STUDIES ON THE SECONDARY METABOLISM OF SOME PLANT TISSUE CULTURES

by

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A thesis submitted in partial fulfilment of the requirements of the University of London for the degree of Doctor of Philosophy

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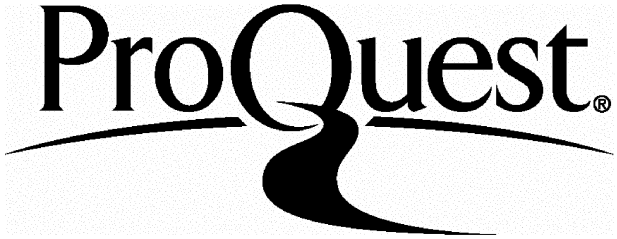
February 1993



ABSTRACT

In the first part of this work, tissue cultures of the herb *Galium verum* were established under a variety of light and hormone regimes. Cultures flourished under all regimes attempted. All culture lines produced pigments, with an inverse relationship between growth and pigment production being readily apparent. The acid-hydrolysed extracts of these cultures were examined by high performance liquid chromatography using an ultra-violet (diode-array) detector over the wavelength range 190-370nm. This detected over 80 different compounds, mostly of the same class. Comparison between these extracts with those similarly prepared from roots and flowers of the parent plant indicated a number of compounds to be present in both roots and cultures. Ten compounds were isolated from the acid-hydrolysed extracts of the cultures. Eight of these were characterised by mass spectrometry and ^{1}H NMR spectroscopy as anthraquinone pigments, some of these being unreported in the literature.

In a concurrent study the metabolism of monoterpenoid epoxides in plants and cultures was investigated. ^{14}C-labelled limonene-1,2-epoxide was synthesised in four steps from 4-acetyl-1-methylcyclohex-1-ene. Following purification, this compound was fed to both the plant and to especially established tissue cultures of *Pelargonium fragrans*. In these preliminary studies very poor incorporations were noted and neither of the hoped-for products, namely, ^{14}C-borneol and ^{14}C-camphor were detected. Thus no evidence was obtained for epoxides as biosynthetic intermediates leading to cyclic monoterpenoids in either higher plants or their tissue cultures.

The synthesis of ^{14}C-linalool-6,7-epoxide, from 7-methyl-1,6-octadien-3-one was also attempted, although problems with the epoxidation of linalool could not be fully resolved. A synthetic route to the two furan isomers of 6,7-epoxylinalool was also developed.

Acknowledgements

I should like to thank my supervisor, Dr. D. V. Banthorpe for his enthusiasm, advice throughout the course of this study and critical appraisal of this work. I am also grateful to other members of the department for their help, in particular, Mr. S. T. Corker for assistance with HPLC separations and Mr. C. J. Cooksey for double-checking many of the structure determinations.

I am indebted to Professor M. L. McGlashan for awarding me a departmental research studentship; Professor C. R. Ganellin and Mr. W. Tertiuk for permitting the use of HPLC equipment; Mr J. Janes for enabling access to the GC and GC-MS facilities at Bush Boake Allen Ltd; Dr. J. E. Parkin for the use of a study room; Dr. C. O. Chan and Mr. A. Yusuf for proof-reading of this manuscript; Dr. P. J. Barrie for molecular drawings; and my brother Tom for the loan of a computer.

Many thanks to Dr. S. A. Edwards, for not only much practical advice, but also for the many words of support and encouragement which he and Ms. S. Brooks gave.

Finally, I wish to thank my parents for their patience and understanding throughout my time at college.

Abbreviations

Most abbreviations are given in full at their first occurrence in the text. Other abbreviations for reagents and solvents are those which are widely accepted. Standard abbreviations related to spectroscopic techniques are frequently used.

Contents

CHAPTER ONE: INTRODUCTION

Section 1.1 Primary and secondary metabolism

The study of natural products began with the isolation, identification and classification of compounds occurring in living material. More recently the study of biosynthesis - the pathways by which these complex molecules are built up from simple starting materials - has enabled a new classification of natural products to be made based on common biosynthetic routes. A number of these pathways are shared by all organisms and enable the synthesis and utilisation of certain compounds such as amino acids, sugars, fatty acids and nucleotides and the polymers derived from them (proteins, carbohydrates etc) which are essential for the well being of the organism. This is primary metabolism and these compounds are primary metabolites. In most organisms other pathways exist, producing compounds which have no apparent utility. These compounds are known as secondary metabolites and the pathways of their synthesis and degradation constitute secondary metabolism. It should be remembered that the dividing line between the primary and secondary metabolisms is an artificial one with certain compounds being classified as primary metabolites when they are definitely secondary metabolites (e.g. certain obscure amino acids) and *vice versa* (e.g. many steroid alcohols).

Section 1.2 The functions of secondary metabolites[1]

For many years the functions of secondary metabolites have been regarded as obscure. For instance, it has been suggested that they are the products of "overflow" metabolism formed when the substrate level is in excess of that needed for primary metabolism. Other explanations

are that they be the products of minor side-routes or are detoxification products. The levels of secondary products are related to the stage of development of an organism, nutritional condition or environmental stress. In evolutionary terms any metabolite of a plant which deters, attracts (for assistance with pollination) or inhibits the growth of competitors will help the species succeed. The field of ecological biochemistry seeks to investigate these chemical interrelationships between competing species. A few examples of these interactions illustrate the great variety of roles assumed.

(i) In the semi-arid desert of southern California two species of shrub *Salvia leucophylla* and *Artemesia californica* dominate. These produce monoterpenoids, primarily camphor and 1,8-cineole whose scent emanates from the plant. The airborne molecules of these compounds fall to earth and are absorbed onto soil particles and this inhibits germination of other herbs. This reduction in competition from other species by affecting their germination or growth is known as allelopathy. It is only after the occurrence of a bush fire (which causes the destruction of soil- and air-borne monoterpenoids) that other species occur in any numbers.

(ii) The contrast in the colour of a flower against the general leafy-green background can act as a visual signal to pollinators such as insects, bats or birds which feed on the nectar found within the flowers. Several classes of pigment are responsible for flower colour including the flavonoids, carotenoids and (to a lesser extent) the quinones and betalin alkaloids. Often plants need to be able to respond rapidly to changing pollinators - an example of which is provided by scarlet gilin (*Ipomopsis aggregata*). In populations of this plant growing near Flagstaff, Arizona it has been observed that a number of plants shift in flower colour during the flowering season

from red through shades of pink to white. This shift correlates precisely with the coincident emigration of humming birds which are the primary pollinators in mid-July and with the need to be attractive to the remaining pollinator, a hawkmoth *Hyles lineata*.

(iii) The bombardier beetle possesses internal storage organs containing hydroquinone, methyl-hydroquinone and hydrogen peroxide. Adjacent to these are glands which contain catalases and peroxidases. In response to attack the reagents are forced into these glands producing a mixture of quinones and oxygen which is released as a hot (up to 100°C) gas-propelled spray.

Section 1.3 Biosynthesis of secondary metabolites in plants[2]

Primary and secondary metabolism are interconnected because the former provides a number of small molecules which are the starting materials for all the important biosynthetic pathways of secondary metabolism. There are four main pathways leading to secondary metabolites:

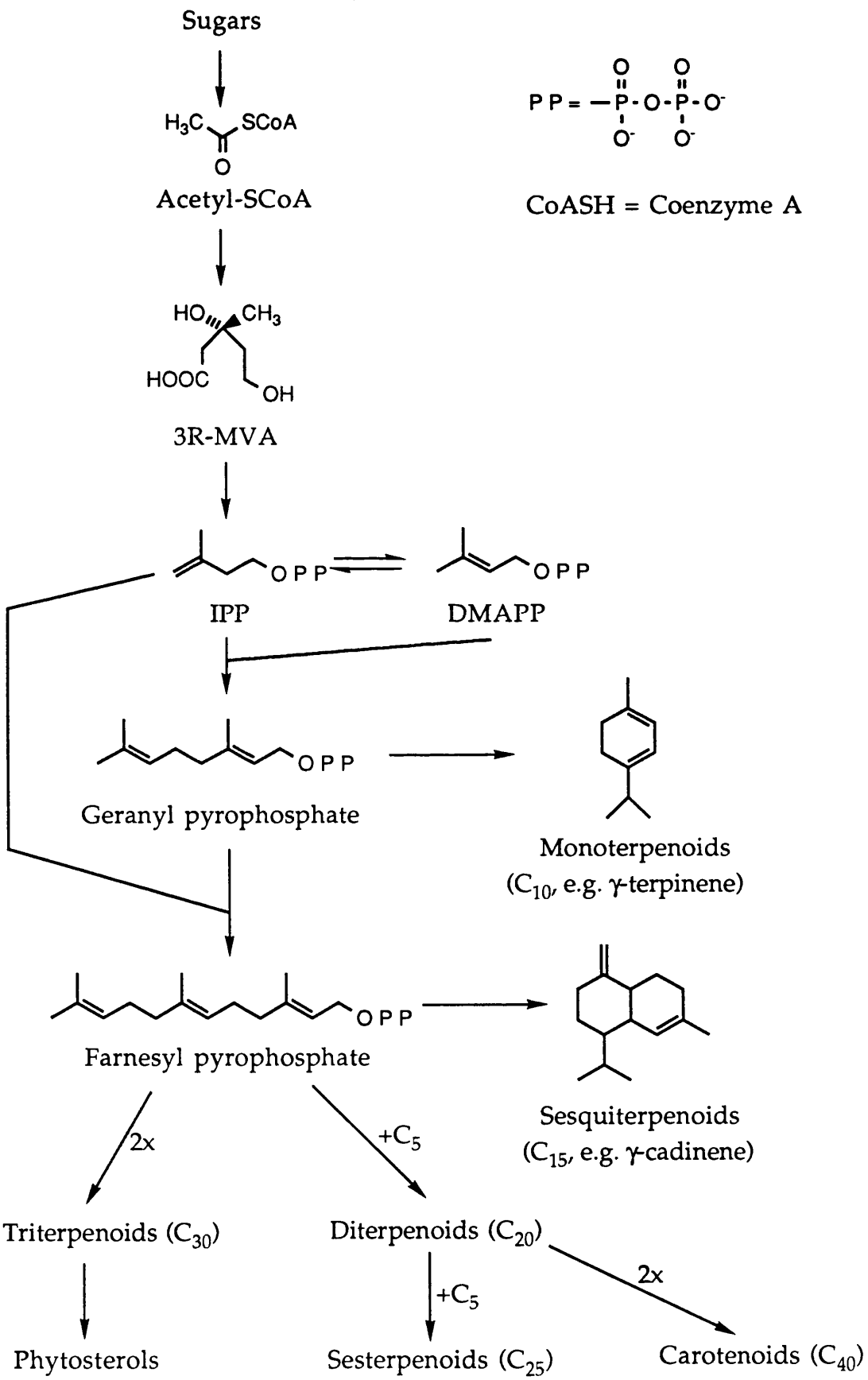
1. The terpenoid pathway.
2. The polyketide pathway.
3. The shikimate pathway.
4. The pathway from amino acids.

Since this work involves products derived from the first three pathways these will be discussed further.

Section 1.3.1 The terpenoid pathway

The terpenoid pathway (figure 1.1) begins with the linkage of three acetyl-SCoA units to form mevalonic acid (MVA) which is decarboxylated to Δ^3-isopentenyl pyrophosphate (IPP). Most IPP is isomerised to 3,3-dimethylallyl pyrophosphate (DMAPP) which acts as a

Figure 1.1 The terpenoid pathway



starter for chain elongation and condenses with further IPP units in a head-to-tail fashion to form chains from C_{10} through to C_{25}. Each of these chains can then undergo some form of modification, such as cyclisation, oxygenation or rearrangement, to yield a number of the large class of terpenoids. The phytosterols and triterpenoids are derived from a tail-to-tail linkage of two C_{15} farnesyl pyrophosphate chains. The carotenoids are formed via condensation of two molecules of geranylgeranyl pyrophosphate, the C_{20}-progenitor of the diterpenoids.

Section 1.3.2 The polyketide pathway

The polyketide pathway (figure 1.2) also utilises acetyl-SCoA as a starting material. The net effect of the first few steps is to couple C_2 acetyl subunits head-to-tail to form a chain of variable length. Complete reduction of the keto groups leads to the simplest polyketides, the fatty acids. Dehydration of these saturated compounds leads to the unsaturated fatty acids and polyacetylenes.

Alternatively the polyketo intermediate can be cyclised, leading to the flavonoids, some anthraquinones and some other plant phenolics.

Section 1.3.3 The shikimic acid pathway

The shikimic acid pathway (figure 1.3) is the other main route to plant phenolics. Erythrose-4-phosphate and phosphoenolpyruvic acid are combined into a molecule of dehydroshikimic acid. Further reactions incorporating a second molecule of phosphoenolpyruvic acid, lead to one of two possible aromatic amino acids, phenylalanine and tyrosine. Many aromatic natural products are derived from these two compounds, including the coumarins and phenylpropanoids. The stereospecific elimination of ammonia from either phenylalanine or tyrosine is

Figure 1.2 The polyketide pathway

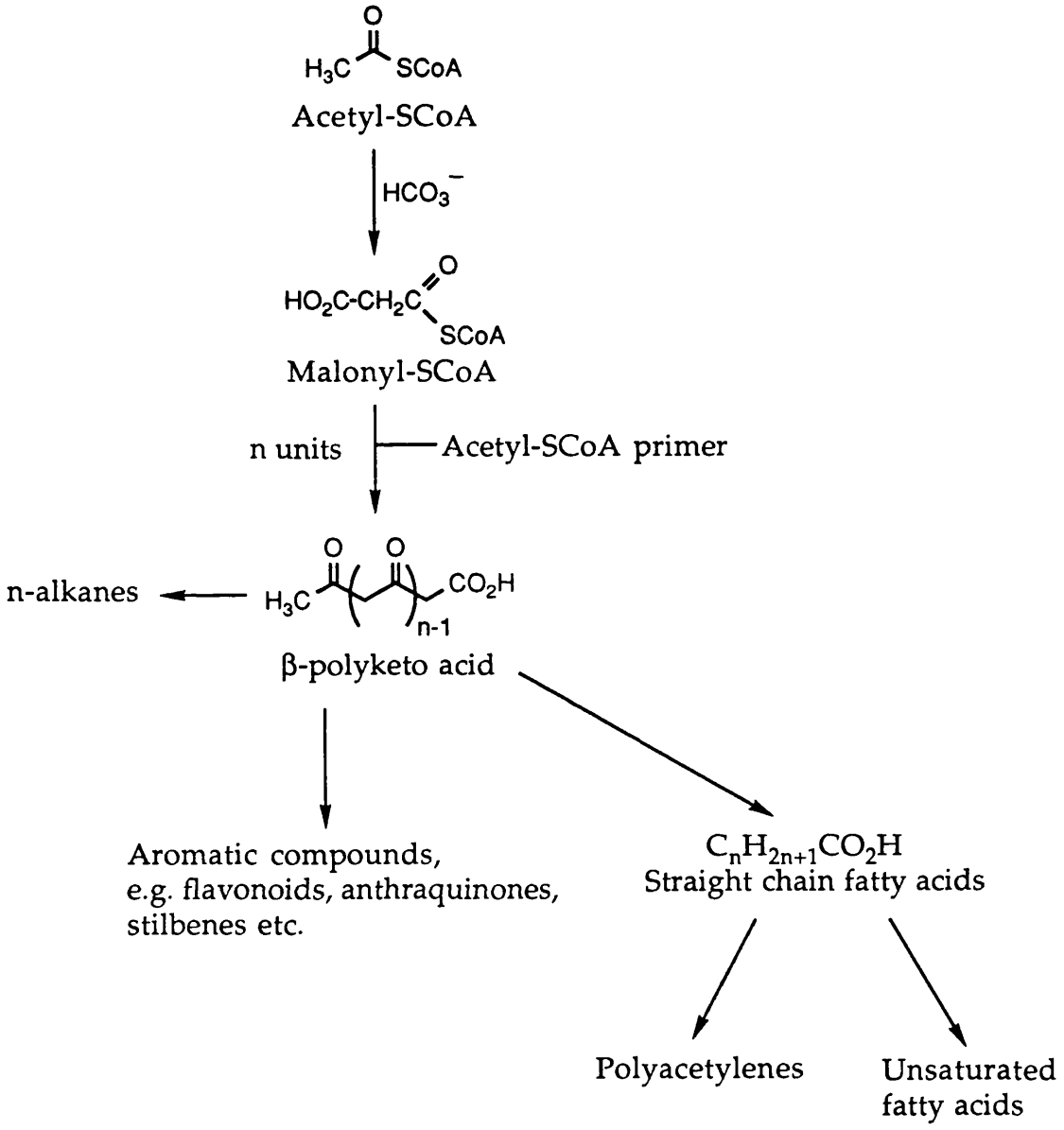
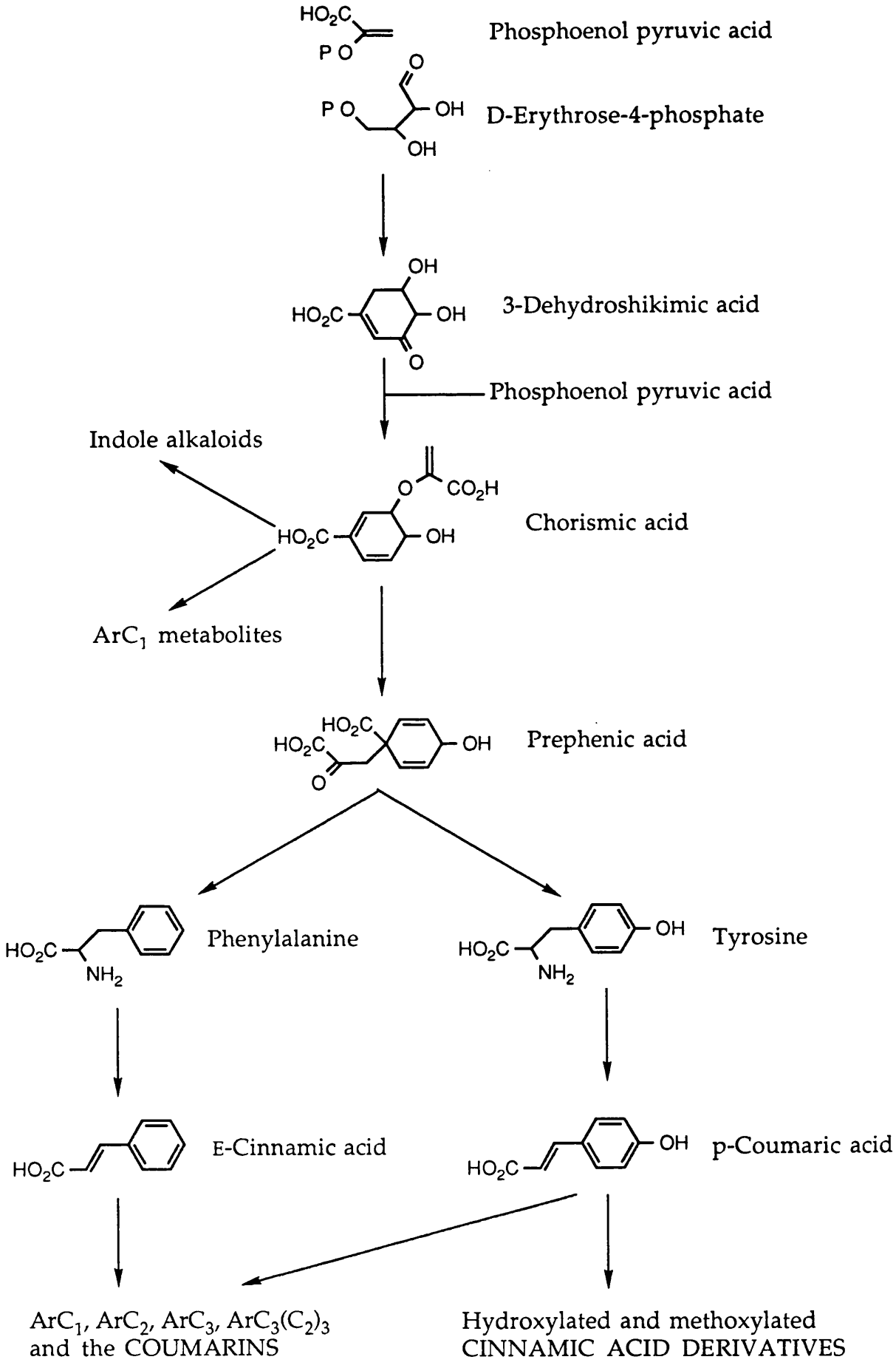


Figure 1.3 The shikimic acid pathway



facilitated by the enzyme phenylalanine ammonia lyase (PAL). The regulation of this enzyme has received much attention since phenylalanine is also important in protein production and hence the PAL enzyme controls a branch point between primary and secondary metabolism.

Section 1.3.4 Biosynthesis of anthraquinones

A number of anthraquinone pigments were isolated during the course of this work and hence a brief discussion is given here of the biosynthetic pathways leading to this class of natural products.

In 1953 Birch and Donovan[3] proposed that two routes lead to anthraquinones and that one of these involves acetate. Indeed two pathways have now been established[4,5]:

(i) **Polyketide-derived anthraquinones.** In this pathway one molecule of acetyl-SCoA is extended by seven C_2 units (derived from malonyl-SCoA) to form an (hypothetical) octaketide which can fold and aromatise in different ways giving rise to anthraquinones with characteristic substitution patterns (see figure 1.4). This pathway occurs mainly in microorganisms and in a few plants e.g. *Rhamnus frangula* and *Rumex alpinus* (both *Polygonaceae*).

(ii) ***o*-Succinylbenzoic acid-derived anthraquinones.** These compounds are widely distributed in higher plants and are formed from shikimic acid, α-ketoglutaric acid and mevalonic acid with *o*-succinylbenzoic acid as a key intermediate (see figure 1.5). In contrast to the polyketide-derived anthraquinones, the anthraquinones formed by this latter pathway are (with only a few possible exceptions) substituted in one aromatic ring only. Feeding experiments have demonstrated that the anthraquinones occurring both in plants and cultures of the family Rubiaceae, which includes *Galium verum* (the herb used in this study),

Figure 1.4 Formation of polyketide-derived anthraquinones

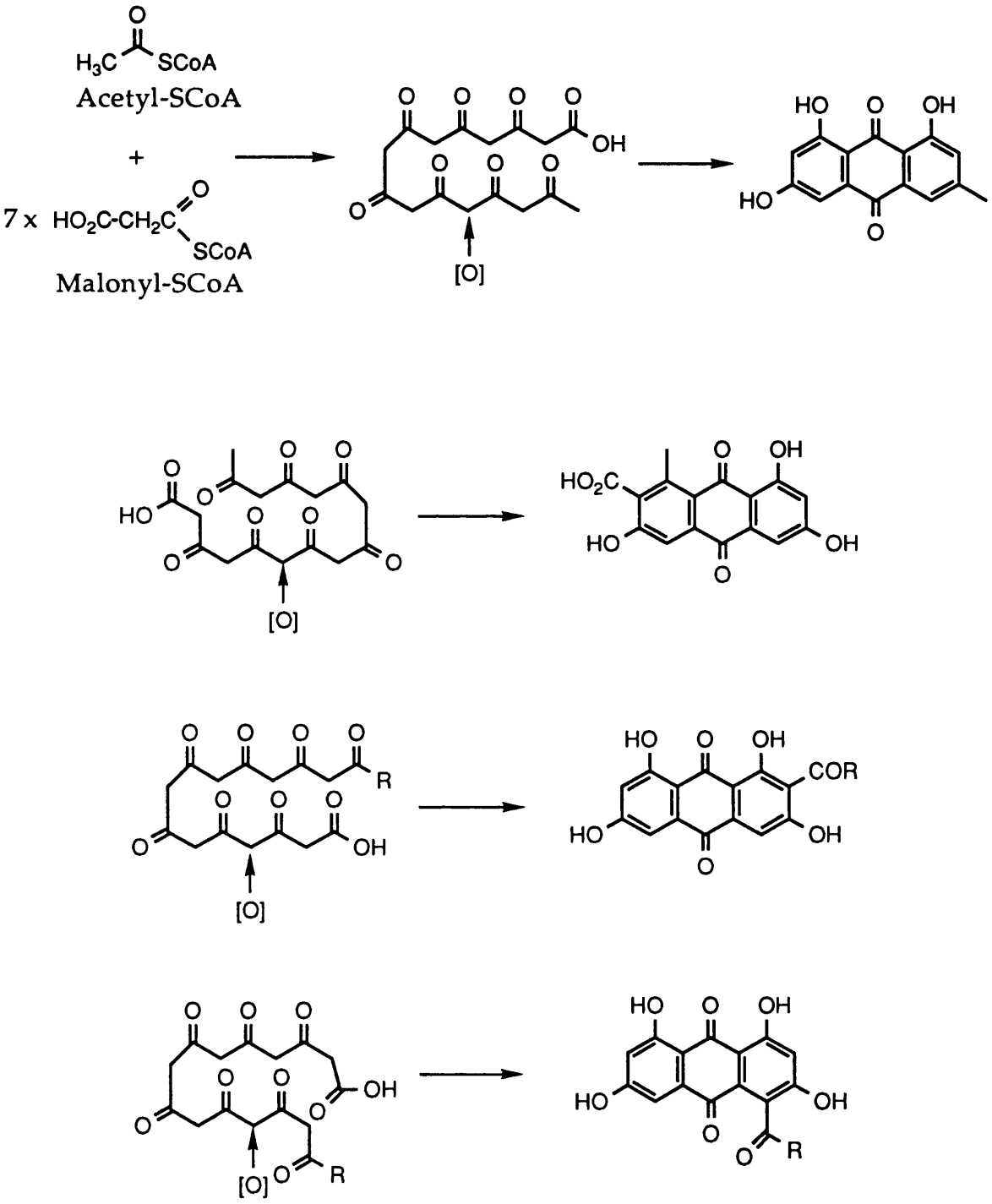
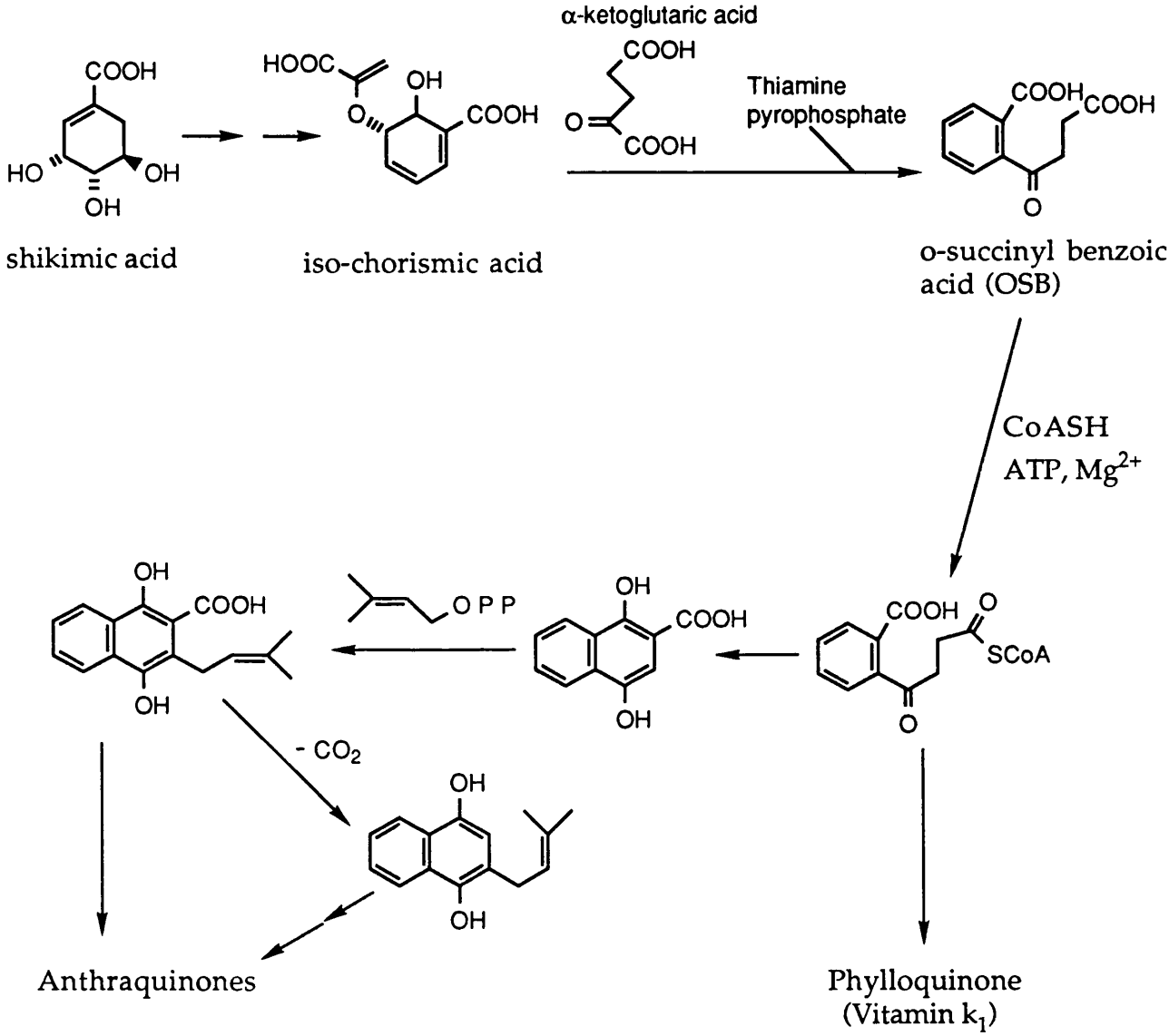


Figure 1.5 Formation of o-succinylbenzoic acid-derived anthraquinones



are derived via this pathway. The naphthoquinone, phylloquinone (vitamin K_1) occurs in the chloroplasts of green plants and some tissue cultures and shares a common biosynthetic pathway with these anthraquinones (see figure 1.5). Studies on cultures which produce both demonstrate that conditions which trigger anthraquinone synthesis switch off phylloquinone synthesis and *vice versa*.[5] How this metabolic shift is achieved and which regulatory phenomena are involved are of great interest.

Simple biochemical modifications (e.g. *o*-methylation, side-chain oxidation, decarboxylation) of the basic skeletal structures formed as shown above by the two pathways account for a large number of those naturally-occurring anthraquinones encountered.

Section 1.4 Plant tissue culture

Section 1.4.1 History of plant tissue culture

In 1902 Haberlandt made the first attempts to culture plant cells aseptically *in vitro*.[6] Although no cell division was observed, he was able to maintain plant cells for up to three weeks on a primitive nutrient medium. It was not until the discovery of the plant hormone indole-3-acetic acid (IAA) and the appropriate vitamins that it became possible to perpetuate plant cells in culture. Later the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA) were developed; the cytokinin, 6-furylaminopurine (kinetin) was discovered; and its synthetic analogue 6-benzylaminopurine (BAP) was made. The use of these growth regulators together with undefined natural mixtures as additives (e.g. coconut milk) greatly increased the potential of plant tissue culture.

These early experiments produced callus cultures which consisted of an amorphous mass of unspecialised (parenchymatous) cells growing

on a solid agar medium. They resembled the undifferentiated wound tissue produced by whole plants.

The next development was suspension culture. These are derived from callus cultures by dispersing calli in a shaken liquid medium and are composed of cell aggregates (up to about 100 cells) as well as individual cells. They are typically even less differentiated than callus cultures.

Today, many other types of plant tissue culture are possible. As well as cultures consisting of undifferentiated cells and tissue it is now possible to grow cultures of particular organs (e.g. roots or shoots). Pollen cells, protoplasts and embryos may also be maintained.

Section 1.4.2 Applications of plant tissue culture

Section 1.4.2.1 Applications of plant tissue culture: Plant propagation

By varying the levels of auxins and cytokinins in a tissue culture medium it is possible to induce root or shoot formation, and hence, plant regeneration. Moreover, as few viral diseases affect the plant apex (which is rapidly dividing), virus-free cultures (and hence plants) can be established by the culture of apical meristems.[7]

Plant propagation is the principal reason for commercial interest in tissue culture with many household plants being produced in this way. The technique can be used as a means of rapid propagation since the plantlets can be divided at intervals into segments which can then be grown individually. The whole procedure can be carried out in a fraction of the time taken by more conventional means (e.g. nursery) and is particularly useful for the propagation of infertile plants. Recently conservationists at Kew Gardens have begun to use the technique to propagate rare plants before reintroducing them back into the wild. The presumption is made, which usually seems to be the case,

that the genetic capability of the propagated plant is the same as that of the parent plant.

Section 1.4.2.2 Applications of plant tissue culture:

As a source of chemicals

Plant tissue culture has several advantages as a source of plant-derived compounds over traditional means:

(i) it is independent of various environmental factors e.g. climate, pests, disease, geography and seasonal and political upheavals and variations;

(ii) conditions are easier to control;

(iii) As the extent of expression of secondary pathways in the intact plant and the corresponding culture are quite different compounds can accumulate in cultured cells which have not been detected in the parent plant (e.g. the dimeric indole alkaloids Voafrine A and Voafrine B, which have not been found in nature but do occur in cell suspension cultures of *Voacanga africana Stapf.*[8]);

(iv) it may be more suitable in cases where plants are difficult to grow or propagate;

(v) growth, processing and recovery can all be on-line.

Higher plants produce a great variety of natural products such as flavours, fragrances, pharmaceuticals, dyes and gums and indeed plants are still the sole source of about 25% of prescribed medicines.[9] Considering the above advantages it is not surprising that there is a great deal of interest in the alternative production of such compounds by plant cell cultures.[10-13]

In general, low yields or undetectable amounts of secondary metabolites from plant tissue cultures are usually reported. However, in comparing yields for the plant with those for the culture it should

be noted that the former are usually for that part of the plant which is harvested, for example the capsule for alkaloids extracted from the opium poppy. It might be more useful if these yields were averaged over the whole plant. Moreover, wet-weight yields are generally quoted, but since culture tissue has a much higher water content than that of the plant, comparison of dry weights might be more instructive.

The following procedure as proposed by Deus and Zenk[14] can be used to increase the yield of secondary metabolites:

1 Establish cultures from high yielding plant variants;[15]

2 Select high-producing cells;

3 Establish stable cell lines from the selected cells;

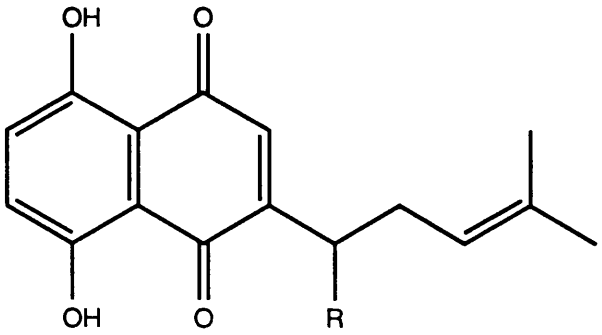
4 Optimise production of chemicals from these lines.

High-producing cells can be selected for in several ways by: (i) visualisation, if the compounds produced are coloured; (ii) ultra-violet/ fluorescence, if the compounds produced are UV-active or fluoresce - although this may have side effects (see section 1.4.3.2); (iii) specific radioimmunoassay;[16] (iv) visualisation, if the compound produced becomes coloured upon secretion into the media; and (v) selection by resistance to a toxic analogue of the compound.

After a high-producing cell line has been selected it has to be continuously monitored as cell lines can be unstable with respect to secondary metabolite production. It is generally accepted that such instability is genetic in origin, although Berlin[13] has suggested that selection is often carried out before the cells have become fully adapted to the conditions imposed and thus physiological variants have been selected for and not true ones.

Once a stable cell line has been established, production of the secondary metabolite may be optimised by making variations in the

Figure 1.6 The shikonin pigments



R = H, OH or aliphatic acids

media composition and culture conditions such as light and temperature.[17]

Commercially, shikonin pigments are produced using plant tissue culture by Mitsui Petrochemical Industries in Japan.[18] These compounds - which are a type of naphthoquinone (see figure 1.6) - are produced in the roots of *Lithospermum erythrorhizon* and are used both as antiseptics and dyestuffs in Japan. The plants have to grow for 5-7 years before a yield of 1-2% shikonins is reached in the roots. Previously about 10,000 kilos of roots were extracted each year giving some 150 kilos of shikonins now almost half of this amount is produced by plant tissue culture. The only other example of a natural product which is produced commercially using plant tissue culture is the antibacterial alkaloid berberine.[19] The reasons for the lack of commercial exploitation of plant tissue culture are not technological - plant cells can now be cultivated on a large scale (30,000 litres) for several months aseptically. The problem is fundamentally a biological one, in that plant cells in culture rarely produce

compounds in levels comparable to those produced in the field grown plant *(see section 1.4.4)*.

Section 1.4.2.3 Applications of plant tissue culture:
In the study of growth and metabolism

As cultures are grown under easily defined conditions it is a simple matter to measure the effect of varying particular nutrients, vitamins and hormones on growth processes such as cell division and differentiation. e.g. White's (not the present author!) work on tomato cultures.[20] Similar studies can provide information on aspects of primary metabolism such as DNA-, RNA-, and protein-synthesis.[21]

Most studies are concerned with natural-product synthesis by plant tissue cultures as this has great commercial potential as mentioned above. Research has focused mainly on ways of improving yields of these products by means akin to those already described for studying growth. Relatively little work has investigated the biosynthesis of secondary metabolites in plant tissue cultures and yet it is through a better understanding of these pathways that the commercial potential of cultures will probably (if at all) be realised. Cultures have been used to study certain aspects of secondary metabolite enzymology.[22] In fact, our understanding of phenylalanine ammonia lyase (PAL), a key enzyme in phenylpropanoid metabolism, comes largely from experiments with parsley cultures.[23] By noting the reactions that occur when certain chemicals are fed to cultures i.e. by observing biotransformations, one is able to explore indirectly the regio- or stereo-specificity of some of the reactions of biosynthesis.[24] Cell-free extracts of cultures can similarly be used.[25]

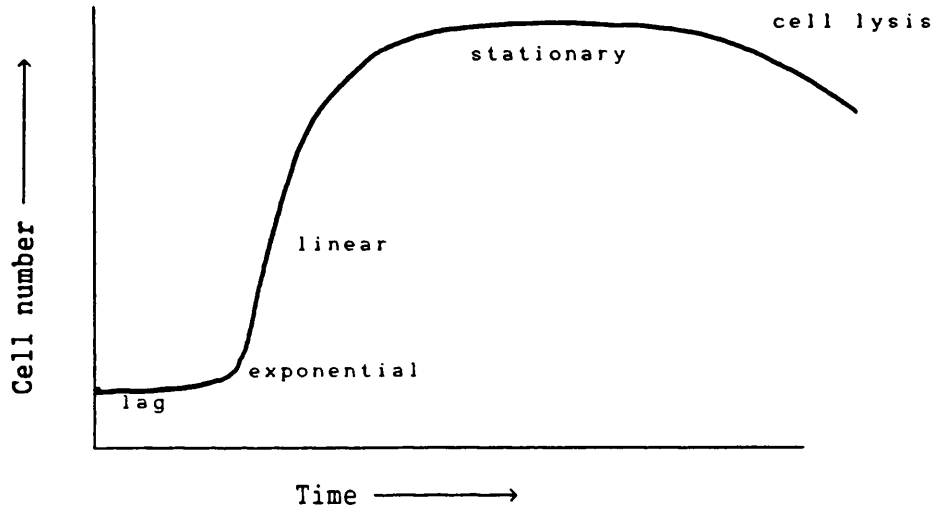
Section 1.4.3 Techniques of plant tissue culture[26]

Plant tissue cultures must always be handled under sterile conditions since plant cells grow much more slowly than microorganisms yet tissue culture media appeal equally to both. The first step in the establishment of a culture is the sterilisation of the plant material. This usually takes place in two stages: a pre-sterilisation in ethanol (about 30 seconds) followed by shaking in sodium hypochlorite (3% w/v). It is preferable to use young tissues rather than mature material, and the exact nature of these is dependent upon the type of culture being initiated e.g. root, shoot, etc. Germination of sterilised seeds under aseptic conditions will often provide more suitable tissue.

The sterilised explant of plant tissue is then placed onto a nutrient medium contained in a conical flask. This transfer should be carried out in a lamina air-flow cabinet. Such cabinets provide a purging stream of ultrafiltered air enabling manipulations to be made in a sterile environment. The instruments used (scalpel, spatula, etc) are sterilised in a flame or using a hot-bead steriliser. The media, contained in flasks plugged with cotton wool and capped with aluminium foil, is sterilised in an autoclave before use. After transfer the flask is resealed and left to develop callus tissue in an incubator under controlled conditions of light and temperature. When sufficient callus has developed (usually one to four weeks) it is excised from the plant material and transferred onto fresh medium to sustain growth. This process of subculture can be continued indefinitely provided infection or regeneration to the plant are prevented. It serves two key purposes: to provide a fresh supply of nutrients and to remove toxins secreted into the medium. A typical growth curve illustrating the cycle undergone by most batch cultures is shown in

figure 1.7. Since all cells are not in equivalent environments, diffusion gradients of nutrients form. Consequently cells exist in a variety of states of growth and differentiation.

Figure 1.7. A generalised growth curve for plant tissue culture



Suspension cultures can be initiated from callus cultures by placing callus in a liquid medium. This is then shaken continually to disperse and aerate the callus. Suspension cultures may be subcultured using a pipette fitted with a wide-bore sterile tip. After several such subcultures fine suspensions consisting of individual or small aggregates of cells can usually be obtained. As most of the cells are in close proximity to the nutrient supply, less pronounced gradients than those found in callus cultures exist. As the solution becomes depleted of nutrients all cells suffer equally. Thus the timing of subculture is more important than for callus cultures - as too is the inoculum size. Suspension cultures grow more rapidly than callus cultures and hence after several cycles (unless special measures are taken) the culture will consist of those cells which grow most rapidly

which are not necessarily those which produce the most secondary metabolites.

Section 1.4.3.1 Techniques of plant tissue culture: Tissue culture media

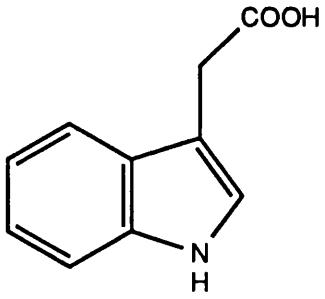
It is necessary for the medium to contain components which meet all the nutritional requirements of the culture. For convenience, media may be considered as containing two types of components: the inorganic salts and the organic constituents.

Plant tissue cultures have salt requirements similar to those of the plant, and since these are similar for most plants only a few mixtures are used. e.g. those of Murashige and Skoog,[27] of White,[20] and of Heller.[28]

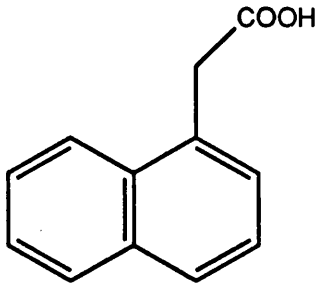
A great variety of organic constituents are used. Since most cultures are not autotrophic a carbon source has to be incorporated into the medium. Many potential sources have been tested including: glucose, sucrose, fructose, maltose, starch, molasses and sorbitol. Of these glucose and sucrose seem to support growth best. Thiamine (Vitamin B_1) is a critical factor for most cultures. Other vitamins are sometimes included e.g. pyroxidine and nicotinic acid. A nitrogen source is frequently added e.g. L-amino acids and ammonium salts. The most critical organic constituents are the growth regulators (phytohormones). Either an auxin (NAA and IAA) or cytokinin (kinetin and BAP), or more usually both, are needed for callus growth. The optimum concentration and type of auxin and/or cytokinin varies from one species to another. The synthetic growth regulator, 2,4-D, acts as both an auxin and cytokinin.[29] Some common growth regulators are shown in figure 1.8.

