td>
<td>483</td>
<td>55</td>
</tr>
<tr>
<td>Propanol</td>
<td>150</td>
<td>46</td>
</tr>
<tr>
<td>Butanol</td>
<td>33.4</td>
<td>19</td>
</tr>
<tr>
<td>Pentanol</td>
<td>11.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Hexanol</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Heptanol</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Octanol</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>Nonanol</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Decanol</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Figure 5.7 – Change in transition temperature for suspensions of distearoylphosphatidylcholine (DSPC) (dashed line) and DPPC (solid line) as a function of ethanol concentration. Also shown are the electron density profiles for DPPC in the L$_p$ phase at 40 mg/mL and in the interdigitated phase at 60 mg/mL. Taken from (207).
5.1.2.3b Alkanes

It has been shown using $^{31}$P NMR that alkanes have minimal effect on the ordering of the glycerol backbone. This suggests that alkanes do not interact with the headgroup and accumulate more deeply in the lipid bilayer, aligning themselves to the lipid hydrocarbon chains (148,149). However, phospholipid carbons 2 to 10, which are more ordered, are entropically less accessible for alkanes. Thus the more disordered bilayer centre is the favoured location of hydrophobic solutes (148,149).

Short chain alkanes, such as octane or hexane, are able to fit easily into this region. This has been shown using X-ray diffraction experiments where accumulation of the short chain alkanes in between the lipid acyl chain terminal methyl groups can be seen by the shallower and flatter nature of the terminal methyl group dip in electron density profiles (see section 1.3.3.2b). This results in thickening of the bilayers as indicated by an increased repeat period in the density profile (169).

However, as the alkane chain-length increases a point is reached when the entire alkane molecule cannot be located in this disordered lipid region and must expand into the more ordered region (148,264) and align parallel with the lipid acyl chains (169). Electron density profiles of DPPC and egg PC bilayers from X-ray experiments confirm this since longer alkanes (C12-C16) each increase bilayer width by exactly an amount consistent with the reduction of chain tilt from approximately 30° to 0°. This loss of chain tilt is also seen by specific effects on wide angle reflections produced by the bilayers. In addition the terminal methyl trough of the electron density profiles of these bilayers is not significantly affected (169).

Due to the interaction with the more ordered region of the acyl chains (i.e. carbons 2 to 10), these longer chain alkanes tend to be less soluble in the bilayer (264). For example the maximum amount of hexadecane that can be solubilised into phosphatidylcholine bilayers is 1 molecule per six to ten molecules of lipids whilst accumulation of up to 1 mol of hexane per mol of lipid has been reported (148). However alkanes lipid solubility is also dependant on lipid acyl chain length with solubility increasing with the increase in lipid acyl chain length (264).

n-Alkanes are found to show similar trends in their effect on the gel to liquid-crystalline transition temperature as their corresponding alkanols. Long chain alkanes (C ≥ 12) are found to increase the temperature and the enthalpy of the $T_C$ for DMPC and DPPC, but with no effect on the width of the phase transition peak. This
temperature increase occurs because the longer alkanes intercalate between the lipid acyl chains resulting in increased hydrocarbon chain interaction (i.e. they have a rigidifying effect on the bilayer) (77,149,169). This is similar to that of long chain alkanols (77,149).

On the other hand short chain alkanes (i.e. more than four methylene groups shorter than the lipid in question) were found to decrease and broaden the $T_C$ as well as decreasing the enthalpy. This occurs because shorter alkanes disturb the Van der Waals interaction between the phospholipid acyl chains which are primarily responsible for determining the phase transition temperature (77,149,169).

Another important effect that alkanes have on the $T_C$ of lipids such as DPPC and DMPC is the formation of multiple peaks (either two or three) within the main phase transition and the removal of the pre-transition for DPPC by longer chain alkanes but not shorter ones. The formation of multiple peaks by the longer alkanes suggests that several phases exist during the phase transition. It has been suggested that formation of two peaks results from separate solvent free and solvent saturated phases existing in the bilayer and is due to there not being enough hydrocarbon molecules in the bilayer to interact with all the lipid molecules. Three peaks are normally only seen when the alkanes chain length is great than the chain length of the lipid and their origin is still unknown (169).

It should also be noted that in general n-alkanes are more potent in destabilizing the bilayer structure than their corresponding n-alkanols. As the alkanols interact with the phospholipid headgroup area they will not only increase the tail area but also the headgroup area and are thus less effective in promoting an inverted lipid phase than the corresponding alkanes (148). However due to their low water solubilities hydrocarbons have a lower bioavailability to microorganisms.

5.1.2.3c Terpenes and Other Compounds

The effects of small solvent molecules like benzene, toluene and chloroform on the $T_C$ of lipid bilayers have also been investigated. Solvent such as these have been found to reduce the $T_C$ of DPPC liposomes (148). However, in some instances this only occurs where the small solvent molecules are at very high concentrations (261). These types of molecules being less hydrophobic than corresponding n-alkanes are expected to accumulate in between the lipid acyl chains near the headgroup area.
In the case of benzene there is currently disagreement regarding the phospholipid regions where it resides. Some studies have indicated that benzene molecules tend to partition into the hydrophobic region (276) whereas other studies have showed that benzene adsorbs at the headgroup interface (148). However, it has been recently shown, using fluorescent probes, that cyclic hydrocarbons such as toluene and cyclohexane primarily accumulate into the interior of the bilayer rather than into its peripheral regions and as a result are also able to increase the thickness of the membrane (92).

More polar molecules have been shown to anchor at the phospholipid headgroup in a similar fashion to n-alkanols. For example, benzyl alcohol, which has been shown to both decrease and broaden the $T_C$ of DPPC bilayers as well as effecting lipid packing arrangements (277), is thought to be anchored by its alcohol group to the phospholipids headgroups. $^1$H NMR studies have confirmed this and have also shown that above the phase transition the interaction of the alcohol with the headgroup can be regarded as a weak complex. In this situation the aromatic ring interacts with the bilayer interior very near the headgroup water interface. It has been suggested that below the phase transition benzyl alcohol is bound to the headgroup via the hydroxyl moiety but with the aromatic ring virtually excluded from the hydrocarbon chain region of the bilayer (265).

This is in direct contradiction to X-ray diffraction studies which have indicated that benzyl alcohol had no detectable effect on bilayers when added above the phase transition temperature. However below the transition temperature it produced marked effects on hydrocarbon chain packing and decreased membrane thickness. This suggests that benzyl alcohol is most likely anchored to the lipid water interface by its OH moiety and extends to a depth of only three to four methylene groups into the acyl chain region of the bilayer. Consequently the chains must bend around the benzyl alcohol because creation of large holes in the acyl chain region is energetically unfavorable. The perturbation of the chains induces formation of additional kinks in the acyl chains which in turn reduce the thickness of the bilayer. Because the interaction between chains is highly cooperative (see section 5.1.2.1) this effect is expected to be maximal for bilayers below their transition. For bilayers above their transition temperature the chain disorder should already compensate for holes
created by benzyl alcohol and hence there is no reduction in membrane thickness (277).

Anaesthetic molecules are also known to affect $T_C$ (27,163,278,279). The amine anaesthetics procaine, benzocaine, tetracaine and dibucaine were found to lower the $T_C$ of dipalmitoylphosphatidylethanolamine (DPPE) and DPPC with varying effectiveness (280). This disordering of lipid bilayers by anesthetics has in the past been generally considered to be crucial to the anesthetic affect. A more recent study on anesthetic steroids has confirmed this, where it was found that alphaxalalone and its inactive congener $\Delta$16-alphaxalalone differed in their effects on $T_C$. Alphaxalalone [I] was found to reduce and broaden the $T_C$ while $\Delta$16-alphaxalalone [II] increased the $T_C$ slightly. Additional data obtained from solid state $^{13}$C-NMR showed that the bilayer disordering alphaxalalone was fully incorporated into the bilayer while the membrane ordering $\Delta$16-alphaxalalone was only partially so. These results also highlight the importance of slight structural changes in molecules and their effects on membrane order, since the only difference between these two molecules is the absence of one double bond in the C16 position on the molecule (163).

Investigation into the membrane effects of insecticides have also shown the importance of small molecular changes on the positioning of molecules within the lipid bilayer and hence their affects on $T_C$. For example it has been found that the $\alpha$-[III] and $\beta$-endosulfan [IV] isomers, organochlorine insecticides differing only by stereochemistry two Cl groups and two ring hydrogens, each remove the pre-transition as well as decreasing and broadening the main phase transition of DPPC bilayers as a function of concentration. However, the $\beta$-isomer induces a shoulder in the main peak which appears at a lower temperature and increases in area as the main peak decreases, while the $\alpha$-isomer does not. This formation of a new phase transition which coexists with the original transition has been suggested to occur as a result of lateral phase separation due to non-random distribution of the insecticide. It has also been suggested that the shifted main transition peak is a result of lower levels of this isomer while the second peak represents domains of higher concentration (281). According to the parameters set out by Jain and Wu (261) and further data using fluorescent probes (281) it was suggested that for these two insecticides the $\beta$-isomer tended to localise near the phospholipid headgroups at the lipid/water interface while
the α isomer tended to localise deeper into the bilayer, most likely around the C1-C9 atoms of the lipid acyl chains. Similar data on membrane disordering affects and membrane localisation of other insecticides, including DDT, has also been investigated (282-291).

![Chemical Structures]

Studies into the membrane effects of the antidepressants diethazine, chlororpromazine and despramine derivatives have yielded similar phase transition profiles to that of the organochlorine compounds above. Hence it has been similarly concluded that these molecules localise near the phospholipid headgroup of the DPPC bilayer and cause the formation of coexisting phases which are thought to differ in their additive concentrations (164,181).
Phase transition temperature effects may again be shown for important drug molecules, such as morphine and several derivatives. The importance of slight structural modifications to the effect of these drugs on $T_C$ is also apparent. Modifications of the alkyl side chain of these molecules affected phase transitions of DPPC (see Figures 5.8 & 5.9) with the $T_C$ decreasing with decreasing alkyl side chain length (181). Another example where structural changes can effect transition temperatures and profiles is seen with the $\alpha$-phenyl alcohols where increasing the number of methylenes between the terminal OH and the benzene ring leads to greater decrease in $T_C$ (180).

Many other compounds have been studied for their membrane disordering potential. The reasons for this are many and varied, however some of the compounds not mentioned here in detail include; cholesterol, halofrantrine (an antimalarial agent), quercetin, umbeliferone, methoxypsoralen, alkanoic acids, oxidative phosphorylation uncouplers, multivalent cations, chaotrope anions, ionic and non-ionic surfactants, ionophores, quaternary ammonium compounds, phenylalkyl alcohols, beta-adrenergic receptor blocking drugs, abietic acid, various proteins and fluorescent probes (108, 162, 175, 179, 180, 182, 185, 187, 206, 223, 261, 265, 292-298).

![Chemical structures](image)

**Figure 5.8 – Structures of morphine derivatives affecting phase transition of the dipalmitoyllecithin-water systems shown in Figure 5.9.** Taken from (181).
Figure 5.9 – DSC heating curves of the dipalmitoyllecithin-water system with various morphine derivatives added. a) dipalmitoyllecithin; b) dipalmitoyllecithin/M II; c) dipalmitoyllecithin/M III; d) dipalmitoyllecithin/M IV; e) dipalmitoyllecithin/M V. (50:50 molar ratio drug:lipid). Taken from (181)
Recently the skin permeabilising effect of terpenes has been investigated and has been attributed to the effects of these terpenes on lipids. Limonene, 1,8-cineole and nerolidol have each been shown to decrease the $T_C$'s of the three main phase transitions in model systems of the stratum corneum (86,299,300). Whilst limonene only affected the $T_C$, 1,8-cineole also decreased the $\Delta H$ and nerolidol caused peak broadening (i.e. caused reduction in lipid cooperative unit size) (86,300). Further studies have subsequently shown that terpenes such as thymol and menthol which are able to hydrogen bond will also enhance transdermal uptake of drugs such as ibuprofen (301). Again this was suspected to be linked to terpene effects on lipids. A similar permeabilising effect has also been shown for terpinen-4-ol, $\alpha$-terpineol and carveol when applied to real stratum corneum samples where water and ethanol permeation was increased by these compounds (302).
Many of the examples discussed here illustrate the important relationship between effects on lipid bilayer packing and the various functions or activities of a variety of compounds. However, to our knowledge, no work has been done on terpenoids and how their affects on lipid bilayers relate to their antimicrobial activity.

In this set of experiments the effects of various terpenoids on the gel to liquid crystalline phase transition temperature and profile of DPPC bilayers were investigated. The importance of molecular structure on the lipid disordered ability and antimicrobial activity of the terpenoids will be discussed. Localisation of the terpenoids in DPPC bilayers was also able to be determined from DSC data.

5.1.3 Results and Discussion (Part A)

5.1.3.1 Effects of Terpenoids on \( T_C \) of DPPC

Of the 60 compounds initially tested for antimicrobial activity (see chapter 3) 41 were screened (see Table 5.3) for their effects on the \( T_C \) of DPPC lipid bilayers. The results in Table 5.3 show clearly that each of the terpenoids tested significantly reduced the \( T_C \) of DPPC by between 10-22°C. This shows that terpenoids are able to effectively increase the disorder (i.e. fluidity) of DPPC bilayers. It is therefore probable that terpenoids also increase disorder in lipid bilayers of microbial membranes systems. This type of action of terpenoids (as already discussed) would disrupt the overall function of the cell membrane and inhibit cell growth.

Interestingly, the terpenoids determined previously to be inactive in MIC tests (e.g. limonene) are also found to dramatically affect the \( T_C \) of DPPC. This apparent disparity can be easily explained by the fact that lack of antimicrobial activity for the terpenoids was found to be associated with H-bonding and water solubility (see Chapter 4). In this experiment water solubility and to a large extent H-bonding effects are negated by the fact that terpenoids were added directly to the lipid, thus being able to mix readily with the bilayer, before the addition of water to the system. This data also shows therefore that the relatively inactive terpene hydrocarbons and acetates may exhibit antimicrobial activity provided they can be transported across the initial water barrier.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Antimicrobially Active (+)/Inactive (-)</th>
<th>Reduction in Temperature of $T_c$ (°C)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-(+) - Citronellal</td>
<td>+</td>
<td>14.4</td>
<td>0.08</td>
</tr>
<tr>
<td>β-Citronellol</td>
<td>+</td>
<td>19.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Geraniol</td>
<td>+</td>
<td>20.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Geranylacetate</td>
<td>-</td>
<td>11.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Linalool</td>
<td>+</td>
<td>14.8</td>
<td>0.19</td>
</tr>
<tr>
<td>Linalool Oxide</td>
<td>+</td>
<td>17.4</td>
<td>0.64</td>
</tr>
<tr>
<td>Linalyl acetate</td>
<td>-</td>
<td>11.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Nerol</td>
<td>+</td>
<td>15.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Neryl acetate</td>
<td>-</td>
<td>10.5</td>
<td>0.00</td>
</tr>
<tr>
<td>(-)-cis-Myrtanyl amine</td>
<td>+</td>
<td>19.5</td>
<td>0.55</td>
</tr>
<tr>
<td>(1R)-(+) - Myrtenal</td>
<td>+</td>
<td>14.4</td>
<td>0.03</td>
</tr>
<tr>
<td>(1R)-(+) - Myrrtenol</td>
<td>+</td>
<td>13.0</td>
<td>0.02</td>
</tr>
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<td>(1S)-(+) - Verbenone</td>
<td>+</td>
<td>10.9</td>
<td>0.01</td>
</tr>
<tr>
<td>(1S2S5S)-(+) - Myrntanol</td>
<td>+</td>
<td>12.9</td>
<td>0.01</td>
</tr>
<tr>
<td>(S)-(+) - β-Pinene</td>
<td>-</td>
<td>19.5</td>
<td>0.01</td>
</tr>
<tr>
<td>(S)-cis-Verbenol</td>
<td>+</td>
<td>11.7</td>
<td>0.26</td>
</tr>
<tr>
<td>(+)-α-pinene</td>
<td>-</td>
<td>11.7</td>
<td>0.08</td>
</tr>
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<td>(-)-α-pinene</td>
<td>-</td>
<td>18.1</td>
<td>0.19</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>+</td>
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<td>0.07</td>
</tr>
<tr>
<td>(-)-Limonene</td>
<td>-</td>
<td>20.1</td>
<td>0.17</td>
</tr>
<tr>
<td>(-)-menthol</td>
<td>+</td>
<td>10.6</td>
<td>0.11</td>
</tr>
<tr>
<td>(-)-menthone</td>
<td>-</td>
<td>16.6</td>
<td>0.02</td>
</tr>
<tr>
<td>(-)-Perillaldehyde</td>
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<td>17.0</td>
<td>0.11</td>
</tr>
<tr>
<td>(+)-Limonene</td>
<td>-</td>
<td>20.1</td>
<td>0.35</td>
</tr>
<tr>
<td>(α)-(±)-Terpinylacetate</td>
<td>-</td>
<td>13.8</td>
<td>0.02</td>
</tr>
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<td>(R)-(+) - Carvone</td>
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<td>14.6</td>
<td>0.00</td>
</tr>
<tr>
<td>(S)-(+) - Perylalcohol</td>
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<td>22.0</td>
<td>0.01</td>
</tr>
<tr>
<td>1,4-Cincole</td>
<td>-</td>
<td>21.5</td>
<td>0.28</td>
</tr>
<tr>
<td>1,8-Cincole</td>
<td>-</td>
<td>22.3</td>
<td>0.15</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>-</td>
<td>19.8</td>
<td>0.05</td>
</tr>
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<td>α-Terpineol</td>
<td>+</td>
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<td>0.00</td>
</tr>
<tr>
<td>α-Terpinolol</td>
<td>-</td>
<td>20.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>+</td>
<td>18.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Dihydrocarveol</td>
<td>+</td>
<td>14.4</td>
<td>0.01</td>
</tr>
<tr>
<td>Dihydrocarvone</td>
<td>+</td>
<td>14.6</td>
<td>0.01</td>
</tr>
<tr>
<td>γ-Terpine</td>
<td>-</td>
<td>18.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Limonene oxide</td>
<td>+</td>
<td>19.3</td>
<td>0.01</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>-</td>
<td>19.7</td>
<td>0.26</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>+</td>
<td>15.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Thymol</td>
<td>+</td>
<td>17.7</td>
<td>0.06</td>
</tr>
<tr>
<td>(+)-pulegone</td>
<td>+</td>
<td>15.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Menthylacetate</td>
<td>-</td>
<td>13.8</td>
<td>0.01</td>
</tr>
</tbody>
</table>

