THE BIOSYNTHESIS OF TERPENOIDS IN TISSUE CULTURE:

SYNTHESES OF LETHAL METABOLITES AND TOXICITY STUDIES

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for the degree of
DOCTOR OF PHILOSOPHY

by

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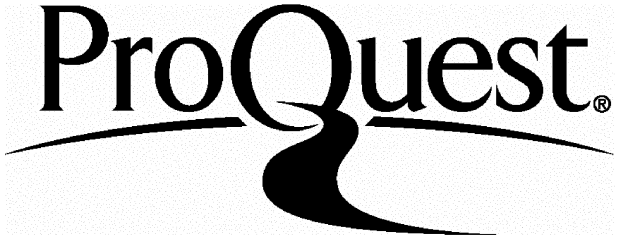

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Martin.

Abbreviations

All the abbreviations used in this work are those that are commonly accepted. They are given in parentheses as they appear in the text but the following list provides a summary:

ATP - Adenosine triphosphate
BAP - Benzylaminopurine
CAD - Collisionally activated dissociation
CFE - Cell-free extract
c.m.c. - Critical micellar concentration
CoA - Coenzyme A
c.p.m. - Counts per minute
2,4-D - 2,4-Dichlorophenoxyacetic acid
DMAPP - Dimethylallyl pyrophosphate (diphosphate)
d.p.m. - Disintegrations per minute
EDTA - Ethylenediaminetetra-acetic acid
EI - Electron-impact
ER - Endoplasmic reticulum
EtMgBr - Ethylmagnesium bromide
ETMSA - Ethyltrimethylsilyl-acetate
FAB - Fast atom bombardment
FDA - Fluorescein diacetate
FFR1 - First field-free region
FFR2 - Second field-free region
FPP - Farnesyl pyrophosphate
GC/MS - Gas chromatography interfaced to a mass spectrometer
GPP - Geranyl pyrophosphate
HMDS - Hexamethyldisilazane
HMGCoA - 3S-Hydroxyl-3-methyl glutaryl-CoA
HPLC - High Performance liquid chromatography
IPP - Isopentenyl pyrophosphate
LCC - Liquid column chromatography
LDA - Lithium diisopropylamide
LD_{50} - the concentration of a compound that is required to kill half the population of a sample.
LSC - Liquid scintillation counting
mM - millimolar ($mmol.dm^{-3}$)
MS - Mass spectrometry
MVA - 3R-Mevalonate
MVAPP - 3R-Mevalonate pyrophosphate
NAA - α-Naphthaleneacetic acid
NAD - Nicotinamide adenine dinucleotide (oxidized form)
NADH - Nicotinamide adenine dinucleotide (reduced form)
NADP - Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH - Nicotinamide adenine dinucleotide phosphate (reduced form)
NBS - N-bromosuccinimide
NCS - N-chlorosuccinimide
NCI - Negative chemical ionisation
NFPT - N-fluoropyridinium triflate

NMR	- Nuclear magnetic resonance
PCI	- Postive chemical ionisation
PCV	- Packed-cell volume
TLC	- Thin-layer chromatography
RDA	- Reverse-Diels Alder reaction
SDS	- Sodium dodecyl sulphate
SID	- Surface-induced decomposition
SIMS	- Selected-ion monitoring
TBAF	- Tetrabutylammonium fluoride
TBABF	- Tetrabutylammonium bifluoride
THF	- Tetrahydrofuran
TMS-I	- Trimethylsilyl-iodide

Abstract

The work described can be conveniently divided into four related but distinct sections.

Part One describes a set of experiments that follow on from a previous study of the incorporation of 1-^{14}C-Isopentenyl pyrophosphate (1-^{14}C-IPP) into terpenoids by cell-free extracts from cultures of *Lavandula angustifolia.* Of the total incorporations (*ca.* 5%) most (70%) of the label was present in the farnesols. The addition of NADP caused an increase of incorporation into the sesquiterpenoid hydrocarbons caryophyllene (13%) and humulene (30%), with a concomitant decrease of incorporation into the farnesols. By using enriched cell-fractions the site of sesquiterpenoid biosynthesis was found to be associated with the microsomal fraction.

Part Two describes a series of experiments carried out on cell-suspension cultures of *Pelargonium fragrans.* A statistically reliable and novel method of estimating cell-viability was developed to study the toxicities of some common terpenoids as such toxicity may account for the lack of accumulation of terpenoids in culture. All compounds were toxic (in the range 1-5 mmol.dm^{-3}) and the toxicity (LD_{50}) was greatest during the exponential-period of culture-growth. The cultures could however, be habituated to the terpenoids over a number of subcultures. The inclusion of a surfactant in the culture-medium lowered the toxicity of the terpenoids and therefore provided a model storage mechanism (sink) for these compounds in a single-phase culture. The polyethoxylate-surfactants were found to be the most suitable for this purpose. Two subsidiary studies deal with the toxicity of some fluorinated compounds to tissue cultures and the reactions of exogenous terpenoids with the culture medium.

Part Three describes the syntheses of five fluorinated monoterpenoids. Two fluorinated linalools (4-fluoro- and 9-fluoro-) were prepared by treatment of the respective fluoro-6-methyl-hept-5-en-2-ones with vinylmagnesium bromide. A number of methods of introducing fluorine into 6-methyl-hept-5-en-2-one were attempted; the most successful method involved fluorination of the trimethylsilyl-enol ethers using N-fluoro-pyridinium triflate. Linaloyl, neryl and geranyl fluorides were prepared by

treatment of the corresponding chlorides with anhydrous tetrabutylammonium bifluoride. ^{19}F-, ^{1}H- and ^{13}C- nuclear magnetic resonance and mass spectrometry were used to characterise the products. Some unexpected results are discussed in detail.

Part Four describes studies that were used to interpret the fragmentation patterns in the mass spectra of three monoterpenoid acetates occurring in the oil of *L. angustifolia* that was studied in Part One. Linaloyl, neryl and geranyl acetates all showed identical electron-impact mass spectra. A combination of linked-scanning and deuterium-labelling experiments were used in order to characterise the fragmentation patterns. Other methods of ionisation (fast atom bombardment and chemical ionisation in the positive and negative modes) were also used to confirm the patterns.

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PART 1 The Incorporation of 1-^{14}C-IPP into Lower Terpenoids by Cell-free Extracts of *Lavandula Angustifolia*

1-^{14}C-IPP is the accepted abbreviation of 1-^{14}C-Isopentenyl pyrophosphate (*ie.*, a diphosphate).

Chapter 1 Introduction

1.1 The Biosynthesis of Terpenoids

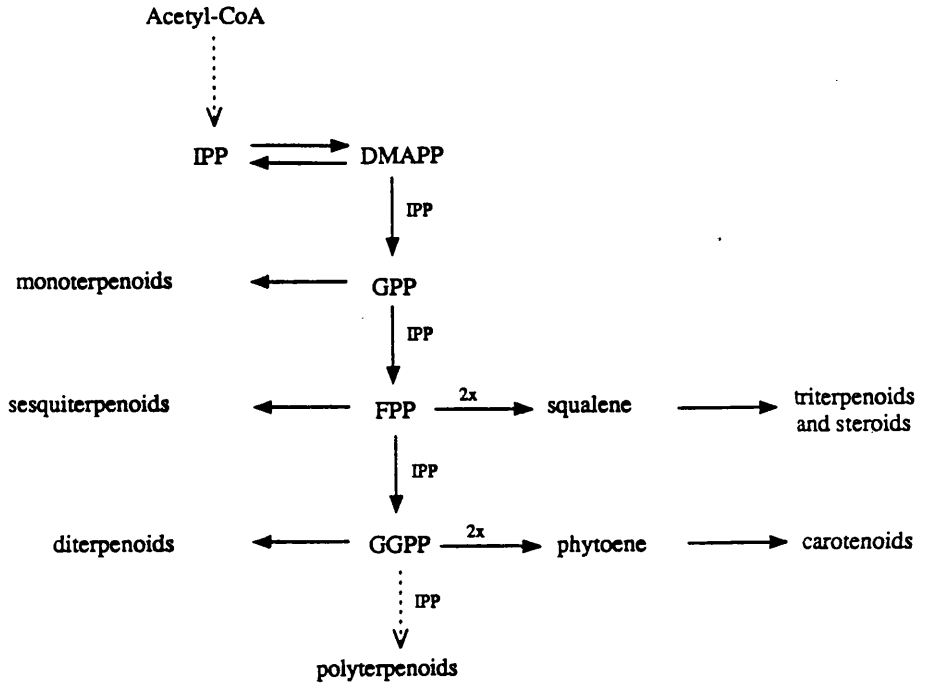
The terpenoids are a group of secondary metabolites (the biological significance of which is discussed in Section 3.1) that are built-up from one or more C_5 units, although some members of this family contain a non-integral number of such units owing to further modification of their newly-formed parents. A particular class of terpenoid can be distinguished by its prefix *eg.*, hemi-(C_5), mono-(C_{10}), sesqui-(C_{15}), di-(C_{20}), sester-(C_{25}) or tri-(C_{30}) terpenoid. The last is the parent class for steroids. There are two more common members of this family; the carotenoids (C_{40}) and the polyisoprenoids [$(\text{-}C_5\text{-})_n$; $n \rightarrow 1000$)]. In 1953 Ruzicka put forward the Biogenetic Isoprene Rule to unify the very large number of structural types that had been found by then. This rule essentially stated that all the members of a particular class of terpenoids are related by simple functionalisation, cyclisation and rearrangements and that all members of the class are derived from a common precursor. The various branches of terpenoid biosynthesis (Scheme 1.1) show how the precursors of each class are themselves related.

This entire pathway has been shown to be intimately related to amino acid and fatty acid biosynthesis in plants[1] and animals.[2] Terpenoids are thought to originate from acetate (an assimilate associated with primary metabolite precursors) that was shunted into the isoprenoid pathway at times of stress, cessation of growth, or senescence (Scheme 1.2).

The first section of this chapter outlines some of the most recent research on the regulation of mono- and sesquiterpenoid biosynthesis at the enzymic level in plants (and animals). The second section covers some work carried out on the sites of synthesis and accumulation of these compounds, and the way in which these

parameters may regulate the levels of these compounds produced both *in vivo* and *in vitro*.

Scheme 1.1 The Major Branches in the Biosynthesis of Terpenoids

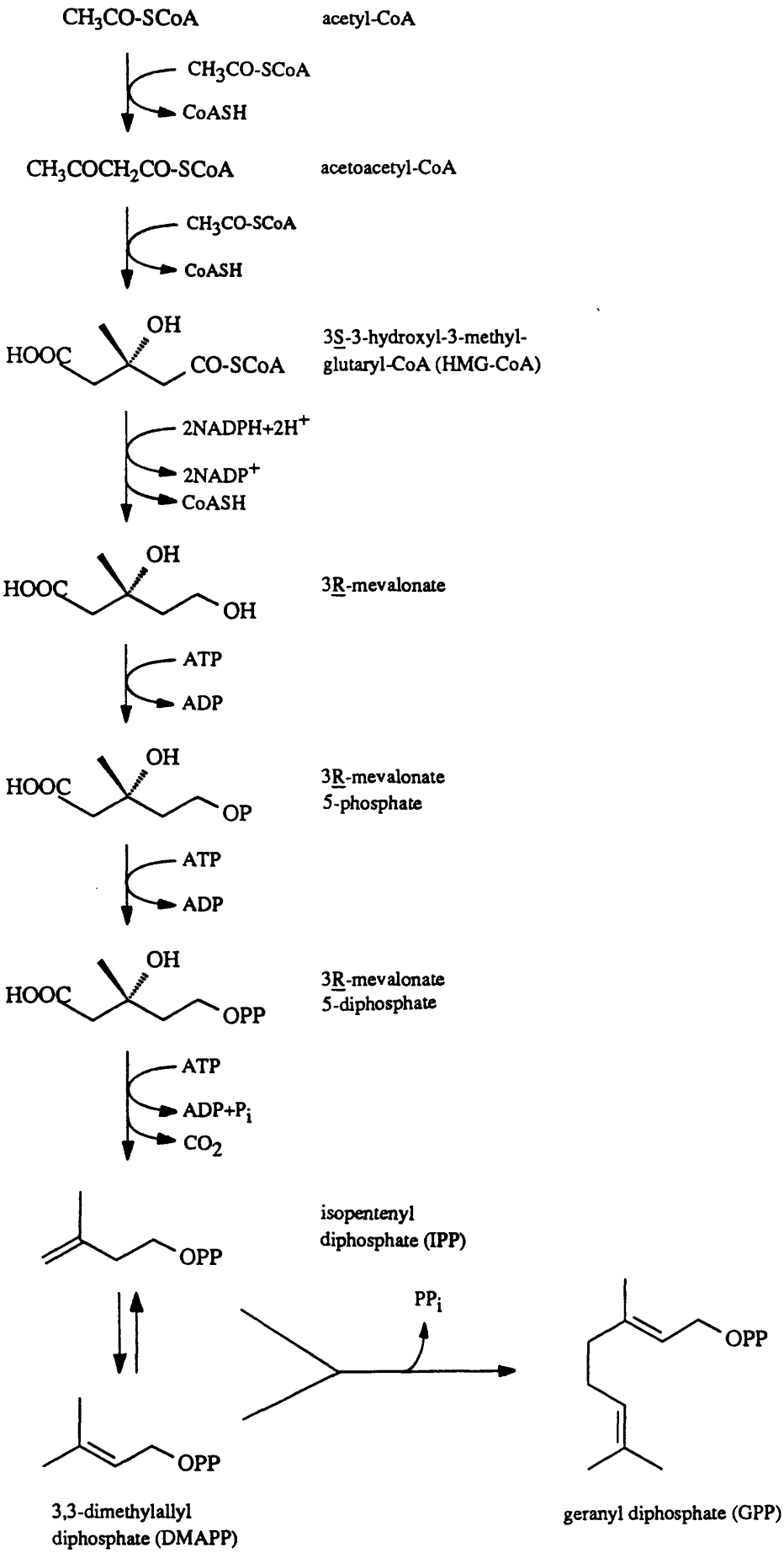


1.2 The Regulation of Terpenoid Biosynthesis by Control of Enzymatic Activity

The effects reported later (Chapter Two) of NADPH and NADP on incorporation levels of 1-^{14}C-IPP into terpenoid products are probably related to the mechanisms that control biosynthesis of terpenoids at the various branch-points along the pathway (but not at the HMG-CoA step because the precursor used, IPP, is formed subsequent to this).

Plants accumulate a wide range of terpenoid compounds which may be produced by different tissues or by different organelles at the cellular level. With limited amounts of assimilates available for these processes to occur, the regulation of certain synthetic steps plays a key role in determining the class and skeleton of the end-products. The compounds that accumulate in the whole plant or derived callus-culture represent the balance between synthesis and degradation. Tissue cultures normally produce much

Scheme 1.2 The Mevalonate Pathway to Geranyl Diphosphate (Geranyl Pyrophosphate; GPP)



lower levels of terpenoids than the parent plants which suggests that either degradation plays a more dominant role (because callus does not show sufficient differentiation to store these products) or cultures do not have the correct enzymic complement to synthesize terpenoids. It is convenient that use of cell-free extracts may be used to separate synthesis from degradation.

In the last decade, the use of cell-free extracts[3] prepared from whole plants and tissue cultures has enabled many of the biosynthetic steps to be explored. More recently, partly-purified prenyltransferases[4] and cyclases[5] have been used to study the mechanisms and stereochemistries of individual steps of terpenoid biosynthesis.

The biosynthetic pathway leading to terpenoids was first discovered in yeast:[6] the initial steps that lead to the C_5 precursor, isopentenyl pyrophosphate are summarised in Scheme 1.2. The Claisen-type condensation of two molecules of acetyl-CoA to form acetylacetyl coenzyme A is carried out by acetylacetyl-CoA synthetase. An aldol-type reaction is then responsible for the addition of a third acetyl-CoA molecule to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). This addition is catalysed by the transfer of acetyl-CoA from a reactive cysteine-residue of the enzyme HMG-CoA synthetase.[7] A stereospecific reduction (utilising NADPH) of 3S-HMG-CoA to 3R-mevalonic acid (MVA) is brought about by HMG-CoA reductase and this reaction is known to have a regulatory role in the pathway: thus, in mammals the activity of HMG-CoA is known to be rate-limiting for the sequence and subject to feedback inhibition by sterols.[8] The regulatory significance of the enzyme has been investigated in plants and mevalonic acid and a number of structurally-related compounds[9] have been shown to reduce the activity of the enzyme. Several monoterpenoids from plants are also known to be potent inhibitors[10-12] of HMG-CoA activity. However some of these reports are contradictory (*eg.*, mevalonate does not inhibit activity in some cases)[13] and this may be owing to more than one form of HMG-CoA reductase associated with different intracellular sites and classes of isoprenoid.[14] NADP is known to be a competitor[15] with the cofactor NADPH which binds to HMG-CoA and is responsible for the reduction; thus, the ratio of NADP:NADPH could regulate the activity of the enzyme.

The next step in the pathway is the diphosphorylation of mevalonic acid by two ATP-dependent kinases. The resulting MVA-5-diphosphate (MVAPP) is decarboxylated by the action of a third ATP-dependent enzyme (MVAPP decarboxylase) to yield isopentenyl diphosphate (or pyrophosphate; IPP). Although the kinases are not believed to have any regulatory role,[16] the activity of the decarboxylase has been shown to correlate with the onset of sesquiterpenoid production in some plants[17] and so is thought to modulate the levels of end-products.

The isomerisation of IPP to 3,3-dimethylallyl diphosphate (DMAPP) is brought about by the action of isopentyl diphosphate Δ^3-Δ^2-isomerase, the activity of which is known to be controlled by inorganic phosphates and several prenyl diphosphates.[18]

Prenyl transferases are responsible for the condensations of IPP and allylic diphosphates (DMAPP, GPP, FPP *etc.*) and these lead to the prenyl diphosphates which are precursors of the various classes of terpenoids shown in Scheme 1.1. Most prenyl transferases catalyse a sequence of steps (*eg.*, IPP $\rightarrow$ GPP $\rightarrow$ FPP *etc.*) but specific FPP synthetases (GPP transferases)[19] and GGPP synthetases have been isolated. These enzymes are at primary branch-points and they commit the incorporation of IPP into the various classes of terpenoid. In the intact plant, the activities of these branch-point enzymes (and hence the class of terpenoid produced) are considered to be regulated by their compartmentation and the availability of assimilates[20] such as sucrose. Thus, incorporation of radioactive tracers is usually low.[21] However, in cell-free extracts these restraints are removed and incorporation levels may depend only on the differing affinities of the various prenyl transferases towards exogenous IPP and the levels of enzyme surviving extraction. Some prenyl transferases are known to be associated with cyclases and other enzymes responsible for secondary transformations on so-called metabolic grids or on multienzyme complexes.[22] Cyclases may be branch-point enzymes and therefore have a regulatory role in the biosynthesis of lower terpenoids. Croteau[23] has demonstrated that activity of bornyl diphosphate synthetase (a cyclase) is rate-limiting in the formation of camphor in cell-free extracts of *Salvia* species. Such results have fuelled attempts to control cyclase activity.

1.3 The Regulation of Terpenoid Biosynthesis by Compartmentation

The notoriously low accumulation of terpenoids in most tissue cultures is usually attributed to the lack of specialised storage structures for their accumulation and synthesis *in vitro*[24] and also possibly to the toxic effects of these unsequestered chemicals on the cells (see Part Two). Consequently in the latter case, for the culture to survive and be observed and studied there must be degradative enzymes that remove the unwanted compounds. Only a few sesquiterpenoids[25-6] have been recorded from tissue cultures, although a number of toxic sesquiterpenoid phytoallexins have been isolated by first treating cultures with bacteria.[27] In the whole plant, certain reaction sequences are compartmentalised at the cellular and subcellular level. There are currently two opposing theories concerning the subcellular compartmentation of terpenoid biosynthesis. The first considers that all organelles (*eg.*, plastids and mitochondria) are capable of supporting the whole terpenoid pathway[28] while the other considers that IPP is first synthesised in the cytoplasm and then transferred to the various organelles which are responsible for producing specific classes of terpenoids.[29]

Monoterpenoid-biosynthesis has been shown to be associated with leucoplasts[30] and chromoplasts (*eg.*, chloroplasts)[31] by preparation of cell-fractions enriched in these organelles. The former were found to contain GPP synthetase and monoterpenoid cyclase activity. However, cell-free systems prepared from the cytoplasm[32] of some plants have been shown to contain cyclase activities. Similarly, many of the enzymes associated with terpenoid-functionalisation are known to be located in this part of the cell.[33-35] Studies on the biosynthesis of sesquiterpenoids and diterpenoids have demonstrated that specific prenyltransferases and cyclases are associated with the endoplasmic reticulum[36] and plastids[37] respectively.

Croteau *et al.*,[38] have shown that isolated leaves of *Mentha* species, when administered with labelled MVA, incorporate the substrate into a mixture consisting of mainly sesquiterpenoids even though monoterpenoids are almost 50 times more abundant in the parent plant. Other such studies have demonstrated that the biosynthesis of monoterpenoids and some diterpenoids[15] at the cellular-level occurs in physiologically-isolated compartments such as glandular trichromes,[39] resin ducts[40] and resin cavities[18] which are not readily accessible to assimilates such as glucose, acetyl-CoA and allylic

diphosphate precursors. Consistent with this a number of cyclases, hydroxylases and oxides have been isolated from glandular trichromes.[41-2] The biosynthesis of components such as carvone from GPP (cyclisation → hydroxylation → oxidation) by *Mentha* species is known to be restricted to these structures.

The main subject of this thesis is the metabolism of terpenoids in plant tissue cultures. In order to develop the techniques for the later studies an initial investigation was undertaken on such metabolism in *Lavandula angustifolia* (lavender) that repeated and extended some previous work carried out by Dr. D.G. Watson[43] (hereafter referred to as DGW) which was briefly mentioned in a recent publication.[44] This work is of considerable interest in itself.

We can take studies on *L. angustifolia* to be typical for a wide range of herbaceous plants that have been extensively investigated in tissue culture in the U.C.L. and many other laboratories.

1.4 Summary of Previous Work:

The studies of DGW are summarised in Table 1.1;

Table 1.1 The Effects of Cofactors and a Phytohormone on the Incorporation of 1-^{14}C-IPP into Terpenoid Products by Cell-free Extracts of *L. angustifolia*

Additive	Concentration (mmol.dm^{-3})	Total Incorporation (%)	Incorporations (% of Total) within R_f bands on TLC		
			1	2	3
None		25	86	-	14
NADP	1.8	37	7	-	93
NADPH	1.8	40	10	-	90
ATP	2.0	32	14	-	86
2,4D-*	*	35	82	17	1

1. R_f 0.20 - 0.30
2. R_f 0.70 - 0.75 } silica gel TLC plates (C_6H_{14}:EtOAc; 85:15)
3. R_f 0.80 - 1.00

* 2,4-Dichlorophenoxyacetic acid in medium (1.0 mg.dm $^{-3}$)

Data reproduced from: Watson, D.G., PhD Thesis, University of London (1981)

Analysis of Products: DGW maintained callus of *Lavandula angustifolia* for a period of two months on MS medium (see 13.1). A cell-free extract, prepared from these cultures, was treated with 1-^{14}C-IPP and was found to incorporate the substrate into a mixture of products that separated into two distinct bands when analysed by radio-TLC (12.1.a; system 1.i). The first band probably consisted of the terpenoid alcohols geraniol, nerol and farnesol together with some polar diterpenoids. The second band consisted of a mixture of unidentified terpenoid hydrocarbons but neither set of compounds were characterised.

Incorporation Levels: The addition of NADP (1.8 mmol.dm^{-3}) or NADPH (1.8 mmol.dm^{-3}) to the cell-free extract resulted in 10-fold increases of incorporation into the terpenoid hydrocarbons with a concomitant decrease of incorporation into the alcohols by almost 80%. When exogenous ATP (2 mmol.dm^{-3}) was administered to the cell-free extracts the opposite effect on incorporation levels was observed; *viz.* an 8-fold increase of incorporation into the alcohols and a decrease of incorporation into the hydrocarbons by 80%. Various other findings were reported *eg.*, cell-free extracts prepared from cells grown on medium supplemented with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in place of α-naphthaleneacetic acid (NAA; see 13.1) gave increases of incorporation levels into both product-groups. In addition, TLC-analysis of this mixture revealed a third band of products (Table 1.1). Cell-free extracts prepared from callus grown on medium containing less sucrose (half the usual concentration) showed increased incorporation levels into both bands by as much as 50%. In general, the biosynthetic capacity of the cultures in their twenty-sixth passage (*ie.*, after two years), showed negligible incorporation of labelled 1-^{14}C-IPP into terpenoid products. This work, if valid could be very important.

Chapter 2 Results and Discussion

Aims and Summary of Previous Work:

Aims: This chapter is sub-divided into two sections. Section 2.1 (analytical methods) describes four experiments which were used to identify the main components of a parent plant and of tissue cultures of *L. angustifolia* (and thus indicate any ability of the callus cultures to accumulate terpenoids). The experiments describe how we cross-matched the chromatograms of these products with the chromatograms of the products formed by the cell-free systems. One of the preliminary experiments involved feeding C_5, C_{10}, C_{15} and C_{20} precursors to the cell-free systems so that the products could be screened by class. The identifications we obtained were all consistent with those made by DGW.

Section 2.2 (incorporation results) describes experiments to reproduce and verify the effects observed by DGW of nicotinamide cofactors on the incorporation levels of the ^{14}C-IPP tracer. Other additives to the cell-free system were also studied, together with incorporation studies on cell-free extracts derived from two new cell-lines of lavender.

Apart from reproducing the results of DGW and confirming the identities of the products, other experiments show (i) how incorporation depends on the growth-cycle of the culture from which the cell-free extract was prepared and (ii) the cell - fractions associated with biosynthesis in extracts of *L. angustifolia*. More rigorous attempts to identify the products formed were also necessary.

2.1 The Analysis of Terpenoids produced by the Intact Plant, Callus Cultures and Cell-free Extracts of *L. angustifolia*

This section describes a series of experiments that were used to indicate the ability of the callus cultures to accumulate terpenoids, the identification of these products by chromatographic methods and the use of these identifications in analysing the low levels of ^{14}C-labelled products formed by the cell-free extracts.

The results are then summarised in Section 2.1.3 and used in the discussions in Section 2.2 on the biosynthetic power of the cell-free extracts. The following table summarises the analyses carried out in this work.

Table 2.1 Assays for Terpenoids Carried out in this Work

Extraction	Analytical Method	Purpose
1) Parent Plant	HPLC & GC/MS	To provide chromatographic standards for assays 3,4
2) Commercial Oils	HPLC & GC/MS	As above
3) Callus	GC/MS & TLC	To Indicate accumulative power of callus
4) CFE of Callus	Radio-TLC*	To Indicate synthetic power of callus

* GC/MS not possible with ^{14}C-labelled products because of possible contamination of available instruments.

2.1.1 The Analysis of Three Callus-lines of *L. angustifolia* for Evidence of Terpenoid Accumulation and Comparison with the Parent Plant

A cell-line of *L. angustifolia* used in the previous study[43] was maintained on growth medium and two new cell-lines were initiated from explants of *L. angustifolia v. Mill.* The media used are given in section 13.1. A summary of this is as follows:

The leaves and flowerheads from the specimen of *L. angustifolia* (from which the three callus-lines had been initiated) were pulverised and extracted by steam distillation (13.6) using a potassium phosphate buffer (the solution used for the incubation buffer). The hexane extract was analysed by TLC (12.1.a; system 1-3) and GC/MS (12.2; systems 1,2). The three callus-lines were analysed by solvent extraction and TLC/GLC methods as previously. The GC-trace of the extract of the callus (line A) showed peaks corresponding to 24 major components. The phellandrenes,

Table 2.2 Origins of Cell-lines

Cell-line	Source	Auxin	Cytokinin
A	DGW	NAA (2 $mg.dm^{-3}$)	Kinetin (0.2$mg.dm^{-3}$)
B	DGW	2,4-D (2$mg.dm^{-3}$)	Kinetin (0.2$mg.dm^{-3}$)
C	New	NAA (2$mg.dm^{-3}$)	Kinetin (0.2$mg.dm^{-3}$)

Origin: A (DGW, maintained since 1980); B (DGW, As for A but transferred in 1988 onto medium with the supplements shown); C (newly initiated in 1988)

terpinenes and myrcene were identified in the GC-trace of this extract (by cross-matching with standards) but it was not possible to fully analyse such solvent extracts of calli (at B.B.A. Ltd., London) because of the presence of high molecular-weight compounds and plastisizers which would have damaged the GC capillary-columns. Some of these compounds may have been high molecular weight terpenoid resins.[45a] It has recently been shown that some tissue cultures accumulate waxes[45b] and these may well be responsible for the difficulties encountered with the present analyses. However, it was possible to cross-match 15 compounds in the TLC chromatograms of the extracts prepared from calli (lines A,B and C) and the distillate of the flowerheads of the parent plant. This established that the cultures did have the power to accumulate terpenoids that were characteristic of the intact plant. In order to identify the components within this set of compounds that were common to all three extracts the distillate of the flowerheads was analysed by GC/MS and some 20 major components were identified in total. Some of these are shown in Table 2.3.

Most other components (a further 50) such as geraniol, nerol, carvone, phellandrenes, pinenes, germacrene and humulenes were present individually in less than 1% abundance. The compounds which were identified are present in all lavender oils in varying proportions (good quality oil has a 1:1 ratio of linalool to linaloyl acetate). Oil compositions of new hybrids of *Lavandula* are reported annually[46-50] but many of the differences found may well result from the different extraction processes employed (*eg.*, linaloyl acetate is known to eliminate and / or rearrange during steam distillation,

even in buffer solutions at pH 7.0).[51]

Table 2.3 Major Steam-volatile Components of the Flowerheads of *L. angustifolia*

Compound*	% Abundance (from areas of GC-peaks)
Linaloyl acetate	50
Linalool	20
Lavendulyl acetate	5
Borneols	5
Terpineols	5
Caryophyllene	5
Ocimenes	3
Others (see text)	7

* See Appendix for Structures of these Compounds

2.1.2 The Pre-fractionation and Analysis of Oils from Three Hybrid Lines of *L. angustifolia*

Many of the products that could possibly be formed by the cell-free systems could not be obtained as commercial standards. However, commercial lavender oils could be obtained and were analysed to identify many of the minor components and isolate them by HPLC for re-analysis by GC/MS. We obtained steam distillates of three different lavender hybrids (*ex.* Norfolk Lavender Farms, King's Lynn, Norfolk) from which we tried to separate individual components that were not available as laboratory standards. The three distillates were first analysed by GC/MS (12.2a; system 2); the major components of the oils are shown in Table 2.4.

Table 2.4 The Major Components of the Distillates Obtained from Three Hybrids of *L. angustifolia*

Component	A %	B %	C %
Myrcene	0.5	0.7	0.7
Ocimenes	2.6	3.4	6.7
Linalool	19.2	18.7	26.3
Oct-1-en-3-yl-acetate	3.3	4.1	4.5
Borneol	2.1	1.9	1.4
Terpinen-4-ol	2.1	2.0	2.2
Linaloyl Acetate	46.3	45.2	35.6
Lavendulyl Acetate	6.4	5.9	5.7
Caryophyllene	3.7	3.6	2.0
Caryophyllene Oxide	0.6	0.5	0.2

More than 70 components were present in each sample but only 20 of these could be observed on TLC chromatograms which had been eluted in the three systems given in section 12.1a and visualised by the appropriate sprays.

Table 2.5 summarises the pre-fractionation procedures used to separate the components of the distillate.

HPLC gave separation of the major components but these were still contaminated by minor components; the fractions were collected and re-eluted using a less polar solvent mixture in order to optimise the separation of isomers within each fraction that had been obtained from the first elution. However, although good separation of structural types and classes was achieved (*eg.*, C_{15} from C_{10} and hydrocarbons from oxygenated compounds) it was not possible to separate many of the structural isomers (which most of the minor components were). Even elution of the fractions on reverse-phase HPLC and preparative GC did not achieve this. The compounds that were separated easily happened to be those standards that were available commercially.

Table 2.5 Pre-fractionation Procedures

Method	Result
1) TLC (Systems i, iii, iv)	20 spots
2) TLC ($AgNO_3$ impregnated)	Separates C_{10}-hydrocarbons from C_{15}-hydrocarbons
3) TLC (Preparative)	Separates hydrocarbons from alcohols and acetates in 12 fractions
4) HPLC (Normal-phase, system 2)	15 fractions of greater than 50% purity in main component
5) GC/MS (Systems 1 and 2)	Establishes composition of fractions in 1-4. Approx. 70 terpenoid components in total

Summary: By characterising each fraction and re-eluting on TLC (in three solvent systems) it was possible to determine where each major component eluted in the chromatogram and similarly, which minor components were likely to co-elute in the same R_f range.

2.1.3 Preliminary Analysis of the TLC-chromatograms of the Products formed by Cell-free Extracts of *L. angustifolia*

Having established that the three cell-lines were accumulating terpenoids characteristic of the parent plant the next step was to determine whether cell-free systems derived from these cell-lines were capable of biosynthesizing the same or related products.

Cell-free extracts were prepared from the three tissue culture lines of *L. angustifolia v. Mill.* When analysed by TLC (system 1.i), the products separated into three bands which were detected by liquid scintillation counting (LSC) and autoradiography. A fourth band containing the C_5 alcohols (resulting from phosphatase activity on the 1-^{14}C-IPP) was also observed. The bands are referred to as 1-3 (in order of the R_f on TLC). Table 2.6 shows the total incorporation of the substrate into the three bands. The majority of the label (*ca.*, 80%) was found in band 1 from extracts prepared from all three cell-lines. Autoradiographs were also recorded for the solvent extract of the spent cell-free extract which had been incubated with apyrase and alkaline phosphatase. The quantification of the incorporation levels will be described in

Table 2.6 The Incorporation of the 1-^{14}C-IPP into Terpenoid Products by Cell-free Extracts of 3 Lines (A,B,C) of *L. angustifolia*

Cell-line	Total Incorporation† (%)	Mean Incorporation (% of Total) within R_f Bands on TLC		
		1	2	3
A	6	83	15	2
B	11	78	16	6
C	6	67	15	18

1. R_f 0.20 - 0.30
2. R_f 0.30 - 0.80
3. R_f 0.80 - 1.00

Silica gel TLC plates (C_6H_{14}: EtOAc; 85:15)

† $2\sigma = \pm 10\%$

Section 2.2. In total, six major products were formed by the cell-free system. We assigned these as shown in Table 2.7.

Section 2.2 reports that the addition of certain additives enhanced the incorporation of the tracer into those products in bands 2 and 3. This phenomenon was used in order to observe these products by autoradiography.

The separation of the C_{10} hydrocarbons could not be achieved efficiently; an attempt to feed a large-scale (1 dm^3) cell-free extract with unlabelled IPP and analyse the products directly by GC failed, owing to the presence of high molecular weight contaminants (described previously) in the product-mixture. The only methods available for unequivocal characterisation of products formed by cell-free extracts involve multi-step degradations, and to date these have only been used for studies where only one product is formed.[52] However, the identifications made here are consistent among the TLC chromatograms that were recorded, and verify the identifications made by DGW. It is interesting to note that the cell-free systems produce the farnesols as the main products whereas these were not detected in the oils from the callus or the flowerheads.

Table 2.7 The Products formed by the Cell-free Extracts of *L. angustifolia*

Compound	Class	Comments
2*E*-farnesols	C_{15}	elute as one spot
2*Z*-farnesols	C_{15}	elute as one spot
Caryophyllene	C_{15}	
Humulene	C_{15}	
Unknown	C_{15}	eluted to within 0.04 R_f of humulene in all TLC systems
β-Pinene	C_{10}	plus other isomers

Note: percentage yields are not recorded because these were not required to make the assignments
The incorporation levels of the tracer are dealt with in section 2.2

2.1.4 The Confirmation of Product-identity by Class: the Incorporation of DMAPP, GPP, into 4,8,12-^{14}C-FPP and 4,8,12,16-^{14}C-GGPP into Terpenoid Products

A cell-free extract of callus line A was prepared (protein assay 0.4-0.5 mg.cm^{-3}) and incubated separately with the following precursors;

(i) Unlabelled DMAPP (10 nmol.cm^{-3}; 180 min.)

(ii) Unlabelled GPP (10 nmol.cm^{-3}; 180 min.)

(iii) 4,8,12-^{14}C-FPP (0.02 nmol.cm^{-3}; 10, 20, 35 min.)

(iv) 4,8,12,16-^{14}C-GGPP (0.10 nmol.cm^{-3}; 10, 20, 35 min.)

A control experiment (incubation of substrate in buffer only was set up for each of the above. The results of the various incubations are summarised in Table 2.8.

The results of this experiment support the previous finding that the cell-free extract of lavender is principally a system that produces farnesol as the main product. No incorporation of either FPP or GGPP into hydrocarbons was observed.

Table 2.8 The Incorporation of Other Substrates

Substrate	Origin	Products
DMAPP	Synthetic	C_5 only
GGP	Synthetic	C_{10} alcohols
^{14}C-FPP	Pea CFE[53]	90% Farnesols (ratio 2*E*:2*Z* was 5:1); some nerrolidol and phytol
^{14}C-GGPP	Pea CFE	Phytol and farnesols

The labelled FPP and GGPP substrates were found to be contaminated with GPP since control experiments (hydrolyses) gave small quantities of the respective alcohols of each substrate.

2.2 Levels of Incorporation of 1-^{14}C-IPP into those Products formed by Cell-free Extracts of *L. angustifolia*

2.2.1 Comparison of the Biosynthetic Capabilities of Extracts Prepared from Different Cell-lines of *L. angustifolia*

Cell-free extracts were prepared from callus-material of the three cell-lines (A,B,C; in stationary-phase of growth) B and C of which had been maintained for 10 passages since initiation and line A, which was four years old. Our methods were slightly different from those used by DGW. In particular, incubations were routinely carried out under hexane (1 cm^3) to facilitate continuous removal of labelled products as they were formed and reduce losses due to volatilisation. The incorporation levels are shown in Table 2.9 together with the protein assay of each extract (determined by the method by Bradford; see 14.3).

The products of each incubation were co-chromatographed against phytol and a distillate of lavender oil and they were assayed by LSC (see 14.5). A record of the TLC plate was made by autoradiography.

Table 2.9 Comparison of the Biosynthetic Capabilities of Cell-free Extracts Prepared from Cell-lines A,B,C of *L. angustifolia*

	A	B	C
Protein Assay ($mg.cm^{-3}$)	0.5	0.4	0.7
Total Incorporation (%)	15.1	11.0	3.0
C_5	2	1	2
2*E*-Farnesols] 2*Z*-Farnesols]	80	74	53
Humulene	12	15	8
C_{15}- hydrocarbon	2	1	0
Caryophyllene	1	1	2
cis-Ocimene/Myrcene	0	1	15
β-Pinene/Limonene	0	<1	1

$2\sigma = \pm 10\%$ (all values are an average of four experiments)

The incorporation levels (for culture line A) are similar to those of DGW. Extracts prepared from our recently-initiated callus (line C) showed the lowest overall incorporation of the substrate (3%). All three extracts incorporated most of the substrate into the 2*E*-farnesols with smaller levels of incorporation into the 2*Z*-farnesols. Extracts of lines A and B also incorporated the substrate (12-15% of total) into a product which co-chromatographed with humulene in all three TLC systems and a second product (*ca.* 2% of total) of a compound which remains unidentified but which eluted to within R_f 0.04 of humulene in all three TLC systems (possibly an isomer of humulene *eg.*, isohumulene, farnesene or germacrene). We could not detect this compound in an extract of the steam distillate of lavender oil. All these extracts produced small amounts (*ca.* 1% of total) of the C_{15} hydrocarbon caryophyllene, together with the monoterpenoid hydrocarbons *cis*-ocimene, myrcene, limonene and β-pinene. The incorporation of the substrate into the monoterpenoid hydrocarbons was significantly greater (*ca.* 15% of total) in extracts of line C.

2.2.2 The Dependency of Incorporation Levels on the Optimum Conditions for Preparation and Assay of Cell-free Extracts

The purpose of this experiment was to determine (i) the optimum stage of growth of callus from which to prepare a cell-free extract, (ii) the optimum incubation time with the substrate using extracts prepared from cell-line A.

Table 2.10 shows the incorporation levels of the substrate into products formed by cell-free extracts prepared at two-day intervals throughout the growth-cycle. The results for the time-course of incorporation by extracts prepared during the stationary-phase of growth are shown.

Table 2.10 The Dependency of Incorporation on the Stage of Growth of Calli (Line A)

Day	1	3	5	7	9
Protein Assay ($mg.cm^{-3}$)	0.2	0.2	0.3	0.2	0.3
Total Incorporation (%)	4.0	4.5	4.1	7.5	6.4
C_5	2	4	2	4	2
2*E*-Farnesols] 2*Z*-Farnesols]	90	86	90	86	86
Humulene	2	8	4	8	8
C_{15} -hydrocarbon	1	1	1	1	1
Caryophyllene					
cis-Ocimene/Myrcene	3	0	0	0	0
β-Pinene/Limonene	2	1	3	1	3

All incubations 180 mins.
$2\sigma = \pm$ 10% (all values are an average of four experiments)

Protein assays were routinely 0.2-0.3 $mg.cm^{-3}$ throughout the experiment. The protein assays did not reflect the increase in enzyme-activity over the growth period; the total incorporation of the substrate increased from day 1 (4%) to day 14 (8%). The major products were the 2*E*-farnesols, into which were incorporated most of the label (80%)

in all extracts. Notably, the incorporation of the substrate into humulene and the monoterpenoid hydrocarbons was least in extracts prepared from cultures in the lag-phase of growth. The high incorporation of the label into the farnesols may reflect the role of FPP as a precursor in the sequence FPP → squalene and phytosterols (a sequence which is known to play a regulatory role in cell-wall biosynthesis).[53] We carried out incubations of the extract with the substrate for 15, 30, 45, 120 and 180 minutes. Negligible incorporation occurred in the first 60 minutes of incubation, after which the levels rose steeply and became constant at 180 minutes.

2.2.3 The Incorporation of ^{14}C-IPP into Terpenoids by Different Cell-fractions: Preliminary Zonal Studies

This experiment describes an attempt to associate different cell-fractions with the biosynthesis of particular types of terpenoid.

The subcellular compartmentalisation of terpenoid biosynthesis was mentioned in 1.3. There is plenty of experimental evidence for the association of certain organelles with the synthesis of specific classes of terpenoids although the compartmentalisation of terpenoid biosynthesis may well vary from species to species. No such exploratory work has been carried out for lavender or similar herbaceous species so we prepared four fractions enriched in different cellular organelles from our cultures of *L. angustifolia* (line A).

The cell-fractions were separated by differential centrifugation (12.2d) and the respective pellets re-suspended in incubation buffer and mixed with the substrate in the usual way (14.1). The centrifugation force and protein assays for each fraction are shown in Table 2.11.

The volume of each incubation was adjusted to give a protein concentration of 0.4 mg.cm^{-3} (*ie.*, to match the lowest assay and therefore standardise all incubations). Table 2.12 shows the percentage incorporation of the substrate into terpenoid products by the various cell-fractions.

Table 2.11 Protein Assays of Cell-fractions that were Isolated by Differential-centrifugation (Line A)

Fraction	Centrifugation Conditions		Protein Assay
	g x 1,000	time (min)	$mg.cm^{-3}$
Plastids	3	10	0.86
Mitocondria (i)	10	15	0.64
(ii)	15	10	
Post-mitocondrial/light ribosomes	50	60	0.40
Supernatant	50	60	0.60
(a + b + c)†			0.85
a + b + c in (d)			0.50

All protein assays adjusted to 0.4 $mg.cm^{-3}$ prior to incubation with substrate.
† Suspended in incubation buffer

Table 2.12 The Incorporation of 1-^{14}C-IPP into Terpenoids by Different Cell-fractions: Preliminary Zonal Studies (Line A)

	Plastid	Mitochondria	Post-Mitochondria	Supernatant	All
Total	2.9	<1.0	1.3	1.1	<1.0
C_5	8	19	6	22	19
2*E*-Farnesols] 2*Z*-Farnesols]	83	59	20	66	52
Humulene	5	4	5	0	12
C_{15}-hydrocarbon	0	0	4	6	0
Caryophyllene	1	7	65	3	5
cis-Ocimene/Myrcene	1	-	0	0	5
β-Pinene/Limonene	2	11	0	3	7

$2\sigma = \pm$ 10% (all values are an average of four experiments)

The plastid-fraction gave the largest incorporation into the 2*E*-farnesols (78% of incorporated label). The most significant result was the large increase of incorporation into the caryophyllene by the post-mitochondrial (105,000 x g) pellet. This pellet may have contained the so-called light ribosomes of the endoplasmic reticulum (ER). Note that the incorporation of the substrate into the 2*E*-farnesols is correspondingly lower in this microsomal-enriched fraction. From these results it appears that a sesquiterpenoid cyclase is present in the cell-fraction. The low incorporation of the substrate into caryophyllene by the supernatant (from 105,000 x g) suggests that sesquiterpenoid cyclase and dehydrase activity in *L. angustifolia* is associated with structures of the cell that form a pellet below 105,000 x g (possibly the light ribosomes). Microsomal fractions are normally isolated at 100,000 x g. The supernatant gave the largest incorporation of the substrate into the C_5 alcohols (*ie.*, the smallest prenyltranferase activity) and indeed gave the lowest overall incorporation level.

It is not profitable to extend the conclusions of this experiment beyond the limits of its accuracy. Clearly the cell-fractions were only enriched (not pure) because they all gave incorporation of the label into the products that were characteristic of the entire cell-free system.

2.2.4 The Enhancement of Incorporation Levels of 1-^{14}C-IPP by Additives to the Cell-free Extracts

The previous experiments aided the selection of optimum conditions from which to prepare an extract and have indicated with which parts of the cell the compounds that were analysed in section 2.1 are associated. The following set of experiments were carried out to verify the effects observed by DGW of exogenous additives on incorporations of the tracer into the terpenoid products. The experiments were carried out four times with extracts prepared from each cell-line.

Cell-free extracts of the three lines of *L. angustifolia* (from the calli in the stationary-phase of growth) were prepared and the resultant protein assays were found to be 0.4-0.6 mg.cm^{-3} (lines A and B) and 1.0 mg.cm^{-3} (line C). The additives shown in Table 2.13 were added to different aliquots (1 cm^3) of each extract 30 minutes prior

Table 2.13 The Enhancement of Incorporation (as % of Total) of 1-^{14}C-IPP by Additives to the Cell-free Extract (Line A)

	Control	NADPH	NADP	NAD	ATP	(NADPH + NAD + ATP)	2,4-D
Total Incorporation (%)	**6.5**	**4.5**	**5.2**	**19.3**	**3.9**	**20.4**	**12.1**
C_5	2	2	1	3	1	2	3
2*E*-Farnesols] 2*Z*-Farnesols]	82	48	36	89	77	61	93
Humulene	12	3	30	4	11	1	2
C_{15} -hydrocarbon	2	1	1	3	8	1	2
Caryophyllene	1	19	13	1	1	21	0
cis-Ocimene/Myrcene	0	17	13	0	1	10	0
β-Pinene/Limonene	1	10	6	0	1	4	0

$2\sigma = \pm 10\%$ (all values are an average of four experiments)

to incubation with the substrate to give the final concentration (2 mmol.dm^{-3}). All the incubations were carried out for 180 minutes under a layer of hexane to ensure continuous removal of products. The latter were analysed by TLC with three different solvent systems (12.1a). Autoradiographs were recorded which were compared with the chromatograms for products from the control incubation (line A). The chromatogram (formed by solvent system i; section 12.1a) was assayed for radioactivity by liquid scintillation counting.

Extracts from all three cell-lines showed very similar effects of the additives on incorporation levels of the substrates compared with the control incubation. The results for extracts prepared from line A are shown in Table 2.13. The total incorporation levels (7 %) were increased by NAD and the phytohormone 2,4-D by 3-fold and 2-fold respectively. However, these additives only increased the overall incorporation into the 2*E*-farnesols; few other compounds were produced.