Undefined natural mixtures such as coconut milk, protein hydroxylates, yeast and malt extracts are often added to the medium to

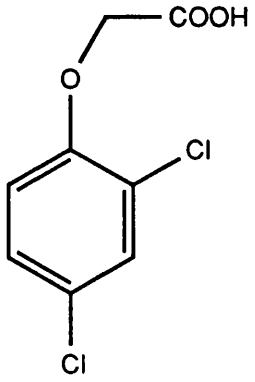
Figure 1.8 Some common growth regulators



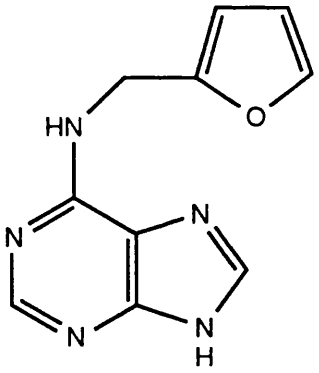
IAA



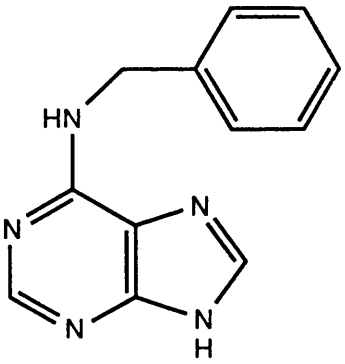
NAA



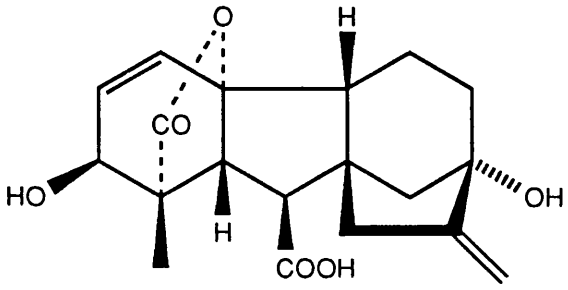
2,4–D



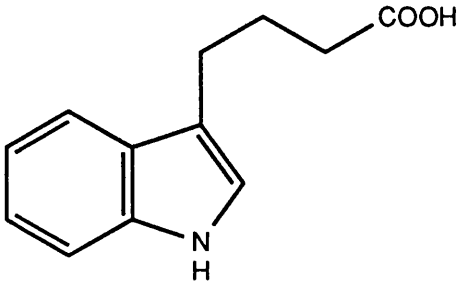
Kinetin



BAP



(+)-Gibberellic acid (GA_3)



3-Indolebutyric acid (IBA)

promote growth. The use of these mixtures is to be avoided as an important feature of plant tissue culture is the ability to define the conditions under which cultures are grown. As new growth regulators are discovered, possibly from examining these mixtures, the use of undefined mixtures should diminish. Even when it is necessary to initiate cultures using these mixtures, the latter can frequently be omitted at later subcultures with little adverse affect.

Ready-made nutrient mixtures are commercially available. These greatly simplify media preparation and are supplied with instructions. Such advances aid the reproducibility of results.

Section 1.4.3.2 Techniques of plant tissue culture: Variation of illumination and temperature

The intensity, quality and duration of illumination is known to affect both the growth of, and secondary metabolite production in, plant tissue cultures. Chloroplasts may be formed especially when a photoperiod is used, although chlorophyll synthesis and photosynthetic carbon fixation are known to be inhibited by sucrose.[30] The synthesis of flavonoids in parsley (*Petroselinum hortense*) cultures is influenced by light. As long as these cultures are kept in darkness cells multiply but do not form flavonoids. Once exposed to light the glucoside apiin can be detected. In these cultures the syntheses of PAL and UDP-apiose synthetase, which are two key enzymes in flavonoid formation, are under photo-control.[31] Monoterpenoid production by *Pelargonium* cultures is also affected by the photoperiod.[32] A further example is provided by experiments carried out on *Haplopappus gracilis* cell cultures - irradiation for forty eight hours with blue light resulted in a 400 percent rise in enzyme activity, whereas irradiation with far-red light had no effect.[33]

The temperature at which cultures are maintained is probably just as important as the light conditions, although this has received little investigation and most cultures have been incubated at 25°C.

Finally, it is interesting to compare present day techniques with those used by Haberlandt. In his pioneering studies at the turn of the century Haberlandt was able to maintain mesophyllic, (rather than the more preferable meristematic) plant cells on a simple nutrient medium which contained no vitamins or hormones, using only primitive precautions to guard against bacterial and fungal infection. Furthermore, Haberlandt predicted correctly that embryo sac fluids (e.g. coconut milk) would have a stimulative role for cell growth. The most significant aspect of Haberlandt's discussion is his statement that he strongly believed artificial embryos could be cultivated from vegetative cells - what has now come to be known as "totipotency".

Section 1.4.4 Secondary metabolism in plant tissue culture

In the discussion of secondary metabolism in culture three types of behaviour can be identified: accumulation of at least some metabolites of the whole plant; no accumulation of secondary metabolites; and accumulation of novel secondary metabolites. It is common to find either no accumulation or low levels of accumulation of secondary metabolites in plant tissue cultures and it is the possible reasons and solutions to this on which this discussion will concentrate.

On use of the strategy outlined in section 1.4.2.2 one will, more often than not, obtain cultures which accumulate low levels of secondary metabolites. Cultures are in theory totipotent - that is they retain all the genetic information to regenerate the whole plant. Such regenerated plants have similar activities to the parent plant

and hence the low yields observed in culture could be due to a lack of gene expression under these conditions.

One possible reason for the low levels of secondary metabolites accumulated by cultures is that cultures are unable to store the secondary metabolites they produce, unlike the parent plants which contain special organs (e.g. glandular hairs to store monoterpenoids). The absence of such structures may repress by means of autotoxicity accumulation of the compounds. This has recently been observed for *Pelargonium* cultures.[34] It follows that cells producing zero or low levels of such compounds may be selected - unknowingly - instead of active lines. This theory is supported by work performed on *Atropa belladonna*: root differentiating calli are capable of producing tropane alkaloids, whilst non-differentiating calli are not.[35] This link between degree of differentiation and ability to accumulate secondary metabolites has been made by several authors.[36] It is now generally thought that the greater the morphological differentiation the greater the yield of secondary products and indeed in the intact plant secondary products normally appear in cells which have undergone cell expansion and differentiation rather than in meristematic tissue. Interestingly, small electric currents (1-2μA) have recently been used to induce differentiation in tobacco tissue cultures, although any changes in secondary metabolism were not reported.[37] Although it is thought by many workers that secondary products are formed as a consequence of differentiation there are a number of examples where secondary product accumulation occurs in the absence of specific morphological structures.[38]

It is possible to immobilise plant cells on inert supports (e.g. polyurethane sponges). This is claimed to increase the production of secondary metabolites.[36,32] In these immobilised cells there is a high

degree of cell to cell contact. Physical and chemical gradients similar to those in the intact plant are encouraged and may act to "switch-on" secondary metabolism.

Accumulation might not occur because of competition between the synthetic and degradative enzymes of secondary metabolism. Epoxidases and hydratases which can degrade terpenoids are known to exist in plant tissue cultures.

Bohm in his interestingly titled paper "The inability of plant cell cultures to produce secondary products"[39] concludes that "The lack of specific enzymes represents the most important reason for the absence of secondary metabolite formation". There is a lot of evidence against this proposition: cell-free extracts are capable of performing multistep reactions. Thus, cell-free extracts of *Rosmarinus officinalis*[40] (rosemary), prepared by three different techniques, could sustain biosynthesis of monoterpenoids from IPP. Also, enzyme activities some 300-fold greater than those that could be extracted by the same techniques from the parent plant were found. Importantly, the original cultures did not accumulate or secrete detectable amounts of monoterpenoids.

Secondary metabolism is fully integrated into the developmental programme of the plant, and is thus strictly controlled. Hence, secondary pathways may only be expressed at certain stages of plant development. In plant tissue cultures an inverse relationship between growth and secondary metabolite accumulation is often found with accumulation (and differentiation) at its greatest near the end of the growth cycle (see reference 17 for examples of culture-lines which follow this relationship as well as of ones which do not). The primary and secondary pathways always compete for common substrates and during growth there appears to be an inhibition of the latter pathway.

Researchers, largely interested in cultures for commercial reasons, have added to our otherwise limited knowledge of the regulatory systems involved by attempting to effect a "switch" from primary to secondary metabolism in order to improve yields. Inhibition of protein and RNA synthesis using streptomycin sulphate causes such a switch.[41] It is possible to follow the switch from primary to secondary metabolism by the use of radioactive precursors. Phillips and Henshaw[42] have successfully switched from protein synthesis (primary metabolism) to the synthesis of polyphenols (secondary metabolism) by manipulating the phosphate levels in suspension cultures of sycamore (*Acer pseudoplatanus* L.) and have followed this switch using labelled phenylalanine which is metabolised by phenylalanine ammonia lyase (PAL), an enzyme common to both pathways. Nutrients, growth regulators and nitrogen levels also effect this switch. A similar study using cell cultures of *Catharanthus roseus* noted that the increased formation of indole alkaloids was accompanied by a 10-fold increase of trytophan decarboxylase (TDC), an enzyme connecting primary and secondary pathways.[43]

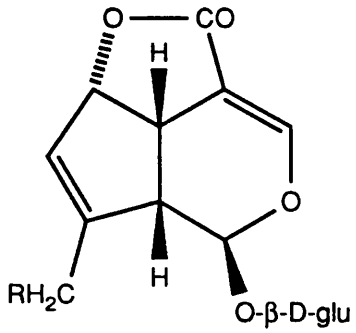
Until quite recently, investigations of secondary metabolism in plant tissue culture were chiefly concerned with establishing high producing cell lines more or less by chance. Phytoalexins, which are secondary metabolites produced by cultures in response to either a real or simulated attack by fungi or bacteria demonstrate that dormant or repressed cells can be stimulated to produce secondary metabolites.[44] Preceding this, increases in specific m-RNA activity and subsequent increases in specific enzyme levels have been demonstrated. It is by studying examples of gene expression such as these which will, hopefully anyway, lead to a greater unravelling of the regulatory controls of secondary pathways.

Section 1.5 **Galium verum : a short description**

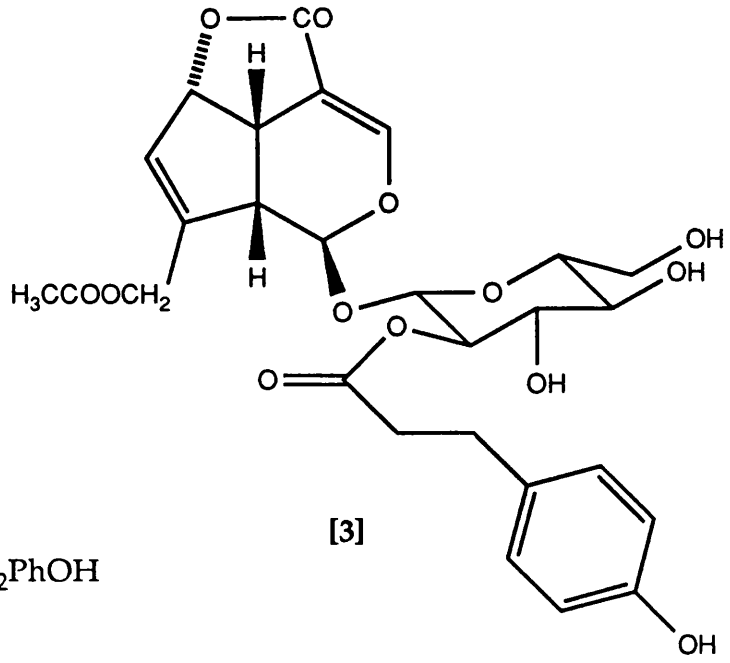
Galium verum L., the herb used in this study, is an erect herbaceous plant that is a native of Great Britain and blooms in July and August. This herb is frequently seen growing wild by the roadside in waste land from the coast to the hills. It is a member of the Rubiaceae (madder) family which contains over three hundred and fifty genera and in excess of five thousand species distributed mainly in tropical regions.[45] Of those that do grow in the northern part of Europe a large proportion of these possess tinctorial properties.[46] The term Galium is derived from the Greek word "gala" which means milk - the early Greeks strained milk through linen containing flowers of the plant to impart a rich colour to the milk. Furthermore, most of the members of this genus are able to curdle milk. The rich colour of Cheshire cheese was originally derived from flowers of *Galium verum* whilst the roots furnish a dye which has been used in the Hebrides for dyeing woolstuffs red. In Tudor times the flowers were used to dye hair yellow and it is probably from this that the common English name of this plant "Lady's Bedstraw" originates, alternatively it may be derived from the Christian legend that this was one of the cradle herbs used in the manger at Bethlehem.

Medicinally, the plant has been used as a popular remedy in stone and urinary diseases, as well as for epilepsy and internal bleeding.[47,48] In the curiously titled "Leechdom's Wortcunning and Star Craft of Early England" the use of a number of species of the genus Galium for dyeing and medicinal purposes is described.

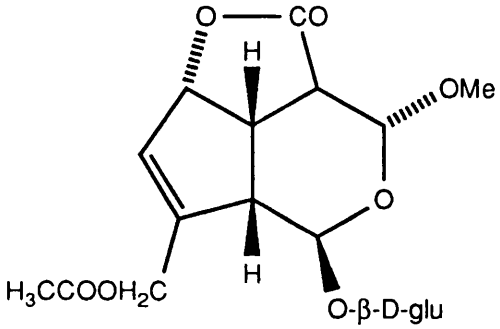
The chemical composition of *Galium verum* is now quite well established with a number of iridoids,[49-54] flavonoids[50] and quinones[55-59] having been isolated. One of the chief components of the plant is the iridoid glycoside asperuloside [1] which was first



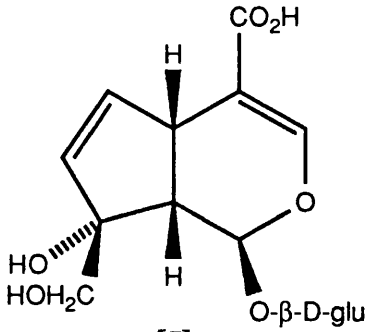
[1] R = $OCOCH_3$
[2] R = $OCOCH_2CH_2PhOH$



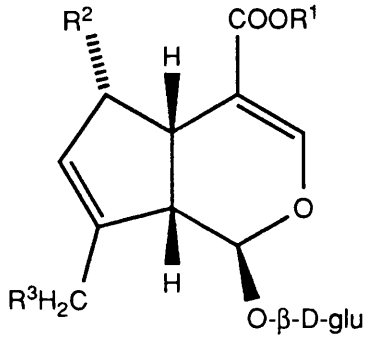
[3]



[4]



[5]

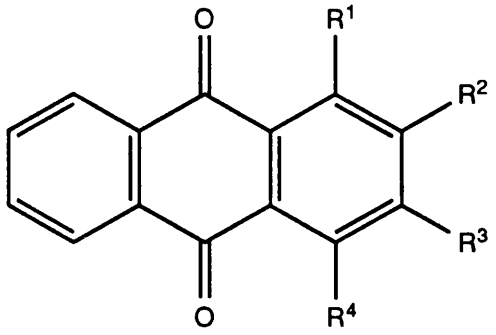


	R^1	R^2	R^3
[6]	H	β-OH	OH
[7]	H	β-$OCOCH_3$	OH
[8]	H	H	OH
[9]	H	α-OH	$OCOCH_3$
[10]	H	α-OH	OH
[11]	CH_3	β-OH	OH
[12]	CH_3	H	OH
[13]	CH_3	α-OH	$OCOCH_3$
[14]	CH_3	α-OH	OH

detected in the aerial parts of the plant in flower by Hérissey in 1926.[49] Since then a total of fourteen iridoids have been detected namely, the so-called V_1 [2], V_2 [3] and V_3 [4] iridoids, monotropein [5], scandoside [6], 6-acetyl-scandoside [7], geniposidic acid [8], asperulosidic acid [9], deacetylasperulosidic acid [10], scandoside methylester [11], geniposide [12], daphylloside [13] and deacetyl-daphylloside [14]. It has been suggested that a number of these are artifacts produced by the hydrolysis or methanolysis of asperuloside [1] during extraction.[60] Nonetheless some workers have reported the presence of two of these suggested artifacts (Daphylloside [13] and deacetylasperulosidic acid [10]) even when the extraction is carried out using acetone (in which asperuloside [1] is stable[51]). Others[53] believe the methanolysis of asperuloside [1] occurs too slowly to form these artifacts in significant amounts during the course of extraction.

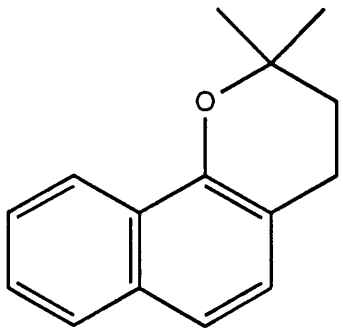
Although the roots of *Galium verum* have been used for dyeing purposes the pigments received little attention apart from the work of Hill and Richter[55-57] (who identified glycosides of pseudopurpurin [27], rubiadin [24] and alizarin [17]) prior to that of Burnett and Thompson in 1968.[59] In this study which examined the occurrence of anthraquinones and related naphthalenic compounds in *Galium* spp. and *Asperula odorata* two naphthalenic {[28] and [29]} and thirteen anthraquinones {[15] to [27]} were isolated from the roots of the plant and characterised. The coexistence of these two types of quinones provided early evidence for the biogenesis of some anthraquinones from shikimate and mevalonic acid.

A number of flavonoids have been isolated from the aerial parts of the plant.[50] Two of these have been characterised as palustroside [30] (diosmetin-O-β-D-glucopyranosyl-(6⟶1)-O-α-L-arabopyranoside) and

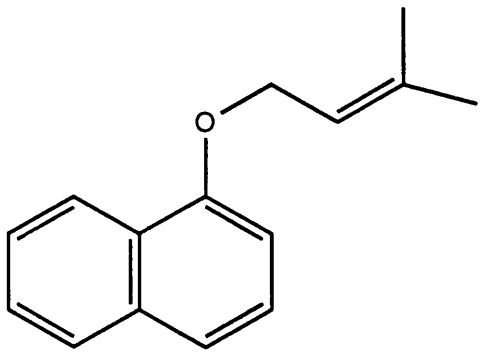


	R^1	R^2	R^3	R^4
[15]	H	OH	H	H
[16]	H	OCH_3	H	H
[17]*	OH	OH	H	H
[18]	OH	CH_3	H	H
[19]	OCH_3	OH	H	H
[20]	OH	OCH_3	H	H
[21]	OCH_3	CH_3	H	H
[22]	OH	H	OH	H
[23]	OH	CH_2OH	OH	H
[24]*	OH	CH_3	OH	H
[25]	OCH_3	H	OCH_3	H
[26]	OH	OH	H	OH
[27]*	OH	OH	CO_2H	OH

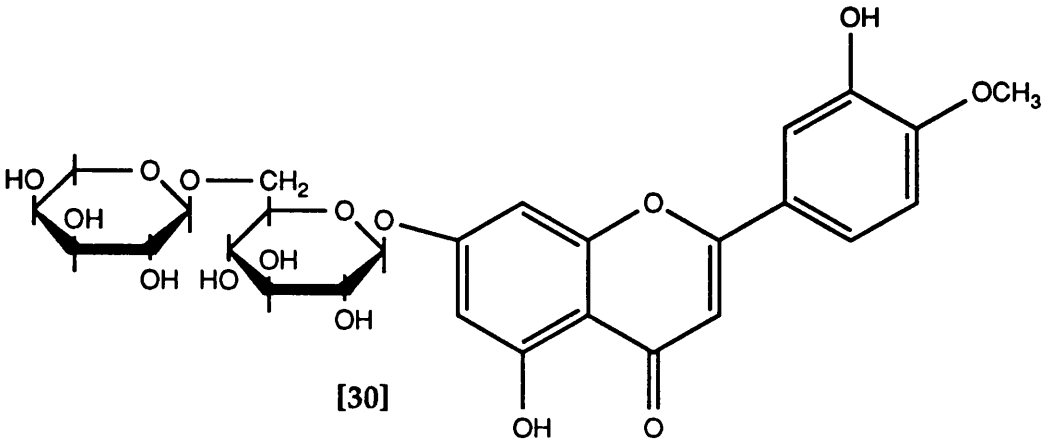
* Present both free and as a glycoside



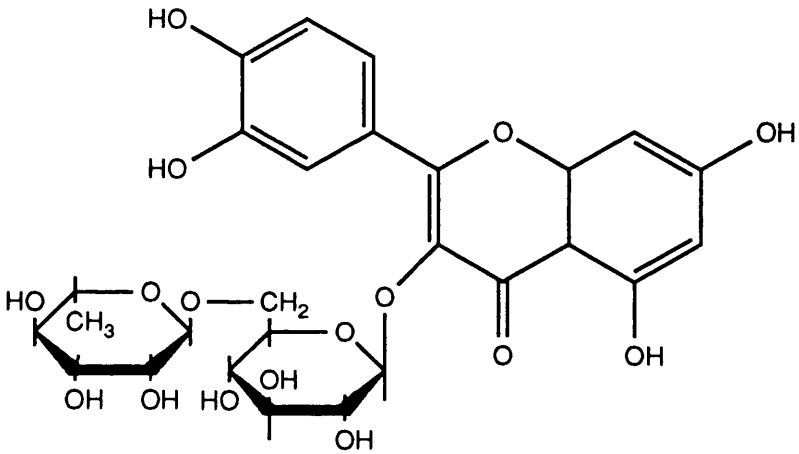
[28]



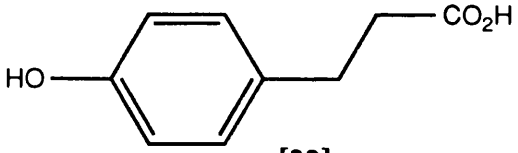
[29]



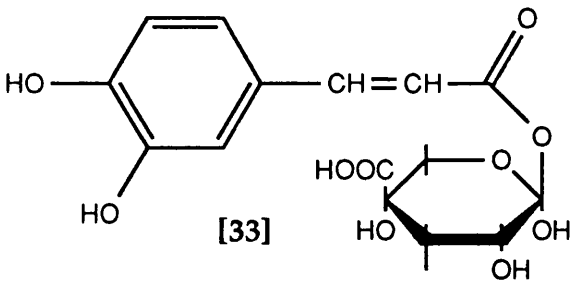
[30]



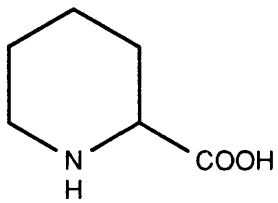
[31]



[32]



[33]



[34]

the widespread quercetin glycoside rutin [31] and are thought to be the major colouring substances of the flowers.

Two phenylpropanoids phloretic[61] [32] and chlorogenic[50] [33] acids have been isolated. The former of these is the first reported isolation in the plant world of the free form of phlorizin. However, the extraction was made using boiling benzene followed by boiling ethanol and it may be that this is a decomposition product (for example of iridoid V_1 [2]). Finally pipecolic acid [34] (piperidine-2-carboxylic acid) has also been isolated.[62]

Section 1.6 Reasons for studying tissue cultures of Galium verum

The herb *Galium verum* (L., Rubiaceae, Lady's Bedstraw) is known to produce anthraquinone pigments. The aims of this work are to establish cultures of this plant, identify any pigments produced and to investigate some of the factors (in particular light conditions and phytohormone type and level) which affect the type and level of any pigments produced.

Cultures have been raised from *G. verum* before but these studies made no attempt to identify the pigments formed.[63,64] Although secondary products are usually produced in small amounts in cell cultures, anthraquinones are often produced in large amounts.[65] This is surprising since they originate from very different biosynthetic precursors, e.g. acetate or phenylalanine, tyrosine and/ or mevalonic acid. It has been noted that cultures of a closely related species, *G. mollugo*, contain anthraquinones but no iridoids and yet the plant itself contains both.[66] Iridoids are derived from mevalonic acid and sugars which also contribute to anthraquinone biosynthesis. The addition of *o*-succinylbenzoic acid (a known precursor of shikimic acid-derived anthraquinones - see figure 1.5) to these cultures

stimulated anthraquinone formation suggesting that mevalonic acid and sugars are present at non-limiting levels. A further aim of this study is to identify any iridoids produced in cultures of *G. verum* in addition to the main aim which is to identify any pigments formed.

CHAPTER TWO: GENERAL EXPERIMENTAL METHODS

Outlined in this chapter are general details of the spectroscopic and chromatographic methods used in chapter three. More specific experimental details (including experimental results) are described, section by section, towards the end of this thesis (pages 184-225).

Section 2.1 Chromatography

The analysis and purification of most of the compounds encountered in this study was carried out using thin-layer chromatography, column chromatography and high performance liquid chromatography.

Section 2.1.1 Thin Layer Chromatography (TLC)

TLC was used both as an analytical method and as a preparative method of purification. A variety of solvent systems were employed and these are described in the experimental accompanying each relevant section.

Section 2.1.1.1 Analytical TLC

Plastic-backed TLC plates pre-coated with silica gel 60 were utilised for analytical work (ex. Camlab Ltd, Cambridge). Plates were cut prior to use with a scalpel to a size appropriate to the number of samples being investigated at any one time. Samples were applied as individual spots at the baseline, about 1cm above the edge of the plate, with a microlitre syringe. Usually, samples of approximately 1μl of a 10% solution in an appropriate solvent were applied to the plates, care being taken to ensure that the spots were as small as possible. Plates were eluted in appropriate solvents at room

temperature in tanks lined with filter paper to aid equilibration of solvents.

After development compounds which could not be detected by eye were located by spraying plates with a 10% (w/v) solution of phosphomolybdic acid in ethanol. After heating at 100°C for about 5 minutes, compounds appeared as blue spots on a yellow background. Where applicable, compounds detected by TLC were provisionally identified by comparison of R_f values with those of authentic samples. The R_f of a compound is defined as: $\frac{\text{distance moved by compound}}{\text{distance moved by solvent front}}$

Section 2.1.1.2 **Preparative TLC**

Glass plates, 20x20cm, coated with a 1000μm thick layer of silica gel (60Å pore size, 10-12μm particle size) were used (ex. Whatman Lab. Supplies Ltd., Maidstone, Kent). Plates were washed with methanol prior to use - although the plates were used shortly after purchase this washing was found to remove a large amount of extraneous matter. Samples were dissolved in methanol, applied as narrow bands and then the plates developed with the appropriate solvent mixture.

As preparative-TLC was used only for the purification of coloured compounds, the location of bands containing the required compounds could be achieved by eye without recourse to spray reagents. The coloured bands were scraped off using a spatula (this procedure was carried out in a fume cupboard) and the pigments eluted from the silica gel by washing with redistilled methanol. Typically, 100mls of solvent was used per band scraped. Scraping the bands whilst the silica gel was still damp was found to limit greatly the hazard due to silica dust.

Section 2.1.2 Column chromatography

Column chromatography was used as a preliminary method of purification with both silica gel (60-80 mesh) and Sephadex LH-20 being employed as adsorbents.

Glass columns of various lengths and diameter were used with a sintered glass disk (number 3 porosity) at the base, a tap and a capillary outlet of low dead volume. Columns were packed by standard procedures using a thin slurry of the adsorbent in the eluting solvent. When Sephadex LH-20 was used as adsorbent it was pre-swollen with methanol (the eluting solvent) overnight.

Samples dissolved in a small volume of the eluting solvent, were applied to the top of the column by means of a capillary pipette. The column flow rate and the volume of the fractions collected depended on the resolution of the column. TLC was used to analyse fractions eluted by column chromatography.

Section 2.1.3 High Performance Liquid Chromatography (HPLC)

HPLC was found to be the most powerful of all the chromatographic methods employed in this work. The HPL-chromatograph used in this study consisted of a Gilson binary gradient system (two model 303 pumps, model 802C manometric module and a model 811 dynamic mixer) controlled by Gilson 714 software run on an IBM PS/2 model 30 computer, connected to either an LKB model 2140 ultraviolet (UV) photodiode array detector or a Bischoff model 3110 refractive-index (RI) detector. An integrated chromatogram for data collected using either of the two detectors was produced using the Gilson 714 software. Data collected by the UV detector was further analysed using a Tandon computer running LKB Wavescan software.

Separations were achieved on both an analytical and preparative

scale using both normal- and reverse-phase columns. A variety of mobile phase solvent systems were employed and these are described in the experimental accompanying each relevant section. Analytical and preparative scale separations were performed using flow rates of 1 and 4.9 mls/min respectively. In all cases samples dissolved in the appropriate solvent were filtered through 0.2μm Millipore filters prior to injection. Reverse-phase HPLC was performed on an analytical scale using a column (250×4.6mm) packed with Spherisorb ODS2 (5μm particle size), whilst a larger column (250×10mm) packed with the same material was used for preparative-scale separations. A guard column (10x4mm) also packed with Spherisorb ODS2 was used to protect both of these columns from contamination by impurities present in either the solvents used or the sample injected. In normal-phase HPLC either an analytical column (250×4.6mm) packed with silica gel (Nucleosil-100, 5μm particle size) or a preparative column (250×10mm) packed with the same material was used. These two columns were protected by a guard column (50x4.6mm) packed with Nucleosil-100.

Section 2.2 Spectroscopic techniques

Section 2.2.1 Mass spectrometry

Section 2.2.1.1 Electron impact (EI) mode

Mass spectra were routinely measured in the EI mode using an electron beam energy of 70eV and were compared, by computer, with those spectra held on the database of the National Bureau of Standards.

It is possible to record the variation in the intensity of a given ion with time as it is detected by the mass spectrometer - i.e. to obtain an ion profile. Such measurements were taken in this work at six second intervals for the first few (up to ten) minutes after a

sample was introduced into the spectrometer. If the profiles of two ions are superimposable, or nearly so, then this suggests that they are derived from the same molecule. Conversely, when a sample containing a mixture of compounds is analysed by this method then (unless the components of this mixture have very similar thermal volatilities) the profiles of some of the ions in the mass spectrum will not be superimposable. Hence the ion profiles of the major ions in the mass spectrum of a sample were routinely inspected using this technique (known as linked scanning) to confirm sample purity. Linked scanning cannot be used in conjunction with either chemical ionisation or fast atom bombardment techniques (see below) as the ions produced by these procedures possess, in general, very similar thermal volatilities.

Section 2.2.1.2 Mass spectrometry: Chemical ionisation (CI) mode

This modified form of the EI procedure is one of the so-called "soft" ionisation methods and results in little fragmentation of the sample being analysed. Hence it is useful in establishing the molecular mass of an unknown. In CI mass spectrometry the sample is introduced at about 1 torr pressure with a carrier gas - ammonia in this work. The ammonia is ionised by electron impact to form primary ions which react with excess of ammonia to give secondary ions:

$$NH_3 + e^- \longrightarrow NH_3^{+\cdot} + 2e^-$$

$$NH_3^{+\cdot} + NH_3 \longrightarrow NH_4^+ + NH_2^{\cdot}$$

$$NH_4^+ + NH_3 \longrightarrow NH_4^+NH_3$$

which react with the sample (M):

$$\text{e.g.} \quad M + NH_4^+ \longrightarrow [M + H]^+ + NH_3$$

$$\text{or} \quad M + NH_4^+ \longrightarrow [M...H...NH_3]^{+*} \xrightarrow{NH_3} [M + NH_4]^+ + NH_3^*$$

As these (M + 1) and (M + 18) ions are chemically produced, they do not have the great excess of energy associated with ionisation by electron impact, and they therefore undergo less fragmentation. Some compounds also undergo a substitution reaction of the type:

$$M + NH_4^+ \longrightarrow [M + NH_4 - HX]^+ + HX$$

the net effect of which is the replacement of a substituent X by NH_3. For example, in alcohols the substitution ion formed corresponds to $[M + NH_4 - H_2O]^+$ and is isobaric at low resolution with $M^{+\cdot}$. Although the relative intensity of ions formed by the above processes is dependent on the gas pressure and a combination of electronic and steric effects in the substrate, a CI spectrum obtained using ammonia as the reagent gas usually contains the pseudo-molecular ion as the base peak, in addition to a small $(M + 18)^+$ peak and other small peaks derived from substitution reactions. These latter peaks are helpful in establishing which functional groups are present in the molecule. The reactions, mechanisms and applications of ammonia CI mass spectrometry are covered in an excellent review by Westmore and Alauddin.[67]

Section 2.2.1.3 Mass spectrometry: Fast atom bombardment (FAB) mode

CI mass spectrometry is usually able to provide a mass spectrum of samples of medium to high thermal volatility (i.e. non-polar molecules of molecular weights of up to 1000 daltons or medium polarity molecules of molecular weight up to 300 daltons), whereas FAB mass spectrometry is useful for samples of low thermal volatility such as polar molecules of molecular weight up to 200 daltons or molecules of high molecular weight (800-10,000 daltons). In this procedure the

sample is dissolved in a few microlitres of a liquid matrix of low volatility and the solution is then bombarded by a beam of fast xenon atoms. In this work either thioglycerol or *m*-nitrobenzoic acid were used as matrices. When the fast xenon atoms impact into the solution of the sample in the matrix, the sample is desorbed, often as an ion, by momentum transfer. Ionisation by translational energy minimises the amount of vibrational excitation and this results in less destruction of the ionised molecules. The polar solvent promotes ionisation and allows diffusion of fresh sample to the surface. The beam of sample ions is analysed in the usual way. Adduct ions such as $[M + H]^+$ are usually seen rather than the molecular ion itself. The interpretation of FAB spectra are discussed in detail by Watson.[68]

Section 2.2.2 Nuclear magnetic resonance (NMR) spectroscopy

Section 2.2.2.1 ^{1}H NMR spectroscopy

^{1}H NMR spectra were acquired on either a Varian VXR400 or Jeol GSX FT500 machine (^{1}H resonances at 400 and 500 MHz respectively) over a wide spectral width (δ = -1 to 16ppm) using d-chloroform as solvent. It was necessary to use high-grade d-chloroform (minimum isotopic purity 99.96 atom %D, ex. MSD Isotopes Inc., Montreal, Canada) as the sample size was small (typically < 0.5mg). For a number of samples d_6-benzene (99.5 atom %D, ex. Aldrich Chemical Company Inc., Gillingham, England) and/or d_4-methanol (99.8 atom %D, ex. Aldrich Chemical Company Inc., Gillingham, England) were also used.

Section 2.2.2.2 ^{1}H NMR spin simulation experiments

Signals in the aromatic region of the ^{1}H NMR spectra of most of the pigments isolated in this work were second order and could not easily be interpreted by inspection. A computer program (written by

Varian Associates Inc., Palo Alto, California for use with the VXR400 spectrometer and based on the FORTRAN program LAME) which could include second-order effects was used to simulate the spectra of coupling systems occurring in this region. The program required four key pieces of information (i) the type of spin system to be simulated (e.g. ABCD), (ii) the frequencies of the lines forming this system in the observed spectrum, (iii) estimated values of the chemical shifts of the protons in the system and (iv) the estimated values of the coupling constants. The program first calculates the root mean square error between the observed spectrum and one simulated using estimated values of the chemical shifts and coupling constants for the designated type of coupling system. The program then makes small changes to both the chemical shifts and coupling constants used to generate the simulated spectrum and produces a new calculated spectrum. This procedure is repeated automatically until the root mean square error between the observed and calculated spectra is minimised. A comparison of the values of the chemical shifts and coupling constants obtained from the simulation program with those in correlation tables enabled the (partial) structure of a number of the pigments to be elucidated. These conclusions could not be made simply by inspection of the observed spectrum as second-order effects were significant.

Section 2.2.2.3 ^{13}C NMR spectroscopy

These spectra were routinely obtained using d-chloroform as the NMR solvent with either broadband decoupling of the proton region or by performance of an "Attached Proton Test" (APT) experiment.[69,70] In this latter technique (also known as J-modulated spin echo spectroscopy) the proton decoupler is turned off during a short period

τ. If τ is chosen to be equal to $1/J(^{1}H-^{13}C)$ then analysis of the magnetisation in the rotating frame shows that CH and CH_3 signals will be aligned along the -X axis while CH_2 and quaternary signals will be along the +X axis. Although this procedure does not distinguish between CH_3 and CH or between CH_2 and quaternary carbon peaks this partial information when combined with chemical shift (and intensity) values often suffices to characterise the signals.

Section 2.2.2.4 Quantitative ^{13}C NMR spectroscopy

Quantitative or semi-quantitative ^{13}C NMR spectra (i.e. ones where the area of a signal is proportional, or nearly so, to the number of carbon atoms causing that signal) are desirable in structural determinations, where it is clearly useful to know if a signal is due to more than one shift-equivalent carbon. Broadband decoupled spectra are not usually quantitative. There are two reasons why this is so. Firstly, carbon atoms with long relaxation times may not be able to return to an equilibrium distribution between pulses. Secondly, there is a large variation in the nuclear Overhauser effect (nOe) enhancement experienced by carbon atoms.

Three methods were employed in this work in an effort to obtain quantitative broadband-decoupled spectra: gate decoupling, long pulse delays, and the addition of paramagnetic relaxation reagents. Inverse-gated decoupling can be used to create a more quantitative spectra by minimisation of the nOe enhancement for all carbon atoms.[70] In this experiment the decoupler is turned on only during the pulse and for the period of the acquisition time. Since the free induction signal decays quickly in an exponential fashion, while the nOe factor slowly builds up in an exponential fashion, a proton-decoupled ^{13}C spectrum is obtained without nOe enhancement of signals. Furthermore

the use of longer delays than usual between pulses can significantly reduce signal area discrepancies caused by longer relaxation times. Alternatively, paramagnetic relaxation reagents (e.g. $Cr(acac)_3$ which was used in this work) may be added to the sample. These reagents reduce all of the spin-lattice relaxation times and level-off all of the nOe factors. The obvious disadvantage of this method is that if the sample is to be recovered then the added reagent has to be removed by chromatography.

Section 2.2.3 Infrared (IR) spectroscopy

Spectra were recorded to a resolution of one wavenumber using a Nicolet 205 FT-IR spectrometer over the range 4000 to 400 cm^{-1}. Samples were dissolved in ethanol-free chloroform and run as solutions in a microcell of pathlength 0.1mm.

Section 2.2.4 Ultraviolet-Visible (UV-VIS) spectroscopy

Spectra were recorded using a Perkin Elmer Lambda 16 UV/VIS spectrometer with the sample dissolved in benzene-free ethanol. A scan speed of 60nm/min, slit width of 0.5nm and pathlength of 1cm were used.

CHAPTER THREE: PIGMENT PRODUCTION BY TISSUE CULTURES OF GALIUM VERUM

Section 3.1 Effect of sucrose levels upon growth rate and pigment production: A brief study

Secondary metabolite accumulation and cell growth are both affected by the level of the carbon source (usually sucrose) contained in a plant tissue culture medium.[17] There is a general consensus of opinion that (within limits) increasing the sugar concentration results in a progressive increase in the accumulation of secondary metabolites with a corresponding decrease in callus growth (see reference 35 and references therein). Several studies performed on cultures of the Rubiaceae plants have found that, for levels of sucrose between 2 and 8% (w/v), anthraquinone accumulation and growth are inversely related with the former being at its lowest with low levels of sucrose (i.e. 2% w/v).[71,72] Another study,[63] however, has suggested (based on a detailed investigation of the anthraquinone production by cell cultures of 19 Rubiaceae species) that "There is absolutely no general pattern in the induction of secondary product formation within the plant cell cultures derived from members of one family and not even of the same genus" and hence that "Production media for secondary metabolites must be elaborated specifically for each individual plant species and probably even for subspecies and variants of one and the same species".

A brief study was undertaken in collaboration with Dr. J. Tampion of the Polytechnic of Central London on the above suggested correlations of growth, anthraquinone accumulation and sucrose levels using callus cultures of *Galium verum*. These cultures were readily initiated on a Murashige and Skoog medium supplemented with sucrose (3% w/v), NAA (0.5 mg/l), kinetin (0.25 mg/l), IBA (2.5 mg/l), GA_3

(2mg/l) and agar (1% w/v) and were stable on this medium with respect to both growth, callus morphology and pigment production - orange coloured pigments were produced spontaneously.

After five subcultures, callus material (of known mass) was transferred onto one of four different media each containing different levels of sucrose (3, 6, 9 and 12% w/v). After 21 days callus material was weighed, the pigments extracted exhaustively with a known volume of 80% ethanol and the UV-VIS spectrum of this crude extract recorded. The molar extinction coefficient of the anthraquinone alizarin [17](ε = 5,500$dm^3mol^{-1}cm^{-1}$ at 434nm) and the observed value of the $O.D._{max}$ in the region 400-450nm enabled the pigment concentration (expressed in μmol per gram of fresh weight) in the callus cultures to be calculated. Five culture flasks were used for each experiment (i.e. sucrose level) and the individual growth rates and pigment concentrations measured for each of these five were averaged to give mean values of both the growth rate and pigment concentration. This method for determining the pigment concentration in tissue cultures was originally used by Zenk[65] and is based on the small variation in values of the molar extinction coefficient for the band occurring at highest wavelength in the UV-VIS spectrum of anthraquinones of the Rubiaceae.