To determine the importance of functional group on the lipid disordering capacity of the terpenoids, alcohols, ketones, aldehydes, acetates and hydrocarbons.
were grouped together. The resulting plot (see Figure 5.11) indicated that the alcohols, ketones, aldehydes and acetates had only small differences in their affect on $T_C$, while the hydrocarbons were slightly more effective at reducing $T_C$ than the other functional groups.

![Box plot showing reduction in $T_C$ of DPPC for different terpene functional groups](image)

**Figure 5.11 – Effect of terpene functional group on the gel to liquid-crystal phase transition ($T_C$) of DPPC. Alcohols (n=15), Ketones (n=5), Aldehydes (n=3), Acetates (n=5), Hydrocarbons (n=9)**

The main point illustrated in Figure 5.11 however is that there is a relatively large spread of temperatures within each of the functional groups. This suggests that slight structural differences between molecules with a particular functional group are just as important as the actual functional group present when it comes to the lipid disordered capacity of the terpenoids. This is especially so for terpene alcohols and hydrocarbons since they have the largest spread of results. However, as stated earlier, both water solubility and hydrogen bonding factors are the main molecular parameters associated with antimicrobial activity. Thus functional group will be important in the terpenoid’s access to the lipid bilayer even if it does not affect the disordering capacity of the compound. This suggests that the important parameters involved in lipid disordering are more complex and are probably a combination of the effects of
terpene structure, functional group orientation and positioning of terpenes within lipid bilayers.

5.1.3.2 Effects of Terpene Molecular Structure on Concentration Dependence of $T_C$ Depression

The influence of terpene structure and functional groups on $T_C$ was investigated more closely using 14 terpenoids selected from the original screening group. Investigation of the influence of terpene concentration on $T_C$ provides two pieces of information. These are the maximum disordering capacity of the terpenoid and the rate at which it reaches the concentration where this occurs. These two factors are vital in discerning how structural changes affect $T_C$ and how this relates to antimicrobial activity or inactivity. This is because the total capacity to disorder a membrane, and the initial amount of terpenoid required to produce an effect, both impact on antimicrobial activity. In particular the rate at which a compound reaches its maximum disordering capacity would be expected to affect activity since for any compound to be active a balance between availability to the membrane (influenced by water solubility and hydrogen bonding capacities) and the amount required to cause a physiologically significant change to the membrane is crucial.

The significance of structural variation on this balance is seen with the comparison of the three ketones; carvone [V] (active), dihydrocarvone [VI] (active) and menthone [VII] (inactive). Figure 5.12 shows that carvone and dihydrocarvone, which differ only by the level of saturation within the ring structure, have an almost identical concentration dependent effect on $T_C$. Both compounds reduced $T_C$ rapidly before a minimum $T_C$ was reached. However, menthone, while showing a similar type of trend, required a greater concentration to achieve the same reduction in $T_C$. This may contribute to menthone's inactivity since it is less soluble than the other two ketones yet greater amounts of the compound need to come into contact with the bilayer to have the same effect on membrane order.
Figure 5.13 shows the concentration effect of carveol [VIII] (active), carvone [V] (active) and carvacrol [IX] (active) on $T_C$. These three compounds were selected as they are structurally related compounds varying mainly in functional group. It is evident from this data that carvacrol is by far the most effective at disordering the lipid bilayer. However, at lower terpene concentrations carvone gives a similar $T_C$ reduction. This data also shows, that carvone is more effective at disordering the lipid bilayer at both high and low concentrations than carveol. This is somewhat surprising given that these two compounds are very similar in their antimicrobial activities against *E. coli*, *S. aureus*, *P. aeruginosa* and *C. albicans*. The fact that each of these three compounds shows very different effects on $T_C$ yet show similar antimicrobial activity patterns suggests that lipid disorder is not the only factor affecting activity. This is not surprising considering that overall effects on membrane fluidity are governed by three major factors (i.e. order, stability and permeability).

The third set of terpenoids selected for comparison were perilla alcohol [X] (active) and perilla aldehyde [XI] (active). Figure 5.14 shows that both perilla alcohol and perilla aldehyde have similar types of trends (i.e. initial rapid decrease in $T_C$ followed by saturation if $T_C$ depression as concentration increases). However, perilla aldehyde does not decrease $T_C$ as effectively at lower or higher concentration as perilla alcohol. This suggests that the OH functional group allows for more effective membrane disorder. This may be related to hydrogen bonding capacity.
Figure 5.12 - Effects of varying mole fractions of carvone, dihydrocarvone and menthone on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Figure 5.13 - Effects of varying mole fractions of carvone, carvacrol and carveol on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Figure 5.14 - Effects of varying mole fractions of perilla aldehyde and perilla alcohol on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Comparison of two alcohols, carveol (active) and menthol [XII] (active) showed the influence of the position of the alcohol function on $T_C$ depression. Figure 5.15 shows that both carveol and menthol show a similar trends in $T_C$ depression with menthol being not quite as effective as carveol. Hence the overall saturation or position of the alcohol group seems to affect the ability to disorder the lipid bilayer. Limonene [XIII] was also compared to carveol as its functionless non-polar and inactive structural analogue. It can been seen from Figure 5.15 that limonene has a similar type of trend in $T_C$ depression to that of carveol. However, as for menthone, the amount of limonene required to produce the same $T_C$ depression is initially larger even though the $T_C$ at saturation is lower. Thus even though hydrocarbons like limonene may disorder lipid bilayers slightly more effectively than oxygenated terpenoids a greater amount is required to reach saturation of $T_C$ depression. In a natural system this is of course hampered by the low water solubility of hydrocarbons like limonene.

![Chemical structures](image)

The effect of the alcohol function being on or off the p-menthane ring structure is also seen by comparison of terpinen-4-ol [XIV] (active) and $\alpha$-terpineol [XV] (active). It can be seen in Figure 5.16 that although terpinen-4-ol and $\alpha$-terpineol show similar type of trends in $T_C$ depression $\alpha$-terpineol is more effective at disordering the lipid bilayer at both low and high concentrations. Hence the position of the alcohol group on the molecule is again shown to influence the terpenoids.
effectiveness to disorder the bilayer. This result is surprising since both of these compounds show identical antimicrobial activity against three of the four test organisms. This suggests that in this case the increased capacity of α-terpineol to disorder the lipid bilayer does not result in an increased antimicrobial activity. The increased activity of terpinen-4-ol against *P. aeruginosa* is also not the result of its lipid bilayer disordering capacity since it has been shown (see chapter 6) that activity against *P. aeruginosa* is governed by the ability of these two terpenoids to penetrate the impermeable outer membrane of this organism. It is extremely important to note however that the lack of correlation between capacity to disorder lipid bilayers and antimicrobial activity in this case does not negate the contribution of the bilayer disordering ability of these compounds towards their effectiveness as antimicrobial agents. Rather these results suggest that either the crucial level of lipid bilayer disorder is able to be achieved effectively by both compounds in a natural system or that other factors are also involved in determining their antimicrobial activity.

The final set of terpenoids compared were the acyclic terpenoids geraniol [XVI] (active) and citronellol [XVII] (active). These compounds were selected due to their difference in saturation along the main carbon chain. It can been seen in Figure 5.17 that both geraniol and citronellol have a virtually identical effect on $T_c$. This shows that in this case that the slight structural change between these two compounds has no effect on $T_c$ depression.

![Chemical structures](image-url)

[XIV]  [XV]  [XVI]  [XVII]
Figure 5.15 - Effects of varying mole fractions of carveol, menthol and limonene on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Figure 5.16 - Effects of varying mole fractions of terpinen-4-ol and α-terpineol on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Figure 5.17 - Effects of varying mole fractions of geraniol and citronellol on onset temperature of DPPC order/disorder phase transitions. Error bars represent standard deviation.
Comparison of the data from several sets of terpenoids shows that each of the terpenoids has a similar type of general trend in $T_C$ depression versus concentration. In particular it is noted that each of the terpenoids tested showed a rapid decrease in $T_C$ which then plateaued, the mole fraction at which this occurred being dependent on the terpenoid. It was also observed through comparison of the p-menthane and acyclic terpene alcohols studied (see Figure 5.18) that the final $T_C$ achieved decreased as the OH functional group position tended toward the end of the molecule. Indeed a correlation (see Figure 5.19) was found to occur between the polarity (calculated – see section 2.1.4) of the p-menthane alcohols and $T_C$ depression. This relationship between polarity and the ability of p-menthane alcohols to disorder the lipid bilayer may be related to how they are able to orientate themselves within the bilayer. As the polarity increases for the p-methane alcohols they will tend to act more like n-alkanols which are known to orient themselves with their functional group attached to the lipid water interface and their hydrocarbon chains aligning with the lipid acyl chains. This will increase the interaction of the p-methane alcohols with the lipid acyl chains of the bilayer and hence increase their ability to disorder the bilayer (75,148,22). This is supported by the fact that both geraniol and citronellol (acyclic terpenoids) have the lowest final $T_C$ except for that of perilla alcohol.
Figure 5.18 - Effects of various terpene alcohols (and carvacrol) on onset temperature of DPPC order/disorder phase transitions.
Figure 5.19 - Relationship between calculated polarity of p-menthane alcohols and onset temperature of DPPC order/disorder phase transitions.

\[ y = -0.0527x + 5.6358 \]

\[ R^2 = 0.8025 \]
5.1.3.3 Effects of Terpenoids on DPPC gel/liquid-crystal Transition Profiles

According to Jain and Wu (261) (see section 5.1.2.2a) the characteristics of the transition profile changes caused by the terpenes should provide information as to the orientation of the molecules in the membrane along with their effects on lipid cooperative unit size. Therefore the effects of 14 terpenoids on transition profiles were investigated. Figures 5.20-5.26 show the changes in transition profiles of each of the selected terpene in respect to concentration. From these figures it is immediately apparent that each of the selected terpenes has a significant effect on the transition profile of DPPC.

Inspection of these transition profile changes, using the criteria set out by Jain and Wu (261) indicates several important and interesting points. Firstly, each of the oxygenated terpenes except α-terpinyl acetate show the same type of profile changes and hence are each affecting the bilayer order in a similar fashion albeit with different sensitivity based on concentration.

For the oxygenated terpenes the most obvious profile change is an immediate loss of the pre-transition peak at low terpene concentration. In addition to this the parent peak of the pure DPPC transition (1°) becomes broader in Half Height Width (HHW) initially but then becomes narrower and reduces in area as concentration of terpene increases (see figures 5.20-5.26 & Table 5.5). As the 1° transition is seen to decrease in area a second parent (2°) transition peak emerges at a lower onset temperature than the 1° parent and increases in area as terpene concentration increases such that in some cases (e.g. carveol) the 1° parent disappears completely leaving only the 2° transition. It is important to note that although the area of the 2° peak increases as terpene concentration increases the HHW of this transition remains constant within experimental error and is similar in HHW and shape to that of the original 1° transition of pure DPPC (see Table 5.5).

The transition profile changes described above do not fit any one of the phase transition profiles outlined by Jain and Wu (261) but are rather indicative of both A↓ and D↓ profiles. In particular the initial broadening of the 1° HHW is characteristic of A↓ type profiles while the appearance of the 2° transition which is increased in area but is similar in HHW and shape to the 1° transition is characteristic of D↓ type profiles.
### Table 5.4 - Effects of Terpenes on gel to liquid crystal phase transition profiles of DPPC

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<th>Compound</th>
<th>Mole Fraction Of Terpene</th>
<th>1° Peak Ave HHW SD</th>
<th>2° Peak Ave HHW SD</th>
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<td></td>
</tr>
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<td></td>
<td>0.2 4.19 0.09</td>
<td>P -</td>
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</tr>
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<tr>
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<td>0.2</td>
<td>4.46</td>
<td>0.19</td>
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</tbody>
</table>

p = peak present but HHW unable to be accurately determined due to small size of peak and/or peak convolution.

n = no detectable peak present

# = new peak also appears at lower temp and has similar HHW and peak shape as and original 1° peak. Does not have similar shape of HHW to the pre-transition of 1°.

+ = Peaks appear to be directly on top of one another so HHW most likely reflects wider 1 peak.

* = new peak also appears at higher temp than 2°. HHW not able to be determined for this peak but shape suggests similar to 2 and original 1°.
Similar A↓D↓ profile have been shown previously for α-endosulfan (see section 5.1.2.3c) by Videira et al (281) who suggested that this molecule also localises at the lipid/water interface especially at higher concentrations. It was also suggested by Videira et al (281) that the reduction in the cooperative unit resulting in such additives is caused by the presence of two simultaneously existing domains in the bilayer. The first, the down shifted main transition, resulted from areas of low insecticide concentration while the second, the new down shifted transition, resulted from domains of high insecticide concentration. Due to the similarity of the results between the study by Videira et al (281) and our results it is possible that this occurs for the oxygenated terpenoids investigated in this study.

It is suggested therefore that the oxygenated terpenes interact at more than one site in the lipid bilayer depending on concentration. At low terpene concentrations the terpene act more like type A profile compounds and orientate within the bilayer around the C1-C8 methylene groups of the lipid acyl chains. This results in 1° transition broadening inferring that the cooperative unit undergoing transition at any one temperature is small thus the effect of the terpene on lipid order is restricted in terms of the entire bilayer. As the terpene concentration increases, however, the transition profiles tend more towards D↓ type profiles suggesting that the oxygenated terpenes become more localised at the phospholipid headgroup and cause the formation of a new phase which has a similar cooperative unit but distinctly different packing characteristics to the 1° transition. This type of profile was not expected for the oxygenated terpenes as D type profiles are mainly associated with ionic compounds (261) though not exclusive to them. The exact reason for this unusual localisation is unclear, however, it is possible that at higher terpene concentrations it becomes more energetically viable for the oxygenated terpenoids to orient closer to the lipid water interface than deeper within the bilayer and in close association with one another. This apparent coating type effect of the oxygenated terpenes would be expected to affect the interaction of other species with the membrane due to changes in the lipid/water (i.e. lipophilic/hydrophilic) interface.