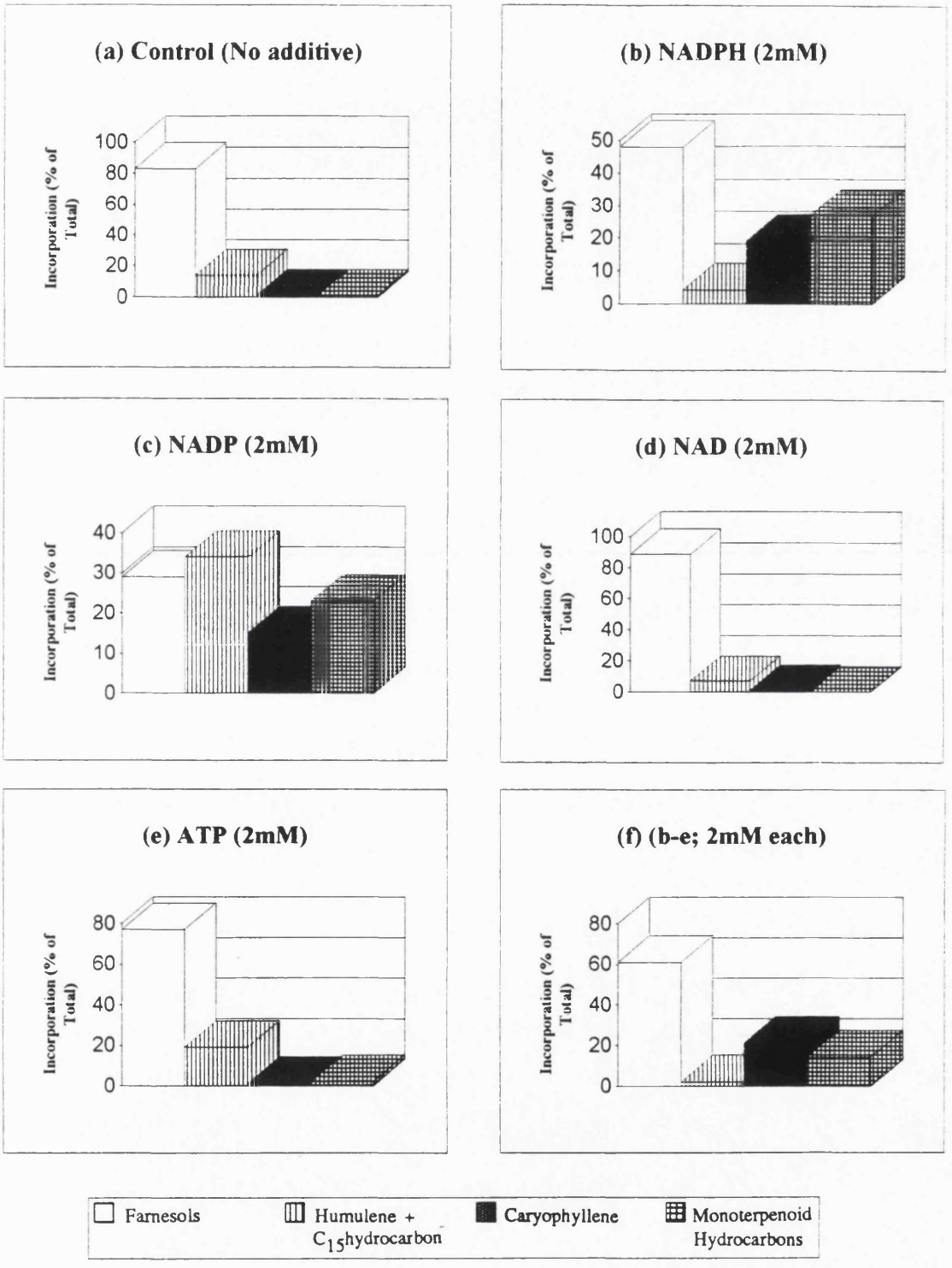
Extracts that were pre-incubated with the cofactors NADP and NADPH did not increase the total incorporation level of the substrate but changed the pattern of products that were formed. These results are summarised as follows:

Table 2.14 Summary of the Effects of NADP and NADPH on Incorporation (% of Total)

Compound	Control(%)	NADP(%)	NADPH(%)
Farnesols	82	29	47
C_{15} -hydrocarbons	16	48	26
C_{10} -hydrocarbons	2	23	27

Total results above are mean incorporations from four experiments with $2\sigma = \pm 10\%$

Diagram 2.1 The Enhancement of Incorporation Levels of 1-^{14}C-IPP into Terpenoids by Additives to the Cell-free Extract



Both cofactors enhanced the levels of the monoterpenoid hydrocarbons but addition of NADP enhanced incorporation into the sesquiterpenoid hydrocarbons, caryophyllene, humulene and an unknown sesquiterpenoid hydrocarbon whereas much less incorporation into the 2*E*-farnesols occurred. The bar charts in Diagram 2.1 show the semi-quantitative differences in the products that were formed. NADPH did not enhance the levels of humulene and the unidentified sesquiterpene hydrocarbon although a decrease of incorporation into the 2*E*-farnesols was observed. These cofactors may operate at the GPP and FPP branch points by funnelling these metabolites into the C_{10} and C_{15} products. The detection of cyclic and acyclic hydrocarbon-products suggests that the cofactors may regulate cyclase and diphosphorylase-dehydrolase activities.

It is possible that the addition of NADPH activates an isomerase-cyclase which is responsible for the sequences GPP → NPP → monoterpenoid hydrocarbon and 2*E*-FPP → 2*Z*-FPP → sesquiterpenoid hydrocarbon. This is a reasonable surmise because the interconversion of the pyrophosphate precursors may involve redox steps. The latter mechanism would very probably be dependent on the ratio of NADP:NADPH concentrations in the extract. Thus, if the rate of the conversion GPP → NPP was increased by the addition of exogenous NADP, the yield of cyclic monoterpenoids could similarly increase. Alternatively, exogenous NADPH could slow down or inhibit the conversion, and thus lead to acyclic monoterpenoid hydrocarbons.

The incorporation results for extracts treated with NADPH verify the effects observed by DGW but they also show that NADP and NADPH have very similar effects on monoterpenoid biosynthesis but different effects on sesquiterpenoid biosynthesis. Incubations containing both cofactors (*eg.*, Diagram 2.1f) show the additive effects of NADP and NADPH. The fact that the increased incorporation into the hydrocarbons (particularly C_{15}) correlate with a significant decrease in incorporation into the farnesols, suggests that the exogenous nicotinamide cofactors do regulate enzyme activity at the cyclase levels, although whether this is as a result of enhanced cyclase activity or suppressed prenyltransferase activity is not clear.

It is impossible to say whether the cofactors regulate the FPP branch-point by inhibiting farnesyl transtransferase and squalene synthetase because the corresponding

C_{15} and C_{30} compounds are not produced by extracts which have not been administered with the cofactors. Indeed NADPH would be expected to enhance squalene synthetase activity.

These cofactors may operate at the IPP → DMAPP level. Changing the ratios of IPP and DMAPP (the alkylating agent), by the addition of exogenous cofactors may well attenuate prenyltransferase (alkylation) and cyclase activities in favour of the latter. At first sight this seems unlikely but Threlfall and Whitehead[54] found that cell-free extracts of *Nicotiana tabacum* that were treated with NADP showed large increases in squalene synthetase activity. Treatment of their cultures with cellulase prior to formation of the cell-free extract resulted in almost exclusive incorporation of the substrates into sesquiterpenoid alcohols and two unidentified compounds with suppression of squalene synthetase activity. The proportion of alcohol was increased by pre-treating the extract with NADPH. The oxygen was thought to arise from molecular oxygen (unlike our extracts, the incubations were not carried out under hexane).

The addition of the phytohormone 2,4-D to our extracts increased the total incorporation by 2-fold (12%) but the mixture consisted mainly of the farnesols (90 %). We found one report in the literature of relevance; Croteau[55] demonstrated that application of cytokinins to leaves of *Lavandula, Mentha* and *Salvia* species caused a 2-fold rise in monoterpenoid content, accompanied by a 20-fold rise in monoterpenoid cyclase levels. In comparison, we have observed an increase in the levels of farnesols produced by an extract treated with an auxin.

2.2.5 Summary and Conclusions

The results of these experiments confirm those obtained by the previous worker DGW. The mature callus of *L. angustifolia* accumulated some monoterpenoids (0.05% w/w; *cf.* 0.5% w/w in the intact plant) although the cell-free extracts produced from the former essentially produced farnesols. The total incorporation (5-6%) found here was lower than that found by DGW (15-17%) in his preliminary work on this topic.

The incorporation level was clearly dependent on the availability of NADP and NADPH. Both these cofactors significantly increased the yields of the sesquiterpenoid hydrocarbons caryophyllene and humulene. DGW also observed an increase in the yield of diterpenoid hydrocarbons although this was not observed in the present study. Similarly, DGW observed that the nicotinamide cofactors increased the overall incorporation by over 10-fold. We found that the overall incorporation remained constant (5-6%) and that the increase of incorporation into the C_{15} hydrocarbons was mirrored by a decrease of incorporation into the C_{15} alcohols, indicating some type of stimulation of dehydrase activity. If the work of DGW was correct it was likely that these cofactors were also stimulating prenyl transferase activity in addition to control at the branch-points of terpenoid biosynthesis.

NAD did increase the overall incorporation by 4-fold and the effects of NAD and NADPH were found to be additive thus giving large incorporation into the C_{15} alcohols and C_{15} hydrocarbons. Our study has also shown that in cell-free systems of *L. angustifolia* the biosynthesis of alcohols is associated with the plastid/mitochondrial fraction of the cell whereas that of the C_{15} hydrocarbons is associated with the post-mitochondrial fraction. At this stage it was decided to break-off the work. The trends and product patterns have been demonstrated and confirmed but conclusive proof of all the products would have involved prohibitive and repetitive labours in the absence of a GC/MS or HPLC available for radiochemical samples. A future study could examine the effects of the nicotinamide cofactors on the separate cell-fractions and the incorporation of the label into different classes of substrate by these fractions.

PART 2 The Toxicities of some Terpenoids to Tissue Cultures of *Pelargonium fragrans*

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PART 2 The Toxicities of some Terpenoids to Tissue Cultures of Pelargonium fragrans

Chapter 3 Introduction

3.1 Toxic Terpenoids

Many plants and animals possess natural defence mechanisms based on terpenoids that protect them from fungi, insects and animal predators. In addition, many plants produce inhibitors that are terpenoids to prevent the growth of other plant species in the immediate environment. The best examples are the volatile oxygenated monoterpenoids growth-inhibitors from the leaves of *Salvia leucophylla*[56] and from creosote bushes. These inhibitors are so potent that soils in which the shrubs grow are barren and devoid of other plants. Monoterpenoids are well known to be cytotoxic to plants[57] causing a fall in the number of intact mitochondria and Golgi bodies,[56] inhibiting respiration and photosynthesis[58] and decreasing cell-wall permeability.[57] Cyclic monoterpenoids are thought to inhibit HMG-CoA reductase.[59] Previous workers have demonstrated the toxicities of some monoterpenoids to tissue cultures of *Pelargonium fragrans*[57] and in this study we have used the same plant clone as used by the previous workers to make comparisons possible.

Many monoterpenoids are constitutive biocides that accumulate in response to infection or stress and the pinenes[60] are well known examples. Certain species of wild tomato are known to contain toxic sesquiterpenoids within glandular trichomes.[61] Other plants produce biocides in response to infection or physical attack. Myrcene and car-3-ene are produced by tissue cultures of *Abies grandis*[62] infected with fungus. Compounds produced in this way are collectively known as phytoallexins[63] or stress compounds and these are probably multi-site toxicants that disrupt membrane systems, particularly the plasmalemma.[64] Cells killed by treatment with the sesquiterpenoid rishitin[65] were discovered to accumulate rapidly the non-vital stain Evan's Blue as a result of an increase in cell-wall permeability.[66] Some non-mevalonoid phytoallexins produced by *Phaseolus vulgaris*[67] were found to cause inhibition of respiration and subsequent cell-death in cell-suspension cultures of the same plant.

3.2 Detoxification by Biotransformation

The rate at which an organism detoxifies such biologically active terpenoids (if indeed it can) will modify the toxicities of the latter. The reactions that lead to detoxification can be divided into three groups;[68] (i) oxidation, reduction and hydrolysis (ii) conjugation with a single endogenous substrate *eg.*, glycosylation and (iii) the reaction with more than one endogenous substrate.

The latter two processes could increase the water solubility of lipophilic compounds leading to their transport, compartmentalisation and thus detoxification. Such mechanisms could explain the occurrence of monoterpenoid glucosides in whole plants and tissue cultures.[69] Cells in a number of suspension cultures have been shown to glycosylate exogenous terpenoid alcohols in yields of up to 70%.[70] Rose petals are known to contain high concentrations of monoterpenoid glucosides. This may be due to the lack of specialised storage cells[71] associated with monoterpenoid biosynthesis in *Rosa* species. The glycosylation of 2-phenylethanol by cultures of *Rosa* species has also been shown to occur.[72] Such detoxification may be viewed as a special case of biotransformation *ie.*, the process whereby exogenous metabolites are (claimed to be - see Chapter 7) enzymatically modified by addition to plant cell-cultures - usually suspensions. There are numerous reported biotransformations of substrates by callus and suspension cultures *eg.*, hydroxylations[73] reductions of aldehydes to alcohols,[74] isomerisations,[75] oxidations,[76] double bond saturations[77] and ring-openings.[78]

3.3 "Biotransformation" or Reaction with the Medium?

Although biotransformations were not the subject of this work, it is worth mentioning some factors that we studied, that could in principle account for some of the reactions listed in the last paragraph. Could some "biotransformations" be brought about by the pH or by the components of the tissue culture medium, rather than by the cells? And indeed, does the pH of a culture generally vary during growth? This is a pertinent question as attempts to grow cultures in buffered media have not been very successful.[79a,b] Does the culture medium behave as a solution capable of supporting redox reactions? (the formulation contains the ions, Mn^{2+}, Mo^{6+}, Cu^{2+}, Co^{2+}).

Some workers[80] have found that aged samples of monoterpenoid hydrocarbons contain high concentrations (up to 0.4 $mol.dm^{-3}$) of peroxides or hydroperoxides. Such compounds are not always readily detectable by GLC (probably because they decompose at the high injector temperatures or on the column and thereby lead to a broadened peak in the GLC trace). Thus, a culture administered with a monoterpenoid contaminated with peroxides may well yield a product-alcohol, leading an observer to conclude that a biotransformation of a hydrocarbon to an alcohol has occurred when in fact a simple reduction of the peroxide effected by the culture medium has taken place. Indeed the unsuspected peroxide may kill all or a significant fraction of the cells.

3.4 The Provision of a Sink to Accumulate Secondary Metabolites Produced by Suspension Cultures

One way of lowering the toxicity of an end-product or preventing its detoxification *via* biotransformation is to add a "sink" to the culture medium which can perform the function of the storage cells in the intact plant - storage cells that are usually lacking from the culture. This also helps in the isolation and convenient collection of any oil from the culture.

Those aspects of differentiation responsible for the synthesis, transport and accumulation of secondary products in the whole plant and *in vitro* have been described in section 1.3. Although a wide variety of secondary metabolites have been isolated from tissue cultures grown on solid media,[81] fewer compounds have been isolated in appreciable yields (compared with the whole plant) from cell-suspensions. Ozeki[82] has attributed this phenomenon to a lack of metabolic differentiation required for secondary product-synthesis. However, a number of cell-suspensions are known to possess the batteries of enzymes responsible for the biosynthesis of a wide range of natural products.[83] Immobilisation of suspension cultures on inert polymer supports is known to encourage aggregation and differentiation of cells which may be responsible for the synthesis and storage of secondary metabolites.[84a,b] The manipulation of phytohormone-levels to encourage organogenesis of storage tissue (*eg.*, hairy root cultures) is also a standard method of eliciting product-formation, but neither of these methods necessarily overcomes the problems of toxicity, bioconversion

and instability of the end-products within the culture medium. As a consequence of this, methods have been developed to grow cultures in media containing a second-phase (solid or liquid) for the continuous accumulation of compounds excreted by the plant cells; this sink provides an equivalent of differentiation that is required for the storage of secondary products. Yoshikawa[85] demonstrated that a small quantity of agar powder added to suspension cultures of *Lithospermum* species induced the accumulation of a shikonin pigment that was characteristic of cultures of the same plant that had been grown on solid media. The addition of activated charcoal to suspension cultures of *Lithospermum* species induced the formation of a benzoquinone.[86] The presence of activated charcoal was thought to remove toxic phenolic substances[87] that accumulate in tissue cultures and inhibited differentiation.

The addition of an inert lipophilic phase to suspension cultures of *Matricaria* species[88] is an effective method for enhancing the accumulation of terpenoids by partitioning them (depending on their polarity) between the aqueous medium and the secondary phase. The most commonly used liquid is Miglyol 812 (Dynamit, *ex.* Nobel Industries; a triacylglycerol with C_8 and C_{10} fatty acid chains)[89] although hexadecane has recently been shown to perform the same function.[90] Cultures of *Thuja occidentalis* have produced increased yields by up to 3-fold of monoterpenoids in the presence of a secondary phase of hexadecane. The use of a lipophilic phase of Miglyol in biotransformation-studies has facilitated extraction of newly-formed products which are not obtained in single-phase cultures that have been treated with the same substrates as controls.[91] Claims that biosynthesis and metabolism can be induced in two-phase cultures may be explained by the accumulation of products that usually evaporate from a single-phase culture (*eg.*, see 7.3).

However, problems with the aeration of two-phase suspension cultures and product-separation have yet to be overcome.[92] Becker[93] has recently shown that a modified silica gel used in reverse phase HPLC columns (Lichroprep R8, Merck, Dorset) is effective in stabilising products that are excreted into the culture medium[70] and which would otherwise break down.

3.4.1 The Potential of Surfactant Micelles to Accumulate Secondary Metabolites in Single-phase Cultures

We considered the use of surfactant micelles ("surface-active agents") could provide a storage mechanism for terpenoids synthesized *de novo*, in addition to providing a means of administering substrates to cell-cultures for biotransformation. This appears to be a novel approach. Some surfactants are known to form micelles at very low concentrations and if growth of tissue could be sustained in cultures containing these surfactants the latter could provide an efficient sink within a single-phase system for the continuous accumulation of products.

Surfactants have been traditionally used in biochemistry for the solubilisation of membrane proteins[94] and they are known to cause an increase in permeability of cell-membranes.[95] In addition, most surfactants can solubilise a wide range of small organic molecules in aqueous conditions. We considered that these two properties may increase the biosynthesis, excretion and accumulation of terpenoids (and other secondary metabolites) in suspension cell-cultures when grown in media containing surfactants, especially as some surfactants are capable of solubilising oils at very low concentrations (*eg.*, 0.0002 mol.dm^{-3} of surfactant in water).

Surfactants are amphiphilic substances having hydrophilic and hydrophobic parts. When they are mixed with aqueous solutions they associate into organised, roughly spherical aggregates called micelles. This process results from the entropy-unfavourable contact between water and the hydrophobic part of a surfactant. Micelle-formation occurs at a well defined concentration, the critical micelle concentration (c.m.c). These micelles are transient species but a typical spherical micelle may contain up to 100 monomers. Addition of electrolytes (*eg.*, culture media?) to solutions containing surfactants causes a drastic reduction in the c.m.c., an increase in micelle size and an increased solubilising capacity. Indeed, the selectivity of micelles towards certain substrates can be increased.

Three of the most common types of surfactants (distinguished by the nature of their hydrophilic head groups) are (i) anionic; *eg.*, sodium dodecylsulphate (ii) cationic; *eg.*, trimethylammonium bromide and (iii) polyether; *eg.*, polyoxyethylene-[20]-sorbitol

monolaurate. Recently, zwitterionic[96] and carbohydrate-derived surfactants[97] have become available. We grew cultures of *Pelargonium fragrans* in media containing a range of surfactants (16 in all) from each of the above classes and monitored the cell-viabilities over the growth period.

In conclusion, we see that the toxicity of terpenoids could play a crucial role in:
(i) the establishment of product cell-lines, (ii) the study of biotransformations and (iii) the introduction of two-phase or micellar suspensions for harvesting secondary products
Hence, reproducible methods for determining toxicities (*ie.*, the measurement of cell-populations after different treatments) is essential before qualitative assessment of (i)-(iii) can be made.

Chapter 4 Results and Discussion: Toxicity Studies Using Terpenoids as Additives to Suspension Cultures

Aims: This chapter describes a set of novel experiments to measure the toxicities of nine available terpenoids and to study; (i) at which stage in the growth-cycle of the culture the toxic effect is greatest; (ii) which class of terpenoid is most toxic; and (iii) which functional group endows the most toxicity on the molecule.

We start with the methods used to monitor cell-viability, including a new method for counting individual alive and dead cells in a single sample of cells removed from culture. The statistical methods used are detailed in the experimental section (13.3b).

4.1 Determination of the Growth Rate and Viability of Suspension Cultures of *P. fragrans*

Numerous methods have been developed to measure the growth and metabolism of tissue cultures. We decided to use (i) cell-counting with a haemocytometer to estimate the total number of cells per unit volume of culture; (ii) counting of viable and non-viable cells within a single population of cells (iii) fresh- and dry- mass measurements (commonly referred to as fresh- and dry-weights); (iv) total-culture mass measurement; and (v) packed cell-volume. The experimental details are given in section 13.3a. Methods (i) and (ii) had the advantage of monitoring the growth of a single culture throughout its passage of the culture-cycle and therefore provided the best methods for screening the effects of exogenously administered compounds. A summary of the statistics described in section 13.3b is as follows:

Table 4.1 Glossary of Terms

Term	Symbol	Comments
Population Mean	μ	Population refers to the whole culture. Subscripts denote live (x) and dead (y)
Sample Mean of Live Cells	**X**	Sample refers to the aliquot of cells removed from the culture
Sample Mean of Dead Cells	**Y**	
Sample Variance	**s**	Subscripts denote live (x) and dead (y)
Population Standard Deviation	σ	As above
Population Proportion	**P**	As above and expressed as percentage viability when referring to the population proportion of live cells (P_x)
Sample Proportion	*p*	As above
Number of Experiments	**n**	Each sample was divided into n smaller samples
Number of Cells Counted	**N**	used with haemocytometer

For greater clarity when describing means and proportions, the nouns "sample" and "population" may be used as adjectives (*eg.*, sample means, population proportion)

4.1.1 Estimation of Cell-numbers

(i) Estimation of Population Proportions of Viable and Non-viable Cells using a Novel Cell-dissociation and Staining Technique.

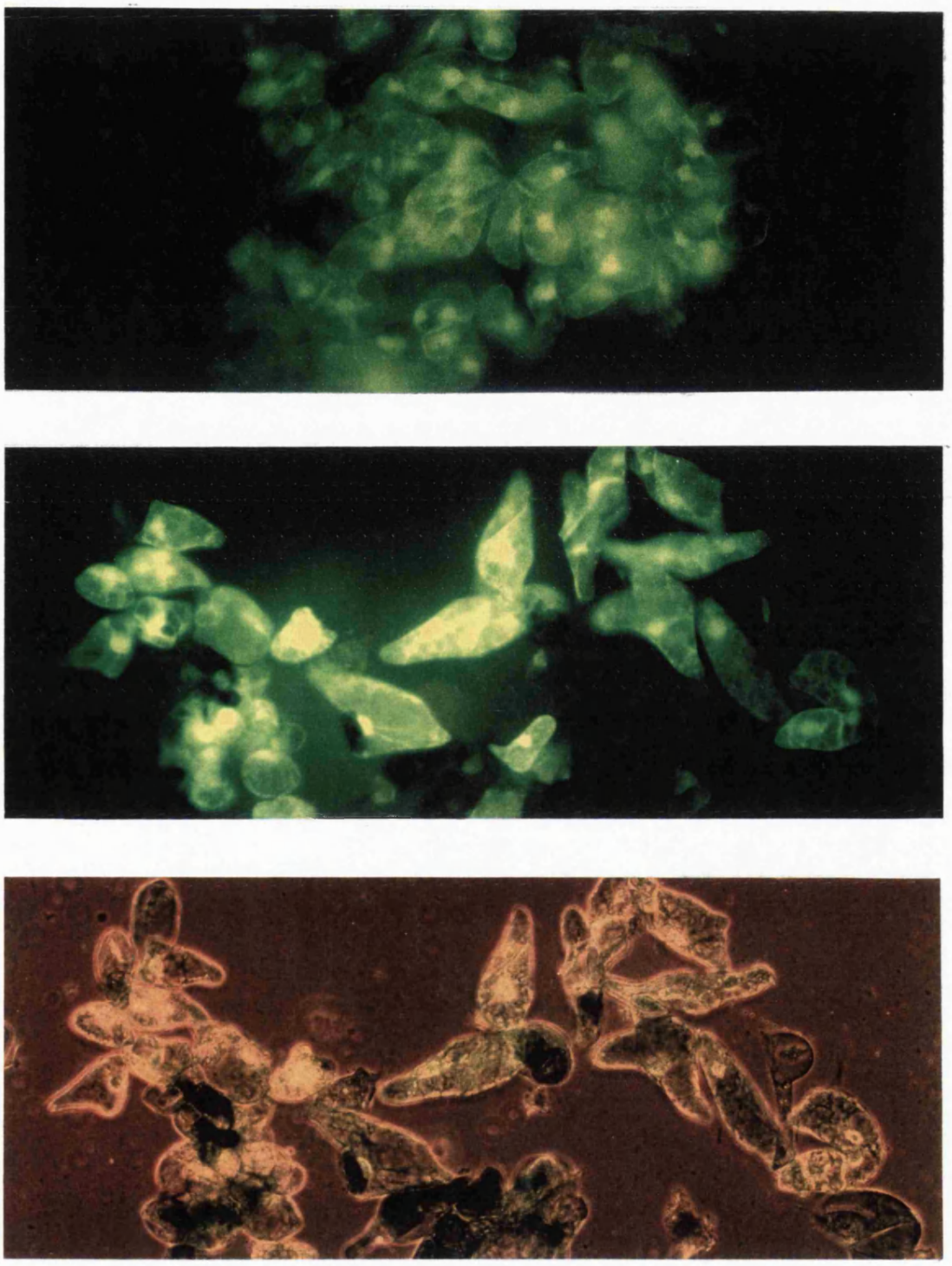
Previous workers[98] have found that suspension cultures that grow as clumps (*ie.*, suspended callus rather than fine cell-suspensions; *eg.*, from rice and soybean) were difficult to monitor by cell-counting. Our cultures of *Pelargonium fragrans* were no exception; stock lines (on medium described in section 13.1) grew as green undifferentiated clumps of approximately 0.5 cm diameter (although cultures habituated to a sub-lethal dose of caryophyllene or grown in medium containing

Table 4.2 Estimated Population Means (μ) and Population Proportions (P) of Live (x) and Dead (y) Cells from Suspension Cultures of *P. fragrans*

Day	X	s_x	Y	s_y	n	μ_x	μ_y	P_x(%)	P_y(%)
2	313	121	54	23	6	X ± 97	Y ± 19	85 ± 1	15 ± 1
4	315	145	65	16	6	X ± 117	Y ± 13	83 ± 1	17 ± 1
6	679	202	125	46	4	X ± 237	Y ± 54	84 ± 1	16 ± 1
8	840	223	150	58	4	X ± 262	Y ± 68	84 ± 1	16 ± 1
10	1619	761	202	127	4	X ± 895	Y ± 149	89 ± 1	11 ± 1
12	1070	220*	139	120*	1	X ± 1389	Y ± 756	88 ± 1	12 ± 1
14	1215	828	256	128	2	X ± 3734	Y ± 577	83 ± 1	17 ± 1

X ≡ mean number of live cells in samples
Y ≡ mean number of dead cells in samples
s ≡ Variance
n ≡ number of experiments
* Estimated Variance for n = 1
Population proportions (P) are estimated with 99% confidence whereas population means (μ) are estimated with 90% confidence. Note that the sample means (X and Y) are not good estimates of the true population means (μ) in this type of experiment.

Plate 4.1 Dual-staining of Dissociated-cells from a Suspension Culture of *P. fragrans*



Magnification: 2.5 x 20

(a) *Top:* Sample of cells removed from the culture and stained with fluorescein diacetate (FDA) and visualised through blue-light fluorescence filters

(b) *Middle:* Sample of live-cells within a sample of cells that had been removed from the culture and dissociated prior to staining and counting as in (a)

(c) *Bottom:* The same sample of cells as in (b) but viewed under white-light to visualise the dead cells that are stained by Evan's Blue

surfactant micelles both yielded much more dispersed cell suspensions (*ca.* 2mm diameter; see sections 4.2.3 and 4.2.4). One solution to this problem is to boil the cells in solutions of hydrochloric or perchloric acids[79b] which separate the cells, but such treatment obviously yields only a total cell-count because the cells are killed in the process. We considered using an enzymatic method (cellulase and maceroenzyme)[99] but the method is costly and time consuming (*eg.*, the time between removing a sample of cells and counting them was at least one hour). Consequently, we decided to use a (balanced salt) cell-dissociation fluid (*ex.* Sigma, Poole, Dorset) which is claimed to produce fine cell-suspensions (*ca.* < 2mm aggregates) when added to cultures. The manufacturers would not provide us with the composition of this solution, but we think it contains EDTA which is known to dissociate cell-clumps.

We developed a rapid method of using this solution to produce fine suspensions of individual cells and small cell-clusters of no more than 10 cells. We attempted to add the solution directly to the cells as they were viewed under the microscope but this resulted in severe plasmolysis. The optimum method involved removal of a small sample (*ca.* 5 cm^3) of freshly agitated culture and replacement of some of the medium (*ca.* 2 cm^3) with dissociation fluid. Gentle agitation by shaking or by bubbling argon through the sample produced a suspension of dissociated cells within 5 minutes. Full experimental details are given in section 13.3a. The cells could be stained with fluorescein diacetate (FDA) and live cells counted with a tally counter. Our attempts to use dual-staining of the cells with FDA and Evan's Blue provided an excellent method for obtaining (i) a count of viable cells (ii) a count of non-viable cells and (iii) a total cell count from a single-sample of the population. Plate 4.1a shows viable cells (fluorescent green) removed from a suspension of *P. fragrans* (on day 8 of a 14 day growth-cycle) that have been stained and viewed under the microscope (12.2e; system 2). Plate 4.1b shows a second sample of cells (from the same culture), which have been dissociated prior to staining and Plate 4.1c shows this same sample viewed under white light to show the non-viable (blue) cells. In fact, the fluorescent cytoplasma of the viable cells can still be observed under white light. A microscope slide containing 12 counting wells (*ex.* Flow Labs, Dewsbury) was used to count 12 samples such as those shown in plate 4.1(b) 4.1(c) from which the mean viabilities and population proportions (within a 99% confidence interval) could be

calculated for viable and non-viable cells. Table 4.2 gives the mean values (X and Y) for alive and dead cells respectively, together with the population proportions p_x and p_y. From this data it is clear that the culture maintained 80-90% viability over the 7 sampling intervals (14 days). As our results indicate, the large sample sizes used yielded estimated population proportions to within 0.5% using a 90% confidence interval and to within ± 1% with 99% confidence. Previous workers[57] estimated population proportions (as per cent) from 6 replicate measurements of 100 (n=600) cells chosen at random. By application of the equations in section 13.3b their results have a sampling error of between 6% and 10% (for 99% confidence).

(ii) Estimation of Total Population of Living Cells (per cm^3) of Culture using a Haemocytometer

The method developed in (i) was adapted as described in 13.3c so that an estimate of the mean number of living cells (per cm^3 of culture) could be obtained at successive intervals of two days in order to determine the true growth-curve of the culture. Previously, the haemocytometer has been used only to determine the total cell-count (per cm^3) from heat and acid-treated cells.[79b] Five counts were recorded at each interval from which the estimated mean numbers of living cells (per cm^3 of culture) were calculated. These are shown in Table 4.3 and graphically in Diagram 4.1.

Diagram 4.1 Estimated Mean Numbers of Live Cells (per cm^3) in a Suspension Culture of *P. fragrans*

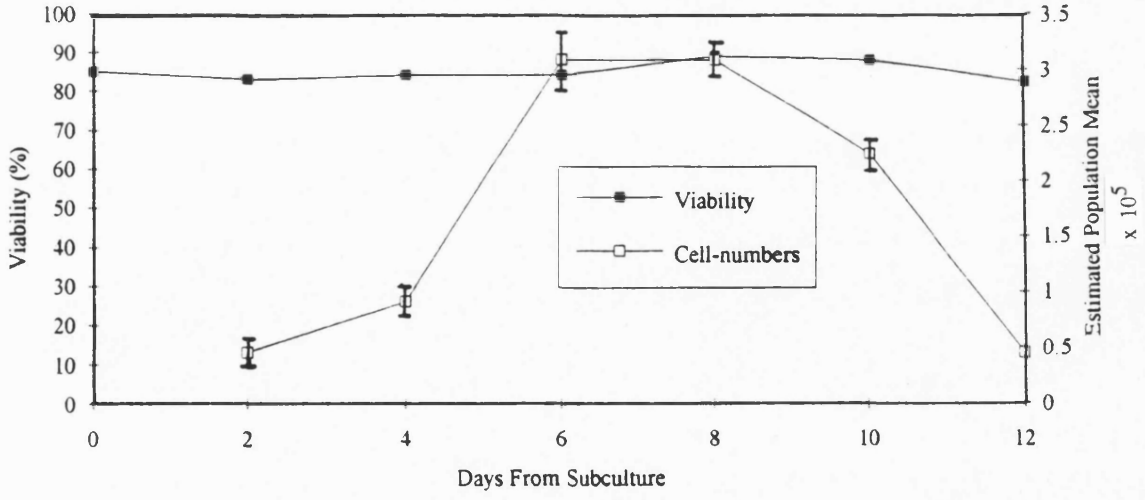


Table 4.3 Sample Mean and Estimated Population Mean Numbers of Live (x) Cells from a Suspension Culture of *P. fragrans*

Day	n	X	s_x	μ_x (per cm^3 of culture)
2	5	5.0×10^4	8.9×10^3	$(5.0 \pm 8.5) \times 10^3$
4	5	9.4×10^4	1.4×10^4	$(9.4 \pm 1.3) \times 10^4$
6	5	3.1×10^5	1.2×10^5	$(3.1 \pm 1.1) \times 10^5$
8	4	3.1×10^5	5.4×10^4	$(3.1 \pm 6.4) \times 10^4$
10	5	2.3×10^5	3.9×10^4	$(2.3 \pm 3.7) \times 10^4$
12	7	1.4×10^5	1.7×10^4	$(1.4 \pm 1.2) \times 10^4$

n ≡ number of experiments
X ≡ mean number of live cells in samples
s_x ≡ sample variance of live cells
μ_x ≡ Mean number (estimated with 99% confidence) of live cells in the culture

Notice the exponential-phase of growth between days 4 and 6 after which the numbers of living cells remained steady until day 8. From day 8 onwards the number of living cells declined. The error bars drawn in Diagram 4.1 show this was a real effect and this is not surprising since the graph represents the counts of only living cells, and not the total cell-density. A graph of the latter would be expected to show a stationary-phase extending from day 6 to day 12 since the number of cells remains constant once the stationary-phase is reached even though the proportion of living cells decreases. Perhaps the growth-curve of a tissue culture should be redefined in respect of this. For example, it is generally supposed that secondary product-formation occurs during the stationary-phase; *ie.*, the enzymes required for their biosynthesis are "switched on" when mitosis is complete. From Diagram 4.1 the maximum number of living cells occurred at day 7 and therefore secondary product-synthesis may have been maximal at this interval and not at day 10 (during the stationary-phase).

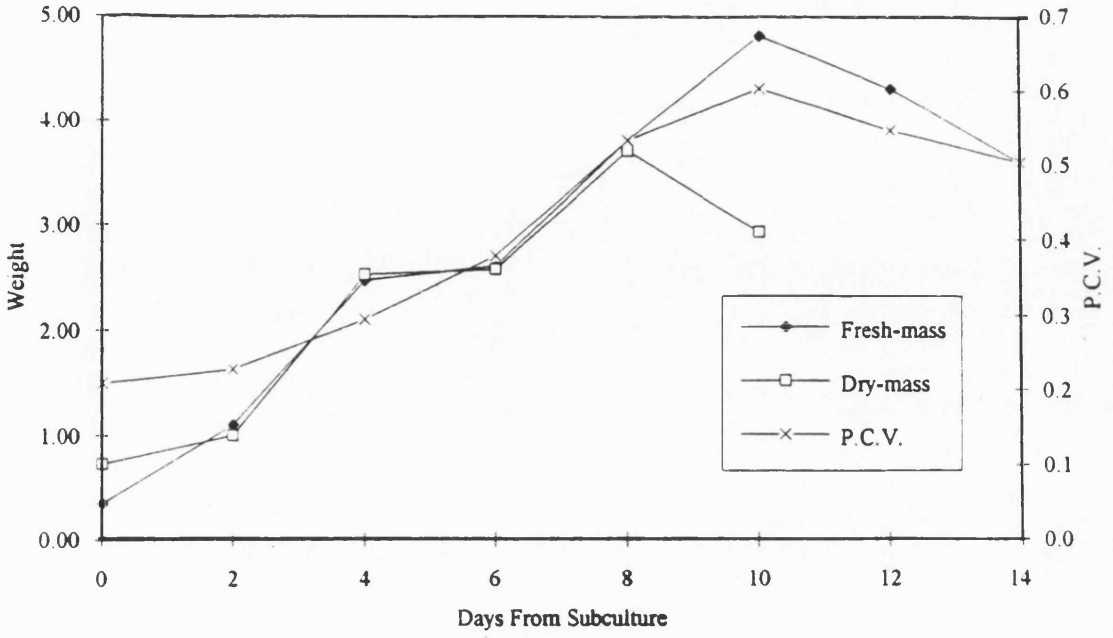
4.1.2 Determination of Growth Rates by Measurements on Fresh- and Dry-Masses and Packed-cell Volumes

Measurements were recorded at intervals of 2 days as given in section 13.3e. Since our initial aim was to select a non-destructive technique of monitoring growth that could be used repeatedly on a single culture throughout its passage, we decided to keep a record of total culture-weight at 2-day intervals for 14 days from the date of subculture. This simply involved weighing the flasks and their contents. Three culture-populations were prepared by subculturing a single culture using a pipette fitted with one of three dispensers cut to a specific diameter, so that one flask contained an inoculum of fine cells and another flask contained an inoculum of larger cell-clumps. Four flasks of each culture-type were prepared (together with four control-flasks that had been subcultured in the normal way; see section 13.2b). They were then re-weighed at intervals of 48 hours (± 2 hours). This experiment was carried out on suspension cultures of *Lavandula officinalis* and *Pelargonium fragrans*. The culture of lavender had a more heterogenous spread of cell-clumps (*ie.*, < 1mm to 5mm). No increase in weight was observed over the 14-day period; in fact all the cultures decreased in weight by approximately 4g. Thus, we turned to the more tedious determination of packed cell-volume (PCV) and fresh- and dry-masses. The results are shown in Diagram 4.2. The dry-masses must be considered with some caution since sucrose derived from the medium may contribute to the values.

The results correlate with the measurements of cell-viability across the growth-cycle (4.1.1.i) although the stationary-phase was not reached until days 9-10. Counting of cells estimates the rate of increase in cell-numbers (max. by day 7) whereas fresh-mass and PCV measurements monitor the growth-rate (equivalent to the increase in cell-number and in cell-size) of the culture. Thus, the maximum cell-number may be reached after 7 days, followed by cell-growth during days 8-10. We chose measurement of cell-number (of live cells) and estimation of population proportions as reliable indicators of culture-growth. In some of the following experiments, fresh- and dry-mass measurements were recorded to support our conclusions.

Up to this point we have developed and calibrated the cell-counting method and statistics so that the growth of the cell-population can be followed over a culture cycle. These methods could be used in the assay of the toxicities of terpenoids, surfactants and other additives to the cultures.

Diagram 4.2 Fresh- and Dry- Masses and Packed Cell-volumes from a Suspension Culture of *P. fragrans*



4.2 Viabilities of Suspension Cultures of *P. fragrans* after Treatment with Terpenoids

A sample of limonene (B.B.A. Ltd., London) was shaken with a saturated solution of sulphite (13.4a) to reduce any contaminating peroxides to the corresponding alcohols. The sample was then purified by column-chromatography (12.1b, system 1) and the solvent removed to yield the purified monoterpenoid. When analysed by TLC (12.1a; system 1.i) the sample showed a major spot corresponding to limonene and 2 minor spots at low R_f probably corresponding to traces of alcohol. When a second TLC plate was developed with a spray reagent (ii;12.1a) neither of these spots turned red, confirming that the sample was peroxide-free.

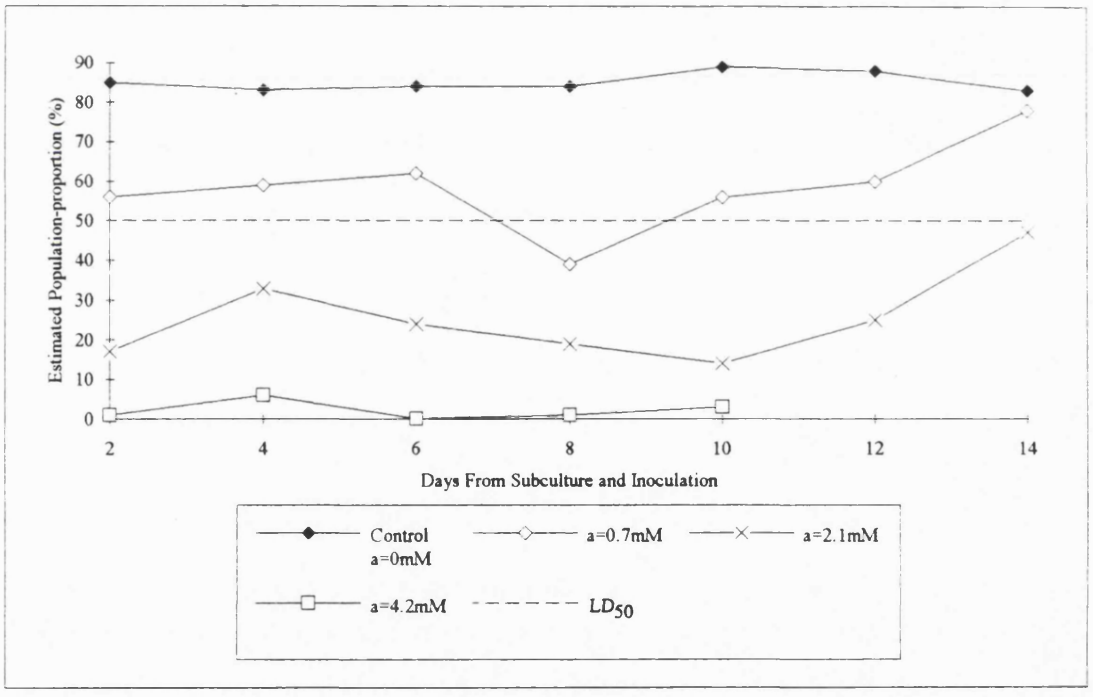
4.2.1 Viabilities of Cultures after Treatment with Limonene during the Lag-phase of growth.

Three cultures of *Pelargonium* were freshly subcultured and administered with different quantities of limonene (in freshly redistilled methanol as described 13.4b) to give final "concentrations" of 0.7mM, 1.5 mM and 4.2mM). Control cultures

containing (i) an aliquot of methanol, and (ii) MS medium containing neither methanol or limonene were incubated with the three test-flasks.

Dual-counts (*ie.*, in the same sample) of alive and dead cells were recorded (section 13.3a) from samples of the culture taken at intervals of 2 days from the date of subculture to the end of the growth-cycle (14 days as determined in section 4.1.1). The number of cells counted ranged from *ca.* 1500 (day 2) to *ca.* 7000 (day 10) *ie.*, depending on the stage of growth of the culture. Table 4.4 shows the calculated sample means and proportions of live and dead cells for each interval and dose of limonene. These viabilities are shown graphically in Diagram 4.3.

Diagram 4.3 Estimated Proportions of Live Cells in a Suspension Culture of *P. fragrans* that had been treated with Limonene (a_{mM}) at Subculture (Day 0)



Measurements recorded for a control population (untreated culture) are also shown. Clearly, at the 4.2 mM level the terpenoid was toxic, resulting in estimated viabilities of less than 10%. Cultures administered with the two sub-lethal doses of limonene

(administered at day 0) showed the same pattern of cell-viability over the growth-cycle; although these cultures were inoculated with the oil during the lag-phase of growth, the toxicity was not realised until the period of exponential-growth began whence the viability decreased to two-thirds of the value at the lag-phase. It then steadily increased once the stationary-phase of cell-growth was reached. Thus, although any dose of limonene decreased cell-viability compared with a control culture the effect was most marked during the period of cell-division and possibly cell-growth. Another way of expressing this is to say that the LD_{50} for limonene increased with the age of the culture; by the end of the growth-cycle the dose of limonene must be increased by 3-fold in order to kill half the population. This suggests that prolonged treatment of a cell-line with a terpenoid selects a sub-population of cells that is capable of storing it or else breaking it down.

Table 4.4 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with Limonene (a mM) on Day 0

Control (a = 0 mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	2.2×10^3	0.85	0.15	85 ± 1	15 ± 1
4	2.2×10^3	0.83	0.17	83 ± 1	17 ± 1
6	3.2×10^3	0.84	0.16	84 ± 1	16 ± 1
8	4.2×10^3	0.84	0.16	84 ± 1	16 ± 1
10	7.3×10^3	0.89	0.11	89 ± 1	11 ± 1
12	1.2×10^3	0.88	0.12	88 ± 1	12 ± 1
14	2.9×10^3	0.83	0.17	83 ± 1	17 ± 1

Limonene (a = 0.7 mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	1.6×10^3	0.56	0.44	56 ± 3	44 ± 3
4	2.5×10^3	0.59	0.41	59 ± 3	41 ± 3
6	2.6×10^3	0.62	0.38	62 ± 2	38 ± 2
8	2.6×10^3	0.39	0.61	39 ± 2	61 ± 1
10†	4.8×10^3	0.56	0.44	56 ± 2	44 ± 2
12	4.6×10^3	0.60	0.40	60 ± 6	40 ± 6
14	2.3×10^3	0.78	0.22	78 ± 2	22 ± 2
16	8.9×10^3	0.81	0.19	81 ± 3	19 ± 3

Limonene (a = 2.1 mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	8.8×10^3	0.17	0.83	17 ± 3	83 ± 3
4	1.5×10^3	0.33	0.67	33 ± 3	67 ± 3
6	1.3×10^3	0.24	0.76	24 ± 3	76 ± 3
8	2.6×10^3	0.19	0.81	19 ± 2	81 ± 2
10	3.5×10^3	0.14	0.86	14 ± 2	86 ± 2
12	6.1×10^3	0.25	0.75	25 ± 5	75 ± 5
14	2.1×10^3	0.47	0.53	47 ± 3	53 ± 3
16‡	1.9×10^3	0.53	0.47	53 ± 3	47 ± 3

Limonene (a = 4.2mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	3.9×10^2	0.01	0.99	1 ± 1	99 ± 1
4	7.4×10^2	0.06	0.94	6 ± 2	94 ± 2
6	7.2×10^2	0.00	1.00	0	100
8	3.3×10^2	0.01	0.99	1 ± 1	99 ± 1
10	5.4×10^2	0.03	0.97	3 ± 2	97 ± 2
12*	-	-	-	-	-
14*	-	-	-	-	-

P ≡ proportion (Estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of cell-type in the samples
N ≡ number of cells counted
† LD_{50} at day 10
‡ LD_{50} at day 16
* Cultures were dead

Day refers to the number of days after subculture when the compound was added to the culture

4.2.2 Viabilities of Cultures after Treatment with Limonene at Different Stages in the Growth-cycle

The estimated population proportions (with 99% confidence) of alive and dead cells are shown in Table 4.5. The viabilities are shown graphically in Diagram 4.4. The effect of adding the terpenoid during the exponential- or stationary-phases of growth was to increase the observed toxicity compared with adding this compound during the lag-phase of growth. If limonene was a potential inhibitor of cell-wall synthesis it would have to be added during the period of rapid cell-division when the cell walls were most susceptible to damage.

Diagram 4.4 Estimated Proportions of Live Cells in a Suspension Culture of *P. fragrans* that had been treated with Limonene (a_{mM}) 48 Hours prior to counting

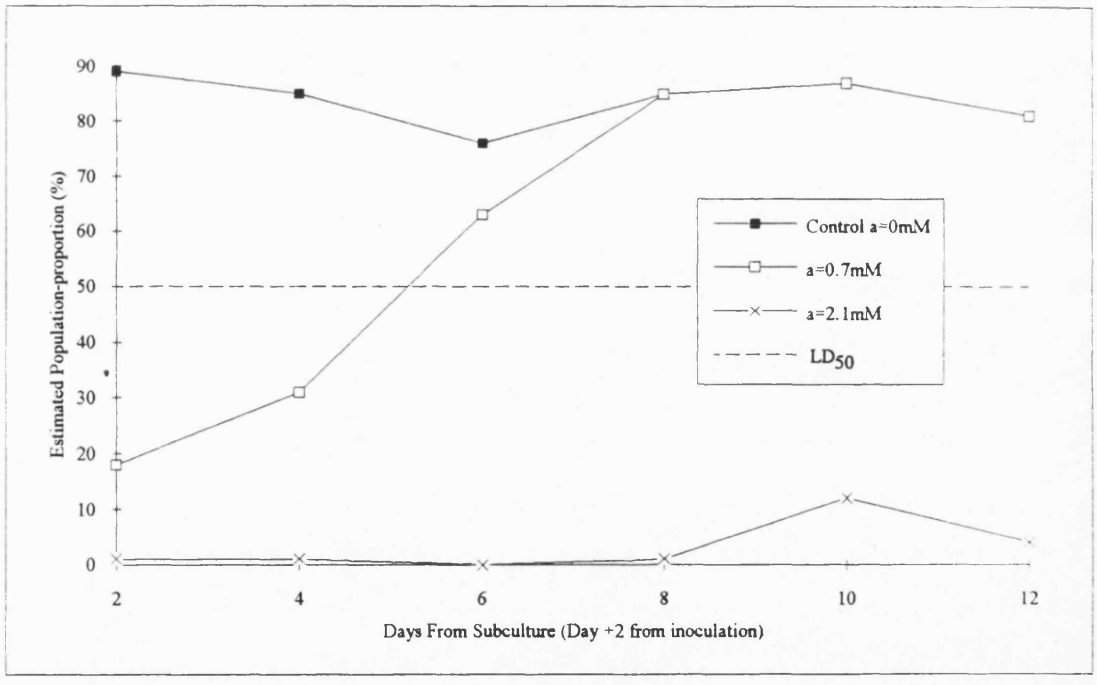


Table 4.5 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with Limonene (a mM) 48 Hours Prior to Counting

Control (a = 0)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	1.1×10^3	0.89	0.11	89 ± 2	11 ± 2
4	5.5×10^3	0.85	0.15	85 ± 4	15 ± 4
6	1.2×10^3	0.76	0.24	76 ± 3	24 ± 3
8	7.7×10^2	0.85	0.15	85 ± 3	15 ± 3

Limonene (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	1.9×10^3	0.18	0.82	18 ± 2	82 ± 2
4	3.0×10^3	0.31	0.69	31 ± 2	69 ± 2
6	2.4×10^3	0.63	0.37	63 ± 3	37 ± 3
8	1.2×10^3	0.85	0.15	85 ± 3	15 ± 3
10	1.5×10^3	0.87	0.13	87 ± 3	13 ± 2
12	1.7×10^3	0.81	0.19	81 ± 2	19 ± 2

Limonene (a = 2.1mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
2	1.9×10^3	0.01	0.99	1 ± 0.6	99 ± 0.6
4	2.1×10^3	0.01	0.99	1 ± 0.6	99 ± 0.6
6	4.4×10^2	0.00	1.00	0	100
8	4.1×10^2	0.01	0.99	1 ± 1	99 ± 1
10	3.6×10^2	0.12	0.88	12 ± 4	88 ± 4
12	1.4×10^3	0.04	0.96	4 ± 1	96 ± 1

N ≡ number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

4.2.3 Viabilities of Cultures after Treatment with α-Pinene, β-Pinene, Nootkatone and Caryophyllene

The estimated population proportions (with 99% confidence) of alive and dead cells are shown in Table 4.6. The effects of α- and β-pinene were qualitatively similar but both were less toxic than limonene. We decided to determine whether the functional group of a terpenoid was significant in determining its toxicity and so we treated our cultures with nootkatone and caryophyllene which are a sesquiterpenoid ketone and hydrocarbon respectively. The viabilities of cultures grown in media containing the sesquiterpenoids (at the 0.5 mM level) were comparable with the results of using α- and β-pinenes. However, cultures treated with the ketone (at 1.5mM) were entirely dead whereas those treated with the hydrocarbon (at 1.5 mM) showed no decreased viability compared with those grown at the lower concentration (0.5mM).