The dependence of both the mean growth rate and mean pigment accumulation on the concentration of sucrose contained in the culture medium are shown in figures 3.1 and 3.2 respectively. It can be seen quite clearly from these graphs that, within the range 3 to 12% sucrose, growth decreases as the concentration of sucrose in the culture medium is raised whereas pigment accumulation increases. These results agree well with the findings of Ibrahim[71] who investigated the effect of sucrose levels upon the accumulation of anthraquinones in

Figure 3.1 Effect of sucrose concentration upon growth of callus cultures of Galium verum

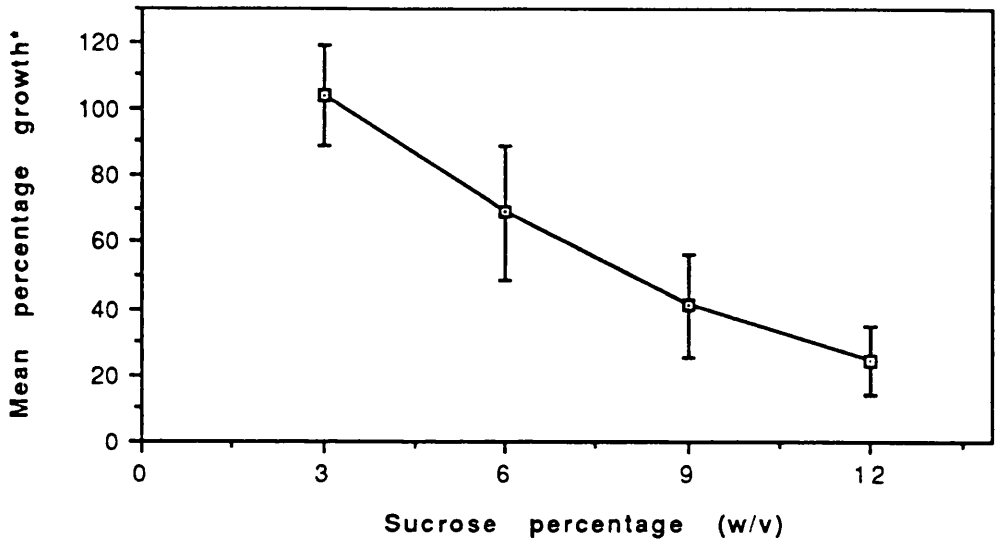
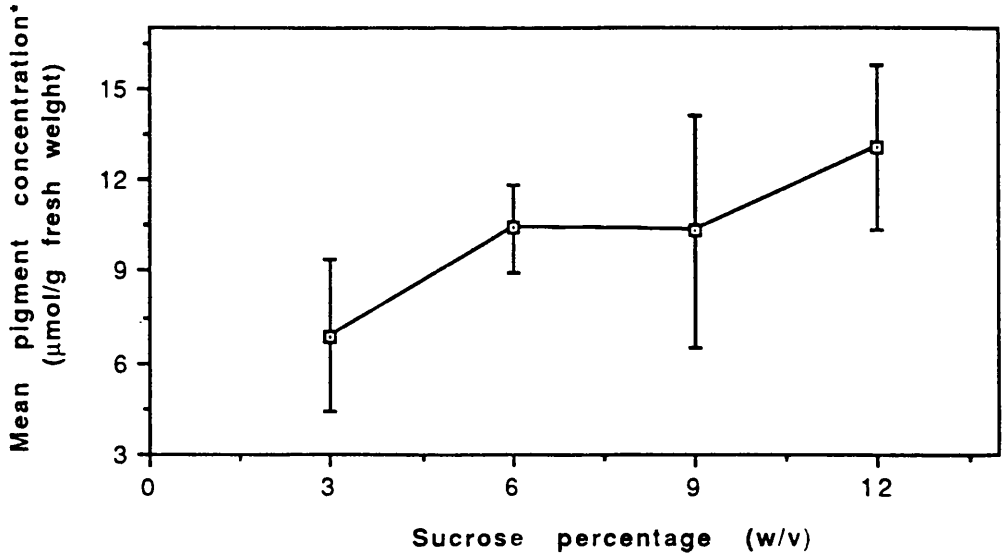


Figure 3.2 Effect of sucrose concentration upon pigment accumulation by callus cultures of Galium verum



* Error bars are calculated as the standard deviation of the individual values from the mean results

cultures of *Cinchona succirubra*. Each data point in figures 3.1 and 3.2 is the mean of the results for the individual flasks used in each experiment. There was a large variation in the amount of initial callus material used (0.12-0.66g) and the error bars associated with each data point in the two figures are affected by the magnitude of this variation since the amount of initial callus material will affect the measured values of both growth rate and pigment concentration. An improved procedure might be to use a greater number of culture flasks for each experiment (20 say, rather than 5) with a smaller mass range of callus material contained in each flask. The method used in this study to estimate pigment concentration assumes anthraquinones are the only compounds present in the cultures which absorb in the region 400-450nm. This is a possible source of error as other compounds, such as tannins (which are present in necrotic cells), might also be present. Obviously the slowest growing culture lines are also those which are most likely to contain such necrotic cells (and hence polyphenolics) and this may contribute to the high pigment concentrations recorded for these lines.

Section 3.2 Effect of illumination and hormone regimes upon pigment production

The procedure used in the previous section had been of some use in correlating anthraquinone accumulation with the level of sucrose contained in the medium, although the method is subject to error when other classes of pigment are present in the culture material. Hence, it was decided to undertake a more detailed study, the major aim of which was to characterise the individual compounds produced by callus cultures of *Galium verum* under various light and hormone regimes.

Section 3.2.1 **Initiation and maintenance of callus tissue cultures**

Tissue cultures were initiated using stem material from a single plant. Explants were surface-sterilised and transferred to agar medium containing Murashige and Skoog vitamins and minerals together with the supplements detailed in table 3.1. Explants on these nine media were then divided between two incubators: one maintaining a 16 hours/8 hours light/dark cycle and the other continuous light conditions. The temperature of the incubators were regulated at 24°C. Typically callus appeared within seven days and after four weeks this was transferred onto fresh media. Thereafter the cultures were subcultured at three week intervals. In this way cultures were established under all the conditions attempted.

With two exceptions the morphology of the various cultures remained stable over all passages (culture line eight produced shoots and rootlets during initiation, but this trend ceased after the first subculture). Initially, selection at subculture between non (or less)-pigmented and pigmented calli had been anticipated, but little such selection was made since for any one of the growth conditions the callus tissue appeared similarly pigmented. The appearance, relative growth of callus and yields of extract for each culture line are summarised in table 3.2. Cultures maintained under photoperiod conditions had a greater tendency to "greening" than their counterparts illuminated continuously (table 3.2, column 3). The two exceptions to stable callus morphology were found in culture lines thirteen and fourteen (which both contained 0.1mg/l kinetin and 1mg/l 2,4-D). These two lines had a tendency to form shoots and rootlets, especially the latter type where periods of slow growth were interspersed with shoot/rootlet formation. In general, cultures grown under conditions of continuous illumination grew faster than those

under a photoperiod (table 3.2, columns 4 and 5). The presence of 2,4-D greatly inhibited growth - culture lines eleven and twelve (containing the highest levels of 2,4-D) were the slowest growing of all the cultures. An inverse relationship between growth and pigmentation was easily noticeable. The fastest-growing culture (culture line three - continuous light and high NAA levels) was also the lightest coloured, whilst the slowest growing (culture line twelve - photoperiod and high 2,4-D levels) was also the darkest.

One of the cultures (culture line five) caused a red colouration of the agar media from inception. Although its counterpart maintained under photoperiod conditions showed no such inclination, four other cultures did so (culture lines two, six, seven and eight), although to a much lesser extent. Thus, five out of seven lines containing the hormone BAP (and sixteen in total) secreted pigment into the media. Efforts were made to extract from the agar medium the substance or substances giving rise to the colouration. The agar medium was liquefied by warming but the colouring matter could not be extracted into an organic solvent even when the aqueous layer was either acidified or made alkaline. A red to yellow colour change was observed on acidifying the aqueous phase. It is likely that the colouration was due to a pigment present in a bonded (i.e. glycosidic) form, although this possibility was not investigated further.

After nine subcultures when a large biomass had been obtained, the various cultures were extracted with methanol which is commonly used for the extraction of anthraquinones.[73] All callus material, except a little of culture line three, was used in the extraction thereby terminating all lines but one. Details of this extraction are given in table 3.2. Owing to a mishap, the callus material from cells of culture lines nine, eleven and thirteen were lost on the day of

extraction and so no further results are available for these. Some cultures of line three were maintained for over three more months, throughout which they remained morphologically stable.

Table 3.1 The various hormone and light regimes under which cultures were grown

Culture line	Light regime	Hormones (mg/l)					Casein (g/l)
		GA_3	BAP	NAA	Kinetin	2,4-D	
1	c	10	0.5	2	-	-	-
2	p	10	0.5	2	-	-	-
3	c	-	-	2	0.2	-	-
4	p	-	-	2	0.2	-	-
5	c	-	1	-	-	1	3
6	p	-	1	-	-	1	3
7	c	-	5	1	-	-	-
8	p	-	5	1	-	-	-
9	c	-	-	2	0.1	-	-
10	p	-	-	2	0.1	-	-
11	c	-	-	-	-	2	-
12	p	-	-	-	-	2	-
13	c	-	-	-	0.1	1	-
14	p	-	-	-	0.1	1	-
15	c	-	-	1	1	-	-
16	c	-	0.5	0.1	-	-	-

Key: c = continuous light, 24°C

p = photoperiod (16 hours light, 8 hours dark), 24°C

Table 3.2 Appearance and yields of callus material from the various culture lines

Culture line[a]	Appearance of callus	Greening of callus[b]	Mass of callus (g)	Relative Growth[c]	Mass of extract (g)	% Yield (w/w)
1	orange	-	33.59	**	1.30	3.88
2	brown, media coloured	*	26.84	*	1.66	6.18
3	orange, white hairs	-	160.70	*****	5.59	3.48
4	orange, white hairs	-	81.66	***	3.18	3.89
5	dark red, media coloured	-	15.04	*	0.60	4.01
6	orange/ brown, media coloured	*	3.66	*	0.24	6.58
7	yellow/ orange, media coloured	-	22.26	*	0.56	2.52
8	orange/ brown media coloured	**	6.87	*	0.18	2.60
9	yellow/ orange, white hairs	-	-	-	-	-
10	orange, white hairs	-	22.75	*	1.04	4.59
11	yellow/ orange	-	-	-	-	-
12	dark brown, some orange rootlets	-	1.52	*	0.09	6.14
13	yellow/ orange, some shoots & rootlets	-	-	-	-	-
14	almost black, some orange, some shoots	-	2.86	*	0.17	6.06
15	orange, white hairs	-	14.29	*	0.56	3.89
16	orange/ brown rootlets	-	17.81	*	1.18	6.63

[a] see table 3.1 for growth conditions; [b] - = no greening, * = some greening, ** = significant greening; [c] * = 0-20% ** = 20-40%, *** = 40-60%, **** = 60-80%, ***** = 80-100% of the growth of the fastest growing culture line.

Section 3.2.2 Analysis of crude extracts by TLC

Qualitative analyses of the thirteen different extracts by TLC should denote any major differences, and the size and intensity of the spots should indicate relative amounts. Numerous unsuccessful attempts were made to develop a suitable solvent system to separate the pigments by silica-gel TLC using various mixtures of (i) ethyl acetate/ methanol/ water, (ii) toluene/ methanol/ acetic acid, (iii) toluene/ ethyl acetate/ acetic acid and (iv) ethyl acetate/ petroleum spirit/ acetic acid. The first of these mixtures (in the combination 100 : 16.5 : 13.5 v/v) has been used before to effect a separation of a mixture of free and glycosidic anthraquinones.[74] Nonetheless a satisfactory separation could not be achieved using this system in this work, primarily owing to heavy tailing of compounds. The addition of acid to some of the solvent mixtures (which can help to produce discrete spots) did little to improve the appearance of the chromatograms.

In addition to using silica gel as the stationary phase, cellulose and PEI-cellulose were also tried in combination with the solvent mixtures described above.

Section 3.2.3 Analysis of acid-hydrolysed extracts

Such broadening and tailing of TLC spots as described above is often indicative of the presence of glycosides. A particular pigment may be present in a variety of glycosidic forms (i.e. with variation in the number and type of sugar residues attached) and as such derivatives frequently possess similar chromatographic properties to one another, a broad diffuse spot is often seen rather than a number of distinct ones. Furthermore, hydrogen bonding between these glycosides and the stationary phase material results in tailing of the

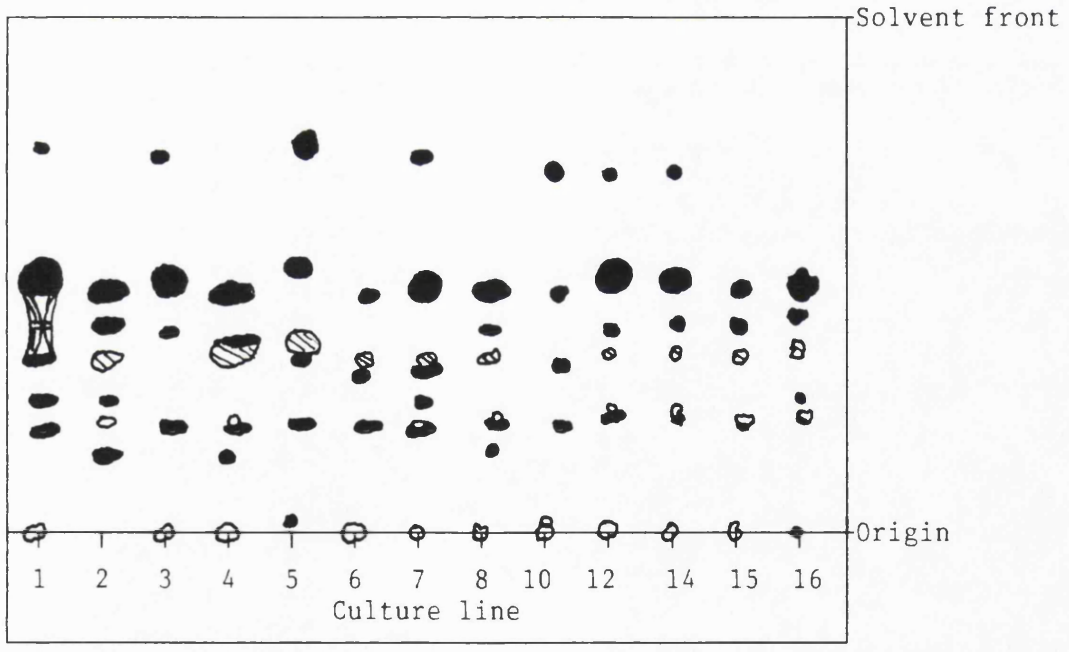
TLC spots. Modification of the mobile phase (e.g. the addition of acid as tried above) or the stationary phase (e.g. making up plates using dilute aqueous tartaric acid for the slurry)[75] can decrease the degree of tailing. An alternative, though more extreme, solution is to cleave the sugar linkage using either dilute acid or enzymatic methods. Acid cleavage is generally preferred to enzymatic cleavage as the latter is often too specific and so may not cleave all of the glycosides present in a crude extract. Cleavage of the sugar linkage is advantageous not only because it leads to the formation of compounds which are easier to chromatograph, but also because it produces an extract which contains fewer components than the mixture of glycosides from which it was formed. The chromatographic separations achieved at this stage were so poor that it was judged further modification of either the mobile or stationary phases would produce insignificant improvement. A successful trial hydrolysis was made on one of the extracts using dilute hydrochloric acid by the same method as described by Inouye *et al*[76] for a number of anthraquinone glycosides. The other extracts were subsequently treated in the same way.

Section 3.2.3.1 Analysis of acid-hydrolysed extracts by TLC

The acid-hydrolysed extracts were analysed by TLC using a wide variety of solvent systems, the best of which was found to be: methanol : toluene : acetic acid (10 : 90 : 0.5 v/v) as shown in figure 3.3. Close inspection of the developed plates showed some spots to consist of several close-running components. Two-dimensional TLC partially resolved these components (system one: acetonitrile : toluene : acetic acid (50 : 50 : 0.5 v/v); system two: methanol : toluene : acetic acid (10 : 90 : 0.5 v/v)). It was at this stage that the regular use of an HPL-chromatograph became available. It was not

until after the HPLC work had been started that the probable reason for the close running of some components was unearthed: i.e. solvent demixing.[77] This can happen during development when a small concentration of a strongly eluting solvent is used with a weakly eluting one. At the beginning there is a tendency for preferential adsorption of the strongly eluting solvent, and the front can consist of 100% weakly eluting solvent. As the sorbent becomes saturated with the strongly eluting solvent, the second front consisting of the mixture appears. Thus two solvent fronts are seen on the layer - as was the case.

Figure 3.3 TLC of acid-hydrolysed extracts



(Key: ▮ = yellow, ▨ = orange, ▯ = red).

Section 3.2.3.2 Analysis of acid-hydrolysed extracts by HPLC

HPLC offers several advantages over TLC for the analysis of mixtures. These include: (i) greater resolution of components; for instance, compounds of widely-differing polarities can be separated by the use of solvent gradients (ii) when a reverse phase column is used irreversible adsorption is virtually eliminated and peak-broadening (tailing) greatly reduced and (iii) the attachment of an integrator to the detector used enables greater quantification.

As the major pigments found in the plant material of *Galium verum* are anthraquinones it is likely that the same is true of the cultures. Anthraquinones absorb in both the ultra-violet and visible regions of the electromagnetic spectrum, although the extinction coefficients for the former are greater. Hence the extracts were analysed by HPLC using an ultra-violet detector (which is also more sensitive than a refractive index type). A diode-array detector (DAD) was used. Such detectors allow the simultaneous measurement of absorptions at several wavelengths in the ultra-violet region of the electro-magnetic spectrum of the eluate as it exits the column. The particular detector used allows absorptions at 256 different wavelengths in the region 190-370nm to be measured thus enabling the ultra-violet spectrum of individual compounds in the mixture to be measured after they have been separated analytically on the column. Obviously, detection in this region of the electromagnetic spectrum will also reveal any other UV-active compounds present in the extract (e. g. other phenolics such as phenylpropanoids, anthocyanins, flavonoids, tannins and chalcones) and this was borne in mind.

One of the extracts was analysed using a gradient run of 0 to 100% acetonitrile over sixty minutes with water as the co-solvent and octadecylsilane as the bonded (reverse phase) stationary phase. The

chromatogram obtained showed considerable tailing of peaks, which makes their integration of limited value. In reverse-phase HPLC, standard-bonded phase columns are packed with silica particles that have an organic coating (octadecylsilane in this work) bonded to the surface. Unreacted silanol groups on the surface of the silica give rise to peak tailing.[78] There are two types of silanol interactions, one specific for bases and the other for acids. Thus, the addition of acid or base (as appropriate) to the mobile phase can be used to reduce these interactions as these mobile-phase additives have a stronger interaction with the silanol functional groups at the surface than have the sample compounds. Since it is likely that the pigments contained in the extract are mostly hydroxyanthraquinones, which are slightly acidic, trifluoroacetic acid (TFA, 0.1% v/v) was added to the solvents used. This greatly reduced peak-tailing. The other extracts were then analysed using this modified solvent system as rapidly as possible using the same samples of solvents (degassed by helium sparging) and with allowance for equal time intervals between runs (thirty minutes) to aid reproducibility.

The diode-array detector records an ultra-violet spectrum every three seconds yielding a great deal of data (over 1,200 spectra in a typical run!). The data for one of the extracts (culture line two) is shown, in part, in figure 3.4. This is a 3D-plot of wavelength (190-370nm) vs. time (15-55 minutes) vs. absorbance and shows quite graphically similarities and differences between components. However, more abundant components can obscure lesser ones and so the type of plot shown in figure 3.5 (known as an isogram or contour plot) is more useful. This represents data in a form analogous to a geographical map with wavelength and time being along either axis and absorbance represented by contour lines. The plot is an expansion of the region

Figure 3.4 A "topogram" showing the chromatographic separation achieved by analytical HPLC of the components in the acid-hydrolysed extract of culture line number two

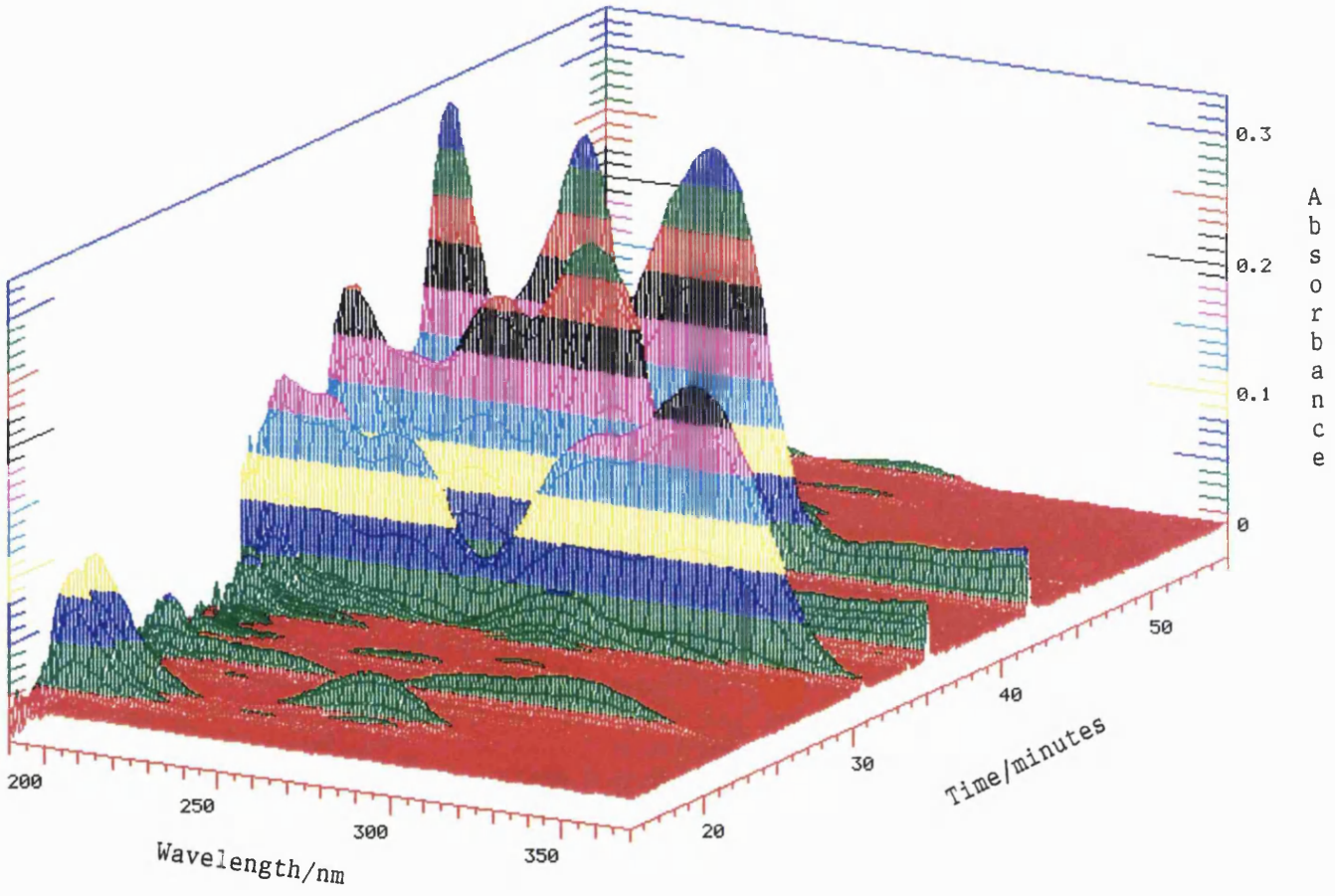
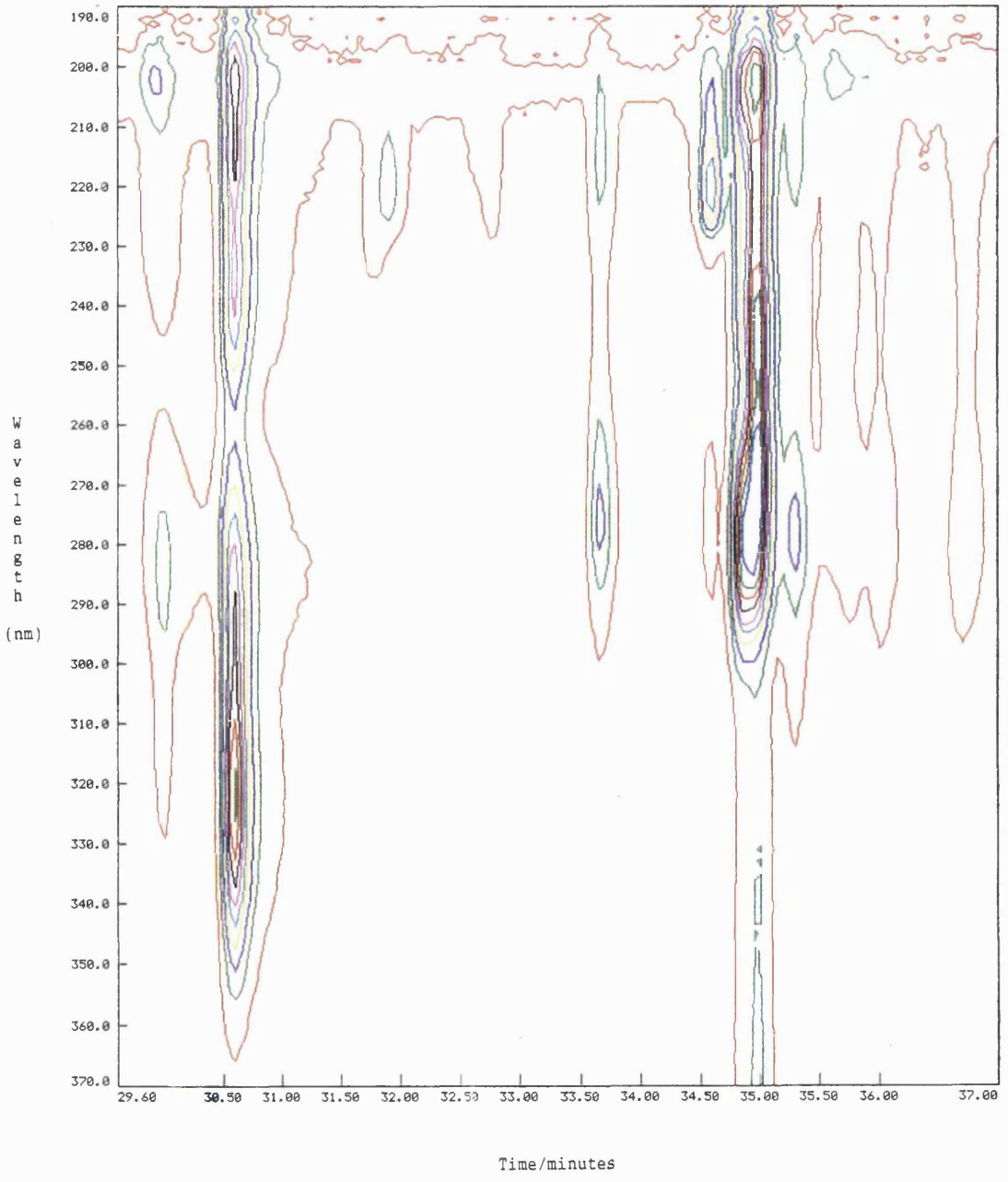


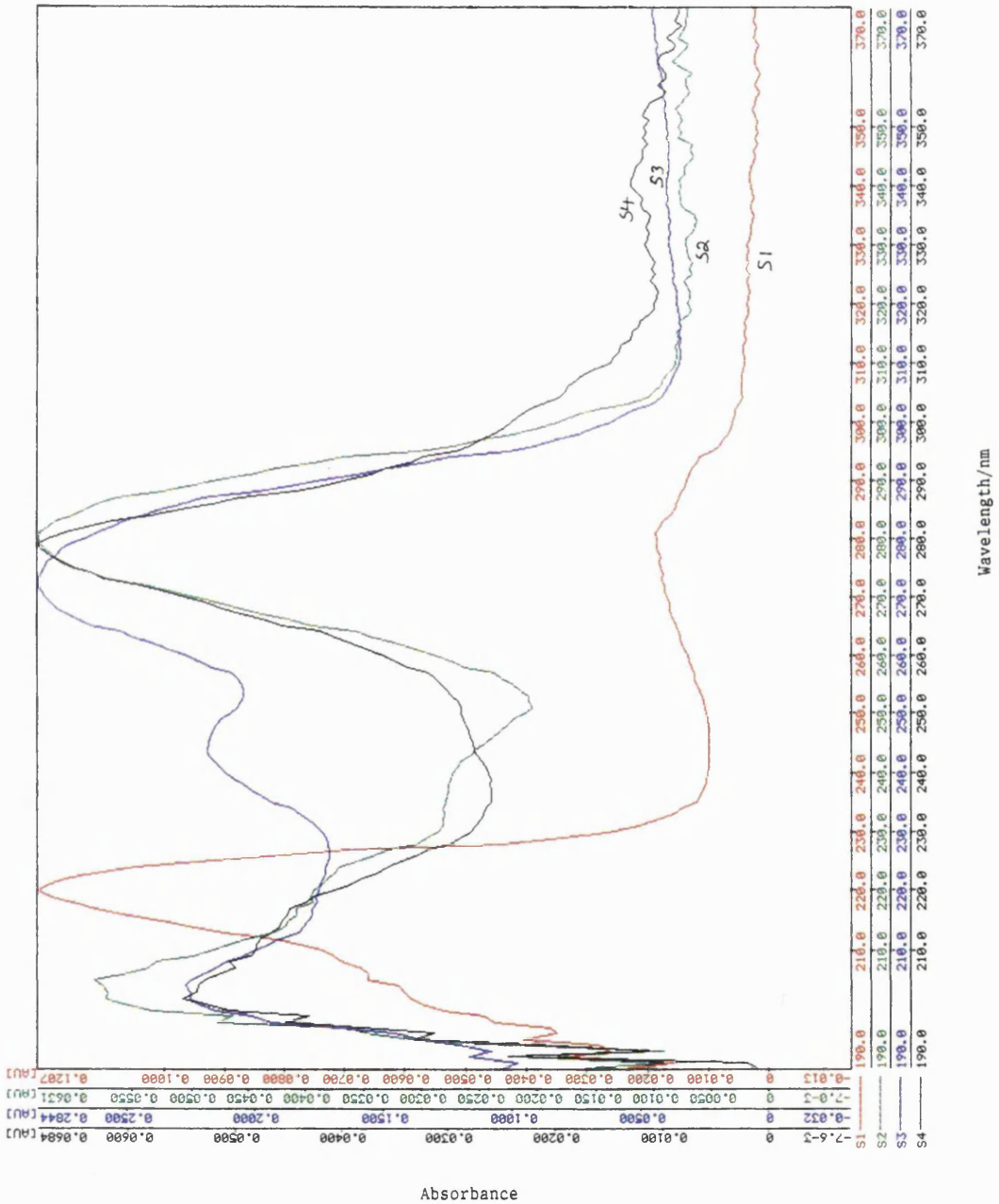
Figure 3.5 A "contour" plot showing the components present in a portion of the analytical HPLC chromatogram of the acid-hydrolysed extract of culture line number two



containing the first and second of the three most abundant peaks in figure 3.4. The peak at retention time 33.60 minutes is easily discernible in the contour plot although it is hidden in figure 3.4.

For each extract, a chromatogram (i.e. a plot of absorbance vs. time) was also recorded at 240nm (at which a large number of the components have a significant absorbance). This showed only one broad peak at 35.1 minutes in the period R_t= 34.50-35.5 minutes - although it is clear from examination of the contour plot that at least three compounds elute in this region. In contrast, the peaks at R_t= 30.60 and 33.60 in figure 3.5 are quite symmetrical indicating the purity of these two peaks (the slight distortion of the first of these is caused by peak tailing). Chromatogram, isogram and ultra-violet spectra can be displayed on the screen simultaneously, enabling the quick examination of the ultra-violet spectra of all the components in a mixture. This split mode is particularly useful: - the ultra-violet spectra for a pure compound is examined across the chromatographic peak (upslope, apex and downslope) then once these have been normalised they should be very nearly superimposable. Such "peak profiles" were not the same for all the peaks in the extracts, thus indicating a number of components to be co-eluting. An example (in the region R_t= 34.50 to 35.50 minutes in figure 3.5) is shown in figure 3.6. Spectra were extracted at four points (R_t= 34.59, 34.78, 34.95 and 35.29 minutes). Not only are the two components (spectra S1 and S4) either side of the major component (spectra S3) clearly distinguishable, but a previously hidden component is also revealed (spectra S2). There is a gradual rise and fall in absorbance as measured at 240nm moving from spectra S1 to S4 accounting for the appearance of these as one broad peak in the chromatogram.

Figure 3.6 The ultra-violet spectra of some of the components present in the acid-hydrolysed extract of culture line number two



Some distortion of the baseline is common in all gradient-elution procedures.[79] Although degassed (HPLC-grade) solvents had been used baseline problems were still noticeable - especially below 220nm. Fortunately these problems can be minimised by digitally subtracting a solvent blank. This was done for all the extracts. Impurities in the organic mobile phase (the ubiquitous plasticisers, for example) can accumulate at the head of the column during equilibration. When the gradient elution is begun these artifacts are eluted giving rise to spurious peaks. No such peaks were observed in the solvent blank.

The elution profiles were monitored at 240nm. The chromatograms obtained were integrated and the results tabulated. Even if it were possible to reproduce the same operating conditions for all the extracts, some retention times would still differ as the relative proportions of incompletely-resolved components affects the measurement of these times. The ultra-violet spectra of the components in all extracts were examined using the data-handling system for the diode-array detector as a precaution against wrongfully grouping different components together into a set. This precaution also showed no compounds were present which absorbed at other wavelengths. 83 different compounds which absorb at 240nm were altogether detected, although only sets with one or more members comprising 1% or more of the total amount of the extract (as measured by peak areas) were tabulated (47 in total). The UV spectra of these compounds are tabulated in table 3.3. It is evident from the data contained in this table that several classes of compound are present. A large number of these compounds exhibit absorptions at 240 and 280nm, and this suggests that these are of the same class. Whilst another group possess (amongst other bands) a band in the region 305-325nm. Indeed a class separation seems to have been achieved with the former group

mostly being eluted from the column after 36 minutes, whilst the latter group are all eluted before this time. Table 3.4 lists values which are the percentage of the total area of the chromatogram (measured at 240nm) the particular component occupies.

Section 3.2.4 Comparison of pigments produced by cultures with those produced by roots and flowers

As many of the secondary metabolites present in the field-grown plant are well established, a comparison of crude plant extracts with those of the cultures should greatly aid identification of the major compounds produced in the latter. Methanolic extracts of both flowers and roots of the plant were analysed by analytical HPLC. It is likely that the hydrolysed extracts of the cultures contain a number of those compounds present in the roots and flowers but in a free form. Hence, a portion of both of these plant extracts was acid-hydrolysed in the same way as for the cultures (see section 3.2.3). It was necessary to extract, hydrolyse and obtain analytical HPLC traces of the flowers and roots in the same day as on standing the crude non-hydrolysed flower extract quickly decomposed with a black precipitate being formed. The ultra-violet spectra of the components of these plant-derived extracts are tabulated in table 3.5.

All components in the flower extract were eluted from the HPLC column between 8 and 23 minutes. It was clear that this flower extract contained three classes of UV-active compounds and that the HPLC solvent system used was able to achieve a class separation. Five similar compounds (each with $\lambda_{max} \simeq$ 235nm) were eluted between 8 and 16 minutes, followed by two of another class (3 bands $\lambda_{max} \simeq$ 325, 290, and 244nm) and finally four components each with $\lambda_{max} \simeq$ 253 and 349nm. The hydrolysed extract of the flower had most components eluted between 11

Table 3.3 Ultra-violet spectral data of the major components present in the acid-hydrolysed extracts of the various cultures

Compound at R_t (mins)	λ_{max} (nm)
16.9	203
17.6	225
18.1	204
18.8	280 (100), 227 (26)
21.1	226 (100), 278 (82), 307 (72)
22.6	322 (100), 290 (sh, 67), 237 (62)
23.2	226 (100), 275 (18)
23.5	270
25.5	255
25.8	256
27.3	215 (100), 275 (70),
30.1	307 (100), 290 (81) 223 (63)
30.7	322 (100), 291 (74), 236 (62)
31.2	223 (100), 279 (42)
31.9	270
32.3	235 (100), 279 (48)
33.7	276 (100), 215 (66)
35.1	mixture (i) 280; (ii) 245
35.4	279
35.7	202 (100), 246 (74), 268 (66), 338 (18)
36.1	292 (100), 219 (8)
36.8	276
37.4	225 (100), 258 (38)
38.7	252 (100), 281 (sh, 38)
40.0	256
40.3	245 (100), 281 (88)
40.7	220 (100), 279 (14)
41.0	204 (100), 241 (63)
41.3	225 (100), 257 (sh, 40), 288 (12)
41.8	279 (100), 242 (99), 203 (92)
42.4	203 (100), 268 (76), 242 (70)
43.1	204 (100), 275 (70), 242 (68)
43.8	202 (100), 246 (84), 267 (67)
44.5	276
45.0	278 (100), 241 (80)
45.5	203 (100), 276 (93), 242 (75)
45.9	244 (100), 281 (93)
47.4	250
47.9	255
48.6	255 (100), 275 (sh,74)
51.5	240 (100), 277 (81)
51.9	280 (100), 241 (93)
52.4	201 (100), 278 (45), 240 (41)
53.8	253 (100), 220 (67), 278 (sh, 49)
54.4	244 (100), 278 (100)
54.8	281 (100), 241 (93)

Footnote: The figures in brackets are absorbance values expressed as a percentage of the most intense band

Table 3.4 Levels of components present in the acid-hydrolysed extracts of the various cultures (continued overleaf)

R_t mins	Culture line												
	1	2	3	4	5	6	7	8	10	12	14	15	16
16.9	3	3	1	-	-	-	2	-	13	4	-	5	6
17.6	2	4	43	6	-	-	-	-	5	7	3	2	3
18.1	7	5	23	3	2	-	3	-	2	6	-	7	7
18.8	52	4	194	-	2		3	19	37	2	42	5	24
21.1	8	4	11	8	-	-	2	16	13	3	6	16	13
22.6	7	16	3	59	1	-	3	-	1	5	4	13	5
23.2	5	7	8	12	17	-	9	11	5	13	11	10	8
23.5	-	-	13	6	-	15	-	-	-	3	-	-	-
25.5	5	5	-	6	11	12	4	-	3	-	5	4	1
25.8	13	8	32	10	4	21	5	4	13	-	-	-	8
27.3	3	1	-	2	18	-	-	-	2	16	-	17	10
30.1	11	17	-	16	-	-	5	-	4	1	2	9	10
30.7	122	135	11	294	4	-	38	14	12	20	6	128	51
31.2	-	-	9	-	5	-	11	33	4	-	6	-	-
31.9	1	3	17	12	4	2	1	14	4	2	3	16	4
32.3	1	-	12	10	7	4	-	12	3	2	-	-	-
33.7	4	10	3	2	12	18	9	9	1	7	-	0	4
35.1	78	223	8	62	85	457	198	244	35	84	45	74	73
35.4							45						
35.7	14	22	7	42	35		12		44	123	94	76	87
36.1	25	7	20	3	7	22	12	19	51			32	50

Footnotes: Growth conditions are detailed in table 3.1. Entries have been multiplied by ten to save space and make it easier to detect differences at a glance. Figures quoted in parentheses indicate that the integrator was unable to distinguish between components in a mixture although their presence could be seen from examination of data in the split mode. The row in which these figures are placed indicates that the major component of the mixture occurs at this retention time.

Table 3.4 (continued) Levels of components present in the acid-hydrolysed extracts of the various cultures

R_t	Culture line												
mins	1	2	3	4	5	6	7	8	10	12	14	15	16
36.8	6	12	9	2	11	-	3	3	2	5	-	-	14
37.4	5	6	12	17	2	10	3	-	25	30	43	33	36
38.7	5	10	3	5	71	37	21	32	1	2	15	4	3
40.0	1	-		-	59	-	1	131	1	5	-	-	-
40.3	4	-	15	4	14	-	3		6		-	5	
40.7	1			22	50	25	26	-	2	138	-		5
41.0	2	9	7	4	-	38		18	3	7	-		5
41.3	5	6	-	17	-	-		-	27	10	-	5	23
41.8	508	353	307	148	226	151	332	161	526	218	623	397	448
42.4	-	-	-	-	38	-	70	-	-	-	-	-	-
43.1	-	-	12	-	9	16	-	-	-	8	-	-	-
43.8	0	2	28	28	-	-	12	5	-	16	2	46	2
44.5	11	10		12	21	-	31	8	5	-		-	13
45.0	-	-	-	4		10	6	-	-	7	-	1	-
45.5	18	25	29	66	83	37	28	41	53	102	27	27	26
45.9	4	10	12	9	70	29	30	33	7	30	8	11	8
47.4	-	-	0	12	3	-	-	3	22		-	1	-
47.9	1	1	1	-	4	-	5	9	-	18	-	-	-
48.2	1	3	0	-	6	-	4	19	-		-	-	-
48.6	11	13	9	5	20	16	13	58	4	15	6	7	4
51.5	-	7	4	-	2	-	2	-	1	13	-	2	1
51.9	7	5	7	-	1	-	3	-	4		14	3	3
52.4	5	3		50		-	4	-	3	1	-	3	5
53.8	2	3	2	3	43	12	6	16	1	-	-	-	-
54.4	1	2	1	-	2	2	1	26	0	6	-	-	-
54.8	2	13	5	-	1	-	3	11	3	26	-	-	2

Table 3.5 Ultra-violet spectral data of compounds present in extracts of the field-grown herb *Galium verum* (continued overleaf)

Compound at R_t (mins)	λ_{max} (nm)
	Flowers
8.8	234
10.3	236
12.1	236
14.3	237
15.7	**236**
16.0	**327 (100), 246 (78), 292 (73)**
16.7	326 (100), 290 (sh,53), 243 (48)
17.4	251 (100), 348 (81)
17.9	234
20.5	**254 (100), 350 (87)**
21.2	**254 (100), 348 (100)**
22.1	252 (100), 351 (95)
	Hydrolysed flowers
11.3	281 (100), 225 (17)
17.1	216 (100), 258 (34), 294 (15)
17.4	322 (100), 287 (69), 239 (62)
18.0	279 (100), 225 (17)
19.4	324 (100), 212 (84), 291 (sh,72), 242 (50)
20.1	225 (100), 288 (94)
20.8	227
22.9	227 (100), 288 (72)
24.5	322 (100), 214 (84), 287 (73), 240 (58)
25.0	218 (100), 258 (38), 291 (21)
25.5	327 (100), 237 (49)
26.2	220 (100), 275 (22)
28.0	347 (100), 255 (80)
28.8	309 (100), 224 (76)
29.6	322 (100), 287 (66), 236 (63)
31.2	225

Footnotes: The figures in brackets are absorbance values expressed as a percentage of the most intense band. Major components are shown in **bold type**. These extracts were examined by HPLC several months after those of the cultures and retention times were not comparable. Hence two of the acid-hydrolysed extracts of the cultures were rerun enabling comparisons to be made.

Table 3.5 (continued) Ultra-violet spectral data of compounds present in extracts of the field-grown herb *Galium verum*

Compound at R_t (mins)	λ_{max} (nm)
	Roots
8.7	234
10.3	234
13.0	236
14.2	233
15.2	286 (100), 314 (97), 232 (82)
15.6	236
16.0	228 (100), 300 (91)
16.3	224 (100), 274 (37)
17.4	224 (100), 274 (36)
20.3	228 (100), 282 (16)
20.7	252 (100), 350 (34)
21.0	237 (100), 323 (84), 287 (sh, 59)
21.5	276 (100), 336 (79)
22.3	227 (100), 274 (23)
23.1	263 (100), 242 (80)
24.3	263 (100), 242 (88)
24.8	214 (100), 255 (93)
25.1	227 (100), 278 (31)
26.1	225 (100)
26.9	267 (100), 242 (65)
29.9	225 (100), 286 (21)
33.9	278
34.6	238
39.4	216 (100), 238 (73)
43.3	276 (100), 216 (93), 239 (89)
45.8	218 (100), 242 (72), 268 (44)
	Hydrolysed roots
11.2	280 (100), 227 (17)
13.4	249
15.7	252
18.0	279 (100), 226 (25)
20.7	227
21.4	320 (100), 214 (76), 236 (70), 288 (sh, 68)
21.6	228 (100), 281 (47)
22.7	227
24.4	221 (100), 278 (47)
28.2	227 (100), 341 (100)
28.6	226
28.9	267 (100), 242 (69)
29.5	322 (100), 287 (sh, 71), 234 (68)
30.5	213 (100), 257 (58)
31.1	225
33.8	mixture (i) 272, (ii) 243
34.1	280
38.8	215 (100), 261 (72)
40.1	242 (100), 279 (98)
43.3	240 (100), 275 (87)

and 32 minutes. The UV spectra of these components were, not surprisingly, similar to those in the non-hydrolysed extract, although over half of the components were eluted later than the last component of the non-hydrolysed extract. This is consistent with cleavage of a glycosidic linkage, leading to less polar components, which are therefore eluted later (in reverse phase HPLC).

Examination of the ultra-violet spectra of the components present in the root extract revealed several classes of UV-active compound to be present. The three most common classes of compound either had maximum absorptions at $\lambda_{max} \simeq 235$, or at 225 and 275, or at 242 and 267nm. The acid-hydrolysed extract of the roots when examined by HPLC correlated with the non-hydrolysed root extract in the same way as did the hydrolysed and non-hydrolysed flowers. Namely, components were later eluting in the hydrolysed extract of the root, but had UV-spectra similar to the components of the non-hydrolysed extract of the root.