Jain and Wu (261) also suggested that the 2° transition in D type profiles was indicative of a more tightly packed bilayer structure provided the 2° transition was at a higher transition temperature. While the oxygenated terpenes induced a 2° transition at a lower temperature Atomic Force Microscopy studies of supported DPPC bilayers
(see section 5.2.4.3 for more details) indicate that they also result in a more tightly packed bilayer. Such a change in the packing profile could be detrimental to membrane bound enzyme since a more condensed packing arrangement may distort crucial enzyme structure or reduce structural flexibility of such enzymes.

Two exceptions to the A↓-D↓ profile of oxygenated terpenes discussed above were found to occur. The first occurred at high concentrations of menthol. In this case after the disappearance of the 1° transition a third transition (3°) appeared at an even lower temperature than the 2° transition. This 3° transition then increased in area but had a similar HHW to that of the original 1° transition. It would seem, therefore, that at high concentration the menthol is able to induce another change in packing characteristic of the lipid bilayer which has a similar cooperative until to that of the original untreated bilayer. The reason for this is not discernible from this data.

The second exception to the A↓-D↓ transition profile was found to occur with α-terpinyl acetate [XVIII]. Unlike the other oxygenated terpene α-terpinyl acetate was not found to affect the pre-transition of the lipid bilayer. This suggests that α-terpinyl acetate does not interact with the lipid acyl chains in such a way as to force them to align vertically and thereby remove the pre-transition. The significance of this difference in effect is uncertain in terms of overall effects of bilayer order. However it should be noted that limonene, the only hydrocarbon tested, which is also relatively inactive compound also did not affect the lipid bilayer pre-transition.

![Chemical Structure](image)
Figure 5.20 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) terpinen-4-ol and (B) α-terpineol. X-axis represents temperature (°C).
Figure 5.21 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) geraniol and (B) β-citronellol. X-axis represents temperature (°C).
Figure 5.22 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) (-)-carvone and (B) carvacrol. X-axis represents temperature (°C).
Figure 5.23 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) perilla aldehyde and (B) perilla alcohol. X-axis represents temperature (°C).
Figure 5.24 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) carveol and (B) menthone. X-axis represents temperature (°C).
Figure 5.25 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) $\alpha$-terpinyl acetate and (B) dihydrocarvone. X-axis represents temperature ($^\circ$C).
Figure 5.26 – Phase transition profiles of DPPC liposomes modified by the addition of increasing mole fractions of (A) (-)-menthol and (B) (+)-limonene. X-axis represents temperature (°C).
Limonene was the only terpene investigated which showed a different transition profile to the A↓-D↓ profile. Instead limonene caused a broadening of the 1° transition (characteristic of A↓ profile) followed by the appearance of a 2° transition at lower temperature than the 1° transition which increased in area and HHW as terpene concentration increased (characteristic of B↓ profile). This data suggests that limonene also interacts at more than one site in the bilayer, dependent on concentration. At low concentration limonene acts as an A↓ type compound and thus will tend to localise around the C1-C8 methylene groups of the lipid acyl chain. As the concentration increases limonene acts more like B↓ type compound and thus localise more toward the glycerol backbone. This movement of localisation of limonene in the bilayer is expected to some extent as the C1-C8 methylene groups may become saturated with limonene at higher concentration. At higher concentration limonene would also be expected to partition more toward the centre of the bilayer and hence induce C↓ type profiles. This profile may also exist for limonene at high concentration and must not be ruled out, since presence of limonene at C1-C8 and the glycerol backbone would mask this profile.

5.1.4 Outcomes : Part A

This set of experiments has clearly shown that terpenoids are able to effectively disorder, hence increase fluidity, of DPPC lipid bilayers. The results also show that those terpenoids which are antimicrobially inactive may still able to affect the bilayer order provided they are able to gain access to the bilayer, something which is normally precluded by their low water solubility and H-bonding. Structural changes in the terpenoids were found to affect the maximum bilayer disordering capacity and the concentration dependence of the disordering effect. For the terpene alcohols the maximum bilayer disordering capacity was found to be related to the polarity of the molecules which is in turn related to the positioning of the OH functional group on these molecules.

In addition to affecting the T_C of DPPC bilayers directly, the terpenes were also found to localise at two different regions of the bilayer depending on their concentration. Oxygenated terpene tended to orient at the C1-C8 methylene groups of the lipid acyl chains at lower concentration then localise at the phosphorylcholine
headgroup as concentration increased. This apparent coating type effect displayed by the oxygenated terpenes would be expected to affect the interaction of other species with the membrane due to changes in the lipid/water (i.e. lipophilic/hydrophilic) interface. The hydrocarbon limonene also showed a similar type of multisite localisation dependent on concentration, but oriented at the C1-C8 methylene groups at low concentration then at the glycerol backbone and possibly lower end of acyl chains at higher concentrations.
5.2 Introduction Part B: Effects on Bilayer Stability (lipid Packing)

Most studies on the effects of compounds on bilayer stability have to date been done using artificial membranes. Traditionally, bilayer stability has been estimated using the $T_{\text{LH}}$ of PE bilayers or the susceptibility of formation of $H_{\text{II}}$ phases in PC bilayers. However, the recent advent of Atomic Force Microscopy (AFM) has seen its use in monitoring stability and packing arrangements of lipid bilayers under near physiological conditions.

5.2.1 Formation of the Hexagonal II ($H_{\text{II}}$) Phase

Egg phosphatidylethanolamine (PE) has a transition from lamellar ($L_o$) to inverted hexagonal ($H_{\text{II}}$) phase when the temperature is raised to 30°C (148,149,303). The temperature at which this phase transition occurs is termed $T_{\text{LH}}$. Since various types of PE readily form the $H_{\text{II}}$ phase, the temperature at which this occurs being dependent on the fatty acid composition of the PE (201), much of the work on bilayer stability studies has been done using these lipids. However, PC’s (e.g. DPPC) have also been found to form $H_{\text{II}}$ phases upon the addition of $n$-alkanes (200) and certain ceramides (294). This effect was noted to be highly dependent on the size of the ceramides and $n$-alkanes as well as the lipid acyl chain.

It has been proposed that $H_{\text{II}}$ phase stabilisation is achieved by two different mechanisms. The first is by reducing $R_o$, the spontaneous radius of curvature that the lipid monolayers of a system would adopt in the absence of other constraints under given conditions. The principal constraint is that of having to pack the interstices between monolayer cylinders or spheres with hydrophobic moieties. In one component systems, these interstices must be filled by entropically disfavored stretching of the phospholipid acyl chains. This is an unfavourable contribution to the $H_{\text{II}}$-phase chemical potential. Hence lipids that form stable lamellar phases have near infinite value for $R_o$, while lipids that form the $H_{\text{II}}$ phase have small values of $R_o$. The tendency of a system to form inverted phases is increased by additions of components that decrease $R_o$ (304).

The second way in which hydrophobic impurities can stabilize the $H_{\text{II}}$ phase is by partitioning into the interstices between the $H_{\text{II}}$ tubes. This relieves the entropically
disfavored stretching of the phospholipid chains. Low concentration of moderate to long chain alkanes stabilize HII phases by partitioning into the hydrophobic interstices between HII tubes (304).

5.2.2 Compounds which Affect TLH

5.2.2.1 Alkanols

It has been shown that partitioning of long chain alkanols (C ≥ 6) into PE bilayers decreased the TLH. In other words, the bilayer structure was made less stable by these alkanols. This observation may be again explained by the fact that the alkanols orient themselves with their hydroxyl group anchored at the headgroup of the phospholipids and their hydrocarbon chains aligned with the phospholipid acyl chains. Although this accumulation will increase the headgroup area 'a' of the phospholipids, they also have a pronounced effect on the lipid volume 'v', thus resulting in an increase of v/l/a and hence promote the formation of the HII lipid phase (148,303).

However, shorter chain alkanols (C < 6) were found to have a bilayer stabilising effect on PE bilayers. For example, ethanol, which partitions in membranes especially in the headgroup area of the phospholipids, will increase the area 'a'. According to the lipid packing theory an increase in headgroup area 'a' will oppose the formation of a HII lipid phase. This is indeed observed since the addition of ethanol to egg PE liposomes results in an increase of the TLH (148,303). However as discussed in the introduction (section 1.3.3.2b) microorganisms require a balance of between bilayer and non-bilayer forming phospholipids for various functions which require the HII phase of the lipids, hence the stabilizing effects of alkanols like ethanol may be unfavorable for cell growth (148).

When comparing the effects of alkanols on both TC and TLH it is interesting to note that short chain alcohols (C ≤ 3) decrease the TC (i.e. decrease lipid ordering) and increase the TLH (i.e. increase the bilayer stability), while long chain alkanols (C > 10) results in more rigid (increased TC) but less stable bilayers (decreased TLH). The alkanols with chain length between C4 and C10 decrease the lipid ordering and destabilize the bilayer structure (decreased TC and TLH) (148).

The interaction of short chain alkanols with the membrane apparently stabilizes the bilayer structure since the TLH increases. However, it has been
demonstrated that these short chain alkanols (C ≤ 3) can promote an unusual phospholipid aggregation structure, the interdigitated phase (L_{p1}). In the interdigitated phase the lipid acyl chains from the opposing monolayers are fully interpenetrated, thereby exposing the terminal methyl groups (Figure 5.29). These short-chain alkanols (C ≤ 3) will anchor with their polar moiety to the phospholipid headgroup, and with the non-polar part between the phospholipid acyl chains. Since the non-polar moiety is short compared with the fatty acid acyl chains, these molecules would potentially cause voids between the lipid chains in the bilayer interior. As energy of formation of holes between hydrocarbons is extremely large, the lipids respond by forming the interdigitated phase (148,149,207,274).

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Please refer to print copy

Short chain alkanols are not the only molecules to induce the interdigitated phase in lipids. Compounds such as benzyl alcohol, chlorpromazine, tetracaine 2-phenylethanol and 4-phenyl-1-butanol can also induce interdigitation. This effect is again attributed to the compounds interaction with the lipid headgroup, via the hydroxyl group of the molecule, and the shallow penetration of the hydrocarbon portion of the molecule into the bilayer (274,305).

Although the interdigitated phase has been observed in various phospholipids, this lipid phase is not frequently observed in PE systems. As PE is the major phospholipid in many microorganisms, while PC’s are not abundant in eubacteria, the microbiological significance of the interdigitated phase is unclear. In addition
interdigitation is only observed for bilayers in the gel phase (148) and since most of
the phospholipids in microorganisms are generally in the liquid-crystalline phase
(148,197) it is unclear whether the interdigitated phase will occur naturally or can be
induced by solvents in microorganisms at all (148).

5.2.2.2 Alkanes

It has also been shown that n-alkanes are able to dramatically lower the $T_{\text{LH}}$ of
PE (Figure 5.30) (149,303). Various $^{31}$P-NMR studies have shown that increasing the
chain length of the alkanes enhances this effect and that alkanes are twice as potent as
corresponding alkanols (303). This trend extends even as far as eicosane (C20) (149).

The mechanism by which n-alkanes lower $T_{\text{LH}}$ is not well understood but it
has been suggested that the lipid acyl chain disorder created by alkanes partitioning
into the lipid bilayer will make the $H_{\text{II}}$ phase more energetically desirable (303).
Further experimental evidence using NMR, calorimetry and X-Ray diffraction (199)
has indicated this is achieved by the second method proposed by (304), that is,
partitioning of the alkanes into the interstices between the $H_{\text{II}}$ tubes.

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5.2.2.3 Other Compounds

Whilst alkanes and alkanols have been the focus of much of the research on $T_{\text{LH}}$ various other important molecules have also been found to affect this phase transition. For example cholesterol has been shown to decrease the $T_{\text{LH}}$ for soya bean PE (198) thus destabilising the PE bilayer. This evidence is supported by the almost complete absence of cholesterol in bacterial membranes which consist mainly of PE’s.

Investigation into the effect of low levels of diglycerides has shown that they also affect the $T_{\text{LH}}$ and consequently stabilise the $H_n$ phase. The mechanism of this stabilisation differs from solvent such as alkanes, however, as these compounds have been shown to reduce $R_o$ of dioleoylphosphatidylcholine (DOPC) and N-monomethylated dioleoylphosphatidylethanolamine (DOPE-Me) lipids rather than partitioning into the hydrophobic interstices between $H_n$ tubes (199).

The influence of PC’s on PE $T_{\text{LH}}$ have also been investigated and found to profoundly influence the $T_{\text{LH}}$ temperature. This suggests that a balance of PE and other lipid types may be crucial to the stability and versatility of PE based membranes (198).

Antibiotics such as gramicidin have also been shown to induce non-lamellar phase in lipid bilayers. This action is considered to contribute to its overall activity (306).

5.2.3 Atomic Force Microscopy

While investigation of $T_{\text{LH}}$ has been used extensively in the past to monitor bilayer stability, more recently AFM seems to be increasing in popularity as a tool to investigate not only the packing and stability of artificially created bilayers but also the surfaces of living microorganisms (209,211,212,216-221,223-226).

This has arisen since AFM provides several advantages over other high resolution imaging techniques and the more traditional monitoring of $T_{\text{LH}}$. These advantages include; its relatively non-intrusive nature, the ability to monitor the lipid bilayer in solution and under near physiological conditions, molecular markers are not required, and imaging can be done in real time. In addition AFM can image down into the nanometer region and thus image individual phospholipid headgroups, hence it is extremely useful for viewing the affects of the terpenes on lipid bilayer packing and stability.
However, the recent application of AFM supported lipid bilayer and living cell membranes has brought to light the problems involved in high resolution imaging of soft surfaces (211,224). These include the dependence of imaging resolution on probe-sample interactions and the apex structure of the probe itself (224). It has also been shown that the type of lipid used to make bilayers and the pH at which imaging is conducted also affect their ability to resist deformation due to the AFM probe (217).

It is therefore considered extremely important to minimise the probe force on the samples since it has been reported that, even at the low forces involved with AFM imaging, probe induced sample destruction does occur. Force minimisation can be achieved in two ways. The first is by use of Tapping Mode AFM. In this mode the AFM probe tip is oscillated vertically at a high frequency as it moves over the sample, resulting in intermittent contact between the tip and the sample. This is in contrast to Contact Mode AFM where the probe tip is in direct contact with the sample surface at all times (211). The second method of minimising contact force is to use a force minimisation curve. In this method the probe tip is manually raised from the sample surface until the point immediately before the force on the probe tip reduces to zero indicating that it is not longer in contact with the sample surface. This method allows for the minimal possible force to be applied to the sample.

5.2.3.1 Effects of Compounds on Bilayer Stability and Packing as Monitored Using AFM

With the realization of AFM as a powerful tool for investigating lipid bilayer properties various workers have also investigated the effects of several known bilayer partitioning compounds. For example, both ethanol and 2-propanol were found to reversibly induce interdigitation in DPPC bilayers. This was deduced due to distinct changes in height profiles within the lipid bilayer indicating the appropriate thickness required for an interdigitated phase. In the case of ethanol DPPC interdigitation was found to occur only at high concentration (200mg/mL) and after long contact times. At extremely high concentrations of ethanol however (300mg/mL) the DPPC bilayer structure was no longer able to be maintained and small globular structures appeared presumably formed by DPPC molecules. In contrast to ethanol, 2-propanol was found to induce interdigitation of DPPC at much lower concentrations (100mg/mL). This
increased effectiveness of 2-propanol was attributed to its larger hydrophobic chain which should result in a more favorable partitioning into the lipid bilayer. Similar effects on distearoylphosphatidylcholine (DSPC) bilayers have also been shown for 2-propanol although no such effects were evident for dilaurylphosphatidylcholine (DLPE) bilayers (220). Clearly the formation of interdigitated areas in lipid bilayers would be detrimental to microorganisms because of the many functions of membranes that rely on the presence of a stable bilayer.