4.2.4 Viabilities of Cultures Habituated to Sub-lethal Doses of Limonene, Caryophyllene and Phytol

Suspension cultures of *P. fragrans* were maintained on sub-lethal doses of limonene (0.7mM) and caryophyllene (0.5mM) which were added to the culture media (section 13.2c; method 1) immediately before subculture of the plant tissue. Cultures were exposed to these doses for 12 passages (24 weeks) during which time regular measurements of cell-viability were made (counts were recorded on day 4 of the growth-cycle for each measurement).

The estimated population proportions (within a 99% confidence interval) of alive and dead cells are shown in Table 4.7. The appearance of the cultures were different after 4 passages of exposure to the terpenoids. Cultures treated with limonene contained larger cell-aggregates (1mm-5mm) and showed increased greening relative to controls. The cell-aggregates were tough and resistant to breakage by prolonged shaking of the culture flask. When examined under the microscope (after treatment with the cell-dissociation fluid as in 13.3.a) the cells showed considerable thickening of their walls. This thickening was not restricted to those cells on the margins of a cell-aggregate (at the cell surface-medium interface).

Table 4.6 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with Terpenoids (a mM) on Day 2

α-Pinene (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.1×10^3	0.88	0.12	88 ± 3	12 ± 3
6	1.3×10^3	0.78	0.22	78 ± 3	22 ± 3
8	1.2×10^3	0.76	0.24	76 ± 3	24 ± 3

α-Pinene (a = 2.1mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	5.2×10^2	0.17	0.83	17 ± 4	83 ± 4
6	5.1×10^2	0.33	0.67	33 ± 5	67 ± 5
8	7.1×10^2	0.45	0.55	45 ± 5	55 ± 5

β-Pinene (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	8.4×10^2	0.83	0.17	83 ± 3	17 ± 3
6	8.1×10^2	0.77	0.23	77 ± 4	23 ± 4
8	7.4×10^2	0.68	0.32	68 ± 4	32 ± 4

β-Pinene (a = 2.1mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	4.6×10^2	0.15	0.85	15 ± 4	85 ± 4
6	5.4×10^2	0.10	0.90	10 ± 3	90 ± 3
8	4.0×10^2	0.22	0.78	22 ± 5	78 ± 5

Nootkatone (a = 0.5mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	8.2×10^2	0.75	0.25	75 ± 4	25 ± 4
6	4.8×10^2	0.17	0.29	71 ± 5	29 ± 5
8	8.9×10^2	0.65	0.35	65 ± 4	35 ± 4

Nootkatone (a = 1.5mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	4.2 x 10	0.00	1.00	0	100
6	4.4 x 10^2	0.00	1.00	0	100
8	5.1 x 10^2	0.00	1.00	0	100

Caryophyllene (a = 0.5mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.2 x 10^3	0.76	0.24	76 ± 3	24 ± 3
6	1.1 x 10^3	0.66	0.34	66 ± 4	34 ± 4
8	7.2 x 10^3	0.70	0.30	70 ± 4	30 ± 4

Caryophyllene (a = 1.5mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	4.8 x 10^2	0.64	0.36	64 ± 6	36 ± 6
6	9.1 x 10^2	0.65	0.35	65 ± 4	35 ± 4
8	9.0 x 10^2	0.74	0.26	74 ± 4	26 ± 4

N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

Cultures treated with caryophyllene and phytol showed much less greening than control cultures and were fine cell-suspensions (particle size 0.2mm-2mm). The viabilities of cultures habituated to limonene and phytol had increased (by 30%) relative to cultures initially exposed to these terpenoids (but showed the same cell-viability as control cultures). Moreover, the phytol-habituated culture could be maintained with high viability (80 %) in medium containing an increased dose (10-fold) of the diterpenoid. Cultures habituated to caryophyllene showed no increase in cell-viability over the experimental period. Cultures remained viable (80%) throughout the experiment. However, such a constant high viability may well indicate tolerance to the particular dose so we decided to expose these cultures to increasing doses of caryophyllene in the thirteenth passage. Table 4.7 shows the estimated population proportions of living cells at Day 4 in cultures grown in the presence of caryophyllene. We anticipated that cultures may have died in media administered

Table 4.7 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Habituated to Doses (a mM) of Terpenoids Administered over Z Weeks

Limonene (a = 0.7 mM)

Week (Z)	N	p_x	p_y	P_x(%)	P_y(%)
0	2.5×10^3	0.59	0.41	59 ± 3	41 ± 3
8	6.1×10^2	0.57	0.43	57 ± 5	43 ± 5
12	5.2×10^2	0.80	0.20	80 ± 5	20 ± 5

Caryophyllene (a = 0.5 mM)

Week (Z)	N	p_x	p_y	P_x(%)	P_y(%)
0	1.2×10^3	0.76	0.24	76 ± 3	24 ± 3
8	2.0×10^3	0.90	0.10	90 ± 2	10 ± 2
12	2.3×10^3	0.67	0.33	67 ± 3	33 ± 3
24	$4\,0 \times 10^3$	0.80	0.20	80 ± 5	20 ± 5

Caryophyllene (Z = 24)

Week (Z)	N	p_x	p_y	P_x(%)	P_y(%)
0.5	4.0×10^2	0.80	0.20	80 ± 5	20 ± 5
1.5	3.0×10^2	0.82	0.18	82 ± 5	18 ± 5
2.1	5.0×10^2	0.65	0.35	65 ± 6	35 ± 6
4.2	4.1×10^2	0.47	0.53	47 ± 6	53 ± 6

Phytol (a = 0.4 mM)

Week (Z)	N	p_x	p_y	P_x(%)	P_y(%)
0	-	-	-	-	-
8	2.9×10^3	0.58	0.42	58 ± 2	42 ± 2
12	1.2×10^3	0.66	0.34	66 ± 4	34 ± 4
24	2.7×10^2	0.82	0.18	82 ± 6	18 ± 6

N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

All the cultures used in this experiment were inoculated with the terpenoids at subculture and counts were recorded on day 4.

with an increased dose of caryophyllene (3 mM; previously such cultures died- see section 4.2.3). We were surprised to find that cultures treated with an even higher dose (5 mM) of caryophyllene were still viable (50 % *ie.*, LD_{50} of caryophllene was 5mM) at day 4 of the passage. This was clear indication of habituation to the dose of caryophyllene.

4.2.5 Viabilities of Cultures after Treatment with Camphor, Camphene, 3-Bromo-camphor and Camphor-surfactant Mixtures

Solutions of the terpenoids (in dimethoxyethane) were prepared and administered to the cultures following the method given in section 13.4b; method 2. The solubility of those terpenoids in the surfactant solution was proven by filtration of the solution through a Millipore filter unit (used for sterilisation of the solutions administered to culture) and examination of the porous membrane contained within; no crystals of the terpenoid were recovered when the membrane was washed with organic solvent (which was then evaporated to dryness). The estimated population proportions (with 99% confidence) of live and dead cells are shown in Table 4.8. Cultures treated with 3-bromo-camphor and solutions of SDS detergent were killed by day 8 of the growth-cycle. However, although cultures could not tolerate camphor (at 7 mM) they were successfully maintained (at 55% viability) when treated with a lower dose (6 mM; *ie.*, LD_{50}) of a solution of camphor containing SDS detergent. Similarly, our cultures were tolerant to car-3-ene (at 3.5mM) in the presence of this surfactant whereas in its absence cultures died. A tolerance to chrysanthemyl alcohol (at 3.5mM) was observed in the absence of surfactant.[100]

4.2.6 Discussion

The results of section 4.1 have shown that our novel cell-counting technique enables an estimate of cell-viability to be carried out within 5 minutes of taking the sample. This is a quick, simple and cheap method of counting exact numbers of alive and dead cells in a sample, and in some experiments we could count as many as 8000 cells to gain an accurate estimate of the viability of the whole culture. Previous workers[57] counted a maximum of 300 cells per experiment, and these were often from selected (*ie.*, non-random) clumps on a microscope slide. They also did not use a

Table 4.8 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with Solutions (a mM) of Camphor-related Terpenoids 48 Hours Prior to counting.

Camphor (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	7.3×10^2	0.65	0.35	65 ± 5	35 ± 5
6	5.1×10^2	0.81	0.19	81 ± 4	19 ± 4
8	6.3×10^2	0.76	0.24	76 ± 4	24 ± 4

Camphene (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.1×10^3	0.72	0.28	72 ± 3	28 ± 3
6	4.5×10^2	0.69	0.31	69 ± 6	31 ± 6
8	8.7×10^2	0.76	0.24	76 ± 6	24 ± 4

3-Bromo-camphor (a = 0.7mM)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.4×10^3	0.73	0.27	73 ± 3	27 ± 3
6	1.3×10^3	0.72	0.28	72 ± 3	28 ± 3
8	5.0×10^2	0.00	1.00	0	100

Control a (10µl DME)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.0×10^3	0.89	0.11	89 ± 3	11 ± 3
6	5.1×10^2	0.81	0.19	81 ± 4	19 ± 4
8	6.1×10^2	0.67	0.33	67 ± 5	33 ± 5

Control b (stock)

Day	N	p_x	p_y	P_x(%)	P_y(%)
4	1.1×10^3	0.91	0.09	91 ± 2	9 ± 2
6	1.2×10^3	0.89	0.11	89 ± 2	11 ± 2
8	1.5×10^3	0.78	0.22	78 ± 3	22 ± 3

N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

dual-staining technique. In addition we have made use of Binomial statistics[101] to give reliable estimates of population (*ie.*, total culture-population) means and proportions.

The results in section 4.2 have shown that cell-suspension cultures when exposed to pre-determined doses of terpenoids show decreased cell-viabilities. This effect was greatest if the terpenoids were administered during the lag-phase of cell-growth (*ie.*, the LD_{50} was least).

The results are summarised as follows:

Table 4.9 The Toxicities of Terpenoids to Suspension Cultures: Summary of Results

Additive	Comments
None	Proportion of live cells remained at *ca.* 80%-90% throughout growth-cycle although the absolute number increased to a maximum by the stationary-phase of growth
Monoterpenoid	LD_{50} depended on stage of growth of culture: compounds were most toxic when added during growth rather than before. Cultures became more resistant to toxic effects with age. The pinenes were less toxic than limonene.
Sesquiterpenoids	The hydrocarbon was less toxic than the ketone
Mono-, sesqui-, and diterpenoids	Prolonged exposure of cultures to terpenoids over a number of growth-cycles caused thickening of cell walls and greatly increased viabilities which indicated habituation of the cultures to each compound

Conclusion: No clear correspondence of toxicity and class/structural-type. Toxicity probably a membrane-effect since all compounds are toxic during cell-division

The monoterpenoid hydrocarbons were more toxic to the cultures than the sesquiterpenoid hydrocarbon, caryophyllene. Within the monoterpenoids, the ketone (camphor) was less toxic than the hydrocarbons. However, the sesquiterpenoid ketone, nootkatone, was more toxic than caryophyllene so there seems no clear correspondence of toxicity with functional group. The inclusion of a small quantity of the detergent (sodium dodecylsulphate) caused a decrease in the toxicity of the terpenoids. All the compounds were toxic within the range 1mM to 5mM and it is likely that this apparent toxicity was no more than a physical (membrane) effect of oil causing the cells to clump and die, rather than a chemical effect. Cultures treated with limonene over an extended period (13 passages) did form much larger clumps than control cultures. These cultures were shown to be more tolerant to a dose of limonene. The effect could be reproduced with cultures habituated to phytol; cultures treated with caryophyllene maintained high viabilities during all experiments.

Do terpenoids administered to suspension cultures (as unstable oil-in-water emulsions) penetrate the cell-walls? And if so, what happens to the oil droplets once inside the cell? The toxicity of a dose of terpenoid may be a simple membrane-effect or a real inhibition of some biochemical process. The latter would require the terpenoid to cross the cell-wall and previous workers[102] have shown that this may occur.

In the intact plant, the cytoplasm must be protected from the deleterious effects of terpenoids synthesized *de novo*. In these cells special relationships occur between the outer membrane of the plastid (site of synthesis) envelope and the smooth endoplasmic reticulum (ER). This makes possible the transfer of the terpenoid from the site of synthesis to the site of accumulation (extracelluar site) without contact with the cytoplasm. The sites of terpenoid synthesis were discussed in Part One.

One example of how the toxicity of a terpenoid towards a cell membrane may manifest itself is noteworthy: mitochondria differ from the Golgi apparatus in that they do not receive their proteins and phospholipids in small vesicles from the ER.[103] Instead phospholipids are transported by water-soluble transport proteins. It is conceivable that such "naked" phospholipids are a source of natural surfactant which could solubilise oil droplets (terpenoids) present in the cell and in fact behave as quasi-vesicles, entrapping the oil and transporting it to the mitochondria (where

terpenoids have been detected). Our results show that when terpenoids are administered to the suspension cultures as surfactant-solutions, the cells are tolerant to a higher dose of oil. In fact the dose of oil could be increased 10-fold before the culture was killed.

Suspension cultures do not usually accumulate terpenoids. This may be owing to (i) the less aggregated (and hence less differentiated) nature of suspension cultures compared with callus-cultures (so that specialised storage cells do not develop) and (ii) the toxicity of any terpenoids that are synthesized, to the small, thin-walled cell aggregates and fine cells of a suspension culture. The inclusion of a surfactant in the culture medium could conceivably provide an artificial storage mechanism for terpenoids that are synthesized *de novo* and thus lead to greater accumulation. Our observations indicate that surfactants also serve to break-up the cell-aggregates, thus leading to a truly fine cell-suspension. This may be beneficial in its own right and aid in the selection of a specific cell-type from the culture. The next section surveys 16 surfactants as possible additives to a suspension culture.

The results above have obvious very important consequences for (i) cultures that produce terpenoids but cannot store them and (ii) the doses of terpenoids used in biotransformation studies.

Chapter 5 Results and Discussion: Toxicity Studies using Terpenoids as Additives to Suspension Cultures Grown in Media Containing Surfactants at their Critical Micellar Concentrations

Aims: We previously solubilised camphor and related compounds in surfactant-solutions for addition to suspension cultures. In the present experiments we established 16 cultures in media containing surfactants from each of the six classes: (i) anionic (ii) cationic (iii) zwitterionic (iv) carbohydrate-based (v) polyether and (vi) terpenoid with a view to selecting one or more surfactants in the presence of which the cultures could grow with viabilities equal to those in control cultures. These surfactants could then be tested as potential sinks for the accumulation of terpenoids produced by the culture and for the continuous extraction of products formed in biotransformation-reactions.

5.1 Selection of a Suitable Surfactant

Sodium dodecylsulphate (SDS) has been recently used in studies of the acid-catalysed rearrangement of linaloyl acetate[104] (where the presence of SDS micelles were shown to exert considerable product-selectivity in the solvolysis reaction. Table 5.1 shows the viabilities (expressed as percentage population proportions with 99% confidence) of cultures grown in media containing surfactants from the six classes of surfactant that we surveyed. Carbohydrate and terpenoid-based compounds were chosen because cultures are known to secrete polysaccharides[105] and certain high molecular-mass terpenoids (*eg.*, the triterpenoid, ursolic acid) into the culture medium and these compounds may also perform a transport function within the cell (ursolic acid may be a transporter of other terpenoids within the cell). Alternatively, such compounds may just be waste products or they may be natural detergents produced by the culture to solubilise potentially-harmful secondary metabolites. Saponin triterpenoids have been isolated from the seed pods of *Acacia*[106] where they are believed to act as surfactants. Semi-aqueous terpenoid-based solutions are known to have surface-active properties.[107] In our initial experiments we administered the cultures via a Millipore filter unit (on day 0 *ie.*, at time of subculture) with a small volume of a concentrated surfactant solution (of known concentration such that the final concentration in the culture medium was the critical micellar concentration). A count of cell-viabilities (and of total cell-numbers using a haemocytometer) was recorded for each culture

Table 5.1 Estimated Population Proportions of Live (x) Dead (y) Cells From Suspension Cultures of *P. fragrans* Grown in a Mcdia Containing Surfactants at Critical Micellar Concentrations (c.m.c.)

SURFACTANT	CLASS [a]	c.m.c. (mmol.dm^{-3})	DAY	*n*	P_x(%)	P_y(%)
DEOXYCHOLIC ACID	A	5.00	12	4.8×10^2	0	100
GLYCODEOXYCHOLIC ACID	A	2.00	12	3.6×10^2	0	100
SODIUM DODECYL SULPHATE	A	8.27	12	2.9×10^2	0	100
HEXADECYLTRIMETHYLAMMONIUM BROMIDE	C	0.03	12	7.2×10^2	40 ± 5	60 ± 5
CETYLTRIMETHYLAMMONIUM BROMIDE	C	0.33	12	7.6×10^2	0	100
3-[(3-CHOLAMIDOPROPYL)-DIMETHYLAMMONIO]-1-PROPANE-SULFONATE	Z	8.00	2	6.2×10^2	0	100
			4	4.3×10^2	0	100
n-DODECYLGLUCO-PYRANOSIDE	C	0.19	2	1.8×10^2	91 ± 6	9 ± 6
			4	3.4×10^2	89 ± 4	11 ± 4
			12	4.8×10^2	80 ± 5	20 ± 5
POLYOXYETHYLENE-[9,10]-p-t-OCTYL PHENOL	P	0.24	12	5.2×10^2	0	100
POLYOXYETHYLENE-[20]-SORBITOL MONOLAURATE	P	0.05	2	3.3×10^2	89 ± 4	11 ± 4
			4	7.2×10^2	79 ± 4	21 ± 4
			12	8.1×10^2	92 ± 2	8 ± 2
POLYOXYETHYLENE-[20]-SORBITOL MONOPALMITATE	P	0.002	2	2.9×10^2	86 ± 5	14 ± 5
			4	4.2×10^2	0	100
POLYOXYETHYLENE-[20]-SORBITOL MONOOLEATE	P	0.001	2	2.2×10^2	89 ± 4	11 ± 4
			4	2.2×10^2	10 ± 5	90 ± 5
POLYOXYETHYLENE-[10]-LAURYL ETHER	P	0.15	12	2.3×10^2	0	100
POLYOXYETHYLENE-[23]-LAURYL ALCOHOL	P	4.80	4	2.2×10^2	10 ± 5	90 ± 5
POLYOXYETHYLENE-[10]-OLEYL ETHER	P	-	2	3.3×10^2	0	100
			4	4.8×10^2	0	100
URSOLIC ACID	T	-	12	6.1×10^2	2 ± 1	98 ± 1
GUM ACACIA	T	-	12	6.4×10^2	5 ± 2	95 ± 2
CONTROL	-	-	12	1.2×10^3	91 ± 1	9 ± 1

a ≡ class of surfactant: anionic (a); cationic(c); zwitterionic (z); carbohydrate (c); polyether (p); Terpenoid (t).
c.m.c. ≡ Critical micellar concentration
DAY ≡ Day (in growth cycle) on which sample was taken.
n ≡ number of cells per cm^3 of culture
P ≡ proportion (estimated with a 99% confidence) of the cell-type in the culture

(and compared with a control culture) on the final day of the growth-cycle. Those cultures which did not survive or showed drastically reduced viabilities were discarded and the surfactants ranked in order of the viability of the respective culture.

5.1.1 Viabilities of Cultures after Treatment with Anionic and Cationic Surfactants

Table 5.1 shows that the ionic surfactants were the least satisfactory with the exception of hexadecyltrimethylammonium bromide. Cultures grown in the presence of this surfactant maintained limited viability (40%). Generally, for cultures treated with anionic surfactants the low viabilities may be due in part to (i) the high c.m.c. value and (ii) a decrease in the activity coefficients (and hence effective concentration) of the ions in the MS medium owing to (counter-ion binding)[108] between the surfactant micelles and oppositely-charged ions. If cell-death is due only to the high c.m.c. value of a surfactant, the problem could be overcome. The c.m.c values given in Table 5.1 are expressed as $mol.dm^{-3}$ in distilled water.[109-10] Of course, the culture medium is an electrolyte (the ionic strength of medium prepared as in section 13.1, but not brought to pH 5.50, is 1.37×10^{-1} $g.dm^{-3}$) and the c.m.c. of ionic surfactants is known to be drastically reduced in electrolyte-solutions.[111]. We attempted to measure the c.m.c. of sodium dodecylsulphate (SDS) in distilled water and extend this to measure the c.m.c. of SDS in a solution of ionic strength equal to that of MS medium (but lacking hormones). Measurements of the electrical conductance and surface tension of SDS solutions were made. The latter method was too insensitive to obtain reproducible results. Conductance measurements (using a platinum electrode) were recorded by titrating a known volume (1 dm^3) of double-distilled water with a concentrated solution of SDS. A graph of conductance against concentration of SDS showed an exponential increase of conductance with concentration of SDS but no inflexion point associated with micelle-formation at the c.m.c. In a second attempt we added pre-weighed aliquots of SDS to water (double-distilled water; $5dm^3$) and measured the conductance (as in section 13.5b) separately. A graph was plotted which showed an inflexion point corresponding to $8.1 \times 10^{-3}_{\Lambda}$ moles of SDS: (the c.m.c. has been previously reported to be 8.3×10^{-3} $mol.dm^{-3}$).[109] The addition of a small quantity of sodium chloride (17mmol) to a solution of SDS at the c.m.c value caused the conductance to rise beyond the gain-setting of the instrument. The gain was increased (100-fold) to compensate for this, but this resulted in such poor sensitivity of the instrument that further

readings, upon addition of more SDS, remained constant. Persson *et al.*[112] have used ^{13}C-NMR chemical shifts of aqueous surfactants to determine micelle-aggregation numbers (the number of surfactant monomers making up the micelle). A concentration-dependence of ^{13}C-NMR shifts was observed. Although we could use this method to estimate the micelle-size in MS medium, it would not allow us to calculate the c.m.c. of a surfactant in the medium.

5.1.2 Viabilities of Cultures after Treatment with Terpenoid-derived Surfactants

Cell-viability was not maintained in media containing the terpenoids that were selected. Gum acacia is the principle emulsifier of aqueous-based terpenoid solvents so we used a solution (5%) of this compound in the liquid medium (solubilisation could be effected during autoclaving of the medium) but this resulted in low viability (5%) of the cultures by the end of the passage.

The second terpenoid we used, ursolic acid is thought to be soluble (400 ppm) in the cell-vacuoles of some plants and it could be a transporter of terpenoids within the cell. We administered a solution of ursolic acid (10% w/w in MeOH; 2 cm^3; via a Millipore filter) to our cultures. However, as shown in Table 5.1, only very low viability (2%) could be maintained by day 12. Geranyllinalool is thought to be a membrane lipid that is responsible for enhancing GGPP synthase activity in cell-free extracts of *Curcubita pepo*.[113] This compound is believed to solubilise the substrate by the formation of micelles thereby increasing the yield of diterpenoids.

5.1.3 Viabilities of Cultures after Treatment with Polyether- and Carbohydrate-derived Surfactants

Some of the cultures grown in media containing the polyether- and the carbohydrate-derived surfactants did maintain high cell-viabilities throughout the growth period. The experiments were repeated for those cultures showing greater than 80% viability at day 12. Measurements were recorded on days 2, 4 and 12 in order to determine if cell-viability was constant or was reduced in earlier stages of growth and later masked by a recovery in cell-viability by day 12 (as was reported in section 4.2.2). We found that maximum cell-viability was maintained in cultures containing polyoxyethylene-[20]-sorbitol monolaurate. The estimated viabilities matched those of our control cultures. This must be due to the low c.m.c. value (0.05 $mol.dm^{-3}$) of

this surfactant. The electrostatic repulsions that destabilise ionic surfactants are absent for polyethoxylate-surfactants and monomer association is greater: this results in a much lower c.m.c. Polyoxyethylene-[20]-sorbitol monolaurate is commercially known as Tween 20 and has recently become available as a biochemical reagent (containing no antioxidants) as a solution (10%) in water in ampoules sealed under nitrogen (*ex.* Pierce Ltd., Europe BV, The Netherlands) which we used. As far as we know, there are no reports on the selectivity of Tween 20 micelles towards monoterpenoids so we designed a preliminary experiment to test if monoterpenoids were solubilised when administered to a suspension culture containing Tween 20 at the c.m.c. This is described in the following section.

5.2 The Effect of Polyoxyethylene-[20]-Sorbitol Monolaurate on the Toxicity of Monoterpenoid Peroxides Administered to Suspension Cultures of *P. fragrans*

One way to test if a surfactant can sequester the terpenoids that could produced by a culture would be to add a toxic dose of terpenoid and see if this toxicity was the same in the presence of the surfactant. A variation would be simple evidence that the surfactant was performing a storage function. We decided to use monoterpenoid peroxides (surely toxic!) to kill our cultures, and then to repeat these experiments in the presence of the title-surfactant. This would also test another theory: previous studies[57] on the toxicities of monoterpenoid hydrocarbons showed that β-pinene and α-terpinene were particularly toxic: it seemed that the toxicities of these hydrocarbons could well be due to the presence of peroxides in the samples because the standards they used were not purified prior to use.

We prepared peroxide-cocktails of both hydrocarbons by standard photochemical oxidation[114] to test the idea above (see section 15.4). We administered cell-suspension cultures of *P. fragrans* with solutions of (i) β-pinene (purified as in section 13.4a); (ii) β-pinene-hydroperoxide mixture (iii) α-terpinene-hydroperoxide mixture by the method described in the experimental (13.4b; method 1). A separate group of cultures containing polyoxyethylene-[20]-sorbitan monolaurate (at c.m.c) were also administered with the same solutions. Table 5.2 shows the estimated viabilities (expressed as percentage population proportions with 99% confidence) on days 2 and 4 of the growth-cycle.

Table 5.2 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with Terpenoid Hydrocarbons and their Peroxide/ Hydroperoxide Derivatives (a mM) 48 Hours Prior to Counting.

β-Pinene

Day	Dose (mM)	n	p_x	p_y	P_x(%)	P_y(%)
2	1.8	3.9×10^2	0.10	0.90	10 ± 4	90 ± 4
	3.0	6.1×10^2	0.00	1.00	0	100
4	1.8	4.8×10^2	0.15	0.85	15 ± 4	85 ± 4
	3.0	4.8×10^2	0.00	1.00	0	100

β-Pinene (CULTURE TREATED WITH POLYOXYETHYLENE-[20]-SORBITOL MONOLAURATE AT c.m.c.)

Day	Dose (mM)	n	p_x	p_y	P_x(%)	P_y(%)
2	1.8	3.7×10^2	0.49	0.51	49 ± 7	51 ± 7
	3.0	2.5×10^2	0.24	0.76	24 ± 7	76 ± 7
4	1.8	6.1×10^2	0.27	0.73	27 ± 5	73 ± 5
	3.0	3.4×10^2	0.13	0.87	13 ± 5	87 ± 5

β-Pinene (HYDROPEROXIDE MIXTURE)

Day	Dose (mM)	n	p_x	p_y	P_x(%)	P_y(%)
2	0.6	-	0.00	1.00	0	100
	1.8	-	0.00	1.00	0	100
	3.0	-	0.00	1.00	0	100
4	0.6	-	0.00	1.00	0	100
	1.8	-	0.00	1.00	0	100
	3.0	-	0.00	1.00	0	100

cont.

β-Pinene (HYDROPEROXIDE MIXTURE; CULTURE TREATED WITH POLYOXYETHYLENE-[20]-SORBITOL MONOLAURATE AT c.m.c.)

Day	Dose (mM)	n	p_x	p_y	P_x(%)	P_y(%)
2	0.6	3.1×10^2	0.93	0.07	93 ± 4	7 ± 4
	1.8	3.2×10^2	0.58	0.42	58 ± 7	42 ± 7
	3.0	-	0.00	1.00	0	1
4	0.6	2.8×10^2	0.91	0.09	91 ± 4	9 ± 4
	1.8	3.2×10^2	0.94	0.06	94 ± 3	6 ± 3
	3.0	-	0.00	1.00	0	100

α-Terpinene (HYDROPEROXIDE MIXTURE)

Day	Dose (mM)	n	p_x	p_y	P_x(%)	P_y(%)
2	0.6	5.3×10^2	0.49	0.51	49 ± 6	51 ± 6
	1.8	-	0.00	0.00	0	100
	2.4	-	0.00	0.00	0	100
4	0.6	3.4×10^2	0.40	0.60	40 ± 7	60 ± 7
	1.8	-	0.00	1.00	0	100
	2.4	-	0.00	1.00	0	100

c.m.c. ≡ critical micellar concentration
N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

Cultures administered with β-pinene showed cell-viabilities which were comparable with those of cultures administered with limonene (4.2.1). However, all the cells in culture were killed by a much smaller concentration (0.6mM) of the hydroperoxide mixture formed from β-pinene. We were surprised to find that cultures were considerably more tolerant to the hydroperoxide mixture formed from α-terpinene. Since the latter is known to consist mainly of ascaridole we concluded that our cultures may have been more tolerant to endoperoxides than to the hydroperoxides formed from the photooxygenation of β-pinene. We attempted to prepare a mixture of peroxides from caryophyllene but failed to obtain any products using the reaction conditions described.

Table 5.2 shows that those cultures grown in medium containing polyoxyethylene-[20]-sorbitol monolaurate when grown in the presence of β-pinene (2.2 mM) showed an increase (5-fold) in cell-viability relative to untreated cultures and even managed to maintain their viability in the presence of an increased concentration (3.5 mM) of β-pinene. Moreover, they were also tolerant to the hydroperoxide mixture derived from β-pinene; high cell-viability (93%) was recorded for cultures grown in a concentration (0.6mM) of the mixture that previously killed other cultures (grown in the absence of surfactant micelles). The cultures were still alive (58% viability) when the concentration of hydroperoxide was increased (to 1.8 mM). These results must be attributed to the solubilising-effect of the surfactant. The data of Table 5.2 indicates that micelles of polyoxyethylene-[20]-sorbitol monolaurate do solubilise both non-polar hydrocarbons such as β-pinene and their hydroperoxide derivatives. The micelles may be more selective to the latter which are more polar in nature because the proportions of live cells in culture were drastically increased in the presence of surfactant. This may result from an increased solubility of peroxides within the micelles, or from hydrogen bonding between the peroxides and the polar ethylene oxide head groups of the surfactant.

The data certainly suggests that the reported[57] high-toxicities of the pinenes and terpinenes were due to contaminating peroxides, because when purified, these hydrocarbons were no more toxic than other monoterpenoids.

5.3 Discussion

The results of the preliminary experiments indicate that the solubilisation of exogenously-administered terpenoids by the inclusion of a non-toxic surfactant in the culture medium is a good model-system for the collection and storage of terpenoids synthesized by cultures. The following table summarises the results so far:

Table 5.3 Surfactants as Additives to Suspension Cultures: Summary of Results

Compound	Toxic†	Comments
Anionic surfactants	Yes	Cultures entirely dead
Cationic Surfactants	Yes	Very low viability
Zwitterionic Surfactants	Yes	Cultures entirely dead
Polyethoxylate Surfactants	No	Very high viabilities that match control cultures. This effect may be due to much lower c.m.c. and non-ionic nature compared with classes above.
Carbohydrate and Terpenoid-based Surfactants	Yes	Surprising since such compounds perform the function of a surfactant in nature
β-Pinene	Yes	LD_{50} *ca.* 1.4 mM (days 2-4)
β-Pinene - in cultures containing Polyethoxylate Surfactants	Yes	LD_{50} *ca.* 2.1 mM (days 2-4)
Monoterpenoid Peroxides	Yes	Kill cells at >1 mM
Monoterpenoid Peroxides in cultures containing Polyethoxylate Surfactants	Yes	LD_{50} *ca.* 2.1 mM

†surfactants were added to cultures at the critical micellar concentration

Surfactants could provide a much cheaper and reliable alternative to traditional secondary phases, for the accumulation and storage of secondary products in tissue cultures. We have grown the first single-phase cultures that incorporate an extracelluar storage site which does not interfere with the aeration or growth of the cultures (in that cell-viabilities are not affected by the presence of the surfactant).

5.4 Future Work

Future work could involve the use of mixed micellar systems to develop differing selectivites of surfactants to certain classes or structural type of secondary product. Preliminary work on the selectivity of micelles in catalysed reactions is already reported[112] and could be easily applied to biotechnology. Cyclodextrins are another group of compounds that could be studied for storage properties in culture. They are already used in the food and drinks industry for solubilising flavours.[115] Similarly, controlled-release systems[116] used in the pharmaceutical industry could be applied to plant biotechnology to serve as a method for introducing compounds to tissue cultures at constant sub-toxic dose-rates.

Chapter 6 Results and Discussion: Toxicity Studies using Fluorinated Substrates as Additives to Suspension Cultures

Aims: We decided to grow our cultures in media containing fluoroacetate and other fluorinated compounds in order to estimate their toxicities using our novel statistical and staining methods of determining cell-viability.

The cytotoxicity of fluoroacetate to animals is well-known.[117] Fluoroacetate poisoning is brought about by blocking the tricarboxylic acid cycle *in vivo*. The substrate is converted into fluorocitrate which then blocks the enzyme aconitase which is responsible for the conversion of *cis*-aconitate into either citric acid or isocitric acid in the mitochondria. Accumulation of citric acid is therefore a characteristic feature of fluoroacetate poisoning. Fluoroacetate has been isolated from more than 25 species of plants at concentrations that are extremely toxic to herbivorous animals.[118]

6.1 Viabilities of Cultures after Treatment with Sodium Fluoroacetate

Eight stock solutions of sodium fluoroacetate in distilled water were prepared and administered to our cultures to give the final concentrations shown in Table 6.1. The estimated viabilities (expressed as percentage populations with 99% confidence) on days 4 ,6 and 8 are shown. Cultures that had been grown in solutions containing the lowest concentration (0.01 gdm^{-3}; 0.1 $mmol.dm^{-3}$) of the substrate were still viable (50%) by day 4. At day 6 this viability could be maintained at a higher concentration (0.4 $g.dm^{-3}$; 4.7 $mmol.\ dm^{-3}$) and by day 8 the concentration could be increased further (2 $g.dm^{-3}$; 23.8 $mmol.dm^{-3}$) before the cell-viability was seen to fall (below 40%). We repeated our experiment by increasing the concentration (5 gdm^{-3}; 59.5 $mmol.dm^{-3}$) of fluoroacetate before measuring cell-viability. The cultures showed cell-death (0% viability) when the concentration had been increased (3 $g.dm^{-3}$; 35.7 $mmol.dm^{-3}$).

Some tissue cultures have been found to accumulate fluoroacetate[119] when grown in media supplemented with sodium fluoride. However, the origin of the metabolite is the subject of some controversy: it may be synthesized by bacteria growing on the

Table 6.1 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Cultures of *P. fragrans* Administered with Sodium Fluoroacetate 48 Hours Prior to Counting.

Day	Concentration ($g.dm^{-3}$)	N	p_x	p_y	P_x(%)	P_y(%)
4	1.0×10^{-1}	9.7×10^2	0.49	0.51	49 ± 4	51 ± 4
	1.0×10^{-2}	1.4×10^3	0.60	0.40	60 ± 3	40 ± 3
	1.0×10^{-3}	1.2×10^3	0.62	0.38	62 ± 4	38 ± 4
	1.0×10^{-4}	4.4×10^2	0.77	0.23	77 ± 5	23 ± 5
	1.0×10^{-5}	1.3×10^3	0.64	0.36	64 ± 3	36 ± 3
6	2.0×10^{-1}	1.2×10^3	0.66	0.34	66± 4	34 ± 4
	3.0×10^{-1}	7.8×10^2	0.56	0.44	56 ± 5	44 ± 5
	4.0×10^{-1}	3.0×10^3	0.58	0.42	58 ± 2	42 ± 2
8	2.0×10^{-1}	9.1×10^2	0.38	0.62	38± 4	62 ± 4

N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

intact plant and these bacteria may well be carried over *in vitro*. Whatever the cause, either the bacteria or the callus would need to be impermeable to fluoride or fluoroacetate and/or possess a specialised transport and accumulation mechanisms to prevent deleterious cytochemical effects. It is unlikely that our cultures possessed any such mechanisms when first cultured in the presence of fluoroacetate although they may have possessed the potential to select for them. It is therefore likely that the apparent induced tolerance of our cultures to the substrate depended on the impermeability of the cell-walls to the ions. At much higher concentrations cell-death may have been caused by the diffusion of fluoroacetic acid across the cell wall or by extensive cell-plasmolysis (an osmotic withdrawal of water from the cells)

owing to the high ionic strength of MS medium containing added electrolyte (3 $g.dm^{-3}$).

However, if fluoroacetate were formed within the cell it is possible that its breakdown to acetate and inorganic fluoride preceded incorporation of the acetate moiety into the carboxylic acid-cycle. Ward[120] observed that the majority of sodium fluoroacetate administered to a lettuce plant was broken down into inorganic fluoride and only a small quantity (2%) was incorporated into the lethal 2-fluorocitrate. Seedlings of *Acacia georginae* are known to degrade fluoroacetate to more than 50 fluorine-labelled metabolites.[121] It is possible that cultures (such as *P. fragrans*) that are capable of synthesizing and metabolising terpenoids[122] divert 2-fluoro-acetyl-CoA (which is non-toxic *per se*) into secondary product-synthesis yielding a range of fluorinated-terpenoid derivatives *eg.*, 2,4,9-trifluorogeraniol (derived from three 2-fluoroacetyl CoA units). The presence of a fluorine in the un-ionised form of acetyl-CoA in the acetoacetyl-CoA thiolase step ($FCH_2COSCoA$) and HMG-CoA synthetase steps ($FCH_2COCH_2COSCoA$) of monoterpenoid biosynthesis[123] would labilise both intermediates towards aldol-attack by the acetyl-CoA anion and yield compounds such as 9-fluoro-geraniol. Alternatively, the culture could detoxify fluoroacetate at the 3-HMG-CoA stage of terpenoid-biosynthesis by conversion of fluoro-HMG-CoA to fluoro-acetoacetate, catalysed by HMG-CoA cleavage enzyme.[124]

6.2 Viabilities of Cultures after Treatment with 2-Fluoroethanol, 2,2,2-Trifluoroethanol and Sodium Fluoride

The title-compounds were administered to our cultures to give the final concentrations shown in Table 6.2. Those cultures treated with 2-fluoroethanol showed cell-viabilities that matched the control culture (containing no fluoro-additive). High cell-viability (90%) was maintained in a solution (3 $g.dm^{-3}$;46.9 $mmol.dm^{-3}$) of the substrate. Cultures showed a similar tolerance to 2,2,2-trifluoroethanol within the confidence interval (99%) used. The table shows that both compounds were less toxic to the culture than sodium fluoroacetate. We did not anticipate these results: fluoroethanol is known to be as toxic as fluoroacetate owing to its possible conversion to this or 2-fluoroacetyl-CoA within the cell. Unless a possible detoxification route can operate, we can only conclude that 2-fluoroethanol was too polar to cross the

Table 6.2 Sample Proportions and Estimated Population Proportions of Live (x) and Dead (y) Cells From Suspension Cultures of *P. fragrans* Administered with 2-Fluoroethanol, 2,2,2-Trifluoroethanol and Sodium Flouride, 48 Hours Prior to Counting.

Compound	Day	Concentration ($g.dm^{-3}$)	μ_x	N	p_x	p_y	P_x(%)	P_y(%)
SODIUM	4	1.0	$4.4k \times 10^4$	2.5×10^2	0.40	0.60	40 ± 8	60 ± 8
FLUOROACETATE	6	1.0	$3.7k \times 10^4$	3.9×10^2	0.34	0.66	34 ± 6	66 ± 6
	6	3.0	0	5.0×10^2	0.00	1.00	0	1
	6	5.0	0	3.6×10^2	0.00	1.00	0	1
2-FLUORO-	4	6.4×10^{-1}	4.0×10^4	6.4×10^2	0.75	0.25	75 ± 4	25 ± 4
ETHANOL	6	6.4×10^{-1}	1.40×10^5	2.8×10^2	0.84	0.16	84± 6	16 ± 6
	6	1.96	1.62×10^5	6.4×10^2	0.85	0.15	85 ± 4	15 ± 6
	6	3.20	1.44×10^5	1.1×10^3	0.87	0.13	87 ± 3	13 ± 3
	9	3.20	1.34×10^5	8.1×10^2	0.43	0.57	43 ± 4	57 ± 4
2,2,2-TRIFLUORO-	4	1.0	5.0×10^4	6.2×10^2	0.70	0.30	70 ± 5	30 ± 5
ETHANOL	6	1.0	1.0×10^5	4.9×10^2	0.66	0.34	66 ± 6	34 ± 6
	6	3.0	1.7×10^5	9.2×10^2	0.84	0.16	84 ± 3	16 ± 3
	6	5.0	1.8×10^5	6.4×10^2	0.82	0.18	82 ± 4	18 ± 4
	9	5.0	1.3×10^5	6.8×10^2	0.51	0.49	51 ± 5	49 ± 5
SODIUM FLUORIDE	6	2.1	0	4.3×10^2	0.00	1.00	0	1
CONTROL	4	-	7.0×10^4	7.1×10^2	0.88	0.12	88 ± 3	12± 3
	6	-	2.07×10^5	2.6×10^2	0.86	0.14	86 ± 6	14 ± 6

N ≡ Number of cells counted
P ≡ proportion (estimated with 99% confidence) of the cell-type in the culture
p ≡ proportion of the cell-type in the samples

Day refers to the number of days after subculture when the compound was added to the culture.

cell-wall. Similarly, 2,2,2-trifluoro-ethanol would thus not permeate the cell. In any case the conversion of the latter into 2,2,2-trifluoroacetic acid would not lead to the lethal synthesis of 2,2,2-trifluorocitric acid since the presence of three fluorine atoms is known to prevent the former from being recognised as acetic acid by the enzyme systems associated with the Krebs cycle. The lower toxicity of the two fluoroethanols compared with 2-fluoroacetate may be attributable to their evaporation from the culture medium during the experiment or alternatively because they did not interfere

with the osmoregulation of the plant cells.

Table 6.2 shows that a culture administered with sodium fluoride showed 100% cell death by day 6 of the growth period. The concentration of salts in the growth medium was less than the lethal-concentration of fluoroacetate used in section 6.1 and so the result reflects more than just the effect of added electrolyte on cell-viability. The fluoride ion is known to be absorbed and accumulated by some plant cells[125] and causes loss of photosynthetic activity.[126] The mechanism is thought to involve inhibition of photophosphorylative mechanisms by a direct effect on ATP-ase activity.[127] Some whole plants and their tissue cultures are known to incorporate inorganic fluoride into fluoroacetate. Gröbelaar[128] reported accumulation (*ca.* 1g) of fluoroacetate in cultures grown in media containing fluoride (0.25 $g.dm^{-3}$). Massel[129] reported a six-fold increase in the accumulation of limonene in conifers exposed to fluoride although no explanation for this phenomenon was given.

6.3 Discussion

Our results suggest that the apparent non-toxicity of the fluoroacetate ion to the cultures was due to a simple membrane-effect. If a fluorinated-terpenoid could be introduced to a culture that is capable of metabolising terpenoids it is possible that fluoroacetate could be produced *in situ.* This could be a method for selecting a sub-population of cells that have the ability to store the fluorinated-terpenoid (or any other terpenoid) because they would prevent the lethal synthesis of fluoroacetate from occurring and would thus survive. The chemical syntheses of some fluorinated terpenoids is reported in Part Three.

Chapter 7 Results and Discussion: General Consideration of some Fundamentals that are Overlooked in many Studies of Biotransformations

Aims: To determine whether some claims of biotransformations (or detoxifications) could be explained by simple chemical conversion of terpenoids by the culture medium. The experiments include monitoring the pH of a culture of *P. fragrans* and studying the effect of pH on monoterpenoids mixed with culture media.

A number of common biotransformations of substrates by tissue cultures were mentioned previously (3.2). For example, cultures of *Cannabis sativa*[130] were found to interconvert exogenous geraniol to nerol and *vice versa.* The same cultures were also found to oxidise these primary-allylic alcohols to citral a and citral b (in approximately 40% yield). Other examples include the reduction of menthone to neomenthol by cultures of *Mentha*[131] and the reduction of various monoterpenoid aldehydes to their alcohols by cultures of *Lavandula angustifolia.*[73]

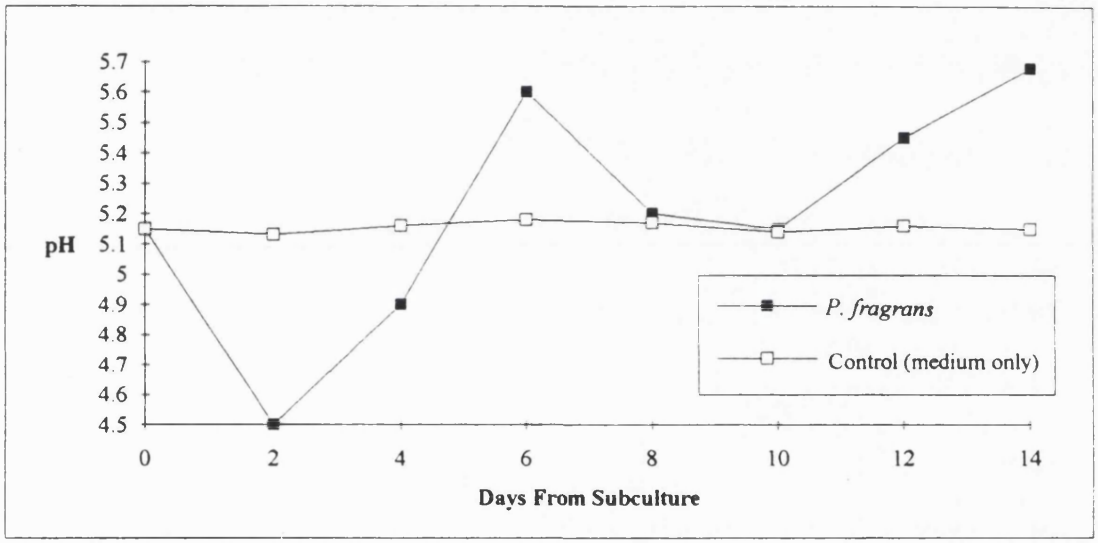
Although many of these biotransformations can be reproduced by feeding monoterpenoid pyrophosphates to cell-free extracts,[132a,b] it is possible that some of these redox reactions and hydrolyses can be accounted for by reaction of a substrate with the culture medium (an acidic electrolyte-solution of transition metal-ions). Some plant cells are known to excrete large amounts of oxidative and hydrolytic enzymes into the liquid medium[133] although the most likely cause of a fall in pH is the excretion of H^+ (to maintain electrical balance as NH_4^+ is absorbed) by the cells as they grow. Such a drop in pH could be the cause of many rearrangements and conversions. Workers who report biotransformations normally run controls by incubating the terpenoid with the culture medium (no cells) at pH 5.8 for the length of the experiment. Rather, they should incubate with the final or average pH of the medium (it may be pH 3.0; *ie.*, 102-fold more acid!). The spent medium could be used by removing the cells by centrifugation.

7.1 Variation of the pH of a Culture During its Growth-cycle

We decided to monitor the pH of a suspension culture of *P. fragrans* on consecutive days throughout the growth-cycle (by the method described in section 13.3g). The

pH of a control batch of the same medium (kept within the culture cabinet at the same temperature as the experimental flask) was also monitored. The results are shown in Diagram 7.1. Both flasks contained medium at pH 5.15 at the time of subculture (the medium was adjusted to pH 5.50 before sterilisation in the autoclave) but the pH of medium in the experimental flask fell to 4.50 as the growth of the culture remained in lag-phase (Diagram 7.1).

Diagram 7.1 The Variation of the pH of a Suspension Culture of *P. fragrans* over the Growth-cycle



With the onset of exponential growth, the pH of the culture increased to a maximum of 5.60 by day 6, after which the value fell to the initial pH at the time of subculture. The pH finally increased as the culture advanced to the stationary-phase of growth. Apart from the most likely cause of a decrease in pH (see above) these observations could be explained by the action of auxin on plant cells.[134] They are known to activate ion-pumps within the cell-membrane causing an efflux of protons from the cell[135] and a relaxation of the cell-wall.[136] This decrease in pH is thought to stimulate cell-wall-loosening enzymes which catalyse cell-elongation growth. From

our results we may conclude that these ion-pumps are switched on by the culture immediately before the exponential growth-phase begins and they are switched off as the culture reaches stationary-phase.

The drift of the pH towards pH 5.70 as the culture advanced into this stage of growth could be explained by the release of OH^- as the cells removed NO_3^- from the medium. pH has recently been referred to as a "second messenger"[137] in plants, having a vital role in the biosynthesis of fatty acids, lipid oxidation and even stress-signalling. A few reports have described the inclusion of organic buffers[79b,80] and metal chelates in tissue culture media, to maintain a constant pH, but some of these were shown to affect growth and morphology of the tissue. It is possible that the pH falls even lower than the value we observed (pH 4.50) in some plant cultures particularly if acidic metabolites (*eg.*, phenolics) are excreted into the growth medium. (For example, our agar cultures of *Rosa damascena* accumulated a red pigment which was found to be a mixture of anthocyanins (metabolites which are known to be acidic) when analysed by HPLC (12.1c; methods 3 and 4). This fluctuation in pH may well account for the range of conversions (that are reported to occur in suspension cultures administered with terpenoid substrates) for which more elegant explanations have been advanced. We decided to add a few terpenoids to MS medium and store them for periods of up to 3 months alongside our tissue cultures in the incubator.

7.2 The Reaction of Some Terpenoids with the Culture Medium

These experiments show how the pH of the medium can be responsible for some of the conversions that may occur long before the compounds enter the plant and are biotransformed. The results may well explain some claims of true bioconversion, although studies on biotransformations are normally made over 2-4 weeks and the present experiments were carried-out over 12 weeks.