Table 3.6 lists those compounds which are present in the acid-hydrolysed extracts of the cultures and one or more of the four plant-derived extracts. The flower extract did not contain any components present in the acid-hydrolysed extracts of the cultures. Most notably, the acid-hydrolysed extracts of the roots compared well with those of the cultures with up to eleven components in common. Since the major components of the roots are anthraquinones it is therefore likely that these common components are also anthraquinones with a number being identical to those present in the roots. However, two factors - the irreproducibility of retention times and the similarity between the UV-spectra of the components - indicated that such correlations should be treated with caution. One of the major components of the acid-hydrolysed extracts of the cultures with a

retention time of 30.7 minutes was present as a significant component in the hydrolysed extracts of both the roots and flowers. This suggests the component is not an anthraquinone, an iridoid or a flavonoid compound since the references relating to the chemical composition of the plant (see section 3.1) indicate these compounds are located in the roots or aerial parts respectively.

Table 3.6 Components common to the acid-hydrolysed extracts of the cultures and one or more of the extracts of the plant

Compound at R_t (mins)	Component present in the acid-hydrolysed extracts of the cultures and		
	Roots	Hydrolysed roots	Hydrolysed flowers
18.8	-	*	*
22.6	-	****	-
23.2	****	-	-
30.7	-	*****	**
35.1	-	****	-
35.4	-	**	-
36.1	-	?	-
41.8	-	****	-
43.8	-	?	-
44.5	-	?	-
45.5	*	***	-
45.9	-	?	-

Footnote: *,** etc indicate approximate levels of components, where *** is approximately three times the level of * etc.
? indicates it is uncertain whether or not this component is present in the acid-hydrolysed extract of the roots.

Section 3.2.5 Isolation of pigments from acid-hydrolysed extracts

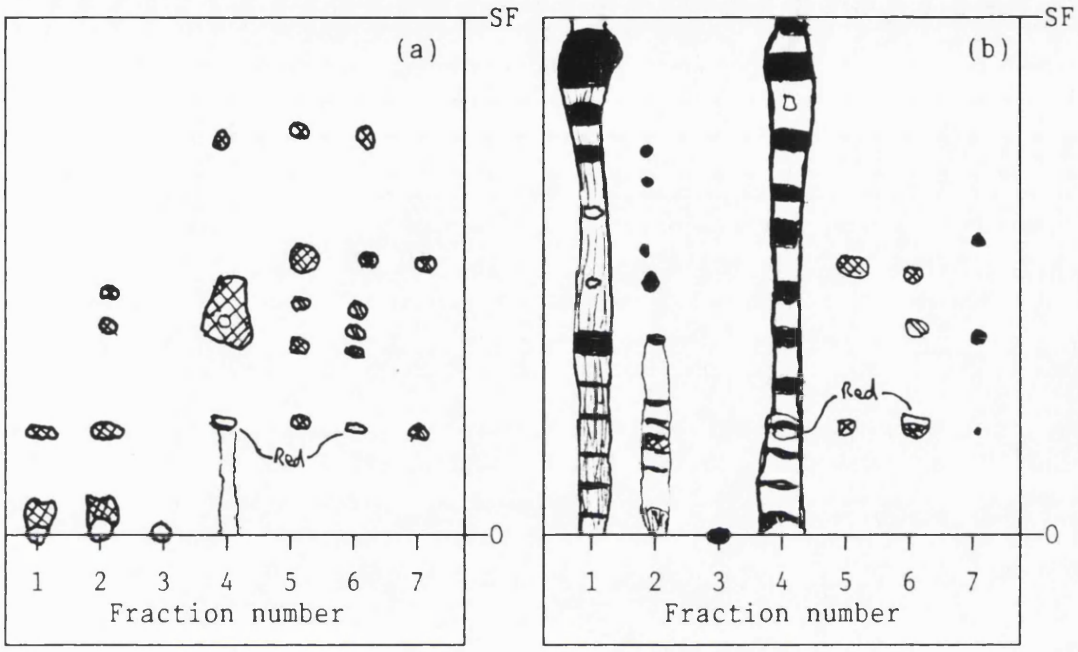
The strategy of the purification procedure was to separate the pigments using chromatographic techniques such as column and thin layer chromatography with only the coloured bands or fractions being collected. Only when separation of these pigments had been achieved were universal detection methods (e.g. universal spray reagents) to be used to aid removal of non-coloured compounds. Such a procedure saves time.

Section 3.2.5.1 Isolation of pigments from acid-hydrolysed extracts by a combination of gel filtration, silica gel column and thin-layer chromatography

In general, *Sephadex* LH-20 separates compounds by gel filtration i.e. in order of decreasing molecular size. It is, therefore, very useful for the removal of high molecular mass and polymeric material from a sample. It has, however, also been used to separate compounds of much the same molecular mass (e.g. anthraquinones[80]). A major advantage over other chromatographic techniques (e.g. silica-gel chromatography) is the high recoveries encountered i.e. little irreversible adsorption occurs. For these reasons it was used as an initial purification step.

The combined methanolic extracts were chromatographed through the gel using methanol as solvent. The three coloured (yellow / orange) fractions collected were each recycled to give a total of seven coloured fractions which were analysed by TLC (see figure 3.7). Some separation of the pigments had been achieved, but (more of interest) the fifth and sixth coloured fractions appeared to contain only pigments - possibly due to these compounds being of similar molecular mass.

Figure 3.7 TLC of pigments after separation using Sephadex LH-20

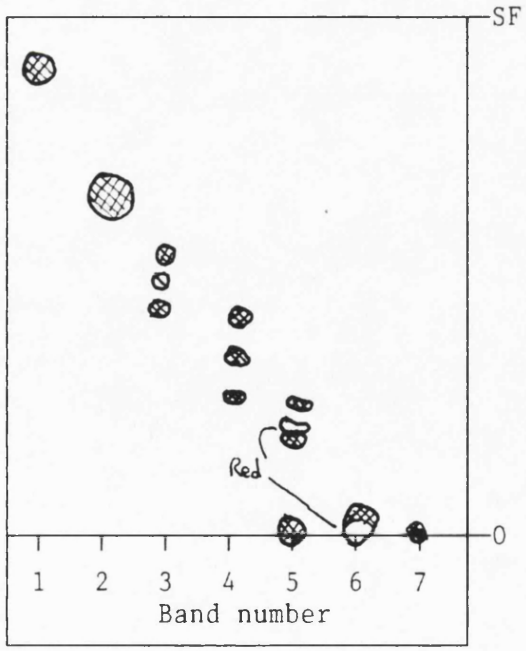


(a) before spraying, (b) after spraying plates with a solution of phosphomolybdic acid and heating.

▨ = yellow, ▧ = orange, □ = red, ■ = compounds revealed by spraying.

SF = solvent front, 0 = origin.

Figure 3.8 TLC of pigments after separation by preparative-TLC



The fractions obtained using *Sephadex* LH-20 were far from pure and hence a separation by silica-gel column chromatography was attempted. The mixture contained in the fifth fraction from the *Sephadex* LH-20 separation was applied to a silica-gel column and eluted with a mixture of toluene, methanol and acetic acid (90 : 10 : 0.5 v/v) giving two apparently pure pigments (R_f= 0.84 and 0.59 in figure 3.7). The other fractions contained mixtures which could not be separated effectively by column chromatography using any of a variety of methods - for example, dissolving in, or eluting with different solvent systems, as well as pre-equilibrating the column with various solvents.

Band broadening (tailing) as well as the inability to reproduce the separations achieved by analytical TLC had limited the application of column chromatography to this separation. Since the pigments had been found to be well separated by analytical TLC, attempts were made to separate them by preparative-TLC. The adsorbent bound to such plates is usually only four times thicker than that of analytical ones and hence over-loading easily occurs. Thus a large number of plates had to be used. Seven bands were harvested when the impure fractions obtained from the silica gel column separation were subjected to preparative-TLC. These bands were analysed by analytical TLC (figure 3.8). An improved separation of pigments had occurred and so the other fractions from the *Sephadex* LH-20 separation were similarly purified. The polar bands (R_f< 0.4) from these separations were still mostly unresolved and so were submitted to further purification by preparative TLC using a more polar solvent system.†

† Trials were made to concentrate further the band the sample is applied as by allowing the migration of a polar solvent (methanol) to about 2cm above the applied band. This modified technique gave a poorer separation and was not used any further.

Whilst these purifications were in progress the use of an HPLC-chromatograph became available. The fractions (about sixty in total) from the above separations were consequently analysed by reverse-phase HPLC and all but a few found to consist of complex mixtures. This illustrates the limitations of the conventional isolation methods - the use of which underpins most natural product studies.

Section 3.2.5.2 Isolation of pigments from acid-hydrolysed extracts by HPLC

The crude half-portions of the thirteen culture extracts, retained after the hydrolysis, were examined by analytical HPLC (see section 3.2.3.2). When these results were compared with those for the partially-purified pigments, it could be seen that some of the main components of these extracts were not present in appreciable amounts in any of the sixty fractions! This suggests decomposition or irreversible adsorption had occurred during the purification process. Indeed, during both silica gel column chromatography and preparative-TLC some of the pigment had remained bound to the silica. These problems, as well as those caused by band broadening, are greatly diminished in reverse-phase HPLC. For these reasons it was decided to purify the main pigments in these crude extracts by reverse-phase HPLC, with the inclusion, where appropriate, of those which had already been partially purified as described in section 3.2.5.1.

Purification by reverse-phase HPLC enabled six pigments to be isolated, with a further four being obtained by a combination of normal and reverse-phase HPLC. These isolated pigments were examined by both normal and reverse-phase analytical HPLC (at least three

systems) with peak profiling (at least four profiles) in order to establish purity. The compounds appeared to be greater than 95% pure using an UV-detector and this was confirmed by the use of a refractive index-type detector.

Section 3.2.6 Characterisation of compounds isolated from callus cultures

The characterisation of these compounds was achieved largely by the use of ^{1}H NMR spectroscopy and mass spectrometry. The key features of both these and the other spectroscopic techniques used during the characterisation of the isolated compounds are as described in the previous chapter. Two books by R. H. Thomson[81,82] and a review by R. Wijnsma and R. Verpoorte[83] were invaluable as sources of reference data and also provided information on the natural occurrence of some of the characterised compounds.

Most of the purified compounds were established to be anthraquinones. Analysis of all but the most abundant of the isolated compounds by ^{13}C NMR spectroscopy would have required very long acquisition times and as the time available on the instrument was limited only the main compound was examined by this technique.

Interpretable IR spectra could not be obtained for all of the purified compounds because of experimental difficulties arising from the coincidence of solvent-derived overtone bands with those of the sample. IR spectra were acquired using an FT-IR instrument and a microcell containing a solution of the sample in ethanol-free chloroform. An attached data-station allowed subtraction of a spectrum obtained for the neat solvent from one of the sample + solvent to give a difference spectrum equivalent to a spectrum of the pure sample. Unfortunately, as was the case here, when the sample size is very

small the difference spectrum often contains residual bands belonging to the solvent. A number of alternative solvents (CCl_4, CH_2Cl_2 and $CDCl_3$) were also (unsuccessfully) tried.

Compounds were characterised in order of availability and are numbered below as assigned in figure E1 (see experimental).

Section 3.2.6.1 Characterisation of pigment [IX]

The main component of the acid-hydrolysed extract of the cultures was isolated as an orange/yellow powder (19.3mg). The EI mass spectrum showed amongst others a base peak at m/z 252 and two weak peaks at m/z 269 and m/z 284. As fragment peaks or those due to impurities can often be mistaken for the molecular ion peak, ammonia chemical ionization (CI), which is a "soft" ionization technique was used. The CI mass spectrum of pigment [IX] showed a prominent peak at m/z 285 and another of approximately half the intensity at m/z 270. This suggests either the peak at m/z 270 is a fragment of the quasi-molecular ion at m/z 285, or that a mixture of two compounds (one containing nitrogen) of molecular masses 284 and 269 daltons are present. Both of these peaks were present in the normal mass spectrum. The peak profiles of the ions at m/z 252, 269, 284 and the other chief ions in the mass spectrum were superimposable, or nearly so, which suggests a single compound of molecular mass 284 daltons is present. It is likely that the peak at m/z 270 in the CI mass spectrum is formed from the molecular ion by a reaction of the following type:

$$M + NH_4^+ \longrightarrow [M + NH_4 - HX]^+ + HX$$

This is a common reaction in CI spectrometry and the presence of a peak at $[M - 14]^+$ indicates the leaving group "X" to be a methoxyl group. Accurate mass measurements of the ions at m/z 284, 269, and 252 in the normal (EI) mass spectrum gave values corresponding to the

formulae $C_{16}H_{12}O_5$, $C_{15}H_9O_5$ and $C_{15}H_8O_4$ respectively. The latter two ions are formed by loss of methyl and elimination of methanol respectively from the molecular ion.

In the ^{1}H-decoupled ^{13}C NMR spectra (figure 3.9) the majority of signals lay in the region usually associated with unsaturated carbon atoms (δ 100-160ppm). The region of the spectrum containing the two signals furthest downfield (δ 186.87 and 182.21ppm) is that region usually associated[70] with either an unsaturated aldehyde or ketone or alternatively with a saturated carboxylic acid, but the absence of peaks in the region usually associated with saturated carbon centres eliminates the latter possibility. Carbon atoms bonded to oxygen in either alcohols or ethers absorb in the same region as the two upfield signals observed at δ 68.92 and 59.36ppm.[70]

Although some structural information had been obtained from the ^{1}H-decoupled ^{13}C NMR spectrum, the total number of carbon atoms was not easily discernible, primarily because of the variation in intensity of signals. Hence an inverse-gated ^{1}H-decoupled ^{13}C NMR spectrum was acquired using a longer delay than usual between pulses. The resulting spectrum (see figure 3.10a) was a significant improvement upon the normal ^{1}H-decoupled spectra. An expansion of the region δ 108-136ppm is given in figure 3.10b. Two signals are seen at around δ 110ppm. In the region δ 133-135ppm four signals are apparent with that furthest downfield being due to two shift-equivalent carbon atoms (deduced by examination of peak areas). In total, signals due to sixteen carbon atoms were discernible. This is consistent with the molecular formula given by mass spectrometry measurements, i.e. $C_{16}H_{12}O_5$.

A more sophisticated pulse sequence known as J-modulated spin echo spectroscopy or the Attached Proton Test (APT) was used to

Figure 3.9 The ^{1}H-decoupled ^{13}C NMR spectrum of pigment [IX]

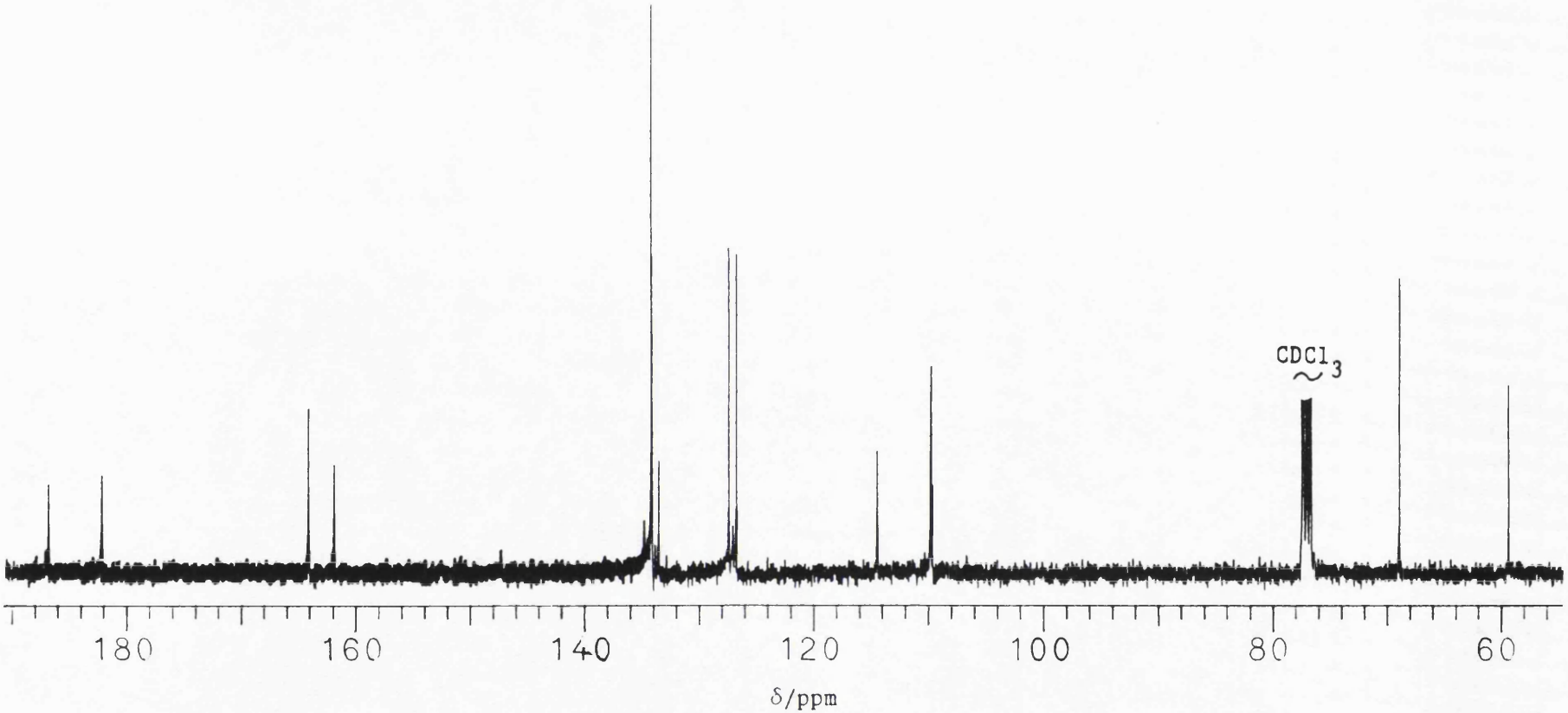
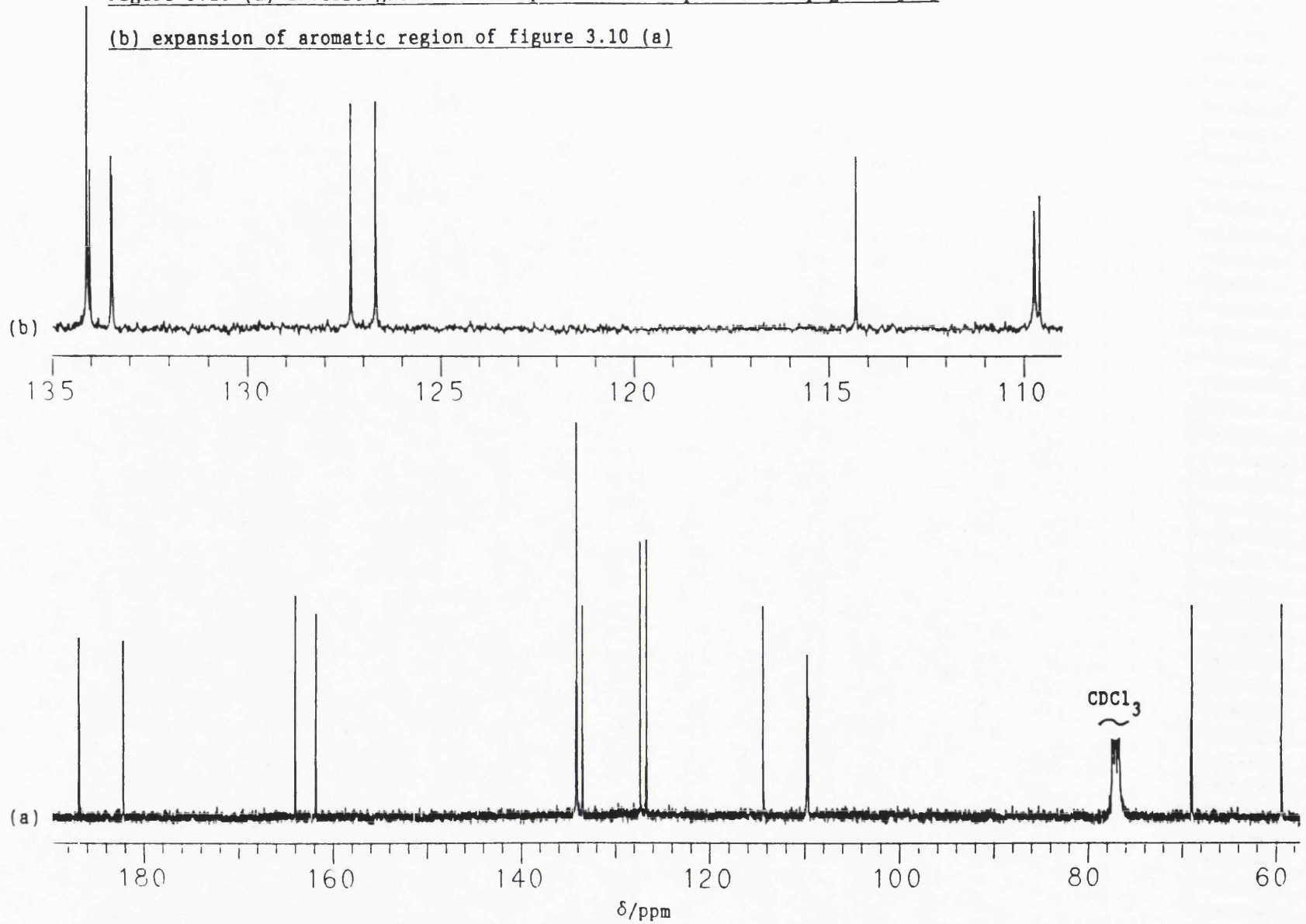


Figure 3.10 (a) Inverse-gated ^{1}H-decoupled ^{13}C NMR spectrum of pigment [IX],

(b) expansion of aromatic region of figure 3.10 (a)



increase further the information obtainable from the ^{13}C spectra. The resulting spectra obtained is shown in figure 3.11. This technique gives peaks due to carbon in CH_2 or quaternary positions on the +X axis and those signals due to carbon in either CH_3 and CH positions on the -X axis. When combined with chemical shift information and signal intensity (quaternary carbons are usually very weak) a distinction between CH_3 or CH, and CH_2 or quaternary signals can usually be made. Hence the following could be deduced: (i) the signal at δ 59.36ppm is due to a methyl group attached to an oxygen atom - i.e. a methoxyl group; (ii) all the signals which are shown in the -X axis between δ 100 and 140ppm are due to alkene/aromatic carbon atoms each with one proton attached; (iii) the two carbonyl carbons (δ 186.87 and 182.21ppm) are ketonic and not aldehydic.

The number of unsaturated carbon centres (twelve) and the presence of two ketonic centres suggested that the pigment might be an anthraquinone. An examination of the literature for ^{13}C data for anthraquinones (see appendix III of reference 81 for a list of salient work) revealed that the two most upfield chemical shift values observed were consistent with an anthraquinone substituted with an hydroxyl group in one of the α-positions. The hydrogen of this hydroxyl group chelates with the neighbouring ketone function giving rise to a downfield shift from the usual value of δ 180-183ppm to δ 185-187ppm for one of the ketonic carbon atoms.

In the ^{1}H NMR spectrum with d-chloroform as solvent, a methoxyl group (δ 3.55ppm), an isolated methylene group (δ 4.91ppm), five aromatic protons (δ 7.28, 7.75 and 8.24ppm) and two hydroxyl protons at δ 9.37 and 13.27ppm (both of these signals disappeared on shaking with D_2O) were evident (see figure 3.12a). Although the chemical shift of hydroxyl protons vary greatly even for a particular class (and are

Figure 3.11 The J-modulated spin-echo ^{13}C NMR spectrum of pigment [IX]

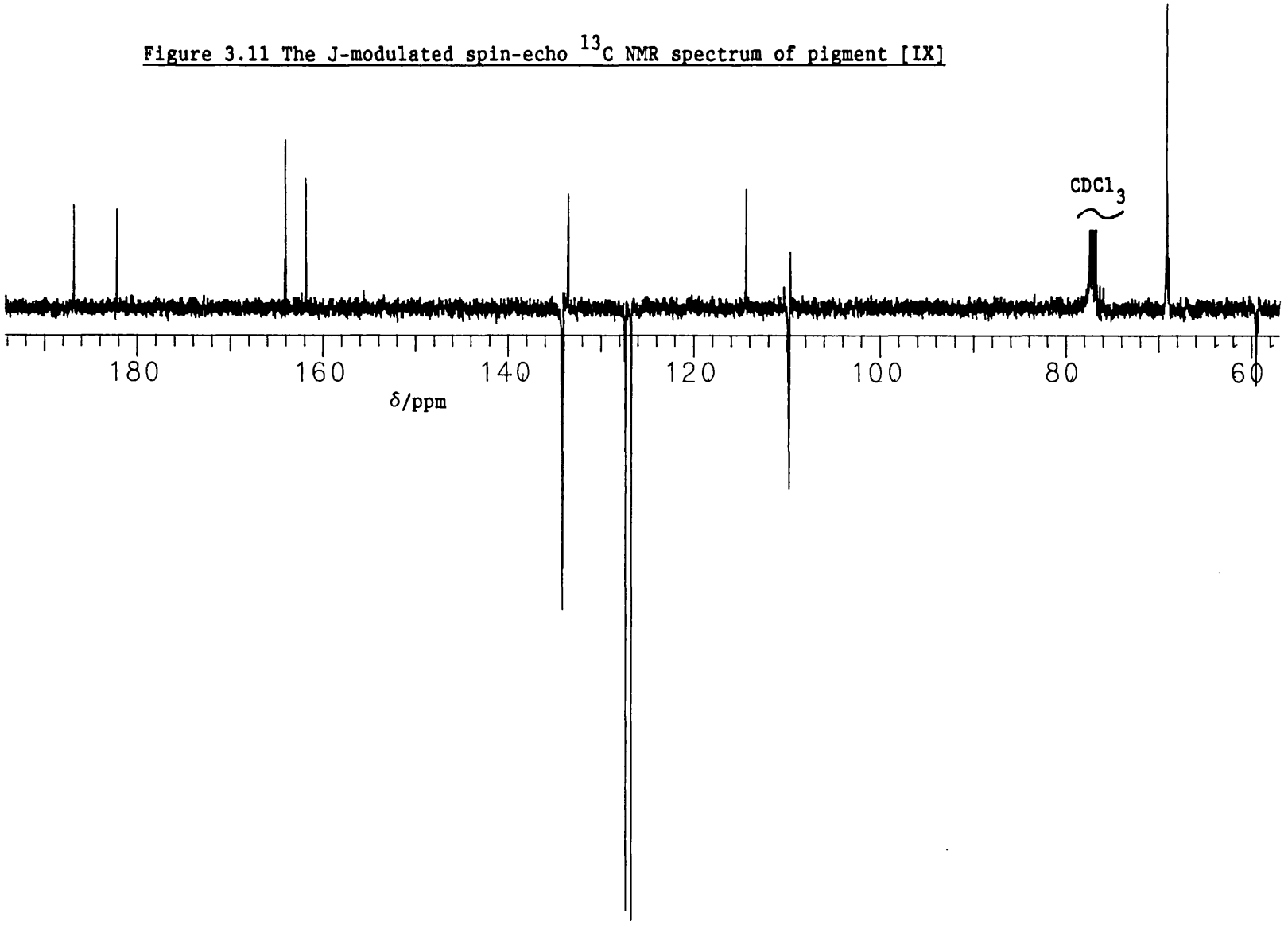
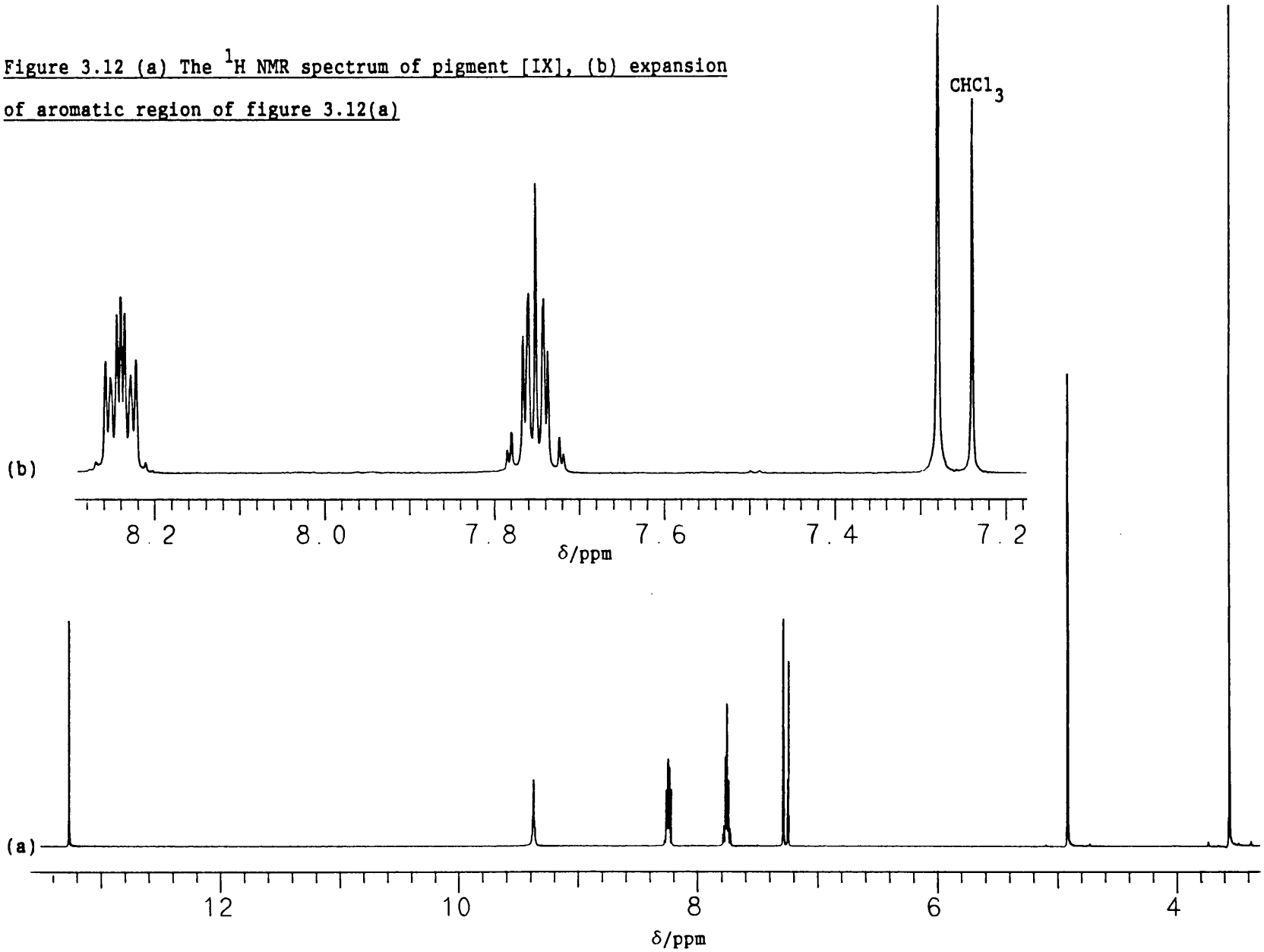


Figure 3.12 (a) The ^{1}H NMR spectrum of pigment [IX], (b) expansion of aromatic region of figure 3.12(a)



concentration dependent) one signal (δ 9.37ppm) is nonetheless suggestive of a phenolic proton, whilst the signal at δ 13.27ppm indicates either a phenolic proton subject to intramolecular hydrogen bonding or a carboxylic acid. An examination of the aromatic region (figure 3.12b) revealed, in addition to a signal due to an isolated aromatic proton (δ 7.28ppm), two multiplets each with an integral of two units at δ 7.75 and 8.24ppm which are characteristic of an unsubstituted A-ring of an anthraquinone.

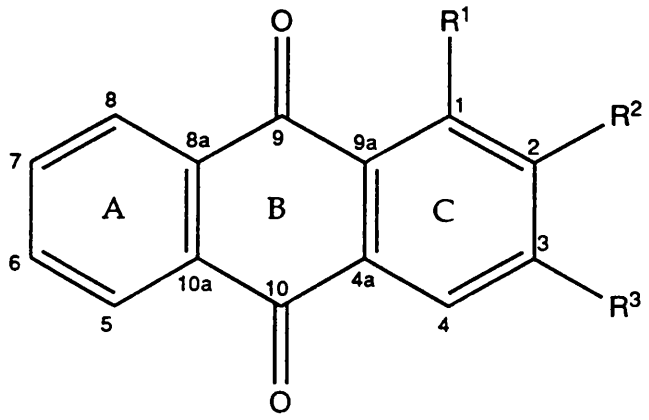
The infrared spectrum revealed a chelated and non-chelated carbonyl frequency (at 1625 and 1670cm^{-1} respectively) which indicates the presence of one α-hydroxyl group. An intramolecularly hydrogen bonded hydroxyl group was also detected (3180cm^{-1}).

From the above information it can be deduced the pigment is an anthraquinone unsubstituted in one ring and with an α-hydroxyl group in the other ring. The second hydroxyl group must be in the 2- or 3-position because of the presence of a non-chelated carbonyl frequency in the infrared spectrum. The UV-VIS spectrum recorded in alkaline ethanol revealed a value of λ_{max}= 502nm for the longest wavelength maxima. The position of this maximum gives information about the number and location of hydroxyl groups attached to the anthraquinone skeleton[83] and is only affected to a small degree (± 20nm) by other substituents. The values of this maximum for 1,3- and 1,2-dihydroxyanthraquinone are 485 and 576nm respectively[83] which suggests pigment [IX] is a 1,3-dihydroxyanthraquinone. Finally the methylene and methoxyl groups must be linked to give a CH_2OCH_3 group which must be attached to the anthraquinone skeleton in either the 2- or 4-position. This anthraquinone is unsubstituted in one ring and hence is likely to be derived biosynthetically from shikimic acid (see section 1.3.4). Anthraquinones formed by this pathway are usually

substituted by alkyl groups in the 2- and not the 4-position and thus it is likely the CH_2OCH_3 group of pigment [IX] is also in the 2-position. In addition the UV-VIS spectrum of the pigment (λ_{max}= 242(sh), 246, 282, 334 and 415nm) resembles that of lucidin [23], a 1,3-dihydroxyanthraquinone (λ_{max}= 242, 246, 280, 330, 415nm)[83] rather than that of alizarin [17], a 1,2-dihydroxyanthraquinone (λ_{max}= 248, 264, 277, 330(sh), 432nm).[83] On the basis of the above spectroscopic data, pigment [IX] is proposed to be 1,3-dihydroxy-2-methoxymethyl-anthraquinone, [35].

1,3-dihydroxy-2-methoxymethylanthraquinone, [35] which is the ω-methyl ether of lucidin [23], has previously been isolated from the roots of *Galium album*,[84] *Rubia cordifolia*,[85] *Morinda parvifolia*[73] and the heartwood of *Faramea cyanea*[86] which are all members of the *Rubiaceae* family. It has been suggested[81-83] that this ω-ether could be an artefact formed by the reaction of lucidin [23] with methanol (which was used in this work as solvent for the extraction of culture material). The benzylic alcohol function of lucidin [23] is known to be very reactive forming an ω-ethylether simply by boiling with chloroform containing the normal 2% ethanol.[82]

The UV-VIS, IR, mass spectrometry and ^1H NMR data obtained for pigment [IX] are in good agreement with the published values.[84-86,73] Although no ^{13}C spectral data have been reported for lucidin-ω-methylether [35], that of the ethylether has been analysed.[87] An examination of the ^{13}C chemical shift values and assignments reported for the ω-ethylether, together with the ^{13}C data obtained for the pigment [IX] (both ^1H-decoupled ^{13}C chemical shift values and relative peak areas) and the results of the APT experiment enable the peaks to be assigned (see table 3.7).



	R^1	R^2	R^3
[23]	OH	CH_2OH	OH
[17]	OH	OH	H
[35]	OH	CH_2OCH_3	OH

Table 3.7 ^{13}C NMR data for pigment [IX], lucidin-ω-methylether

δ/ppm	Assignment	δ/ppm	Assignment
186.87	C-9	127.33	C-5[c]
182.21	C-10	126.69	C-8[c]
164.04	C-1[a]	114.32	C-2
161.84	C-3[a]	109.75	C-4
134.12	C-6	109.60	C-9a
134.12	C-7	68.92	$\underline{C}H_2OCH_3$
134.03	C-4a	59.36	$CH_2O\underline{C}H_3$
133.50	C-8a[b]		
133.46	C-10a[b]		

a,b,c assignments may be reversed.

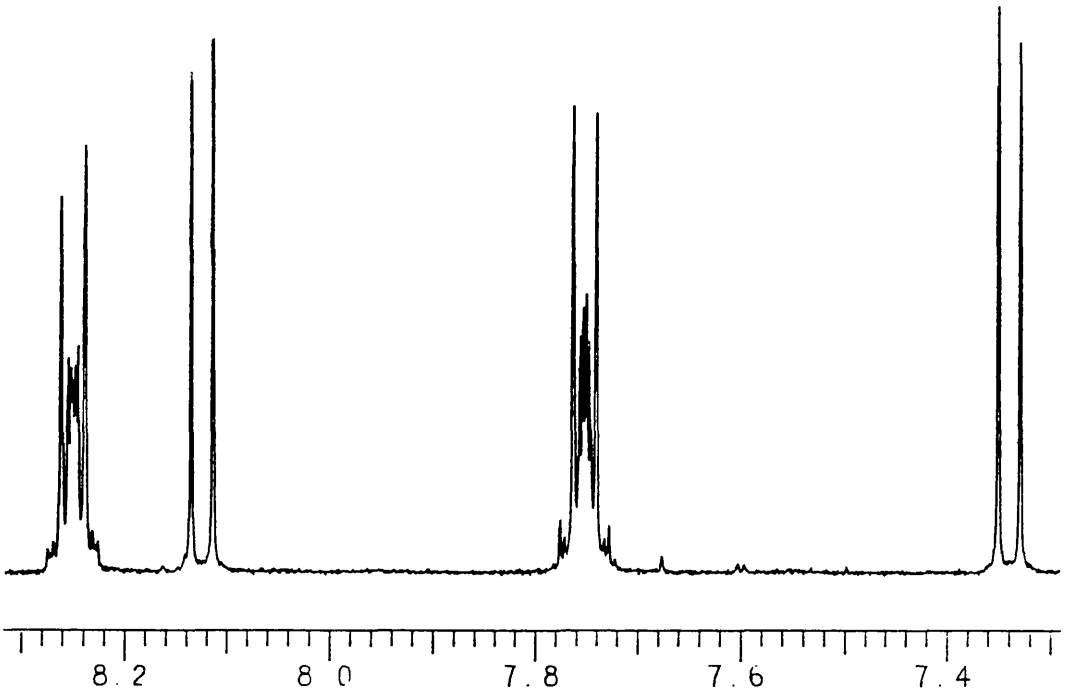
Section 3.2.6.2 **Characterisation of pigment [I]**

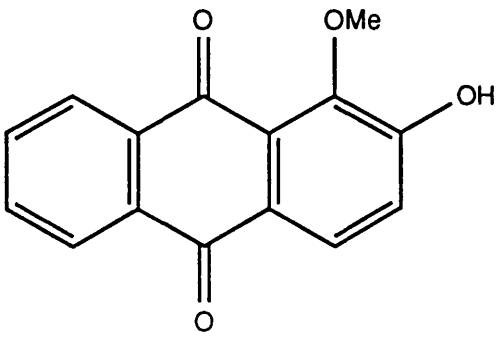
The pigment [I] was isolated as an orange/yellow powder (4.9mg). The CI mass spectrum of [I] had m/z 255 as the base peak with only three other ions evident (these three were all less than 15% of the intensity of the base peak). In the conventional EI mass spectrum the most prominent ions were at m/z 254, 236 and 208 (the base peak). These two spectra indicated the pigment to have a molecular mass of 254 daltons with the other two prominent ions in the EI spectrum corresponding to successive loss of H_2O and CO. An accurate mass spectrum of the molecular ion gave a value corresponding to the composition $C_{15}H_{10}O_4$.

The ^1H NMR spectrum of [I] revealed a methoxyl group (δ 4.02ppm), an hydroxyl proton (δ 6.65ppm, which disappeared on shaking with D_2O), two doublets each with an integral equivalent to one hydrogen atom and a coupling constant of 8.5Hz (δ 7.34 and 8.13ppm), as well as two multiplets each with an integral of two (δ 7.72-7.78 and 8.22-8.28ppm). The coupling constant of the two doublets, 8.5Hz, is a typical value for a coupling between two aromatic protons on adjacent carbons (i.e. an aromatic *ortho* coupling).[70] The chemical shifts of these doublets are consistent with this assignment. The integral and chemical shift values of the two multiplets as well as their complex nature, suggested these signals might be caused by the four protons of an unsubstituted A-ring of an anthraquinone (see figure 3.13). Although these signals had a different splitting pattern from the previously identified anthraquinone pigment [IX] (see figure 3.12b), it was considered, and later confirmed (see section 3.2.6.8) that such differences might be caused by small differences in either coupling constants and/or chemical shifts.

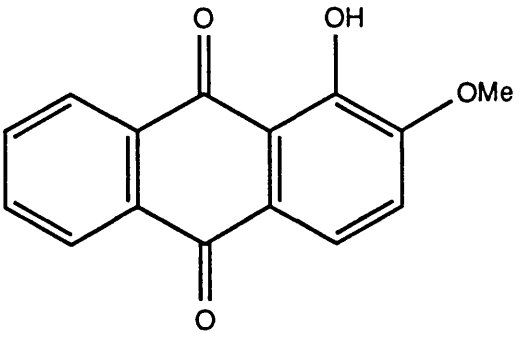
On the assumption that the pigment is an anthraquinone

Figure 3.13 Aromatic region of the ^{1}H NMR spectrum of pigment [I]





[19]



[20]

substituted in one ring only and with two *ortho* protons in the other ring (as suggested above), then the methoxyl and hydroxyl groups would occupy the two remaining sites. Two structural isomers are consistent with these assumptions, namely the 1- and 2-methyl ethers of alizarin, [19] and [20] respectively, both having the required molecular formula of $C_{15}H_{10}O_4$, although the absence of a peak at around δ 13ppm in the ^1H NMR spectrum (indicating an hydroxyl proton that was intramolecularly hydrogen bonded) would seem to exclude [20] as a possibility. A comparison of the EI mass spectrum of pigment [I] with reference spectra[83] for [19] and [20] indicated the pigment to be alizarin-1-methyl ether [19]. The EI mass spectra of both methyl ethers of alizarin show an intense peak at m/z 236 corresponding to loss of water from the molecular ion. This peak was also prominent in the mass spectrum of pigment [I]. The presence of a dominant $M^+ - H_2O$ fragment in the mass spectra of anthraquinones is indicative of either α-methoxy or o-hydroxy-methoxy substitution, but as both isomers incorporate one or both of these features, distinction between them cannot be made by examination of this peak. However, the 1-methyl ether isomer shows m/z 208 as the base peak, as did pigment [IX], whereas this is only a minor peak in the mass spectrum of the other isomer.