The membrane effects of antibiotics such as filipin, a polyene antibiotic with antifungal activity and belonging to the same family as amphotericin B and nystatin (307), have also been studied using AFM (222). In particular it has been shown that filipin induced lesions in DPPE bilayers. Densely packed circular protrusions, suspected to be aggregates of filipin, were also found to form in the DPPE bilayers along with doughnut-shaped lesions. While filipin did not completely breakdown the bilayer structure such lesions and aggregates of filipin upon the bilayer surface is likely linked to its membrane oriented antibiotic activity (222).

AFM has also helped to detect the effects of compounds which had before been assumed as harmless to membranes. One example is the commonly used buffer tris(hydroxymethyl)aminomethane (tris). Tris was found to induce a ripple phase in supported unilamellar DPPC bilayers at room temperature. This ripple structure showed various types of domains that could extend to several micrometers in length. Two different periodic ripples were found to coexist. This ripple phase was found to exist for a wide temperature range especially at higher tris concentrations (221). This shows the care which must be taken to avoid artifacts caused during sample preparation for such techniques as AFM. This is supported in a review by Shao et al (208) who provides several other examples similar to tris and states that 'much more attention should be paid to the effect of small membrane-interacting molecules present in solution.' Hence the bilayer preparations described in this thesis were modified to exclude the use of any additives.

5.2.3.2 Measurement of Bilayer Packing Using AFM

In addition to determining the direct visual effects of various compounds on lipid bilayers, AFM has also been used for the direct imaging of the phospholipid headgroups of lipid bilayers. For example, the headgroups of dimyristoyl-L-α-
phosphatidyl-DL-glycerol (DMPG) were imaged under physiological conditions after preparation via Langmuir-Blodgett deposition. These headgroups were found to not only have areas similar to that estimated by other techniques but also long range positional as well as orientational order. Headgroups were found to exist in rows with similar repeat distances such that the arrangement could be described as hexagonal in nature. In addition protein-lipid interactions were able to be imaged (212).

In another example dymystroyolphosphatidylethanolamine (DMPE) bilayers produced by Langmuir-Blodgett deposition onto mica were successfully imaged under water at room temperature. The lattice structure of DMPE was visualised with sufficient resolution that the location of individual headgroups could be determined. The headgroups of the lipids were similarly found to be relatively ordered into a hexagonal type arrangement. The sizes of the headgroup areas were also found to correspond to those estimated using other techniques such as x-ray diffraction (209).

More recently 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (diC15-PC) headgroups were imaged at room temperature in bilayers formed using a vesicle spreading technique. However, it was discovered that when using Tapping Mode imaging, the lipid headgroups did not show the same ordered matrix as found for DMPE. Indeed it was shown that only when using low-force Contact Mode imaging, that an ordered matrix was formed after repeated scans (218). This data suggests that the actual native arrangement of the lipids in a bilayer is random and disordered rather than a hexagonal type matrix and that this matrix is purely an artifact of the scanning probe. However, the fact that the overall headgroup areas match that estimated by other techniques means that the actual headgroup images are realistic in their packing requirement in terms of area. Hence, it has been suggested that low force contact mode is appropriate for determining the headgroup areas and hence packing of lipid in lipid bilayers. In fact the artificial ordering of the lipids will enhance the quality of imaging.

It is expected that any of the terpenoids which affect the order, permeability or stability of lipid bilayers would also affect the lipid headgroup spacing. Changes in such headgroup area should also provide some information on the localisation of the terpenes within the bilayer since 1) those compounds residing in the space between the two monolayers should only change the bilayer thickness and not change the headgroup area significantly, 2) those compounds localised at the glycerol backbone
or aligned with the lipid acyl chains would be expected to increase the headgroup area, 3) those compounds which were localised at the phospholipid headgroup would tend to decrease the headgroup area (261).

In this study the physical stability and lipid packing of dipalmitoylphosphatidylcholine (DPPC) supported lipid bilayers was monitored using AFM before and after treatment with oxygenated monoterpenes. This was achieved by monitoring the gross physical degradation of the bilayer structure and the effects of terpenes on the DPPC headgroup areas.

5.2.4 Results and Discussion

5.2.4.1 Imaging DPPC Bilayers

Unilamellar DPPC bilayers prepared through the vesicle fusion technique were initially imaged using contact mode. However, it was immediately evident that after repeated scans the AFM probe tip was damaging the prepared bilayers (data not shown). To avoid such damage further scans were then performed in Tapping Mode so as to reduce the actual contact time involved between the tip and bilayer. Unfortunately after repeated scans the AFM probe tip was still found to degrade the bilayer structure over the time period required for monitoring the effects of terpenes on the bilayers. Since this was not adequate, force minimisation was performed before each sample was imaged. This treatment was found to be successful in that no significant changes to the bilayers, after repeated scanning for up to 40 minutes (approx 20 scans) occurred. The maximum duration for any one experiment never exceeded this limit. To confirm the stability for the bilayer itself four scans of each sample were run before the addition of any terpenes to the system.

Initial imaging of DPPC bilayers showed large areas (up to 15×15μm²) of flat and relatively featureless surfaces. The formation of a unilamellar bilayer was confirmed for each sample before the beginning of each experiment. This was achieved by measuring the bilayer thickness through natural defects in the bilayers which are common in such supported lipid bilayer preparations (210,218,220). Those samples accepted as being unilamellar bilayers were those having a thickness between 5-8nm (see Figure 5.29) which is the estimated thickness of DPPC and similar PC bilayers (218,220).
Figure 5.29 – Cross section of DPPC supported lipid bilayer showing depth of bilayer as approximately 5nm. Holes varied in depth from 5-8 nm depending on sample.
5.2.4.2 Effects of Terpenes on DPPC Bilayer Structural Stability

Since the terpenes have very similar structures it was postulated that their effects on supported lipid bilayers would be the same. However, our results indicate that each of the terpenes tested showed slightly differing membrane effects. In the case of carvone (see figures 5.30-5.32) it can be clearly seen that the number and size of holes (dark areas) dramatically increases as carvone concentration is increased. This data suggested that not only are the natural defects in the untreated bilayer increased in size but new defects in the form of holes are also formed. The ability of carvone to create breaches in the bilayer would be expected to affect membrane permeability and the overall stability as size and number of the holes increases. However, the ability of carvone to also increase the size of natural membrane defects may also be important in its membrane activity as it would be expected that slight deformations in the bilayer structure would exist directly surrounding membrane imbedded proteins. If such areas are part of the site of action of carvone then not only would permeability and structural stability be expected to be compromised but also the function of these proteins.

It should be noted that each of these images is scanned from bottom to top as those scans from top to bottom occurred during the addition of increasing terpene into the system. This slight flow (1ml in 1 minute) of new solution into the system creates enough disturbance to reduce image quality. Hence each of these images shown are approximately 1.2 minutes apart at the same point on the image. Therefore the time scale involved for carvone to significantly affect the DPPC bilayer structure is around 15 minutes which is well within the time frame required for microbial cell death (see chapter 6) and K⁺ leakage from E. coli (see section 5.3.1). It should also be noted that while the number of holes rapidly increases at a carvone concentration of as low as 81 ppm (see Figure 5.30) the main increase in hole size occurs at around 325 ppm.

Carveol, the alcohol analogue of carvone, was also found to affect the stability of the supported lipid bilayer. Unlike carvone however, carveol did not induce formation of new holes in the bilayer, though it did again increase the diameters of existing holes. Instead carveol seemed to attack the membrane over its entire surface causing loss of entire bilayer in places and the top lipid layer in others (see Figure 5.33). This difference in effect may be a result of the carveol actually dissolving the lipid bilayer in a detergent type manner. This theory is supported by the fact that this
Figure 5.30 – DPPC bilayer untreated (A) and exposed to carvone at 81 ppm (B)
Figure 5.31 – DPPC bilayer exposed to carvone at; 160 ppm (C) and 325 ppm (D)
Figure 5.32 – DPPC bilayer exposed to carvone at 490 ppm (E) and 820 ppm (F)
Figure 5.33 – DPPC bilayer untreated (A); exposed to carveol at 838 ppm for 2 minutes (B); and exposed to carveol at 838 ppm for 4 minutes (C).
effect was not seen to occur to any great extent until higher levels of carveol (ca. 840 ppm) were used and left exposed to the bilayer for an extended period of time. After the last image shown in Figure 5.33 no further images were able to be obtained.

Carvacrol, the phenol analogue of carveol, was also found to have a similar gross membrane effect to carveol. However, this effect was so dramatic that the entire bilayer was seen to completely relax within a 30 second period, thus coating the AFM tip and preventing further imaging (data not shown).

The similarity of the action of carveol and carvacrol was initially thought to be linked with the OH functional group. However, perilla alcohol (see Figure 5.34) was found to increase the number of holes in the membrane in similar fashion to that of carvone. The main difference between carvone and perilla alcohol was that perilla alcohol produced fewer and smaller holes than carvone. It is not known if this is a result of the lower maximum concentration of perilla alcohol able to be used (maximum concentration was restricted by terpene water solubility). Existing holes were again found to increase in size and lipid defects were also found to be the origin of the formation of many larger holes. It should also be noted that the largest increase in size and number of holes occurred at ca. 500 ppm perilla alcohol, which is comparable to that of carvone. Hence it is suggested that while perilla alcohol does not affect the membrane as dramatically it does have a similar membrane perturbing action as carvone.

Interestingly, perilla aldehyde, which is also antimicrobially active found to cause no significant visible structural damage to the supported lipid bilayer. This may be attributed to the lower solubility, hence the lower maximum level of this compound able to be applied to the lipid bilayer. However, even at the low concentration of 220 ppm perilla aldehyde was still found to affect lipid headgroup packing areas (see section 5.2.4.3). This data suggests therefore that at least at low concentrations antimicrobial activity of perilla aldehyde is not associated with perturbing the physical structure of the lipid bilayer.
Figure 5.34 – DPPC bilayer untreated (A); exposed to perilla alcohol at 98 ppm (B) and 500 ppm (C)
5.2.4.3 Lipid Headgroup Packing Area

Similar force minimisation was used for imaging of phospholipid headgroups to that performed in Contact Mode, as the image quality able to be obtained using Tapping Mode was not adequate. Low-force Contact Mode did not seem to affect the bilayer within the much shorter scan times (max of 5 minutes at anyone area) and much smaller scan areas.

Successful headgroup imaging was confirmed using untreated bilayers by visual presence of individual raised areas on the images and by determining the area of each headgroup. Headgroup areas for the DPPC bilayers were found to be 47.9Å² (see Table 5.5) which is comparable with that previously determined for DPPC (212) and other similar PC’s (218).

<table>
<thead>
<tr>
<th>DPPC Treated with;</th>
<th>Concentration (ppm)</th>
<th>Lipid Headgroup Area (Å²)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-</td>
<td>47.9</td>
<td>3.2</td>
</tr>
<tr>
<td>Perilla aldehyde</td>
<td>220</td>
<td>29.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Perilla alcohol</td>
<td>500</td>
<td>40.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Carvone</td>
<td>820</td>
<td>35.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Carveol</td>
<td>840</td>
<td>30.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>400</td>
<td>32.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

5.2.4.3a Effects of Terpenoids on Lipid Packing

Imaging of lipid headgroups was undertaken to determine the effect of terpenes on the packing of lipids in the bilayer. While imaging was successful (Figure 5.35) and headgroup areas were measured (Table 5.5) it should be noted that the actual packing lattice is a known artifact of the AFM tip continuously moving across the lipid bilayer surface (218). Despite this the tightness of the lipids packing should be a indicator of the physical state of the bilayer.

The results in Table 5.5 show that the terpenes (even perilla aldehyde) decreased the headgroup packing area. It has been suggested that compounds which localise at the phospholipid headgroup will indeed decrease lipid packing area (261). Therefore this result confirms the inferences made from Tc profiles that the oxygenated terpenes studied here localise around the lipid headgroups (see section
5.1.3.3). However, this reduction of lipid headgroup area creates an apparent contradiction, since a decrease in lipid headgroup area should result in a more densely packed bilayer. This alone would be expected to reduce permeability, but the decrease in headgroup area will also increase size of existing holes and possibly cause new holes to form (as seen in larger scans) which will obviously increase total membrane permeability.

However, this does not explain the result obtained for perilla aldehyde since it was found to reduce the DPPC headgroup area to the greatest extent. Further studies are required therefore to ascertain how the terpenes reduce headgroup packing areas so that this information may be linked more conclusively with structural stability data.

Figure 5.35 – Image of DPPC headgroups in water using force minimised Contact Mode.
5.2.5 Outcomes Part B: Membrane Stability

AFM images of supported lipid bilayers have shown that antimicrobially active terpenoids are able to cause significant bilayer disruption in the form of lesions and/or changes in lipid packing density. These types of structural damage and changes to lipid packing would be expected to affect functioning of a microbial membrane by increasing small ion permeability and affecting membrane bound enzyme function. This may lead to cell lysis in the long term. Decrease in lipid packing area, as measured by imaging of phospholipid headgroups, confirms suggestions that the oxygenated terpenes studied do localise at the phospholipid headgroups. However, reduced lipid packing area does not explain the effects of the terpenes on lipid stability and further investigation is required.
5.3 Introduction Part C: Effects on Membrane Permeability

As discussed in previous sections, the accumulation of various solvents has an effect on the $T_C$ of lipid bilayers and by inference on biological membranes. Studies with both liposomes and intact cells have shown repeatedly that membrane permeability is linked to $T_C$ (148,152), hence anything affecting $T_C$ will also influence permeability. For example disturbance of the optimal lipid ordering caused by the incorporation of lipids with a reduced chain length results in an increased permeability of a membrane (148). An increase in permeability has also been seen upon accumulation of membrane disordering solvents like ethanol, narcotics, antibiotics such as nisin and aromatics (148,308).

Recently it has been shown that the toxicity of cyclic aromatic hydrocarbons for microorganisms is caused by an increase in the membrane permeability induced by these hydrocarbons. Accumulation of various cyclic hydrocarbons, including β-pinene, was found to enhance the proton permeability of whole cell and of liposomes derived from *E. coli* phospholipids. As a result of this increase in proton permeability the proton motive force of the cell was significantly decreased and in some instances completely dissipated. For each of the hydrocarbons tested a maximum increase in the permeability of the membrane was observed at a membrane concentration of approximately 1 solvent molecule per 2 lipid molecules (92,148).

Terpenes have also been noted recently for their permeability enhancing effects on human skin and model stratum corneum membranes (see section 5.1.2.3c). This has been directly related to their effects on lipid bilayer packing and/or lipid bilayer entropy (84,86,87,300). The fungicidal activity of cinnamaldehyde against *Saccharomyces cerevisiae* has also been attributed to its membrane permeabilising effect (309). Even more recently tea tree oil, the main component of which is the terpene alcohol terpinen-4-ol, were found to stimulate leakage of intracellular $K^+$ from *E. coli* cells. This $K^+$ leakage shows the effects of this oil on membrane permeability. $K^+$ leakage was found to be greater for exponential growth phase cell than stationary phase cells. This has been attributed to changes in the inner and outer membrane as cells enter the stationary phase (79). The fact that tea tree oil, whose major active constituents are oxygenated terpenes, causes $K^+$ leakage in *E. coli* suggests that
individual terpenoids may also affect K⁺ leakage in this organism, given that they also seem to act at the site of the membrane.

In this study seven oxygenated terpenes, of known antimicrobial activity against *E. coli* AG100 (see chapter 3), were selected and their effect on *E. coli* AG 100 membrane permeability was monitored by determining levels of K⁺ leakage.

**5.3.1 Results and Discussion**

As can be seen in Table 5.6, of the seven terpenes tested, only five showed a greater than 10% increase in K⁺ leakage over the period of 60 minutes. Table 5.6 also shows the MIC values of these compounds against *E. coli* and it is immediately apparent that the five most active compounds are also those which cause the greatest K⁺ leakage. This data suggests therefore that the antimicrobial activity of these compounds arises in part from their ability to increase membrane permeability to small ions such as K⁺ which will in turn act to de-energise the cell by removing the electric potential across the lipid bilayer.