A mixture (1:1:1) of limonene, linaloyl acetate and caryophyllene was administered to MS medium (100cm^3) by the method described in section 13.4b; method 1. A small sintered funnel containing powdered activated charcoal was placed on top of the culture flask and the two glass rims sealed together with plastic film. A foil cap was placed on the top of the funnel and the culture flask placed in the incubator for 12 weeks. At the end of this period, the medium and the charcoal were washed with

diethyl ether and the samples were analysed by GC/MS (section 12.2a; system 2).

We found that in the presence of the tissue culture-medium for 12 weeks the aliquot of caryophyllene had been converted into caryophyllene oxide with no traces of the hydrocarbon remaining. No limonene was present but α-terpineol and carvone were present. A peak corresponding to linalool was observed together with a smaller proportion of linalool oxide.

Oil (410 mg) was recovered from the charcoal washings, containing eight major compounds; limonene (73%); linalool (10%); α-terpineol (10%); carvone (1%); caryophyllene (1%); isocaryophyllene (1%) and a monoterpenoid peroxide (0.5%).

In a second experiment we administered linaloyl, geranyl and neryl acetates to separate culture media adjusted to varying acidic pH values (pH 3-5). Our GC/MS analyses indicate that during the course of 7 days the composition of the geranyl and neryl acetate doses remained constant over the pH range. However, linaloyl acetate was converted into a mixture of limonene (61%), β-pinene (20%) and γ-terpinene (7%).

7.3 Discussion

Our experiments indicate that the culture medium is capable of supporting chemical interconversion of terpenoids that could be mistaken as biotransformations. Tertiary allylic compounds such as linaloyl acetate are most susceptible to hydrolysis, elimination, cyclisation and rearrangement (to form monoterpenoid alcohols and hydrocarbons) while the monoterpenoid hydrocarbons are likely to form ketones, epoxides and peroxides (which may kill some cells). The former processes are likely to be dependent on the pH of the solution (for example linalool is known to form mixtures containing α-terpineol, terpinenes and limonene in acid solutions).[138] The oxidative reactions are probably mediated by redox equilibria occurring between transition metal-ions that are present in the medium-formulation. Our crude head-space analysis demonstrates that significant losses of monoterpenoid hydrocarbons can occur from tissue cultures. Some workers[131] have observed such losses of monoterpenoid substrates from cell-cultures (plant-tissue and medium) of *Mentha* species and have attributed this to enzymatic glycosylation without further examination.

PART 3 Syntheses of some Fluorinated Monoterpenoids: Preparation of Five Fluorinated Derivatives

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PART 3 Syntheses of some Fluorinated Monoterpenoids: Preparation of Five Fluorinated Derivatives

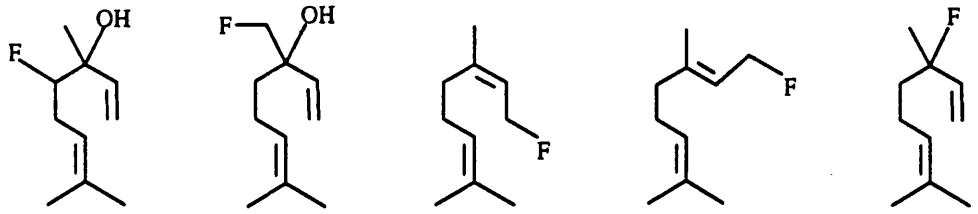
Chapter 8 Introduction

8.1 Scope and Reasons for Study

The introduction of fluorine into the steroid nucleus is now well established[139] and provides a useful probe for the study of the metabolic fate of these molecules in the mammalian body by ^{19}F-NMR spectroscopy. Many fluoro-steroids are known to be powerful drugs with anti-inflammatory, anti-phlogistic, anti-allergic, glucocorticoidal and anabolic properties.[140] The preparation of fluorinated analogues of lower terpenoids has not received the same degree of attention and, to date, few examples are recorded (see 8.3). In addition to their potential as biologically-active probes, the presence of a fluorine atom in these molecules may express an entirely different organoleptic property to the unfluorinated analogue.

The five fluoro-monoterpenoids we prepared are shown as follows:

Diagram 8.1 The Five Fluoro-terpenoids Prepared in this Work



These can serve as precursors to a variety of analogous compounds that can be prepared by established conversions (section 8.3.4). For example, treatment of either 4-fluoro- or 9-fluoro-linalool with boron trifluoride etherate[141] would yield the corresponding fluorogeraniols and fluoronerols. Alternatively, treatment with 30 % sulphuric acid[142] would yield fluoro- myrcenes, dipentenes, terpinolenes, *p*-cymenes,

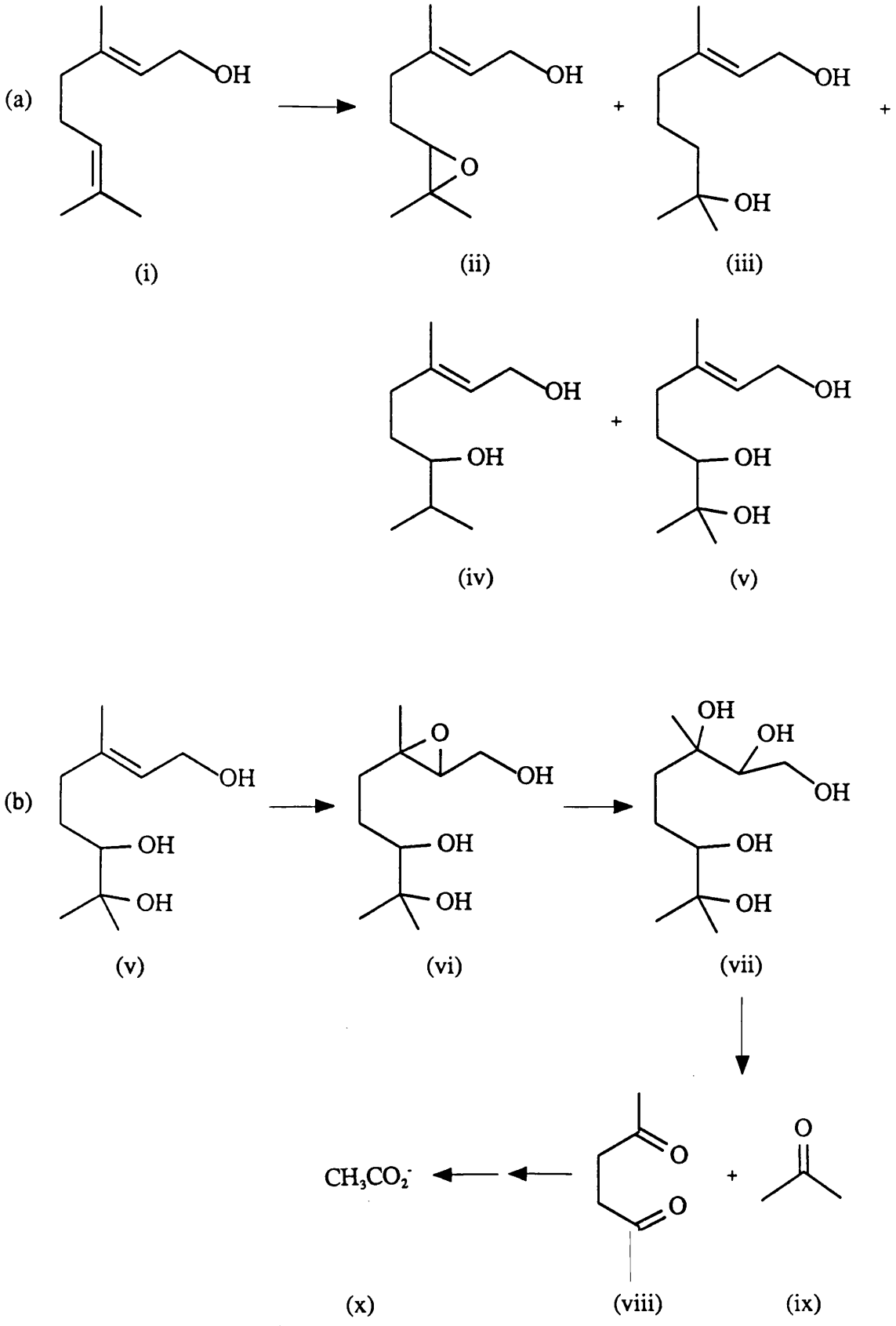
α-terpineols and 1,4- and 1,8- cineoles. In fact, a whole new class of fluorinated monoterpenoids for toxicity-structure correlations, not to mention a new chapter in the mass spectrometry of isoprenoids, could be tapped by application of known preparative (and industrial) chemistry.

8.2 Potential of Fluorinated Monoterpenoids as Metabolic Probes

Recent work on the oxidative metabolism of ^{3}H- and ^{14}C-labelled terpenoids[143-4] has shown products such as (ii)-(v) (Scheme 8.1a) to be key intermediates in the catabolism of the substrates. The ultimate product in this sequence is thought to be acetate (Scheme 8.1b). The high water-solubility of the products has made attempts to separate them for analysis difficult. However, the fate of a fluorinated substrate (which should still be recognised by the catabolic enzymes because the fluorine atom is isosteric with the hydrogen atom) could be monitored by recording ^{19}F-NMR spectra at intervals. For example, a spectrum recorded after five minutes may show a signal corresponding to the substrate, but a spectrum recorded after one hour may show signals corresponding to various products of metabolism including fluoroacetate.

An important approach that is relevant to our previous tissue culture studies is as follows: fluoroacetate formed by the decomposition of a fluoromonoterpenoid is not cytotoxic but its conversion into fluorocitrate in the aconitase step of oxidative metabolism proves fatal to both animal and plant cells. Thus, a plant culture metabolising *eg.*, fluorogeraniol (with a fluorine substitution at the correct carbon) would effectively "commit suicide" by the lethal synthesis[145] of fluoroacetate. Tissue cultures do not usually store terpenoids because of the degree of differentiation required to do so (see Part Two). However, a cell-line showing this differentiation could be selected by administering the culture with a fluoroterpenoid; over successive passages of growth, those cells that metabolise the substrate (to fluoroacetate) would die-off, leaving only cells that are capable of storing terpenoids (or metabolising them to products other than acetate). This technique could provide a way of selecting a sub-population of storage cells to accumulate any terpenoids that are produced by the culture. However, the sub-lethal dose of a non-fluorinated terpenoid to a tissue culture must first be determined, and the effect of this dose on morphology of the culture should be known. Any increased dosage of a terpenoid above normal metabolic levels (*eg.*, 10 μg/g fresh mass of callus) may favour selection of a

Scheme 8.1 The Oxidative Metabolism of Geraniol



completely different cell-line; *eg.*, a cell-line with thickened cell-walls to prevent penetration of the compound into the cells. The optimum interval and method of administration must be determined; the toxicity of the terpenoid may be greater at different stages in the growth-cycle *eg.*, during cell-division (these factors are the objectives of the study of the toxicities of mono-, sesqui- and diterpenoids to tissue cultures and their solubilisation in the culture-medium; Part Two).

8.3 Methods for the Introduction of Fluorine to Monoterpenoid Molecules

The industrial and biological importance of fluorinated organic molecules has led to the development of a number of routes for the introduction of one or more fluorine atoms into an organic molecule.[146-7] The earliest recorded fluorination can be traced back to 1835 with the preparation of fluoromethane, although the simple metathesis studies of Swarts laid the foundation of modern organofluorine chemistry.[148] There are numerous reviews on the general methodology and various classes of reagent[149] *eg.*, free radical, electrophilic and nucleophilic: (i) two of the most widely used classes of reagent in the synthesis of natural product analogues containing fluorine are compounds based on sulphur tetrafluoride *eg.*, (a) diethylaminosulphur trifluoride (DAST);[150] or (b) the recently developed morpholinosulphur trifluoride.[151] Other reactions are: (ii) compounds with nucleophilic fluorine such as tetrabutylammonium bifluoride[152] (hereafter referred to as TBABF) and triethylamine trihydrofluoride[153] (iii) compounds with N-F bonds such as N-fluoropyridinium triflates and N-fluoro sulphonamides.[154]

There are eight possible sites for replacement of one hydrogen (C-H) by fluorine in an acyclic monoterpenoid alcohol. Replacement of the hydroxyl group gives a further site for introduction of fluorine. Poulter[155] has synthesised 9-fluoro-geraniol by *syn*-addition of (4-methyl-3-en-1yl)-copper reagents to derivatives of ethyl-2-butynoate bearing appropriate functional groups at C_4. Some other preparations of fluorinated terpenoids have been reported.[156]

A simple disconnection of linalool yields the cheap and commercially-available 6-methyl-hept-5-en-2-one (1; hereafter referred to as methyl-heptenone) and a two-carbon synthon provided by vinylmagnesium bromide, vinyl lithium or vinyl chloride.

The first vinyl reagent was the basis of the classical synthesis of linalool by Normant.[157] Thus, fluorination of methyl-heptenone at any of six available sites should provide a simple and efficient synthesis of various fluorinated linalools, and hence fluorogeraniols and fluoronerols and their bifluorinated analogues. Scheme 8.2 outlines the routes that we followed and the following section briefly introduces each method, together with some other possibilities which incorporate known steps from the literature.

8.3.1 Introduction of Fluorine to Positions C_1 and C_3 of 6-Methyl-hept-5-en-2-one

The terms "kinetic" and "equilibrium" used hereafter as adjectives for products refer to the kinetically- or thermodynamically controlled reaction conditions employed for the formation of enolates and trimethylsilyl-enol ethers.

(a) An equilibrium mixture of enolates (2,3) can be produced by addition of a molar-excess (20%) of methyl-heptenone to a solution of a strong base such as lithium diethylamide[158] or triphenylmethyl lithium[159] (trityl-lithium; Scheme 8.2a). The latter should be particularly useful since it is known to suppress aldol-condensations and has the advantage of being coloured so the reaction can be run as a titration until the colour disappears. These enolates or their enol acetates could be brominated by the addition of a molar-equivalent of N-bromo-succinimide and fluorination effected by nucleophilic substitution using TBABF to yield 3-fluoro-6-methyl-hept-5-en-2-one (5); (see Results and Discussion section 9.3 and 9.4). Alternatively, the enolates can be fluorinated directly by use of acetyl hypofluorite.[160]

(b) The kinetic enolate could be conveniently prepared using a strong hindered base (Scheme 8.2b) such as lithium diisopropylamide (LDA);[161] (see Results and Discussion 9.1). Epoxidation of the enol acetate with a peroxy-acid[162] and subsequent hydrolysis would yield an α-hydroxy-ketone which could then be selectively-fluorinated to the 1-fluoro-6-methyl-hept-5-en-2-one (8) using DAST.

(c) The equilibrium- or kinetically- derived β-trimethylsiloxyethers (trimethylsilyl-enol ethers; hereafter referred to as TMS-enol ethers; 9,10,11) can be prepared by

quenching the respective enolates with TMS-chloride,[163] or by specific reaction of methyl-heptenone with TMS-iodide[164] (see Results and Discussion section 9.5 and 9.7) or ethyltrimethylsilyl acetate,[165] (Results and Discussion section 9.6) to generate equilibrium and kinetic TMS-enol ethers respectively (Scheme 8.2 c,d). These can be brominated and then fluorinated as in (a) or fluorinated directly with N-fluoropyridinium triflate[154] (hereafter referred to as NFPT) to yield the 3-fluoro- or 1-fluoro- methyl-heptenones (5 and 8; see Results and Discussion section 9.8).

The presence of a geminal fluorine may labilise the protons in 5 and 8 towards further enolisation on treatment with one of the reagents above and in consequence very high yields of α,α-difluoro-ketones could be prepared. Dehydrofluorination from the 3,3-difluoro-methyl-heptenones by passage through a column of alumina[166] would yield the conjugated 3-fluoro-methyl-heptenone (and subsequently the fluoro-linalool, geraniol and nerol derivatives of derived this compound).

8.3.2 Introduction of Allylic Fluorine to Positions C_4 and C_7 of 6-Methyl-hept-5-en-2-one

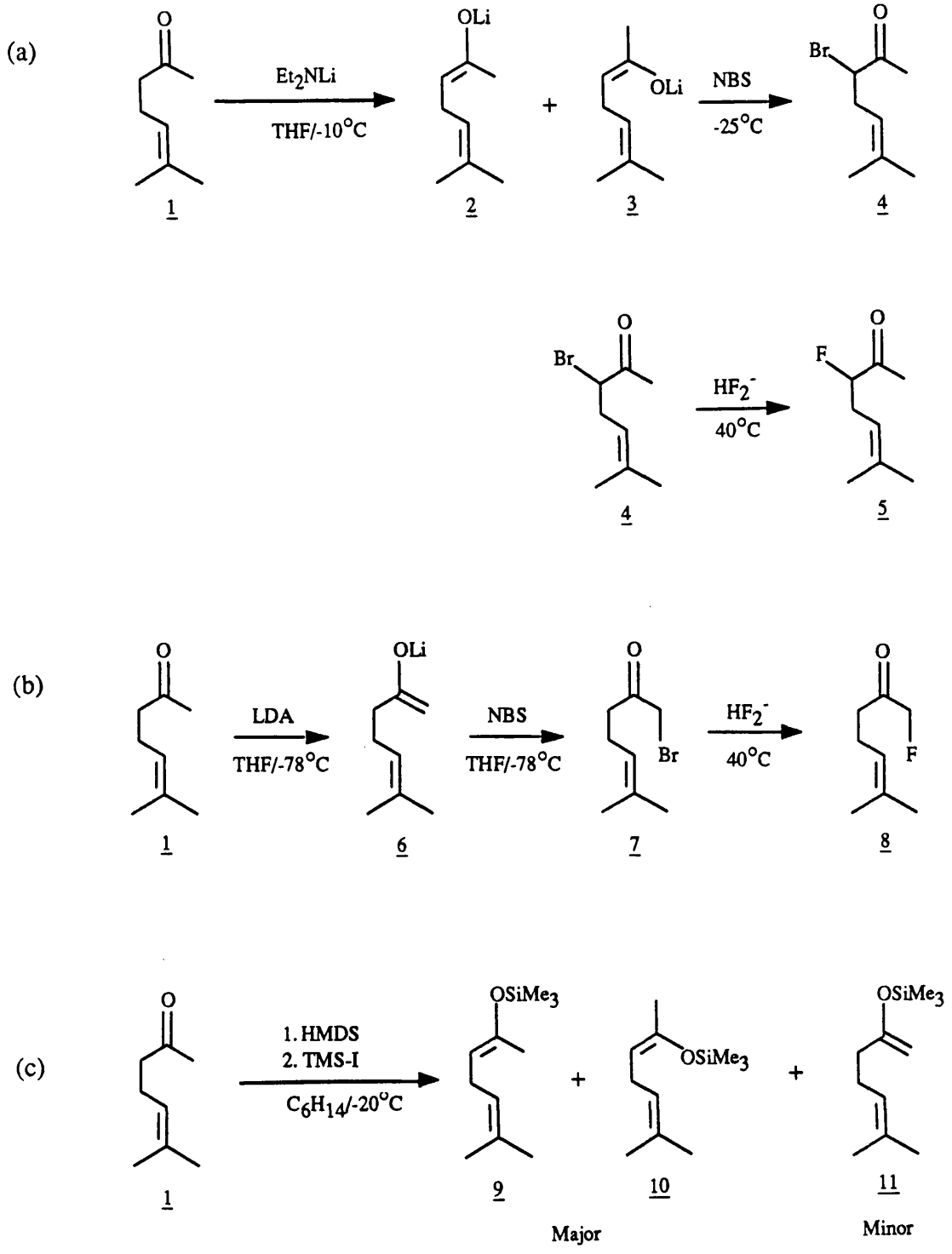
Bromine can be introduced at position C_4 by the classical treatment with N-bromo-succinimide (NBS)[167] followed by displacement of bromide by fluoride.[168]

8.3.3 Introduction of Fluorine to Position C_5 of 6-Methyl-hept-5-en-2-one

(a) 5-Fluoro- (and 5-hydroxy-) methyl-heptenones could be obtained by ring-opening of the 5,6-epoxide with triethylamine hydrofluoride,[169] and subsequent dehydration (or dehydrofluorination) with an acid catalyst.

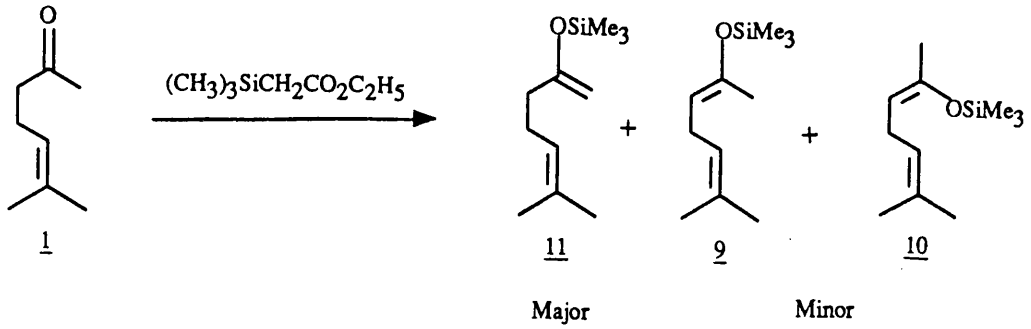
(b) Selective vinylic-fluorination at C_5 could be attempted using the method of Burdon *et al.*[170] by saturation of the double bond over $KCoF_4$ to form the vicinal difluoride followed by dehydrofluorination to yield the 5-fluoro-methyl-heptenone (thus, not by direct replacement). The product may alternatively be prepared by dehydrobromination of the vicinal bromofluoride produced by bromofluorination[171] of methyl-heptenone using NBS and tetrabutylammonium fluoride (TBAF).

Scheme 8.2 Steps that We Used for the Preparation of Fluorinated-Linalools and Related Compounds

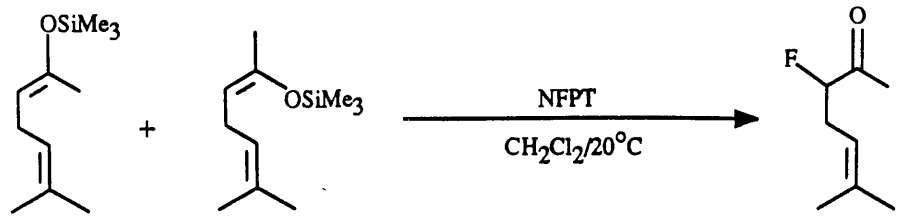


HMDS - Hexamethyldisilazane; TMS-I - Trimethylsilyl Iodide; NBS - N-Bromosuccinimde; LDA - Lithium Diisopropylamide;

(d)

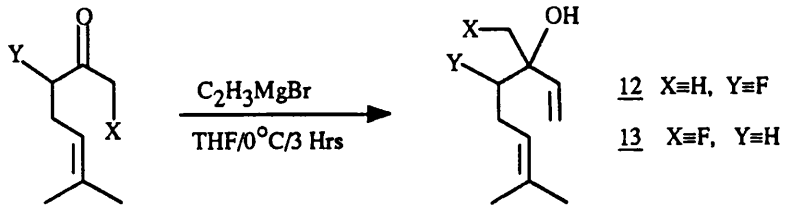


1 → 11 (Major) + 9 + 10 (Minor)



9 + 10 → 5

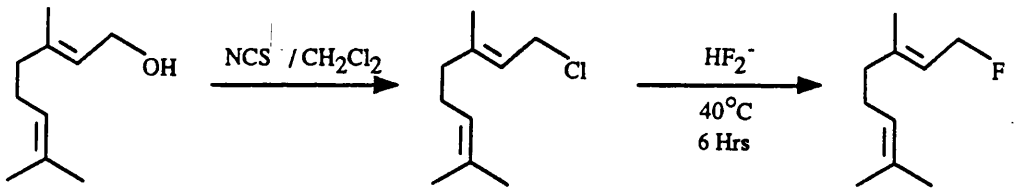
(e)



12 X≡H, Y≡F

13 X≡F, Y≡H

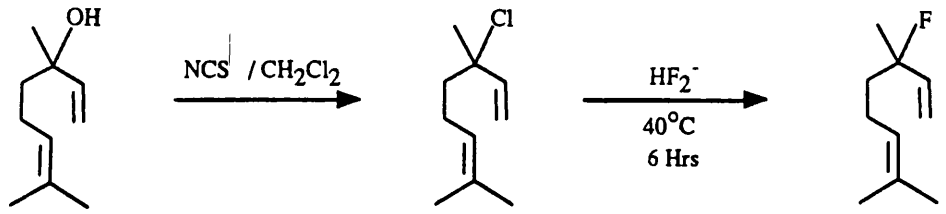
(f)



14 2*E*-
15 2*Z*-

17 2*E*-
18 2*Z*-

(g)



16 → 19

NFPT - N-Fluoropyridinium Triflate; NCS - N-Chlorosuccinimide

8.3.4 Reactions of Organometallic Reagents with Position C_2 of Fluoro-6-methyl-hept-5-en-2-ones

(a) Routes to fluorolinalools and fluorodihydrolinalools are provided by the reaction of the fluoroketone with vinylmagnesium bromide,[172] vinyllithium,[173] vinyl chloride and ethylmagnesium bromide respectively (Scheme 8.2e).

Modified reaction conditions are probably required in each case (see Results and Discussion section 9.9 which discusses the effect of fluorine on the reaction of 1-fluoro- and 3-fluoro-methyl-heptenones with vinylmagnesium bromide). Alternatively the two-step acetylenation- hydrogenation[174-8] (dissolving metal[179] or Lindlar catalyst[180]) may be employed.

Intuitively, a fluoro-Grignard reagent can introduce the fluorine atom to methyl-heptenone but there is no evidence for the formation of such reagents and they cannot be obtained commercially. This may be attributable to the decomposition of these reagents to form fluorocarbenes.

(b) Thioacetylisation[181] of the fluoromethyl-heptenone would be a particularly elegant preparation of the fluorogeraniol and fluoronerol regioisomers providing the intermediate thioethers do not undergo nucleophilic displacement of ethyl sulphide during the hydrolysis of the trimethylsilyl-moiety.

(c) Conversion of the fluorolinalools into geranyl and neryl analogues could be conveniently carried out by treatment of the fluorolinalool with *p*-toluenesulphonic acid,[182] boron trifluoride etherate[141] or by the more recent method of Fujita *et al.*[183] by refluxing with $O{=}W[OSi(C_2H_5)_3]_4$.pyridine complex.

(d) The target fluoro-monoterpenoid alcohols may be derivatised for administration to cell-free extracts as the respective diphosphate esters[184-5] or phosphonates.[186] Conversion of the alcohols to the corresponding glucosides for administration to cell-cultures can be carried out by the modified Königs-Knorr procedure of Banthorpe *et al.*[187]

8.3.5 Fluorination of a Monoterpenoid Alcohol

Geranyl fluoride has been prepared[188] by treatment of geraniol with methyllithium and *p*-toluenesulfonyl fluoride. Substitution[189] of halogen by fluorine in either geranyl bromide or -chloride (and the neryl and linaloyl analogues), should provide a simple conversion to the corresponding fluorides (Scheme 8.2 f, g; see Results and Discussion section 9.10). Although reagents such as DAST and triethylamine hydrofluoride provide a single- step conversion (with inversion of configuration) of an alcohol to the corresponding fluoride, these reagents are costly and difficult to handle. Treatment of an activated derivative of the alcohol with TBABF would be a simple method for obtaining the fluoride in good yield, with retention of configuration at the carbon bearing the functional group.

The methods selected from the above that were finally attempted are presented in the Results and Discussion in Chapter Nine. The complete experimental details are given in section 15.1.

Chapter 9 Results and Discussion

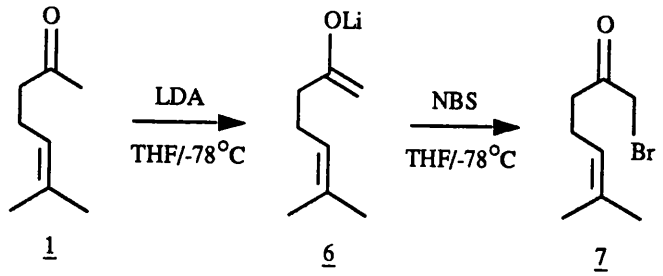
Aims: This chapter describes those routes that were outlined in Chapter Eight which were chosen to make the five fluorinated terpenoids that we eventually prepared.

The preparation of lithium enolates 2,3 and 6 was the initial method of choice for the selective introduction of fluorine at C_1 or C_3 of 6-methyl-hept-5-en-2-one. TMS-enol ethers were also formed under conditions of equilibrium and kinetic control and brominated and fluorinated to yield the respective fluoromethyl-heptenones. The equilibrium TMS-enol ethers were fluorinated directly with NFPT. The fluorolinalools were prepared by treatment of the fluoroketones with vinylmagnesium bromide using modified reaction conditions.

Linaloyl, neryl and geranyl fluorides were prepared from the respective chlorides which in turn we obtained by a modified Corey-Kim procedure described in the Experimental Section (15.1j).

Since many of the reaction products were isomeric, the mass spectra and NMR spectra are discussed in detail to confirm structural assignments. Also, few ^{19}F-NMR spectra of terpenoids are reported and interpreted in the literature and no such spectra have been analysed for our compounds. The discussions also include some schemes to account for fragmentations observed in the mass spectra of the products.

9.1 Generation and Bromination of the Kinetically-controlled form of the Enolates of 6-Methyl-hept-5-en-2-one

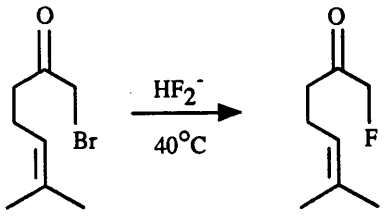


1 6 7

The 1-bromo-ketone (7) was prepared by the slow addition of a solution of methyl-heptenone in THF to a solution of lithium diisopropylamide[161] at -78°C to yield the kinetically-controlled lithium enolate (6), which was then brominated with NBS, (32 %; see 15.1g). This product gave characteristic fragmentations in its mass spectrum; cleavage of the C_2-C_3 bond gave ions at *m/z* 121: *m/z* 123 (1:1); cleavage of the C_3-C_4 bond gave ions at *m/z* 135: *m/z* 137 (1:1). The absence of an ion at *m/z* 125 or quasi-molecular ions [*ie.*, $(M+1)^+$] associated with the 3-bromo-ketone (section 9.3) support the 1-bromo-structure.

The ^{1}H-NMR showed a singlet at 4.61 ppm corresponding to the methylene protons of C_1. It is surprising that no contribution of the 1,2-enol form of 7 was observed in the ^{1}H-NMR spectrum. Calculations predict a doublet at 6.08 ppm for the vinylic proton of C_1 in this form and a 0.12 ppm shift upfield for the methylene protons of C_3 with a four-bond coupling between the protons of C_1 and C_3, of approximately 2 Hz. depending on the geometry of the C_1-C_2 bond.

9.2 Preparation of 1-Fluoro-6-methyl-hept-5-en-2-one from 1-Bromo-6-methyl-hept-5-en-2-one



7 8

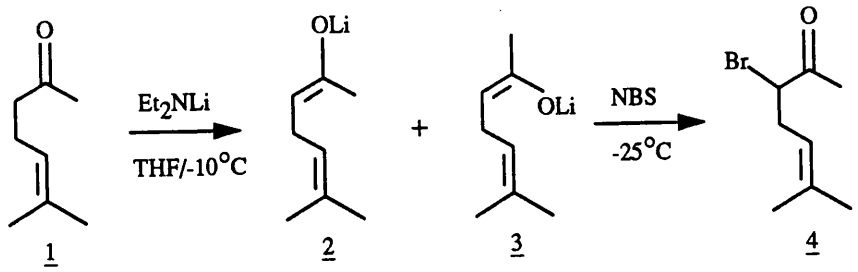
Substitution of bromine by fluorine was achieved by the addition of 7 to vacuum-dried TBABF at 40°C for 5 minutes to yield the 1-fluoro-ketone (8; 10 %). A molecular ion (*m/z* 144) was observed for this product. Fragmentations of bonds C_1-C_2 and C_2-C_3 were indicated by ions at *m/z* 111 and *m/z* 83 respectively. It is interesting that a peak for *m/z* 68 was observed (15 %) which can only be accounted for by a distonic process (distonic ions have charge and radical-site formally separated; section 9.7.2). The ^{1}H-NMR spectrum of the 1-fluoro-ketone revealed a downfield shift of the methylene signals of C_1 of 2.65 ppm (relative to 1) to 4.79 ppm with a four-bond proton-fluorine coupling of 2.75 Hz. The origin of this is discussed in section 9.8.

Although the formation of a kinetically-controlled mixture of lithium enolate (6) was synthetically useful for the preparation of almost pure 1-bromo-ketone, it could not be readily converted into the equilibrium enolates (2 and 3). Kinetically-controlled mixtures are formed in relatively non-polar solvents such as THF and DME with lithium amide bases.[190] Abstraction of the proton at C_1 of methyl-heptenone is typically 20-fold greater than the abstraction of the protons at C_3. The analysis by House[191] of enolate equilibria revealed the more highly-substituted enolates to be less stable than the kinetic regioisomer because of the large steric bulk of the covalent lithium-oxygen association. This contrasts to an increased proportion of equilibrium regioisomers for enolates generated from potassium bases.

However, in a subsequent communication House[192] reported that when equilibrium conditions were achieved (in the presence of an excess of the ketone) a greater proportion of equilibrium regioisomers were present for lithium enolates because the covalent lithium-oxygen bond behaved in a manner analogous to a carbocation which favoured alkyl substitution. However, lithium enolates typically require increased reaction times and elevated temperatures to achieve equilibrium conditions.

The kinetic enolate could be equilibrated by the standard methods; *ie.*, conversion into the corresponding enol acetate followed by treatment with *p*-toluene-sulphonic acid[193] or methyllithium.[194]

9.3 Generation and Bromination of the *E*- and *Z*- Equilibrium-controlled Forms of the Enolates of 6-Methyl-hept-5-en-2-one



1 2 3 4

The equilibrium enolates (2 and 3) were prepared by treatment of a solution of lithium diethylamide[158] with methyl-heptenone at -25°C for two hours. The lithium

reagent had been prepared in a separate flask from the reaction of butyllithium[195] with diethylamine and was transferred to the reaction flask by means of a ground-glass connecting tube filled with glass wool using an argon pressure bleed. This procedure removed any unreacted lithium from the reaction pot.

A solution of NBS in freshly-redistilled THF was added slowly over the course of a further hour at low temperature (section 15.1f) to yield the 3-bromo-ketone (4; 40%). The 3,3-dibromo-ketones were formed as the major by-products of dibromination (analogous to dialkylation products) which would be expected from an equilibrium mixture of enolates. Clearly, the presence of the first bromine made the 3-bromo-ketone labile towards further enolisation and bromination at C_3. The relative rates of kinetic and equilibrium deprotonations are probably similar whereas there is a 20-fold difference in deprotonation of these sites in the parent methyl-heptenone (1). It has been reported[191] that dialkylation of 2-methyl-3-heptanone can be reduced by alkylation of the kinetic enolate *in situ*. However, *in situ* bromination with NBS would not be suitable owing to the tendency of this reagent to enolise and so consume the lithium base.

The product (4) underwent quasi-chemical ionisation (*ie.*, self-chemical ionisation; CI; by a bimolecular process) under GC/MS conditions (an open EI source was used *ie.*, a source that was not specifically sealed to prevent leakage of a CI-reagent gas) yielding two $(M+1)^+$ molecular ions at, *m/z* 205:207 (1:1). A bimolecular ion-molecule process must account for this; hydrogen bromide is probably eliminated from a first molecular ion and then protonates a second. A fragment-ion at *m/z* 125 corresponding to elimination of hydrogen bromide from the molecular ion was observed. Such processes are not unusual in GC/MS since the source-pressure may be 10-fold higher than that used for probe-sample introduction owing to the helium carrier gas. Ballentine *et al.*,[196] have observed this phenomenon for various α-substituted carbonyl compounds by comparison with CI spectra of these compounds. The process was rationalised by one or more of the fragment-ions behaving as a CI-reagent gas which protonates the intact neutral molecule. For this to happen, this molecule must have a greater proton-affinity than the fragments. Some ketones have indeed been shown to form ion-molecule complexes[197] sufficiently long-lived to be detected.

Fragment-ions were also observed at *m/z* 161 and *m/z* 163 (1:1) corresponding to C_2-C_3 cleavage. No ions resulting from the C_3-C_4 cleavages of 4-bromo-, 5-bromo- or 6-bromo-products of allylic bromination by NBS were detected.

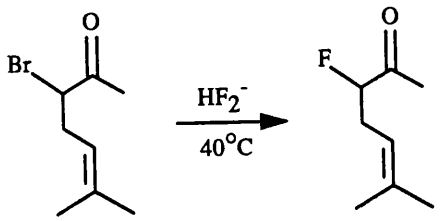
The reconstructed ion-current (RIC; an interpretation by the data system of the total ion-current with time) for the GC/MS spectrum of the product indicated three dibrominated product-peaks (20% GC-yield) with retention times 1.5 relative to the product 3-bromo-ketone (4). One peak showed molecular ions at *m/z* 282:284:286 (1:2:1) corresponding to the 1,3-dibromo-product. Peaks at *m/z* 203:205 (1:1) indicated loss of bromine from the molecular ion. The other two compounds also showed peaks at *m/z* 203:205 but no molecular ions. The spectra of these compounds were consistent with the structures of α,α- and α,α'-dibromo-6-methyl-5-hepten-2-ones. The molecular ion of a dibrominated product may readily interconvert to enol-forms by hydrogen atom or proton-transfer: this is analogous to the ionisation of most ketones under electron-impact (see 9.7.2). The interconverting enol-forms would be expected to stabilise the molecular ion and they would also form the precursors to a series of distonic ions. Such ions readily account for the fragmentations producing the ions observed in the mass spectra (a full discussion of distonic ions is given in section 9.7.2). The spectra showed signals at *m/z* 202, 203, 204, 205, 206, 207, *m/z* 148:150 for ions resulting from loss of $Br^{\cdot}$ and HBr (for both ^{79}Br and ^{81}Br) from the molecular ions M^+ (282;284;286; 1:2:1) and for the $(M+1)^+$ even-electron molecular ions (283:285:287; 1:2:1).

Only one enol-form can be drawn for the molecular ion of the 3,3,-dibromo-ketone in which both bromines are allylic. Clearly, this would be prone to lose the two allylic bromine atoms to yield the two fragment-ions $(M-^{79}Br)^+$ and $(M-^{81}Br)^+$ which were observed in the mass spectrum. The base peak in the spectrum of this compound was the ion at *m/z* 124 corresponding to $(M-Br-Br)^+$.

Two enol forms can be drawn for the molecular ion of the 1,1-dibromo-ketone: the major form has both bromine atoms in vinylic positions. As expected, the spectrum of this isomer also shows an ion $(CHBr_2)^+$ resulting from cleavage of the C_1-C_2 bond.

Hoffman[198] has correlated the fragmentation patterns of α,α-dibromo-ketones with the tendency of these molecules to enolise in solution (by treatment with Zn/Cu couple in methanol). He argued that compounds with greater alkyl substitution were more likely to form an α-methoxy-ketone than would an unsubstituted ketone because of the greater lifetime of the enolate-structure in the former. Similarly, the prolonged lifetime of the enolate-like molecular ions of these compounds resulted in a number of rearrangement ions (eg. M^+-Br; M^+-Br_2; M^+-Br-CO; M^+-HBr_2) with total ion-intensity proportional to the quantity of methoxy-ketone formed in solution. However, his theory would predict the absence of rearrangement-ions such as $(M\text{-}HBr_2\text{-}CO)^+$ for compounds such as 1,3-dibromo-methyl-heptenone which do not readily enolise in solution. This compound actually showed a very intense peak for such a rearrangement-ion, which is probably due to the contribution of two enol-forms to the stability of the molecular ion. Hoffman's theory did not take account of the latter.

9.4 Attempted Preparation of 3-Fluoro-6-methyl-hept-5-en-2-one from 3-Bromo-6-methyl-hept-5-en-2-one



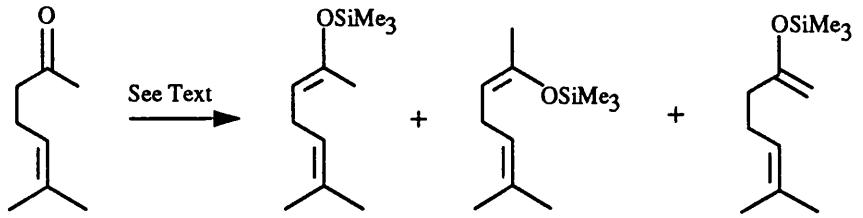
4 5

Substitution of bromine by fluorine using vacuum-dried TBABF as reagent failed and resulted in dehydrobromination to yield a product showing an intense molecular ion at *m/z* 124, corresponding to an α-β unsaturated diene. It is likely that the conjugative stability of this compound favoured elimination from the 3-bromo-ketone over substitution. Fluorination at room temperature did not work since the liquid TBABF soon solidified. Other methods and conditions were thus attempted; *eg.*, (i) TBAF in THF (ii) potassium bifluoride; (iii) caesium fluoride; (iv) potassium fluoride-16/8 crown ether; but these methods also failed. *In situ* fluorination was attempted by the addition of a mixture of TBAF (2 mol. equiv.) and NBS added to

the enolate at -25°C in the previous manner. However, the presence of the fluorinating reagent decomposed the lithium-enolate probably by destruction of the $Li^{+}OR$ ion-pair.

To overcome this problem it was possible that the enolate could be trapped as the corresponding TMS-enol ether. Direct mild fluorinations of trimethylsilyl enol ethers[154] and sulphides[199] have been reported.

9.5 Attempts to Prepare Kinetically-controlled and *E*- and *Z*-Equilibrium-Controlled Trimethylsilyl-enol Ethers of 6-Methyl-hept-5-en-2-one by Classical Methods



1 9 10 11

Silyl-enol ethers can be prepared by a number of methods[200-201] and have become invaluable synthetic intermediates over the last 20 years. Four "classical" methods were employed to form a mixture of equilibrium TMS-enol ethers (9,10) from methyl-heptenone.

(a) The method of Stork *et al.*[202] (which involved refluxing methyl-heptenone with a dispersion of sodium hydride in glyme in the presence of an excess of triethylamine and trimethylsilyl-chloride) gave only limited, conversion (5%) to a mixture of enol ethers, with considerable polymeric aldols as by-products. Proton-abstraction is thought to proceed only in the presence of traces of alcohols (and the corresponding alkoxides) which are the proton-transfer agents.[203]

(b) A modification of (a) utilising the coloured triphenylmethyl-lithium (formed from the reaction of triphenylmethyl-chloride with lithium)[159] as base was employed in an attempt to observe enolate-formation by colorimetric titration of methyl-heptenone. This procedure was not reproducible: even warming the reaction mixture to -25°C

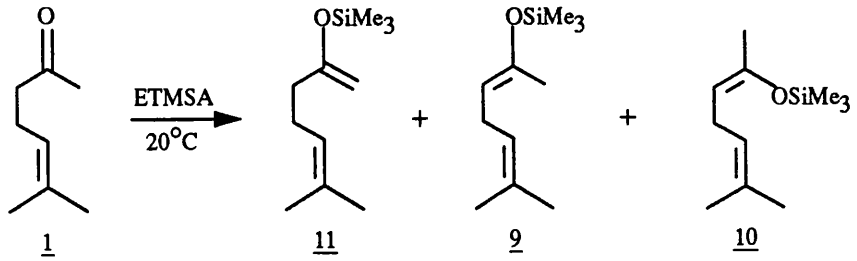
failed to initiate the reaction. This suggested that aldol condensations may even occur at -78°C! The preparation was repeated by adding the lithium-reagent to a solution of the ketone and trimethylsilyl chloride and (2 mol. equiv.), triethylamine (2 mol. equiv.) cooled to -78°C, but was again unsuccessful. Tomboulian *et al.*[159] have found that significant quantities of aldols were formed in the enolisation of acetone and cyclohexanone, and they considered that excess of lithium in the reaction pot was responsible for the complex mixture of products formed during the enolisation of benzaldehyde.

Two further techniques[163] for the production of trimethylsilyl-enol ethers from ketones were attempted:

(c) The first involved generation of the equilibrium enolate using lithium diethylamide and quenching of this enolate with trimethylsilyl chloride and triethylamine. This was not reproducible for the formation of 2 and 3. It was possible that the diethylamine (produced by formation of the enolate) was consumed by reaction with the trimethylsilyl-chloride because the kinetic enolate (formed from the reaction of the more hindered lithium diisopropylamide with 1) could be readily quenched to give the trimethylsilyl-enol ether in good yield (58 %).

(d) The final conventional technique we used involved reaction of methyl-heptenone with trimethylsilyl-chloride and triethylamine under reflux (equilibrium) conditions. The products (9 and 10) could not be obtained at temperatures lower than 100°C within 48 hours. The optimum yield was 20%. Nearly thirty successive attempts failed to optimise reaction conditions; these included (i) using triethylamine as solvent and HCl acceptor and with excess (50%) of trimethylsilyl-chloride; (ii) addition of trimethylsilyl- choride to a solution of methyl-heptenone and triethylamine in dimethylformamide after 1 hour of reflux; (iii) increased reaction time (60 hours) at 90°C; (iv) decreased reaction time (12 hours) at 130°C.

9.6 Preparation of the Kinetically-controlled Trimethylsilyl-enol Ether of 6-Methyl-hept-5-en-2-one using Ethyltrimethylsilyl-acetate



1 11 9 10

Major Minor

The kinetic trimethylsilyl-enol ether (11) could be prepared from methyl-heptenone in good yield (total 92%: 11, 93%; 9,10, 7%) with an excess of ethyltrimethylsilyl-acetate (ETMSA; containing TBAF as catalyst) at -25°C (note; careful work-up; see section 15.1h).[165]

The enolisation of methyl-heptenone proceeds by formation of a tetrabutylammonium-enolate of ETMSA which then abstracts an α-proton of the ketone in a kinetically-controlled manner.[204] The deprotonation of ETMSA by fluoride to produce the anion $(Me_3SiCHCOOR)^-$ instead of the desired $(CH_2COOR)^-$ is unlikely as such ions have previously been shown to undergo addition to carbonyl compounds.[205] The enolate is then silylated by trimethylsilyl-fluoride. At increased reaction times the yield of the kinetic enol ether was decreased but the proportion of equilibrium geometric isomers increased; TBAF has been previously reported to cleave enol ethers to the parent ketones.[206] Equilibration of 11 with 1 would therefore occur at increased reaction times. This was minimised by the addition of excess of ETMSA. Nakamura reported[207]* *Z*- stereoselectivity (97%) for the equilibrium products from initial kinetic selection. This contrasts to *E*-stereoselectivity for the formation of lithium-enolates (section 9.2). The difference may be attributable to the differing mechanisms; (i) a six-centred transition state in lithium-enolates where the steric repulsion between the amine moiety (of the lithium amide) and the alkyl group of the ketone leads to *E*-stereochemistry and (ii) a five-centred transition state for enolates generated by ETMSA (where the ammonium-cation plays no part) in which steric repulsion between the alkyl groups of the ketone leads to *Z*-stereoselectivity.

Table 9.1 Yields of Kinetic TMS-enol Ether

Reaction Time (hrs.)	Yield: (%)[a]	11(%)[b]	9(%)[b]	10(%)[b]
0.25	98	93	6	1
1.00	91	77	{22}*	
3.00	79	53	{46}*	
24.00	65	42	{58}*	

a Yield by weight
b GC-Yield using Peak-areas
* mixture of 9 and 10 (see Ref. 207)

The term kinetic refers to the conditions employed for the formation of the lithium enolate.

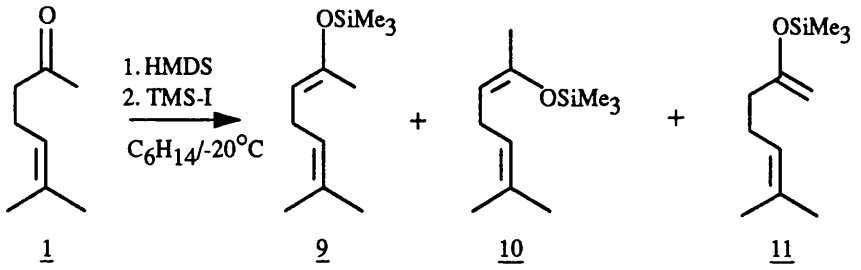
9.6.1 Discussion of ^{1}H-NMR Spectrum

The ^{1}H-NMR spectrum of 11 showed the two vinylic signals for the protons of C_1 at 4.05 ppm and 4.06 ppm with separate four-bond couplings to the protons of C_3 of 2.41 Hz. and 1.58 Hz. respectively.

9.6.2 Discussion of Mass Spectrum

The mass spectrum of the product showed two intense ions at *m/z* 130 (70%) and *m/z* 115 (80%) which can only be explained by a quasi-McLafferty rearrangement; the terminal C_1-C_2 double bond of the kinetic enol ether probably abstracts the hydrogen at C_5 by six-membered ring formation. For this to occur the C_6-C_7 double bond must migrate (by a distonic process) to the C_7-C_8 position. If equilibrium occurs there may only be a small proportion of ions showing this bond-migration but their rapid fragmentation by the McLafferty rearrangement would drive the equilibrium towards their formation. House[163] has previously reported a quasi-McLafferty rearrangement of this type for the kinetic TMS-enol ether of 2-heptanone in which the rearrangement can occur directly. This would substantiate the required hydrogen rearrangements in its unsaturated analogue.

9.7 Preparation of *E*- and *Z*- Equilibrium-controlled Trimethylsilyl-enol Ethers of 6-Methyl-hept-5-en-2-one Using Trimethylsilyl-iodide



1 9 10 11

Major Minor

An equilibrium mixture was successfully prepared by treatment of methyl-heptenone with hexamethyldisilazane (HMDS) and trimethylsilyl-iodide[164] in pentane to yield 9 (82 %); 10 (16 %); 11 (2 %; total 86 %). The predominance of the *E*-stereoisomer suggests there is a considerable energy barrier to formation of the *Z*-isomer due to loss of orbital-overlap caused by steric interaction of the bulky trimethylsilyl group. This parallels the proportions of kinetic and equilibrium lithium-enolates mentioned previously.