The UV-VIS spectrum of [I] in ethanol showed maximum absorptions at 247, 270, 284(sh), 330 and 381nm, which agree with literature values[83] for alizarin-1-methyl ether [19] (λ_{max}^{EtOH} 242(sh), 249, 270, 284(sh), 385nm) when the error in taking measurements is considered (approximately ± 5nm). The values for alizarin-2-methyl ether [20] are quite different (λ_{max}^{EtOH} 223, 250, 425nm). On addition of sodium hydroxide solution to an ethanolic solution of [I] the colour of the solution changed from yellow to orange ($\lambda_{max}^{EtOH/OH^-}$ 249, 268(sh), 315,

498nm). A basic ethanolic solution of alizarin-2-methyl-ether [20] is purple ($\lambda_{max}^{EtOH/OH^-}$ 232, 515nm).[83] On the basis of this information pigment [I] was characterised as alizarin-1-methyl-ether [19].

Alizarin-1-methyl-ether [19] has been isolated from extracts of over twenty plant species (all Rubiaceae), including *Galium verum* and six other members of the *Galium* group.[81-83] This pigment has also been isolated from tissue cultures of several of these plants.[81-83]

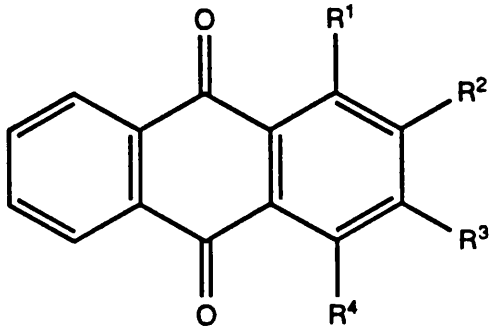
Section 3.2.6.3 **Characterisation of pigment [II]**

This pigment was isolated as a yellow solid (0.7mg). The CI mass spectrum indicated the molecular mass of the pigment to be 284 daltons (m/z 285 as the base peak) with another peak at m/z 255 probably derived from a small amount of pigment [I]. The conventional EI mass spectrum showed an intense molecular ion, ions at m/z 269, 267 and 266 (base peak) corresponding to the loss of ·Me, ·OH and H_2O from the molecular ion, as well as a large number of other ions at lower masses which suggested impurities were present. Accurate mass measurements indicated a molecular formula of $C_{16}H_{12}O_5$.

The ^1H NMR spectrum of [II] indicated an anthraquinone unsubstituted in one ring (two multiplets at δ 8.23 and 7.74ppm, two hydrogens each), an isolated aromatic proton (δ 7.70ppm) and two methoxyl groups (δ 4.08 and 3.99ppm). A signal derived from an hydroxyl proton was not observed in the ^1H NMR spectrum although an hydroxyl group must be present in this pigment to be consistent with the empirical formula given by mass spectrometry. Hydroxyl protons present in an α-position of the anthraquinone skeleton give rise to sharp signals in ^1H NMR spectra, whereas those present in a β-position are frequently not observed.[83] This is because the former are strongly intramolecularly hydrogen-bonded to the neighbouring carbonyl oxygen

and exchange very slowly on the NMR timescale whereas the latter are not so bonded and exchange rapidly. Hence the hydroxyl group of pigment [II] is likely to be present in the β-position. During the identification of some of the other pigments it had been noted that signals derived from hydroxyl substituents appear sharper when d_6-benzene is used as the NMR solvent rather than d-chloroform. This may be because d_6-benzene contains less residual water than d-chloroform and hence the rate of exchange of hydroxyl protons is slower in the former solvent. When the sample was rerun with d_6-benzene being used as solvent a signal due to an hydroxyl proton was noted (δ 5.80ppm, this signal disappeared on shaking with D_2O). The presence of a small amount of pigment [I] which had first been noted in the analytical HPLC chromatogram of [II] was also noted in both of these spectra (approximately 5% by comparison of peak areas).

The mass spectra and NMR data described above suggested pigment [II] was a dimethoxy-monohydroxy-anthraquinone with all three substituents in the same ring and with the hydroxyl substituent occupying a β-position. Three structures are consistent with this analysis namely [36], [37] and [38]. Although [38] has been made synthetically[88] it is not reported to have been isolated from a natural source, unlike [36] and [37] which have both been isolated from Rubiaceous plants.[83] It is conceivable however that [38] might be biosynthesised from purpurin [26] which is widely spread in Rubiaceous plants and hence any of the three isomers are likely on biosynthetic grounds. The ^1H NMR data for pigment [II] are in closest agreement with those given for [36] as shown in table 3.8. However, the UV-VIS and mass spectra of pigment [II] differ considerably from those published for [36] or indeed [37] or [38]. As impurities present in the sample might account for these discrepancies the sample was



	R^1	R^2	R^3	R^4
[36]	OCH_3	OH	OCH_3	H
[37]	OCH_3	OCH_3	OH	H
[38]	OCH_3	OH	H	OCH_3
[26]	OH	OH	H	OH
[19]	OCH_3	OH	H	H

Table 3.8 ^{1}H NMR data for pigments [II], [36], [37] and [38][a]

[II]	[36]	[37]	[38]
3.99 (3H, s)	3.99 (3H, s)	3.99 (3H, s)	3.98 (3H, s)
4.08 (3H, s)	4.11 (3H, s)	4.03 (3H, s)	3.99 (3H, s)
7.70 (1H, s)	7.72 (1H, s)	7.60 (1H, s)	6.98 (1H, s)
7.71-7.78 (2H, m)	7.75 (2H, m)	7.75 (2H, m)	7.66-7.80 (2H, m)
8.20-8.27 (2H, m)	8.26 (2H, m)	8.25 (2H, m)	8.15-8.25 (2H, m)

[a] all recorded in $CDCl_3$, using TMS as internal standard at 400, 300, 300 and 200MHz for [II], [36], [37] and [38] respectively. See reference 83 for spectral data for [36] and [37] and reference 88 for [38].

repurified by HPLC with heartcutting of the major peak. In this procedure the front and tail of a peak are discarded and only the central portion is collected, generally enabling (a smaller amount) of a more pure sample to be obtained. Unfortunately, the UV-VIS, NMR and mass spectrometric data obtained for this rechromatographed sample indicated that the sample size had become so small that impurities (e.g. plasticisers) derived from the HPLC and NMR solvents used had reached significant levels masking any signals derived from the sample.

In the mass spectra of both [36] and [37] the ion at m/z 269 is reported[83] to be very intense relative to the ion at m/z 266, whereas in the mass spectrum of [II] the reverse is true. However, a methoxy substituent in the *peri* position of an anthraquinone usually gives rise to a quite intense ion corresponding to the loss of water.[89] Furthermore, the loss of water from the molecular ion will also occur when a hydroxyl group is positioned *ortho* to a methoxyl group.[90] A compound possessing both of these features, and for which the mass spectral data are well established[83] is alizarin-1-methyl ether [19]. This compound shows an intense peak (43% of the base peak) corresponding to the loss of water from the molecular ion whilst the peak corresponding to the loss of a methyl radical is virtually undetectable. Hence it is possible that the pigment [II] is indeed anthragallol 1,3-dimethyl ether [36] and that some of the published spectral data are incorrect.

Section 3.2.6.4 Characterisation of pigment [III]

The pigment [III] was isolated as a bright yellow solid (1.6mg). The CI mass spectrum showed four prominent ions at m/z 271, 285, 300 and 315, with the peak at m/z 285 as the base peak. In the same mass

region of the conventional EI mass spectrum ions at m/z 270, 282, 283, 284, 285, 299 and 314 were observed. Peak profiling of these ions was inconclusive: the profiles of the peaks at m/z 282, 283, 284 and 285 were superimposable, although the ions at m/z 270, 299 and 314 were too low in abundance to enable reliable profiles of these peaks to be acquired. The fragmentation pattern (both mass losses and relative intensities) observed in this spectrum were analogous to those noted for lucidin-ω-methyl-ether [35] (see table 3.9), indicating the pigment [III] to be of similar structure to lucidin-ω-methyl-ether [35] but having a molecular mass of 314 daltons with another pigment of molecular mass 270 daltons present as a minor impurity.

Measurement of the UV-VIS spectrum in alkaline ethanol can provide useful information as to the substitution pattern of anthraquinones. The position of the band occurring at highest wavelength is largely dependent on the number and position of hydroxyl groups attached to the anthraquinone skeleton.[83] The similarity between this value when measured for pigment [III] and lucidin-ω-methyl ether [35] (505 and 502nm respectively) suggested pigment [III] was also a 1,3-dihydroxyanthraquinone.

The ^{1}H NMR spectrum of [III] revealed two hydroxyl resonances (δ 13.39 and 9.30ppm), an isolated aromatic proton (δ 7.28ppm), an isolated methylene group (δ 4.91ppm), two methoxyl groups (δ 3.96 and 3.55ppm) and a series of multiplets at δ 8.20ppm (1H; d, J = 8.6Hz), δ 7.68 (1H; d, J = 2.7Hz) and δ 7.23 (1H; dd, J = 8.6, 2.7Hz). This latter signal was partially obscured by a signal derived from residual chloroform in the NMR solvent. A number of these signals (δ 13.39, 9.30, 7.28, 4.91 and 3.55ppm) occur at either the same or similar chemical shift to signals present in the spectrum of lucidin-ω-methyl-ether [35]. The three multiplets in the aromatic

Table 3.9

Mass spectral data for lucidin-ω-methyl-ether [35] and pigment [III]

Lucidin-ω-methyl ether [35] m/z (rel. int.)	Assignment	Corresponding ion in mass spectrum of pigment [III] m/z (rel. int.)
284 (7)	M^+	314 (7)
269 (5)	M^+ - Me	299 (6)
255 (4)	M^+ - CHO	285 (9)
254 (27)	M^+ - CH_2O	284 (67)
253 (22)	M^+ - CH_3O	283 (25)
252 (100)	M^+ - CH_3OH	282 (100)
226 (2)	254 - CO	256 (3)
225 (4)	253 - CO	255 (5)
224 (7)	252 - CO	254 (6)
196 (21)	224 - CO	226 (8)
168 (9)	196 - CO	198 (2)
139 (13)	168 - CHO	

Table 3.10 Solvent-induced shifts[a] in ^1H NMR of pigment [III] and lucidin-ω-methyl-ether [35]

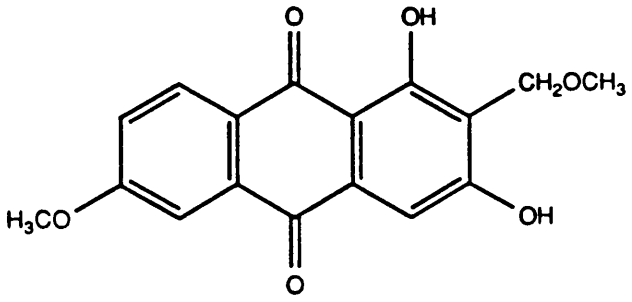
Pigment [III]			Lucidin-ω-methyl-ether [35][b]		
δ_{CDCl_3}	$\delta_{C_6D_6}$	Δ^c	δ_{CDCl_3}	$\delta_{C_6D_6}$	Δ^c
13.39	13.96	-0.57	13.27	13.71	-0.44
9.30	9.34	-0.04	9.37	9.37	0.00
8.20	8.14	+0.06			
7.68	7.67	+0.01			
7.28	7.75	-0.47	7.28	7.68	-0.40
7.23	6.77	+0.46			
4.91	4.52	+0.39	4.91	4.50	+0.41
3.96	3.10	+0.86			
3.55	2.69	+0.86	3.55	2.70	+0.85

[a] all entries are in ppm, [b] see experimental to section 3.2.6.1

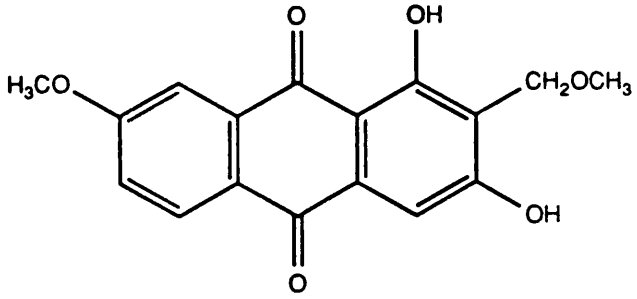
[c] $\Delta = \delta_{CDCl_3} - \delta_{C_6D_6}$

region of the spectrum are consistent with ones derived from those protons present in one of the aromatic rings of an anthraquinone which is monosubstituted in a β-position. i.e. two protons which are *ortho* coupled to each other (J $\simeq$ 8Hz), one of which (δ 7.23ppm) is additionally *meta* coupled (J $\simeq$ 2.7Hz) to another proton (δ 7.68ppm). When d_4-methanol was used as the NMR solvent the aromatic region of the spectrum obtained clearly showed signals due to four protons with multiplicities and coupling constants consistent with those measured above using d-chloroform. The sample was also reexamined using d_6-benzene as the NMR solvent. The observed changes in the chemical shift values for a number of the signals (the so called "solvent shifts") were similar to those noted for lucidin-ω-methyl ether [35] (see table 3.10).

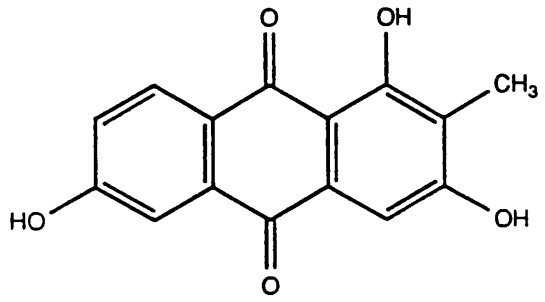
The spectral data for pigment [III] thus suggest that the compound is an anthraquinone substituted in the C ring in the same way as the C ring of lucidin-ω-methyl-ether [35] with the A ring containing a methoxyl group as the β-substituent. Two structures are consistent with this, namely anthraquinones [39] and [40]. Both of these structures differ from the other pigments isolated in this work in that both benzylic rings are substituted. This suggests the pigment is formed via the polyketide pathway, although the substitution patterns present in either [39] or [40] do not correspond to any of the common ones for polyketide-derived anthraquinones (see section 1.3.4 and figure 1.4). However a number of anthraquinones which are substituted in both rings in a similar way to [39] or [40] have been shown (by radioactive feeding experiments) to be derived from o-succinylbenzoic acid.[91,92] Although neither [39] or [40] have been reported in the chemical literature the spectroscopic data for [III] are consistent with values given for similarly substituted



[39]



[40]



[41]

Table 3.11

^{1}H NMR data for 2-methyl-1,3,6-trihydroxyanthraquinone [41] and [III][a]

[41]	Pigment [III]
8.06 (1H, d, J = 8 Hz)	8.15 (1H, d, J = 8.3 Hz)
7.44 (1H, d, J = 3 Hz)	7.59 (1H, d, J = 2.6 Hz)
7.13 (1H, s)	7.28 (1H, dd, J = 2.6, 8.3 Hz)
7.12 (1H, dd, J = 8, 3 Hz)	7.18 (1H, s)
2.10 (3H, s)	

[a]both spectra were recorded using CD_3OD as solvent and TMS as internal standard, at 100 and 400MHz for [41] and [III] respectively. Some signals in the spectrum of [III] were obscured by the intense signals of residual methanol.

anthraquinones.[81] For example, the ^{1}H NMR spectrum of 2-methyl-1,3,6-trihydroxyanthraquinone [41] which has been isolated from *Rubia cordifolia* and *R. akane* (both Rubiaceae) is very similar to that of [III] as shown in table 3.11. Hence [III] is likely to be either [39] or [40], although as no synthetic samples were available for either of these compounds both possibilities are left open for this pigment.

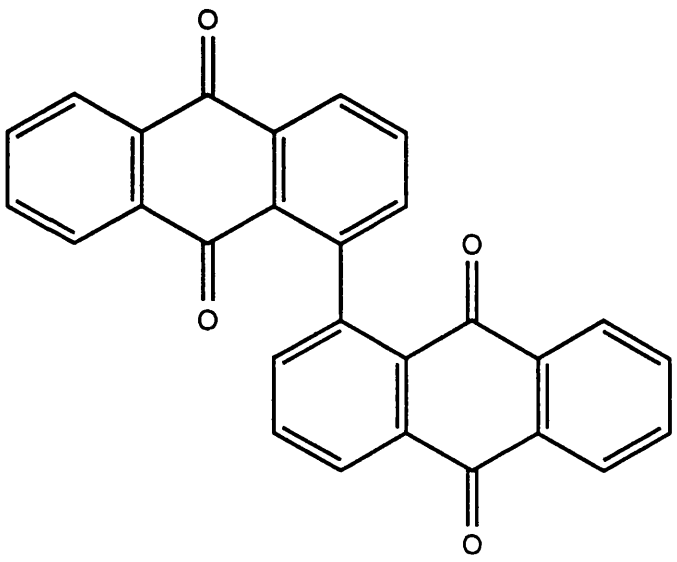
Section 3.2.6.5 Characterisation of pigment [IV]

Pigment [IV] was isolated as a yellow solid (0.7mg). No major ions were detected by either CI or FAB mass spectrometry for this compound which is surprising as a wide range of compounds (non-polar to polar, low to high molecular mass) can be analysed using one or other of these two techniques.[70] Although ions were seen in the conventional EI mass spectrum at m/z 129, 149, 167, 253, 277, 278, 279, 305 and 320 the peak profiles of most of these ions were dissimilar, indicating a mixture to be present. A number of these ions occur at mass numbers usually associated with (solvent-derived) plasticiser impurities (m/z 129, 149, 167 and 279).[93] It is unlikely that these plasticiser impurities had originated from the HPLC purification procedure as no such impurities had been noted during the characterisation of other pigments purified using solvents of the same origin. It is more likely that the sample was accidentally contaminated during part of the mass spectrometry procedure, possibly by the use of a contaminated probe source. The sample was repurified by HPLC and reexamined by EI, CI and FAB techniques but similar poor results were obtained. It is unlikely that [IV] is a simple anthraquinone as the anthraquinone pigments characterised in this work were easily analysed by EI and CI mass spectrometry. FAB mass

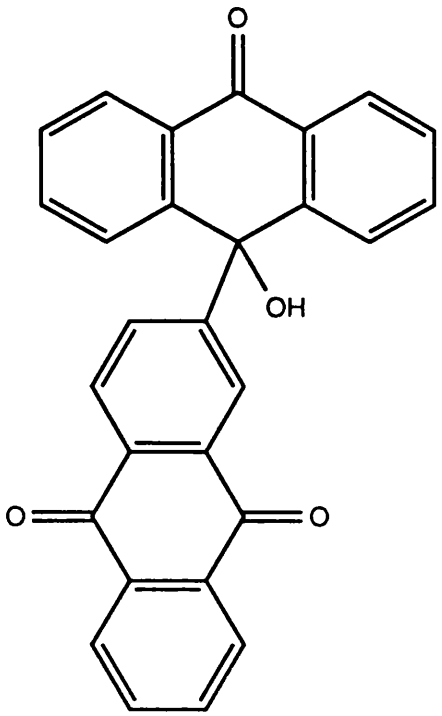
spectrometry is the most troublesome of the three techniques and hence it is possible that [IV] is a compound which would normally respond to analysis by this technique but did not in this case for some reason (e.g. poor solubility in the liquid matrix).

The ^{1}H NMR spectrum of [IV] showed a proton subject to hydrogen-bonding (δ 13.20ppm, s), two multiplets (δ 8.22-8.30 and 7.76-7.84ppm, each with an integral of four units) and two further singlets (δ 7.35 and 5.45ppm, each with an integral of two units).

The UV-VIS spectrum of [IV] in ethanol showed maxima at 246, 278, 335 (sh) and 410nm. After the addition of a few drops of base maxima were observed at 222, 260 (sh), 312, 348 (sh) and 502nm. These spectra together with the chemical shifts of the two multiplets in the ^{1}H NMR spectrum indicate [IV] is an anthraquinone derivative. The magnitudes of the integrals for the signals in the ^{1}H NMR spectrum of [IV] suggest the pigment is not a simple monomer but possibly a dimer with a basic skeleton such as [42] or [43]. These do occur naturally although chiefly in insects and fungi and only in a few plants of which none belong to the Rubiaceae family.[81] In any case such dimers usually show an abundant molecular ion in the mass spectrum.[81] The available sample size meant other analytical techniques (e.g. ^{13}C NMR, IR or elemental analysis) could not be used to elucidate the structure of this pigment and hence this pigment was not further characterised.



[42]



[43]

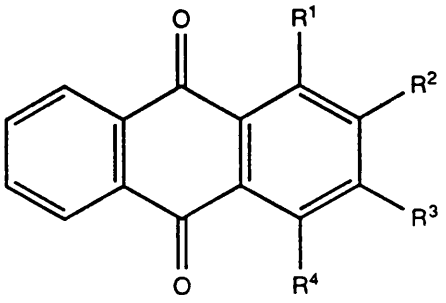
Section 3.2.6.6 **Characterisation of pigment [V]**

The pigment [V] was isolated as a yellow powder (3.9mg). The CI mass spectrum showed a single prominent ion at m/z 255 indicating a molecular mass of 254 daltons. In the conventional mass spectrum the molecular ion at m/z 254 was the base peak. Other ions were observed at m/z 236 (corresponding to the loss of water from the molecular ion) and at m/z 226, 225, 208, 197, 180, 169, 152 and 141 (corresponding to the successive loss of CO or CHO from either the molecular ion or the ion at m/z 236). The occurrence in this spectrum of the molecular ion as the base peak together with ions corresponding to successive loss of CO indicates [V] is an anthraquinone.[83] Accurate mass measurements indicated a molecular formula of $C_{15}H_{10}O_4$.

In the ^1H NMR spectrum two multiplets (δ 8.22-8.30 and 7.74-7.80ppm), each with an integral of two units, indicated [V] was

an anthraquinone unsubstituted in one ring. The substituents of the other aromatic ring were established as a methyl group (δ 2.23ppm, s), an isolated proton (δ 7.27ppm, s), and two hydroxyl groups - one in the α-position (δ 13.19ppm, sharp singlet which disappeared on shaking with D_2O) and the other in a β-position. Although no signal was observed for this second hydroxyl proton (the protons of β-hydroxyl groups are frequently not seen in the ^1H NMR spectra of anthraquinones due to fast exchange with residual water in the NMR solvent),[83] the presence of this group is required to be consistent with the molecular formula given by mass spectrometry measurements.

Four structural isomers ([24], [44]-[46]) are consistent with the spectral data described above, although only two of these ([24] and [46]) have previously been isolated from a natural source.[81-83] None of the reported naturally-occurring anthraquinones feature both an α-positioned methyl group and an unsubstituted benzylic ring - such as is present in structures [44] and [45]. This is understandable as both [44] and [45] are unlikely to be formed by either of the biosynthetic routes leading to anthraquinones: an α-positioned methyl group suggests formation via the polyketide pathway, whereas an unsubstituted benzylic ring suggests formation via the shikimic acid pathway (see section 1.3.4). Hence an anthraquinone containing both of these features seems unlikely to have been formed naturally. Moreover, examination of the ^1H NMR data[81,82] measured for the twelve reported naturally occurring anthraquinones containing a methyl group in the α-position (eight of insect origin and four from plants of the Lilaceae family) indicates these methyl protons resonate in the region δ = 2.60-3.00ppm, whereas a signal derived from the methyl group of [V] was observed at δ = 2.23ppm. The chemical shift of this signal and the others in the ^1H NMR spectrum of [V] agree well with those



	R^1	R^2	R^3	R^4
[44]	OH	H	OH	CH_3
[24]	OH	CH_3	OH	H
[45]	OH	OH	H	CH_3
[46]	OH	OH	CH_3	H

Table 3.12 ^{1}H NMR data[a] for pigment [V] and rubiadin [24]

Pigment [V] δ/ppm	Rubiadin [24] δ/ppm
13.19[b] (1H, s)	-
8.22-8.30 (2H, m)	8.21-8.31 (2H, m)
7.74-7.80 (2H, m)	7.74-7.82 (2H, m)
7.27 (1H, s)	7.22 (1H, s)
2.23 (3H, s)	2.22 (3H, s)

[a]recorded at 400MHz using TMS as internal standard with $CDCl_3$ and ($CDCl_3$ + CD_3OD) as solvents for [V] and [24] respectively. See reference 94 for spectral data for [24].
[b]exchangeable with D_2O.

reported for [24] as is shown in table 3.12 (no NMR data have been published for [46]). Whether pigment [V] has the structure [24] or [46] can not be ascertained reliably by mass spectrometry as [V], [24] and [46] have very similar mass spectra. The UV-VIS spectrum of [V] (λ_{max}^{EtOH}: 242(sh), 245, 279, 310 and 414nm) does however resemble that of [24] (λ_{max}^{EtOH}: 241(sh), 245, 279 and 414nm) more so than that of [46] (λ_{max}^{EtOH}: 249, 268, 283(sh), 331 and 436nm). On the basis of this data pigment [V] is characterised as [24], rubiadin. This anthraquinone occurs[81-83] in over forty members of the Rubiaceae family of plants including *Galium verum*, as well as tissue cultures of several of these plants. It has also been isolated from the heartwood of *Tectona grandis* (Verbenaceae) and roots of *Cassia multijuga* (Leguminosae).

Section 3.2.6.7 **Characterisation of pigment [VI]**

Pigment [VI] was isolated as a yellow solid (1.2mg). The CI mass spectrum showed only one intense ion at m/z 255 indicating a molecular mass of 254 daltons. In the conventional EI mass spectrum the ion at m/z 254 was the base peak with all other ions 10% or less of the intensity of this peak. A number of these other ions correspond to successive loss of CO from the molecular ion. Accurate mass measurements indicated a molecular formula of $C_{15}H_{10}O_4$.

The ^1H NMR spectrum of [VI] contained a signal at δ 13.06ppm which indicated an hydroxyl proton subject to intramolecular hydrogen bonding. This spectrum also revealed a series of doublets at δ 8.24 (1H, J = 8.4Hz), 7.71 (1H, J = 7.6Hz), 7.63 (1H, J = 2.7Hz) and 7.49ppm (1H, broad, J = 7.6Hz), a doublet of doublets at δ 7.20ppm (1H, J = 2.7, 8.4Hz), as well as a singlet at δ 2.36ppm (3H) corresponding to a methyl group attached to an aromatic ring. It can be deduced from this data that two proton-coupling systems are present

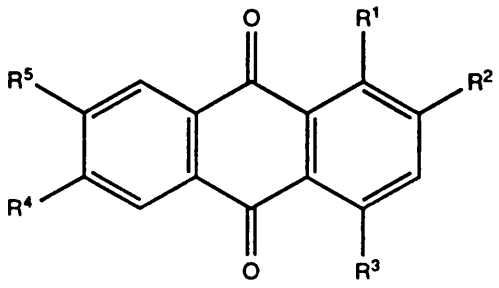
in the pigment molecule, namely, one derived from a 1,2,4-trisubstituted aromatic ring and the other from two *ortho* coupled protons. The appearance of one of these latter pairs of doublets as a set of broad signals indicates that this proton is additionally coupled (long-range) to one or more other protons.

An intense molecular ion and other ions corresponding to successive loss of CO units in the mass spectrum suggest this pigment is an anthraquinone. The molecular formula, presence of an α-chelated hydroxyl group (as indicated by the signal at δ 13.06ppm in the ^{1}H NMR spectrum) and the constraints imposed by the proton coupling systems observed limit the structure of this anthraquinone to one of six possibilities [47]-[52].

Although there are no references to either [51] or [52] in the chemical literature a dozen naturally occurring anthraquinones with a methyl substituent in an α-position have been isolated.[81,82] The ^{1}H NMR spectra of these compounds show a signal derived from an α-positioned methyl group in the region δ = 2.60-3.00ppm, whereas a signal derived from the methyl group of [VI] was observed at δ = 2.36ppm. Hence [VI] is unlikely to be either [51] or [52].

The UV-VIS data of [VI], together with those reported for a synthetic sample[95] of [47] and for [49][81] are given in table 3.13. It can be seen from this table that the UV-VIS spectra of [VI] and [49] are very similar, but quite different from the spectrum of [47]. The spectra of [VI] and [49] were recorded using different solvents (methanol and ethanol respectively) and this may account for the differences between the two spectra.

The ^{1}H NMR data for [VI] and those reported for synthetic samples of [47] and [49] are given in table 3.14. The spectrum of [VI] does not correspond exactly to either of the synthetic samples although it



	R^1	R^2	R^3	R^4	R^5
[47]	OH	OH	H	CH_3	H
[48]	OH	OH	H	H	CH_3
[49]	OH	CH_3	H	OH	H
[50]	OH	CH_3	H	H	OH
[51]	OH	H	CH_3	OH	H
[52]	OH	H	CH_3	H	OH
[53]	OCH_3	CH_3	H	OH	H
[15]	H	OH	H	H	H

Table 3.13 UV-VIS spectral data[a] for pigments [VI], [47] and [49]

[VI]	[47]	[49]
λ_{max}^{MeOH}	λ_{max}^{MeOH}	λ_{max}^{EtOH}
220	231	220
247		245
270	261	271.5
283 (sh)	284	280 (sh)
294 (sh)		292 (sh)
	331 (sh)	337.5
388 (sh)		395 (sh)
412		411
433 (sh)	426	425 (sh)

[a]all entries are in nms.

does contain the same number of signals with the same multiplicities and similar coupling constants to those in the spectrum of [49]. d-Chloroform and d_6-DMSO were used as solvents to record the spectra of [VI] and [49] respectively and this may account for the discrepancies between the two spectra. The spectrum of [VI] and [47] were both recorded using d-chloroform as solvent and yet these spectra are quite different. In particular, the multiplet present at δ = 8.11ppm in the spectrum of [47] (which results from coupling of one benzylic proton with two other ring protons and three methyl protons) is absent in the spectrum of [VI].

The UV-VIS and ^{1}H NMR data for [47] and [48] would be expected to be similar to one another (and likewise for [49] and [50]) and hence on the basis of the spectral data discussed above [VI] is deduced to be either [49] or [50]. Indeed a pigment which was characterised as being either [49] or [50] has been isolated from callus cultures of another Rubiaceous plant, *Cinchona pubescens*.[96] The ^{1}H NMR data obtained for this pigment are also shown in table 3.14. All but one of the signals in the ^{1}H NMR spectrum of this pigment (recorded in a $CDCl_3$/ CD_3OD mixture of unspecified proportions) agree within 0.06ppm to those observed in the spectrum of [VI]. The anomalous signal is that corresponding to H-7 of [49] where a value of δ 7.63ppm compares with one of δ 7.20ppm in the spectrum of [VI]. This latter value is closer to those given[83] for the proton resonances of the analogous structures [53] and [15] (δ 7.16 and 7.21ppm respectively) which suggests the published value of δ 7.63ppm is erroneous. It is not possible with the spectral data available to distinguish further between the two possibilities ([49] or [50]) for pigment [VI]. The compound soranjidiol [49] has been isolated from the wood and roots of a number of Rubiaceous plants.[81-83] Although this compound is

substituted in both benzylic rings radioactive feeding experiments have indicated[91] that it is made biosynthetically via the shikimic acid pathway.

Table 3.14 ^{1}H NMR data[a] for [VI], [47], [49] and a pigment isolated from callus cultures of *Cinchona pubescens.*

Pigment [VI][b]	[47][c]	[49][d]	Pigment[e] ex. C. Pubescens cultures
δ	δ	δ	δ
13.06 (s)[f]	12.89 (s)	13.11 (s)	
8.24 (J = 8.4)	8.19 (J = 8.1)	8.10 (J = 8.8)	8.23 (J = 7.0)
7.71 (J = 7.6)	8.11 (m)	7.64 (J = 7.7)	7.71 (J = 7.5)
7.63 (J = 2.7)	7.84 (J = 8.2)	7.58 (J = 7.7)	7.63 (dd J = 7.0, 2.0)
7.49 (J = 7.6)[g]	7.58 (J = 8.1)[g]	7.46 (J = 2.6)	7.57 (J = 2)
7.20 (dd J = 2.7, 8.4)	7.26 (J = 8.2)[g]	7.23 (dd J = 2.6, 8.8)	7.50 (J = 7.5)
	6.24 (s)		
2.36 (3H, s)	2.54 (3H, s)	2.28 (3H, s)	2.38 (3H, s)

[a] all resonances arise from single protons and are doublets unless otherwise indicated. Chemical shift values are in ppm relative to TMS as internal standard and coupling constants are in Hertz.

Spectra recorded at [b] 400, [c,d] 200 or [e] 300 MHz using either [b,c] $CDCl_3$, [d] d_6-DMSO or [e] ($CDCl_3$ + CD_3OD) as solvents.

[f] exchangeable with D_2O.

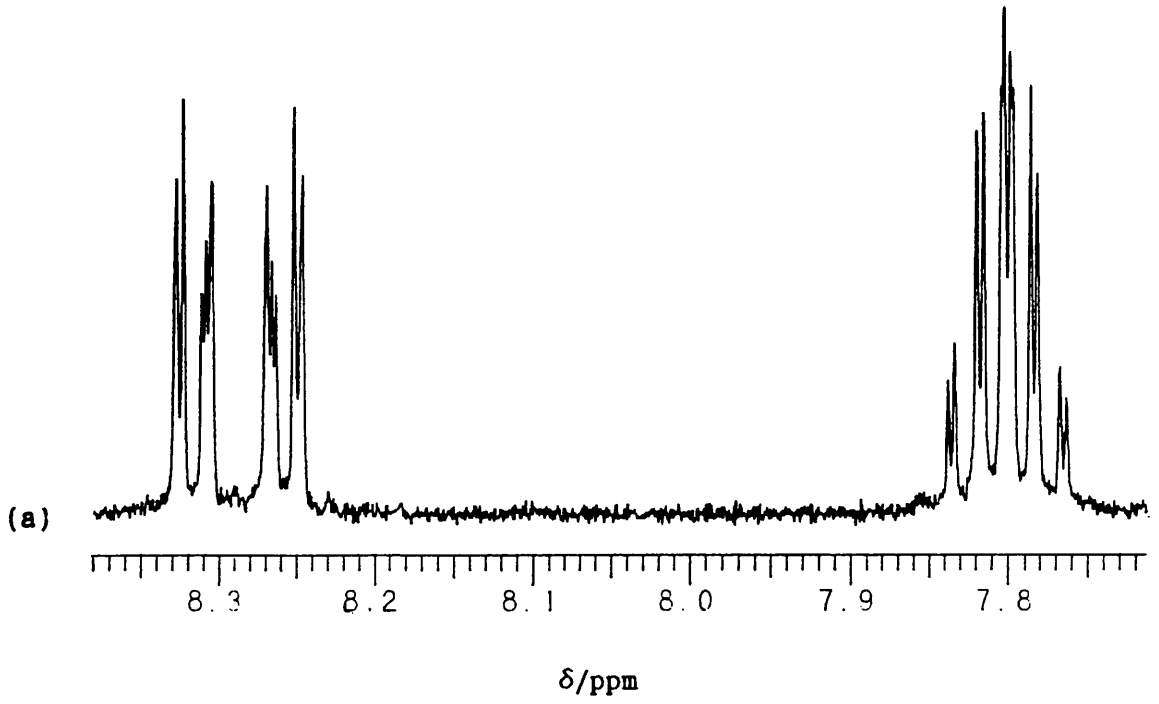
[g] broad signals.

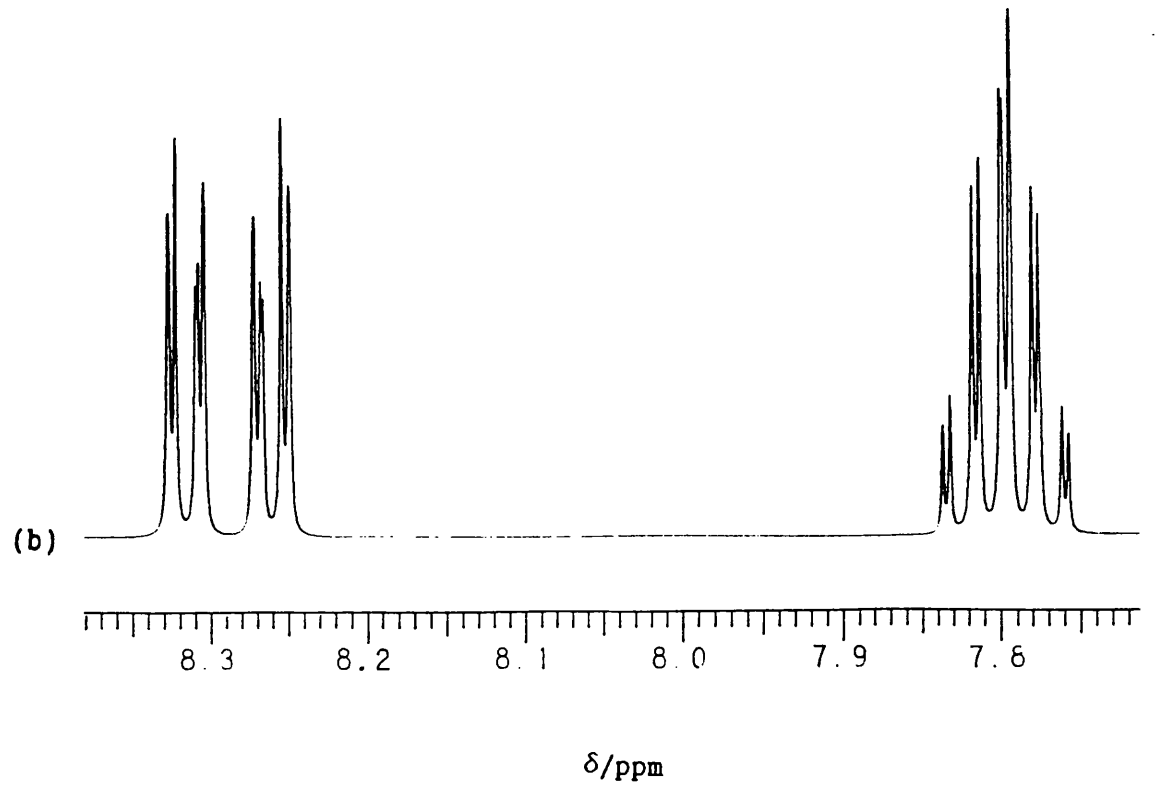
Section 3.2.6.8 **Characterisation of pigment [VII]**

The pigment [VII] was isolated as a yellow powder (3.2mg). In the CI mass spectrum only the base peak at m/z 299 and a small peak at m/z 284 (12% of the intensity of the base peak) were evident. This suggested the molecular mass of the pigment to be 298 daltons and that this pigment contained a methoxyl substituent (see section 2.2.1.2). The conventional EI mass spectrum revealed the molecular ion (m/z 298) and the base peak at m/z 266 (corresponding to the loss of methanol from the molecular ion). Also apparent were ions consistent with the successive loss of CO (m/z 238, 210, 182, 154, 126) from the base peak. An accurate mass spectrum showed the pigment to have the empirical formula $C_{16}H_{10}O_6$. The mass spectral data thus indicated the pigment [VII] to be a quinone (loss of CO units), containing hydroxyl (loss of more than two CO units) and one methoxyl (loss of methanol) substituents.

The ^1H NMR spectrum of [VII] revealed two chelated hydroxyl protons (δ 14.81 and 12.64ppm), an isolated aromatic proton (δ 7.38ppm), two aromatic multiplets (δ 8.27-8.33 and δ 7.76-7.84ppm, two protons each) and a methoxyl group (δ 4.06ppm). An examination of the region of the spectrum containing the multiplets suggested the observed pattern might be derived from an AA'BB'-type coupling system see figure 3.14a). As these multiplets were quite different in appearance to those found for the previously characterised anthraquinones a spin-simulation experiment was undertaken assuming an AA'BB'-type system, using values of the chemical shift that were estimated from inspection of the second order spectra for the four protons (δ = 3326, 3304, 3125 and 3112Hz, for A, A', B and B' respectively) and values for coupling constants which had been produced for pigment [IX] using a similar simulation program. The

Figure 3.14 (a) A portion of the aromatic region of the ^{1}H NMR spectrum of pigment [VII], (b) A spectrum produced by a spin simulation experiment for pigment [VII] (same spectral width as 3.14a)



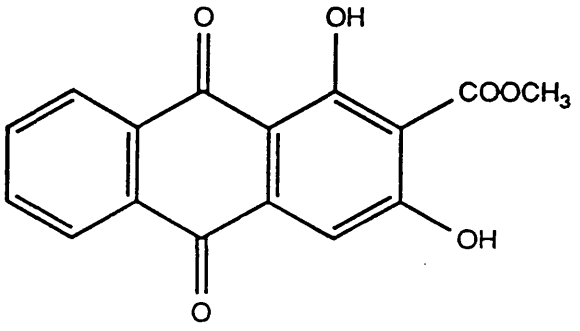


observed and simulated spectra obtained for pigment [VII] (figures 3.14a and 3.14b respectively) are very similar in appearance. The values of the chemical shifts and coupling constants provided by the simulation program are shown in table 3.15. The magnitude of these coupling constants are typical[70,93] of those associated with *ortho*- (7.74, 7.56 and 7.58Hz), *meta*- (1.35 and 1.56Hz) and *para*- (0.35Hz) coupled benzylic protons and indicate an *ortho*-disubstituted aromatic ring to be present such as is seen in an unsubstituted aromatic ring of an anthraquinone.

Table 3.15 Results of spin simulation experiment

Estimated chemical shifts δ/ppm		Estimated coupling constants J/Hz	
δ_A	= 8.32	$J_{AA'}$	= 0.35
$\delta_{A'}$	= 8.26	J_{AB}	= 7.74
δ_B	= 7.81	$J_{AB'}$	= 1.35
$\delta_{B'}$	= 7.78	$J_{A'B}$	= 1.56
		$J_{A'B'}$	= 7.56
		$J_{BB'}$	= 7.58

To conform both to the empirical formula of $C_{16}H_{10}O_6$ given by mass spectrometry and be consistent with the ^1H NMR and mass spectrometry data observed the pigment [VII] must be an anthraquinone unsubstituted in one ring and containing either (amongst other substituents) a carboxylic acid or methyl ester functionality in the other ring. The data for the pigment [VII] agree very closely with



[54]

Table 3.16 ^{1}H NMR data for pigment [VII] and munjistin methyl ester [54]

Pigment [VII][a] δ/ppm	Munjistin methyl ester [54][a,b] δ/ppm
14.81[c] (1H, s)	14.82[c] (1H, s)
12.64[c] (1H, s)	12.64[c] (1H, s)
8.24-8.33 (2H, m)	8.23-8.39 (2H, m)
7.76-7.84 (2H, m)	7.76-7.87 (2H, m)
7.38 (1H, s)	7.40 (1H, s)
4.06 (3H, s)	4.08 (3H,s)

[a] $CDCl_3$ was used as the solvent in both cases.

[b] taken from reference 97.

[c] Exchangeable with D_2O

those given for munjistin methyl ester [54][81,83,97] (especially ^{1}H NMR data - see table 3.16). This anthraquinone has been isolated from the roots of *Rubia tinctorum*[97] and the heartwood of *Morinda lucida*[81] (both *Rubiaceae*) and the roots of *Tectona grandis* (Verbanaceae).[81] There appear to be no other references to this compound in the chemical literature.

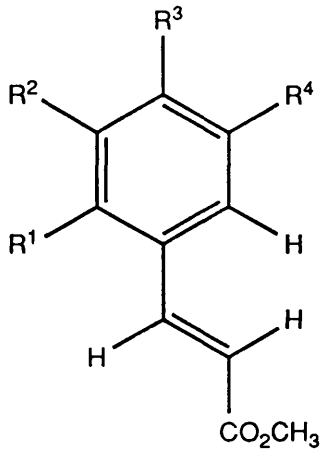
Section 3.2.6.9 Characterisation of [VIII]

This compound was isolated as a white solid (2.1mg). The characterisation of [VIII] was greatly aided by the identification of one of the components of the acid-hydrolysed extracts of the flowers (retention time = 24.5 minutes in table 3.5) - which had a very similar ultra-violet spectrum - as *trans*-caffeic acid methyl ester [55] (see experimental for the isolation and identification of this ester).