It is also apparent from this data that significant K⁺ leakage is found to occur at sub MIC levels. This suggests that although the terpenes are able to increase permeability of the membrane to K⁺ the organism must still be able to recover or in some way compensate for this. Such a process must take effect sometime beyond 1 hour but within the 24 hours of the MIC test. Higher levels of terpene were not able to be tested due to their limited water solubility and detrimental effects oil water emulsions will have on an ion selective electrode.

In order to investigate structure function relationships several terpenes were compared. For example α-terpineol and terpinen-4-ol, structural analogues differing only in the position of the OH functional group, were examined. Figure 5.36 shows that both these compounds have a very different effect on the rate of K⁺ leakage at 1000ppm. In addition when these two compounds were compared at 500ppm α-terpineol was found to cause negligible leakage while terpinen-4-ol caused greater leakage. This indicates that terpinen-4-ol has a greater capacity to induce K⁺ leakage from *E. coli* cells and will initiate K⁺ leakage at lower concentrations than α-terpineol. This may be crucial to terpinen-4-ol activity against an organism such as *P. aeruginosa* whose impermeable outer membrane will reduce the amount of these
terpenes able to access the cytoplasmic membrane (see section chapter 6 for more details).

Table 5.6 – Percentage leakage of potassium ions from E. coli (AG100) cells over a period of 60 minutes (expressed as percentage of potassium ions of the total potassium ion content of the cells).

<table>
<thead>
<tr>
<th>Terpene</th>
<th>Concentration (ppm)</th>
<th>MIC Against E. coli (AG100) (ppm)</th>
<th>K⁺ leakage after 60 minutes (% of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpinen-4-ol</td>
<td>1000</td>
<td>1900</td>
<td>73.6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>1000</td>
<td>1900</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Carveol</td>
<td>1000</td>
<td>1500</td>
<td>32.0</td>
</tr>
<tr>
<td>Myrtenol</td>
<td>700</td>
<td>950</td>
<td>30.8</td>
</tr>
<tr>
<td>Carvone</td>
<td>800</td>
<td>1900</td>
<td>30.3</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>800</td>
<td>9100</td>
<td>11.3</td>
</tr>
<tr>
<td>Menth-6-ene-2,8-diol</td>
<td>1000</td>
<td>9500</td>
<td>1.5</td>
</tr>
</tbody>
</table>

In contrast comparison of carvone and its alcohol analogue carveol showed only slight effects on K⁺ leakage (Figure 5.37), the only difference being in the apparent ten minutes K⁺ leakage lag time when exposed to carveol (1000 ppm). After this point however there was a rapid increase in the rate of K⁺ leakage which stayed consistent up to 60 minutes and was almost identical to that of carvone. In contrast E. coli cells exposed to carvone, even at the slightly lower concentration of 700 ppm, immediately started to leak K⁺ at an increased rate that remained fairly constant up to 60 minutes. This data suggests that carvone is able to more effectively permeabilise the E. coli membrane to K⁺ ions than its counterpart carveol. This may be a direct result of the ability of carvone to form holes within lipid bilayers even at low concentrations as opposed to carveol which tends to act more in a detergent like fashion and requiring a greater amount of time to take effect (see section 5.2.4.2). It should be noted that the time taken (10-15 minutes) for a visible effect of carveol on DPPC bilayers is comparable with the initial 10 minute lag time of K⁺ leakage from E. coli cells. It should also be noted that the time frame required for carveol, carvone, α-terpineol and terpinen-4-ol to induce significant K⁺ leakage from E. coli cells is comparable to the time required to induce a 1 log reduction in cell numbers of E. coli by other terpenes of comparable antimicrobial activity (see chapter 6 for details).
Figure 5.36 - Potassium ion leakage (expressed as % of total K⁺ ions in cells) from E. coli AG100 exposed to terpinen-4-ol (1000 ppm) and α-terpineol (1000 ppm)
Figure 5.37- Potassium ion leakage (expressed as % of total K⁺ ions in cells) from *E. coli* AG100 exposed to carvone (700 ppm) and carveol (1000 ppm)
In order to determine whether the membrane action of the oxygenated terpenes was restricted to p-menthane type terpenes the 2.1.1 bicyclic terpene myrtenol was also tested. Figure 5.38 shows that indeed myrtenol is also able to increase $K^+$ leakage from *E. coli*. The rate and overall level of $K^+$ leakage after 60 minutes was found to be similar to carvone. Likewise *E. coli* cells did not experience a $K^+$ leakage lag time when exposed to myrtenol. This data shows that the ability to cause $K^+$ leakage from *E. coli* cells is a general feature of oxygenated terpenes which are antimicrobiially active against this organism.

This is supported by $K^+$ leakage data obtained for menth-6-ene-2,8-diol [XIX] and 1,8-cineole [XX], two oxygenated but relatively inactive terpenoids. In the case of Menth-6-ene-2,8-diol there was no significant increase in the $K^+$ leakage from *E. coli* cells up to 60 minutes (see Figure 5.38). In the case of 1,8-cineole a small initial rapid increase in $K^+$ was found to occur however the subsequent rate of $K^+$ leakage from *E. coli* cells was found to remain similar to that of the untreated *E. coli* cells. This suggests that 1,8-cineole while inducing a rapid but small loss in $K^+$ ions from the *E. coli* cells does not actually increase the permeability of the cell membranes to $K^+$ ions. The small initial loss of $K^+$ ion from *E. coli* cell may result from an initial rebalance of osmotic pressure upon addition of the 1,8-cineole to the cells or could be a baseline correction error due to interaction of the 1,8-cineole and the ion electrode.
Figure 5.38 - Potassium ion leakage (expressed as % of total K⁺ ions in cells) from E. coli AG108 exposed 1,8-cineole (800 ppm), p-menth-6-ene-2,8-diol (1000 ppm) and myrcenol (700 ppm)
5.3.2 Outcomes Part C: Membrane Permeability

The results from K⁺ leakage experiments showed that those compounds which are antimicrobially active against *E. coli* cells tended to induce K⁺ leakage to a greater extent and at a greater rate than less active compounds. In addition, it was also found that slight structural changes in terpenes, such as between terpinen-4-ol and α-terpineol, can affect the concentration at which K⁺ leakage from *E. coli* cells is affected. Finally, the time frame in which the active terpenes cause significant leakage from *E. coli* cells is consistent with both membrane stability effects as viewed by AFM and *E. coli* cell death rates. It is therefore suggested that terpene induced K⁺ leakage from *E. coli* cells does play a role in their antimicrobial activity against this organisms.

5.4 Chapter Conclusion

The three main experiments described in this chapter were carried out in order to investigate the effects of terpenoids on membrane order, stability and permeability. These experiments have shown that terpenoids interact with artificial lipid bilayers and cell membranes. The way in which terpenes affect membranes is to reduce membrane order, in some cases compromise membrane structure and therefore stability, affect lipid bilayer packing and increase membrane small ion permeability. These effects, combined and individually, are expected to have a significant bearing on the antimicrobial activity of the terpenes.

Of equal importance is the fact that small changes in molecular structure of the terpenoids can produce very different magnitudes of these effects and therefore further study is required to understand more clearly why such structural changes lead to different membrane and antimicrobial activities.
Chapter 6
The Outer Membrane and Resistance of *P. aeruginosa* and *E. coli* to Terpenoids

6.1 Introduction

In enteric bacteria the outer membrane has been shown to provide protection from the detergent action of bile salts and degradation of digestive enzymes (110). In other Gram negative bacteria, as well as some enteric bacteria, the outer membrane has been shown to be an effective permeability barrier to many antibiotics that are otherwise effective against Gram positive bacteria (110,112). Recently it has also been shown that the outer membrane of *P. aeruginosa* NCTC 6749 significantly contributes to its resistance to tea tree oil and several of its components (310). Hence, the impermeability of the outer membrane of Gram negative bacteria is considered crucial for their overall resistance. Even where the diffusion of such antibiotics is merely slowed by the presence of the outer membrane the bacteria can then inactivate the small amount of penetrating antibiotic rather than try to inactivate the almost infinite amount of antibiotic present in the medium (110,112). The role of the outer membrane as a permeability barrier to substances such as antibiotics can be studied by the use of a membrane permeabilising agent. Many different compounds have been studied for their permeabilising action on the outer membrane and these are covered in detail in various reviews (112,127,129,311).

Polymyxin B nona-peptide (PMBN), a polymyxin derivative which lacks the fatty acid tail and terminal diaminobutyric acid, is one such outer membrane permeabilising agent (312). PMBN when used at low concentrations has been shown to permeabilise the outer membrane of Gram negative organisms without causing cell death (311). The molecular mechanism of this permeabilising activity is suspected to be similar to its parent antibiotic but without its destructive nature since it is known that PMBN interacts with LPS on the surface of the outer membrane and expands the surface area of the outer membrane as indicated by characteristic protrusions seen by
electron microscopy (313). It is also known that the permeabilising action of PMBN does not result in the release of LPS (313) or periplasmic proteins (314).

PMBN has been shown to sensitize Gram negative bacteria to hydrophobic probes (314) and normal human serum (315) and some hydrophobic antibiotics (316). Specifically it has also been shown to sensitize E. coli to serum complement (317) and Salmonella typhimurium and E. coli to hydrophobic antibiotics (315). However PMBN did not increase sensitivity to hydrophilic antibiotics such as ampicillin which are believed to traverse the outer membrane by porin channels (312).

It is uncertain how monoterpenes, which are generally considered to be insoluble in water but actually have solubility ranging from 1-7000 ppm (318-320) (Table 3.1), enter the Gram negative cells. It is possible that the more water soluble terpenes enter via the hydrophilic pathway while those less soluble would enter via the hydrophobic pathway (see section 1.3.1.1c). However, increased solubility does not always lead to increased antimicrobial activity, α-terpineol and terpinen-4-ol are one example (see Table 3.1). Hence, it is also possible that terpenoids are able to enter via the self promoting pathway, possibly by complexing with divalent metal cations such as Ca$^{2+}$ and Mg$^{2+}$ thus destroying the LPS cross bridging and permeabilising the outer membrane (see section 1.3.1.1c).

Investigations into the perception of hot and cold have shown that compounds such as EDTA (321) and l-menthol (322-325) create a cooling effect through their prevention of calcium ions from penetrating nerve-terminal membranes. In both cases this calcium ion blocking is attributed to the calcium complexing ability of these molecules. Hence it is possible that terpenes such as l-menthol are able to permeate the outer membrane by the self promoting pathway.

In this set of experiments pairs of structurally similar monoterpenes that belong to different antimicrobial activity groups were examined for their ability to kill the Gram negative organisms P. aeruginosa and E. coli with and without the presence of the outer membrane permeabalisng agent PMBN. The role of the outer membrane in the resistance of these two organisms to certain terpenoids is discussed. The possibility that outer membrane permeability of these organisms to terpenes was related to their ability to complex with calcium ion was also investigated.
6.2 Results and Discussion

6.2.1 Outer Membrane and Resistance

To examine the role of the outer membrane in resistance to terpenoids and how this relates to terpenoid structure and molecular properties several pairs of terpenoids were selected. These pairs of compounds were selected because they showed very different antimicrobial activity against either *P. aeruginosa* or *E. coli* yet were similar in structure. The structural difference highlighted by use of these particular pairs were; positioning of the functional group (terpinen-4-ol [I]/α-terpineol [II]); level of ring saturation (carvone [III]/dihydrocarvone [IV]); type of functional group present (perilla alcohol[V]/perilla aldehyde [VI]) and level of chain saturation in an acyclic terpenoid (geraniol [VII]/citronellol [VIII]). Limonene [IX] and geranyl acetate [X] were also examined to determine the role of the outer membrane of both *E. coli* and *P. aeruginosa* against terpene hydrocarbon and acetates.
Figures 6.1-6.3 show the kill rates of α-terpineol, terpinen-4-ol, carvone, dihydrocarvone, perrilla alcohol and perilla aldehyde against *P. aeruginosa* at the MIC of the more active terpene of each pair. It can been seen from Figure 6.1 that the overall number of cells killed and initial death rate caused by α-terpineol is less than that of terpinen-4-ol suggesting that terpinen-4-ol has a greater killing capacity and possibly greater outer membrane permeability.

When cells were pre-treated with PMBN the initial rate and overall numbers of cells killed was found to increase for both compounds. The increase in overall numbers of cells killed by α-terpineol, as a result of the effective removal of the *P. aeruginosa* outer membrane as a permeability barrier, would suggest that the outer membrane of this organism is responsible for providing some resistance for *P. aeruginosa*. This resistance is still present though less effective against terpinen-4-ol. It should also be noted that the initial rate of cell death was found to increase as well as total numbers of cells killed. Since the initial rate of cell death must be related to the rate at with a compound can reach its active site(s) in or on the organism this data suggests that the initial rate of contact of α-terpineol and terpinen-4-ol with their active site(s) is also affected by the outer membrane of *P. aeruginosa*.

Figures 6.2 and 6.3 show a similar situation for the carvone/dihydrocarvone and perilla aldehyde/perilla alcohol pairs. In contrast to α-terpineol and terpinen-4-ol, whose killing action began immediately, carvone, dihydrocarvone, perrilla alcohol and perilla aldehyde were found to be ineffective against *P. aeruginosa* over the first five minutes. Pretreatment of the cells with PMBN increased the initial killing rate of cells for each of the compounds showing that this delay in cell death is directly attributable
Figure 6.1 - Averaged rates of *Ps. aeruginosa* (NCTC 6749) cell death, treated with PMBN (10ug/mL) and untreated, when exposed to terpinen-4-ol (0.7% v/v) and α-terpineol (0.7% v/v). Error bars denote standard deviation.
Figure 6.2 - Averaged rates of *P. aeruginosa* (NCTC 6749) cell death, treated with PMBN (10ug/mL) and untreated, when exposed to carvone (0.4% v/v) and dihydrocarvone (0.4% v/v). Error bars show SD.
Figure 6.3 - Averaged rates of *P. aeruginosa* (NCTC 6749) cell death, treated with PMBN (10µg/mL) and untreated, when exposed to perilla alcohol (0.3% v/v) and perilla aldehyde (0.3% v/v). Error bars show SD.
to the outer membrane. These results are not unusual since it has been previously reported that terpene aldehydes and ketones are generally less active than their alcohol counterparts (6) (see Table 3.1) and this is supported by the slower overall rate of cell death when no PMBN was added.

The structural differences between the pairs of compounds tested does lead to subtle differences in several molecular properties. For example, terpinen-4-ol has a lower surface area and polarity than α-terpineol yet a larger H-bond donor capacity (see Appendix I). Differences in properties such as hydrogen bond donor capacity, molecular volume, molecular surface area and hydrophilic/lipophilic balance have been associated with activity against *P. aeruginosa* (see chapter 4). These subtle differences in molecular properties must therefore be associated with how effectively the outer membrane of *P. aeruginosa* can protect the organism from the killing effect of α-terpineol and terpinen-4-ol. In physical terms the role these molecular properties play is unclear.

Geraniol and citronellol differed from the compounds previously discussed in that they are acyclic monoterpenes whose MIC values differ (see chapters 3 and 4) against *E. coli*. When cells were pre-treated with PMBN (Figure 6.4) the kill rates caused by both compounds was found to increase, but with citronellol still having a slower killing rate than geraniol. This suggests that the overall killing capacity of citronellol is less than that of geraniol. However, the fact that the cell death rate caused by geraniol is increased by the PMBN pretreatment shows that geraniol is still slightly hindered by the outer membrane of *E. coli*. In the absence of PMBN, citronellol was found to have no significant effect on cell numbers yet, with addition of PMBN, cell numbers were reduced to below detection limits. This indicates that the outer membrane of *E. coli* is providing effective resistance to the action of this compound.
Figure 6.4 - Averaged rates of \textit{E. coli} (AG100) cell death, treated with PMBN (10\,ug/mL) and untreated, when exposed to geraniol (0.2\% v/v) and citronellol (0.2\% v/v). Error bars show SD.
Geranyl acetate and limonene, which have been previously (see chapters 3 and 4) shown to have MIC values of >2% v/v against *E. coli* and *P. aeruginosa*, were found to have no significant killing capacity over two hours (Figures 6.5-6.8) when used alone against these organisms. However, both types of cells when pretreated with PMBN were susceptible to geranyl acetate with *E. coli* cells more so than *P. aeruginosa*. It is interesting to note that *E. coli* cells died at almost a constant rate after an initial delay whilst *P. aeruginosa* cells began to die immediately but continued to die at a slower rate.