9.7.1 Discussion of ^{1}H-NMR Spectra

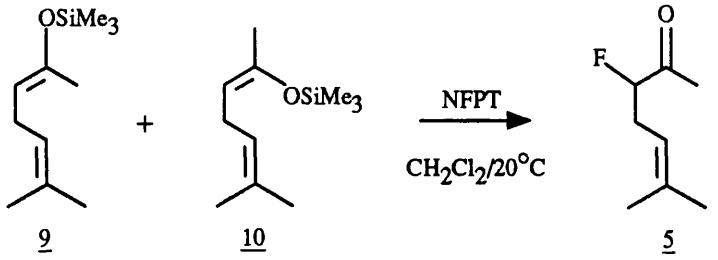
The ^{1}H-NMR spectrum of the *E*-stereoisomer (9) showed that the β-vinylic proton of C_3 occurred at 4.38 ppm with a *trans*-allylic four-bond coupling (0.94 Hz.) to the protons of C_1. Further, the signal for the protons of C_4 was shifted downfield by 0.40 ppm (relative to 1). The signal was observed as a distorted triplet with a three-bond coupling (7.11 Hz.) to the protons of C_2. The signal for the β-vinylic proton of the *Z*-stereoisomer (10) occurred at 4.18 ppm with a *cis*-allylic four-bond coupling of 1.84 Hz.

9.7.2 Discussion of Mass Spectra

The mechanism proposed by House[163] for the fragmentation of molecular ions of the equilibrium TMS-enol ethers of 2-heptanone explained the formation of diagnostic ions at *m/z* 130 and *m/z* 115 by cleavage of the C_4-C_5 bond. The equilibrium TMS-enol ethers of methyl-heptenone (9,10) cannot undergo cleavage of the vinylic C_4-C_5 bond in the same way although the fragment ions at *m/z* 130 and *m/z* 115 were observed in the mass spectra. Moreover, these ions were characteristic of the kinetic

TMS-enol ether (11) and so they were probably derived from a population of interconverting distonic molecular ions. This situation probably involved initial isomerisation of the molecular ions via a common distonic keto-molecular ion. The lower abundances (50% of that of the kinetic isomer) of ions at *m/z* 115 and *m/z* 130 in the spectra of the equilibrium TMS-enol ethers are evidence for the required isomerisation (via the keto-ion) being required to occur before fragmentation. Distonic ions are common in the rearrangements of ionised carbonyl compounds.[208] Asymmetric ketones such as 1 form distonic ions[209] by 5- and 7-membered ring rearrangements[210-12] giving rise to distonic enol-ions. Clearly, for 9 and 10 these ions could readily interconvert to a series of other distonic molecular ions. The formation of such a population at equilibrium conditions is probably competitive with unimolecular dissociation[213] (*eg.*, the quasi- McLafferty abstraction of the hydrogen at C_5). Distonic ions readily undergo 3-,5-,6- and 7- membered processes[214-16] to produce cyclic intermediates or transition states. Even 4-membered rings have been reported to be formed from the molecular ions of some ketones.[217] Methyl-heptenone (1) shows a peak at *m/z* 108 corresponding to $(M-H_2O)^+$. Sigsby *et al.*[218] have shown dehydration of the molecular ion of protonated hexanone to occur by a 4-membered ring-rearrangement. Loss of water from the molecular ion of methyl-heptenone (1), under normal EI conditions, probably occurs via the same mechanism but from a distonic (*versus* protonated) molecular ion (equivalent to a protonated molecular ion for this purpose). The critical energies of many skeletal fragmentations are dependent on the strain energies of such cyclic transition states.[219]

9.8 Preparation of 3-Fluoro-6-methyl-hept-5-en-2-one by Direct Fluorination of *E*- and *Z*-Equilibrium-controlled Trimethylsilyl-enol Ethers using N-Fluoropyridinium Triflate



9 10 5

A mixture of the enol ethers 9 and 10 was refluxed with a suspension of N-fluoropyridinium triflate in anhydrous dichloromethane for 12 hours to give the 3-fluoro-6-methyl-hept-5-en-2-one (5 ; 26 % yield). This contrasted with the yields reported by Unemoto *et al.*[154] (90 %), and may reflect the lability of the fluoroketone towards elimination in the presence of the triflate ion (under reaction conditions favouring E2; refluxing in dipolar aprotic solvent).

9.8.1 Discussion of NMR Spectra

We observed coupling between hydrogen and fluorine nuclei in the NMR spectra of 5. This is an infrequently reported phenomenon, and there are few reports that mention such coupling in fluoroterpenoids. This section describes our observations and an attempt at interpretation has been made, based on current theories. The ^{1}H-NMR spectrum of 5 showed a signal at 2.51 ppm for the methylene protons at C_3 with a two-bond proton-fluorine coupling of 50.04 Hz. and a three-bond proton-proton coupling of 6.62 Hz. Table 9.2 shows the experimental coupling constants for the fluoroketones 5 and 8. Notice that $^2J_{HF}$ was larger in the 3-fluoro-ketone because of the differing substituent effects operating at C, compared with C_3 (the methylene group of C_1, in 8 is more deshielded than the methine group of C_1 in 5)

Phillips and Wray[220] have calculated the dependence of $^2J_{HF}$ values on various substituents and observed a decrease of 4 Hz. when an α-carbon is replaced by sulphur. Pople[221] has observed a small dependence of $^2J_{FH}$ on the proton-fluorine internuclear separation and the dihedral between the two nuclei.

Table 9.2 Observed Proton-fluorine Coupling Constants for 1-Fluoro- and 3-Fluoro-6-Methyl-hept-5-en-2-one

H-C_n	$^nJ_{HF}$ (Hz.) (1-Fluoro)	$^nJ_{HF}$ (Hz.) (3-Fluoro)	HF Internuclear Separation (nm)*	$^nJ_{HF}$ (Hz.) (1-Fluoro)	$^nJ_{HF}$ (Hz.) (3-Fluoro)
1	$^2J_{HF}$ = 47.76	$^4J_{HF}$ = 4.74	0.23	$^1J_{CF}$ = 184.9	-
2	$^4J_{HF}$ = 2.75	$^2J_{HF}$ = 50.04	0.23	-	-
3		$^3J_{HF}$ = 19.43	0.25	-	$^1J_{CF}$ = 185.9
4				-	$^2J_{CF}$ = 20.80
				-	$^3J_{CF}$ = 3.50

* Estimated value from scale model | Note: numbering (n) refers only to those carbons attached to protons

Both 5 and 8 exhibit unexpected $^4J_{HF}$ coupling. The former showed signals for the C_1 methyl-group as a doublet (2.22 ppm). The splitting of this signal probably resulted from through-bond and through-space effects: $^4J_{HF}$ values have previously been reported to be a consequence of F-H hydrogen bonding. Through-space effects are well documented for rigid systems.[222] This phenomenon was also observed by Mooney[223] in the ^{1}H-NMR spectrum of a rotamer population of $(CH_2F)_3CF$ and was greatest for the T_{FFF} form (*ie.*, all protons equivalent). An empirical converging-vector rule[224] has been formulated to predict the phenomenon. $^nJ_{FH}$ (n>3) occurs if a vector directed along the C-F bond converges upon and intersects with a vector drawn along an angular C-H bond. Construction of a molecular model of 5 demonstrated that the minimum internuclear distance between the fluorine nucleus and the γ-protons of C_1 was no greater than the internuclear distance of the α-protons of C_3 from the fluorine nucleus (*ca.* 0.23 nm) and less than the β-proton-fluorine distance (*ca.* 0.25 nm). Furthermore, the converging-vector rule was only satisfied by the γ-protons of C_1. Hilton and Sutcliffe[225] have reported through-space coupling at distances greater than 0.22 nm. Three mechanisms were postulated for this phenomenon: two of these assume overlap between rear lobes of C-H bonds but the mechanism of Anet *et al.*[226] requires an intervening atom to be present (such as oxygen) to transmit the coupling

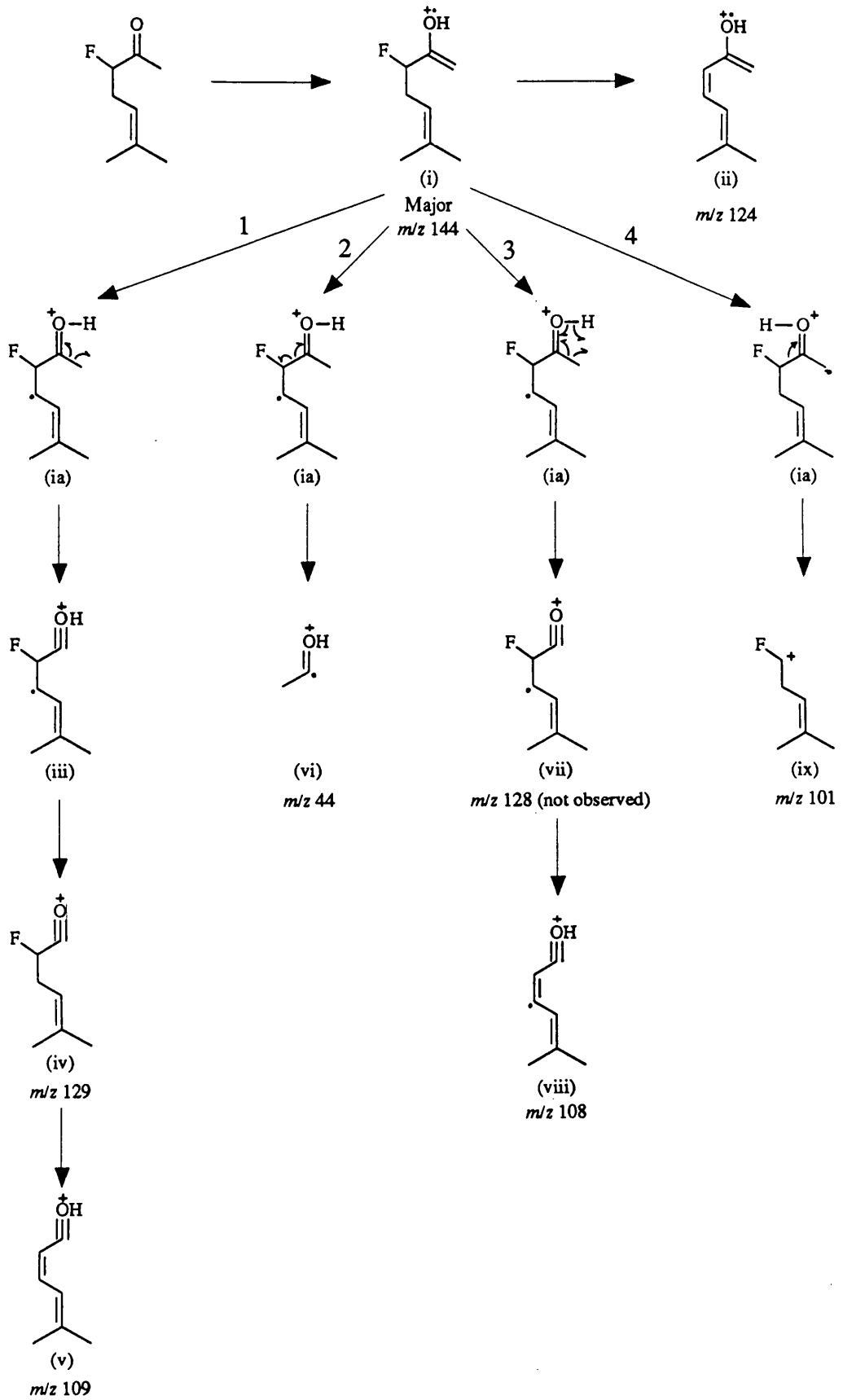
via lone-pairs of electrons. This mechanism could explain the $^4J_{FH}$ coupling observed in our work. It is possible that the fluorine lone-pairs contributed sp^2 character to the C_2-C_3 bond thus decreasing the internuclear β-proton-fluorine distance. Construction of a model with both C_2 and C_3 sp^2, gave an internuclear distance *ca.* 0.20 nm. No theoretical verification of this mechanism has been advanced. No coupling between the γ-protons of C_1 with the protons of C_3 was observed in the parent-ketone (1). The 1-fluoro-ketone (8) also showed a through-space $^4J_{FH}$ coupling of 2.75 Hz. (see 9.2). The internuclear separation between the fluorine and the γ-hydrogen of C_5 in the 3-fluoro-ketone was estimated to be 0.17 nm and the orientation of these nuclei also satisfied the converging-vector rule. $^5J_{HF}$ has been reported to be as large as 3.0 Hz. in *syn*-3-fluoro-anti-bromo-exo-tricyclo-[3.2.1.0]-octane.[227] Construction of a scale model demonstrated the internuclear distance in this molecule to be approximately 0.15 nm.

Table 9.2 shows C-F coupling constants. These are typically complex and previously calculated values have often not agreed with experimental results.[228] The observed value of J_{CF} may have three contributions, possibly varying in sign as well as magnitude, and therefore substituent and structural effects are large and complicated. Some long-range $^3J_{CF}$ and $^4J_{CF}$ values have been reported for aliphatic compounds.[229] These are thought to be conformationally dependent.

9.8.2 Discussion of Mass Spectra

The mass spectrum of 5 showed a molecular ion at *m/z* 144 (2%). Dehydrofluorination of the molecular ion (i) to yield an ion (ii) (*m/z* 124;58 %) was observed and this was probably the conjugated structure (ii) shown in Scheme 9.1. This scheme also shows an alternative fragmentation pathway for the molecular ion (i). The distonic ion (ia) may be formed by a 5-membered hydrogen-atom transfer. Ions of this structure may undergo alkyl- or alkane cleavage[213] at bond C_1-C_2 or bond C_2-C_3 as shown. It appears that C_1-C_2 cleaved only by a radical-mechanism to produce an ion such as (iv) at *m/z* 129, with expulsion of methyl (mechanism 1), because an ionic process expelling methane would yield ion (vii), *m/z* 128 (mechanism 3). Loss of HF from ion (iv) would yield the ion (v), *m/z* 109 (obs. 90%). Bond C_2-C_3 probably cleaves by an ionic mechanism to yield ion (ix), *m/z* 101 (mechanism 3). Loss of HF from this ion would yield an ion *m/z* 81 (obs. 70 %). Note that ion

Scheme 9.1 Proposed Fragmentation Pathways to Account for the Diagnostic Ions in the Mass Spectrum (EI) of 3-Fluoro-6-methyl-hept-5-en-2-one (5)



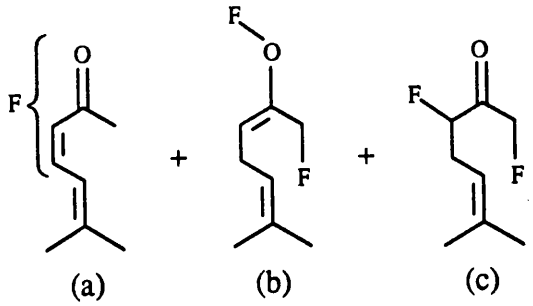
(ia) could either cleave at bond C_3-C_4 to yield ion *m/z* 68 (obs. 20 %) or form a distonic ion with the radical centre stabilised at the C-F bond which could cleave similarly to yield the familiar terpenoid fragment at *m/z* 69 (obs. 85 %).

9.8.3 Mechanism of Fluorination with NFPT

The fluorination of enol ethers 9 and 10 may proceed by (i) direct attack of the fluorine cation at C_3 with concomitant expulsion of the trimethylsilyl-group or (ii) by a mechanism analogous to the α-fluorination of sulphides,[199] which is thought to occur by initial attack of the fluorine at sulphur followed by a 3-centred rearrangement to yield the product. However, it is extremely unlikely that the second mechanism would operate in the fluorination of TMS-enol ethers owing to the steric hindrance of the TMS-group towards proton-abstraction by triflate. The standard theories of O- and C-acylation and alkylation of enolates[230] could explain the mechanism; NFPT can be considered as an unreactive fluorinating reagent (due to the large N-F bond energy)[231] and so direct C-fluorination might be favoured.

Alternatively, the HSAB theory[232] would predict fluorination at oxygen: the temperature of the reaction may then favour formation of a thermodynamically more stable α-fluoro-ketone (5) with expulsion of the trimethylsilyl moiety. An attempt to fluorinate the kinetic enol ether (11) yielded a product mixture containing the 3-fluoro-ketone (5; 10%) and a product (90%) showing an intense molecular ion at *m/z* 142 together with a second major ion at *m/z* 100 in its mass spectrum. The spectrum included other peaks at *m/z* 41, *m/z* 57, *m/z* 69, *m/z* 84, *m/z* 111 that were characteristic of the skeleton of 1. The data was consistent with the structure of a

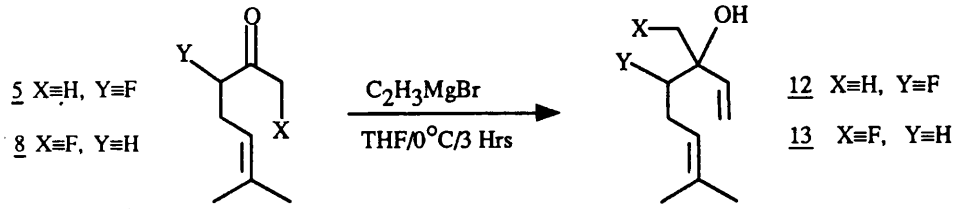
Diagram 9.1 Possible Intermediates in the Fluorination of TMS-enol Ethers (9, 10)



(a) (b) (c)

stepped-diene (Diagram 9.1a). The mechanisms of fluorination outlined here may account for this in the following manner: an intermediate O-fluoro species may be formed by expulsion of the TMS-moiety, which may not readily rearrange (by a mechanism analogous to the fluorination of sulphides) to the 1-fluoro-ketone (8) since this would require formation of a primary carbocation at C_1. Rather, this O-fluoro-species could undergo attack by the basic triflate ion at C_3 with concomitant fluorination at C_1 (fluorination and migration of a double-bond have been observed in the fluorination of 1-methoxy cyclohex-2-ene)[154] to produce the compound shown in Diagram 9.1b. This may then rearrange to the compound shown in Diagram 9.1c which could then eliminate HF to yield the compound shown in Diagram 9.1a which accounts for the mass spectrum of the product that was formed.

9.9 Preparation of 4-Fluoro- and 9-Fluoro-3,7-dimethyl-octadien-3-ols (Fluorolinalools)



Note: this scheme summarizes the conversions 5 → 12 and 8 → 13

The fluoro-linalools (12,13) were prepared by the slow addition of the fluoroketones (5,8) to solutions of vinylmagnesium bromide in THF at 0°C. The products were recovered in relatively poor yield (20%) for this type of reaction (*cf.* up to 80 % in Ref. 157). The major (50%) by-products in each case were compounds with molecular formula $C_{12}H_{20}O$ (*ie.*, substitution of fluoride by the Grignard reagent in the product-fluorolinalool) showing molecular ions at *m/z* 180. It is probable that the other isomers were the geranyl and neryl analogues. The formation of these products can be explained by three possible mechanisms depending on whether substitution of fluoride occurred from the fluoroketone or fluorolinalool:

(i) Migration of the vinyl group by a radical process.[233] Henry[234] proposed the formation of an intermediate epoxide in the reaction of α-chloro-acetone with methyl-

magnesium bromide. Foldi[235] reported normal addition to the carbonyl double bond and replacement of the α-halogen with rearrangement in the reaction of an α-chloro-ketone with ethylmagnesium bromide. The mechanism was not thought to be a simple metathesis (ii) reductive enolisation has been reported for α-halo ketones.[236] Bromoacetomesitylene has been shown to enolise with formation of positive bromine whereas dichloro-mesitylene formed an enolate only by loss of an acidic hydrogen. Clearly, the formation of an enolate of 5 would not be expected to proceed with loss of positive fluorine (iii) the postulate by Howk,[237] that intermediate O-halogen (hypohalite) compounds may mediate dehalogenation could explain defluorination of 5. The intermediate O-fluoro-enolate would be consistent with the O-fluoro species generated in the fluorination of TMS-enol ethers. However, introduction of a second vinyl moiety to give the expected normal addition product at the carbonyl group cannot be rationalised from such an intermediate O-fluoro species.

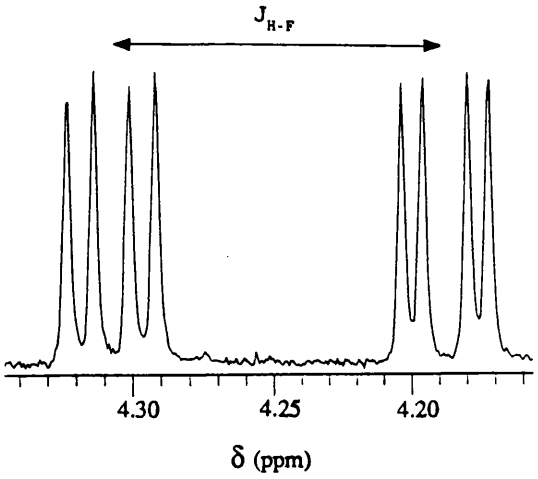
The α-fluorine atom would be expected to labilise the carbonyl moiety with respect to normal addition and so it is likely that defluorination occurred from the 4-fluoro-linalool (12), and not from the fluoroketone (5). However, a simple nucleophilic displacement of fluoride by the vinyl moiety is difficult to rationalise because the tendency of Grignard reactions to occur in non-polar solvents suggests the reagent does not exist as discrete ions capable of S_N^2 substitution even in a moderately polar solvent such as THF (allyl-Grignard reagents are an exception; see Ref. 233).

Addition of the Grignard reagent to a cooled solution of the fluoroketone failed to yield the desired product and resulted in partial conversion of the fluoroketone exclusively to the dehydrofluorinated α-vinyl-ketone. The yield of 4-fluoro-linalool was optimised by addition of the fluoro-ketone to the Grignard solution cooled to -25°C. Careful work-up after 24 hours using methanol/ice-water (80:20) gave the product in adequate yield (40%) in each case (decomposition of the Grignard reagent with saturated ammonium chloride solution yielded no product).

9.9.1 Discussion of NMR Spectra

The ^{13}C-NMR spectrum showed the expected α-effect of fluorine on C_4 of the 4-fluoro- product ($^1J_{CF}$= 178 Hz.) as for the parent 3-fluoro-methyl-heptenone.

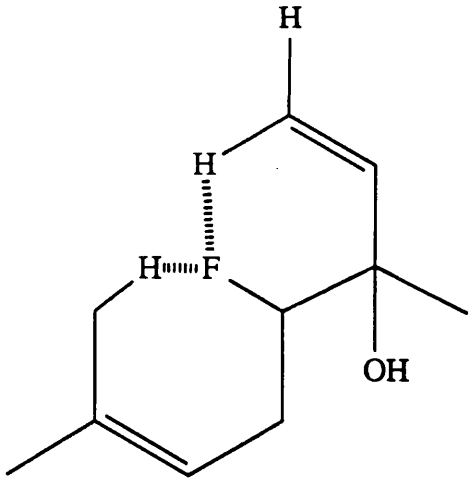
Diagram 9.2 The Expanded ^{1}H-NMR Signal of the Proton at C_4 of 4-Fluoro-linalool (12)



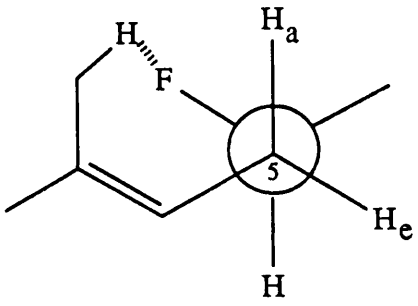
Similar two-bond couplings were observed to C_3 and C_5 ($^2J_{CF}$=21 Hz.). The ^{1}H-NMR spectrum showed a two-bond fluorine-hydrogen coupling ($^2J_{HF}$= 48 Hz.) but did not show the expected signal for the three-bond proton-coupling to the two protons of C_5. The signal was observed as two separate three-bond couplings to each of these protons. This can be rationalised by "through-space" hydrogen-bonding; The C-F bond-length is 27% longer than the C-H bond, which means that the minimum internuclear distance between the fluorine atom and the proton of C_1 in a scale model of the molecule) is approximately 0.075 nm (*cf.* C-H bond-length of 0.109 nm!). In addition, the protons of C_{10} may form shorter hydrogen-bonds to the fluorine to yield a quasi-bicyclic conformer with no steric constraint (Diagram 9.3 a-c). The diagram shows that both protons of C_5 become inequivalent. The measured three-bond couplings to the proton of C_4 are 3.2 Hz. and 9.4 Hz., corresponding (in magnitude) to typical three-bond equatorial-axial and axial-axial couplings in cyclohexane rings.[238] Further evidence is provided by the signal of the protons attached to C_9; no through-space four-bond coupling was observed here (unlike in the parent 3-fluoro-methyl-heptenone) because this methyl is trans-annular with respect to the fluorine atom in the hydrogen-bonded conformer. (In any case, these protons are less acidic than the equivalent protons of the fluoro-ketone). Also, the signal for H_B attached to C_1 was observed downfield (0.20 ppm) relative to this signal in the spectrum of linalool, probably as a consequence of hydrogen-bonding. The signal of the proton attached to C_5 was complex, showing two separate three-bond fluorine-hydrogen couplings, and four separate three-bond proton-proton couplings.

Diagram 9.3 The Hydrogen-bonded Bicyclic Conformation of 4-Fluoro-linalool (12)

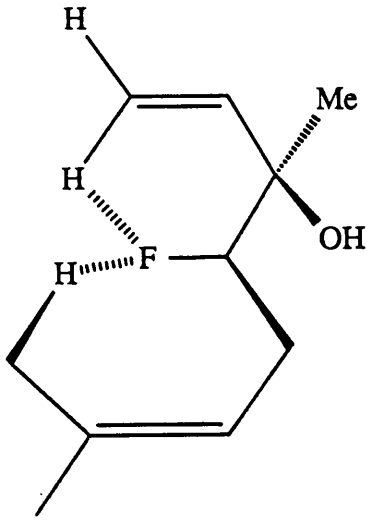
(a)



(b)



(c)



(R-stereoisomer)

Note: the examiners have commented that both C_3 and C_4 are chiral and the reaction mixture probably contained a mixture of diastereoisomers. The signal shown in diagram 9.2 could result from a Nuclear Overhauser Effect occurring between the protons of C_9 and C_5.

Note that through-space four-bond coupling between H_B and the protons of C_9 could not occur in the hydrogen-bonded conformer of 4-fluoro-linalool.

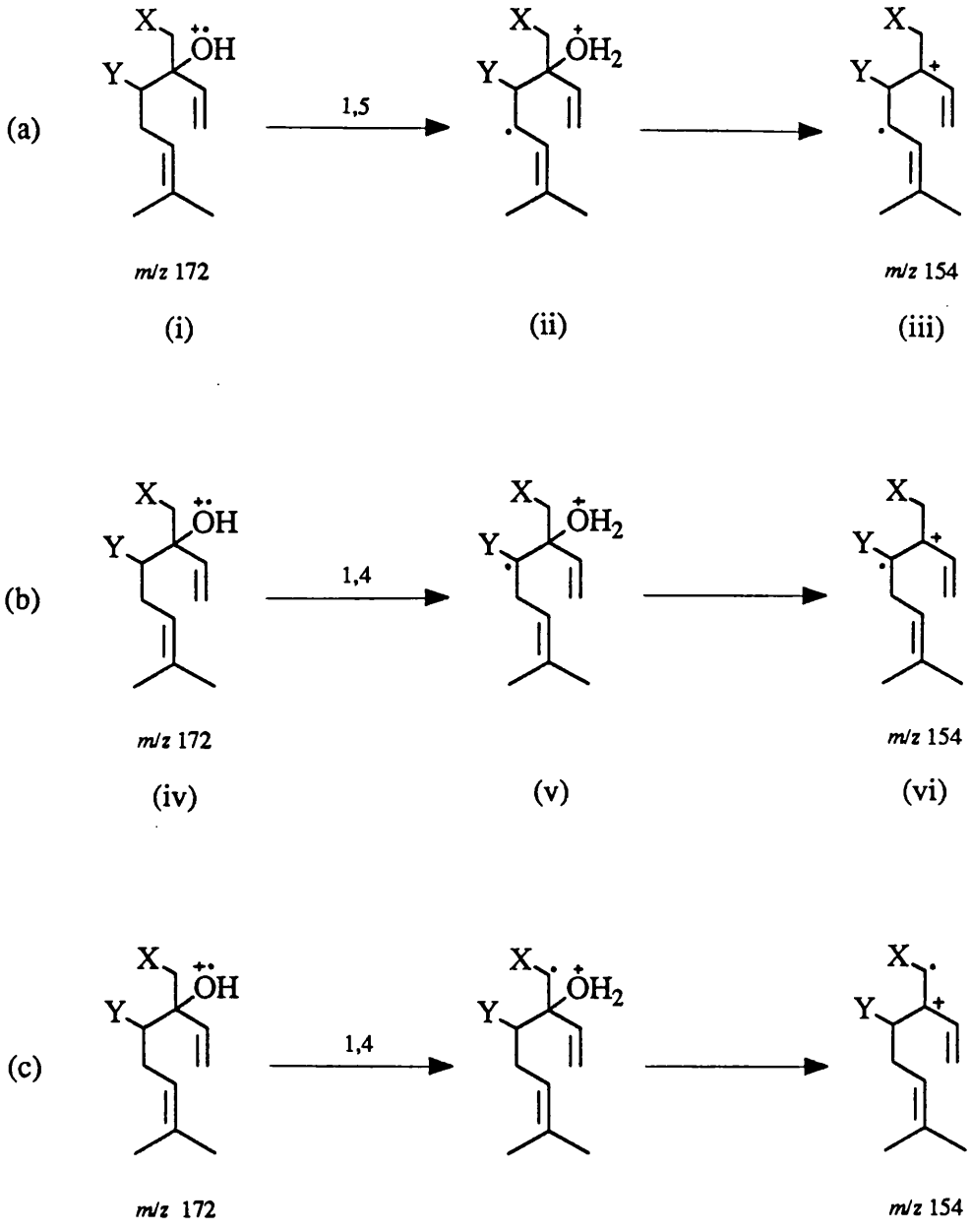
9.9.2 Discussion of Mass Spectra

The mass spectra were characteristic of the oxygenated monoterpenoid skeleton discussed in Part Four. The isomers were differentiated by two diagnostic peaks; the 4-fluoro-product showed cleavage of C_3-C_4 to form an ion at *m/z* 101 that was absent in the spectrum of the 9-fluoro-product, which showed a cleavage of C_3-C_4 yielding an ion at *m/z* 89. Both compounds showed peaks corresponding to $(M\text{-}H_2O)^+$. Since oxygen normally bears the positive charge[239] elimination by an E_i-type process was difficult to rationalise. The mechanism could best be explained by dehydration from one of three interconvertible distonic ions (Scheme 9.2 a-c) as shown. It is reasonable to assume that the distonic site would form at the carbon attached to the fluorine. Thus, existence of the molecular ion of 4-fluoro-linalool may favour fragmentation according to Scheme 9.2b whereas that of the molecular ion of 9-fluoro-linalool may favour Scheme 9.2c. Such ions would be readily interconvertible by four- and five-membered distonic processes discussed in section 9.7.2 (indeed the presence of ions at *m/z* 68 and *m/z* 69 in the spectra of the 4-fluoro-and 9-fluoro- products was evidence for the existence of different populations of distonic ions).

The ions at *m/z* 154 (formed by dehydration of the molecular ion) might undergo cyclisation (section 11.2) to yield three populations of ions (Scheme 9.3 ia-iiia) which are only interconvertible via ion (iia); cyclic distonic or prototopic shifts could not interconvert ions (ia) and (iiia) directly. The presence of identical peaks (absent in linalool, and therefore probably containing fluorine) in the spectra of the two fluoro-linalools could be evidence of a common ion (ii) being formed from both isomers. However, these mechanisms must be considered with some caution for two reasons; (i) ions corresponding to dehydrofluorination (at *m/z* 134 and *m/z* 152), would only be expected for the internal 4-fluoro-isomer, but they were are also present in the spectrum of the external 9-fluoro-isomer. Their formation may parallel the mechanism of dehydration (*ie.*, hydrogen transfer to the heteroatom) or alternatively, result from interconversion of the two fluorine-sites by fluorine-transfer

Scheme 9.2 Distonic mechanisms to Account for the Dehydration of the Molecular ions of 4-Fluoro and 9-Fluoro-linalools (12, 13)

4-fluoro-, X≡H; Y≡F; 9-fluoro-, X≡F; Y≡H



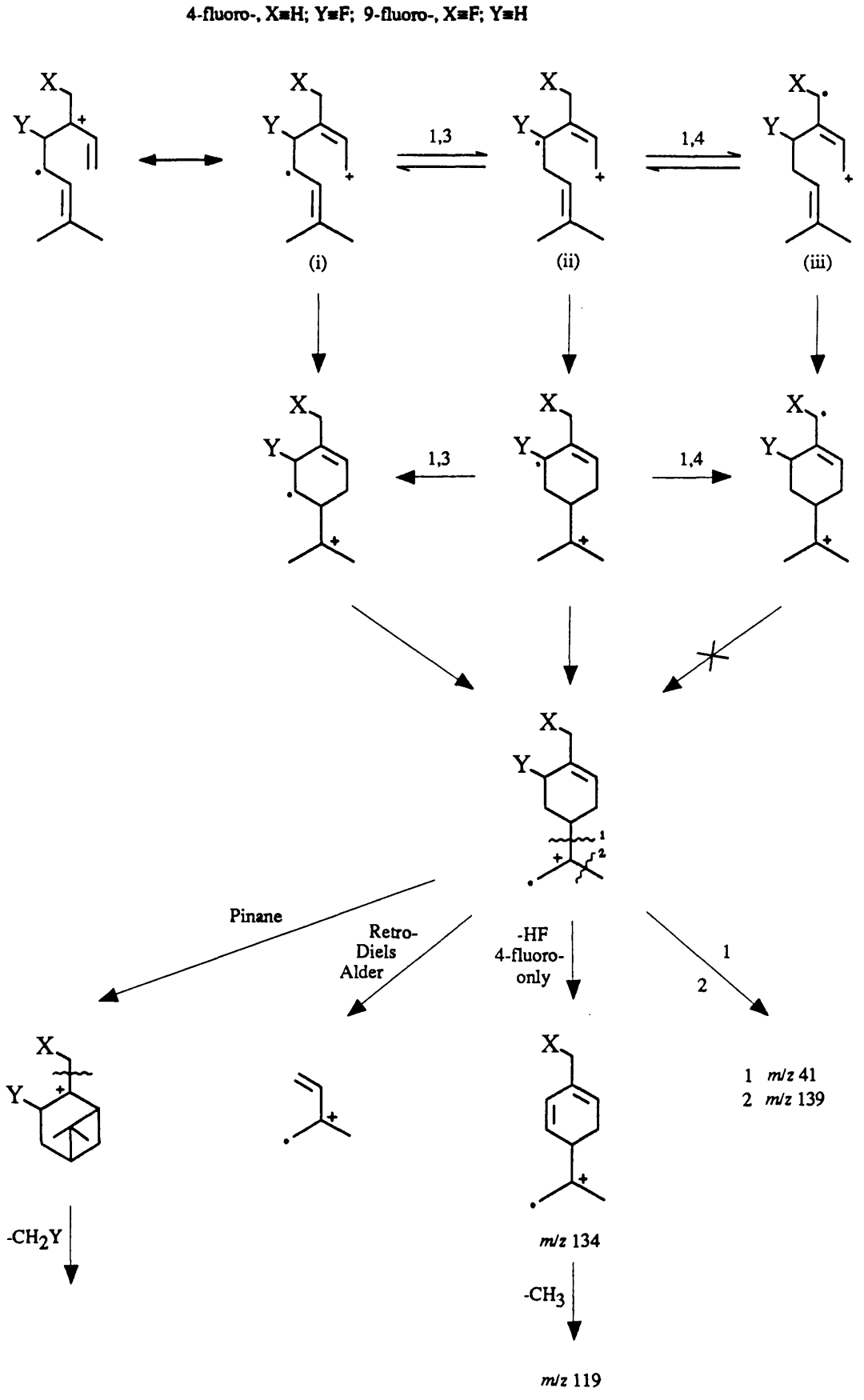
Distonic ions occur where the charge and radical-site are formally separated

(analogous to the sigmatropic migration of fluoride in the pyrolysis of perfluoro-cyclohexadienes);[240] (ii) formation of a pinane skeleton (Scheme 9.3) would yield a further set of complex fragmentation mechanisms, although the initial ring-formation may be sterically crowded and entropically-unfavoured.

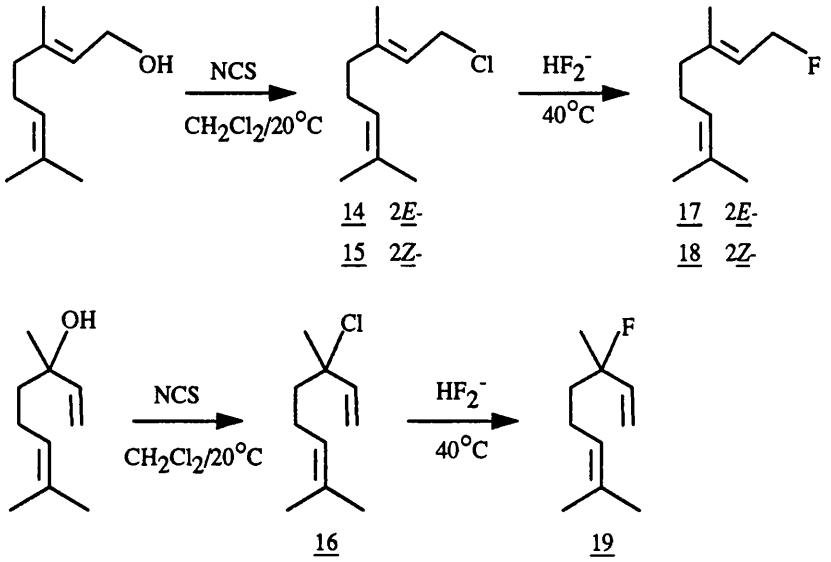
Under conditions of chemical ionisation (CI) the mass spectrum of 4-fluoro-linalool (12) showed no peaks corresponding to $(M\text{-}H_2O)^+$ or $(M\text{-}H_2O\text{-}Me)^+$. Furthermore, a measurement of bond-order of the molecular ion of this isomer determined the number of double bonds to be 1.5 (compared to a measured value of 2.0 under electron-impact). This indicated that under conditions of CI the oxygen did not bear the positive charge (*ie.*, protonation of one double bond occurred). Thus, the distonic processes leading to dehydration (Scheme 9.2) were probably not operative. The spectrum showed no base peak at *m/z* 71; the observed peaks could be accounted for by straightforward fragmentation of the acyclic skeleton. The GC/MS analyses of the reaction mixtures that contained the fluorolinalools (12,13) revealed three further spectra showing molecular ions at *m/z* 172. These were: (i) a compound showing a very intense peak corresponding to $(M\text{-}H_2O)^+$ (45 %) and a second at *m/z* 59 (65 %), both of which indicated the presence of the respective fluoro-α-terpineol in the mixture (*m/z* 59 is diagnostic of of α-terpineol).

Other signals at *m/z* 139 $(M\text{-}H_2O\text{-}Me)^+$ and *m/z* 101 (cleavage of C_3-C_4) suggest this was the terpineol-derivative of 4-fluoro-linalool. (ii) a compound showing no ion at *m/z* 71 (indicative of C_3-C_4 cleavage) and no $(M\text{-}H_2O)^+$ an intense $(M\text{-}HF)^+$ and an intense ion at *m/z* 101 (also indicative of C_3-C_4 cleavage) which may have corresponded to 4-fluoro-geraniol. (iii) a compound showing no $(M\text{-}HF)^+$, a weak $(M\text{-}H_2O)^+$ but a very intense $(M\text{-}H_20\text{-}CH_2F)^+$ which may have corresponded to 9-fluoro-geraniol or nerol.

Scheme 9.3 Mechanisms for the Generation of Common Ions in the Mass Spectra (EI) of 4-Fluoro- and 9-Fluoro-linalools (12, 13)



9.10 Preparation of *E*- and *Z*- Fluoro- 3,7-dimethyl 2,6-octadienes (Geranyl- and Neryl Fluorides) and 3-Fluoro-3,7-dimethyl-1,6-octadiene (Linaloyl Fluoride)



Attempts to prepare the tosylates[241] of geraniol, nerol and linalool failed, probably owing to the decomposition of the former via carbocationic intermediates.[242] Instead, we employed a modified version of the Corey-Kim reaction[243] in which the alcohols were converted (in an aprotic solvent to suppress solvolysis) to the corresponding chlorides. Both geranyl chloride (14) and neryl chloride (15) were formed in excellent yield (83 %). Linaloyl chloride (17) could only be formed in low yield (20%; from ^{1}H-NMR) probably due to the lability of this tertiary-allylic chloride towards rearrangement to a mixture of the primary-allylic geranyl- and neryl-isomers (70 and 30 % respectively).

The fluorides were obtained by addition of the neat chloride to vacuum-dried TBABF followed by stirring at 40°C for 24 hours. Geranyl and neryl fluorides were obtained in good yield (30%) whereas only a poor yield of linaloyl fluoride (<10%) could be achieved.

9.10.1 Discussion of NMR Spectra

The NMR spectra of the products were consistent with the structures we expected. Geranyl- and neryl chlorides were readily differentiated by diagnostic ^{1}H-NMR resonances for the proton at C_6 (0.04 ppm lower field in the *Z*-isomer).

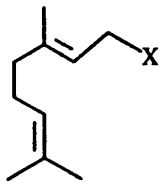
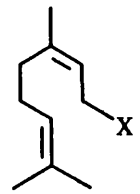
However, the protons at C_8 and C_{10} in both isomers occurred at δ1.58 ppm and δ1.66 ppm respectively. It is interesting to note that an interaction of the chlorine atom with the hydrogen atoms in the pendent isopropylidene plane of neryl chloride could occur and perhaps cause deshielding of the proton-nuclei. Construction of a molecular model demonstrated that rotation of the C_1-C_2 bond in this isomer allowed contact between the chlorine atom and the two-types of hydrogen; as the carbon-chlorine bond was approximately 60% longer than the carbon-hydrogen bond, the chlorine was located between C_6 and C_8 with minimal steric hindrance, with an internuclear hydrogen-chlorine separation estimated to be 0.135 nm. This was similar in magnitude to the covalent bond-length of hydrogen chloride (0.128 nm!). This, the protons of both C_6 and C_8 could conceivably form hydrogen bonds and this might explain the deshielding of the nuclei at C_6.

The *E*- and *Z*- isomers were differentiated by the ^{13}C-NMR resonances for C_4 and C_9, which were probably dependent on the geometry of the C_2-C_3 double bond as observed for the parent alcohols.

The chemical shifts we observed for the β-protons of C_2, C_4 and C_9 were downfield relative to those in the spectra of the parent-alcohols. This can only be attributed to through-bond inductive effects, since the estimated internuclear β-hydrogen-chlorine separations were estimated to be no less than 0.300 nm (owing to the length of the carbon-chlorine bond) which was greater than the γ-hydrogen-hydrogen separation of the three proton-types.

Geranyl- and neryl fluorides (17 and 18) both showed a large α-effect of the fluorine on the protons on C_1 with a two-bond proton-fluorine coupling constant of 47.86 Hz. The three-bond proton-fluorine coupling constants were smaller in magnitude (50 %; see Table 9.3) than equivalent couplings in the fluoro-methyl heptenones discussed in section 9.8.1. This was probably caused by the deshielding effect of the double bond (C_2-C_3) on the vinylic proton (H_b). The methyl group of C_9 also showed five-bond coupling to fluorine. For geranyl fluoride, this may have resulted from through-bond homoallylic and through-space contributions (minimum estimated internuclear-separation from model= 0.138 nm). However, the larger coupling constant observed

Table 9.3 Dependency of ^{1}H-NMR Chemical Shifts and Proton-Proton Coupling Constants on Electronegativity of Substituent X

R-X	X	H-C_n (δ, ppm)				
		1	2	9	8	$^3J_{1,2}$ (Hz)
 Geranyl	Cl	4.08	5.42	1.70	1.58	7.86
	F	4.90	5.49	1.72	1.61	7.17
		$^2J_{HF}$ = 47.85 Hz	$^3J_{HF}$ = 9.28 Hz	$^5J_{HF}$ = 4.73 Hz		
 Neryl	Cl	4.06	5.43	1.75	1.59	8.08
	F	4.86	5.50	1.80	1.60	7.31
		$^2J_{HF}$ = 47.88 Hz	$^3J_{HF}$ = 7.51 Hz	$^5J_{HF}$ = 6.75 Hz		

for neryl fluoride could only result from the through-bond mechanism. Note the dependency of H_a-H_b coupling on the electronegativity of the halogen. No evidence of hydrogen-bonding (described for the chlorides) was observed for geranyl- and neryl-fluorides, probably owing to the shorter C-F bond length (0.138nm *cf.* 0.177nm for the C-Cl bond length) and the smaller covalent radius of fluorine (0.072 nm cf. 0.099 nm for chlorine).

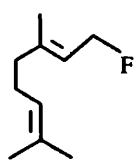
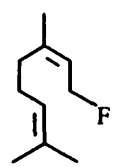
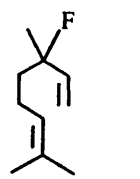
The ^{1}H-NMR spectrum of linaloyl fluoride showed one three-bond proton-fluorine couplings for protons that differed only in the hybridisation state of the carbon to which they were attached. No four-bond proton-fluorine coupling was observed in the spectrum of this compound.

Table 9.4 shows the effect of fluorine-substitution on the ^{13}C-NMR spectra of geranyl and neryl fluorides. The key indicates the alternating (+,-) through-bond effect of the halogen on chemical shifts along the carbon-framework relative to the parent-alcohol of each fluoride. The spectra are readily distinguished by the dependency of the chemical shifts of C_4 and C_9 and the four-bond carbon-fluorine coupling constants on the geometry of the double bond (C_2-C_3). Construction of a molecular model demonstrated that even five-bond carbon-fluorine coupling must be through-bond, and not through-space since the minimum internuclear separation was estimated to be 0.48 nm.

9.10.2 Discussion of Mass Spectra: Comparison of Geraniol with Geranyl Chloride and Geranyl Fluoride

Linaloyl chloride and linaloyl fluoride were both identified by ions corresponding to cleavage of the C_3-C_4 bond (this cleavage produces an ion at *m/z* 71 in the alcohol). The intensities of the ions resulting from such cleavage were significantly greater in the spectrum of the linaloyl halides compared with the geranyl and neryl analogues. Mass spectra of geraniol, geranyl chloride and geranyl fluoride were run consecutively under the standard conditions described (section 12.2a; system 1). The ion-abundances were normalised to observe the quantitative effect of the halogen in the fragmentation of these compounds. As mentioned previously (section 9.9.2) the ionisation of an alcohol occurs at the oxygen atom. Dehydration probably occurs by a distonic process facilitated by hydrogen-transfer from either of C_8 to C_{10} in the skeleton.

Table 9.4 The Effect of Fluorine Substitution on ^{13}C-NMR Chemical Shifts and Coupling Constants

R-F	C_n (δ,ppm)						$^nJ_{CF}$ (Hz.)				
	1	2	3	4	5	9	$^1J_{CF}$	$^2J_{CF}$	$^3J_{CF}$	$^4J_{CF}$	$^5J_{CF}$
	79.35[a]	118.90[b]	144.12[a]	39.51[b]	26.19[b]	14.43[a]	156.50	16.90	11.50	2.60[c] 3.10[d]	3.50
	79.03[a]	119.92[b]	144.25[a]	32.16[b]	26.72[b]	23.47[a]	156.50	17.00	11.50	3.10[b] 2.80[d]	3.30
	-	-	-	40.27[a]	26.76[b]	25.24[a]	-	28.17 22.53	2.82	-	-

a ≡ Downfield relative to OH analogue
b ≡ Upfield relative to OH analogue
c ≡ Four-bond coupling to C_4
d ≡ Four-bond coupling to C_9

Ionisation of the halides is probably very similar. Clearly, such as process is dependent on several factors;

- Ionisation energy of the heteroatom (O, Cl, F)
- Internuclear separation between X and the distonic sites to facilitate hydrogen transfer
- C-X bond energy
- X-H bond energy
- C-H bond energy

Table 9.5 Relative Ion-abundances of Fragment-ions from the Molecular Ions of Geranyl-X (X = OH,Cl,F)

ION	GERANYL-X		
	OH	Cl	F
M^+	900	-	30
$(M-CH_2X)^+$	7600	4600	1600
$(M-HX)^+$	1900	4600	500
$(M-43)^+$	3900	7900	16300
$(M-15)^+$	800	100	500

No molecular ion observed for geranyl chloride

Table 9.5 shows the intensity-data for ions M^+(M-HX)$^+$; (M-CH_2X)$^+$; (M-43)$^+$ and (M-15)$^+$ (for X ≡ OH,Cl,F). Geraniol showed the most abundant molecular ion. However, on the grounds of first-ionisation energy (of X) and C-X bond-strength shown in Table 9.6 the spectrum of geranyl chloride should show the greatest intensity for this ion. Moreover, the intensity of the ion (M-HX)$^+$ was greatest for geranyl chloride even though the H-X bond energy is smallest for hydrogen chloride. If the mechanism of ionisation is similar to that for geraniol, the anomalous behaviour of geranyl chloride must be attributable to the smaller internuclear separation between chlorine and the three potential sites from which hydrogen-transfer

Table 9.6 Bond Data for X (X = OH,Cl,F)

Quantity	X		
	OH	Cl	F
First Ionisation Energy (kJ mol^{-1})	1310	1260	1680
H-X Bond Energy (kJ mol^{-1})	463	431	562
C-X Bond Energy (kJ mol^{-1})	360	1260	1680
Covalent Radius (nm)	0.140	0.180	0.135

Source: Stark V.G., Wallace H.G.
Chemistry Data Book, 2nd Edition, John Murray, London (1984)

occurs such that formation of $(M\text{-}HX)^+$ (requiring hydrogen-transfer) is comparable with formation of $(M\text{-}CH_2X)^+$ (requiring breakage of C_1-C_2). Note also, that the base-peak for geranyl chloride was at *m/z* 41 (*cf.* *m/z* 43 for geraniol and geranyl fluoride). This suggests the most favourable distonic site forms at C_8 or C_{10}. These sites are most accessible in geranyl chloride because of the greater C-Cl bond-length and greater covalent radius of chlorine. However, these distonic sites may form as a result of ion-molecule complexes or molecular ion dimers (section 11.1).