The CI mass spectrum of [VIII] showed two prominent ions at m/z 209 (base peak) and 226 corresponding to the pseudomolecular (M + 1) ion and an ammonium-molecule adduct ion respectively. In the conventional EI mass spectrum the molecular ion at m/z 208 was the base peak with the next most intense ion at m/z 177 corresponding to the loss of a methoxyl radical. Accurate mass measurements indicated the sample to have the molecular formula $C_{11}H_{12}O_4$.

The ^{1}H NMR spectrum of [VIII] revealed two doublets at δ 7.60 and 6.27ppm each with a large coupling of 15.9Hz, a doublet of doublets (δ 7.06ppm, 1H, J = 8.2, 1.9Hz), two further doublets (δ 7.01 and 6.90ppm, 1H each, J = 1.9 and 8.2Hz respectively) and two methoxyl groups (δ 3.91 and 3.78ppm). The magnitude of the coupling constant of the doublets at δ 7.60 and 6.27ppm is a typical value for two protons in a *trans* disubstituted alkene.[70] The similarity between the chemical

shifts of these protons and the alkene protons in a number of derivatives of cinnamic acid methyl ester (typically δ 7.6 and 6.25ppm[98]), as well as the presence of ions corresponding to loss of OMe and COOMe in the mass spectrum suggested [VIII] was also a derivative of cinnamic acid methyl ester. The magnitude of the coupling constants and multiplicities of the multiplets in the spectrum indicate a 1,2,4-trisubstituted aromatic ring. Six derivatives of cinnamic acid methyl ester with the same molecular formula as [VIII] have this substitution pattern [56]-[61]. Although only some of these compounds are known to exist in nature, other naturally-occurring cinnamic acid derivatives containing the same oxygenation patterns as those present in structures [56]-[61] have been reported. The ^{1}H NMR data of [VIII] together with those published for [60](*trans*-isoferulic acid methyl ester) and [61](*trans*-ferulic acid methyl ester) are given in table 3.17. This data suggests [VIII] and [60] share the same structure and indeed the mass spectra of [VIII] and [60] are similar as shown in table 3.18. However, there is insufficient spectroscopic data available in the chemical literature of either natural or synthetic samples of compounds [56]-[59] to eliminate the possibility that compound [VIII] is one of these isomers. The definitive characterisation of compound [VIII] would have required the synthesis of isomers [56]-[61] for which sufficient time was not available.



	R^1	R^2	R^3	R^4
[55]	H	OH	OH	H
[56]	OH	H	H	OCH_3
[57]	OH	H	OCH_3	H
[58]	OCH_3	H	OH	H
[59]	OCH_3	H	H	OH
[60]	H	OH	OCH_3	H
[61]	H	OCH_3	OH	H

Table 3.17 ^{1}H NMR data for pigments [VIII], [60] and [61][a]

[VIII]	[60][b]	[61][c]
δ/ppm	δ/ppm	δ/ppm
7.60 (d, J = 15.9Hz)	7.60 (d, J = 16Hz)	7.61 (d, J = 15.6Hz)
7.06 (dd, J = 8.2, 1.9Hz)		7.17 (d, J = 2.0Hz)
7.01 (d, J = 1.9Hz)	6.70-7.30 (5H, m)	7.06 (dd, J = 2.0, 8.3Hz)
6.90 (d, J = 8.2Hz)		6.80 (d, J = 8.3Hz)
6.27 (d, J = 15.9Hz)	6.25 (d, J = 16Hz)	6.35 (d, J = 15.6Hz)
	5.80 (s, OH)	
3.91 (3H, s)	3.91 (3H, s)	3.89 3H, s)
3.78 (3H, s)	3.78 (3H, s)	3.76 (3H, s)

[a]all resonances arise from single protons unless otherwise indicated and are relative to TMS as internal standard. Spectra were recorded at 400 and 100MHz for [VIII] and [60] respectively.

[b]data taken from reference 99.

[c]data taken from reference 100 (the spectrometer specifications were not given).

Table 3.18 Mass spectral data for [VIII] and [60]

[VIII]	[60][a]
m/z (relative intensity)	m/z (relative intensity)
208 (100)	208 (100)
193 (3)	193 (19)
177 (69)	177 (82)
149 (12)	
145 (27)	
134 (11)	
133 (14)	133 (26)
117 (13)	117 (18)

[a]data taken from reference 101.

Section 3.2.6.10 **Characterisation of pigment [X]**

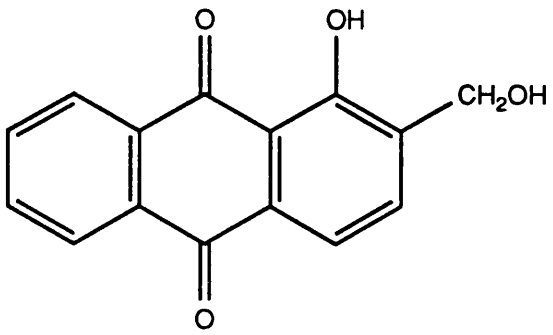
The pigment [X] was isolated as a bright yellow powder (2.0mg). The conventional EI mass spectrum of [X] indicated a molecular mass of 254 daltons. The prominence of this ion (89% of the intensity of the base peak) and ions at m/z 226, 225, 208, 197, 180, 152 and 151 corresponding either to the loss of CO or CHO units from the molecular ion or the ion at m/z 236 (formed by loss of H_2O from the molecular ion) suggested the pigment [X] was an hydroxyanthraquinone. The molecular mass was confirmed by CI mass spectrometry and shown by accurate mass measurements to correspond to a molecular formula of $C_{15}H_{10}O_4$.

The ^1H NMR spectrum of [X] revealed two hydroxyl signals (δ 13.03 and 5.40ppm, which both disappeared on shaking with D_2O), the one furthest downfield suggesting the pigment [X] to be either a carboxylic acid or to contain an intramolecularly hydrogen-bonded hydroxyl proton. In the aromatic region two multiplets (δ 8.28-8.32 and 7.74-7.86ppm, 2H and 4H respectively) were observed. A closer examination of the latter multiplet indicated it to consist of two doublets (δ 7.76 and 7.85ppm, each 1H with J $\simeq$ 8Hz) and a multiplet (centred at δ 7.82ppm, 2H). A singlet at δ 4.85ppm with an integration of two units, indicated a $-CH_2OR$ group bound to an aromatic ring. The ^1H NMR of [X] is thus consistent with an anthraquinone unsubstituted in one ring (two multiplets centred at δ 7.82 and 8.31ppm) with the other ring ortho disubstituted (2H each with J $\simeq$ 8Hz). One substituent is an hydroxyl group in the *peri* position (δ 13.03ppm corresponding to a chelated hydroxyl group) and the other an $-CH_2OR$ group, where "R" must be an hydrogen atom to be consistent with the molecular formula, $C_{15}H_{10}O_4$.

The interpretation of overlapping signals occurring in a very

narrow region of the ^{1}H NMR spectrum (δ 7.74-7.86ppm) had formed an important part of the elucidation of the structure of the pigment [**X**]. As overlapping bands can often be separated by changing the solvent, a new ^{1}H NMR spectrum was acquired using d_6-benzene as the NMR solvent (d-chloroform had been used above). The signals occurring at δ 7.74-7.86ppm in d-chloroform were shifted significantly when d_6-benzene was used, yielding two clearly discernible doublets (δ 7.83 and 7.36ppm, each 1H with a coupling constant of J = 7.8Hz) and a multiplet (δ 6.99-7.03ppm, 2H). Also of note was the simplification of the multiplet previously at δ 8.28-8.32ppm (2H) into two multiplets at δ 8.16-8.19 (1H) and 8.03-8.06ppm (1H).

The NMR and mass spectrometry data discussed above for the pigment [**X**] correspond to the structure of 1-hydroxy-2-hydroxymethyl-anthraquinone [62]. This data together with the UV-VIS spectra (both of a neutral solution of the pigment and when a few drops of alkali had been added) agree well with those published in the literature[81,83,85] as shown in table 3.19. This anthraquinone was first isolated from the leaf material of *Digitalis ferruginea*[102] and given the name digiferruginol. It has also been isolated from the roots and tissue cultures of a number of plant species belonging to the Rubiaceae family[81,83] and from the leaves of *Digitalis orientalis*.[103]



[62]

Table 3.19 Spectral data for [X] and [62][a]

	[X]	[62]
^{1}H NMR data[b] :		
	13.03 (1H, s)[c]	13.03 (1H, s)[c]
	8.28-8.32 (2H, m)	8.30 (2H, m)
	7.74-7.86 (4H, m)	7.80 (4H, m)
	5.40 (1H, broad s)[c]	
	4.85 (2H, s)	4.87 (2H, s)
Mass spectrometric data (m/z (relative intensity)):		
	254 (89)	254 (100)
	236 (17)	236 (15)
	226 (22)	226 (22)
	225 (100)	225 (96)
	208 (19)	208 (18)
	207 (8)	207 (8)
	197 (7)	197 (8)
	180 (14)	180 (15)
	152 (39)	152 (39)
	151 (22)	151 (21)
	139 (14)	139 (14)
UV-VIS data (λ_{max}^{EtOH}, nm):		
	224	224
	245 (sh)	248
	254	254
	280 (sh)	280
	326	328
	407	404
UV-VIS data ($\lambda_{max}^{EtOH/OH^-}$, nm):		
	222	221
	250	250
	272 (sh)	273
		280
	311	314
	504	500

[a]NMR data for [62] was taken from reference 83 whilst all other data was taken from reference 85.

[b]recorded using $CDCl_3$ as solvent at 400 and 100 MHz for [X] and [62] respectively. Chemical shifts are in ppm relative to TMS.

[c]exchangeable with D_2O.

Section 3.3 **Summary**

I have demonstrated in this chapter that tissue cultures of the herb *Galium verum* can be readily established under a wide variety of light and hormone regimes. Cultures flourished under all regimes attempted (18 in total). All culture lines produced pigments and it was found that increasing the level of the carbon source contained in the plant tissue culture medium (sucrose in this case) led to an increase in the accumulation of pigments with a corresponding decrease in callus growth. These results thus agree with those of Ibrahim,[71] amongst others,[17,35,72] who suggested that anthraquinone accumulation and growth are inversely related, whilst anthraquinone accumulation and sucrose concentration (in the plant tissue culture medium) are directly proportional.

The analysis of the crude extracts of the various culture lines by TLC was extremely troublesome, primarily owing to heavy tailing of compounds. Hydrolysis of these extracts with acid did aid analysis somewhat, but it was not until the availability of an HPL-chromatograph fitted with an ultra-violet (diode-array) detector that satisfactory separations were achieved. Over 80 different compounds were detected. Examination of the UV spectra of these compounds indicated two classes of UV-active compound were predominant and these were subsequently identified as the anthraquinone and phenylpropanoid classes of natural products.

The acid-hydrolysed extracts of the cultures were compared, by HPLC, with both non- and acid-hydrolysed extracts prepared from the roots and flowers of the field-grown plant. It had been hoped that such a comparison would aid the the identification of the main components present in the culture extracts, but this was not the case for two main reasons: (i) the irreproducibility of HPLC retention

times; (ii) the great similarity between the UV spectra of a large number of the components present in the acid-hydrolysed extracts of the cultures. Thus the main components of the acid-hydrolysed extracts of the cultures were isolated and characterised. Initially isolation of the main components had been attempted by a combination of Sephadex LH-20 and silica gel column chromatography and preparative-TLC, but this was very time-consuming and led to a large number of fractions each containing a number of components. On the other hand, isolation utilising HPLC throughout gave compounds of high purity in a fraction of the time. The majority of naturally-occurring anthraquinones reported in the literature have been characterised using samples isolated by conventional chromatographic methods such as column chromatography and thus a large number of these structure determinations may be unreliable as they were probably performed on impure samples.

Ten compounds were isolated from the acid-hydrolysed extracts of the cultures, with eight of these being characterised as anthraquinone pigments (table 3.20). Of these eight, only two ([I] and [V]) are reported to be present in the intact plant. There was insufficient time to fully characterise pigments [II], [III] and [VI]. Neither was there sufficient time to perform quantitative HPLC analysis of the acid-hydrolysed extracts of the cultures using the isolated pigments as standards.

Table 3.20 Natural Occurrence of isolated pigments

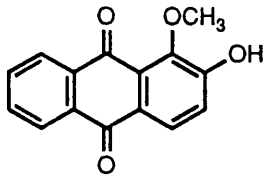
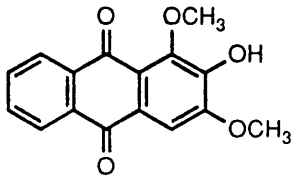
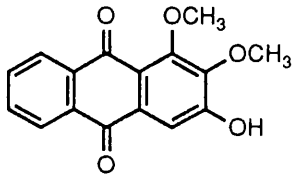
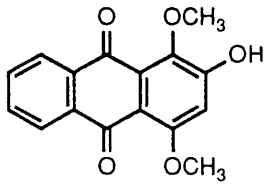
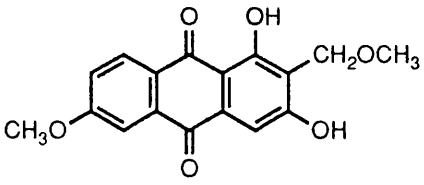
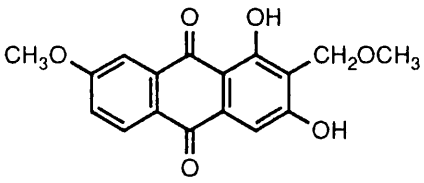
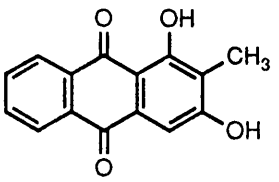
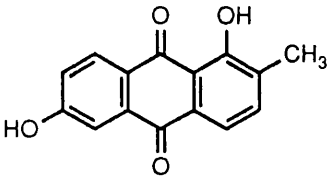
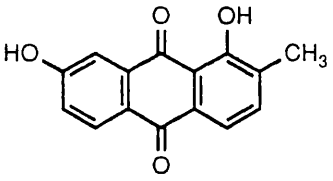
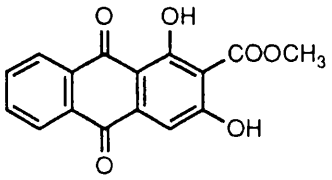
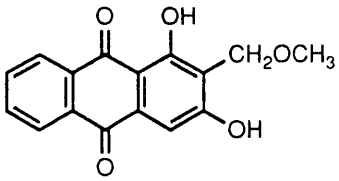
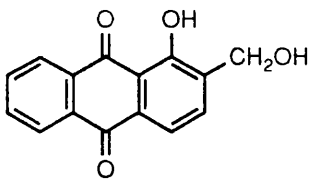
Compound	Structure	Natural occurrence[81-83,96]
I	 [19]	Over 20 plant species (all Rubiaceae) including *Galium verum*. Plant tissue culture (PTC): several species (all Rubiaceae).
II	 [36] or  [37] or  [38]	[36] & [37]: plant material and callus cultures of several species (all Rubiaceae). [38]: there are no references to the natural occurrence of this compound.
III	 [39] or  [40]	Neither [39] or [40] have previously been found in nature.

Table 3.20 (continued) Natural Occurrence of isolated pigments†

Compound	Structure	Natural occurrence[81-83,96]
V	 [24]	Over 40 members of the Rubiaceae family of plants including *Galium verum*. PTC: several species (all Rubiaceae).
VI	 [49] or  [50]	[49] has been isolated from the roots of several plants (all Rubiaceae). PTC: a compound partially characterised as [49] or [50] has been isolated from callus cultues of *Cinchona pubescens* (Rubiaceae).[96]
VII	 [54]	Roots of *Rubia tinctorum*, heartwood of *Morinda lucida* (both Rubiaceae); roots of *Tectona grandis* (Verbanaceae). PTC: no previous references.
IX	 [35]	Roots of *Galium album*, *Rubia cordifolia*, *Morinda parvifolia* and heartwood of *Faramea cyanea* (all Rubiaceae). PTC: callus of *Morinda citrifolia*
X	 [62]	Roots & PTC of several plant species (all Rubiaceae).

† Compounds IV and VIII were isolated but only partially characterised.

PART B: MONOTERPENOID EPOXIDES AS BIOSYNTHETIC INTERMEDIATES IN HIGHER PLANTS

CHAPTER FOUR: INTRODUCTION

Section 4.1 The Biosynthesis of Monoterpenoids in Plants[104-106]

The monoterpenoids are responsible for the characteristic odours of many plants and have been reported to be present in a total of nearly 50 families of flowering plants, although they are best known as constituents of the essential oils of pines, mints and citrus fruits.

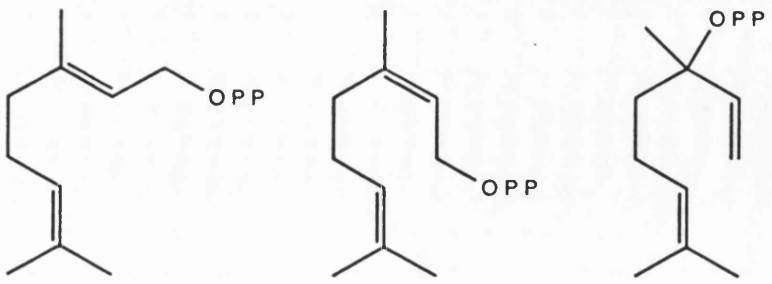
The monoterpenoids are members of the terpenoid class of natural products and diverge from higher isoprenoid biosynthesis at the level of geranyl pyrophosphate (GPP, [63]), the first C_{10} intermediate to arise (see figure 1.1). Although one can envisage the formation of a large number of the acyclic monoterpenoids directly from GPP [63], the majority of natural plant monoterpenoids are cyclic compounds based on the cyclohexanoid ring skeleton and cannot be formed directly from GPP [63] (because of the *trans* double bond at C-2). Studies on monoterpenoid biosynthesis have always been hampered by low incorporation of radioactive precursor into plants, but with the use of cell-free systems (which give somewhat better incorporations) such studies, and in particular the question of the immediate precursor of the cyclic monoterpenoids, have become more approachable. These cyclisation reactions are catalysed by enzymes known as cyclases, a number of which have been partially purified (i.e. freed of competing activities such as phosphatase). Monoterpenoid cyclases are capable of catalysing a multistep process whereby the enzyme carries out an isomerisation to a bound intermediate capable of cyclising, as well as the cyclisation itself. Either GPP [63], neryl pyrophosphate (NPP, [64]) or linalyl pyrophosphate (LPP, [65]) can be used as acyclic precursors by most cyclases. Multiple cyclases each producing a

different skeletal arrangement from the same acyclic precursor often occur in higher plants, while single cyclases, which synthesize a limited variety of skeletal types are also known.

A unified stereochemical scheme for the enzymatic cyclisation of GPP [63] has been given by Croteau[105] (see figure 4.1). It is generally agreed that the cyclase catalyses the initial ionisation of the pyrophosphate moiety to generate an allylic cation. This step is followed by stereospecific *syn* isomerisation to either 3R or 3S linalyl intermediate and rotation about the newly formed C_2-C_3 single bond. Hence the biosynthesis of a large number of monoterpenoids can be viewed as a series of steps: pyrophosphate migration, bond rotation, ionisation, cyclisation, termination diverging enantiospecifically in the isomerisation sequence and involving numerous regiochemical variants in the generation of the various parent skeleta. Croteau's scheme includes the coupled isomerisation component of the multi-step reaction necessary to overcome the geometrical impediment to direct cyclisation. This scheme also accounts for the cyclisation of GPP [63], without free intermediates to all major skeletal types and can obviously rationalise the cyclisation of NPP [64] and LPP [65] as alternate substrates.

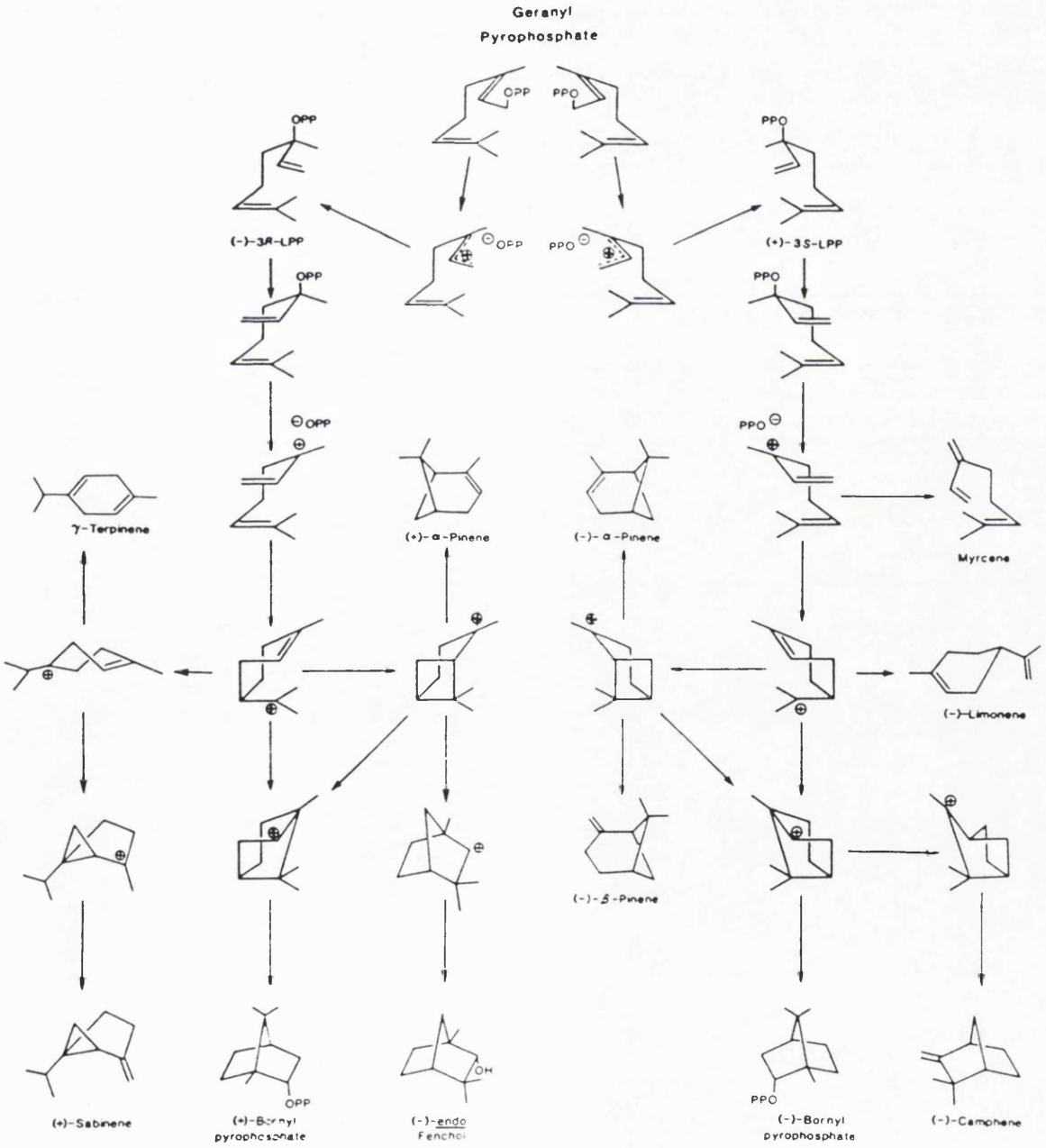
Section 4.2 Epoxides in Plants

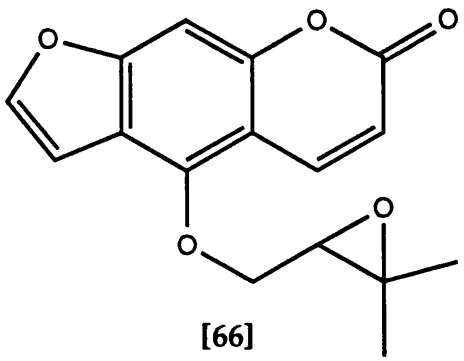
Since the characterisation of the coumarin oxypeucedanin [66] in 1933[107] as one of the earliest recognised epoxide in plants, the presence in higher plants of hundreds of other epoxides have been reported.[108,109] The occurrence of a particular epoxide is often predicted based on the presence of the more stable vicinal diol or hydoxyalkene derivative. For example[110] the monoterpene derivatives [67] to [70] have been isolated from Alexandria grapes (*Vitis*



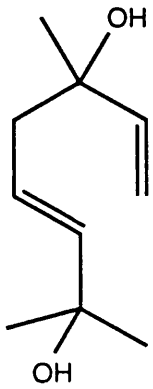
[63] [64] [65]

Figure 4.1 A unified stereochemical scheme for the enzymatic cyclisation of geranyl pyrophosphate (adapted from reference 105).

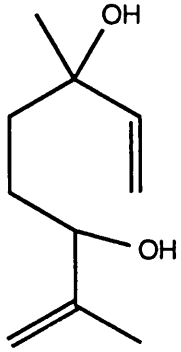




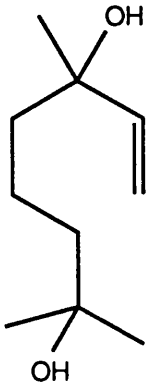
[66]



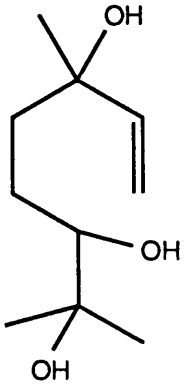
[67]



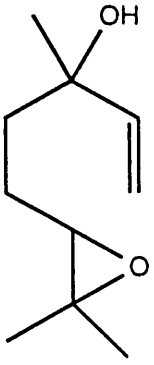
[68]



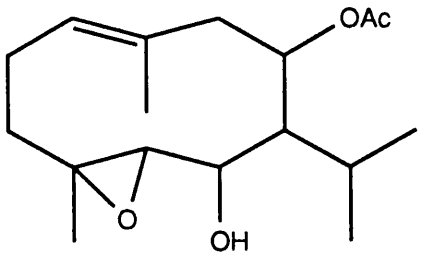
[69]



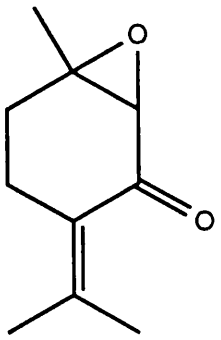
[70]



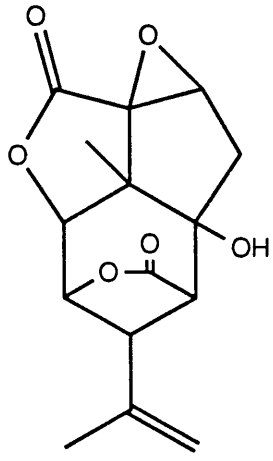
[71]



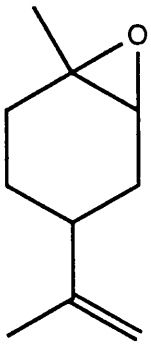
[72]



[73]



[74]

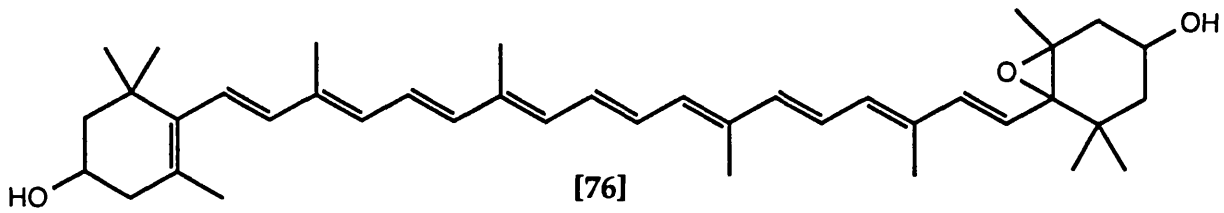


[75]

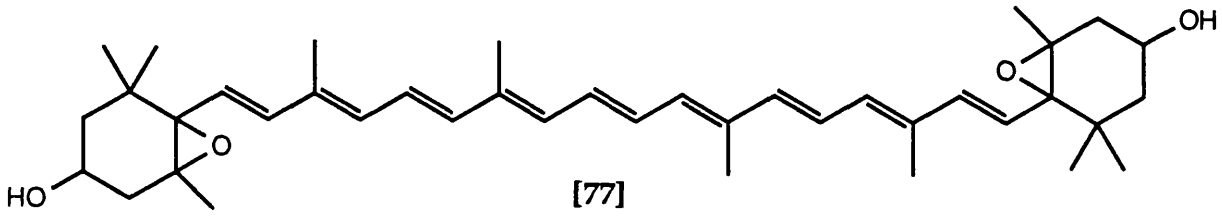
vinifera) and it was postulated that these were derivatives of the same precursor, namely 6,7-epoxylinalool [71]. Another study[111] of bound volatiles from papaya fruit (*Carica papaya* L.) has suggested phosphate-bound 6,7-epoxylinalool [71] is the precursor of the phosphorylated triol [70]. Recently this epoxide has been isolated from European bird cherry flowers (*Padus avium* Mill.) using the moderate conditions of the vacuum headspace method[112] and by extraction of *Carica papaya* fruit at neutral pH.[113,114] Hence with the increasing use of such mild isolation procedures, e.g. supercritical extraction with CO_2, and with improved identification methods, the discovery of many more naturally-occurring plant epoxides is to be expected.

A number of plant epoxides possess feeding-deterrent and toxic properties against insects and mammals and this suggests a defensive role in plant-herbivore interactions for these compounds.[115] For example, the sesquiterpenoid shiromodiol monoacetate [72] which has been isolated from *Parabenzoin trilobum* has feeding-deterrent properties against the insect *Spodoptera littoralis*.[116] Other epoxyterpenoids such the anticholinesterase monoterpenoid, pulegone 1,2-epoxide [73][117] from the medicinal plant poleo (*Lippia steochadifolia*), and the sesquiterpenoid picrotoxinin [74][118] from *Anamirta cocculus* are directly insecticidal. Limonene epoxide [75], which occurs in *Pinus echinata Mill.*, amongst other plants has anticholinesterase activity also[117] and in addition is thought to contribute to host-tree resistance to attack by the southern pine beetle (*Dendroctonus frontalis Zimmmermann*).[119]

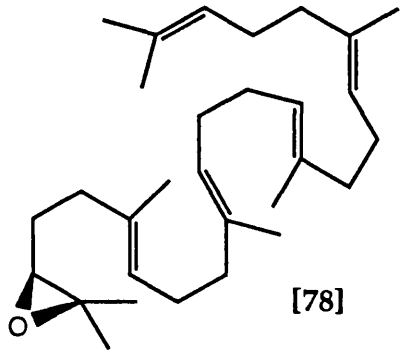
A number of plant epoxides are known to be biosynthetic intermediates in higher plants. Antheraxanthin [76] and violaxanthin [77] are intermediates in the so-called violaxanthin cycle of



[76]

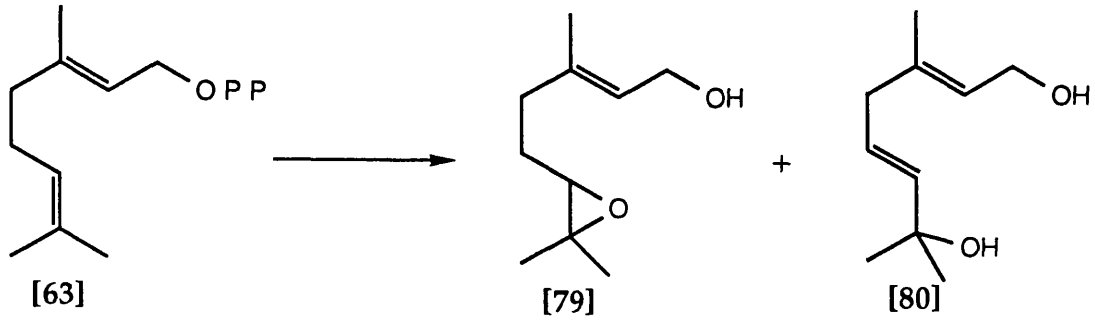


[77]

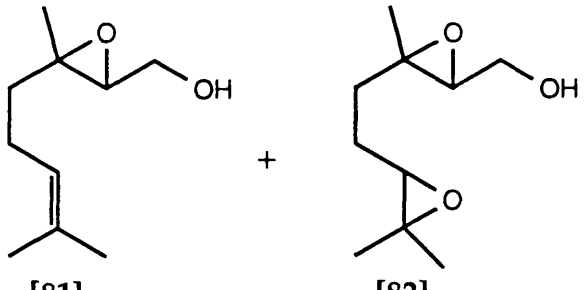


[78]

Figure 4.2



[63] [79] [80]



[81] [82]

chloroplasts[120] and are thus linked to photosynthetic processes. The acyclic precursor of a large number of polycyclic steroids and triterpenoids is squalene monoepoxide [78].[121] The polycyclic structures formed from this epoxide can all be rationalised in terms of the way in which the epoxide may be folded (pseudo chair and boat conformations) on the enzyme surface prior to cyclisation. Cutin, the structural component of plant cuticle is a polymer of C_{16} and C_{18} hydroxy fatty acids and their is direct evidence for the involvement of epoxy fatty acids in the biosynthesis of this structural component.[122] These fatty acid derivatives may also serve as an initial chemical barrier to insect grazing.

The investigation of monoterpenoid biosynthesis in higher plants is often hindered by poor incorporation of presumed C_5 or C_{10} precursors into expected products and even when cell-free extracts are used incorporation rarely exceeds 1%. It has been proposed[123] that an enzyme salvage system regulates unphysiological levels of these precursors by degradation into water soluble products via epoxide intermediates. Alternatively such a system may exist to convert monoterpenoids into water-soluble products for transport and subsequent degradation. Three studies in particular support these proposals:

(i) Cell-free extracts of *Tanacetum vulgare*, *Artemisia annua* and *Santolina chamaecyparissus* have been found to contain seasonally-dependent enzyme systems that convert IPP, DMAPP, GPP and NPP into water soluble-products in up to 96% yield.[123] The bulk of the water-soluble fractions from these incubations were found to comprise of various diols, triols and epoxy compounds. For instance 6,7-epoxygeraniol [79] and 3,7-dimethylocta-*trans*,*trans*-2,5-dien-1,7-diol [80] were the major products (22 and 40% respectively) when GPP

[63] was incubated with extracts from *T. vulgare* (see figure 4.2). Over a dozen other metabolites were detected from this incubation and tentative identifications were made of 2,3-epoxygeraniol [81], 2,3-6,7-diepoxygeraniol [82] and products of their ring-scission (total 23%). Reincubation of mixtures of salvage products with various cell-free preparations from *T. vulgare* provided no evidence that any of the remaining unidentified fractions were protein-bonded intermediates of terpenoid biosynthesis and likewise there was no evidence for terpenoid-sugar conjugates that could be dissociated by pyrogallol or dimethylsulphoxide. Up to 5% (but typically < 1%) of the water-soluble tracer obtained after initial incubations could be cleaved on treatment with β-glucosidase to liberate labelled material with the characteristics (GLC and TLC) of monoterpenoids.

(ii) When ^{14}C-labelled enantiomers of α, β and δ-pinenes ([83], [84], and [85] respectively), (+)-α-pinene epoxide [86], (-)-β-pinene epoxide [87] and (+) and (-)-citronellene-6,7-epoxide [88] were fed to foliage of *Pinus pinaster* the bulk of the tracer in all cases entered pigments (chlorophyll, xanthophylls) and water-solubles.[124] Low conversions into isomeric pinenes and their epoxides were recorded, and diols resulted especially from feeding of the corresponding epoxides. These patterns suggest the sequence: pinenes ⟶ pinene epoxides ⟶ pinene diols ⟶ cleaved and degraded products; eventually leading to water solubles, acetate etc. that can be recycled into pigments even within the short time period of the experiment. The water-soluble fractions from feeding (+)-[83] and (+)-α-pinene epoxide [86] yielded pinonic acid (4-6%, [89]), pinic acid (1-2%, [90]) and norpinic acid (0.5%, [91]). When (-)-[84] was the substrate, about 5% of the water solubles comprised of acids [89] and [90] and 6% of acid [91].

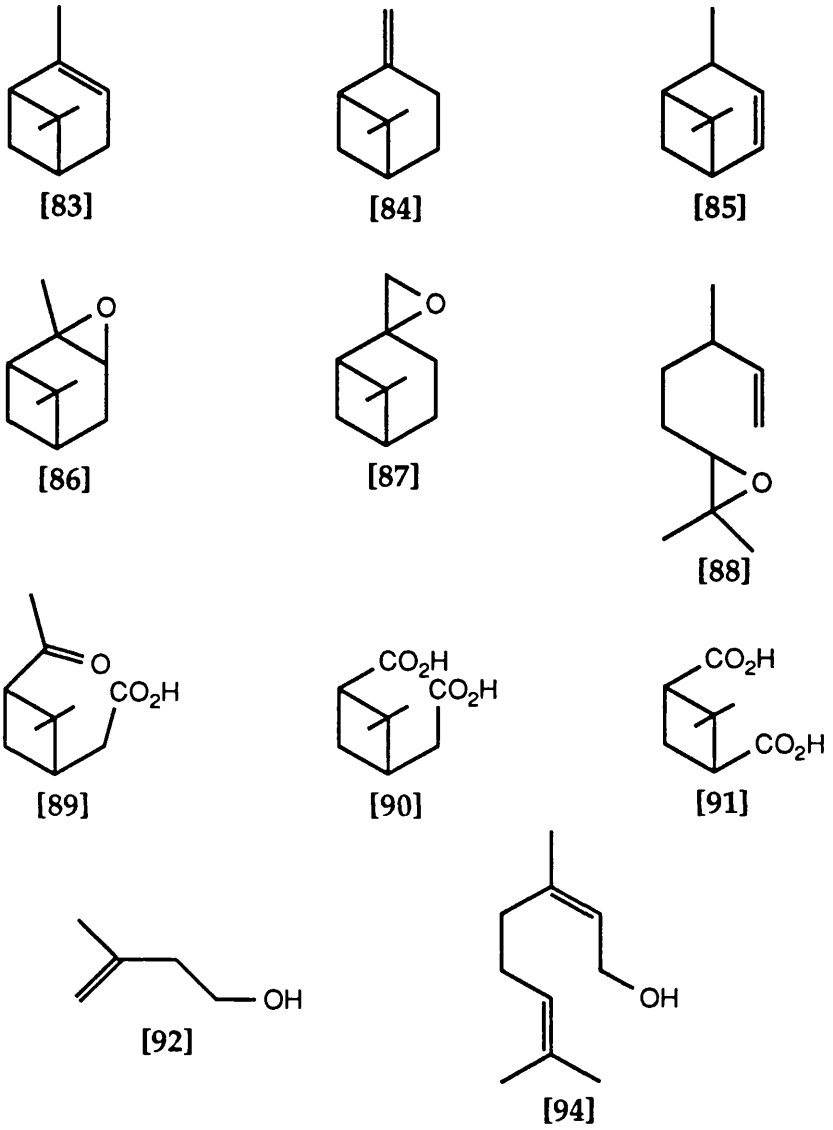
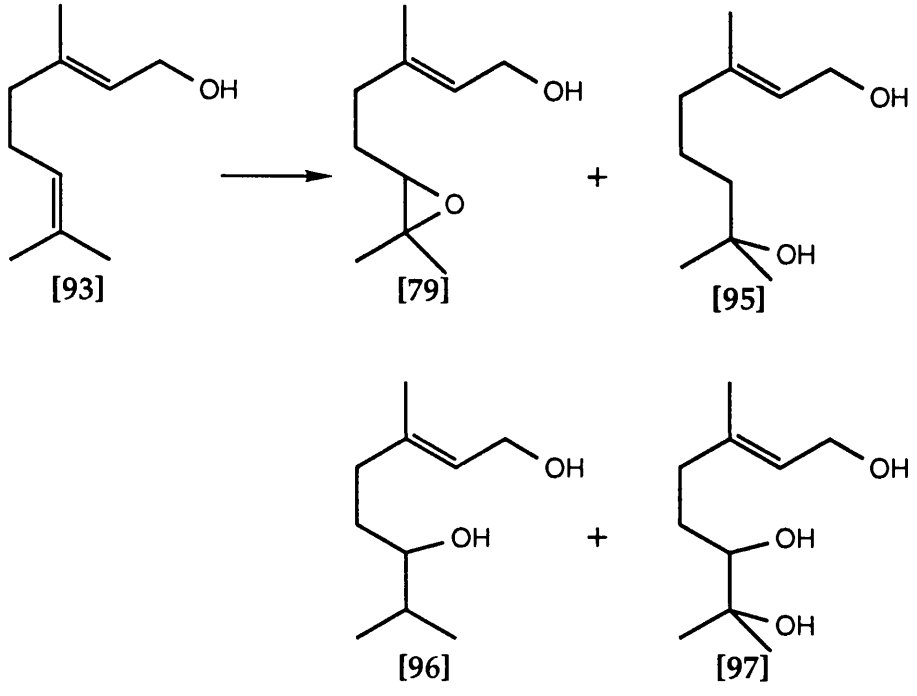

[83]
[84]
[85]
O
[86]
O
[87]
O
[88]
O
CO2H
[89]
CO2H
CO2H
[90]
CO2H
CO2H
[91]
OH
[92]
OH
[94]

Figure 4.3


OH
[93]
OH
O
[79]
+
OH
OH
[95]
OH
OH
[96]
+
OH
OH
OH
[97]

(iii) Cell-free extracts from callus of *Jasminum officinale* have been found to contain epoxidase activities with IPP, isopentenol [92], geraniol [93] and nerol [94] as substrates and also hydratase activities towards the resulting terpenoid epoxides.[125] For instance alcohol [93] was converted into the products [79] and [95]-[97] (0.1, 0.3, 0.3, and 0.3% respectively) all of which were functionalised at the 6,7-bond as shown in figure 4.3. The terpenoid epoxidase and epoxide hydratase activities demonstrated in this study appeared to be sequentially linked as addition of the terpenoid epoxides (alone, or with a variety of possible cofactors) gave no detectable products of ring-opening.

Section 4.3 Aims of this Investigation

Monoterpenoids with the bornane skeleton, such as borneol [98] and camphor [99] are widespread in higher plants and are generally considered[104-106] to be produced from the parent geraniol [93] via a scheme such as that depicted in figure 4.4. However, the route shown in figure 4.5 involving epoxides is a possible alternative. The aim of this investigation was to prepare ^{14}C-labelled [71] and [75], feed these to leaves and stems of mature plants and tissue cultures of *Pelargonium fragrans* (which produce compounds containing the bornane skeleton[126]), or any other suitable and available plant and to see if the labelled material could be incorporated into [98], [99] or their derivatives. The *cis* and *trans* furanoid forms of linalool oxide ([100a] and [100b] respectively) occur frequently in the volatile extracts of higher plants. A further aim of this study was to prepare ^{14}C-labelled [100a] and [100b] and follow the metabolism of these compounds in plants and cultures of *Pelargonium fragrans*.