Similarly, limonene, when used with PMBN, was found to kill *E. coli* and *P. aeruginosa*, with *E. coli* cells being the more susceptible. These results show that again the outer membrane of these two organisms is important to their resistance to the action of terpenes even up to levels as high as 2% v/v.

In chapter 3 it has been shown that terpene hydrocarbons and acetates (see Table 3.1) show little or no activity against bacteria or yeast. The inactivity of these compounds has been associated with their low water solubility, log $K_{ow}$ and hydrogen bonding capacity (see chapter 4). The fact that these properties have been associated with activity is not surprising at least for Gram negative organisms since hydrophobic molecules must enter the cell via the inefficient hydrophobic pathway through the outer membrane. This is corroborated by the fact that PMBN increases the sensitivity of *P. aeruginosa* and *E. coli* to limonene and geranyl acetate as has been seen for hydrophobic antibiotics (316).

For the remaining oxygenated monoterpenes used in this study it is less certain which pathway they will take to enter the cell since they are considerably more soluble (ca. 220-1800 ppm) (318) (see Table 3.1) than the hydrocarbon and acetate. The kill rate experiments have shown that these monoterpenes do show activity alone and therefore these compounds may be able to utilize porins (the hydrophilic pathway) to some extent. However, the fact that PMBN significantly enhances the activity of both the more active and less active compounds from each pair suggests that these compounds do not pass with complete freedom through porins. If they did so then PMBN would not be expected to enhance their activity significantly as is seen for some antibiotics such as ampicillin which are able to effectively utilise porin channels (312).
Figure 6.5 - Averaged rates of *E. coli* (AG100) cell death, treated with PMBN (10µg/mL) and untreated, when exposed to geranyl acetate (2% v/v). Error bars show SD.
Figure 6.6 - Averaged rates of *P. aeruginosa* (NCTC 6749) cell death, treated with PMBN (10ug/mL) and untreated, when exposed to geranyl acetate (2% v/v). Error bars show SD.
Figure 6.7 - Averaged rates of *E. coli* (AG100) cell death, treated with PMBN (10μg/mL) and untreated, when exposed to (-)-limonene (2% v/v). Error bars show SD.
Figure 6.8 - Averaged rates of *P. aeruginosa* (NCTC 6749) cell death, treated with PMBN (10μg/mL) and untreated, when exposed to (-)-limonene (2% v/v). Error bars show SD.
Since the structures of the terpenes investigated are very similar, small changes in molecular properties obviously affect their permeation through the outer membrane. However it is difficult to imagine how these differences would significantly affect their passage through porins. Hence calcium complexing of these terpenes was also determined to ascertain whether this could account for the increased outer membrane permeability since effective calcium complexing agents would be able to pass through the outer membrane by the self promoting pathway. This could possibly account for the difference in the membrane permeability to some terpenes and not others.

6.2.2 Calcium Ion Complexing

Figure 6.9 shows the relative levels of \( \text{Ca}^{2+} \) complexed by the terpenes examined in section 6.2.1. It is immediately apparent from this graph that each of the terpenes tested does complex in a measurable amount with \( \text{Ca}^{2+} \).

It is also clear that the acyclic terpenes are able to complex more \( \text{Ca}^{2+} \) ions per terpene molecule than the \( p \)-menthane terpenes. This may be explained by the greater relative flexibility of acyclic skeletons when compared to \( p \)-menthane skeletons. This suggestion is supported by the reduction in the level of complexed \( \text{Ca}^{2+} \) achieved by geraniol which, having an additional double bond in its main carbon chain, would tend to leave it less flexible than its counterpart citronellol.

The results for citronellol and geraniol also show that in this case greater \( \text{Ca}^{2+} \) complexing may actually be detrimental to activity against \( E. \ coli \) since citronellol is relatively inactive against this organism. By inference then, greater \( \text{Ca}^{2+} \) complexing may also be detrimental to the permeability of this compound through the outer membrane of \( E. \ coli \). It could be envisaged that the increased permeability (hence antimicrobial activity) of geraniol across the \( E. \ coli \) outer membrane may result from a combination of increased levels travelling through porins (resulting from its greater water solubility) and self permeation via calcium complexing. Citronellol on the other hand being much less soluble may rely more heavily on self permeation alone. While citronellol may have a greater capacity to self permeate, the water soluble reservoir available for further free interaction with the inner membrane will be much smaller than for geraniol. In addition the nature of the complexes for both compounds is unknown. The greater flexibility of the citronellol may induce a stronger complex
with this compound and render it inactive as an antimicrobial agent. On the other hand neither compound may significantly complex calcium ions in vivo since a number of important factors are unknown, such as, the affinity of calcium ions to the outer membrane, steric hindrances due to LPS molecules and the strength of the complex with either terpene.

In the case of the p-menthane terpenes, values between structurally similar pairs vary. In the first case carvone and dihydrocarvone seem to show both the lowest Ca\(^{2+}\) complexing and no significant difference between each other. This would suggest that the difference in *P. aeruginosa* outer membrane permeability of these two compounds is not related to their Ca\(^{2+}\) complexing. This data suggests that in the case of carvone and dihydrocarvone outer membrane permeability differences possibly lie in their transport through porins or the hydrophobic pathway. In addition the low levels of Ca\(^{2+}\) complexed by the terpenes when compared to acyclic terpenes suggests they may be less capable of self permeation.

Likewise \(\alpha\)-terpineol and terpinen-4-ol do not show a very substantial difference in Ca\(^{2+}\) complexing. This suggests that the difference in the outer membrane permeability of these compounds is not related significantly to their Ca\(^{2+}\) complexing. It should be noted however that these two compounds may be more capable of utilizing the self permeation pathway across the outer membrane as seen by their significantly higher complexing of Ca\(^{2+}\).

In contrast perilla alcohol was found to complex more Ca\(^{2+}\) than its counterpart perilla aldehyde. This data suggests that in this case increased outer membrane permeability and increased antimicrobial activity against *P. aeruginosa* could be related to Ca\(^{2+}\) complexing. Hence it is possible that the ability of perilla alcohol to complex Ca\(^{2+}\) enables it to self permeate across the outer membrane to a greater extent than perilla aldehyde. It is also interesting to note that perilla alcohol complexes Ca\(^{2+}\) to the greatest extent of all of the p-menthane terpenes. However the fact that perilla aldehyde is no more active overall than terpinen-4-ol and/or carvone suggests that the self permeation pathway is possibly less significant than transport via the hydrophilic pathway (i.e. transport via porins).
Figure 6.9 - Relative amounts of Ca^{2+} ions complexed by various terpenes. Error bars represent standard deviation.
Clearly further work is required to ascertain the importance of each of the transport pathways across outer membranes for terpenes. In addition this data shows only the level of Ca\(^{2+}\) complexed by terpene and gives no indication as to the strength of these complexes since formation constants require the complex stoichiometry to be known. While this has been achieved for various other organic molecule/Ca\(^{2+}\) complexes (326-333) this was not able to be achieved for the terpenes due to time constraints of this project. Data on the strength and stability of terpene/Ca\(^{2+}\) complexes may shed more light onto this problem. The reader should also be reminded that this data is derived from simple solutions and does not take into account the possible steric hindrances and binding capacity of the LPS molecules of the organism.

6.3 Conclusion

In summary, this study has shown that the outer membrane is important for the resistance of *P. aeruginosa* and *E. coli* to some monoterpenes. However, it has also shown that active compounds are not completely uninhibited by the outer membrane. The degree to which a terpenoid’s antimicrobial activity is inhibited by the outer membrane seems dependent on small changes in molecular structure and related molecular properties. Differences in Ca\(^{2+}\) complexing were also investigated and showed that the terpenes can complex Ca\(^{2+}\) to measurable levels. However, the relationship of Ca\(^{2+}\) complexing with antimicrobial activity seems to differ depending on the organisms and compound in question. Thus the pathway by which the terpenes pass throughout the outer membrane is unclear.
References


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98. Troy, F. (1979), The Chemistry and Biosynthesis of Selected Bacterial Capsular Polymers. Annual Reviews in Microbiology, 33, 519-560.


102. Sutherland, I. (1985), Biosynthesis and Composition of Gram-Negative Bacterial Extracellular and Wall Polysaccharides. Annual Reviews Microbiology, 39, 243-270.


_The FASEB Journal_, 3, 1833-1842.

198. Cullis, P. and De Kruijff, B. (1978), Polymorphic Phase Behaviour of Lipid 
Mixtures as Detected by $^{31}$P NMR: Evidence that Cholesterol may Destabilize 
Bilayer Structure in Membrane Systems containing Phosphatidylethanolamine. 
_Biochimica et Biophysica Acta_, 507, 207-218.

by Low Levels of Diglycerides and Alkanes: An NMR, Calorimetric, and X-
ray Diffraction Study. _Biochemistry_, 28, 5010-5019.

Formation in Lecithin-Alkane-Water Systems with Different Acyl Chain 
Unsaturation and Alkane Length. _Biochemistry_, 28, 1323-1329.

201. Cullis, P. and De Kruijff, B. (1978), The Polymorphic Phase Behaviour of 
Phosphatidylethanolamines of Natural and Synthetic Origin: A $^{31}$P NMR 
Study. _Biochimica et Biophysica Acta_, 513, 31-42.

202. Finean, J. and Millington, P. (1955), Low-Angle X-Ray Diffraction Study of 
the Polymorphic Forms of Synthetic $\alpha$-$\beta$ and $\alpha$-$\alpha$-Kephalins and $\alpha$-$\beta$-

203. Papahadjopoulos, D. and Miller, N. (1967), Phospholipid Model Membranes - 
I. Structural Characteristics of Hydrated Liquid Crystals. _Biochimica et 
Biophysica Acta_, 135, 624-638.

204. Engelman, D. (1971), Lipid Bilayer Structure in the Membrane of 
Mycoplasma laidlawii. _Journal of Molecular Biology_, 58, 153-165.


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287. Antunes-Madeira, M. and Madeira, V. (1990), Membrane Fluidity as Affected by the Organochlorine Insecticide DDT. *Biochimica et Biophysica Acta*, 1023, 469-474.


Chapter 7
Determination of Octanol/Water Dispersion Coefficients for Terpenoids Using Reversed Phase-HPLC

7.1 Introduction - Determination of log $K_{ow}$

The relationship of log $K_{ow}$ to various bioactivities, including to some extent the expression of antimicrobial activity versus inactivity has been previously discussed (see section 1.3.2.1b and 4.2.1). However, currently there are a large number of ways in which to determine the octanol/water partition coefficient. These include a vast array of direct and indirect experimental methods as well as theoretical estimation methods (for a review see Lambert (139)). For the purpose of this review only the classical, reversed-phase HPLC and calculation methods are covered, since they are the methods used for this experiment.

7.1.1 Shake Flask Method

This method is the classical method for measuring $K_{ow}$ and is still considered one of the most reliable and accurate methods in use (134,334). In this method the solute is dissolved in one phase and through agitation with the other phase becomes distributed between the two. After separation both phases are then analysed (334). Both phases normally separate under gravity alone, but are usually centrifuged to accelerate the process and to ensure complete separation (134,228). Many variants of this method are now in existence. These variants differ only in the method of equilibration (e.g. agitation, stirring, equilibration by standing) and phase sampling. In general, which-ever method of equilibration is chosen, prolonged or violent mixing is not necessary and often causes emulsion formation (140,228).

Despite the apparent simplicity of this method it does suffer from several complications aside from being labor intensive and slow. Firstly, if the method of solute detection cannot distinguish between impurities in either solvent or the solute
then both solute and solvent must be of extremely high purity. Of course if HPLC or GC are used to analyse the phases this is no longer a concern. Also, distilled water in equilibrium with the atmospheric carbon dioxide causes it to become acidic which can generate problems with acid labile compounds. Secondly, the solute of interest may not be easily dissolved in either octanol or water and thus require the addition of a modifier. Small amounts of a modifier, such as methanol, may be added without affecting the partitioning process but may enhance problems with emulsion formation (132,134). Also solutes with surfactant properties can increase emulsion formation (228). Thirdly, the solvents used in the experiment must be equilibrated with each other before use. Octanol and water are of high enough solubility in one another to affect the volumes of each phase if not pre-equilibrated. Pre-equilibration can take several days (132,134,140). Finally, if the solute has an appreciable vapor pressure, as is common with terpenoids, it may be necessary to consider the amount of vapor space in equilibration vessels (134).

7.1.2 Reversed-Phase HPLC

In this method the solute is injected onto a HPLC column and its retention time will be defined by its partitioning between the non-polar stationary phase, normally octadecylsilane (ODS), and polar mobile phase (132,136,139). These retention times are then converted to log $K_{ow}$ by means of a standard set of solutes for which shake flask log $K_{ow}$ values have already been measured (140,334).

The use of the HPLC method for the measurement of log $K_{ow}$ is growing in use (334). There are many examples in the literature of successful use of the method in determining log $K_{ow}$ for a wide range of compounds (136,335). These log $K_{ow}$ values determined using RP-HPLC have also been successfully used in the correlation with biological data in many instances. One example was given in a review by Carney (336) where the HPLC method was used successfully to correlate the bacteriostatic activity of 10 sulfonamides to the measured capacity factors of each compound. In another example given in the same review the capacity factors of 15 barbiturates were found to be directly correlated to hypnotic effect, O$_2$ uptake activity and arrbacia egg division.

Dorsey (337) also presents several examples where the HPLC method was used to correlate bioactivity with lipophilicity. The activities which correlated with
lipophilicity included protein binding of xanthine derivatives to guinea pig serum albumin, biological activity of cardiac glycosides and steroid hormones, micellar cholesterol-solubilising capacities of bile salts, antituberculous activity of dipyridylsulphides and mosquito repellant properties of amides.

In addition to direct correlations with bioactivity many examples of log K<sub>ow</sub> prediction using HPLC are given by Braumann (338). In one example log K<sub>ow</sub> of 49 ortho, meta and para-substituted toluenes, anilines, phenols, nitrobenzenes and chlorobenzenes were predicted using the HPLC technique. Various other examples are also given in the same review.

### 7.1.2.1 Theory

Since the determination of log K<sub>ow</sub> by reversed-phase HPLC is highly dependent on the retention of solutes the retention mechanisms involved in reversed-phase HPLC are extremely important.

It is now generally accepted that the mobile phase plays a dominant role in the retention process. According to the solvophobic theory the driving force for retention is the unfavorable interaction of a solute with the surrounding water molecules present in the mobile phase. This leads to a net free energy change on exclusion of the solute from the eluent to the non-polar ligands of the stationary phase. Crucial to this model is that the interaction between solute and stationary phase is very weak and non-selective (338).

In HPLC, the affinity of a solute for the stationary phase is characterised by the capacity factor (k'):

$$k' = \frac{t_R-t_0}{t_0}$$

where t<sub>R</sub> is the solute retention time and t<sub>0</sub> is the mobile phase hold time (132,137,336,338-340). Determination of t<sub>0</sub> is often obtained by using a non-retained solvent or salt (132,338).

Since retention volume is directly proportional to retention time at any given flow rate chromatographic retention can be related, via the capacity factor, to the stationary phase-mobile phase partition coefficient (P<sub>sm</sub>) as follows;
\[ k' = P_{sm} \left( \frac{V_s}{V_m} \right) \]

where \( V_s \) and \( V_m \) are volumes occupied by the stationary and mobile phases respectively (139).

The correlation of partition coefficients between various partitioning systems has been suggested by Collander who showed that the partition coefficient for one system could be related to another as follows;

\[ \log K_{ow(1)} = a \log K_{ow(2)} + b \]

where \( a \) and \( b \) are empirical constants which characterize the solvent system in question (139,228,341).

Hence, the capacity factor of a compound on a reversed phase HPLC column, which is directly related to the compounds partitioning between stationary and mobile phases, can be related to \( K_{ow} \) the following manner (132);

\[ \log K_{ow} = a \log k' + b \]

One shortcoming of this relationship derived by Collander is that when the non-aqueous phase in the two partitioning systems are significantly different a poor correlation between two systems is obtained. This occurs especially when the solute set under investigation contains molecules that can and cannot hydrogen bond. Where this occurs ‘plus’ and ‘minus’ deviants can be split into two groups and used to derive individual equations to relate partitioning systems. In most partitioning systems strong hydrogen bond donors are ‘minus’ deviants and strong hydrogen acceptors are plus deviants (100,137,228,342).