9.10.3 Summary

From the possible routes that could be used for the selective introduction of fluorine to either C_4 or C_9 of linalool (section 8.3) we chose those described in Chapter Nine. The formation of β-trimethylsiloxyethers (TMS-enol ethers) from 6-methyl-hept-5-en-2-one proved a suitable method for introducing halogen to the carbon skeleton.

Ethyltrimethylsilyl-acetate and trimethylsilyl-iodide are two new reagents that were used to deprotonate the methyl-heptenone under kinetic- and equilibrium reaction conditions respectively. Both reagents gave good yields (80-90%) of the regiospecific TMS-enol ethers which was an improvement on our attempts to prepare the TMS-enol ethers using the standard methods. The kinetically-controlled mixture was brominated (using N-bromo-succinimide) and fluorinated using tetrabutlyammonium fluoride to give the 1-fluoro-methyl-heptenone (yield *ca.* 5-10%). The equilibrium-controlled mixture of TMS-enol ethers could be directly fluorinated to the 3-fluoro-methyl-heptenone (yield *ca.* 30%) by using N-fluoro-pyridinium triflate. The fluorolinalools were prepared (yield *ca.* 20%) by reaction of the respective fluoro-methyl-heptenones with vinylmagnesium bromide. The NMR and mass spectra of the fluorinated products showed diagnostic features that have been discussed in some detail.

Geranyl, neryl and linaloyl fluorides were prepared in good yields (80%; 80%; 20%) by halex (Finklestein) reaction on the respective chlorides which had been prepared from the alcohols by a reaction based on the Corey-Kim procedure.

9.10.4 Future Work

Both the 4-fluoro- and 9-fluorolinalools could be added to tissue cultures in order to select cells capable of storing terpenoids (because those cells that degrade terpenoids may convert both substrates into the lethal fluoroacetate and therefore they would die). Indeed, cell-free extracts could be formed from those cell-lines that do metabolise terpenoids and these could be used in feeding experiments to determine the ultimate fate of the fluorine.

PART 4 The Interpretation of the Fragmentation Patterns in the Mass Spectra of Linaloyl, Neryl and Geranyl Acetates

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PART 4 The Interpretation of the Fragmentation Patterns in the Mass Spectra of Linaloyl, Neryl and Geranyl Acetates

Chapter 10 Introduction

10.1 Scope

The title-compounds are three monoterpenoids that we identified in the steam distillate of lavender flowers (Part One). Although we could characterise the compounds by their relative retention times on HPLC and GLC (by comparison with authentic standards) we could not readily interpret their mass spectra. This part surveys the electron-impact (EI), chemical ionisation (CI; negative and positive) and fast atom bombardment (FAB) mass spectra of these compounds together with a linked-scan study of selected metastable ions (*ie.*, those ions resulting from metastable decomposition) produced by each compound. The aim was to use these as models of the mass spectrometric identification of isomeric terpenoid acetates in general.

The analysis of the products of cell-free systems of lavender by comparison of these products with HPLC and GLC fractions of a commercial sample of lavender oil was discussed in Part One. Many of the components could not be distinguished by mass spectrometry alone because of the variety of common ions formed in their spectra. For example, fractions containing limonene, myrcene, ocimenes, pinenes, terpinenes and phellandrenes all gave similar fragmentation patterns when analysed by EI. Similarly, the spectra of linaloyl, geranyl, neryl, bornyl and terpinyl acetates were hardly distinguishable. The only way of identifying each compound would be to screen a number of standard terpenoids and identify the components by retention-time on GC. We turned to a commercial perfumery company (Bush Boake Allen Ltd., London) for those analyses given in Part One. The compounds were identified by comparison of their EI-spectra with a computer library of data within a GC retention-time window for each component (matching of ion-intensities of the most abundant eight peaks). These databases are commercially available[244] and are continually updated as new compounds are analysed. Their potential for the rapid screening of complex mixtures of flavours and fragrances by GC/MS is reflected in the high cost of each analysis (currently £50 for each GC peak- many samples of natural oils may contain more than one hundred peaks!).

We decided to attempt to characterise the three isomeric monoterpenoid esters, linaloyl-, geranyl- and neryl acetates by analysis of their mass spectra by using some of the techniques available with our instrument. Spectra generated by EI, and FAB, positive CI were analysed for differences. EI-Spectra were recorded at 70 electron volts (eV.) and 12 eV. for comparison. Selected metastable ions occurring in the spectrum of each isomer were analysed by two linked-scan methods in order to elucidate fragmentation pathways. An attempt was made to determine the structures of ions at *m/z* 136 (resulting from loss of acetic acid from the molecular ion) by comparison of the metastables produced by this ion compared with those produced from the molecular ions (occurring at *m/z* 136) from eight monoterpenoid hydrocarbons. Accurate mass measurements were made for selected ions in order to determine their formulae. In addition, nine deuteriated analogues were prepared and their spectra, generated by the above methods, were used to suggest mechanisms for elimination of acetic acid and other fragmentations. A description of the instrumental techniques is given in a following section with special reference to their application to the study of thermally labile, isomeric, and biological molecules.

10.2 Summary of the Principles of Linked-scanning in the B/E Mode

The following equation relates the mass (*m*) of an ion with charge (*z*) to the magnetic field strength (B), the radius of the ion trajectory (r) and the accelerating voltage (V)

$$m/z = B^2r^2/2V$$

According to this equation if B and E are held constant, a singly-charged ion of mass m_1 will focus to a radius r_1. In practice B is swept to focus all values of m. Ions that are detected in this way are said to be energy- and mass-analysed.[245] In the method of linked-scanning used in this work both B and E were varied but the ratio B/E remained constant. In order to focus a metastable ion m_2 produced from ion m_1, the field strengths must be changed by a factor proportional to m_2/m_1. Thus, to focus all metastables resulting from m_1 the fields must sweep the whole mass range up to m_1, maintaining the B/E ratio. We have also used B^2/E scans to detect parent-ions of chosen metastables to confirm the results of scans in the B/E mode.

10.3 Recent Techniques for the Analysis of Thermally-labile and Isomeric Compounds of Biological Interest

Since the three terpenoid acetates studied here fall into each of the above categories, it seemed logical to draw on several examples from the literature to illustrate the range of experiments possible.

Although EI is the most commonly used source of odd-electron molecular ions $(M)^{+\bullet}$ it has limited use in the analysis of molecules giving short-lived molecular ions or where stereochemical information is required. Molecular ions are produced in excited electronic, vibrational and rotational states and thus fragmentation occurs by a series of unimolecular decompositions. However, the range of ion-energies possible results in some ions fragmenting after leaving the ion-source and thus resulting in peaks at non-integer mass values. The abundances of such metastable ions can be used to differentiate between isomeric compounds. Experiments to detect metastables using the sector region are described later.

"Soft" ionisation methods[246] have been developed to produce molecular ions in an electronic ground state and thus give rise to longer-lived molecular ions. CI and FAB are routinely used in mass spectrometry and are collectively termed secondary-ion mass spectrometry (SIMS)[247-8] because a primary ion must first be generated from another molecule which is introduced into the source with the sample. CI involves the interaction of the neutral sample molecule with odd-electron primary ions generated from a reagent gas by EI, to form an even-electron molecular ion $(M+H)^+$. For positive chemical ionisation (PCI) the reagent gases most frequently used are methane,[249] isobutane[250] and ammonia.[251] Acetone has been used for the production of CI spectra from monosaccharides[252] and has also been shown to be an excellent reagent gas for distinguishing between isomeric alkenes[253] by formation of ion-molecule adducts which then constrain double bond migrations that commonly occur after ionisation.[254] Negative chemical ionisation (NCI)[255] has found widespread application in the detection of chlorinated pesticides in the environment.[256] There are a few reports on the behaviour of diterpenoids[257-8] under NCI. This technique should correctly be termed dissociative electron-attachment since the major process occurring is electron-capture by the sample. The method is thus suited to the analysis

of very polar compounds and has been applied to the detection of esters[259] in volatile oils such as those studied in this Part. Clearly, NCI and PCI spectra can be recorded for a sample using the same reagent gas, by switching the polarity of the instrument.

FAB[260] has been developed in the last decade as a convenient method of ionisation of involatile and thermally-labile biomolecules.[261-2] The sample is dissolved in a glycerol matrix, on to which is focussed a beam of energised xenon atoms (produced by charge-exchange between fast xenon ions and thermal xenon atoms). The actual mechanism of ionisation is not clear, but the glycerol matrix is thought to protonate the sample to produce $(M+H)^+$ ions while the residual RO^- ions of the matrix are thought to deprotonate the sample to form $(M-H)^+$ ions. Compounds with O-X bonds typically give simple α-cleavages and so the technique has been applied to the study of nucleoside phosphates[263] and monosaccharides.[264] FAB/MS can be run in positive and negative modes and has been used in the study of a wide range of polar biological compounds.[265-7] Baldwin *et al.*[268] have distinguished chiral isomers by the formation of molecular-ion dimers in the ion-source.

Many biochemical processes can be studied using substrates with specific incorporations of natural isotopes.[269] The use of ^{14}C- and ^{3}H-labelled substrates (see Part One) is now superseded by non-radioactive ^{13}C- and ^{2}H-isotopes.[270] The latter is relatively cheap and straightforward for structure-analysis of compounds with enolisable hydrogens (see Results and Discussion 11.2.1) although the results can be complicated by hydrogen-rearrangement processes. Djerassi[271] has demonstrated specific fragmentations of terpenoid esters of juvenile hormones by deuterium-labelling methods. Commercially important *p*-menthanes occurring in tobacco have also been studied in this way.[272]

In addition to using the source region of the mass spectrometer for the range of sample-introduction and ionisation techniques discussed above, the analyser region can provide an equally diverse range of experiments to fingerprint a sample. Metastables formed in the first field-free region (FFR1) between the source and the electric sector can be focussed by B/E linked-scanning[273] described earlier. A B/E linked-scan analysis of limonene has been reported[274] and Mellon and Rhodes[275] have

recently used B/E linked-scanning as a rapid method for the identification of products from cell-cultures.

There are two further field-free regions used for metastable ion-analyses. The second field-free region (FFR2) occurs between the electric and magnetic sectors. Since ions in this region of the instrument have already been energy-selected, any subsequent metastable decompositions produce ions with the same *m/z* value as those produced in the source except that the signal occurs as a broad peak. We have observed metastable ions formed in FFR2 in the spectra of monoterpenoid esters (Results and Discussion 11.3).

Thus, a whole range of experiments using different regions of the vacuum system of the mass spectrometer can be employed for complete structure-elucidation.

10.4 Recent Studies on Monoterpenoids

Many of the early mass-spectrometric studies on monoterpenoids were carried out before the advent of double-sector machines and the reports are limited to descriptions of general fragmentations of various classes of compound.[276-7] Much of the rationalisations are speculative but serve as a good introduction to modern work.

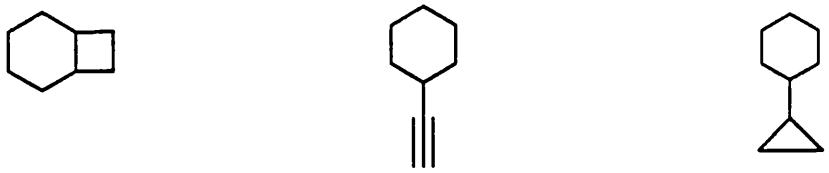
The origins of the ion at *m/z* 68 in the EI-spectrum of limonene have been studied in detail. Boyd *et al.*[278] have rationalised the formation of this ion by a retro-Diels Alder (RDA) process yielding a fragment similar to the molecular ion of isoprene. Tureček[279] reported a symmetrical distribution of charge between the diene fragments although unsymmetrical distributions were reported for RDA occurring from rotationally-excited molecular ions. Vincienti *et al.*[280] have studied the energetics of the RDA-fragmentation of limonene by use of surface-induced decompositions (SID) and angle-resolved mass spectrometry (ARMS); the combination of these techniques allowed higher-energy collisions to induce RDA fragmentation. The low abundance of the ion resulting from the RDA-process at 70 eV. is thought to be owing to partial isomerisation of the molecular ion of limonene to isolimonene, terpinolene, isoterpinolene, α-terpinene and 3,8-*p*-menthadiene. A subsequent CAD-study of ten monoterpenoid hydrocarbons[281] showed the molecular ions of these compounds to

retain their structural integrity with little isomerisation. However, the molecular ions of alloocimene and α-pyronene have been shown to undergo ring-closure and fragmentation through a series of common ions.[282] Peaks corresponding to metastable ions were used to determine the presence of the molecular ion of benzene and the tropylium ion in the spectra of these compounds. Derivatisation of a mixture of monoterpenoids (containing two double bonds) to their monoamino and bisamino alcohols[283] has been shown to yield diagnostic fragmentations of monoterpeneoids with isopropylidene groups (eg terpinolene), methyl substituted endocyclic double bonds (*eg.*, γ-terpinene) and vicinal-disubstituted exocyclic double bonds (*eg.*, β-pinene).

Monoterpenoids containing cyclobutane rings (pinenes, pinanes and their derivatives) have been studied by ammonia-CI.[284] The preferred site of protonation was found to be the double bond and its position influenced the stability and fragmentation of the $(M+H)^+$ ion. The CI-spectra of pinane showed no $(M+H)^+$ ion since no transfer of a proton between the reagent gas and the saturated sample could occur. The mass spectra of monoterpenoids containing a cyclopropane ring has received much interest in the last decade; in particular the group of insecticidal esters derived from chrysanthemic and pyrethric acids.[285] These show characteristic cleavage of the ester moiety with charge-localisation remaining on the ring.

The doubly-charged ion mass spectrum of limonene and nine other monoterpenes[286] have been reported and computations yielded skeletons such as those shown in Diagram 10.1.

Diagram 10.1 Structures to Account for Some Ions Observed in the Doubly-charged Ion Mass Spectrum of Limonene



(a) (b) (c)

The use of deuteriated substrates in the identification of fragments has been used to elucidate fragmentation pathways. The elimination of water from cyclic monoterpenoid alcohols and their deuteriated analogues has been shown to occur by a 1,2-elimination process in hydroxybornanes and dihydrocarveol.[287-289] Dehydration from epimeric menthols[290] has been shown to occur by 1,3- and 1,4-processes in which stereoisomers which possess hydroxyl groups in a 1,3-diaxial relationship to a tertiary hydrogen give rise to more abundant $(M-H_2O)^+$ ions. Maccoll and Mruzek[291] have reported 1,3- and 1,4-elimination of alcohol from menthyl ethers and have identified epimers on the basis of $M^+/(M-ROH)^+$ ion-ratios. A study of the spectra of trifluoroacetic acid esters of menthols[292] showed that a base peak at *m/z* 81 occurred in all but the spectrum of the neomenthyl isomer. This ion was found to be formed in a process involving elimination of the acid and fragmentation of the resultant ion by an RDA-process which could not occur in the molecular ion of the neomenthyl isomer.

As a contribution to the rapidly-growing interest in the reinvestigation of pharmacologically-important essential oils,[293] a number of studies have been aimed at identifying monoterpenoid esters and their higher homologues from mixtures of oils. Bambagiotti[294] has surveyed butyrate, propionate and valerate esters of monoterpenoids and has shown that $(RCO)^+$ ions arise from the acid-moiety involved and these are diagnostic of the ester-type. Lange and Schultz[295] have described a GCMS/PCI technique using ammonia as reagent gas to discriminate between terpenoid alcohols and esters in perfume mixtures by selected ion-monitoring of $(M+H-H_2O)^+$ and $(M+H-acid)^+$ ions. The formation of ion-molecule complexes with ammonia such as $(M+N_2H_6-H_2O)^+$ and $(M+N_2H_6-acid)^+$ produced diagnostic information on the class of the terpenoid moiety. Bambagiotti[296] has also shown the formation of RCOO-ions in the NCI-CAD spectra of bornyl esters: linked-scans for each carboxylate anion were recorded and the acids identified by comparison with authentic samples. In a subsequent communication[297] on the CAD-MIKE (collisionally-activated decomposition mass-induced kinetic energy) spectra of these compounds he claimed that specific fragmentations of the carboxylate anion and the terpenoid fragment occur which demonstrate retention of structural identity prior to dissociation. Lofstedt[298] has analysed moth pheromone acetates by selected-ion monitoring using EI and PCI

techniques. $(M-H_2O)^+$ and $(M-CH_3CO_2H)^+$ ions were chosen as reference ions for comparing pheromones produced by an individual turnip moth before and after mating.

CHAPTER 11 Results and Discussion

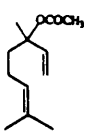
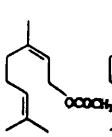
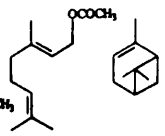
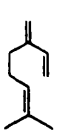
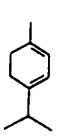
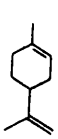
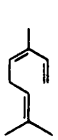
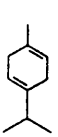
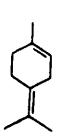
Aims: Linaloyl, neryl and geranyl acetates were components of the lavender-distillate that were analysed in Part One. Although they could be readily distinguished by their retention-times on GLC or HPLC, they behaved in a very similar manner when analysed by EI-mass spectrometry. The following experiments were carried out to try and analyse the behaviours of the three isomers in the mass spectrometer. Some preliminary rationalisations are made, based on the differing fragmentation patterns that were eventually obtained. This chapter is divided into two parts: (i) the elimination of acetic acid from the molecular ions of each compound and (ii) the metastables derived from the terpenoid fragment-ions.

11.1 General Features of the 70, 40, 20 and 12 eV. EI, FAB, PCI and NCI Spectra

Table 11.1 shows the EI-mass spectra (70 eV.) for linaloyl, neryl and geranyl acetates and eight monoterpenoid hydrocarbons. The spectra showed characteristic ions derived from the acid-portions of the molecule (*eg.*, *m/z* 60) and the terpenoid fragments (*eg.*, *m/z* 136 and its daughter-ions which are observed in the mass spectra of the monoterpenoid hydrocarbons). Because the compounds are isomeric, the terpenoid fragments were probably similar or identical, and thus the mass spectra were virtually indistinguishable. The three isomers did not yield detectable molecular ions under normal operating conditions (probe-inlet with source-temperature 180°C), but neryl and geranyl acetates did yield small molecular ions (5 %) when analysed under GC/MS conditions (section 12.2a; system 1) presumably because the helium carrier gas may have removed the excess of vibrational energy, and thus enhanced the molecular ion. The samples were also run at lower ionisation energies 40 eV., 20eV., and 12 eV. At the latter; all three compounds showed a molecular ion. Similarly, quasi-molecular ions could be obtained by FAB and PCI methods of ionisation (Table 11.2).

From Table 11.1 neryl and geranyl acetates showed an ion at *m/z* 69 (derived from the isopropylidene group as the base-peak in the EI-spectra recorded at 70 eV). The spectrum of linaloyl acetate showed an ion at *m/z* 93 as the base-peak. These fragment-ions are also observed as the base-peaks in the corresponding 20 eV. and

Table 11.1 Normalised Ion-abundances (%) in the Mass Spectra (EI ; 70 eV.) of Linaloyl, Neryl, and Geranyl Acetates and Eight Monoterpenoid Hydrocarbons

ION (*m/z*)	 I	 II	 III	IV	 V	 VI	 VII	 VIII	 IX	 X	 XI
196	-	-	-	-	-	-	-	-	-	-	-
195	-	-	-	-	-	-	-	-	-	-	-
181	-	-	-	-	-	-	-	-	-	-	-
155	-	-	-	-	-	-	-	-	-	-	-
154	-	3	0.34	-	-	-	-	-	-	-	-
139	-	-	-	-	-	-	-	-	-	-	-
138	-	-	-	-	-	-	-	-	-	-	-
137	-	4	1	-	-	-	-	-	-	-	-
136	8	12	10	7	12	3	44	23	7	37	70
123	-	3	1	-	-	-	-	-	-	-	-
122	1	2	-	1	1	-	9	2	-	3	-
121	19	17	10	16	14	4	100	23	17	36	100
119	1	1	-	1	-	-	12	1	2	3	5
111	-	-	-	-	-	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-	-	-	-
109	-	2	-	-	-	-	-	-	-	-	-
108	-	2	-	1	2	-	5	6	2	1	4
107	7	6	4	6	5	2	15	20	9	8	18
106	-	-	-	3	-	-	4	1	3	2	5
105	4	3	1	10	3	1	17	5	12	11	20
104	-	-	-	-	-	-	1	-	-	1	1
103	-	-	-	-	-	-	3	-	1	2	3
95	1	5	1	1	3	1	3	9	3	-	1
94	9	11	4	-	18	9	14	25	13	11	9
93	100	44	30	100	100	100	99	71	100	100	96
92	12	12	5	36	11	7	15	21	31	25	10
91	10	7	4	30	2	10	40	18	28	42	40
89	-	-	-	1	1	-	2	1	-	-	-
86	-	2	-	-	-	-	-	-	-	-	-
85	-	10	4	-	-	-	-	-	-	-	-
83	2	3	-	-	-	-	-	-	-	-	-
82	1	3	-	-	2	1	-	21	1	-	-
81	9	16	5	4	4	1	6	14	10	2	4
80	30	33	10	11	14	6	9	14	17	-	7
79	14	10	-	24	30	13	34	35	35	25	42
77	9	7	4	29	27	11	36	21	28	40	35
74	-	-	-	-	-	-	-	-	-	1	1
71	-	3	-	-	-	-	-	-	-	-	-
69	48	100	100	1	54	85	3	9	4	-	1
68	14	81	37	5	4	5	34	100	36	1	4
67	13	30	12	9	14	10	17	60	21	3	13
65	1	4	2	7	8	4	13	10	8	10	12
61	-	-	-	-	-	-	-	-	-	-	-
60	3	1	1	-	-	-	-	-	-	-	-
59	-	1	-	-	-	-	1	-	-	-	-

$2\underline{\sigma} = \pm 12\%$

I, Linaloyl Acetate; II, Neryl Acetate; III, Geranyl Acetate; IV, α-Pinene; V, β-Pinene; VI, Myrcene; VIII, α-Terpinene; VIII, Limonene; IX, *cis*-Ocimene; X, γ-Terpinene; XI, Terpinolene

Table 11.2 FAB and PCI Mass Spectra of Linaloyl, Neryl and Geranyl Acetates

FAB	Linaloyl Acetate			Neryl Acetate			Geranyl Acetate			PCI
Ion (*m/z*)	Scan B	Scan A	PCI	Scan B	Scan A	PCI	Scan B	Scan A	PCI	Ion (*m/z*)
219	13	-	< 1	45	-	1	72	-	1	**216**
199	-	-	-	1	-	-	2	-	-	**215**
195	-	1	-	-	0.10	6	-	0.20	5	**214**
177	-	-	-	-	-	1	1	-	1	**213**
176	8	-	-	12	-	1	17	-	1	**212**
173	2	-	10	2	-	1	5	-	1	**205**
154	[0.40]*	40	-	[0.23]*	22	-	[0.40]*	43	-	**203**
149	1	10	-	-	12	-	1	15	-	**199**
138	-	12	100	-	5	[0.5]*	-	10	[0.5]*	**107**
137	6	37	-	9	25	[0.5]*	8	32	[0.5]*	**196**
136	6	48	-	3	30	-	5	40	-	**195**
135	1	32	15	5	25	15	4	25	25	**154**
123	-	5	100	-	5	100	-	5	100	**137**
121	5	5	20	1	5	8	2	8	10	**123**
119	-	-	15	-	2	13	-	6	12	**121**
109	1	8	15	1	8	9	-	10	6	**109**
108	-	-	24	-	-	19	-	-	18	**85**
107	3	18	43	3	18	44	3	20	42	**81**
106	-	-	10	-	-	19	-	-	22	**78**
105	2	5	24	1	2	11	1	8	11	**70**
97	-	-	10	-	-	12	-	-	14	**69**
95	4	12	35	6	10	15	4	12	15	**58**
94	1	-		-	-		-	-		
93	26	20		13	16		12	17		
92	4	-		2	-		3	-		
91	4	18		5	8		5	13		
90	-	-		-	-		-	-		
83	18	6		13	6		20	6		
81	20	28		26	32		21	29		
69	39	25		59	39		82	43		
67	11	11		10	8		10	8		
55	22	26		15	21		15	21		
53	14	8		15	10		15	10		
51	7	14		7	8		7	10		
43	100	100		100	100		100	100		
41	52	30		67	33		71	33		
39	40	28		48	24		45	36		

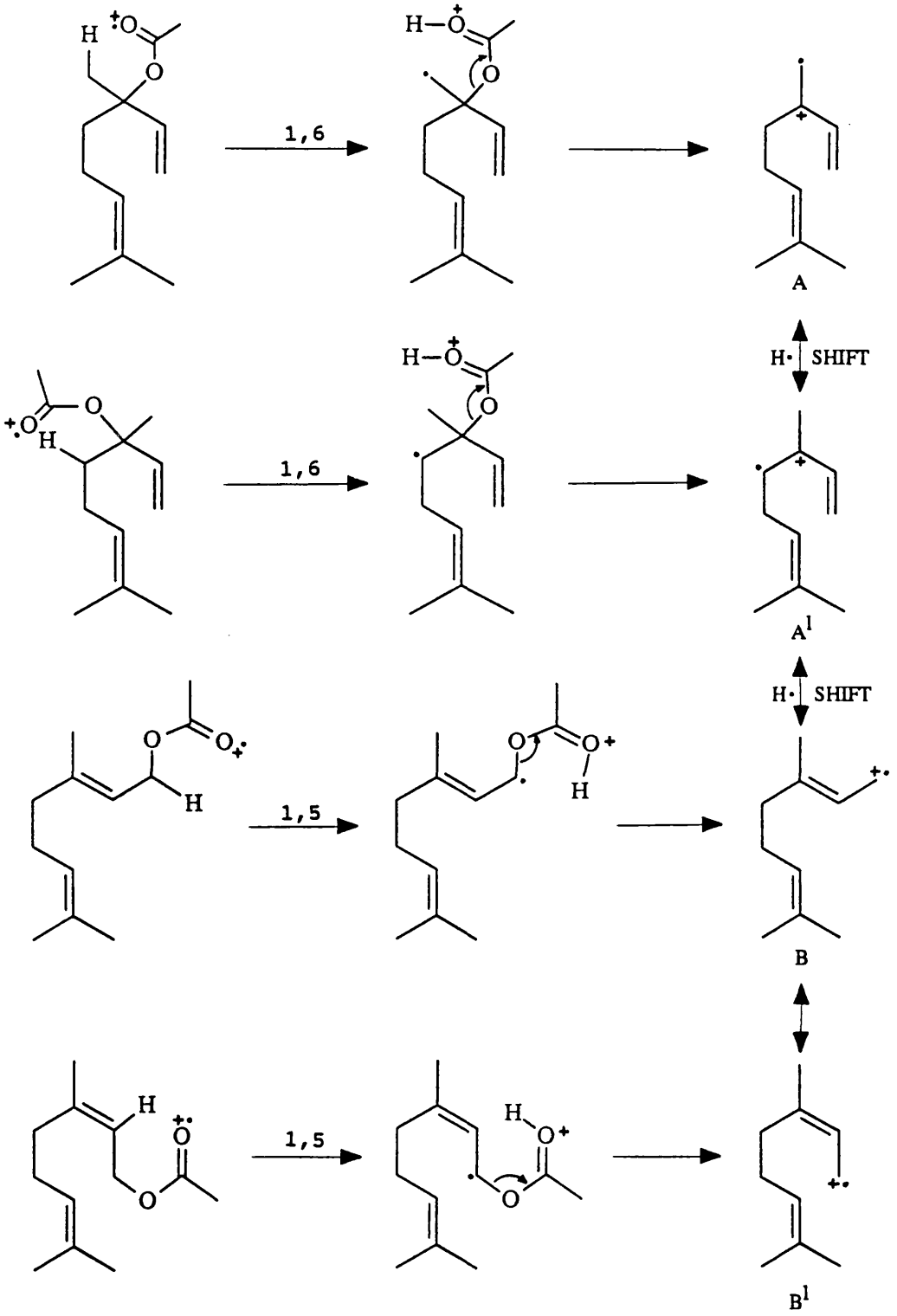
* Low intensity but structurally significant.
$2\sigma = \pm 10\%$
FAB ; fast atom bombardment
PCI ; positive chemical ionisation

12 eV. EI-spectra. Table 11.2 shows the ion-abundance values for the FAB-spectra of the three esters. Data from two separate scans for each compound is shown (scan A and scan B where B=A+40). The early scans (A) showed the appearance of a quasi-molecular ion $(M-1)^+$ in each case, whereas in the later scans (B) this was replaced by the $(M+Na)^+$ molecular ion. The $(M-1)^+$ ion was most abundant in the spectrum of linaloyl acetate but the $(M+23)^+$ ions were most abundant in the spectra of neryl and geranyl acetates. The scan A spectrum of each compound showed abundant ions $(M-CH_2CO)^+$ at *m/z* 154 and $(M-CH_3CO_2H)^+$ at *m/z* 136 although these ions were insignificant in the scan-B spectra which showed the ions $(M+Na-CH_3CO)^+$ at *m/z* 176 and $(M-CH_3CO_2)^+$ in greater abundance. None of the compounds showed an ion corresponding to the molecular ion of acetic acid at *m/z* 60 but all the spectra showed an ion at *m/z* 59, resulting from either (i) cleavage of $(CH_3CO_2)^+$ from the molecular ion or (ii) fast atom bombardment of acetic acid that had been eliminated by pyrolysis of the compounds on the probe tip. This was most abundant in the scan-A spectra. By reference to Table 11.1 and Table 11.2 it is clear that fragmentation of the acid portion of the molecular ion of a terpenoid ester was more pronounced in an even-electron molecular ion generated by FAB than from an odd-electron molecular ion generated by electron impact. On the other hand, the ions characteristic of the terpenoid skeleton were significantly less abundant in the FAB-spectra compared with the EI-spectra. All the FAB-spectra showed a base-peak at *m/z* 43 (corresponding to the ion CH_3CO^+, and some contribution from the isopropyl ion derived from the isopropylidene group) whereas the EI-spectra showed ions derived from the terpenoid-fragment as the base-peak. All three esters showed the ions $(M+NH_3)^+$ in their PCI- spectra and $(M-H)^+$ in their NCI-spectra. The latter was the base-peak in the NCI- spectra of geranyl and neryl acetates although a peak corresponding to $(M-69)^+$ was the base-peak in the NCI-spectrum of linaloyl acetate.

11.2 Ions Corresponding to Elimination of Acetic Acid

The general mechanism for elimination of acetic acid has been shown to follow a 1,2-elimination process.[299] Many such eliminations are thought to involve a series of simple steps involving hydrogen-atom transfers. The elimination of acetic acid from linaloyl acetate can be represented as in scheme 11.1. Two eliminations of acetic acid via formation of a six-membered ring are shown. For the neryl and geranyl analogues a six-membered elimination of acetic acid could not occur. However, by

Scheme 11.1 Generation of a Common Terpene Fragment-ion at *m/z* 136 by Elimination of Acetic Acid from Distinct Molecular Ions of Linaloyl, Neryl and Geranyl Acetates

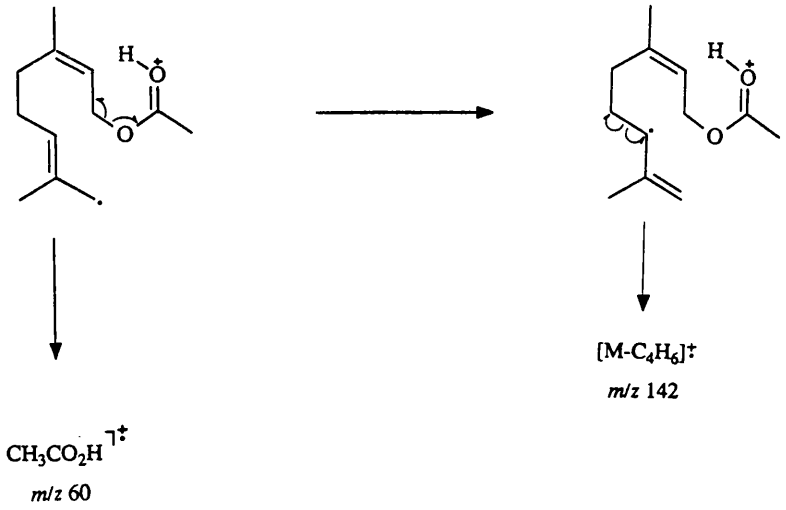


"Terpene" is used here to describe the unsaturated ion that results from the elimination of acetic acid from the molecular ion.

analogy with the results of McAdoo (ref. 208, Part Three) the elimination may proceed via formation of a five-membered ring.

Ions at m/z 142: Neryl acetate was the only isomer of the three studied here to show this ion in the EI (70 eV.) mass spectrum. This can only be accounted for by the transfer of a hydrogen atom from either C_8 or C_9 to the ester-moiety. This is shown in Scheme 11.2.

Scheme 11.2 A Mechanism to Account for the Formation of an Ion at *m/z* 142 in the Mass Specturm of Neryl Acetate



Ions at m/z 136: In the mass spectra of the three acetates an ion at *m/z* 136 was observed and this corresponded with elimination of acetic acid. Scheme 11.1 showed how this common fragment ion could be related to the three skeletal types. The formation of ion-molecule complexes (dimers) could facilitate hydrogen-transfer from other sites and such hydrogen-bonded dimers are commonly encountered in mass spectrometry and readily explain hydrogen-transfer from a remote site in the molecule.

Ions at m/z 68: Neryl and geranyl acetates contain 15 allylic hydrogens that could be transferred by a distonic process. For example, abstraction of hydrogen at C_5 and elimination of the acid would yield a terpenoid-ion which could undergo C_5-C_6 cleavage (this occurs in all terpenoids containing the isopropylidene group) to yield

Table 11.3 Ion-abundances (%) in the Mass Spectra (EI; 12 eV.) of Linaloyl, Neryl and Geranyl Acetates

ION (*m/z*)	Linaloyl Acetate	Neryl Acetate	Geranyl Acetate
196	8	5	9
152	5	9	7
138	5	5	7
137	11	14	14
136	100	100	100
134	11	5	9
122	2	-	25
121	15	14	18
110	2	-	-
109	2	-	-
108	4	5	27
107	15	5	7
94	12	14	25
93	64	41	34
92	27	18	23
80	45	27	20
78	2	-	14
69	2	14	14
68	4	14	13
60	3	9	5
58	2	-	-
56	-	23	9
43	12	23	11
42	32	14	11

$2\sigma = \pm 12\%$

an ion at *m/z* 68 (*viz. m/z* 69 in other spectra). The spectra (70 eV.) showed that this ion was very intense for neryl and geranyl acetates but very weak in the spectrum of linaloyl acetate.

Metastables Occurring in Spectra Recorded at 12 eV: Table 11.3 shows the data for spectra recorded at 12 eV; the base peak for each compound corresponded to the terpenoid fragment resulting from the elimination of acetic acid. A broad peak at *m/z* 99.4 was observed corresponding to the metastable transition for this process which occurred in FFR2. Table 11.4 showed the other expected peaks corresponding to metastable processes and Table 11.5 shows the observed metastable decompositions. These show interesting losses of methyl and isopropyl radicals but they did not distinguish the three acetates under study. The metastable decompositions occurring in FFR1 for the ions at *m/z* 136, *m/z* 121, *m/z* 108 and *m/z* 107 (the terpenoid fragments) are discussed in section 11.3). Ions at *m/z* 154 corresponding to loss of ketene from the molecular ion were not observed in the spectrum of geranyl acetate; instead ions resulting from loss of CH_3COH were observed.

Summary: There were no significant differences in the spectra; all three compounds showed losses of acetic acid from their molecular ions. Elimination of acid produced ions at *m/z* 136. The hydrogen atom that was involved in the elimination process may originate from one of several sites in the molecular ion. This may have influenced subsequent fragmentation *eg.*, most monoterpenoids produce spectra with ions at *m/z* 69 (probably the isopropylidene part of the molecule) but these C_{10}-acetates showed the formation of an ion at *m/z* 68.

11.2.1 Elimination of Acetic Acid from Deuteriated Analogues of Linaloyl, Neryl and Geranyl Acetates

Nine deuteriated analogues shown in Diagram 11.1 were prepared by the methods given in the Experimental Section (15.2). The introduction of one or more deuteriums to C_9 or C_4 of each isomer may help to determine if hydrogen atoms attached to these carbon atoms are associated with the elimination process. This would lead to the elimination of deuteriated acetic acid from the molecular ion and thus give rise to an ion at *m/z* 61. According to Scheme 11.1 geranyl and neryl acetates would not eliminate the deuteriated acid.

Table 11.4 Predicted Metastable Decompositions in the EI-Mass Spectra of the Three Monoterpenoid Esters used in this Study

M_1		M_2	Formula	N	M*
196	→	136	$C_{10}H_{16}$	60	94.4
136	→	121	C_9H_{13}	15	107.4
136	→	108	C_8H_{12}	28	85.8
136	→	107	C_8H_{11}	29	84.2
136	→	94	C_7H_{10}	42	65
136	→	93	C_7H_9	43	63.6
136	→	92	C_7H_8	44	62.2
121	→	107	C_8H_{11}	14	94.6
121	→	94	C_7H_{10}	27	73
121	→	93	C_7H_9	28	71.5
121	→	92	C_7H_8	29	70

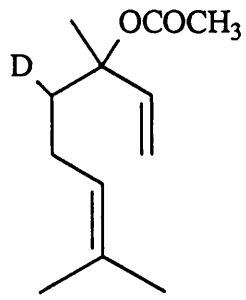
Table 11.5 Observed Metastable Decompositions in FFR2

M_1		M_2	Formula	N	M*
196	→	85	C_6H_{13}	111	37
196	→	69	C_5H_9	127	24
136	→	121	C_9H_{13}	15	107.5
136	→	94	C_7H_{10}	42	64.5
136	→	93	C_7H_9	43	63.5
136	→	92	C_7H_8	44	62.5
136	→	80	C_6H_8	56	47
136	→	42	C_3H_6	94	13

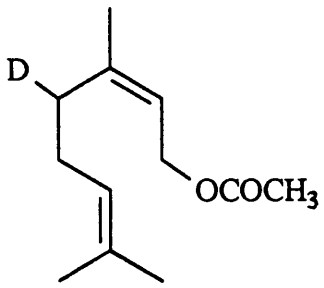
M_1 ≡ Parent ion
M_2 ≡ Daughter ion
M* ≡ Ion observed when metastable-decomposition occurs in the second field-free region (FFR2)
N ≡ Radical produced during metastable-decomposition

Diagram 11.1 Structures of Nine Deuteriated Monoterpenoid Esters Prepared for Analysis by Mass Spectrometry

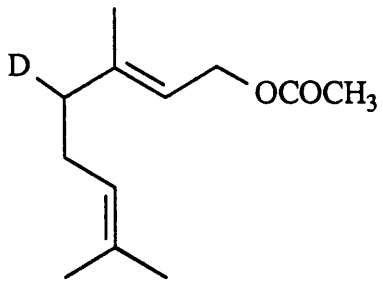
(a) [4-^{2}H]-



27

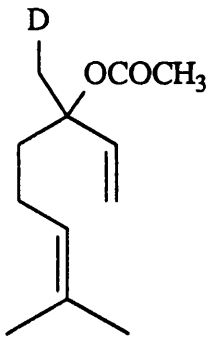


28

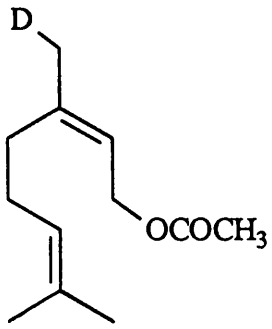


29

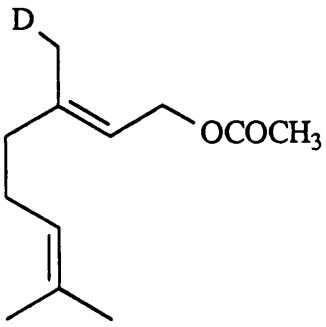
(b) [9-^{2}H]-



30

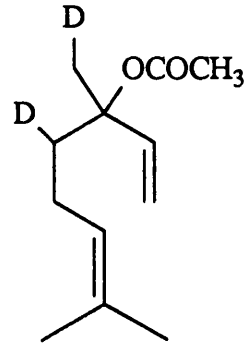


31

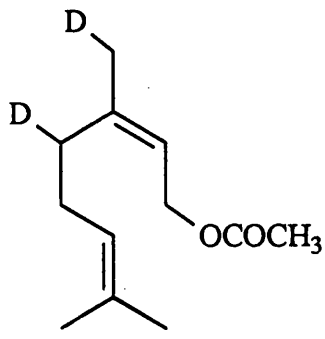


32

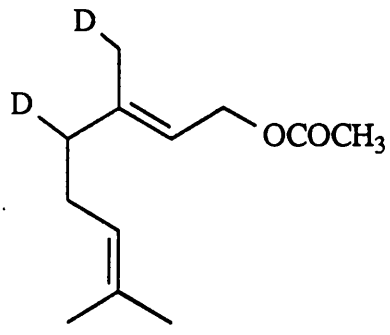
(c) [4,9-$^2H_2$]-



33



34



35

See text for structural assignment by ^{1}H, ^{2}H, ^{13}C NMR.

(i) Preparation of Deuteriated Monoterpenoid Esters and their Characterisation by ^{13}C-NMR, ^{1}H-NMR and ^{2}H-NMR

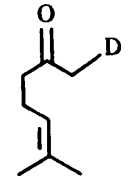
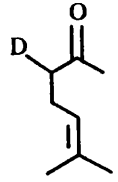
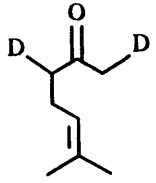
A summary of the general procedure for deuteriation is as follows: deuteriated linalool (formed by a reaction analogous to fluorination in Part Three) was converted in to a mixture of the deuteriated linaloyl, neryl and geranyl acetates in approximately 3:1:3 proportion by a modified version of the method of Babler *et al.* (see Part Three, ref 182). The isomers were separated by preparative HPLC (system 2). The position of the deuterium substitution was dependent on the geometry of the TMS-enol ether which was formed in the first step, and this could be controlled by the appropriate choice of reaction conditions (Section 15.1; method h or i).

House (see Part Three, ref. 191) has previously reported a method for quenching enolates in a mixture of D_2O/CH_3CO_2D for 15 minutes in the presence of a buffer in order to minimise scrambling of the deuterium label. We modified this method by quenching the enol ethers in a mixture of D_2O/CH_3CO_2D containing caesium fluoride (20%) for 10 minutes at 25 °C. Fluoride has been previously shown to cleave TMS-enol ethers. (see Part Three, ref. 206). The introduction of two or more deuteriums involved the formation of the appropriate TMS-enol ether regioisomer from the respective mono-deuteriated 6-methyl-hept-5-en-2-one with subsequent cleavage in the D_2O/CH_3CO_2D medium. These steps were repeated for the introduction of a third deuterium. Since the formation of a TMS-enol ether by either method requires the abstraction of a proton by the base, the hydrogen-kinetic isotope effect discriminates against abstraction of a deuterium introduced at an earlier step.

Table 11.6 shows the ^{1}H-, ^{2}H- and ^{13}C-NMR chemical shifts and heteronuclear coupling constants which confirm the structural assignment of the products in Diagram 11.1. The integral ratios (H_1:H_3) confirm the position of deuterium substitution for the products. In particular, the [1,3-$^{2}H_2$]-ketone is shown not to have been a mixture of the two mono-deuteriated products.

Because the formation of TMS-enol ethers by either method did not involve complete conversion of the ketone exclusively to one TMS-enol ether regioisomer, it was inevitable that some unreacted ketone (and the unwanted deuteriated ketone) would be present in the separated product. These could not be removed by GC or HPLC

Table 11.6 Analytical Data from the ^{1}H-, ^{2}H- and ^{13}C-NMR Spectra and Mass Spectra (EI ; 70eV.) of the Deuteriated 6-Methyl-hept-5-en-2-ones

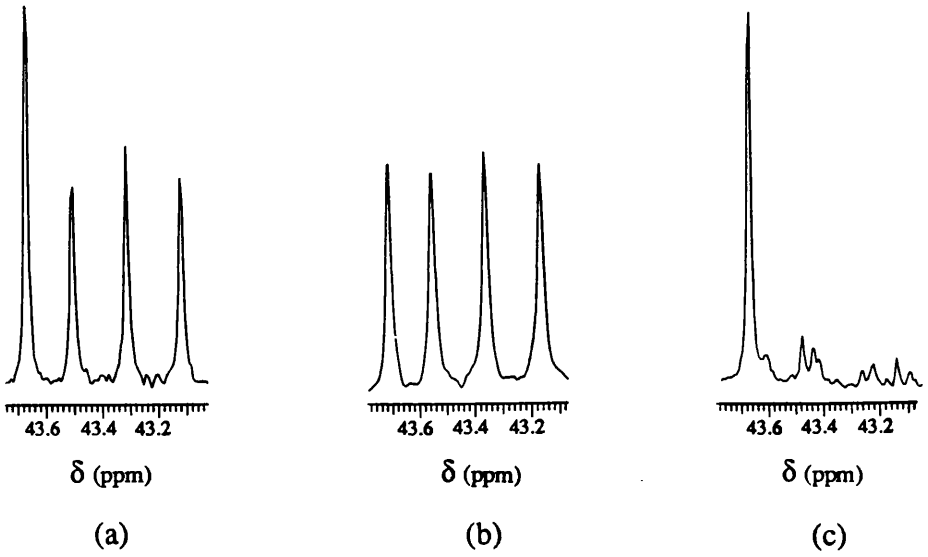
	$\delta_{C\text{-}D}$ (ppm)	$^{1}J_{C\text{-}D}$ (Hz.)	δ_{Hgem} (ppm)	$^{2}J_{H\text{-}D}$ (Hz.)	Integral Ratio $n(H_1 : H_3)$	δ_D (ppm)	M^+ (*m/z*)
	29.55	19.40	2.08	2.00	1:1	2.15	127
	43.22	19.35	2.40	2.09	3:1	2.58	127
	29.61 43.34	19.50 19.40	2.15 2.42	2.00 2.00	2:1	-	128

δ ≡ chemical shift (ppm)
δ_{Hgem} ≡ chemical shift of a proton attached to the carbon bearing the deuterium
J ≡ coupling constant (Hz)
D ≡ deuterium (^{2}H)

$n(H_1:H_3)$ ≡ proportions (integral-ratio) of protons attached to C_1 and C_3; this will be dependent on the deuterium substitution

(because the peaks were not resolved), but the mixture could be adequately quantified by analysis of the ^{13}C-NMR spectra (acquired using a delayed pulse sequence to ensure relaxation of nuclei before a subsequent acquisition). Diagram 11.2a shows the ^{13}C-signal for C_3 of the 3-^{2}H-6-methyl-hept-5-en-2-one recorded under normal operating conditions (D1 = 1.4 and PW = 13.0) and Diagram 11,2b shows that same signal recorded under operating conditions with a reduced pulse-width and an increased period for relaxation of the nuclei (DI = 32.0 and PW = 4.0). The signal for the undeuteriated ketone occurred at 43.66 ppm and the 1:1:1 triplet of the [3-^{2}H]-ketone occurred at 43.31 ppm.

Diagram 11.2 Diagnostic ^{13}C-NMR Signals of Deuteriated 6-Methyl-hept-5-en-2-ones (20, 21)



On inspection of these signals, the sample was seen to contain the desired product (75%). Inspection of the signal for C_1 at 29.55 ppm indicated none of the [1-^{2}H]-ketone was present. Diagram 11.2c shows the signal for C_3 in the [1-^{2}H]-ketone, and clearly, the sample contained a small proportion of the [3-^{2}H]-ketone. An attempt was made to prepare the [3,3-$^{2}H_2$]-ketone and the [1,1,1-$^{2}H_3$]-ketone. Although molecular ions in the mass spectra of the products (at *m/z* 128 and at *m/z* 129 respectively) indicated the correct number of deuteriums had been inserted, the ^{13}C- and ^{1}H-NMR spectra indicated that the pattern of substitution was wrong: the NMR spectrum of a

pure product, thought to be the [3,3-$^2H_2$]-ketone, actually confirmed that 1,3-substitution had occurred. This can be rationalised by the secondary hydrogen-kinetic isotope effect operating when the 3-^{2}H-ketone was added to the base; abstraction of a proton at C_1 would actually be some 20% faster than abstraction of a proton at C_3. The attempt to prepare the [1,1,1-$^2H_3$]-ketone resulted in a mixture containing the [3-^{2}H]-ketone, [1-^{2}H]-ketone, [1,1,3-$^2H_3$]-ketone, the [1,^{2}H]-TMS-enol ether and the [1,1-$^2H_2$]-TMS-enol ether (the more highly substituted isomers).

The products that were made are shown in Diagram 11.1. They did contain some undeuteriated product but this was of no significance because the aim of the present work was to detect the ions resulting from elimination of the deuteriated acetic acid in the mass spectra.

(ii) Electron Impact (70 eV.) Mass Spectra of Deuteriated Monoterpenoid Esters

Table 11.7 shows the spectra of the nine deuteriated acetate esters. From this table it is clear that all three isomers with a deuterium atom at C_4 eliminated the deuteriated acetic acid whereas the isomers with the deuterium substitution at C_9 did not eliminate the deuteriated acid. This suggests that 1,6-transfer of a deuterium atom, followed by elimination occurred (an elimination of this sort shown for linaloyl acetate in Scheme 11.1) although it is not clear how this could take place from the molecular ions of geranyl and neryl acetates unless isomerisation or deuterium scrambling occurred during the elimination process. The introduction of deuterium may well perturb the normal routes of fragmentation (if indeed they do differ for each compound).

Comparison of the ion-abundance ratio $(M\text{-}HAcid)^+/(DAcid)^+$ for each compound showed that elimination of the D-Acid was preferred by those isomers with a deuterium substitution at C_4. For example, this ratio was 8 for [4-^{2}H]-linaloyl acetate and 149 for [9-^{2}H]-linaloyl acetate (*ie.*, elimination of D-Acid was preferred in the [4-^{2}H]-isomer). The corresponding ion-abundance ratio for the [4,9-$^2H_2$]-linaloyl acetate fell between the two previous values (ratio, 53). Similarly, a preference for elimination of D-Acid in [4-^{2}H]-geranyl acetate (ratio, 7) was observed over elimination of D-Acid from the 9-^{2}H- isomer (ratio, 14). Elimination of the D-acid was observed in the spectrum of [4-^{2}H]-neryl acetate and was absent in the spectrum of the [9-^{2}H]- and [4,9-$^2H_2$]-isomers.