Figure 4.4

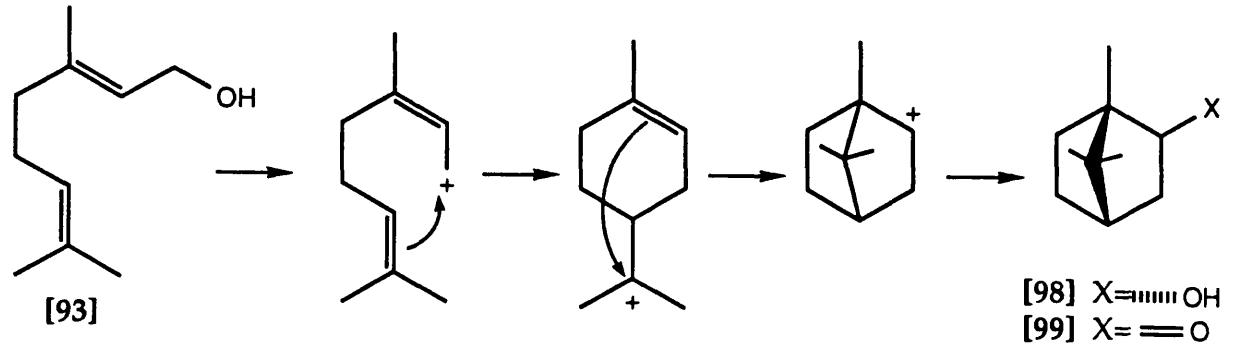
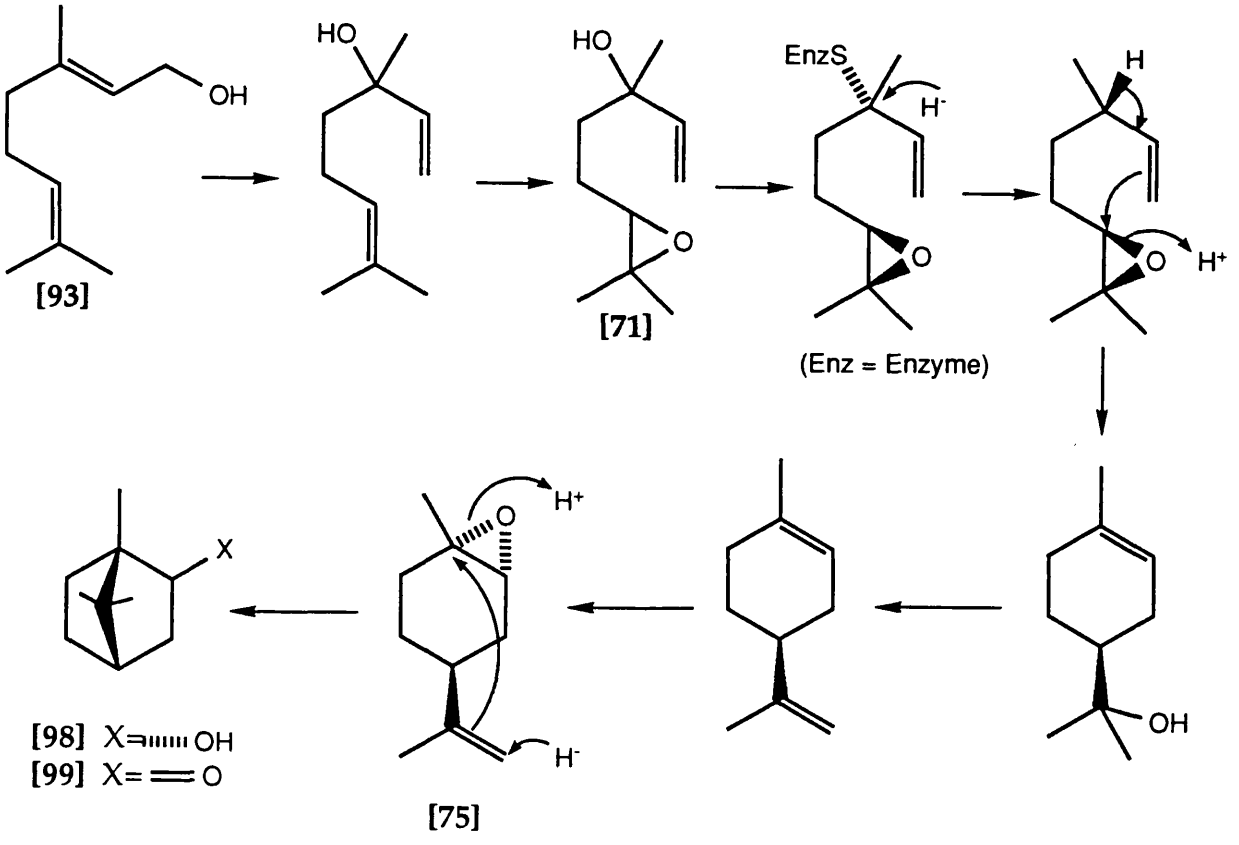
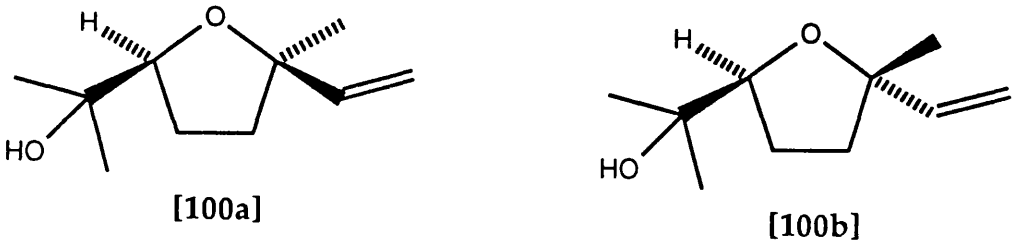


Figure 4.5





CHAPTER FIVE: GENERAL EXPERIMENTAL METHODS

Outlined in this chapter are general details of the experimental methods which were used in chapters six and seven. More specific experimental details (including experimental results) are described, section by section, towards the end of this thesis (pages 184-225).

Section 5.1 Chromatographic methods

The analysis and purification both of radioactive and unlabelled compounds, was carried out using either gas liquid chromatography (GLC), HPLC, TLC or column chromatography.

Column chromatography was used for the purification of large quantities (of the order 100-1000mg) of impure compound. The fractions obtained from column chromatography were monitored by TLC, which was also used to study the course of reactions. These two chromatographic procedures were performed as already detailed in sections 2.1.1 and 2.1.2. The solvent systems employed varied according to the compounds to be separated and are described in the experimental accompanying each relevant section.

Section 5.1.1 Gas Liquid Chromatography (GLC)

GLC was used in conjunction with TLC as an analytical method to monitor the course of reactions involving unlabelled compounds. Additionally, a measure of the purity of the starting materials and products associated with these reactions was provided by GLC. Radioactive substances could not be analysed by GLC as no instrument was available for which the analysis of such substances was permitted because of contamination problems.

A number of analytical GLC systems were employed throughout this

	Instrument	Column specifications	Carrier gas (flow rate)	Column temperature	Injection volume
(i)	Pye Unicam Series 204	OV101 WCOT capillary (26m x 0.32mm i.d.)	Helium (60ml/min)	60-220°C @ 8°C/min then 220°C for 3 mins	0.1µl
(ii)	Ai Gas Chromatography 93 + Hewlett Packard 3394A integrator	FFAP WCOT vitreous silica capillary (25m x 0.22mm i.d.)	"	70-120°C @ 2°C/min 120-190°C @ 4°C/min	"
(iii)	"	"	"	90-190°C @ 4°C/min	"
(iv)	"	"	"	90-120°C @ 2°C/min 120-190°C @ 4°C/min	"
(v)	Perkin Elmer F11	5% di-isodecylphthalate +5% Bentone 34 on Chromasorb AW-DMCS 80-100 mesh (4m x 1/8" o.d.)	Nitrogen (20ml/min)	130°C (isothermal)	"
(vi)	"	10% silicone oil MS200/200 on Chromasorb AW-DMCS 80-100 mesh (4m x 1/8" o.d.)	"	100-200°C @ 10°C/min	"
(vii)	"	"	"	70-200°C @ 8°C/min	"
(viii)	Pye Unicam Series 104	10% Carbowax 20M on Chromasorb AW-DMCS 80-100 mesh (3m x 15mm i.d.)	"	165°C (isothermal)	1µl

TABLE 5.1: GLC analytical conditions

work and these are summarised in table 5.1. All of the instruments used were fitted with flame ionisation detectors maintained at an operating temperature of 250°C. The injector heater units were also maintained at this temperature. Typically, 0.1μl of an approximately 1% solution of the sample in acetone was injected.

Section 5.1.2 HPLC

HPLC was used both as an analytical method and as a preparative method of purification. These techniques were performed using a refractive-index detector and normal-phase (silica gel) columns. The solvent systems employed varied according to the compounds to be separated and are described in the experimental accompanying each relevant section. A number of products were purified using a large preparative column (250x20mm i.d.) packed with silica gel (Nucleosil 100, 7μm particle size). A guard column (50x10mm i.d.) packed with the same material was used to protect the preparative column from contamination by polar substances. Other aspects of the HPLC techniques were employed as already described in section 2.1.3.

Section 5.2 Spectroscopic methods

In reactions involving unlabelled compounds, the majority of products formed were identified by comparison of their chromatographic properties (TLC, GLC and HPLC) with those of authentic samples. For a number of compounds such comparisons gave inconclusive results and hence these compounds (and those for which standards were not available) were analysed by spectroscopic means. Infra-red, UV-VIS, ^{1}H, ^{13}C and mass spectra were obtained as already described in section 2.2. In addition, such compounds were analysed by Gas chromatography - Mass spectrometry (GC-MS) by Mr. Ian Marr at Bush Boake Allen Ltd

using a Varian 3600 Gas Chromatograph coupled to a VG7070H low resolution mass spectrometer (accelerating voltage 4kV; ionisation energy 70eV). The gas chromatograph was fitted with a BP1 (equivalent to OV101) FSOT capillary column (50x0.32mm i.d.). A temperature programme of 40-240°C @ 3°C/min was used. The mass spectral data obtained were compared by computer with those held on a database at Bush Boake Allen Ltd. Gas chromatography - Infra-red - Mass spectrometry (GC-IR-MS) analysis was performed on a number of compounds by coupling a Hewlett Packard 5965A infra-red spectrometer to the GC-MS system described above. The IR spectrometer contained a gold-plated cell (10cm long) and a KBr window operated at 200°C. Three spectra were acquired every second to an optical resolution of $8cm^{-1}$.

Section 5.3 Measurement of radioactivity

The radioisotope used in this work, ^{14}C, decays by the emission of a β-particle:

$$^{14}_{6}C \longrightarrow {}^{14}_{7}N + \beta^{-} \qquad t_{1/2} = 5370 \text{ years}$$

The estimation of the total number of emissions (disintegrations) in a radioactive sample during a given time interval is used to determine the activity (disintegrations per minute, dpm) and specific activity (dpm mol^{-1}).

Section 5.3.1 Liquid scintillation counting

The rate of disintegration of unstable nuclei may most conveniently be determined for most organic materials by liquid scintillation counting. In summary, the radioactive compound is dissolved in a solvent containing one or two organic scintillators as solutes. These scintillators have fluorescent emission spectra in the region for which photomultiplier tubes are most sensitive. Energy

emitted by the radioactive sample is absorbed by the solvent and this energy is then transferred to the scintillator molecules. The excited scintillator molecules emit this energy as photons which are then detected by photomultiplier tubes. After amplification, a count is registered. This is normally expressed as a function of time i.e. counts per minute (cpm).

Section 5.3.1.1 Counting efficiency and quenching

Having determined the rate at which scintillations are detected from the sample it is necessary to determine the relationship between count rates and sample activity. This relationship is known as counting efficiency and is defined as:

$$E = \frac{cpm}{dpm} \times 100\%$$

Interference with the production of light in the liquid scintillant and its transmission to the photomultiplier tubes decreases the counting efficiency. This interference is known as quenching and may result from two principal sources:

(i) the chemical composition of the sample may interfere with the energy transfer from β-particles-to-solvent, solvent-to-solvent and solvent-to-scintillator. This is known as chemical quenching and is a function of electronic structure. For a series of organic compounds it increases in the order: alkenes < alcohols < aldehydes or ketones.

(ii) coloured compounds in the sample absorb photons emitted by the organic scintillant. This is known as colour quenching.

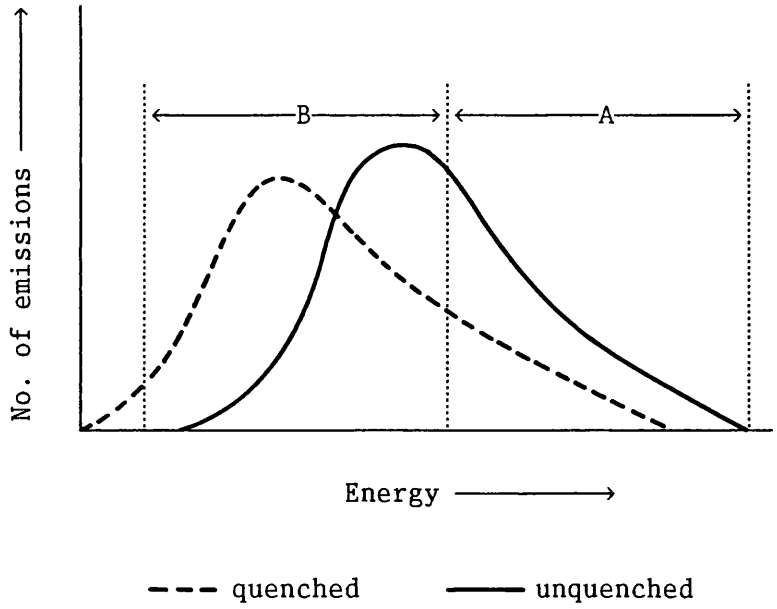
The degree of quenching is also dependent on the liquid scintillation medium being used. Therefore the efficiency of counting should be determined for each system used.

There are several methods which enable the degree of quenching to

be determined. In this work the counting system used (Packard Tricarb model 3255) does this by the external-standards channels-ratio (ESR) method (see below).

The β-particles emitted in a nuclear process have a continuous energy spectrum as indicated in figure 5.1 When quenching occurs, not only does the total number of counts measured decrease, but the energy profile is also shifted. The ESR method exploits this shift by examining the ratio of counts in two channels, e.g. A and B in figure 5.2 Since a relationship exists between the degree of quenching and this shift, the ratios of the total counts determined in band A to that in band B, for both the quenched and unquenched sample, provides a measure of the quenching. To achieve this, ^{226}Ra, a γ-emitting nuclide, is stationed in a shielded position remote from the counter chamber.

Fig 5.1 A β-particle energy spectrum for ^{14}C



The sample is counted as normal and then the γ-radiation source is

transported mechanically to a precise location in the counting chamber where the γ-rays irradiate the counting vial and its contents producing Compton electrons. These are β-particles, and thus cause scintillations which may be counted normally. The counter automatically carries out this routine for each sample and determines the ESR, which is defined by the ratio:

$$\frac{\text{Activity in band A(sample + standard)- activity in band A (sample alone)}}{\text{Activity in band B(sample + standard)- activity in band B (sample alone)}}$$

Section 5.3.1.2 Calibration curves

By using a set of standards of known activity, containing different levels of quenching agent, a plot of counting efficiency vs. ESR can be obtained. From this an efficiency correlation curve can be drawn and used to determine the counting efficiency of samples of unknown activity.

Calibrations were performed using a set of sealed quenched standards (ex. Packard, ten vials each one with specific activity = 203,000 dpm). The results for both channels A and B of the detector were entered into a curve fitting computer program to yield the curves shown in figure 5.2, and the following equations:

$$y = 274.08x - 793.44x^2 + 528.23x^3 + 43.27 \quad (\text{for channel A}, \sigma = 1.331)$$

$$y = 222.01x - 143.84x^2 + 0.33 \quad (\text{for channel B}, \sigma = 1.436)$$

where y = percentage counting efficiency
x = ESR, σ = standard deviation

Both curves are a good fit to the data contained in figure 5.2 with y being a polynomial in x^3 and x^2, for channels A and B respectively. This does not mean that any such physical correlation

Figure 5.2 Calibration curves for scintillation counting

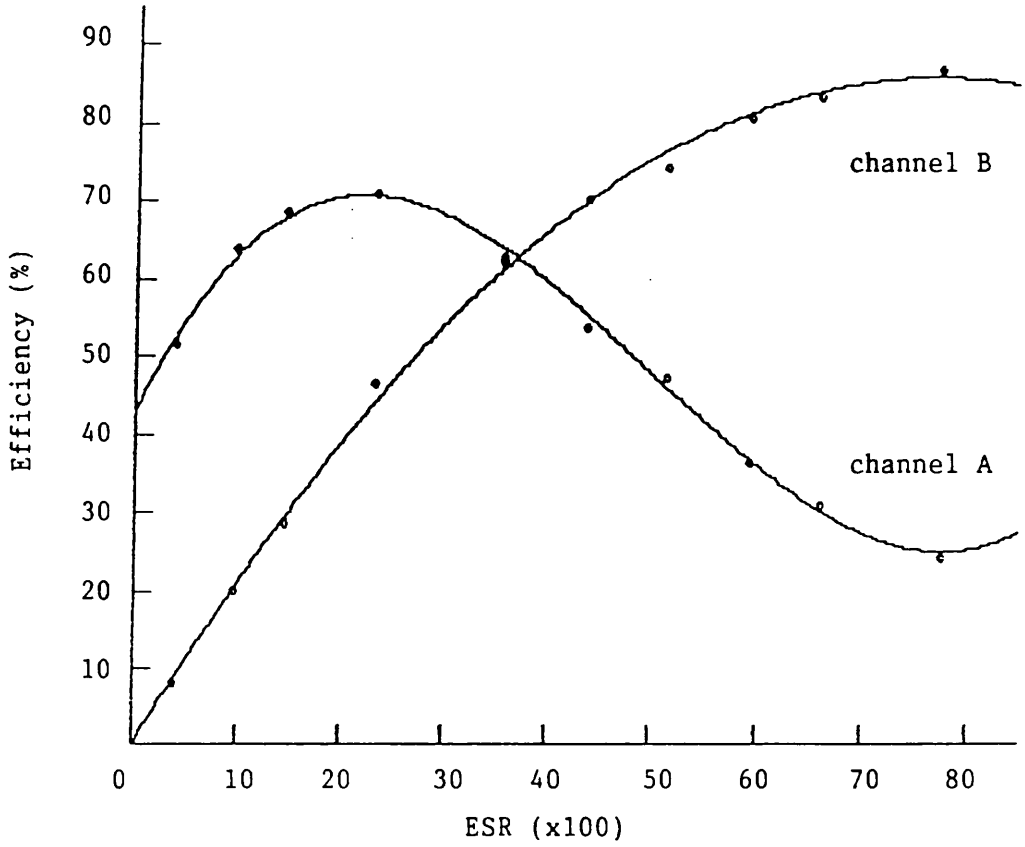
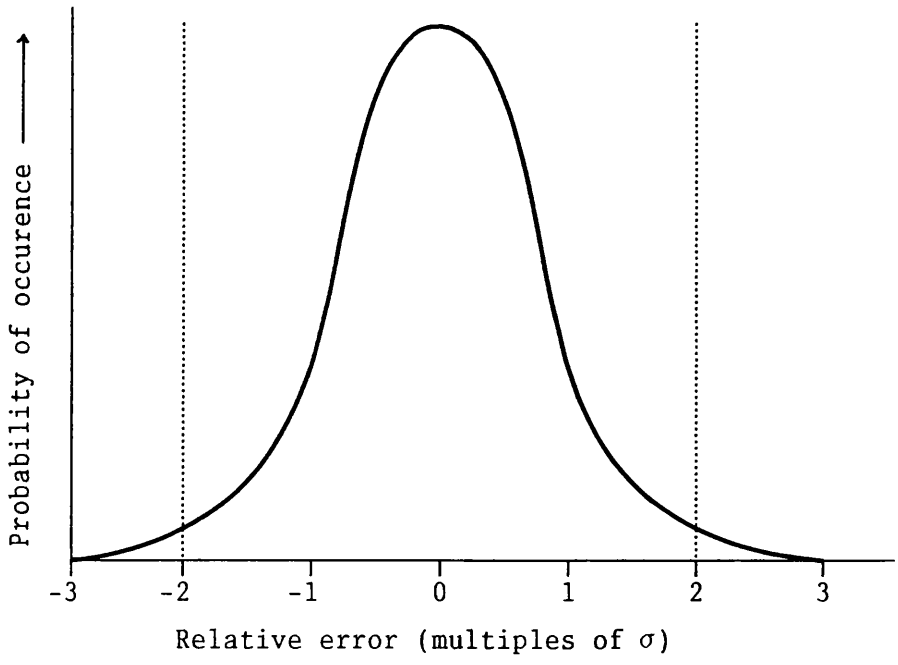


Figure 5.3 Normal distribution curve



exists, only that, in this work, such an approximation is useful. A computer program was written using these equations to calculate a corrected (for both quenching and background radiation) count rate in subsequent experiments.

Section 5.3.1.3 Statistics of counting

The disintegration of unstable nuclei is a random process. The number of disintegrations, in a given time interval varies according to the Poisson distribution, with the standard deviation (σ) of a series of measurements being equal to the square root of the mean counts ($\bar{x}$), i.e. $\sigma = \sqrt{\bar{x}}$ The Poisson distribution can be approximated to a Gaussian or Normal distribution for statistical purposes (fig 5.3). Hence it follows that, for a single determination, there is a 95.5% chance that a measurement will be within two standard deviations of the mean value. Where possible, measurements should be made such that 2σ is ± 1% of x. This entails counting a sample until 40,000 scintillations above background have been recorded.

Section 5.3.2 Radiochromatogram scanning

The radioactivity of samples separated by TLC was measured by scanning the top of the surface of the plate using a Berthold model LB 2723 thin layer scanner, which contains a Geiger-Muller type detector. This records practically 100% of the β-particles leaving the plate which is, however, only 15% (typically) of the total radiation present. Hence, this scanner could only be used to detect compounds of moderately high activity (200cpm minimum). In addition, only qualitative measurements could be made since the machine was fitted with a moving-coil analogue meter and not an XY recorder. This technique was used routinely during the radiochemical syntheses.

CHAPTER SIX: LIMONENE-1,2-EPOXIDE AS A BIOSYNTHETIC INTERMEDIATE IN HIGHER PLANTS

Section 6.1 Model syntheses leading to limonene-1,2-epoxide

A number of synthetic routes leading to labelled limonene-1,2-epoxide [75] were examined. The only source of label available, because of financial constraints, was ^{14}C-methyl iodide and hence all the synthetic routes necessarily involved C-C bond formation. Non-radioactive reagents were used to develop these routes for obvious reasons of cost. Mr J. Janes of Bush Boake Allen Ltd. kindly provided standard samples of most of the compounds associated with the various synthetic routes and this saved a great deal of time and money.

Section 6.1.1 Preparation of limonene-1,2-epoxide via a Wittig reaction

The most direct route to ^{14}C-limonene-1,2-epoxide [75] is via a Wittig reaction as shown in figure 6.1. The starting material [101] is a known compound, for which several methods of synthesis have been reported.[127-130] The Wittig synthesis of limonene [102],[131] together with the epoxidation of [102] to form the desired epoxide [75],[132] are both known reactions and hence this route to ^{14}C-labelled [75] would seem very promising.

Section 6.1.1.1 Preparation of 4-acetyl-1-methylcyclohex-1-ene

(a) by a Diels-Alder reaction[127]

A Diels-Alder reaction between isoprene [103] and methyl vinyl ketone [104] (see figure 6.2) gave, after distillation at reduced pressure, a colourless oil in high yield (85.8%). ^{1}H and ^{13}C NMR data

Figure 6.1

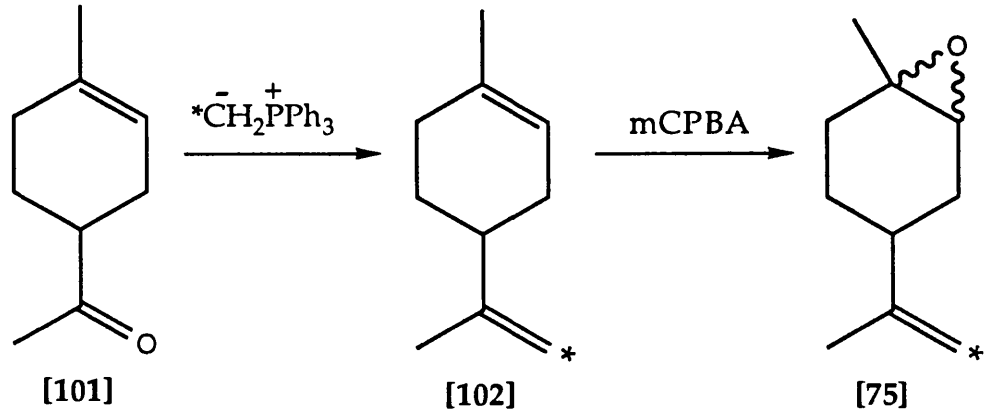


Figure 6.2

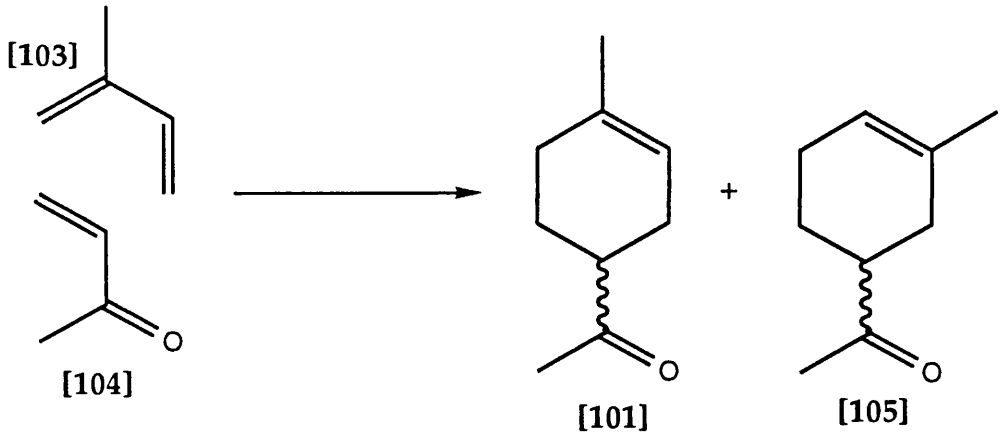
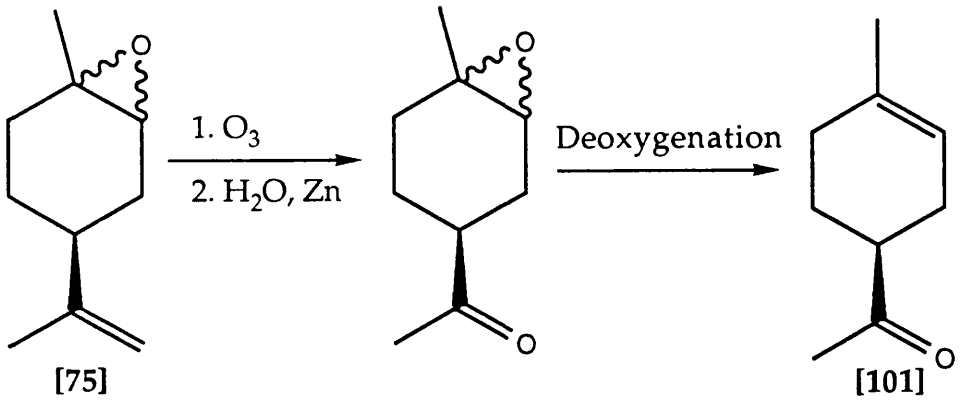


Figure 6.3



revealed that in addition to the desired product [101], the structural isomer [105] had also been formed. In the ^{1}H NMR spectrum of the oil, two multiplets derived from the methine proton adjacent to the acetyl group of the para and meta isomers were observed at δ 2.53 and 2.61ppm respectively. The values of the integrals associated with these signals indicated the *para* and *meta* isomers were present in the ratio 2 : 1 (signals were assigned by comparison with the sample prepared as described in section 6.1.1.1(b)).

The two isomers could not be separated by fractional distillation at reduced pressure and although an analytical GLC separation of the two isomers was achieved, this could not be reproduced on a preparative scale. It is possible to alter the ratio of isomers formed using catalysts (e.g. stannic chloride pentahydrate,[128] *para* : *meta*, 93 : 7; chromatographic grade silica gel,[129] *para* : *meta*, 95 : 5) or alternatively the isomers might be separated via their Girard derivatives by reverse phase HPLC. In either case a racemic mixture would be isolated at best, and hence an alternative preparation was sought which would allow, if desired, either stereoisomer to be made.

Section 6.1.1.1 (b) Preparation of 4-acetyl-1-methylcyclohex-1-ene from limonene-1,2-epoxide

(+)-[101] can be obtained from isoprene and (+)-menthyl acrylate by partial asymmetric synthesis.[130] A more satisfactory route though is from (+)-[75] by ozonisation, followed by deoxygenation (figure 6.3). It is necessary to use the epoxide bridge as a protecting group as the trisubstituted double-bond of limonene [102] is ozonised preferentially.[133] One of the earliest methods for deoxygenation utilised a mixture of zinc, sodium iodide, sodium acetate, and acetic acid.[134] More recently,[135] this mixture has been used simultaneously

to decompose ozonides and deoxygenate epoxides. It was this method which was used to make the desired ketone [101]. Thus a solution of (+)-[75] (11.22g) in dry dichloromethane was ozonised for 8 hours. After removal of most of the solvent, this solution was added dropwise to a suspension of the above zinc mixture and stirred overnight. Work-up and purification by column chromatography afforded ketone [101] (3.11g, 39% yield based on ozonised epoxide) in high purity (>95% by GC/ TLC/ HPLC). Spectral data were fully consistent with the structure assigned.

Section 6.1.1.2 Wittig reaction to synthesize limonene[131]

The reaction is shown in figure 6.4. The ylid was prepared in two stages from methyl iodide and triphenylphosphine via methyl-triphenylphosphonium iodide (see figure 6.5). The phosphonium salt produced by the first reaction was thoroughly dried in an oven at 100°C for two days. In the second stage DMSO was used as a solvent so as to increase the rate of reaction more than that achieved with classical solvents such as diethyl ether or benzene. A methylsulphinyl carbanion - dimethylsulphoxide system was used. This anion, which acts as a base to facilitate ylid formation, was formed from dry DMSO and sodium hydride at 70°C. On cooling to room temperature the solution was treated with the phosphonium salt to yield the Wittig reagent, to which ketone [101] was added. The reaction mixture was stirred at 55°C ± 5°C for 3 hours and then worked-up to give a pale yellow oil. TLC and GLC analyses both indicated that no reaction had taken place and ketone [101] was recovered in 84% yield.

Methyltriphenylphosphonium bromide is more effective as a Wittig reagent than the iodide reagent and hence the Wittig reaction was repeated using this bromide salt which was readily prepared from the

iodide reagent via the nitrate salt. In this attempt at the Wittig reaction, greater care was taken over the drying of reagents: e.g., [101] was dissolved in dry diethyl ether, dried over calcium sulphate, filtered and the solvent removed. Additionally, the phosphonium salt was dried in a vacuum oven for 48 hours. The reaction mixture was worked-up as before and analysed by TLC and GLC which both revealed a large amount of unreacted ketone to be present as well as a small amount of limonene ([102], approximately 3% based on GC areas). The reaction was repeated two further times with similar low yields. Other researchers in this laboratory have also failed to synthesize limonene [102] via a Wittig reaction. One of these workers had formed the base by addition of DMSO prior to sodium hydride. The DMSO solvent was stirred vigorously in an inert atmosphere for 15 minutes (this dispels any dissolved oxygen which can interfere with the reaction), followed by the addition of sodium hydride as a dispersion in mineral oil. The molar ratio of sodium hydride to Wittig reagent appeared to have only a minor effect upon yield of product, with one worker using up to a 6 molar excess of sodium hydride.

The published preparation of limonene [102] via a Wittig reaction[131] had utilised sodium hydride, methyltriphenylphosphonium iodide and the ketone [101] in a molar ratio of 4 : 2 : 1. The aim of these trial preparations was to establish an optimum route to ^{14}C-labelled [75] from ^{14}C-methyl iodide and hence the use of an excess of Wittig reagent (formed from methyl iodide) would not have been prudent.

Although it may have been possible to produce further improvements in yield, e.g. by using different bases (NaOEt, C_6H_6Li), the reaction was considered too unreliable to warrant further investigation: the radiolabelled methyl iodide was supplied in a

Figure 6.4

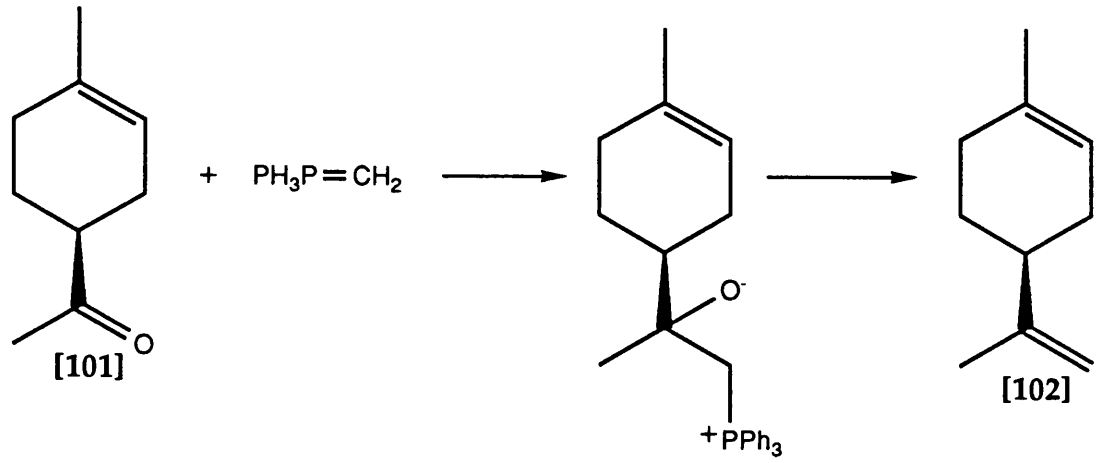


Figure 6.5

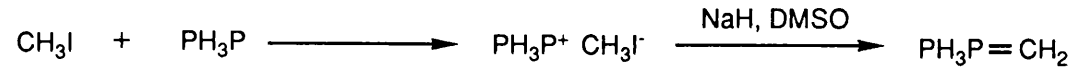


Figure 6.6

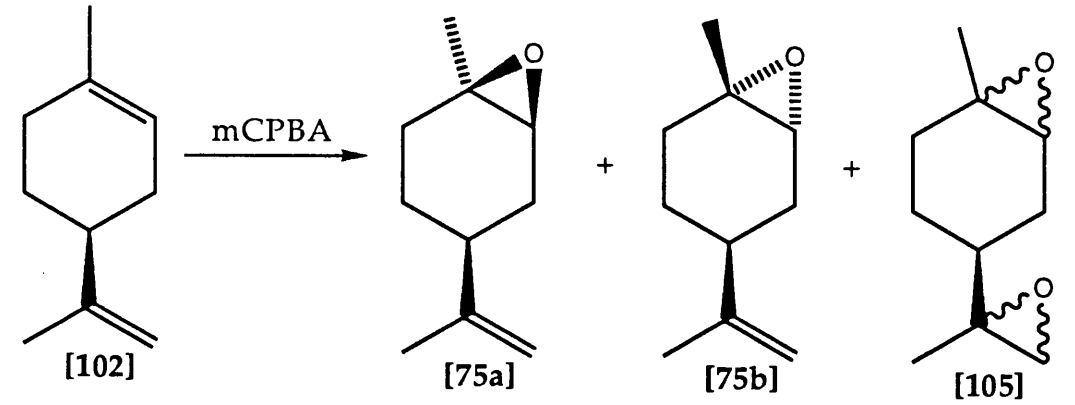
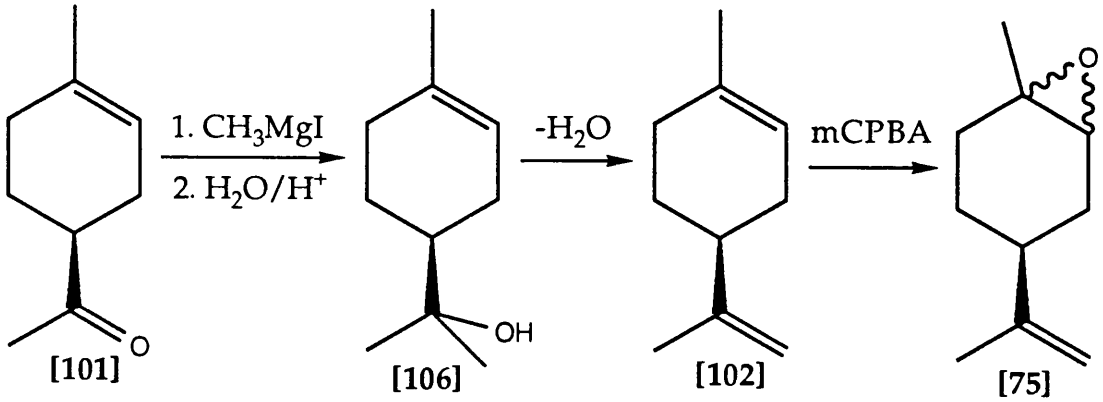


Figure 6.7



sealed vial such that only one attempt to make ^{14}C-labelled [75] would be feasible.

Section 6.1.1.3 **Epoxidation of Limonene**

Two possible epoxidation sites are present in limonene [102]. The preferred site for peracid epoxidation is the endocyclic olefin, as this is the most substituted.[136] Epoxidation of [102] by peracids[137] leads to a 1 : 1 mixture of the *cis* and *trans* isomers ([75a] and [75b] respectively), as well as a small amount of the diepoxide [105] (see figure 6.6). Several methods and reagents have been reported which enable either the pure *cis*[137-138] or *trans*[137-139] isomer to be obtained. Such stereoselective syntheses seem unnecessary in this study however, as it is conceivable that borneol can be formed *in vivo* from either [75a] or [75b]. Additionally, peracid epoxidation gives higher yields of the 1,2-epoxides than these stereoselective synthetic methods and hence it was this former procedure which was attempted.

[102] was epoxidised using m-chloroperoxybenzoic acid (mCPBA) in a biphasic solvent system (dichloromethane/dilute sodium bicarbonate) at 0°C according to the method described by Anderson and Veysoglu.[132] This method uses a sodium bicarbonate buffer to minimise acid-induced opening of the epoxide ring. A stoichiometric amount of mCPBA was used. This amount would be expected to give little or none of the 8,9- or 1,2,8,9-epoxides, only the 1,2-epoxides as there is a large difference in the reaction rates of the di- and tri-substituted double bonds. After work-up and purification by column chromatography an oil was isolated which was identical in its spectral and chromatographic properties with an authentic mixture of the 1,2-epoxides. This oil was isolated in 80% yield based on [102]. GLC and NMR analyses indicated an almost equal proportion of isomers [75a] and [75b] was present.

Section 6.1.2 Preparation of limonene-1,2-epoxide via the dehydration of α-terpineol

Compound [75] can be prepared as shown in figure 6.7 via the dehydration of α-terpineol [106]. A Grignard reaction on (+)-[101] (the methyl ketone prepared in section 6.1.1.1) yields α-terpineol [106]. Dehydration of this alcohol gives [102] which can then easily be epoxidised in high yield (as already shown in section 6.1.1.3) to give the desired product [75]. A number of methods of dehydrating α-terpineol [106] to form limonene [102] have been described.[140]

Section 6.1.2.1 Preparation of α-terpineol

Two methods of preparing α-terpineol [106] from (+)-[101] were investigated. In addition to the Grignard synthesis of [106], an alternative preparation via a Barbier reaction was attempted. The Grignard reaction was found to be the most satisfactory of these two methods. Both of these reactions were carried out using an apparatus such as that illustrated in figure E2 (see experimental), which could be used in two modes: (i) as a vacuum-line to aid transfer of volatile components and (ii) to carry out reactions in an inert atmosphere. Such a set up would permit the easy containment of radioactivity during a "hot" synthesis.

(a) By a Grignard reaction[141]

The Grignard reagent was prepared using magnesium (0.15g) and methyl iodide (0.88g) with diethyl ether as solvent. The solution of the Grignard reagent was cooled, (+)-[101] (0.86g) slowly added, the mixture refluxed gently for an hour and then worked-up to give a yellow oil (0.72g). TLC analysis indicated that this oil consisted almost entirely of α-terpineol [106] with two other faint spots, one

corresponding to the ketone starting material [101]. GLC analysis also revealed three compounds: α-terpineol [106] (97.6% of total area), 4-acetyl-1-methylcyclohex-1-ene [101] (1.1%), and an impurity present in the starting material (1.3%). This gives an overall yield of 74.0% based on methyl iodide. These analyses were confirmed by GC-MS.

Section 6.1.2.1 (b) Preparation of α-terpineol by a Barbier reaction

This reaction developed by P. Barbier in 1898 provides a method for the one-step coupling of halides and carbonyls to form alcohols as shown in figure 6.8. The reaction was developed further by Barbier's pupil, Victor Grignard, into a two-step process, which has found much wider application. There are two main reasons for this: (i) starting the reaction is easier if only magnesium and the halide are present, and (ii) fewer side products are obtained. Recently ultrasonic irradiation of the reaction mixture has been used in an attempt to overcome some of the problems associated with the Barbier synthesis and has been found to improve yields significantly.[142-143] This irradiation is thought to keep both the metal surface clean and remove adsorbed water by cavitation effects. The induction period is greatly reduced as the metal surface remains highly activated and consequently less side products are formed.

A trial Barbier synthesis of α-terpineol was made using a similar set-up and apparatus as already described for the Grignard synthesis (see section 6.2.1.2 (a)). A mixture of magnesium, methyl iodide and ketone [101] were ultrasonically irradiated for 20 minutes. After the usual work-up and purification procedure α-terpineol [106] was isolated in 92% purity (as established by GLC) and 54.3% yield. Longer irradiation times were not found to enhance this yield significantly. The percentage conversion of ketone [101] to alcohol [106] could only

Figure 6.8

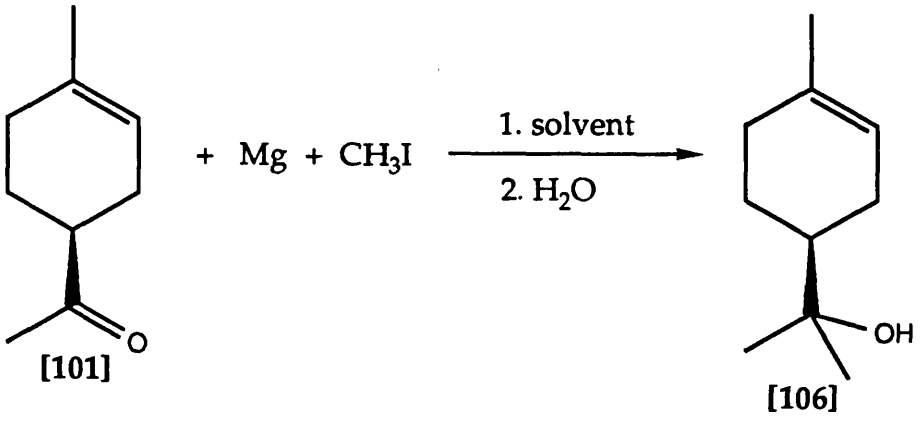


Figure 6.9

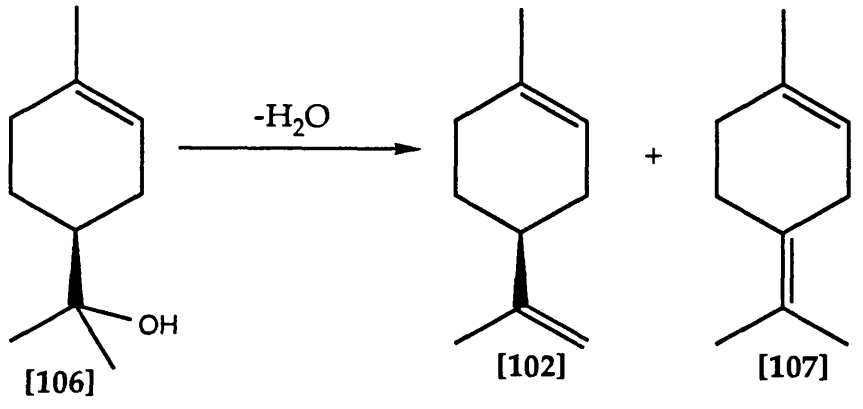
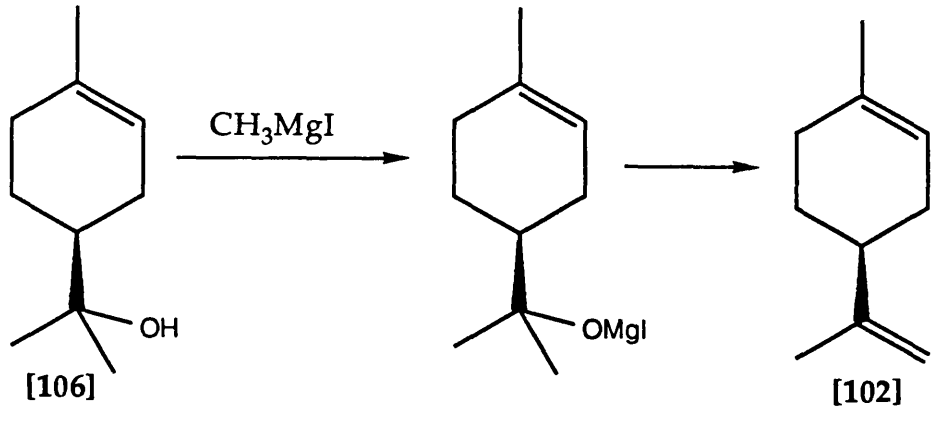


Figure 6.10



be boosted by using an excess of halide (the initial attempt had utilised a 1 : 1 : 1 molar ratio of reactants). The reaction was repeated with lithium (6 molar excess) being used in the place of magnesium, as this has been found by some workers to improve yields significantly in the Barbier reaction.[144] Although an improved yield was observed with this modification (59% as opposed to 54%), this was still less than that observed for preparation via a Grignard preparation (typically 70%).