7.1.2.2 Determination of \( k_w \)

Even though \( k' \) can be related to \( K_{ow} \), \( k' \) will, for a given solute and stationary phase, depend on the composition of the mobile phase mix used in elution (132). Therefore it has been suggested that \( k' \) should be determined using pure water as eluent (\( k_w \)). The advantages of doing this are that the capacity factor is independent of any organic modifier effects, it reflects polar-non-polar partitioning in a manner more
similar to shake flask measurements and it is dependant on the solute's structure and polar functionalities (337). Under most conditions, however, pure water cannot be used as the eluent due to overly long retention times and an organic modifier must be added (136,338).

$k_w$ is still able to be determined if $k'$ is determined over a range of modifier concentrations and extrapolated back to 0% modifier ($k_w$) (136,139). Experimentally it was found that $\log k'$ is related to the volume fraction ($\varphi$) of the organic modifier added to water as follows;

$$\log k' = \log k_w + c\varphi + d\varphi^2$$

where $k_w$ is the capacity factor for pure water as eluent (132,338). However the equation above is a quadratic one. This quadratic relationship can cause significant problems when extrapolating (136).

Lambert (139) discusses extrapolation to $k_w$ and reviews the data for and against such a decision. The general conclusion of the discussion is that methanol should be used if possible as the modifier at one volume fraction, above 25-30% but below 70%, and without extrapolation. This is due to the fact that most of the curvature for methanol occurs at volume fractions below 30% and above 70%. Hence extrapolated $k'$ values determined at moderate volume fractions will deviate from the true $k_w$ value and possibly give poor correlations to $K_{ow}$.

In addition a volume fraction chosen within the range specified above will have a minimal effect on the stationary phase. This is important since it has been suggested that at high modifier levels the eluent is so unlike water that it becomes fairly insensitive to hydrophobicity (139,334,338). Leo (334) outlines one example where HPLC using an eluent of high methanol content did not adequately emulate some commonly accepted properties of hydrophobicity and therefore lead to poor correlation with biological data.

Minick et al. (141) on the other hand come to a different conclusion about extrapolation, giving several examples of the problems associated with monocromatic ($k'$ determined at one organic modifier concentration) as opposed to polycromatic ($k'$ determined at several modifier concentrations) methods. For example at only one modifier concentration there is a limitation to the range of hydrophobicity able to be
measured due to overly long retention times of highly lipophilic compounds. The opposite problem can also occur for highly hydrophilic compounds. Compounds either unretained or retained too long may therefore have to be measured using different column lengths or eluent composition. This retention data must then be cross correlated with the original conditions. This first problem is also compounded by the possibility of peak inversion, which can arise when modifier volume fractions are changed. Peak inversion is where the elution order of two compounds is reversed as a result of the organic modifier volume fraction in the eluent (141).

The study by Minick et al (141) also showed that linear extrapolation when using methanol at multiple volume fractions between 20-80% yielded $k_w$ values that gave similar or better correlation to $K_{ow}$ than if a quadratic extrapolation was used. It also requires far fewer data points (338).

### 7.1.2.3 Organic Modifier Selection

Methanol is by far the most commonly used organic modifier for determining partition coefficients. There are two main reasons for this. The first is because methanol has been found to produce significantly less curvature than acetonitrile and tetrahydrofuran (136,139,141). Solvents such as acetonitrile and tetrahydrofuran both show considerable curvature even at low modifier levels and hence require many data points in order to predict $k_w$ accurately. Thus the use of methanol can minimise the errors in extrapolation to 0% modifier.

Second, methanol is the most water-like of all commonly used HPLC solvents. It is capable of hydrogen bond acceptance and donation and has a solubility parameter considerably closer to water than tetrahydrofuran or acetonitrile. This means that not only the mobile phase but also the stationary phase will be less affected when using methanol. In contrast tetrahydrofuran and acetonitrile are comparatively weak hydrogen donors and acceptors (338) and are known to adsorb to the stationary phase more strongly than methanol (139,338) which has been found to form a monolayer on the surface of ODS stationary phase, the most commonly used stationary phase in RP-HPLC (338). Furthermore acetonitrile may selectively interact with certain solutes via dipole-dipole interactions owing to the high dipole moment of acetonitrile (338). Acetonitrile and tetrahydrofuran are usually only used as modifiers where more
lipophilic compounds are being investigated as they can reduce the retention times and broaden the measurable range of partition coefficients (136).

7.1.2.4 Outliers

Inappropriate selection of organic modifier can lead to the greatest shortcoming of the HPLC method which is the occurrence of outliers. These outliers may be due to the difference in hydrogen bonding capability of the stationary phase relative to octanol. Where this occurs correlations between $k_w$ and $K_{ow}$ can be improved by separating compounds into classes according to their hydrogen bonding properties (141). Effects such as this can occur especially where free silanol groups occur on columns (136).

Silanophilic interactions with basic and polar compounds in particular can severely affect the partitioning behavior of solutes between the eluent and the stationary phases (136). Thus highly endcapped columns are recommended for the reversed-phase HPLC method (139,339,340). Alternatively the addition of a masking agent such as n-decylamine to the mobile phase may suppress the necessary interactions, as shown by Bechalany et al (136), however this did introduce other variables into the method since the masking agent can have selective effects on retention times. Masking agents such as n-decylamine can also ion pair with acidic solutes (139).

To avoid the problem of free silanol groups found in ODS columns altogether, polymeric stationary phase columns have been used. These columns, for example, octadecyl polyvinyl copolymer (ODP), are virtually devoid of free silanol groups (136). In addition, they do not contain trace metals which are associated with silica and are known to cause anomalous retention behavior. They are also stable over a wide range of pH conditions (139).

When comparing ODS with ODP columns in the measurement of octanol/water partitioning Bechalany et al (136) found that ODP was as satisfactory as the ODS phase without the need of an additional masking agent. In the same study however it was also found that acetonitrile should not be used as modifier with this column type.

Another method of avoiding specific interactions of solutes with the stationary phase of the column is to saturate the column with octanol itself and then using as the
eluent octanol saturated water. It is suggested that this method provides a much more realistic estimate of log $K_{ow}$. One limitation of this method however is that compounds of very limited solubility tend to give anomalously low log $K_{ow}$ values (339). Also Braumann (338) argues that when using water-methanol eluents the required dispersive interactions and hydrogen bonding exists in the HPLC system thus precluding the need for coating the stationary phase with octanol.

7.1.2.5 Advantages Over the Shake Flask Method

Despite the possible pitfalls with the HPLC method if care is taken it has many benefits over the shake flask method. This method offers a far more rapid way of determining $K_{ow}$ than the shake flask method, but the main benefit of this method is that quantitative analytical methods are not required as only determination of the retention times is necessary. If standard reference compounds are available and the solutes are compatible with HPLC then this method is faster and more cost effective than the shake flask method (139,342). The HPLC method also usually enables partition coefficients over a wider range than the shake flask method and requires far less sample (139,340,342).

Finally, the success of the octanol/water system as a model for the study of the behavior of bioactive compounds in biological systems has been attributed to its adequate lipophilic-hydrophilic balance brought about by the n-octyl chains, the hydrogen bonding hydroxyl groups and the relatively high water content at saturation. Reversed-phase HPLC also involves both dispersive interactions and hydrogen bonding activity when using ODS as stationary phase and methanol-water eluents.

However, octanol is an isotropic liquid so that the size and shape of the molecule are not determinants of the partition process. This is in contrast to the strong anisotropic nature of a typical biomembrane for which octanol is used as a model. Thus, because of their anisotropic nature, membranes will not be expected to behave as a bulk liquid in its discriminative power with respect to partitioning. Rather the molecular size and shape and orientation of the functional groups of membrane components will contribute to biological partitioning (338). Indeed the surface density of the lipids in bilayers has been found to affect partitioning, with increasing lipid density decreasing the partitioning into the membrane (147).
With HPLC a number of similarities exist between the mobile-phase interface and water-membrane interface which do not occur in octanol/water systems. The chemically bonded phase does not behave as a liquid but resembles much more the ordered array of the membranous hydrocarbon chains. The residual silanol groups, some of them being charged at neutral pH, and the adsorbed layer of hydrogen bonding organic modifier and co-extracted water molecules, may be expected to resemble the polar outer membrane regions.

Thus, although the contribution of these specific interactions to the partitioning process are outweighed by the hydrophobic effect, as reflected by log \( K_{ow} \) relating well to bioactivity, instances where minor structural differences in solutes play an important part in biological partitioning will be detected using HPLC as opposed to octanol/water (338).

### 7.1.3 Calculation Of Octanol/Water Partition Coefficients

One of the greatest obstacles to estimating \( K_{ow} \) is the difficulty of obtaining a reliable result (334). Although log \( K_{ow} \) has been reliably measured for over 7000 compounds this number is small compared to the number of known organic chemicals (228). Hence estimation of \( K_{ow} \) via calculation can be advantageous. The three major types of methods for calculating \( K_{ow} \) are Fragment, Atomistic and Atom/Fragment Contribution methods (334).

#### 7.1.3.1 Fragment Methods

In fragment methods the molecule is regarded as being a combination of a number of chemically recognisable and common atoms or groups of atoms. With the use of a large database of reliable experimental log \( K_{ow} \) data, contributions of fragments to the total log \( K_{ow} \) of molecules can be established.

Rekker (343) and colleagues were the first to publish a method of calculating log \( K_{ow} \) from structural fragments. They employed statistical methods to determine the average contribution to log \( K_{ow} \) of simple fragments such as C, CH, CH₂, CH₃ and OH.

Since then there have been many approaches to defining the contributions of fragments to the total log \( K_{ow} \) of the molecule. Hansch and Leo (230) adopted a constructionalist approach where log \( K_{ow} \) values for a set of small molecules was
determined accurately and then used to calculate chemical fragments from these values. That is, the basic fragments were derived from a small set of the simplest possible molecules. Using this method two important observations were made. First, the value of a fragmental contribution of a functional group depended to some extent on the nature of the chemical environment of the group (i.e., presence and position of other functional groups on the molecule). Second, the contribution of an alkyl side chain depended on both its length and degree of branching. Subsequently appropriate correction factors have been derived to correct for these effects (132).

In summary, the calculated log $K_{ow}$ for a molecule using fragment methods can be represented as follows;

$$\log K_{ow} = \Sigma a_n f_n + \Sigma b_m F_m$$

Where $a$ is the number of occurrences of fragment $f$ of type $n$ and $b$ is the number of occurrences of correction factor $F$ of type $m$ (132, 140). There are several other variants of the fragment method developed by other authors all of which are reviewed by Sangster (132).

7.1.3.2 Atomistic Methods

Rather than considering fragments, atomistic methods are based on contributions of single atoms. Again these single atom contributions depend on the local environment within the molecule. Ghose and Grippen (229) deduced contributions for 110 atom types which was updated to 120 types by Viswanadhan et al (344).

When comparing atomistic methods to fragment methods it was concluded by Mannhold et al. (345) that the Hansch and Leo fragment method generally gave more accurate results than the other methods (132). The Hansch and Leo fragment method was also shown to be accurate in calculating log $K_{ow}$ values for polychlorinated hydrocarbons, although new fragment constants, including interactive and conformational factors were required for this set of compounds (346). In general, however, all calculation methods perform better on simple rather than complicated molecules (132).
7.1.3.3 Atom/Fragment Contribution Method

In the Atom/Fragment Contribution method, log $K_{ow}$ is estimated by initially separating a molecule into distinct atom/fragments. In general, each non-hydrogen atom (e.g. carbon, nitrogen, oxygen, sulfur) in a structure is a "core" for a fragment; the exact fragment is determined by what is connected to the atom. Correction factors are then introduced based on the more complex substructures (such as specific aromatic ring substitutions) in a molecule. The coefficients for individual fragments and groups were derived by multiple regression of more than 2400 reliably measured log $K_{ow}$ values. This “reductionist” fragment constant methodology (i.e. derivation via multiple regression) differs from the “constructionist” fragment constant methodology of Hansch and Leo (230). Meylan and Howard (231) give a more complete description of this methodology and provide a comparison to other estimation methodologies.

7.1.3.4 Advantages of Calculated $K_{ow}$ Values

Calculating $K_{ow}$ should not be used as a substitute for measurement but it can quickly focus on possible errors in measurement and can indicate when other procedures may not be delivering values actually related to the octanol/water partition coefficient.

Leo (334) reported two individual studies where measured log $K_{ow}$ values were misleading. In the first example log $K_{ow}$ values of elasnin and analogues were determined using HPLC. The conclusion drawn from the HPLC data was that hydrophobicity did not account for the elastase enzyme inhibition by elasnin and analogues. However, when calculated values were applied to this data it was found that hydrophobicity was of primary importance. In the second example the log $K_{ow}$ values of N-phenylsuccinimides were obtained using the shake flask method. Again, this data was found to be misleading due to several measured results which were suspected to be erroneous. This obscured relationships between log $K_{ow}$ and nephrotoxicity of these compounds. Subsequent calculation by Leo (334) showed the error and gave results which related better with the nephrotoxicity data than the measured values. Therefore, when determining the octanol/water partition coefficients of any compounds both experimental and calculations methods should be used in combination.
In this study a reversed-phase HPLC method was used, validated against the traditional shake flask method and then compared with atomistic, fragment and atom/fragment contributions methods. The objective of this experiment was to develop a simple RP-HPLC method for analysis of log $K_{ow}$ values for terpenes, which could be used in the investigation into their antimicrobial activity. The results presented here contains possibly the largest collection of log $K_{ow}$ data for the terpenoids and is most certainly the largest internally consistent data set at present.

7.2 Results and Discussion

7.2.1 Precision of RP-HPLC Method

The log $K_{ow}$ values of 57 terpenoids, determined using HPLC, shake flask, atomistic, fragment and atom/fragment contribution methods, are shown in Table 7.1. Table 7.1 also shows the standard error for the HPLC and shake flask methods. It can be seen from this data that the standard error in the HPLC measurements lies between 0.03 and 0.08 while the standard error for the shake flask results lies between 0.01 and 0.06. Hence, the HPLC method has a comparable level of precision to the shake flask technique over the entire log $K_{ow}$ range (1.81-4.48) examined using this technique. Such consistent precision at higher log $K_{ow}$ values is advantageous when compared to other HPLC methods which use octanol treated columns, since such methods are restricted to log $K_{ow}$ values of around 3 due to problems with peak detection (347).

Piraprez et al (348) have also shown the RP-HPLC technique has consistent precision in a study of log $k_{ow}$ values of 96 aroma and flavour compounds. The main difference in the method used by Piraprez et al. (348) and our method was their addition of decylamine to the mobile phase as a masking agent to combat silanotrophic effects on thiazole derivatives, pyrazine derivatives, phenols and sulfur containing compounds which made up the majority of the compounds tested. Although no masking agent was used in our experiments, comparison of log $k_{ow}$ for compounds common to both studies showed good agreement.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Measured log $K_{ow}$</th>
<th>Calculated log $K_{ow}$</th>
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<td>Shake Flask Std. Error</td>
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<td>γ-Terpine</td>
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<td>4.36 3.13</td>
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<tr>
<td>(±)-Limonene</td>
<td>4.38 0.05</td>
<td>4.36 3.13</td>
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<td>4.38 0.05</td>
<td>4.47 3.13</td>
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<td>(±)-α-Pinene</td>
<td>4.44 0.06</td>
<td>4.34 3.13</td>
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7.2.2 RP-HPLC Method Accuracy and Comparison to Calculation Techniques

To determine the accuracy of the HPLC method and calculation techniques, shake flask results were plotted against each of the data sets. The resulting correlation coefficients showed that the HPLC method was the most closely correlated ($r^2 = 0.94$) to the shake flask data followed by the atom/fragment contribution ($r^2 = 0.87$), fragment ($r^2 = 0.80$) and atomistic ($r^2 = 0.66$) techniques.