Table 11.7 Normalised Ion-abundances (%) in the Mass Spectra (EI ; 70eV.) of Deuteriated Linaloyl, Neryl and Geranyl Acetates

m/z	[4-^{2}H] L	[4-^{2}H] N	[4-^{2}H] G	[9-^{2}H] L	[9-^{2}H] N	[9-^{2}H] G	[4,9-$^2H_2$] L	[4,9-$^2H_2$] N	[4,9-$^2H_2$] G
198									
197	-	[0.01][a]	[0.39][a]	-	-	-	-	-	-
083	-	-	-	-	-	-	[0.1][a]	-	-
182	3	-	-	-	-	-	-	-	-
156	-	-	[0.06][a]	-	-	-	-	-	0.53
155	-	2	4	-	-	[0.24][a]	-	-	0.1
138	33	4	10	7	-	1	53	7	18
137	75	29	51	52	5	14	20	1	6
136	17	2	6	1	-	[0.28][a]	68	5	10
123	27	2	5	5	-	[0.22][a]	68	5	10
122	84	25	44	67	3	8	33	2	6
121	28	4	9	7	-	2	3		0.5
110	-	1	3	-	-	-	[0.5][a]	-	1
109	8	2	7	1	-	1	5	1	3
108	29	6	13	3	-	2	6	0.40	2
107	15	3	7	3	-	[0.45][a]	4	0.03	1
95	42	3	6	9	-	[0.47][a]	9	1	3
94	29	8	15	6	[0.16][a]	2	38	17	21
93	75	57	56	49	17	18	34	11	15
92	56	24	38	16	3	7	14	3	6
91	30	6	11	3	-	1	5	0.3	2
80	44	14	21	9	1	3	9	9	13
79	31	31	42	5	6	9	9	4	6
78	38	10	17	4	-	2	9	1	3
69	1	-	1	-	-	[0.03][a]	-	-	0.25
68	6	2	5	[0.01][a]	-	[0.01][a]	2	1	4
67	5	18	43	-	2	9	6	25	53
66	47	100	100	10	100	100	14	100	100
61	9	3	7	[0.35][a]	-	1	1	-	1
60	2	[0.1][a]	1	-	-	-	0.02	-	0.003
41	7	1	3	-	-	1	2	-	1
40	12	5	13	2	-	2	3	1	5
39	100	85	94	100	49	78	100	61	93
38	35	24	52	6	3	10	10	8	19
37	62	84	89	18	44	66	22	54	83

L ≡ Linaloyl Acetate; N ≡ Neryl Acetate; G ≡ Geranyl Acetate
a ≡ Low Abundance but structurally significant
$2\sigma = \pm 10\%$

In all the spectra, the ion-abundance ratio, $(M\text{-}HAcid)^+/(M\text{-}DAcid)^+$ was large (≥6) and reflected the primary kinetic isotope effect associated with elimination of acetic acid in the deuteriated isomers. However, although seven of the isomers showed an ion at *m/z* 61 corresponding to loss of the D-acid, none of the isomers showed an ion at *m/z* 60, corresponding to loss of the H-acid. Loss of ketene was observed (*cf* 11.2) in the spectra of all three geranyl isomers (producing ions at *m/z* 155 for mono-deuteriated and at *m/z* 156 for bis-deuteriated). Clearly deuterium substitution yields little more information than low-energy electron bombardment. Perhaps all three compounds do eliminate acetic acid by a general distonic process which leads to isomeric fragment-ions.

11.3 The Terpenoid Fragment-ion, (M-Acetic Acid)$^+$ and Associated Daughter Ions

The fragment-ion occurred at *m/z* 136 in the EI- and FAB-mass spectra of the undeuteriated isomers and a peak at *m/z* 137 was observed in the PCI-spectra. The EI-spectra of the mono-deuteriated isomers showed this ion at *m/z* 137 and the bis-deuteriated isomers showed this ion at *m/z* 138. The previous section has shown that a common terpenoid fragment-ion at *m/z* 136 may be derived from the molecular ion of each isomer. The following section is concerned with the daughter-ions derived from this ion and whether these verify the likelihood of a common structure of the terpenoid fragment-ion at *m/z* 136. This fragment can be likened to the molecular ions of the monoterpenoid hydrocarbons of the series $C_{10}H_{16}$ which would also occur at *m/z* 136. Some bicyclic monoterpenoid hydrocarbons of the formula $C_{10}H_{16}$ also give rise to ions at *m/z* 136. The aim of the work in this section was to determine whether the terpenoid fragment-ions that occurred in the spectra of the C_{10}-acetates behaved like discrete monoterpenoid hydrocarbons.

Ion-abundance Data: Table 11.8 shows ratios for three pairs of ion-intensities for all eight) monoterpenoid hydrocarbons compared with the monoterpenoid esters. It is noteworthy that the ratios for neryl and geranyl acetates are very similar but they differ from the ratio for linaloyl acetate. There is clear correspondence of the data for the fragment-ions of neryl and geranyl acetates with the data for the cyclic monoterpenoids. The intensity-ratios of the fragment ions derived from linaloyl acetate correspond very well with the data for *cis*-ocimene and α-pinene.

Table 11.8 Abundance Ratios for Ions Occurring in the Mass Spectra (EI ; 70eV.) of Three Monoterpenoid Esters and Eight Monoterpenoid Hydrocarbons used in this study

Compound	Ion-abundance Ratio $(m/z)_a/(m/z)_b$		
	121/136	107/136	93/136
Linaloyl Acetate	2.38	0.83	12.50
Neryl Acetate	1.44	0.50	3.64
Geranyl Acetate	1.00	0.41	2.94
α-Pinene	2.29	0.86	14.29
β-Pinene	1.17	0.42	8.33
Myrcene	1.33	0.67	28.33
α-Terpinene	2.27	0.34	2.25
Limonene	1.00	0.87	3.09
***cis*-Ocimene**	2.42	1.29	14.29
γ-Terpinene	0.97	0.22	2.70
Terpinolene	1.43	0.26	1.37

$2\sigma = \pm 10\%$

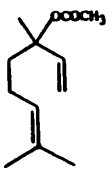
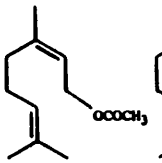
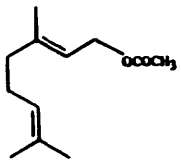
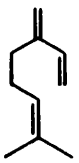
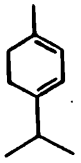
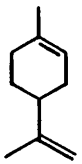
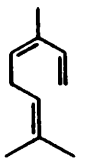
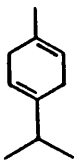
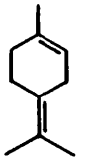
Linked-scanning: Linked-scans (maintaining the B/E ratio constant) were recorded for the ions at *m/z* 136, 121 amd *m/z* 107 for the eight monoterpenoid hydrocarbons and the three monoterpenoid esters. Tables 11.9-11.11 show the normalised ion-abundances (%) for the observed daughter-ions resulting from metastable decompositions in FFR1 (these ions will be referred to as "metastable ions").

Linked-scanning of Ions at m/z 136: As already discussed, this ion resulted from elimination of acetic acid from the molecular ion. The abundance-data in Table 11.9 shows that five common daughter-ions were formed from this fragment. These ions also occurred in the linked-scan spectra of the monoterpenoid hydrocarbons used in this study. This suggests a similarity between the terpenoid fragments (derived from the esters) and the molecular ions of the hydrocarbons. However, none of the ion-intensities were similar which suggests that the skeletons of the terpenoid fragments did not match any of the molecular ions of the hydrocarbons. Indeed, the ion-abundancies in the linked-scan spectra of the esters did not even match, which suggests that they did not form a common-ion at *m/z* 136.

Linked-scanning of Ions at m/z 121 and m/z 107: All the spectra showed the same qualitative pattern of daughter-ions derived from the parent-metastables. There was also a correspondence in the intensities of these daughter-ions: the ions (and their intensities) derived from linaloyl acetate matched those derived from γ-terpinene. The ions (and their intensities) derived from geranyl and neryl acetates were similar to those derived from myrcene.

These results suggest that when the molecular ions of each ester eliminate acetic acid, they form ions at *m/z* 136 which behave like the molecular ions of monoterpenoid hydrocarbons ($C_{10}H_{16}$). However, the intensities of the ions at this *m/z*-value did not match those derived from the hydrocarbons. This can best be explained by the elimination of acetic acid from the C_{10}-acetates to form populations of ions at *m/z* 136 that do not behave like the discrete molecular ion of any single monoterpenoid hydrocarbon. Each C_{10}-acetate may form its own population of ions at *m/z* 136 (which Table 11.9 suggests). For example, Scheme 11.3 shows that the ions at *m/z* 121, *m/z* 107 and *m/z* 93 arise directly from the ion at *m/z* 136 in the spectra of all three acetates. However, only in the spectrum of linaloyl acetate did

Table 11.9 Abundances (%) of Daughter Ions Occurring in the Linked-scan (B/E) Spectra† of Ions at *m/z* 136

ION (*m/z*)	 I	 II	 III	 IV	 V	 VI	 VII	 VIII	 IX	 X	 XI
136[a]	100	100	100	100	100	100	100	100	100	100	100
121[b]	7.12	5.34	10.96	3.65	10.16	5.92	38.97	15.85	4.63	8.24	15.68
108[b]	0.03	-	0.06	0.02	0.26	0.12	2.01	0.71	-	0.09	0.43
107[b]	0.67	0.29	0.79	0.38	0.08	1.62	7.29	8.65	0.89	1.70	2.36
106[b]	-	-	-	-	-	-	-	0.39	-	-	-
105[b]	-	-	-	-	-	-	0.20	0.03	-	0.07	0.15
94[b]	0.16	0.05	0.16	-	0.03	-	2.10	7.15	0.35	0.03	-
93[b]	1.05	0.36	0.66	0.43	1.18	0.92	3.40	2.19	0.09	1.16	0.95
92[b]	0.30	0.01	0.02	2.77	0.49	0.12	1.95	3.93	0.44	1.36	0.10
91[b]	-	-	-	0.01	-	-	-	-	-	0.03	0.03

I, Linaloyl Acetate; **II, Neryl Acetate; III, Geranyl Acetate;** IV, α-Pinene; V, β-Pinene; VI, Myrcene; VIII, α-Terpinene; VIII, Limonene; IX, *cis*-Ocimene; X, γ-Terpinene; XI, Terpinolene

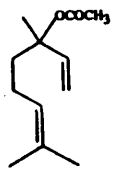
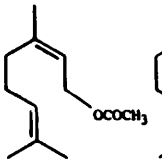
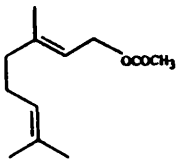
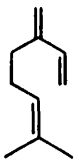
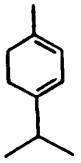
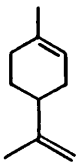
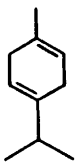
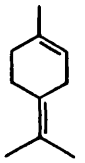
† Electron-impact (70eV.)

a ≡ All ion-abundances for ions at *m/z* 136 were normalised to 1 x 10^6

b ≡ Ion-abundance (%) relative to ion at *m/z* 136

2σ = ± 10%

Table 11.10 Abundances (%) of Daughter Ions Occurring in the Linked-scan (B/E) Spectra† of Ions at *m/z* 121

ION (*m/z*)	 I	 II	 III	 IV	 V	 VI	 VII	 VIII	 IX	 X	 XI
121[a]	100	100	100	100	100	100	100	100	100	100	100
119[b]	1.32	0.07	0.08	0.61	0.04	-	2.14	0.29	1.07	1.32	2.40
106[b]	0.19	-	-	0.02	-	-	0.46	0.03	0.10	0.20	0.48
105[b]	5.36	0.78	0.09	3.20	0.51	0.47	8.36	1.69	5.05	5.57	8.98
103[b]	-	-	-	-	-	-	-	-	-	-	0.02
93[b]	4.53	0.59	0.73	1.58	0.63	0.45	7.43	2.03	2.46	4.33	7.76
91[b]	0.04	-	-	-	-	-	0.42	0.06	0.02	0.17	0.35
80[b]	-	-	0.04	-	-	-	-	-	-	-	-
79[b]	-	-	-	-	-	-	0.03	-	-	-	0.04

I, Linaloyl Acetate; **II, Neryl Acetate; III, Geranyl Acetate;** IV, α-Pinene; V, β-Pinene; VI, Myrcene; VIII, α-Terpinene; VIII, Limonene; IX, *cis*-Ocimene; X, γ-Terpinene; XI, Terpinolene

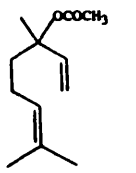
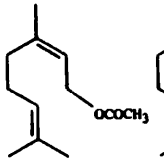
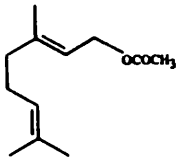
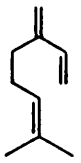
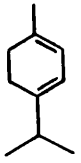
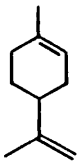
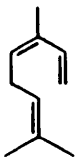
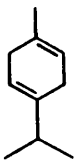
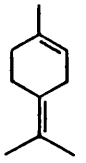
† Electron-impact (70eV.)

a ≡ All ion-abundances for ions at *m/z* 121 were normalised to 1 x 10^6

b ≡ Ion-abundance (%) relative to ion at *m/z* 121

$2\sigma = \pm 10\%$

Table 11.11 Abundances (%) of Daughter Ions Occurring in the Linked-scan (B/E) Spectra† of Ions at *m/z* 107

ION (*m/z*)	 I	 II	 III	 IV	 V	 VI	 VII	 VIII	 IX	 X	 XI
107[a]	100	100	100	100	100	100	100	100	100	100	100
105[b]	2.67	0.38	0.51	4.57	0.28	1.08	3.95	5.05	2.55	1.70	3.20
103[b]	-	-	-	-	-	-	0.06	-	-	-	-
93[b]	0.02	-	-	0.07	-	-	0.09	2.39	-	-	0.02
92[b]	-	-	-	-	-	-	0.03	-	-	-	-
91[b]	2.18	0.34	0.60	6.35	0.34	1.42	5.49	6.24	3.68	2.24	3.43
80[b]	-	-	-	-	-	-	0.05	0.02	-	-	-
79[b]	0.26	-	0.03	2.22	0.01	0.18	2.47	1.69	0.42	0.69	0.95
77[b]	0.04	-	-	0.46	-	-	0.62	0.28	0.03	0.01	0.13

I, Linaloyl Acetate; **II, Neryl Acetate; III, Geranyl Acetate;** IV, α-Pinene; V, β-Pinene; VI, Myrcene; VIII, α-Terpinene; VIII, Limonene; IX, *cis*-Ocimene; X, γ-Terpinene; XI, Terpinolene

† Electron-impact (70eV.)

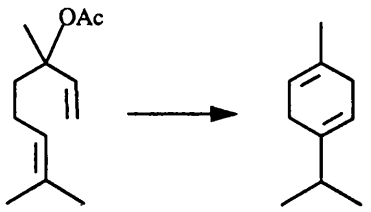
a ≡ All ion-abundances for ions at *m/z* 107 were normalised to 1 x 10^6

b ≡ Ion-abundance (%) relative to ion at *m/z* 107

$2\sigma = \pm 10\%$

the sequence *m/z* 136 → *m/z* 121 → *m/z* 107 → *m/z* 93 also occur. Clearly, linaloyl acetate formed a different population of ions at *m/z* 136 compared with geranyl and neryl acetates. Our results suggest that this population of ions was cyclic.

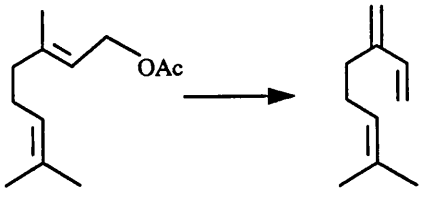
The results may be summarised as follows:
The molecular ion of γ-terpinene and the terpenoid fragment-ion in the spectrum of linaloyl acetate (both occurring at *m/z* 136) do fragment to a common set of ions even though the structures of the two parent ions are not the same. They must, however, be very similar because, the molecular ion of γ-terpinene (ii) can readily be formed by elimination of acetic acid from the molecular ion of linaloyl acetate (i). The linaloyl skeleton is the precursor to a wide variety of cyclic monoterpenoids in nature. It also rearranges to cyclic products in the presence of acid.



(i) (ii)

Note: This is consistent with the results of Section 11.2.1: if the charges are localised and the hydrogen on C_4 is transferred to the oxygen atom during elimination of acetic acid, the result is a terpenoid fragment that is equivalent to the molecular ion of γ-terpinene.

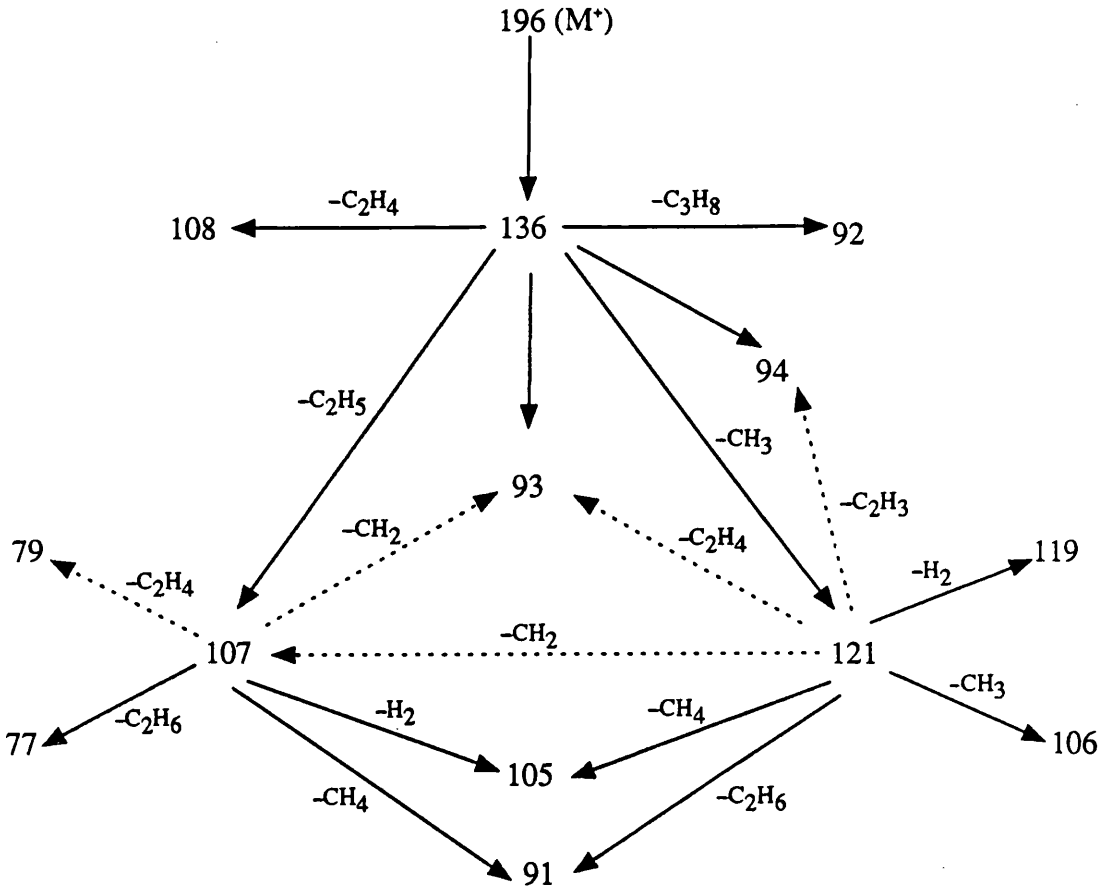
The molecular ion of myrcene and the terpenoid fragment-ions in the spectra of geranyl and neryl acetates (all occurring at *m/z* 136) do fragment to a common set of ions, even though the structures of the parents are not identical. Again, the latter must be related because the molecular ion of myrcene (iv) can be formed by elimination of acetic acid from either geranyl or neryl acetates (*eg.*, geranyl acetate; iii):



(iii) (iv)

Scheme 11.3 Proposed Fragmentation Pathways for the Molecular Ions of Linaloyl, Neryl, and Geranyl Acetates

The numbers in the following scheme are the *m/z*-values observed in the Mass Spectra of the three compounds:



-------- Indicates pathways observed only for the linaloyl- isomer

The two examples above show how measurements on the abundancies of metastable ions that fall at the same *m/z*-value can be used to propose the structures for such ions. However, although the abundancies may differ greatly the structures may differ only in the localisation of charge and radical-centre on the fragment-ion. This explains the virtually-identical spectra that were obtained when the isomeric C_{10}-esters were analysed under normal conditions of electron-impact.

11.4 Conclusion

This brief study has shown that the three monoterpenoid acetates were very difficult to distinguish by usual techniques of electron bombardment because the spectra were complex and almost identical. Under conditions of FAB/MS the spectrum of linalyol acetate was different from the spectra of the other two. It was interesting to note that the fragmentation of the acid-portion of each ester was greater when using this technique. Chemical ionisation also distinguished between the isomers: NCI/MS resulted in the appearance of $(M-H)^-$ ions as the base peaks for the primary acetates whereas a peak corresponding to $(M-69)^-$ was the base peak in the spectrum of linaloyl acetate. The differences may be attributable to the suppression of skeletal rearrangements (via carbocationic intermediates) that seem to characterise the EI-mass spectra of terpenoids.

We prepared and analysed nine deuteriated monoterpenoid esters and deduced that the hydrogen attached to C_4 was involved in the elimination of acetic acid from the molecular ion of each compound.

By using linked-scanning (B/E) it was shown that the terpenoid fragment-ions that were derived by elimination of acetic acid from the molecular ions of each ester behaved like the molecular ions of monoterpenoid hydrocarbons. By the analysis of the daughter-ions produced by fragmentation of chosen metastable-ions we concluded that the molecular ion of linaloyl acetate fragmented to an acyclic ion similar to that produced by the molecular ion of γ-terpinene whereas the molecular ions of geranyl and neryl acetates fragmented to an acyclic ion that was similar to the molecular ion of myrcene. Both proposed structures are isomeric and would occur at the same *m/z*-value, as the terpenoid fragment in the mass spectrum of the ester thus causing the normal EI-spectra of the esters to be hardly distinguishable.

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PART 5 Experimental Methods

Chapter 12 Chromatographic and Instrumental Methods

12.1 Chromatographic Methods

(a) Thin-Layer Chromatography (TLC)

System 1: (Analytical); Silica gel TLC plates (DC-Alufolien Kieselgel 60 plates; 20x20 cm; 200 μm; Merck, Dorset).

System 2: As above but using plates of thickness 1000 μm.

Solvent systems (AR-grade):

(i) Hexane: Ethyl Acetate (85:15)

(ii) Hexane: Ethyl Acetate (90:10)

(iii) Benzene: Ethyl Acetate (70:30)

(iv) Chloroform: Ethanol (99:1)

Spray reagents:

(i) Phosphomolybdic acid (5%) in ethanol. Spots were developed by heating the plate at 80 °C for 5 mins.

(ii) Vanillin (2%), conc. H_2SO_4 (2%) in ethanol. Spots were developed by heating the plate at 110 °C for 5 mins.

(b) Liquid Column Chromatography (LCC)

System 1: Silica gel (70-230 mesh; 60 Å) (column dimensions 60 mm x 360mm). Solvent system (i) above.

System 2: As above (but column dimensions 30 mm x 200 mm).

System 3: As above (but column dimensions 25 mm x 500 mm).

Note: System/solvent compositions referred to in text as TLC System 1(i) etc.

(c) High Performance Liquid Chromatography (HPLC)

All systems fitted with a guard column (50 x 4.6 mm)

System 1: (Normal-phase/analytical); *Column*: 250 mm x 4.6 mm; silica gel (Nucleosil; particle size 5 μm); *Pump*: Waters M6000 (fitted with a Rheodyne injector); *flow rate:* 1 cm^3 min^{-1}; *Detector*: refractive index *Mobile-phase*: hexane: ethyl acetate (90:10)

System 2: (Normal-phase/preparative); as above but using a column with dimensions 2 x(250 x 10mm) and *flow rate*: 5 cm^3 $min.^{-1}$

System 3: (Reverse-phase/analytical); *Column*: 250 mm x 4.6 mm (Spherisorb ODS2; particle size 5 μm) *Pump*: as above; *Detector*: as above; *Mobile-phase*: methanol:water (80:20)

System 4: (Reverse-phase/preparative); As for *system 3* but using column-dimensions and flow rate as shown for *system 2*.

System 5: (Reverse-phase/analytical); *Column*: as for *system 3*; Pump: Gilson High Pressure Binary Gradient, *flow rate*: 1 cm^3 $min.^{-1}$ *Detector*: LKB 2140 Rapid Spectral Detector *Mobile-phase*: acetonitrile:water:trifluoroacetic acid (80:20:0.01).

System 6: (Reverse-phase/preparative); As for system 4 but using column with dimensions and flow rate as shown for *system 2*.

(c) Gas Chromatography (GC)

System 1: (Analytical); Column: BP1 (20 m x 0.22 mm; film thickness 0.25 μm; Thames Chromatography, London); Mobile-phase: helium at a head pressure of 1 Bar; Chromatograph: Pye Series 204 Gas Chromatograph; *Temperatures:* detector (220 °C); injector (200 °C); oven (60-200 °C @ 4 °C min^{-1}).

System 2: (Preparative); As for system 1 but using Carbowax 20 M stationary phase (30 % on Supasorb 60-80 mesh; BDH, Poole, Dorset) packed into a copper column (20 m x 6 mm), and a helium head-pressure of 2 bar.

12.2 Instrumental Methods

(a) Mass Spectrometry

System 1: (GC/MS); *Mass Spectrometer*: VG 7070H (electron-beam 70 eV.; trap-current 200 μA; source-temperature 180 °C; source-pressure $1x10^{-6}$ mbar; accelerating potential (4kV); scan-conditions; 0.2s/decade with a 1.0s

interscan-delay; *Data System*: Finnigan Incos; *Gas Chromatograph*: Pye Series 204 Gas Chromatograph fitted with an OV-101 column (20m x 0.22mm with film thickness 0.25 μm; SGE Scientific) using a temperature programme of 60-220 °C @ 4 °C $min.^{-1}$ (injector-temperature; 200 °C) and head-pressure of 1 bar.

System 2: (GC/MS- peak-matching to terpenoid data-base; B.B.A., Walthamstow, London.); *Mass Spectrometer*: Kratos MS25; *Data System*: Kratos 65-505; *Gas Chromatograph*: Pye Series 204- fitted with an SE-30 column (20% chromosorb W 100-200; 3m x 0.80mm) using a temperature programme 60-160 °C @ 6 °C $min.^{-1}$

System 3: (EI/12 eV.); Mass Spectrometer: Modified AEI MS9 (source-temperature 70 °C and source-pressure 1 x 10^{-6} mbar; sample introduction via syringe (100 μl capacity).

System 4: (FAB); *Mass spectrometer*: as for system 1 but not interfaced to gas chromatograph; sample introduction via probe-inlet and dissolved in glycerol matrix; ionisation by bombardment with argon (2 bar).

System 5: (PCI and NCI); *Mass Spectrometer*: VG ZAB-SE (source-temperature 70 °C; source-pressure 210-4 bar); *Data System*: VG 11250; CI *Reagent Gas*: ammonia.

System 6: (B/E linked-scanning); as for system 1 but B/E ratio held constant and scans aquired over the appropriated mass-range.

(b) Nuclear Magnetic Resonance Spectroscopy (NMR)

Varian VXR 400 NMR spectrometer operating at 399.5 MHz. (^{1}H), 61.4 MHz. (^{2}H), 100.8 MHz. (^{13}C) 376.3 MHz. (^{19}F), or 161.9 MHz. (^{31}P).

(c) Infrared Spectroscopy (IR)

Samples were analysed as solutions in chloroform (2% w/v) on a Perkin Elmer 983 spectrophotometer.

(d) Centrifugation

Samples were centrifuged (4 °C; 0.01 atm) using an MSE Superspeed 65 Mk.2 ultracentrifuge, equipped with an angle-titanium rotor (10x10 cm^3) pre-cooled to 4°C. Centrifugation forces are given in Section 14.1.

(e) Microscopy and Photography

System 1: (Microscopy); *Instrument*: Wild M20 fitted with dual-illuminator for u.v. and blue-light fluorescence and mixed-light illumination; *Light Source*: halogen lamp (50W, 12V; Philips 7027 BRLA1/220); *Filters*: FITC blue-light fluorescence exciting filter (313810; Wild, Heerbrugg) and FITC barrier (313742; Wild) lent to us by Dr. D. Roscoe (Biology Dept. U.C.L.); *Magnification*: 2.5 x 20.

System 2: (Microscopy-photography); *Instrument*: Olympus IMT-2 Inverted Research Microscope lent to us by Dr. C.A. King (Biology Dept., U.C.L.) and fitted with a Hitachi video-monitor and an Olympus OM-2 camera (shutter priority; 1 x 10^{-3} sec.); *Light Source*: halogen lamp (50W, 12V); *Filters*: exciting filter (IF490) and barrier filter (0515+13460); *Magnification*: 2.5 x 20; *Film*: Kodak colour print film (100 A.S.A.). The photographs were colour-matched by CPL (London) to a transparency kindly lent by Dr. J.S. Hyams (Biology Dept. U.C.L.).

Chapter 13 Techniques for the Growth and Analysis of Tissue Cultures

13.1 Tissue Culture Media

All media were based on the Murashige and Skoog formula[300] with sucrose and hormone supplements listed in the appropriate sections below. The medium was purchased as a lyophilised powder (Flow Laboratories, Worcs., England) which was dissolved in distilled water (1 dm^3) to give a stock of basal medium. When diluted (to 1 dm^3) an aliquot (100cm^3) of this stock gave the correct concentration of essential nutrients according to the formulation. The typical procedure was as follows: the stock-medium (100 cm^3) was poured into a volumetric flask and diluted with distilled water (*ca.* 500 cm^3). The auxin and cytokinins were added as solutions from freshly-prepared stocks together with the required quantity of sucrose and the mixture stirred for 10 minutes. Distilled water was then added to bring to the final volume (1 dm^3). The pH of the medium was adjusted until a reading of pH 5.50 was obtained.

For the preparation of suspension cultures, the medium was divided between individual culture flasks (10 x 100 cm^3 of medium into 10 x 250 cm^3 flasks) which were plugged with non-absorbent cotton wool and capped with aluminium foil.

For the preparation of solid agar-medium agar gel (5g) was shaken with the medium and then aliquots (30 cm^3) of the suspension poured into individual culture flasks (33 x 50 cm^3).

All media were autoclaved (121 °C; 1.05 $kgcm^{-2}$; 20 mins.) and allowed to cool prior to use.

(a) ***Lavandula angustifolia. v. Mill***

Source: Dr. D.V. Banthorpe; University College, London

Initiation Media: Solid medium was prepared as above with the following supplements; (i) sucrose (30 $g.dm^{-3}$); (ii) 2,4-dichlorophenoxyacetic acid (2,4-D; auxin; 2 x 10^{-3} $g.dm^{-3}$); (iii) kinetin (cytokinin; 2 x 10^{-4} $g.dm^{-3}$).

Maintenance Media: Suspension and solid media were prepared as in (a) but using NAA in place of 2,4-D.

(b) ***Pelargonium fragrans L.***

Source: Dr. B.V. Charlwood, King's College, London

Initiation Media: Suspension and solid agar-media were prepared as in 13.1 with the following supplements; (i) sucrose (20 $g.dm^{-3}$); (ii) NAA (α-naphthaleneacetic acid; auxin; 1 x 10^{-3} $g.dm^{-3}$); (iii) BAP (Benzylaminopurine; cytokinin; 5 x 10^{-4} $g.dm^{-3}$).

Maintenance Media: Suspension and solid media were prepared as in (a) using BAP (5 x 10^{-3} $g.dm^{-3}$ *vs.* 5 x 10^{-4} $g.dm^{-3}$).

(c) ***Rosa damascena L. (v. trigentipetalla)***

Source: Dr. D.V. Banthorpe, University College, London

Initiation Media: Solid agar-media were prepared as in 13. 1 with the following supplements; (i) raffinose (2.0 g); (ii) 2,4-D (2 x 10^{-5} $g.dm^{-3}$); (iii) kinetin (1 x 10^{-3} $g.dm^{-3}$)

Maintenance Media: Suspensions and solid agar media were made up as in (a). In addition some cultures were transferred to the medium given in 13.1.

(d) Hormone Stock Solutions

2,4-D (2,4-*dichlorophenoxyacetic acid*): (2 x 10^{-2} g) dissolved in NaOH (0.2 $mol.dm^{-3}$; 1 cm^{-3}) and the volume adjusted (to 100cm^3) with distilled water.

NAA (naphthaleneacetic acid): as for 2,4-D

Kinetin: (2 x 10^{-2} g) dissolved in HCl (0.2 $mol.dm^{-3}$; 1 cm^3) and the volume adjusted (to 100 cm^3) with distilled water.

BAP (benzylaminopurine): (2 x 10^{-2} g) dissolved in boiling ethanol (1 cm^3) and the volume adjusted (to 100 cm^3) with distilled water.

13.2 Initiation of Explants and Subculture Techniques

(a) Initiation of Explants

There are numerous excellent laboratory manuals for initiation procedures.[98] Explants were typically surface-sterilised in sodium chlorate (VII) solution (5%) for 15 minutes and transferred to a sterilised laminar flow cabinet and washed several times with sterile distilled water. Leaf petioles and apical meristems were cut lengthwise and

the wounded edge placed on the solid initiation medium. No more than four explants were placed in one flask.

When sufficient callus-tissue had developed (2-3 weeks for lavender and pelargonium; 2-3 months for rose) it was cut from the explant and subcultured on to fresh initiation medium. In general 50% of all explants developed into callus tissue, although for explants of rose the success rate was typically 20%. When the callus had grown in bulk it was subcultured at regular intervals. After 2 passages on initiation medium, the culture could be transferred to the maintenance medium.

(b) Subculturing Techniques

Callus-cultures were subcultured by removing a small sample of callus (*ca.* 2g) from the old culture (typically 10g at the end of the passage).

Suspension cultures were routinely subcultured by gently agitating and pouring a volume (*ca.* 20%) of the old culture into the new flask. For the purposes of growth-curve-analysis and toxicity measurements, a standard volume of culture (30 cm^3) was subcultured by first transferrring the required volume to a pre-sterilised volumetric flask.

(c) Habituation of Suspension Cultures to a Sub-lethal Dose of Terpenoid

Subculturing was carried out (as above) into a fresh flask of medium containing the required dose of terpenoid that had been pre-sterilised by injection through a Millipore filter unit (see 13.4).

13.3 Estimation of Total Cell-numbers and Culture-viability

(a) A Combined Vital and Non-vital Staining Technique for Measurement of Culture-viability

A suspension culture was transferred to the laminar flow cabinet and opened. The flask was gently agitated while pouring a small aliquot (5 cm^3) of culture into a test tube. The contents of this were allowed to settle and then some of the liquid (*ca.* $3cm^3$) was removed and replaced by an equal volume of a fluid (Sigma, Poole, Dorset) that caused dissociation of the cells. A stock solution of fluorescein diacetate (FDA; Sigma, Poole, Dorset) was prepared ($3.3x10^{-2}$ g of FDA in AR-grade acetone

and distilled-water; 1:99 v/v) and an aliquot ($0.5cm^3$) of this solution was added to the sample of cells. Similarly, an aliquot ($0.5cm^3$) of a solution (1% in water) of Evan's Blue was added to the cells and the mixture was gently agitated for 5 minutes in order to dissociate the cells and allow uptake of the stains.
Note: the stock solution of FDA was kept on ice and replaced after two hours.

A plastic pipette which had been cut to a diameter of 4mm was used to remove a small aliquot of the cell-solution and to place this on a microscope slide (Flow Laboratories, Worcs., England) which had been divided into 12 circles (diameter *ca.* 8mm). A cover-slip was lowered onto the slide and the cells viewed on the microscopes described in 5.1.3. Dead cells were stained blue in white light, whereas living cells appeared fluorescent when observed through FITC-fluorescence filters.

Both types of cell were counted (using a hand-held counter), ensuring that at least 200 cells were counted in each chamber in accordance with the counting statistics in the following section.

(b) Statistical Methods used for Estimating Cell Viabilty

We are grateful for the advice of Mr. H. Germes from the Department of Statistics at University College, London for his help in developing the methods used to analyse our data. The proofs of the equations used here are given in standard textbooks on statistics.[301]

Each sample of cells that was removed from culture was placed on a microscope slide that was divided into 12 counting wells.

The number of live cells that can be counted is a discrete random variable (*vs.* continuous random variable). Because cells can either be alive or dead the probability distribution for each type of cell will follow the binomial distribution. The Central Limit Theorem states that for large sample sizes the binomial distribution will approximate to a normal distribution. We have used this to make statistical inferences on the numbers of live and dead cells in our tissue cultures (*ie.*, the populations) from the results of our sampling. The following equations show how this was done:

Mean number of living cells (X) in each sample:

$$X = 1/n \, \Sigma \, x$$

n ≡ the number of experiments (12 in each sample taken from the culture)

x ≡ the number of live cells in an experiment

Estimate of true mean number (μ) of living cells in culture:

$$\mu = X \pm t_{\upsilon} s n^{0.5}$$

t_{υ} ≡ Student's t statistic for a given confidence interval with υ degrees of freedom

υ ≡ (n-1)

s ≡ sample variance:

$$s = (1/n-1)[\, \Sigma \, x^2 - \frac{(\Sigma \, x)^2}{n}]$$

Proportion (p_x) of living cells in each sample:

$$p_x = (\Sigma \, x)/(\Sigma x + y)$$

x ≡ the number of live cells in an experiment

y ≡ the number of dead cells in an experiment

Estimate of the proportion (P_x) of living cells in the culture:

$$P_x = p_x \pm z \, \{[p_x(1- p_x)]/N\}^{0.5}$$

N ≡ the total number of cells in the sample removed from the culture

Z ≡ the standard normal random variable with a value that is dependent on the confidence interval that is chosen

Note: we have chosen to estimate means and proportions with 99% confidence in all our work.

(c) Estimation of Total Cell-number using a Haemocytometer

A solution of dissociated cells that had been prepared and stained as in (a) was transferred to the filling chamber of the haemocytometer (an improved Neubauer type; Weber Scientific International, London) which had been fitted with a cover-slip, by allowing each chamber to fill by capillary-action. Counts of living and dead cells were made for each of the squares in the haemocytometer.

$$\text{Cells.cm}^{-3} \equiv C_m.d \times 10^4$$

$$\text{Total no. Cells} \equiv C_m.d.a.x\ 10^4$$

C_m ≡ mean count of cell type

d ≡ dilution factor

a ≡ volume removed from original culture

(d) Estimation of the Increase in Total Cell-number by Measurement of Fresh- and Dry-mass

Note: fresh- and dry-masses are commonly referred to as fresh- and dry-weights

Three flasks of suspension culture were monitored for each interval (2 days) in the growth-cycle. Seven measurements were made over a 14 day growth-cycle, and thus 21 flasks were used for each measurement.

The tissue from the three flasks was combined and placed on dampened filter paper (of known dry-mass) and dried under suction on a Buchner funnel for 5 minutes until no further water could be extracted. The filter paper was carefully weighed, and then dried (100 °C) to constant mass.

For some experiments fresh- and dry-masses were recorded only at day 0 and day 14 (for a 14 day growth-cycle) and then an estimate of average growth-rate over that period was determined as follows:

$$g\ day^{-1} \equiv (M_{14}-M_0)/14$$

M_0 ≡ mass on day 0.

M_{14} ≡ mass on day 14.

(e) Measurement of Total Weight of Culture

Individual culture flasks under study were weighed at intervals of two days over the growth-cycle.

(f) Estimation of Increase in Total Cell-numbers by Measurement of Packed-cell Volume

Measurements were taken at intervals of two days over a 14 day growth-cycle. One culture was used for each measurement. The cells were transferred to a graduated tube and centrifuged in a bench-top centrifuge for 5 minutes at 2000 rev. min^{-1}. The volume of the cell-pellet remaining in the tube was then recorded.

(g) Measurement of the pH of a Culture Throughout the Growth-cycle

Measurements were recorded at intervals of 2 days throughout the 14 day growth-cycle from a single suspension culture by removing a small aliquot (5 cm^3) of the culture under sterile conditions. The aliquot was then poured into a deep sample tube and a hydrogen electrode (calibrated at pH 4 and at pH 7). used to measure the pH (to ± 0.01 unit).

13.4 Purification of Terpenoids and Administration to Suspension Cultures

(a) Removal of Peroxides

A small quantity (1g) of a terpenoid was vigorously shaken with saturated sodium sulphite solution, dried and recovered by solvent extraction. After distillation at reduced pressure (*ca.* 0.1mm Hg) the terpenoids were stored at -18 °C under argon in sealed ampoules.

The solid terpenoids (the camphor-class) were recrystallised from a mixture of ethanol and water (50:50).

(b) Administration to Suspension Cultures

Method 1: The required quantity of terpenoid (*eg.*, 10-100 µl) was added to the suspension culture as a sterile solution (10%) in freshly redistilled methanol. A Millipore filter-unit (stainless steel, 13mm diameter, Sartorius, Beds., England) containing a cellulose acetate filter (pore size 1.2 µm) was used for this purpose. A glass syringe (1 cm^3) was filled with the terpenoid solution and connected to the filter-unit, and then a sterile hypodermic needle fitted to the other end of the unit. The void-volume of the filter was filled with the solution by depressing the syringe plunger until the sterilised solution emerged from the syringe. The required dose of

terpenoid was then administered to the culture and the flask sealed and returned to the incubation cabinet.

Method 2: Sodium dodecylsulphate (SDS; 1.0g) was autoclaved in a foil wrapper and then added to a suspension which was gently agitated to dissolve the surfactant. The required dose of terpenoid was administered as a solution in hot dimethoxyethane (DME; 2 cm^3) via the Millipore filter as described in method 1.

13.5 Surfactants

(a) Administration of Surfactant-solutions to Suspension Cultures

Surfactants were prepared as stock solutions in distilled water and administered to the suspension culture via the Millipore filter by the technique described in 13.4. The final concentration of surfactant in the suspension medium was adjusted so as to be greater than the reported c.m.c. value shown in Table 5.1 (Results and Discussion, Chapter Five).

(b) Measurement of the Critical Micellar Concentration of SDS

A sub-critical micellar concentration of SDS was prepared in distilled water (11.2641g in 5 dm^3) and the conductance of this solution was measured after the conductance meter had been zeroed at 1 x 10^{-4} S and whilst the solution had been stirred magnetically. Further aliquots of SDS (*ca.* 5 x 10^{-2}g ± 0.001g) were added and the conductance of the solution noted once a steady reading had been achieved. This process was continued until the c.m.c. of SDS had been exceeded (*ca.* 0.50g). The conductance readings were loaded onto a computer and plotted against concentration of SDS using a least-squares programme.

13.6 Extraction Procedures for Callus and Suspension Cultures

(a) Analysis

A small glass sintered-filter (25 cm^3) was filled with activated charcoal (200 mesh; 14g) and wrapped in aluminium foil and autoclaved using the conditions in section 13.1. A flask of suspension culture-medium was opened and a mixture of limonene, linaloyl acetate and caryophyllene (1 cm^3; 1:1:1) was added via the Millipore filter unit described in 13.4b. The flask was taken to a laminar flow cabinet and the

sintered-filter placed in the neck of the flask and sealed into place using polythene film. The sintered filter containing charcoal was covered with a foil cap and the flask incubated at 27 °C for 12 weeks. After this the charcoal was washed with diethyl ether (5 x 100 cm^3), the solvent was removed on a rotary evaporator and the oil (0.41g) was analysed by mass spectrometry (system 2, section 12.2). The culture medium was extracted similarly and the oil analysed by mass spectrometry.

(b) Solvent Extraction of Plant Material

Method 1: Plant-tissue was frozen in liquid nitrogen and ground to a fine powder under redistilled-hexane or diethyl ether containing phytol (2% v/v) as marker. The mixture was then agitated on a laboratory shaker overnight and filtered. The solvent was removed and the oil was eluted through a column of aluminium oxide using hexane: ethyl acetate (70:30). The fractions were combined, dried with potassium carbonate and reduced to a small volume (50 µl) for analysis.

Method 2: A small volume (2 cm^3) of a crude solvent extract was poured into a pear-shaped flask (5 cm^3) containing distilled water (1 cm^3) or a similar quantity of the phosphate buffer used in 14.2. The flask was clamped to one arm of a micro-distillation apparatus and extracted with hexane by the standard method.[302] After 2 hours the organic layer was recovered and dried and the solvent was removed to yield the mixture of oils for analysis.

Chapter 14 The Preparation of Cell-free Extracts of *Lavandula angustifolia* (Lavender)

14.1 General Procedure for the Preparation of Cell-free Extracts

Callus material (200g) was frozen under liquid-nitrogen and was then ground-up under a phosphate extraction buffer (150 cm^3; 14.2) using a pestle and mortar that had been cooled to 4 °C. Batches of polyvinylpyrrolidine beads (200g; Polyclar-AT; Sigma Chemical Co., Poole, Dorset) and Amberlite XAD-4 resin (100g; BDH Chemical Co., Poole, Dorset) were added during the extraction procedure to remove phenolics and lipophilic substances (*eg.*, terpenoids) respectively from the enzyme extract. The XAD-4 resin had been previously washed in acetone followed by dilute acid afterwhich it was stored at neutral pH in distilled water at 4 °C.

The extract was filtered through four layers of cheese-cloth and centrifuged for 30 mins. at 4 °C (see 12.2.d). Previously reported centrifugation forces[43] (27000g) were found to lower the activity of the supernatant. The protein content of the extract was determined by the method of Bradford[303] (see 14.3). An aliquot (1 cm^3) of the extract was poured into a silanized glass test tube (see 14.4) in a water bath at 27 °C. Solutions of labelled isopentenyl pyrophosphate (1-^{14}C-IPP; 0.375 mCi; Amersham Int., Amersham, Bucks.) and exogenous cofactor/additive (see 14.2) were administered to the portions of extract and incubated for 3 hours at 27 °C under hexane (1 cm^3) to facilitate continuous removal of terpenoids that were formed. For the large-scale extract (1 dm^3) unlabelled IPP (4.28 mg) was used as substrate, to give a final concentration of 1 x 10^{-5} $mol.dm^{-3}$. All incubations were carried out in duplicate and a boiled enzyme control was included in each set. Care was taken to ensure consistency in the extraction procedure by starting the incubation of the extract within 30 minutes of macerating the callus-tissue. After incubation of the extract, the solution was quenched with a solution of NaOH (10 µl; 10 $mol.dm^{-3}$) and the hexane-layer was removed and the aqueous portion shaken with a further 3 volumes (3 x 5 cm^3) of hexane which were combined with the first. The aqueous residue was then adjusted to pH 9.0 by the addition of alkaline buffer (Na_2CO_3/$NaHCO_3$; 0.1 mol. dm^{-3}; 9:1 v/v; 1cm^3) and was reincubated with alkaline phosphatase (5 mg; Sigma) and apyrase (5 mg; Sigma) for 50 minutes at 27 °C to cleave products (alcohols) that had been formed as phosphate and pyrophosphate esters. The mixture was then

extracted with hexane (3 x 5 cm^3) and the organic layers combined with the previous washings, dried with potassium carbonate and reduced to a small volume (100 µl). The extract was analysed by TLC and autoradiography as described in section 14.5.

14.2 Extraction and Incubation Buffers

(a) Extraction Buffer

Phosphate buffer (Na_2HPO_4/NaH_2PO_4; 0.2 $mol.dm^{-3}$; 1:1 v/v) at pH 7.0 and containing sucrose (0.25 $mol.dm^{-3}$), sodium metabisulphite (0.005 $mol.dm^{-3}$), ascorbic acid (0.005 $mol.dm^{-3}$) and dithioerythritol (0.001 $mol.dm^{-3}$).

(b) Incubation Buffer

As for the extraction buffer but with the following additions; $MgCl_2.6H_2O$ (0.002 $mol.dm^{-3}$). Usually the extraction buffer is replaced with an incubation buffer by gel-filtration of the extract through a column of Biogel P2 (Bio-rad, England). However, we found that this procedure diluted the protein concentration by as much four-fold, resulting in lower incorporation of the tracer. Consequently we decided to use the incubation buffer for the purposes of extraction and incubation so that the concentration of protein was consistent for all experiments.

(c) Stock Solutions of Cofactors and the Plant Growth Regulator

Aliquots from solutions of the following additives (*ex.* Sigma) were added to the cell-free extracts (1 cm^3) to give the final concentration shown in 2.2.4. All cofactors were incubated with the extracts for 30 minutes prior to addition of the labelled substrate.

14.3 Determination of Protein Concentration in a Cell-free Extract

The method followed the procedure of Bradford *et. al.*[304] Various concentrations of bovine serum albumin (BSA; *ex.* Sigma; 0.1-2.0 $g.cm^{-3}$) in double-distilled water were prepared and aliquots (2 cm^3) of these solutions placed in plastic cuvette-cells. A small volume (100 µl) of a solution (1 $mg.cm^{-3}$) of Coomassie Blue-G (*ex.* Sigma) in distilled water, was added to each solution and the mixture agitated. The absorbance (at 490 nm) of each solution was measured and a calibration curve of

absorbance versus protein concentration was constructed from which the concentration of protein in any cell-free extract could be determined.

14.4 Silanization of Glassware Used in The Preparation and Incubation of Cell-free Extracts

Clean glassware was rinsed with 1,1,1-trichloroethylsilane and dried in an oven at 80 °C for 10 minutes. The apparatus was then rinsed with distilled water and again dried prior to use. This treatment is known to prevent adsorption and subsequent denaturation of enzymes by the acidic siloxy-moieties present in the glass. Our apparatus was resilanized before the preparation of each cell-free extract.