Examination of the data in Table 7.1 shows that the log $K_{ow}$ values derived by the three calculation methods do not always match those obtained by HPLC or shake flask. Additionally, the different methods of calculation do not always give the same result. When the values from each of the calculation methods were plotted against the HPLC data it was found that the atom/fragment contribution method gave the best correlation ($r^2 = 0.76$) followed by the fragment ($r^2 = 0.61$) and then the atomistic methods ($r^2 = 0.26$).

The fact that the atomistic method had the lowest correlation with the HPLC method coupled with the fact that this method also showed poor correlations with the other two calculation methods (data not shown) indicates that the atomistic method is not appropriate for the estimation of log $K_{ow}$ values for terpenoids.

Despite its statistically significant correlation with the atom/fragment contribution method, it is important to examine where the HPLC data deviates from the calculated values. While Figure 7.1 shows that the atom/fragment contribution method has a reasonably consistent variability throughout the log $K_{ow}$ range studied, Figure 7.2 shows that this is not the case for the fragment method. It can be seen in Figure 7.2 that most of the deviation occurs with terpenes that have an alcohol, aldehyde, acetate or ketone functional group.
Figure 7.1 – Comparison of log $K_{ow}$ values measured by RP-HPLC and calculated by the atom/fragment contribution method.

Figure 7.2 – Comparison of log $K_{ow}$ values measured by RP-HPLC and calculated by the Hansch and Leo Fragment method.
Within the oxygenated compounds, aldehyde and ketone terpenoids ($r^2 = 0.66$, range = 1.86-3.78, n = 14) were found to have a better correlation with the fragment method values than alcohols ($r^2 = 0.21$, range = 1.79-3.42, n = 20). Valid correlations for acetates and hydrocarbons were unable to be determined due to clustering of the data.

The disparity between calculated and RP-HPLC values as seen in this study may be a reflection of the complicating factors that oxygenated functional groups introduce to the fragment methods predictive capability when compared with more simple hydrocarbons.

7.3 Conclusions

The reversed phase-HPLC method used in this study was found to accurately determine log $K_{ow}$ values for terpenes without any column treatment. It operated with a consistent precision over a range of log $K_{ow}$ values from 1.8-4.48 and showed good correlation with the shake flask technique. When estimation techniques were used to calculate log $K_{ow}$ values the atom/fragment contribution method was found to be the most accurate followed by the Hansch and Leo Fragment method, with the Atomistic method being the least accurate. In the case of the Hansch and Leo fragment method it was found that the estimated log $K_{ow}$ values for the hydrocarbons was more accurate than for their oxygenated counterparts.
General Conclusion

The antimicrobial activity of essential oils and their major constituents, the terpenoids, is well documented. The level of activity of these compounds has been broadly correlated with the type of functional groups present. Essential oils and terpenes are also known to have a detrimental effect on microbial membranes, a characteristic which has been linked to their antimicrobial activity. This study has confirmed and extended the hypothesis that the antimicrobial activity of terpenoids is primarily a result of their membrane disrupting nature and is structure dependent. This membrane disrupting ability has been shown in this study to be caused by the physicochemical changes that they bring about when they enter the lipid bilayer. Terpenoids were found to adversely affect the three crucial properties essential to an adequately functioning lipid bilayer; stability, order and permeability. Using Atomic force microscopy and differential scanning calorimetry techniques it has been shown that antimicrobially active terpenoids partition into model lipid bilayers or associate with the lipid water interface causing lipid bilayer disorder and in some cases physical damage to the lipid bilayer in the form of lesions or holes. Potassium ion leakage experiments using *E. coli* have also shown antimicrobially active terpenoids increased membrane permeability to small ions. While these effects are largely gross membrane effects, the action of individual terpenoids on specific enzyme systems, especially membrane associated enzymes, should not be ruled out. However, apparent membrane associated enzyme inhibition may be the result of gross effects on membrane physicochemical parameters.

This study has also shown that the presence/absence of antimicrobial activity of terpenoids is affected by their availability to microbial cells, a property which was found to be mainly related to their water solubility. Log $K_{ow}$ was also found to be related to activity/inactivity though to a lesser extent. This weaker association was attributed to the low solubility of terpenoids which render it the limiting factor in bioavailability.

The structure of the microbial cell envelope was also found to affect the antimicrobial activity of the terpenoids. The Gram negative outer membrane of *E. coli*
and *P. aeruginosa* was found to prevent antimicrobial action of many terpenoids as shown by experiments using PMBN.

An understanding of the structure/activity relationships for the terpenoids against microorganisms was also developed. Correlation of antimicrobial activity patterns and molecular parameters of the terpenoids has confirmed that water solubility is the main factor influencing activity/inactivity. The molecular parameters which are most strongly associated with activity against individual organisms were determined for the first time. These were the molecular parameters which discriminated between active and inactive compounds against each organism. Hydrogen bond donor capacity most strongly discriminated activity/inactivity against *P. aeruginosa*. Hydrogen bond acceptor capacity most strongly discriminated between activity/inactivity against *S. aureus* and *C. albicans*, as well as *E. coli*. Activity against Gram negative *E. coli* and *P. aeruginosa* was also associated with a size parameter, with smaller molecules being active. This was not found to be the case for the Gram positive *S. aureus* or the yeast *C. albicans*, which suggests that smaller size is advantageous for compounds to pass through the protective outer membrane of Gram negative organisms. The inadequacies of classifying antimicrobial activity of terpenoids based solely on their functional group, as reflected in the MIC patterns of these compounds, has been demonstrated. Antimicrobial activity is dependent on a combination of interrelated factors namely, availability to the cell, structural defenses of the microorganism and membrane disrupting capacity of the terpenoids.

Based on the data obtained during this investigation it is proposed that the antimicrobial action of terpenoids takes place via a two stage process (Figure 8.1). The first stage is the transfer of the terpenoids into a bioavailable form followed by penetration to the lipid bilayer of the cytoplasmic membrane in sufficient concentrations to initiate membrane disruption. The transfer to a bioavailable form is primarily dependent on the compounds water solubility. The penetration is dependent on the protective structures of the cell, molecular size and hydrogen bonding parameters for Gram negative organisms, and log $K_{ow}$ for all. Terpenoids must therefore have a balance of properties that will allow them to penetrate and accumulate in sufficient concentrations to cause membrane disruption. Thus terpene hydrocarbons and acetates, though having the capability of membrane disruption, are precluded from accessing the membrane in sufficient amounts to exhibit antimicrobial
activity. On the other hand terpene alcohols, aldehydes and ketones have the necessary balance of properties to access and accumulate in the membrane and hence exhibit activity.

The second stage is accumulation in the cytoplasmic membrane which results in the inhibition of cell growth. This can be attributed to the gross membrane effects such as increased bilayer disorder and direct bilayer damage, as shown in model systems, as well as K+ leakage as shown with *E. coli*. These effects will act to de-energize the cell, disturb the osmotic balance of the cell through loss of small ions, render its membrane associated proteins inefficient due to increased membrane disorder and compromise the structural integrity of the cytoplasmic membrane in some cases. In the longer term, depending on the extent of damage, these effects when coupled with any specific effects on membrane systems or detrimental effects on the cytoplasm and or nuclear material will eventually lead to cell death or inhibition of cell growth.
Figure 8.1 - Suggested general antimicrobial mode of action of terpenoids. CP - cytoplasmic embedded protein, LPS - Lipopolysaccharide, PP - porin, PPS - periplasmic space, A - outer membrane protein, LP - lipoprotein, BP - Binding protein
References


275. Nambi, P., Rowe, E. and McIntosh, T. (1988), Studies of the Ethanol-Induced Interdigitated Gel Phase in Phosphatidyocholines Using the Fluorophore 1,6-Diphenyl-1,3,5-hexatriene. Biochemistry, 27, 9175-9182.


287. Antunes-Madeira, M. and Madeira, V. (1990), Membrane Fluidity as Affected by the Organochlorine Insecticide DDT. *Biochimica et Biophysica Acta*, 1023, 469-474.


Appendices
# Appendix I

Table A1 - Molecular properties of terpenoids as calculated using Molecular Modeling Pro.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Surface Area</th>
<th>Molecular Volume</th>
<th>HLB</th>
<th>Polarity</th>
<th>Hydrogen Bonding Capacity</th>
<th>% Hydrophobic Surface Area</th>
<th>Hydrogen Bond Acceptance Capacity</th>
<th>Hydrogen Bond Donor Capacity</th>
</tr>
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<tbody>
<tr>
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<td>14.7487</td>
<td>105.028</td>
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## Appendix I

Table A2 - Molecular properties of terpenoids as calculated using Molecular Modeling Pro.

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<th>Hydrogen Bond Acceptance Capacity</th>
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## Appendix I

Table A3 - Molecular properties of terpenoids as calculated using Molecular Modeling Pro.

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Aspects of Antimicrobial Activity of Terpenoids and the Relationship to their Molecular Structure

By

Shane Griffin

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy – Science

Centre for Biostructural and Biomolecular Research, Faculty of Science Technology and Agriculture, University of Western Sydney, Hawkesbury, Richmond, NSW, Australia. August, 2000.
“Marvelous, what ideas the young people have these days. But I don’t believe a word of it.”

Albert Einstein

[AAfter Heisenberg’s 1927 lecture enunciating the uncertainty principle†]

† In Ehrenberg, *Physic Bulletin*, 1979, 30, pp 262
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Finally my parents for the support they have given me in all areas of life especially my drive to study.
I hereby declare that the work in this thesis was performed by Shane Griffin unless specifically acknowledged, and that it has not been presented elsewhere for any other degree.

Shane Griffin
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Figure 7.2 – Comparison of log $K_{ow}$ values measured by RP-HPLC and calculated by the Hansch and Leo Fragment method.

Figure 8.1 – Suggested general antimicrobial mode of action of terpenoids. CP – cytoplasmic embedded protein, LPS – lipopolysaccharide, PP – porin, PPS – periplasmic space, A – outer membrane protein, LP – lipoprotein, BP – binding protein
List of Abbreviations

AFM – Atomic Force Microscopy
$^{13}$C NMR – Carbon 13 Nuclear Magnetic Resonance Spectroscopy
DA – Discriminant Analysis
DMPE - Dimyristoylphosphatidylethanolamine
DPPC – Dipalmitoylphosphatidylcholine
DPPE - Dipalmitoylphosphatidylethanolamine
DSC – Differential Scanning Calorimetry
GC – Gas Chromatography
HHW – Half Height Width
HII – Inverted hexagonal lipid phase
ISB - Isosensitest Broth
$K_{ow}$ – Octanol/Water Partition Coefficient
$(k_w)$ - capacity factor of a compound on a HPLC column where the eluent is water.
$L_\alpha$ - Lamellar lipid phase
$L_\alpha$-HII – Lamellar to inverted hexagonal phase transition
LDH – Lactate dehydrogenase
LPS - Lipopolysaccharide
MIC – Minimum Inhibitory Concentration
MnIC – Maximum Non-Inhibitory Concentration
ODS - Octadecylsilane
ODP - Octadecyl polyvinyl copolymer
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
$^{31}$P NMR – Phosphorus 31 Nuclear Magnetic Resonance Spectroscopy
PMBN - Polymyxin B Nona-peptide
RP-HPLC – Reversed Phase High Performance Liquid Chromatography
$T_c$ – Gel to liquid-crystalline (order-disorder) phase transition temperature
$T_{1H}$ – Temperature of the lamellar-inverted hexagonal phase transition
Tris – Tris(hydroxymethyl)aminomethane
ZOI – Zone Of Inhibition
Publications Resulting from this Thesis

Research articles


Poster Presentations

S. Griffin, S. G. Wyllie, J. Markham and D. Leach, The role of structure and solubility in determining the antimicrobial activity of terpenoids, Conference for the Phytochemical Society of North America, July, 1998. Also presented at MedAg'98, The 14th National Conference of the medicine and Agriculture Division, Royal Australian Chemical Institute, University of Wollongong, NSW Australia, 22nd-24th November 1998, incorporating additional data.


Oral Presentations

S. Griffin, J. Markham, S. G. Wyllie and D. Leach, The role of structure and solubility in determining the antimicrobial activity of terpenoids, Royal Australian Chemical Institute, Natural Products Group Annual One Day Symposium, Sydney Australia, October 3, 1997.

S. Griffin, J. Markham, S. G. Wyllie and D. Leach, Determining the properties of terpenoids associated with their antimicrobial action, Royal Australian Chemical Institute, Natural Products Group Annual One Day Symposium, Sydney Australia, October 2, 1998.
Abstract

Although the antimicrobial nature of essential oils and their major constituents, the terpenoids, has been widely investigated the mechanism of their antimicrobial action has not been subject to the same scrutiny.

In this study the membrane disruptive nature of the terpenoids has been determined by experiments on the effects of terpenes on both microbial membrane and model lipid bilayer systems. The effects a range of terpenoids on bilayer stability were determined using atomic force microscopy on dipalmitoylphosphatidylcholine (DPPC) supported lipid bilayers. These terpenes exhibited a range of membrane damaging effects. For example, carvone and perilla alcohol were both found to cause the formation of holes in the bilayer structure in addition to increasing the size of naturally occurring defects. In contrast carveol was found to act across the bilayer surface as a whole with the loss of large sections of bilayer from the mica surface. Carvacrol was by far the most membrane perturbing monoterpenene and was found cause complete relaxation of the bilayer structure.

Differential scanning calorimetry experiments also showed that the terpenoids were able to increase disorder in DPPC bilayers. Phase transition profile changes were used to determine the position of localisation of 14 terpenoids within model lipid bilayers. It was found that the majority of the oxygenated terpenoids tested tended to orientate at the C1-C8 methylene groups of the lipid acyl chains when at lower concentrations and then accumulate at the phosphorylcholine headgroup as concentration increased.

Potassium leakage experiments showed that antimicrobially active terpenoids cause increased membrane permeability in living cells. Carvone, terpinen-4-ol, α-terpineol, carveol and myrtenol were each found to cause significant K⁺ leakage from E. coli cells. In contrast the relatively inactive terpenes, 1,8-cineole and p-menth-6-ene-2,8-diol, did not cause significant K⁺ leakage. Slight structural differences between active terpenes were found to affect the rate of K⁺ leakage from E. coli cells. It is postulated that the membrane permeabilising effect of the active terpenes is a significant factor in their antimicrobial activity.
The effect of molecular structure on antimicrobial activity/inactivity and activity differences between each organism were determined using minimum inhibitory concentrations determined for 60 terpenoids, against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Terpenoids with a low water solubility, mainly hydrocarbons and acetates, were found to be inactive. The inactivity of these terpenoids can now be largely ascribed to their inability to access the cell membrane. Log $K_{ow}$ also discriminated between active and inactive compounds though less strongly. This weaker association is considered to be because water solubility is the limiting factor governing the availability of the compound to the cell.

Hydrogen bond donor capacity and hydrogen bond acceptor capacity were found to be the molecular parameters which most strongly discriminated between activities against individual organisms. Activity against Gram negative *E. coli* and *P. aeruginosa* was associated also with a molecular size parameter. This may be connected with the ability of these compounds to pass through their protective outer membrane. This relationship was not found for the Gram positive *S. aureus* or the yeast *C. albicans*.

The protective nature of the outer membrane of *P. aeruginosa* and *E. coli* was also investigated. Kill rate experiments using various pairs of oxygenated terpenes, with closely related chemical structures but considerably different MIC values against these organisms, were found to show differences in rate and number of cells killed when tested at the MIC of the more active compound. Addition of PMBN as an outer membrane permeabilising agent was found to significantly increase the initial rates and overall numbers of cells killed by the inactive compounds, while having a far lesser effect on the active compounds. The hydrocarbon limonene and the ester geranyl acetate, normally inactive, were found to have little killing effect when added alone to cells but did show an enhanced killing capacity upon the addition of PBMB.

The evidence presented in this thesis has enabled the postulation of a two stage process to explain the overall mode of action of these compounds. Stage one consists of the dissolution of terpenes into water and their penetration to the cytoplasmic membrane. Stage two consists of accumulation in the cytoplasmic membrane followed by membrane disruption as a result of gross effects on the physico chemical properties of the cytoplasmic membrane.

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