14.5 Analysis of Products Incorporating the Tracer

(a) Autoradiography

A polythene plate was cut to the size of a standard silica gel plate used for TLC (20 cm x 20 cm) and a grid drawn on it (with each division having approx. dimensions 10mm x 5mm). Aliquots (10 µl) of each standard terpenoid (1% in hexane) were added to the baseline of TLC plates which were then eluted in the three solvent systems shown in section 12.1. (plates were eluted at a temperature of 4 °C in order to standardise conditions and ensure reproducibility in measured R_f values for all experiments. Some of the TLC plates were run in duplicate and the ^{14}C-labelled compounds on the first plate were visualised by autoradiography. A typical procedure for the latter involved storing the TLC plate under photographic film in a darkened box at 30 °C for 3 months. The box was opened in a dark-room and the film developed and washed in water and fixed.

(b) Liquid Scintillation Counting (LSC)

The products (and some endogenous-unlabelled compounds from the tissue culture) on the second TLC plate were visualised using a developer (12.1.i). This treatment probably converted volatile (radioactive) products to involatile derivatives. Using the polythene grid, the plate was divided into small squares (10 mm x 5mm) and the silica from each square dampened with hexane and scraped into a scintillation-vial

containing an organic scintillant (10 cm^3; Optiscint-O; LKB, Croydon). The vials were placed in a liquid scintillation counter). The measured counts per minute (cpm) were corrected to disintegration per minute (dpm) by use of a quench-correction curve. The latter was constructed from measured cpm (from a sample of known dpm) versus the S-value (calculated by the spectrometer to estimate the magnitude of quenching by the scintillant).[304]:

The disintegration of radioactive nuclei is a random process and the probability of disintegration varies according to a Poisson distribution. For a large sample size this distribution can be approximated to the normal distribution.

For a sample for which n disintegrations had been accumulated the standard error (σ) is as follows:

$$\sigma = n^{0.5}$$

Therefore, in order to satisfy the following:

$$2\sigma = \pm 10\%$$

the value of n would have to be 40 000.

Chapter 15 Syntheses of Modified Terpenoids

Notes:

(i) Formats for presentation of ^1H- and ^{13}C-NMR data are as follows; $X(^1H_n)$ and $X(^{13}C_n)$ where X is the measured chemical shift (δ/ppm. from TMS) and n is the numbered carbon (according to I.U.P.A.C.) to which a proton may be attached. Magnetically non-equivalent protons attached to the same carbon are designated appropriate subscripts eg. H_{5a}. ^{19}F-NMR were recorded relative to $CFCl_3$ as external standard.

(ii) All samples in this section were analysed by mass spectrometry (12.2; system 1).

(iii) Purification and drying procedures for starting materials and reaction solvents followed standard methods.[305]

(iv) All reagents and starting materials were obtained from Aldrich Chemical Co. Poole, Dorset, unless stated otherwise.

15.1 Syntheses of Fluorinated Monoterpenoids

(a) Preparation of 3,7-Dimethyl-1,6-octadien-3-ol (Linalool) from 6-Methyl-hept-5-en-2-one. The method followed the industrial procedure developed by Normant.[306] Vinylmagnesium bromide (10 mmol; 1.0 mol.dm^{-3} in THF) was transferred by means of a syringe rinsed in freshly redistilled THF to a flame-dried round-bottom flask (rbf; 25cm^3) containing a magnetic stirrer and kept under a slow stream of argon. The flask was cooled to 0 °C in ice and a solution of 1 (8 mmol; 1.0g) in THF (5 cm^3) was added dropwise with rapid stirring. The mixture was kept at 0 °C for 2 hours and then allowed to warm to room temperature for a further 10 hours. The contents of the flask were then carefully poured into saturated ammonium chloride solution (100 cm^3) and extracted with diethyl ether (3 x 25 cm^3). The organic layer was dried with potassium carbonate and the solvent removed on a rotary evaporator to yield a straw-coloured oil. This was purified by column chromatography (System 1.i) to yield the product (0.98g; 80 %) which gave a single-spot when analysed by TLC (system 1.i).

Analysis: **^{1}H-NMR** (400 MHz.); 5.85 (H_2; q); 5.14 (H_{1b}; d); 5.05 (H_6; t); 4.98 (H_{1a}; d); 1.95 (H_5; m); 1.61 (H_{10}; s); 1.53(H_8; s); 1.49 (H_4; m); 1.20 (H_9; s); **^{13}C-NMR** (100 MHz.); 144.93 (C_2); 131.50 (C_7); 124.40 (C_6); 111.52 (C_1); 73.26 (C_3); 41.93

(C_4); 27.60 (C_5); 26.00 (C_9); 22.65 (C_8); 17.52 (C_{10}); **MS**; (*m/z*) 154 (3%); 139 (10%); 136 (42%); 121 (80%); 107 (25%); 93 (90%); 71 (100%); 43 (90%).

(b) The Preparation of 4-Fluoro-3,7-dimethyl-1,6-octadien-3-ol (4-Fluoro-linalool; 12). The most satisfactory method to circumvent substitution of the halogen by the Grignard reagent involved addition of a solution of the fluoro-ketone (3.07 mmol; 0.50g) in THF (2.5 cm^3) to vinylmagnesium bromide (3.0 mmol of the solution used in (a)) kept at -25 °C with rapid stirring. The mixture was allowed to warm to room temperature and after 1 hour the contents of the flask were poured into a mixture of saturated aqueous ammonium chloride and methanol. This mixture was washed with diethyl ether (3 x 25 cm^3) and the organic layer separated and dried. The solvent was removed to yield a straw-coloured oil (0.10g; 20%) with a woody odour. The product was isolated by preparative HPLC (System 2).

Analysis: **^{1}H-NMR** (400 MHz.); 5.90 (H_2; q); 5.35 (H_{1b}; d); 5.20 (H_6; m); 5.18 (H_{1a}; d); 4.25 (H_4; m; $^2J_{HF}$ = 47.80 Hz.); 2.30 (H_5; m); 1.70 (H_{10}; s); 1.58 (H_8; s); **^{13}C-NMR** (100 MHz.); 140.02 (C_2); 134.50 (C_7); 119.36 (C_6); 114.44 (C_1); 99.64 (C_4) $^1J_{CF}$(177.7 Hz.); 74.69 (C_3) $^2J_{CF}$(21 Hz.); 28.61 (C_5); 25.77 (C_9); 14.13 (C_8); 11.44 (C_{10}); **^{19}F-NMR** (376 MHz.) -33.80 (m); **MS** *(m/z)* 144 (M^+; 2 %); 129 (8 %); 124 (55 %); 109 (100 %); 81 (6 2%); 69 (78 %). *Acc. mass* (EI) 172.1273 ± 5 ppm (calc. 172.1264); *Acc. mass* (CI) 173.1341 ± 2ppm (calc. 173.1342)

(c) The Preparation of 9-Fluoro-3,7-dimethyl-1,6-octadien-3-ol (9-Fluoro linalool;13) The product was prepared as in (b) and was purified by HPLC (System 2) but there was insufficient quantity for analysis by NMR

Analysis: **MS**; *(m/z)* 172 (M^+; 3%); 154 (39 %); 152 (7 %); 139 (90 %); 121(90%); 111 (90 %); 89 (90 %); 71 (80 %); 69 (100%)

(d) The Preparation of 3-Fluoro-6-methyl-hept-5-en-2-one (5)

N-Fluoropyridinium triflate (NFPT; 2.0g; 8.1 mmol) was transferred to a round-bottom flask (50 cm^3) containing a magnetic stirrer bar and fitted with a drying tube and a serum cap. This flask was placed in a desiccator and the contents dried at low pressure (0.1 mm Hg) for 24 hours. Dry CH_2Cl_2 (20 cm^3) was added followed by a

(1.603; 8.1mmol)
solution of the TMS-enol ethers (10,11) in CH_2Cl_2 (15 cm^3). The mixture was stirred at reflux-temperature for 24 hours during which it changed from colourless to brown. The mixture was filtered to remove any solid residue and the solvent removed on a rotary evaporator. The product was taken up in hexane (25 cm^3), filtered, and the solvent removed. This process was repeated until no further solid could be extracted. The filtrate was reduced (to 1 cm^3) and prefractionated by column chromatography (System 2.i). The fraction containing the product was separated by preparative HPLC (System 2) to yield 5 (300 mg; 26 %).

Analysis: **^{1}H-NMR**; (400 MHz.); 5.13 (H_5; m); 4.71 (H_3; m); 2.51 (H_4; dt; $^2J_{HF}$ = 19.43 Hz.); 2.22 (H_1; d) 1.60 (H_7; s); 1.70 (H_8; d); **^{13}C-NMR**; (100 MHz.) 136.33 (C_6); 116.48 (C_5; $^3J_{CF}$ = 3.50 Hz.); 95.62 (C_3; $^1J_{CF}$ = 185.90 Hz.); 30.76 (C_4; $^2J_{CF}$ = 50.04 Hz.); 25.80 (C_7); 26.22 (C_1); 17.88 (C_8); **MS**; *(m/z)* 144 (M^+;2 %); 129 (8 %); 124 (55 %); 109 (100 %); 81 (70 %); 69 (85 %); **I.R.** υ (C=O) 1675 cm^{-1}; υ (C-F) 1049 $cm.^{-1}$

(e) The Preparation of 1-Fluoro-6-methyl-hept-5-en-2-one (8)

Tetrabutylammonium fluoride (TBAF), (2.05g; 7.9 mmol.) was placed in a round-bottom flask (50 cm^3) containing a magnetic stirrer bar. An inlet equipped with a high-vacuum tap and a serum cap were connected to the flask such that the latter could be evacuated and then the bromide added from a syringe, the needle of which was inserted through the vacuum tap. The flask was evacuated (to 0.01 mm Hg) for 48 hours at 40 °C, the vacuum was isolated and argon (50 cm^3) was released into the flask via the tap, using a syringe. The bromoketone (7; 0.54g; 2.65 mmol.) was then added and the mixture stirred for 20 minutes at 40 °C. When the reaction was completed, with the formation of solid tetrabutylammonium bromide, the contents of the flask was re-extracted with methanol (3 x 25 cm^3) and diluted with saturated sodium chloride solution (50 cm^3) at 0 °C. The mixture was shaken with n-hexane (100 cm^3) and the organic layer was recovered and washed a further two times with the sodium chloride solution. The aqueous layer was then recovered and extracted with hexane (3 x 25 cm^3) and the organic fractions combined and dried over anhydrous potassium carbonate. The products were recovered by rotary evaporation and prefractionated by column chromatography (System 2.i), (Yield: 113 mg; 10 %).

Analysis: **^{1}H-NMR** (400 MHz.); 5.08 (H_5; m); 4.79 (H_1; d; $^2J_{HF}$ = 47.76 Hz.); 2.57 (H_3; m; $^4J_{HF}$ = 2.75 Hz.); 2.30 (H_4; dd); 1.68 (H_7; d); 1.62 (H_8; s); **^{13}C-NMR** (100 MHz.); 133.37 (C_6); 122.10 (C_5); 85.02 (C_1; $^1J_{CF}$=184.90); 38.44 (C_3; 38.44); 31.59 (C_7); 22.66 (C_4) ; 14.12 (C_8); **MS**; *(m/z)* 144 (M^+;2%); 111 (10%); 93 (10%); 69 (60%); 41 (100%).

(f) The Preparation of 3-Bromo-6-methyl-hept-5-en-2-one (4) *Method 1:* Lithium wire containing 1 % sodium (0.84g; 120 mmol.) was cut into small pieces and washed with n-hexane followed by a final rinse in methanol. The wire was transferred to a flame-dried round-bottom flask equipped with dropping funnel, low-temperature thermometer and a magnetic stirrer bar and swept with dry argon. Freshly-redistilled tetrahydrofuran (THF; 25 cm^3) was added and the flask was clamped in an ultrasonic bath and 20 drops of a solution of n-butyl bromide (8.22g; 120 mmol.) was added slowly over 5 minutes. The reaction mixture was then cooled (to -10 °C) in a methanol-dry ice bath (kept at -30 °C) when the solution became slightly cloudy after 15 minutes with the formation of bright spots on the lithium. Sequential sonication-cooling-sonication was required to initiate the reaction. The remainder of the n-butyl bromide was then added at a constant rate over an hour with stirring while the temperature was maintained at -10 °C. The pressure of argon was then increased and the dropping funnel replaced with a custom-made side-arm containing a glass-wool plug and connected to a second flame-dried round-bottom flask equipped with a dropping funnel. The reaction mixture was then transferred to the second flask by means of argon pressure and was filtered from unreacted lithium by the glass wool plug. An aliquot (1 cm^3) of this butyllithium solution was then removed using a syringe (rinsed in THF and flushed with argon) and assayed by titration against standard aqueous hydrochloric acid (0.122 $mol.dm^{-3}$). A pH meter was found more useful to locate the end-point than colour-indicators (concentration of butyl lithium in THF was found to be 0.008gcm^{-3}; total 4.4 mmol.). A solution of diethylamine (10 drops; 0.32g/4.4 mmol.) in THF (6 cm^3) were then added to the butyllithium at room temperature. The contents of the flask were then cooled to 0 °C and the remainder of the diethylamine added after a further hour. The solution of lithium diethylamide was then cooled (to -25 °C) and the argon pressure increased sufficiently to prevent leakage of air into the flask caused by rapid cooling. A solution of the ketone (1; 0.63g; 5mmol; 1.1 mol.equiv.) in THF (5 cm^3) was then

added dropwise over 15 minutes followed by the addition a solution (2 cm^3) of NBS (0.89g; 5mmol.) in THF (10 cm^3). The contents of the flask were then cooled (to -25 °C) and the remainder of the N-bromosuccinimide was added over 30 minutes. During the addition the mixture turned deep yellow and a white precipitate of succinimide formed. After a further 3 hours the reaction mixture was allowed to warm to room temperature and the precipitate was removed by filtration. The solvent was then removed and the products dissolved in n-hexane (50 cm^3). The latter solution was then filtered to remove any insoluble NBS and the solvent removed to yield a crude mixture of products. (yield; 40 % by weight).

Method 2: The equilibrated TMS-enol ethers (9,10; 1.60g; 8 mmol.) were syringed into a 50 cm^3 round-bottom flask that has been fitted with a stirrer bar and wrapped in aluminium foil at 4 °C. NBS (1.60g; 8 mmol.) was then added in aliquots (0.1 g) to the stirred mixture. After a further two hours the white precipitate of succinimide was removed by the addition of hexane (3x25 cm^3) followed by filtration. The yellow oil was purified by column chromatography (System 1.i) to yield 4 (70% by weight).

Analysis: **MS**; *(m/z)* 205:207 [$(M+1)^+$ (25%)]; 147:149 (5%); 134:136 (30%); 125 (60%); 121:123 (2%): 43 (100%).

(g) The Preparation of 1-Bromo-6-methyl-hept-5- en-2-one (7)

Method 1: A solution of butyllithium (4.4 mmol.) was prepared as in (f), to which was added a solution of redistilled diisopropylamine (4.4 mmol.) in THF (25 cm^3) and the temperature was maintained at 0 °C for a further hour. A solution of (1; 0.44g; 3.5 mmol; 0 .8 mol equiv.) in THF (5 cm^3) was then added to the of LDA solution which was cooled to -78 °C over the course of 15 minutes and allowed to warm-up to 0 °C, followed by re-cooling to -78 °C. A solution of NBS (0.62g; 3.5 mmol.) in freshly redistilled THF (10 cm^3) was then added to the reaction mixture at -25 °C. The work-up followed the method in (f). (Yield; 0.23g; 32 %).

Method 2: Lithium wire (1.0g; 120 mmol.) was cut and cleaned as in (f) and added to freshly-redistilled THF (25 cm^3) in a flame-dried round-bottom flask fitted with an argon-bleed and a dropping funnel. The flask was clamped in an ultrasonic bath and

a solution of triphenylmethyl chloride (3.50g; 0.012 mmol.) in THF (10 cm^3) was added rapidly. When a red solution of triphenylmethyllithium (trityl lithium) began to appear, the flask was removed from the ice bath and its contents stirred for 4 hours at room temperature afterwhich unreacted lithium was removed as in (f) by filtration into the second reaction flask. The reagent was cooled (to -78 °C) and a solution of 1 (0.63g; 5 mmol.) in THF (5 cm^3) was added over 20 minutes ensuring that the red colour of the lithium-base was not discharged. After a further 10 minutes a solution of NBS (0.89g; 5 mmol.) in THF (10 cm^3) was added over the course of 30 minutes followed by warming of the flask (to 0 °C). The triphenylmethane was removed by column chromatography.

Method 3: The method followed that in (f) method 2, using 11 in place of 9 and 10 to yield 7 (1.28g; 78 %).

Analysis: **^{1}H-NMR** (400 MHz.); 5.06 (H_5; t); 3.85 (H_1; s); 2.63 (H_3; t); 2.25 (H_4; dt); 1.63 (H_8; d); 1.57 (H_7; s); **^{13}C-NMR** (100 MHz.); 201.75 (C_2); 133.30 (C_6); 121.96 (C_5); 39.84 (C_1); 34.42 (C_3); 25.59 (C_7); 22.54 (C_4); 17.59 (C_8); **MS**; *(m/z)* 204:206 (M^+; 10%); 134:136 (2%); 125 (90 %); 121:123 (20%).

(h) Preparation of the Kinetically-controlled Trimethylsilyl-enol Ether (11) of 6-Methyl-hept-5-en-2-one (1) using Ethyltrimethylsilyl-acetate (ETMSA).

Tetrabutylammonium fluoride (TBAF), (0.30g; 0.95 mmol.) was placed in a 3-necked flask equipped with a calcium chloride-drying tube, a serum cap and a thermometer. The flask was placed in a desiccator and evacuated (to *ca.* 0.01 mm Hg.) for 24 hours . By means of a syringe rinsed in freshly-redistilled THF, ETMSA (7.05g; 44 mmol.) was added to the TBAF and the mixture stirred for 10 minutes at -25 °C. The ketone (1; 5.0g; 39 mmol.) was then added to the mixture over the course of 5 minutes (to avoid possible equilibration of the kinetic-enol ether with excess of ketone). After 10 minutes the mixture was allowed to warm to room temperature and became bright orange in colour. The contents of the flask were then diluted with pentane (100 cm^3). The solid TBAF was removed by shaking the mixture with cold saturated bicarbonate solution (5 x 50 cm^3); the organic layer was recovered, dried, and the solvent removed on a rotary evaporator. The products, which were almost pure 11, were eluted through a column of silica gel (System 1) to remove traces of

the catalyst (Note: n-hexane:ethyl acetate 70:30 v/v was used to prevent decomposition of the product on the column). The silyl-enol ether was recovered (yield; 7.10g; 92 %) and GC/MS was used to determine the nature and proportions of kinetic and equilibrium TMS-enol ethers (90:10) by examination of the RIC (Reconstructed Ion-current from the GC-trace) and the mass spectra. The isomers could be separated by preparative HPLC (*System 1*; n-hexane: ethyl acetate 99:1 v/v) (elution order 9,10,11).

Analysis: **^{1}H-NMR** (400 MHz.); 5.13 (H_5; m); 4.06 (H_{1E}; m); 4.05 (H_{1Z}; m); 2.05 (H_4; m); 1.69 (H_8; 1.69; d); 1.62 (H_7; s); 0.21 (OTMS; s); **^{13}C-NMR** (100 MHz.) 146.35 (C_2); 131.56 (C_6); 123.86 (C_5); 89.89 (C_1); 36.67 (C_3); 25.63 (C_7); 25.57 (C_4); 17.62 (C_8); 0.07 (OTMS): **MS**; *(m/z)* 198 (M^+) (8%); 183 (22 %); 155 (30%); 130 (55%); 115 (57%); 73 (90%); 69 (100%).

(i) Preparation of Equilibrium-controlled Trimethylsilyl-enol Ethers (9,10) of 6-Methyl-hept-5-en-2-one using Trimethylsilyl-iodide

A flame-dried flask (500 cm^3) under argon was charged with 1 (2.52g; 20 mmol.), HMDS (3.51g; 4.60 cm^3; 24 mmol.) and pentane (250 cm^3) and cooled to -20 °C with stirring. After 10 minutes, TMS-I (4.40g; 3.14 cm^3; 22 mmol.) was added to the reaction and the mixture was stirred for a further 30 minutes at -10 °C. It was then allowed to warm to room temperature over 2 hours. It was important to maintain the initial temperature at -20 °C (lower temperatures yielded a greater proportion of the kinetic-product). The mixture turned yellow with the precipitation of hexamethyldisilazane hydroiodide, and was quenched in an excess (5 volumes) of cold saturated bicarbonate solution and extracted with pentane (3 x 25 cm^3). The organic layer was dried and the solvent removed to yield almost exclusively a mixture of the enol ethers (9,10). The products were cleaned-up and isolated as in (h). (yield; 3.39g; 86 % overall of which 98 % was equilibrium isomers; 82 % isomer 9; 18% isomer 10).

Analysis: **^{1}H-NMR** (400 MHz.); 5.06 (H_5; m); [*E*-isomer (9); 4.38 (H_3; dt); *Z*-isomer (10); 4.18 (H_3 ; dt)]; 2.65 (H_4; t); 1.74 (H_1; d); 1.66 (H_8; d); 1.60 (H_7; s); 0.21 (OTMS; s); **^{13}C-NMR** (100 MHz.) 145.97 (C_2); 130.80 (C_6); 123.21 (C_5); 107.26 (C_3);

43.32 (C_{4Z}); 36.27 (C_{4E}); 26.54 (C_{1Z}); 25.27 (C_7); 24.05 (C_{1E}); 18.80 (C_8); **MS**; *(m/z)* 198 (M^+;18%); 183 (40 %); 130 (15%); 115 (20%); 73 (100%).

(j) The Preparation of *E*- and *Z*-Fluoro-3,7-dimethyl-2,6-octadienes (Geranyl and Neryl Fluorides; 17,18) and 3-Fluoro-3,7-dimethyl-1,6-octadiene (Linaloyl Fluoride; 19)

(1) The Preparation of Chlorides

General Method: A flame-dried flask equipped with a serum cap, low temperature thermometer and a magnetic stirrer bar was connected to an argon-bleed and cooled to -30 °C whilst ensuring that a positive pressure of argon prevailed during cooling. A solution of N-chlorosuccinimide (4.79g; 42 mmol.) in dry CH_2Cl_2 (120 cm^3) was injected into the flask using a syringe followed by dimethyl sulphide (2.62g; 42 mmol.) which was added dropwise with stirring. The contents of the flask were allowed to warm (to 0 °C) for 5 minutes, and then cooled to -40 °C. A solution of the alcohol (5.0g; 32.50 mmol.) in dry CH_2Cl_2 (20 cm^3) was added by syringe to the milky-white suspension over 5 minutes. The reaction was slowly allowed to warm (to 0 °C) and maintained there for a further hour and during this period a clear colourless solution was formed. The ice bath was then removed and the reaction was stirred at room temperature for 15 minutes before the mixture was poured into a separating funnel (250 cm^3) containing saturated sodium chloride solution (50 cm^3) the aqueous layer was extracted with pentane (3 x 25 cm^3). The organic layers were combined with an additional portion of pentane and washed with saturated sodium chloride solution (2 x 20 cm^3), separated and dried, filtered and the crude product recovered. This was fractionated by column chromatography (System 1.i) and the product stored at -18 °C.

(i) E-Chloro-3,7-dimethyl-2,6-octadiene (Geranyl Chloride; 14) Analysis: (Yield; 5.0g; 83 %); **^{1}H-NMR** (400 MHz.); 5.42 (H_2; m); 5.05 (H_6; m); 4.08 (H_1; d); 2.05 (H_4/H_5; coalescence m); 1.66 (H_{10}; d); 1.58 (H_8; d); 1.70 (H_9; d); **^{13}C-NMR** (100 MHz.); 142.76 (C_3); 131.96 (C_7); 123.56 (C_2); 120.25 (C_6); 41.16 (C_1); 39.43 (C_4); 26.20 (C_5); 25.66 (C_8); 17.67 (C_{10}); 16.10 (C_9); **MS**; *(m/z)* 157 [$(M-15)^+$; 2%]; 136 (10%); 129 (12%); 123 (10%); 69 (90%); 41 (100%).

*(ii) Z-Chloro-3,7-dimethyl-2,6-octadiene (Neryl Chloride; **15**)*

Analysis: (Yield; 5.0g; 83%); **^{1}H-NMR** (400 MHz.); 5.42 (H_2; m); 5.09 (H_6; m); 4.08 (H_1; d); 2.05 (H_4/H_5; coalescence m); **1.66** (H_{10}; d); 1.58 (H_8; d); **1.70** (H_9; d); **^{13}C-NMR** (100 MHz.); 142.66 (C_3); 132.35 (C_7); 123.44 (C_2); 121.11 (C_6); 40.95 (C_1); 31.86 (C_4); 26.46 (C_5); 25.67 (C_8); 17.66 (C_{10}); 23.46 (C_9); **MS**; *(m/z)* 157 [$(M-15)^+$; 2%]; 136 (10%); 129 (12%); 123 (10%); 69 (90%); 41 (100%).

*(iii) 3-Chloro-3,7-dimethyl-1,6-octadiene (Linaloyl Chloride; **16**) Analysis:* **^{1}H-NMR** (400 MHz.); 5.95 (H_2; t); 5.30 (Hz; dt); 5.20 (H_E; dt); 5.05 (H_6; m); 1.61 (H_{10}; s); 1.53 (H_8; d); **MS**; *(m/z)* 136 [$(M-HCl)^+$;13%]; 121 (15%); 93 (40%); 69 (90%); 41 (100%).

(2) The Preparation of Fluorides

General Method: Tetrabutylammonium fluoride (TBABF (2.50g; 9 mmol.) was placed in a round-bottom flask equipped with a high-vacuum tap and the flask was fitted with a serum cap and a magnetic stirrer bar. The flask was evacuated to (0.01 mm Hg) at 40 °C for 48 hours until the crystals liquified. The vacuum was then isolated and argon introduced using a glass syringe inserted through the serum cap and vacuum tap (as in (e)). An argon-bleed was then attached to the flask and the chloride (3.90g; 2.5 mmol.) introduced dropwise with stirring at 40 °C. The mixture was then stirred for a further 6 hours and the products worked-up as in (e). The pure fluorides were isolated by preparative HPLC (System 2) for spectroscopic analysis.

*(i) E-Fluoro-3,7-dimethyl-2,6-octadiene (Geranyl Fluoride; **19**) Analysis:* (Yield; 120mg; 30%) **^{1}H-NMR** (400 MHz.); 5.49 (H_2; m; $^3J_{HF}$ = 9.28 Hz.); 5.10 (H_6; m); 4.90 (H_1; d; $^1J_{HF}$ = 47.85 Hz.); 2.10 (H_4/H_5; coalescence m); 1.69 (H_{10}; d); 1.61 (H_8; d); **1.72** (H_9; d; $^5J_{HF}$ = 4.73 Hz.); **^{13}C-NMR** (100 MHz.); 144. 12 (C_3; $^3J_{CF}$ = 11.50 Hz.); 131.92 (C_7); 118.90 (C_2; $^2J_{CF}$ = 16.90 Hz.); 123.59 (C_6); 79.35 (C_1; $^1J_{HF}$ = 156.50 Hz.); 39.51 (C_4; $^4J_{CF}$ = 2.60 Hz.); 26.19 (C_5; $^5J_{CF}$ = 3.50 Hz.); 25.64 (C_8); 17.65 (C_{10}); 16.43 (C_9; $^4J_{CF}$ = 3.10 Hz.); **^{19}F-NMR** (376 MHz.); -15.00 (m); **MS**; (m/z) 156 [(M^+; 3%)]; 141 (3%); 136 (3%); 113 (10%); 93 (10%); 69 (100%); 41 (80%).

(ii) Z-Fluoro-3,7-dimethyl-2,6-octadiene (Neryl Fluoride; 18)

Analysis: (Yield; 120mg; 30%) **^{1}H-NMR** (400 MHz.); 5.50 (H_2; m; $^3J_{HF}$ = 7.51 Hz.); 5.08 (H_6; m); 4.86 (H_1; d; $^1J_{HF}$ = 47.88 Hz.); 2.11 (H_4/H_5; coalescence m); 1.69 (H_{10}; d); 1.60 (H_8; d); 1.80 (H_9; d; $^5J_{HF}$ = 6.75 Hz.); **^{13}C-NMR** (100 MHz.); 144.25 (C_3; $^3J_{CF}$ = 11.50 Hz.); 132.31 (C_7); 119.92 (C_2; $^2J_{CF}$ = 17.00 Hz.); 123.39 (C_6); 79.03 (C_1; $^1J_{HF}$ = 156.20 Hz.); 32.16 (C_4; $^4J_{CF}$ = 3.10 Hz.); 26.72 (C_5; $^5J_{CF}$ = 3.30 Hz.); 25.64 (C_8); 17.61 (C_{10}); 23.47 (C_9; $^4J_{CF}$ = 2.80 Hz.); **^{19}F-NMR** (376 MHz.); -15.01 (m); **MS**; *(m/z)* 156 [(M^+; 3%)]; 141 (3%); 136 (3%); 113 (10%); 93 (10%); 69 (100%); 41 (80%).

(iii) 3-Fluoro-3,7-dimethyl-1,6-octadiene (Linaloyl Fluoride; 19)

Analysis: **^{1}H-NMR** (400 MHz.); 5.89 (H_2; dq; $^3J_{HF}$ = 14.45 Hz.); 5.46 (H_b; d); 5.20 (H_a; d); 5.20 (H_6); 1.40 (H_9; d; $^3J_{HF}$ = 21.67 Hz.); **^{13}C-NMR** (100 MHz.); 40.27 (C_4; $^2J_{HF}$ = 28.17 Hz.); 26.76 (C_5); 25.24 (C_9; $^2J_{HF}$ = 22.53 Hz.); **^{19}F-NMR** (376 MHz.) -7.30 (m); **MS**; *(m/z)* 156 [(M^+; (21%)]; 141 (3%); 136 (36%); 113 (10%); 73 (2%); 69 (100%); 41 (80%).

15.2 Syntheses of Deuteriated Monoterpenoids

(a) Substitution of One or More Deuterium atoms at C_1 or C_3 of 6-Methyl-hept-5-en-2-one. *General Method:* A mixture of TMS-enol ethers formed under conditions of kinetic- or equilibrium control could be prepared from the parent methyl-heptenone (1) or a deuteriated analogue by one of the specific methods (h) or (i) given previously (15.1). In each case the products were washed with cold D_2O in place of H_2O.

A solution (10 cm^3) of the TMS-enol ether (1.5g; 7.8 mmol.) in freshly distilled DME (20 cm^3) was quenched in deuteriated acetic acid (10 cm^3; prepared from 196 mg of acetic anhydride in 10 cm^3 D_2O) containing caesium fluoride (300mg; it occurred to us that TBAF may decompose the enol ether to form the protonated-ketone). The mixture was stirred at room temperature for 40 minutes and the contents of the flask extracted with dried diethyl ether (5 x 20 cm^3) and washed with saturated bicarbonate solution (5 x 20 cm^3). The organic layers were separated and dried to yield a crude

mixture of deuteriated product and unreacted TMS-enol ether, which could be readily purified by chromatography (System 2.i) for spectroscopic analysis.

*(i) [1-^{2}H]-6-Methyl-hept-5-en-2-one (**20**)*

Analysis: (Yield; 643mg; 65%) **^{1}H-NMR** (400 MHz.); 5.03 (H_5; 1H; t); 2.42 (H_3; 2H; t); 2.22 (H_4; 2H; q); 2.08 (H_1; 2H; t; $^2J_{HD}$ = 2.00 Hz.); 1.64 (H_8; 3H; d); 1.58 (H_7; 3H; s); Integral Ratio H_1:H_3 = 1:1 ; **^{2}H-NMR** (61 MHz.); 2.15 (d; $^2J_{HD}$ = 2.00 Hz.); **^{13}C-NMR** (400 M Hz.); 208.80 (C_2); 132.34 (C_6); 125.52 (C_5); 43.46 (C_3); 29.55 (C_4; t; $^1J_{CD}$=19.40 Hz.); 25.40 (C_7); 17.34 (C_8); **MS**; *(m/z)* 127 [(M^+; 6%)]; 112 (7%); 109 (22%); 66 (55%); 55 (19%); 51 (40%); 40 (100%); 39 (57%); 37 (89%).

*(ii) [3-^{2}H]-6-Methyl-hept-5-en-2-one (**21**)*

Analysis: (Yield; 461mg; 47%); **^{1}H-NMR** (400 MHz.); 5.03 (H_5; 1H; t); 2.40 (H_3; 1H; m; $^2J_{HD}$ = 2.09 Hz.); 2.22 (H_4; 2H; t); 2.11 (H_1; 3H; s); 1.65 (H_8; 3H; d); 1.59 (H_7; 3H; s); Integral Ratio H_1:H_3 = 3:1 ; **^{2}H-NMR** (61 MHz.); 2.58 (d); **^{13}C-NMR** (100 MHz.); 208.87 (C_2); 136.65 (C_6); 122.57 (C_5); 43.32 (C_3; $^1J_{CD}$= 19.35 Hz.); 31.63 (C_1); 25.59 (C_7); 22.40 (C_4); 17.55 (C_8); **MS**; *(m/z)* 127 (M^+) (10%); 112 (17%); 109 (29%); 66 (61%); 55 (30%); 52 (42%); 40 (37%); 39 (100%); 37 (90%).

*(iii) [1,3-$^2H_2$]-6-Methyl-hept-5-en-2-one (**22**)*

Analysis: (Yield; 750mg; 50%) **^{1}H-NMR** (400 MHz.); 5.04 (H_5; 1H; t); 2.42 (H_3; 1H; m); 2.22 (H_4; 2H; t); 2.10 (H_1; 3H; t; $^2J_{HD}$ = 2.00 Hz.); 1.65 (H_8; 3H; d); 1.59 (H_7; 3H; s); Integral Ratio H_1:H_3 = 2:1 ; **^{2}H-NMR** (61 MHz.); 2.58 (D_3; m); 2.15 (D_1; m); **^{13}C-NMR** (100 MHz.); 132.69 (C_6); 122.57 (C_5); 43.34 (C_3; $^1J_{CD}$ = 19.50 Hz.); 29.61 (C_1; $^1J_{CD}$=19.40 Hz.); 25.61 (C_7); 22.45 (C_4); 17.58 (C_8).

*(iv) [1,1,1-$^2H_3$]-6-Methyl-hept-5-en-2-one (**23**)*

Analysis: **MS**; *(m/z)* 129 [(M^+;15%)]; 112 (18%); 111 (44%); 94 (8%); 69 (100%); 55 (61%); 46 (93%).

(b) The Preparation of Deuterium-labelled 3,7-Dimethyl-1,6-octadien-3-ols (Linalools). The general method followed that given in Section 15.1.a

*(i) [4-^{2}H]-3,7-Dimethyl-1,6-octadiene-3-ol (4-^{2}H-Linalool; **24**)*

Analysis: (Yield; 480mg; 78%); **^{1}H-NMR** (400 MHz.); 5.90 (H_2; 1H; q); 5.20 (H_{1B}; 1H; d); 5.12 (H_6; 1H; t); 5.11 (H_{1A}; 1H; d); 1.98 (H_5; 2H; m); 1.67 (H_8; 3H; d); 1.59 (H_{10}; 3H; s); 1.53 (H_4; 1H; m); 1. 24 (H_9; 3H; s); **^{2}H-NMR** (61 MHz.); 1.58 (D_4; s); **^{13}C-NMR** (100 MHz.); 145.93 (C_2); 131.83 (C_7); 124.29 (C_6); 111.59 (C_1); 76.85 (C_3); 41.66 (C_4; $^1J_{CD}$=19.40 Hz.); 29.62 (C_5); 25.63 (C_9); 22.67 (C_8); 17.63 (C_{10}); **MS**; *(m/z)* 155 [(M^+; 1%)]; 137 (8%); 122 (16%); 94 (57%); 83 (16%); 81 (34%); 71 (80%); 55 (18%); 41 (100%)

*(ii) [9-^{2}H]-3,7-Dimethyl-1,6-octadien-3-ol ([9-^{2}H]-Linalool; **25**)*

Analysis: (Yield; 480mg; 78%) **^{1}H-NMR** (400 MHz.); 5.86 (H_2; 1H; q); 5.19 (H_{1B}; 1H; d); 5.11 (H_6; 1H; t); 5.03 (H_{1A}; 1H; d); 2.08 (H_5; 2 H; q); 1.66 (H_8; 3H; d); 1.58 (H_{10}; 3H; s); 1.53 (H_4; 2H; m); 1.24 (H_9; 3H; t; $^2J_{HD}$=1.74 Hz.); **^{2}H-NMR** (61 MHz.); 1.27 (D_1; m); **MS**; *(m/z)* 155 [(M^+;0.1%)]; 137 (9%); 122 (16%); 94 (61%); 83 (20%); 81 (36%); 72 (100%); 69 (57%); 55 (66%).

*(iii) [4,9-$^2H_2$]-3,7-Dimethyl-1,6-octadien-3-ol ([4,9-$^2H_2$]-Linalool; **26**)*

(Yield; 150 mg; 24%); **^{1}H-NMR** (400 MHz.); 5.90 (H_2; 1H; m); 5.20 (H_{1B}; 1H; d); 5.12 (H_6; 1H; t); 5.11 (H_{1A}; 1H; d); 1.95 (H_5; 2H; m); 1.64 (H_8; 3H; d); 1.56 (H_{10}; 3H; s); 1.50 (H_4; 1H; m); 1.20 (H_9; 2H; m); **^{2}H-NMR** (61 MHz.); 1.58 (D_4; s); 1.21 (D_1; s); **^{13}C-NMR** (100 MHz.); 144.93 (C_2); 131.89 (C_7); 124.23 (C_6); 111.64 (C_1; $^1J_{CD}$=19.40 Hz.); 73.35 (C_3;); 41.55 (C_4; $^1J_{CD}$=20.35 Hz.); 22.65 (C_8); 17.65 (C_{10}); **MS**; *(m/z)* 156 [(M^+ 1(%)]; 138 (32%); 123 (49%); 94 (48%); 83 (55%); 81 (39%); 71 (42%); 69 (100%); 55 (18%).

(c) The Preparation of Deuteriated Monoterpenoid Acetate Esters;

General Method: A solution of linalool (10.0 mmol.) in glacial acetic acid (25 cm^3) was added to a mixture of acetic acid (25 cm^3) and acetic anhydride (10cm^3) containing *p*-toluenesulfonic acid monohydrate (600 mg). The reaction was quenched after 20 minutes at reflux temperature by pouring into water (10 volumes). The products were recovered by extraction into diethyl ether (5 x 50cm^3) and purified by column chromatography (System 2.ii) to yield a mixture of linaloyl acetate, neryl

acetate and geranyl acetate (typically 3:1:3 v/v). The isomers (each *ca.* 10 mg) were separated by HPLC (System 2).

*(i) [4-^{2}H]-3,7-Dimethyl-1,6-octadiene-3-yl Acetate ([4-^{2}H]-Linaloyl Acetate; **27**)*
Analysis: **MS**; *(m/z)* 182 (3%); 138 (33%); 137 (75%); 136 (17%); 123 (27%); 122 (84%); 121 (28%); 109 (8%); 108 (29%); 107 (15%); 94 (29%); 93 (75%); 92 (56%); 91 (30%); 80 (44%); 79 (31%); 78 (38%); 68 (6%); 66 (47%); 39 (100%); 37 (62%)

*(ii) Z-[4-^{2}H]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([4-^{2}H]-Neryl Acetate; **28**)*
Analysis: **MS**; *(m/z)* 197 [(M^+;0.01%)]; 156 (0.06%); 138 (4%); 137 (29%); 136 (2%); 123 (2%); 122 (25%); 121 (4%); 109 (2%); 108 (6%); 107 (3%); 94 (8%); 93 (57%); 92 (24%); 91 (6%); 80 (14%); 79 (31%); 78 (10%); 68 (2%); 66 (100%); 39 (85%); 37 (84%)

*(iii) E-[4-^{2}H]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([4-^{2}H]-Geranyl Acetate; **29**)*
Analysis: **MS**; *(m/z)* 197 [(M^+; 0.39%)]; 156 (0.06%); 138 (10%); 137 (51%); 136 (6%); 123 (5%); 122 (44%); 121 (9%); 109 (7%); 108 (13%); 107 (7%); 94 (15%); 93 (56%); 92 (38%); 91 (11%); 80 (21%); 79 (42%); 78 (17%); 68 (5%); 66 (100%); 39 (94%); 37 (89%)

*(iv) [9-^{2}H]-3,7-Dimethyl-1,6-octadiene-3-yl Acetate ([9-^{2}H]-Linaloyl Acetate; **30**)*
Analysis: **MS**; *(m/z)* 138 (7%); 137 (52%); 136 (1%); 123 (5%); 122 (67%); 121 (7%); 109 (1%); 108 (3%); 107 (3%); 94 (6%); 93 (49%); 92 (16%); 91 (3%); 80 (9%); 79 (5%); 78 (4%); 68 (0.01%); 66 (10%); 39 (100%); 37 (18%)

*(v) Z-[9-^{2}H]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([9-^{2}H]-Neryl Acetate; **31**)*
Analysis: **MS**; *(m/z)*; 137 (5%); 122 (3%); 94 (0.16%); 93 (17%); 92 (3%); 80 (1%); 79 (6%); 66 (100%); 39 (49%); 37 (44%)

*(vi) E-[9-^{2}H]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([9-^{2}H]-Geranyl Acetate; **32**)*
Analysis: **MS**; *(m/z)*; 155 (0.24%); 138 (1%); 137 (14%); 136 (0.28%); 123 (0.22%); 122 (8%); 121 (2%); 109 (1%); 108 (2%); 107 (0.45%); 94 (2%); 93 (18%); 92 (7%); 91 (1%); 80 (3%); 79 (9%); 78 (2%); 68 (0.1%); 66 (100%); 39 (78%); 37 (66%)

*(vii) [4,9-$^2H_2$]-3,7-Dimethyl-1,6-octadiene-3-yl Acetate ([4,9-$^2H_2$]-Linaloyl Acetate; **33**)*
Analysis: **MS**; *(m/z)*; 183 (0.1%); 138 (53%); 137 (20%); 136 (1%); 123 (68%); 122 (33%); 121 (3%); 10 9 (5%); 108 (6%); 107 (4%); 94 (38%); 93 (34%); 92 (14%); 91 (5%); 80 (9%); 79 (9%); 78 (9%); 68 (2%); 66 (14%); 39 (100%); 37 (22%)

*(viii) Z-[4,9-$^2H_2$]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([4,9-$^2H_2$]-Neryl Acetate; **34**)*
Analysis: **MS**; *(m/z)*; 138 (7%); 137 (1%); 123 (5%); 122 (2%); 94 (17%); 93 (11%); 92 (3%); 80 (9%); 79 (4%); 66 (100%); 39 (61%); 37 (54%)

*(ix) E-[4,9-$^2H_2$]-3,7-Dimethyl-2,6-octadien-1-yl Acetate ([4,9-$^2H_2$]-Geranyl Acetate; **35**)*
Analysis: **MS**; *(m/z)*; 156 (0.53%); 155 (0.1%); 138 (18%); 137 (5%); 123 (10%); 122 (6%); 121 (0.5%); 109 (3%); 108 (2%); 107 (1%); 94 (21%); 93 (15%); 92 (6%); 91 (2%); 80 (13%); 79 (6%); 78 (3%); 68 (4%); 66 (100%); 39 (93%); 37 (83%)

15.3 The Synthesis of Isopentenyl Pyrophosphate (Diphosphate) for Administration to Cell-free Extracts

The method followed the procedure of Poulter *et. al.*[307] by conversion of the 3-methyl-3-buten-1-yl-tosylate ester into the corresponding diphosphate by treatment of the former with tris(tetra-n-butylammonium) hydrogen pyrophosphate.

(a) Tris (tetra-n-butylammonium) Hydrogen Pyrophosphate

A solution of disodium dihydrogen pyrophosphate (12.25 g (56 mmol.) in aqueous ammonium hydroxide; ; 10% v/v; 50 cm^3) was passed through a column of Dowex AG 50W-X8 (BDH, Poole, Dorset) cation-exchange resin (100-200 mesh, ammonium form). The free-acid was eluted with deionised water (300 cm^3) and the resulting solution titrated to pH 7.3 with aqueous tetra-n-butylammonium hydroxide (40% w/w). The resulting solution was dried by lyophilisation.
^{31}P-NMR (161.90 MHz.) -3.50 ppm.

(b) 3-Methyl-3-buten-1-yl *p*-toluenesulphonate (Isopentenyl Tosylate)

In a flame-dried flask (containing a magnetic stirrer) under argon were combined p-toluenesulphonyl chloride (0.315g; 1.65 mmol.) and 4-(N,N-dimethylamino) pyridine (0.219g; 1.8 mmol.) in dichloromethane (7.5 cm^3). The mixture was stirred during the addition of 3-methyl-3-buten-1-ol (0.129g; 1.5 mmol.). After 2 hours, the contents

of the flask were poured into petroleum spirit (60-80 °C/ 500cm^3) and the resulting precipitate removed by filtration through a large Buchner funnel. The filtrate was concentrated (50 cm^3) by rotary evaporation, diluted with diethyl ether (50 cm^3) and re-filtered. The solvent was removed by rotary evaporation to yield a brown oil (2.52g).

(c) 3-Methyl-3-buten-1-yl Pyrophosphate (Isopentenyl Pyrophosphate)

In a flame-dried flask under argon and containing a magnetic stirrer were syringed 3-methyl-3-buten-1-yl tosylate (0.80g; 3.33 mmol.) and a solution of tris (tetra-n-butylammonium) hydrogen pyrophosphate (9.02g; 10 mmol.) in acetonitrile (12 cm^3). The mixture was stirred for 2 hours at room temperature after which the solvent was removed by rotary evaporation and the resulting white paste dissolved in a mixture of (1:49 v/v) of isopropyl alcohol and ammonium bicarbonate (25 mmol.dm^{-3}). The solution was eluted through a column containing Dowex AG 50W-X8 (ammonium form; 100-200 mesh) which had been pre-equilibrated with two column volumes of the isopropyl alcohol-ammonium bicarbonate mixture. The effluent was lyophilysed to yield a white solid which was dissolved in ammonium bicarbonate (0.1 mol.dm^{-3}; 50 cm^3) and centrifuged (12.2.d). The supernatant was concentrated (to *ca.* 10 cm^3) by rotary evaporation and then lyophilised to yield a white crystalline solid (0.60g; 72%). **^{1}H-NMR** (60 MHz.) 1.82 (H_5;3H); 2.60 (H_3;2H).

15.4 The Preparation of Peroxide Derivatives of β-Pinene and α-Terpinene

The terpenoid hydrocarbon (2.50g) was placed in a round-bottom flask (50 cm^3) together with a catalytic-amount of Rose Bengal (BDH; 10mg) and redistilled methanol (20 cm^3). A magnetic stirrer was placed inside the flask which was fitted with a condenser and placed in a water-cooled glass jacket. The contents were vigorously stirred and irradiated using a sodium lamp (400W) for 8 hours, afterwhich they were removed and purified by column chromatography (system 1.i). The fractions were analysed by TLC (system 1.i) and the peroxide-spots visualised by TLC-developer (ii).

(a) Peroxide Mixture formed from β-Pinene

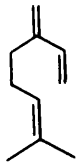
Yield; 769 mg (by weight)

(b) Peroxide Mixture formed from α-Terpinene

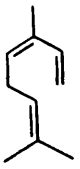
Yield; 820 mg (by weight)

Appendix Structures of Terpenoids Referred to in this Work

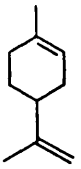
Monoterpenoids (C_{10})



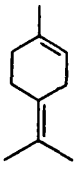
Myrcene



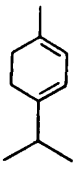
cis-Ocimene



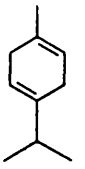
Limonene



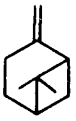
Terpinolene



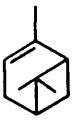
α-Terpinene



γ-Terpinene

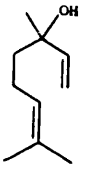


β-Pinene

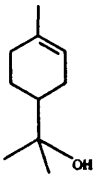


α-Pinene

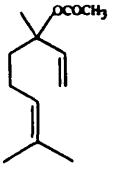
Camphene



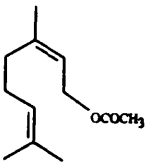
Linalool



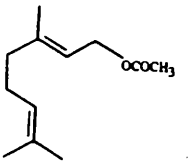
α-Terpineol



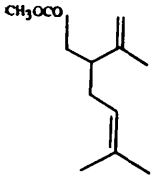
Linaloyl Acetate



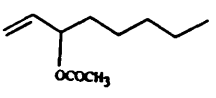
Neryl Acetate



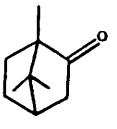
Geranyl Acetate



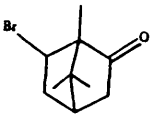
Lavandulyl Acetate



Oct-1-en-3-yl-acetate

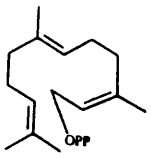


Camphor

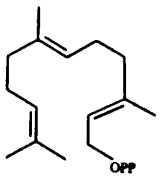


3-Bromo-camphor

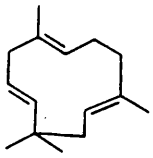
Sesquiterpenoids (C_{15})



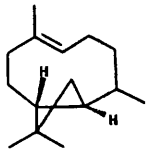
2Z, 6E-Farnesyl Pyrophosphate



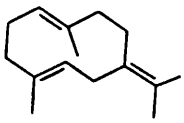
2E-, 6E-Farnesyl Pyrophosphate



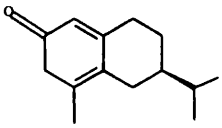
Humulene



Caryophyllene

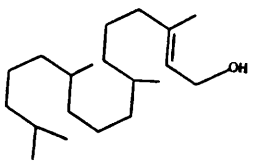


Germacrene



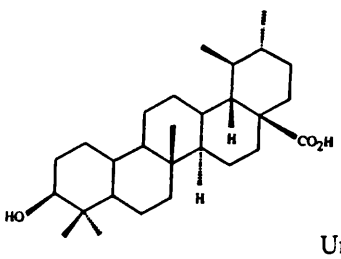
Nootkatone

Diterpenoids (C_{20})



Phytol

Triterpenoids (C_{30})



Ursolic Acid

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