Consequently, the Grignard reaction is the preferred method for preparing ^{14}C-labelled [106] as this should lead to a product in greater yield and/or with greater isotopic content than the Barbier method. Furthermore, when ultrasonic irradiation is used there is an increased chance of stress fracture to glassware and it would obviously be hazardous if such a fracture occurred during a reaction using radioactive materials.

Section 6.1.2.2 Preparation of limonene by the dehydration of α-terpineol

Seven β-hydrogens are present in α-terpineol [106], six of these being terminal and one tertiary. Hence dehydration can occur in two different ways to give either limonene [102] or terpinolene [107], the so-called Hofmann and Saytzeff products respectively (figure 6.9). Generally in the dehydration of alcohols Saytzeff-type elimination predominate with the most stable alkene being formed (i.e. the one with the largest number of alkyl substituents). This would suggest that alkene [107] would be the major product of the dehydration of α-terpineol [106]. In fact a number of reaction methods which proceed chiefly by Hofmann-type elimination have been reported and three such methods are described. Two of these methods led to limonene [102] as the major elimination product, although in too low a yield to be

usefully applied to a preparation using radioactive materials. The first of these three methods is very interesting synthetically as a possible alternative to the Wittig synthesis of alkenes from ketones.

(a) By the use of a Grignard reagent

In 1908 Perkin and Fisher[145] reported the following method for preparing dipentene ((±)-limonene, i.e. [102]):

"α-terpineol (20g) was gradually added to an ethereal solution of magnesium methyl iodide containing 12 grams of magnesium, and, after remaining for four days at the ordinary temperature, the product was decomposed by water and dilute hydrochloric acid ....examination showed that it consisted essentially of dipentene".

Perkin and Fisher based their characterisation on boiling point measurements and elemental analyses alone and hence these results should be treated with caution. Nonetheless, the reaction does seem feasible as shown in figure 6.10 with the Grignard reagent acting as a base and OMgI as the leaving group. This attack to give limonene [102] (the Hofmann isomer), rather than terpinolene [107] (the Saytzeff isomer), can be interpreted in terms of the steric effect: CH_3MgI being a large base would not be able to abstract the Saytzeff hydrogen as it would be hindered by the C_6-ring, whereas the methyl protons are more accessible. Not only might this reaction provide a useful method of forming limonene [102] from α-terpineol [106], but if the above proposed mechanism were upheld, then a one-pot synthesis of [102] from ketone [101] is conceivable (figure 6.11).

The reaction was carried out using α-terpineol [106] as originally described by Perkin and Fisher. After stirring for 4 days the mixture was worked up and the crude oil analysed by GLC. This

showed it to consist almost entirely of unreacted [106] (95%), with a small amount of an unidentified compound (4%) which was neither limonene [102] nor terpinolene [107]. Similarly, when ketone [101] was used as starting material only [106] was formed. These reactions were repeated at an elevated temperature by refluxing the reaction mixtures overnight, but with similar results being observed. It is possible that limonene [102] was formed by Perkin and Fisher during the work-up/purification procedure rather than the reaction itself: Perkin and Fisher distilled the products at 180°C following a wash with acid and it is well known that tertiary alcohols can be dehydrated by heating in the presence of acid. So regretfully, the reaction and the possibility of our developing a new technique of elimination had to be abandoned.

Section 6.1.2.2 (b) Preparation of limonene by the dehydration of α-terpineol using Alumina

Von-Rudloff[140] found that the dehydration of terpene alcohols with neutral alumina to which 1-2% of pyridine had been added gave a single (or only a few) products. e.g. α-terpineol [106] gave limonene [102] in 83% yield. The pyridine acts to neutralise acidic sites present in the alumina matrix and without it a large number of products are reported to be formed.[140] The reaction was carried out at 220°C using this method. GLC analysis indicated that although some limonene ([102], 6%) had been formed, the reaction mixture consisted mainly of unreacted α-terpineol ([106], 80%). The reaction was repeated at higher temperatures with no appreciable increase in yield. Obviously, the Von-Rudloff method is not generally and/or reliably applicable.

Figure 6.11

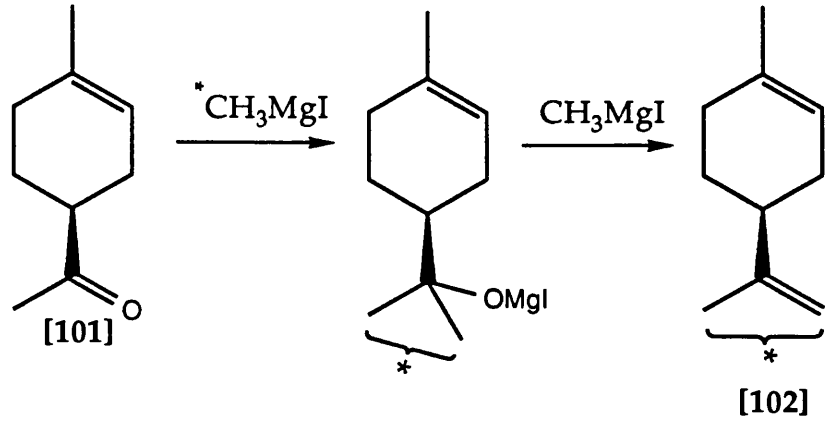


Figure 6.12

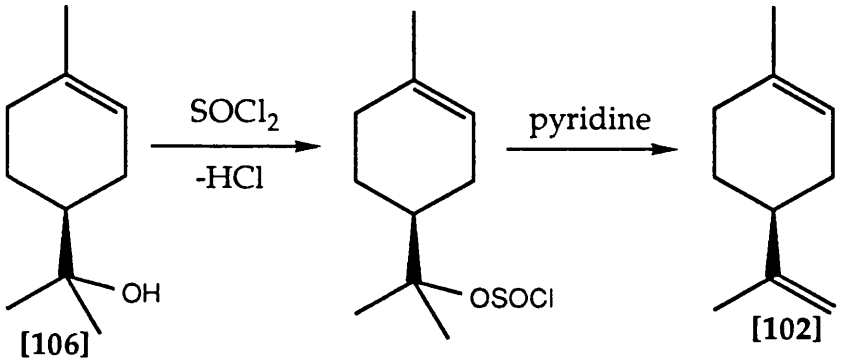
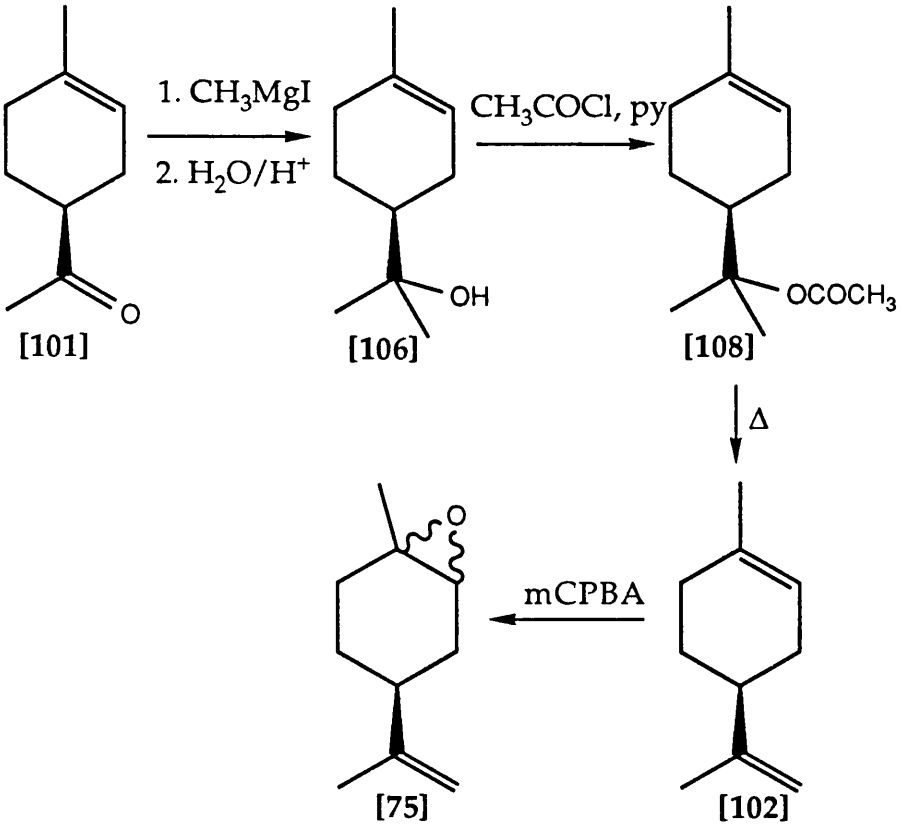


Figure 6.13



Section 6.1.2.2 (c) Preparation of limonene by the dehydration of α-terpineol using thionyl chloride[140,146]

The reaction scheme is shown in figure 6.12. Pyridine is necessary not only for the elimination step, but also to neutralise the hydrogen chloride produced which would otherwise encourage the formation of unwanted rearrangement products. OSOCl is a poor leaving group, hence the transition state resembles C_β-H cleavage (E1cB-like) and is reactant-like with little double bond character. This leaving group effect together with the steric effect will favour formation of the Hofmann product. On the other hand, pyridine is a weak base and so will favour the Saytzeff product. These considerations would seem to suggest that neither isomer will predominate. The reported proportions of products[140] ([102] : [107]; 70 : 21% of total products) appear to be governed by statistical factors: there are six terminal β-hydrogens and one tertiary β-hydrogen.

This reaction was carried out at 0°C and after work up a brown oil was isolated. GLC analysis indicated this residue was heavily contaminated with pyridine, although [102] was also detected (57% of total GLC peak area) in 21.4% yield based on GLC peak areas. Further purification of this mixture by solvent washing resulted in major losses. The reaction was repeated using both longer and shorter reaction times but with similar results. In these subsequent efforts pyridine was removed by column chromatography to give a product of reasonable purity (> 80% by GLC areas, with [107] as the major impurity), although in a reduced yield (not more than 14%).

Section 6.1.3 Preparation of limonene-1,2-epoxide via α-terpinyl acetate

A 4-step synthesis of limonene-1,2-epoxide [75] from (+)-4-acetyl-1-methylcyclohex-1-ene [101] as shown in figure 6.13 was

attempted. The first and last reactions in this scheme had already been carried out successfully as described in sections 6.1.2.1(a) and 6.1.1.3, and hence efforts were focused initially on the second and third reaction steps.

Section 6.1.3.1 Acetylation of α-terpineol

The acylation of tertiary alcohols such as α-terpineol [106] is often a problem as these compounds are less reactive than primary or secondary alcohols and so stronger reagents have to be used. Acetyl chloride and pyridine have been found to provide a good combination for the acetylation of tertiary alcohols.[147] However, when α-terpineol [106] and these reagents were stirred together at 0°C for 3 hours a brown oil was obtained, which consisted mainly of pyridine and unreacted [106]. A second attempt using an elevated temperature (70°C) was visually very different, with brown crystals (pyridine hydrogen chloride) and a white polymer being formed. The products were again heavily contaminated with pyridine, and although greater conversion of products was apparent, a large quantity of unreacted [106] was also present. Hence an alternative preparation of α-terpinyl acetate [108] was sought.

Recently several pyridine derivatives have been used as catalysts in the acylation of unreactive alcohols e.g. 4-N,N-dimethylamino-pyridine (DMAP) for tertiary alcohols.[148] This catalyst together with triethylamine (TEA) and acetic anhydride (Ac_2O) was used to acetylate [106] at 40°C. The course of the reaction was monitored by TLC and after 24 hours none of the starting material was detected. The reaction mixture was worked-up to give an oil which was identical in its spectral and chromatographic properties with an authentic sample of α-terpinyl acetate [108]. This product was greater than 90% pure by

GLC, gave a single spot by TLC and had been formed in high yield (84.5% yield). This result and those of the other attempts to form this acetate are shown in table 6.1. In addition to the higher yield of acetate noted with this final method, another notable advantage is the ease with which TEA is removed during work-up compared to pyridine.

Table 6.1 Acetylation of α-terpineol [106]

Reagent	Temp °C	Time hours	Crude yield %	Reaction mixture[b]	
				% [108]	% [106]
AcCl, py	0	3	51.0[a]	6.3	92.4
" "	70	3	121.7[a]	34.3	63.8
Ac_2O, DMAP, TEA	40	2.5	-	66.0	33.0
" " "	"	5	-	76.2	23.0
" " "	"	7	-	85.7	13.5
	"	24	84.5	93.4	0.6

[a]elevated by pyridine contamination

[b]proportions calculated automatically by glc area

Note: pyridine was eluted too close to the solvent front to be integrated

Section 6.1.3.2 Preparation of Limonene by a pyrolysis reaction

Two products can be formed by the 1,2-elimination of acetic acid from α-terpinyl acetate [108]: limonene ([102], the Hofmann product) and terpinolene ([107], the Saytzeff product). In general, pyrolyses of esters proceed via Hofmann-type elimination with the ratio of products formed being close to that predicted on a purely statistical basis. i.e. if the choice among β-hydrogens for the elimination were purely random. Hence the pyrolysis of α-terpinyl acetate [108] would be expected to yield limonene [102] and terpinolene [107] in, or close

to, the ratio 6 : 1, good enough for the purposes of this work providing there is reasonable conversion with little or no other products. DePuy[149,150] amongst others[151,152] has found that neither the temperature, or conditioning of, or recycling of products through, the pyrolysis column significantly affect the ratio of products formed. The nature of the material used to pack the pyrolysis column (glass helixes/ wool are usually used) also has little effect upon this ratio, although washing of this packing material with acid does lead to an increase in the proportion of the endocyclic product, albeit with considerable charring.

The pyrolysis was performed by passing a solution of [108] (0.40g) in hexane through a heated column (425°C) packed with glass beads with the aid of a dry (and oxygen-free) supply of argon. TLC analysis indicated that after one pass through the heated column, no unreacted starting material was remaining. The column was allowed to cool, washed with a little hexane, the pyrolysate worked-up and purified by column chromatography to give a colourless oil (0.15g).

GLC analysis indicated that the oil obtained consisted mainly of limonene ([102], 67% by area) with a smaller amount of isomer [107] (ratio [102] : [107]; 4.1 : 1 respectively). The reaction was repeated several times in an effort to improve the proportion of [102] formed: The use of higher temperatures was found to increase the proportion of other products. Indeed at 510°C, p-methylisoprenylbenzene (as identified by GC-MS) was the major product. Whilst no increase in amounts of side products was noted with lower temperatures, conversion was incomplete so as to necessitate several passes through the column with subsequent handling losses. Five such passes caused a negligible increase (0.87% by relative peak areas) in the ratio of limonene [102], : terpinolene [107], supporting DePuy's claim[149] that recycling

does not significantly alter this ratio.

Section 6.1.3.3 Synthesis of limonene-1,2-epoxide from 4-acetyl-1-methylcyclohex-1-ene: A Cold Run

The reactions carried out in the previous sections had indicated the epoxide [75] could be prepared from ketone [101] in 4 steps, as shown in figure 6.13. These reactions had, thus far, been carried out in isolation of one another using high-purity starting materials, rather than consecutively (i.e. using the crude product from one step as a starting material for the next step). A series of experiments were conducted to establish if it was necessary to purify any of these crude products, or whether such purifications would lead to unnecessary handling losses. In one experiment preparation of [75] from [101] with purification of the intermediary reaction products gave the epoxide in 5.6% overall yield, whereas in another experiment, when only the final (epoxide) product was purified, an higher overall yield of 15.5% was recorded.

It had been anticipated that, of the 4 individual reaction steps, the pyrolysis and epoxidation reactions would be most affected by impurities present in the crude mixtures used. Experiments showed however, that neither the ratio of limonene [102] : terpinolene [107] formed by the pyrolysis reaction, nor the yield of these products, was any different when either pure or crude acetate product were used. In fact, when losses from the purification procedure were considered, it was better to omit this purification step.

The mixture of isomers [102] and [107] obtained from the pyrolysis reaction could not be separated preparatively by chromatographic means and so epoxidation of the mixture was attempted. The tetra-substituted double bond of [107] would be expected to be

epoxidised in preference to the two double bonds of [102], whilst the trisubstituted double bonds of [102] and [107] would be expected to have similar reactivities. One would expect when an excess of mCPBA is used to obtain limonene-1,2-epoxide [75] and terpinolene diepoxide which are easily separable, providing the reaction is halted as soon as all the limonene [102] and terpinolene [107] had reacted. Hence, the reaction was carried out using an excess of mCPBA, with the reaction being carefully monitored by TLC. When the alkenes [102] and [107] had reacted, the reaction mixture was worked up and the limonene-1,2-epoxide [75] purified by column chromatography. GLC analysis indicated a mixture of the *cis* and *trans* 1,2-epoxide isomers ([75a] and [75b] respectively) had been isolated in greater than 95% purity and this was confirmed by GC-MS.

These experiments showed, therefore, that when crude mixtures of the isolated intermediates were used, epoxide [75] could be prepared from ketone [101] in 15% overall yield, i.e. acceptable for the subsequent radiochemical studies.

Section 6.2 Preparation of ^{14}C-limonene-1,2-epoxide

^{14}C-labelled [75] was prepared using the same general procedure as for the "dry run" (see section 6.1.3.3), except that a modified procedure for the Grignard reaction was used (see experimental). The course of each of the 4 reactions leading to the desired epoxide were monitored by TLC alone as the analysis of radioactive samples was not permitted using the GLC machine available. TLC analysis indicated few side-products were formed from either the Grignard, acetylation, pyrolysis or epoxidation reactions, with α-terpineol [106], α-terpinyl acetate [108], limonene [102]/ terpinolene [107] and limonene -1,2-epoxide [75] respectively as the major products. Following the

epoxidation reaction, the crude products were purified by column chromatography to give limonene-1,2-epoxide ([75], 103.7mg). This was further purified by passage through a second silica column to give the desired epoxide ([75], 66.9mg, 9.2% overall yield). A small amount of this epoxide was analysed by TLC: Radiochromatogram scanning of the developed TLC plate indicated the sample consisted of one component only and this was confirmed by liquid scintillation counting of the material separated on the TLC plate. The specific activity of this epoxide was calculated as 4,231,560 dpm/mmol (1.91 μCi/mmol, 0.071 MBq/mmol).

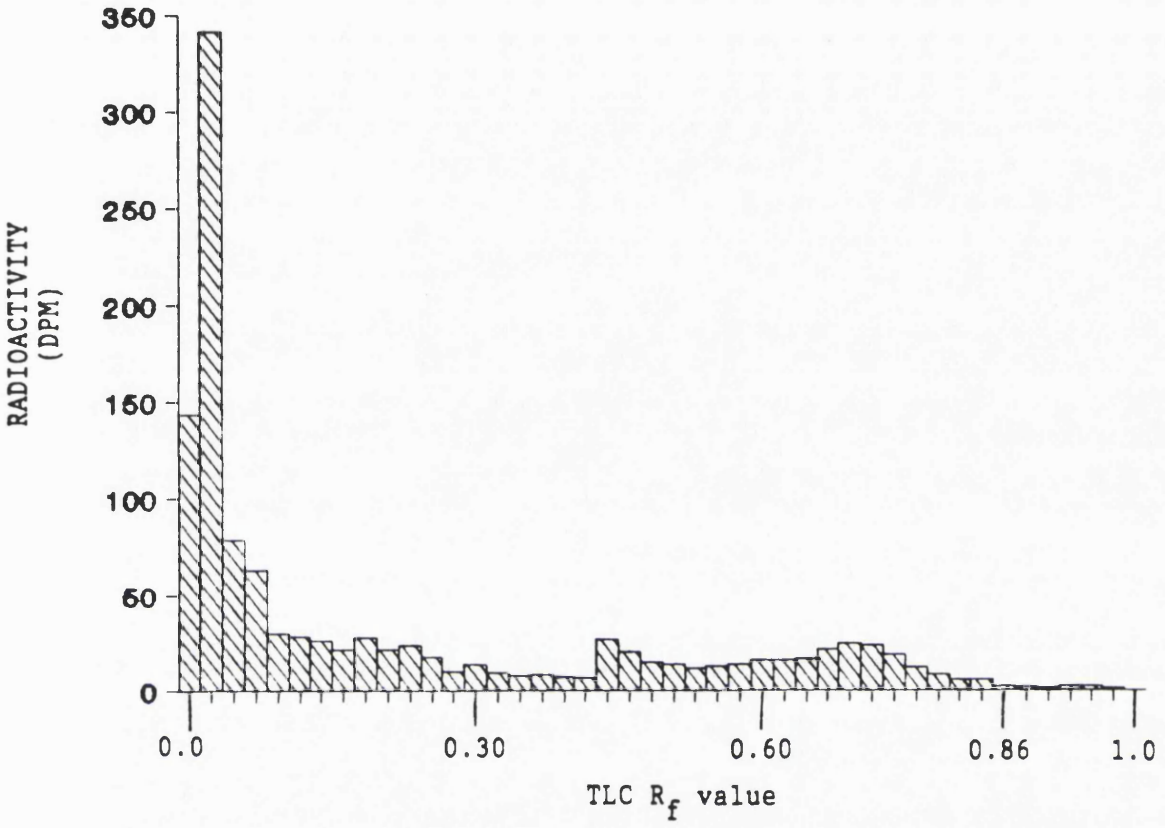
Section 6.3 Preliminary Radioactive feeding experiments

Section 6.3.1 Feeding experiments to plants

^{14}C-labelled limonene-1,2-epoxide ([75], 7μl, 189,595dpm (85mCi) in total) was fed to cuttings of *Pelargonium fragrans* as a solution in ethanol (40μl) by forced transpiration. The cuttings were kept in a fume cupboard (continuous light) for 60 hours, after which they still looked healthy. Following extraction by sonication using diethyl ether as solvent, the extract was analysed by TLC and the material separated on this TLC plate examined by liquid scintillation counting.

The results are shown in figure 6.14 as a plot of disintegrations per minute against R_f value. None of epoxide [75] (R_f= 0.82) was recovered, only a large peak at $R_f \simeq 0.02$ is evident, which being so close to the origin may consist of a mixture of compounds. Borneol [98] and camphor [99] have R_f values of 0.45 and 0.40 respectively and although there is a small peak at $R_f \simeq 0.4$ this is too close to background to be meaningful. In radioactive feeding experiments incorporations of typically 0.1% are noted for products which are a large number of reaction steps from the fed precursor, e.g.

Figure 6.14 Separation of the metabolities formed following feeding of ^{14}C-limonene-1,2,-epoxide to cuttings of *Pelargonium fragrans*



incorporation of label into monoterpenoids when ^{14}C-MVA is the precursor. However, in this case much higher incorporations (ca. 10%) would be expected as the precursor and postulated products are only a few reaction steps from one another. The extraction method used would extract organics and this would give low recovery of degradation products (such as diols etc), but if any of the postulated products (borneol and camphor) were formed these should be recovered in high yield. In fact very low recoveries were noted (0.61% in total). These results thus provide no evidence for the occurence of limonene-1,2-epoxide as a biosynthetic intermediate leading to the

formation of borneol and camphor in higher plants, but instead suggest that this epoxide may be a catabolic metabolite which is broken down *in vivo* into water-soluble products by oxidative degradation.

Section 6.3.2 **Feeding experiments to cultures**

^{14}C-labelled limonene-1,2-epoxide ([75], 8μl, 216,680 dpm (98 mCi) in total) in ethanol (32μl) was injected into a callus culture of *P. fragrans* 12 days into its growth cycle. The culture was incubated under normal photoperiod conditions (see table 3.1) for 60 hours, after which the culture material (12.73g) was worked up (as described for the plant material). The agar medium was also extracted by heating at 70°C until all the medium had liquefied. After cooling to 40°C, the medium was carefully extracted with petroleum spirit. These two extracts were each analysed by TLC and the material separated on these plates examined by liquid scintillation counting.

The results of counting these extracts are shown in figures 6.15 and 6.16. The radiochromatogram obtained for the extract of the agar medium appears similar to that obtained for the plant extract with most of the radioactive material being close to the origin. The data obtained for the culture extract is, however, very different. The main feature is an intense peak at $R_f \simeq 0.95$. This does not correspond to epoxide [75] ($R_f = 0.82$), the proposed products borneol ([98], $R_f = 0.45$) or camphor ([99], $R_f = 0.40$), or monoterpene hydrocarbons (e.g. limonene [102] $R_f = 1.0$) formed by epoxide ring opening and subsequent eliminations. It is possible that this peak is due to a chlorophyll, xanthophyll or caretonoid pigment which would also be extracted with diethyl ether. There is a peak at $R_f \simeq 0.82$ which is likely to be recovered epoxide [75]. The two peaks at $R_f \simeq 0.05$ and 0.08 are close to the origin and hence may be derived from a number of different components (c.f. plant and culture media extracts).

Figure 6.15 Separation of the metabolities present in callus cultures of *Pelargonium fragrans* following feeding of ^{14}C-limonene-1,2,-epoxide

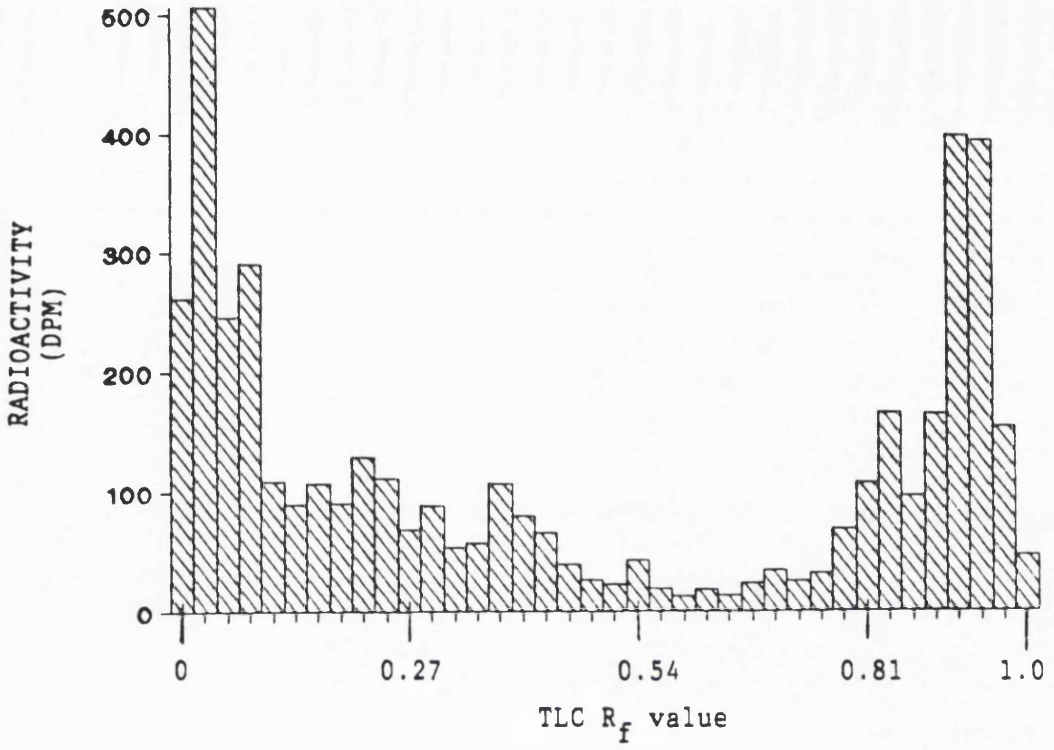
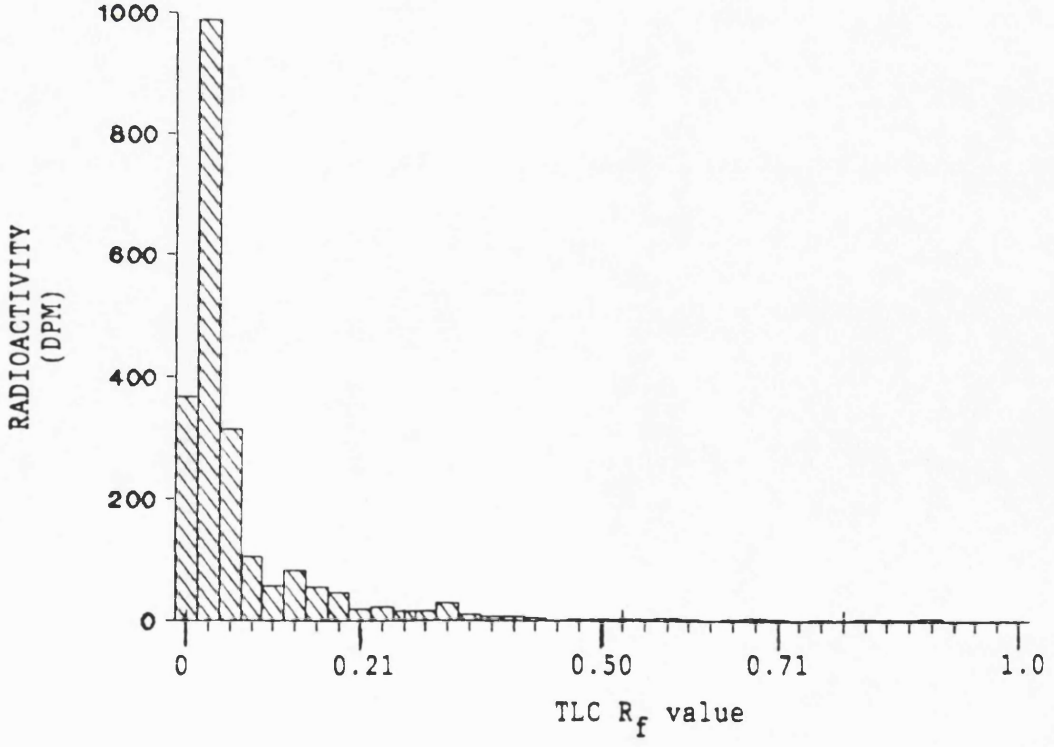


Figure 6.16 Separation of the metabolites present in the medium of *Pelargonium fragrans* cultures following feeding of ^{14}C-limonene-1,2-epoxide



Section 6.4 **Conclusions**

The results of the preliminary feeding experiments described in this chapter indicate that neither borneol nor camphor are derived biosynthetically from limonene-1,2-epoxide. The organic extracts prepared from both callus cultures and plant material following the feeding of the labelled epoxide contained very little activity, with total recoveries of 1.97, 1.04 and 0.61% being noted for the callus culture material, agar medium and plant material respectively. One explanation for such low recoveries is that much of the tracer was converted into water-soluble products that could not be easily extracted into organic solvents, e.g. terpenoid-sugar conjugates, phosphate esters, or polar "salvage" products (formed by the oxidative degradation of the fed epoxide). The isolation of hydrophilic substances is extremely difficult[123] and further work would be to investigate the occurrence of any such products.

Clearly, the experimental data presented in this work does not enable any definite conclusions to be reached as to the fate of the ^{14}C-limonene-1,2-epoxide which was fed to tissue cultures and cuttings of *P. fragrans*. A large portion of the tracer present in the extracts obtained following these feeding experiments was located in that region of the radiochromatogram which is usually associated with diols and other such polar compounds (i.e. ones of low R_f value). The radiochromatogram of the callus material contained significant levels of tracer at $R_f \simeq 0.95$, which is where pigments such as chlorophylls, xanthophylls and carotenoids occur. Hence these preliminary results support the view[123,124] that monoterpenes are turned over *in vivo* by oxidative degradation via the sequence: alkene ⟶ alkene oxide ⟶ alkene diol ⟶ cleaved and degraded products; eventually leading to water solubles, acetate, etc., that can be recycled within the short

time period of the experiment.

In general, monoterpenoids appear to be rapidly turned over *in vivo*[105] and thus it may be that labelled borneol and/or camphor were indeed formed from limonene-1,2-epoxide, but these compounds then went on to form other products during the incubation period. This possibility could be investigated by performing time-course studies. Additional further work would be to use cell-free extracts of plants and callus cultures for feeding experiments, as such extracts (amongst other advantages) tend to give higher (ca. 10-fold) incorporations of tracer.[105]

In summary, a synthetic route to ^{14}C-labelled limonene-1,2-epoxide from 4-acetyl-1-methylcyclohex-1-ene was developed and utilised. The preliminary tracer experiments performed in this work suggest limonene-1,2-epoxide is not a biosynthetic intermediate leading to borneol or camphor in either higher plants or their tissue cultures, but is possibly involved in the degradation of these and related monoterpenoids in a naturally-occurring salvage process.

Chapter 7: MODEL SYNTHESES LEADING TO 6,7-EPOXYLINALOOL AND THE TWO FURANOID LINALOOL OXIDES

The preparation of epoxide [71] and the furans [100a] and [100b] was attempted via the scheme shown in figure 7.1. This scheme is very appealing as it allows the synthesis of all three of the desired compounds in three reaction steps. Another convenient feature is that it utilises the same methodology and apparatus as that which had already been used for the synthesis of limonene-1,2-epoxide [75].

Section 7.1 Preparation of 7-methyl-1,6-octadien-3-one

Section 7.1.1 By the pyrolysis of Nopinone[153]

The synthesis of 7-methyl-1,6-octadien-3-one [109] was attempted by the route shown in figure 7.2. Ozonolysis of β-pinene [84] gave a single compound in 43% yield, which was identified as nopinone [110] by comparison of its spectroscopic data with published values.[154] Some of this product (3.50g) was dissolved in hexane and pyrolysed by passage through a heated column (temperature $\simeq 550^{\circ}C$) over a 45 minute period. TLC analysis indicated the pyrosylate was composed mainly of unreacted [110]. This starting material was removed by column chromatography to leave a mixture of products (0.1g) which were separated by HPLC.

The pyrolysis of nopinone [110] is reported to give the required ketone [109] as the major product (39% of total products), as well as 4-isopropenylcyclohexanone [111](27%), *cis*-2-methyl-3-isopropenylcyclopentanone [112a](14%), *trans*-2-methyl-3-isopropenylcyclopentanone [112b](8%) and a number of unidentified minor products (11% in total). Examination of the ^{1}H and ^{13}C NMR spectra obtained for the purified products from above revealed the desired ketone [109] had been formed

Figure 7.1

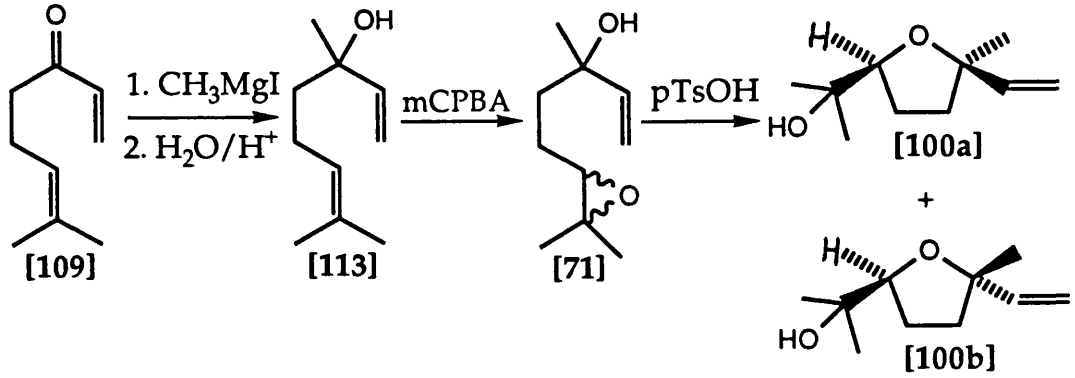
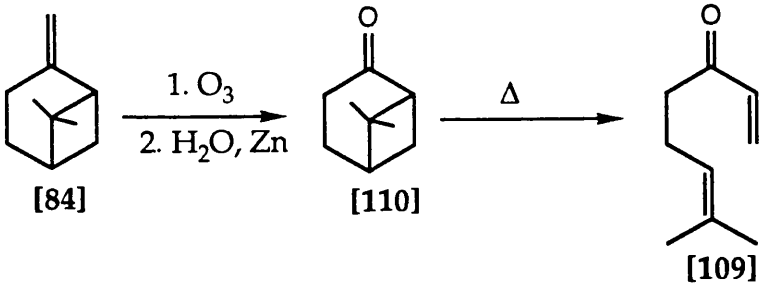


Figure 7.2



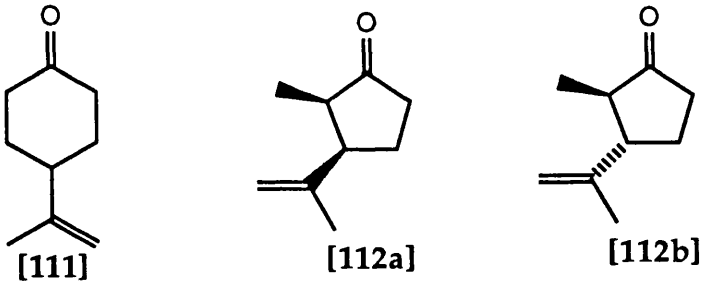
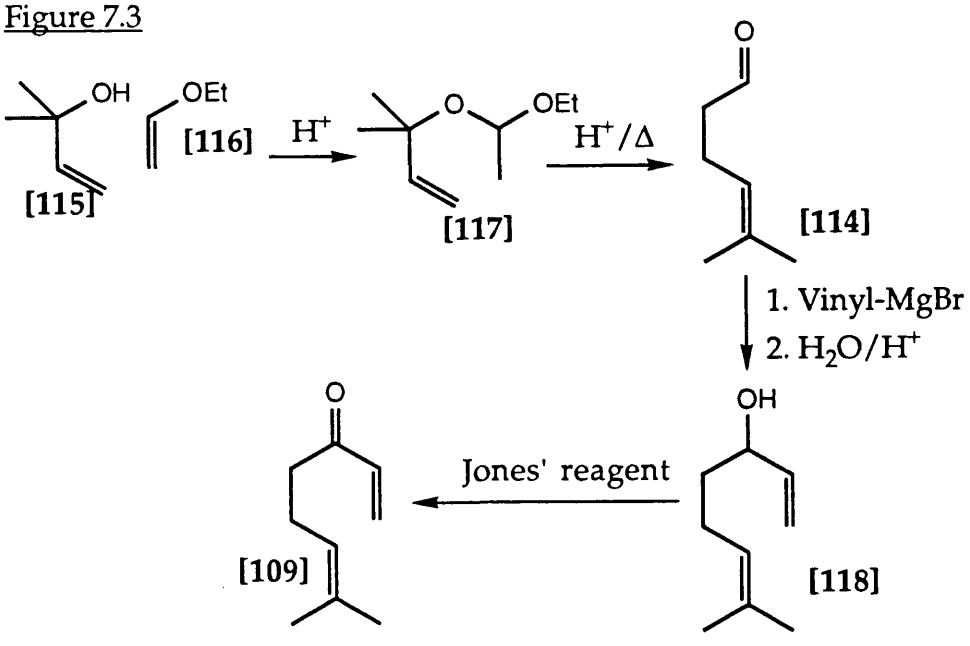


Figure 7.3



as the major product (20mg, 0.57% yield), with smaller amounts of the cyclopentanones [112a] and [112b], as well as 3 unidentified unsaturated polymeric ketones also being formed. Distinction between the 2 epimeric cyclopentanones was based upon the chemical shifts of the methyl group attached to C_2 (δ 0.89 and 1.04ppm for [112a] and [112b] respectively[153]). No 4-isopropenylcyclohexanone [111] was found.

The pyrolysis reaction was repeated two further times using longer passage times (7 and 3 hours) and these attempts did lead to greater conversion of starting material to products. Nonetheless, from a total of 14.67g of ketone [110] used in these attempts only 70mg of [109] was isolated. The rate of reaction of [109] and the relative proportions of products is dependent upon temperature as well as the rate of passage of sample through the column. Previous work in this laboratory by Ms. R. Shah had indicated that a minimum temperature of 526°C was required for any conversion of nopinone into products and that one of 550°C (the upper operating limit of the pyrolyser and the temperature at which the above pyrolyses were performed) was better. Hence this variable could not be optimised further. One frequently-used technique in pyrolyses experiments is to recycle the pyrosylate until all of the starting material has reacted. However in this particular reaction the acyclic dienone [109] is converted substantially (70% at 570°C) into the cyclopentanones [112a] and [112b], whilst the major reaction of [112a] and [112b] is interconversion.

In a trial Grignard reaction performed using the 90mg of ketone [109] obtained from the pyrolysis reactions linalool [113] was formed as the major product. Approximately 1g of ketone [109] would be needed for further trials and the radiosynthesis and as the pyrolysis

reaction had given very low yields of the desired product [109] an alternative method of synthesis was sought.

Section 7.1.2 Preparation of 7-methyl-1,6-octadien-3-one via 5-methyl-4-hexenal

An alternative route to 7-methyl-1,6-octadien-3-one [109] is via 5-methyl-4-hexenal [114] as shown in figure 7.3. An acid-catalysed addition of 3-methyl-but-1-en-3-ol [115] to ethylvinyl ether [116] yields acetal [117] which undergoes an acid-catalyzed Claisen rearrangement to form aldehyde [114].[155,156] This aldehyde can react with a vinyl Grignard reagent to give alcohol [118],[157] which on oxidation with Jones' reagent[158] gives the desired α,β- unsaturated ketone [109]. In an initial attempt to synthesize acetal [117], 3-methylbut-1-en-3-ol [115] and ethyl vinyl ether [116] were refluxed overnight. The reaction mixture was then cooled, neutralised with triethylamine and distilled to give 1,1-diethoxy-5-methyl-hex-4-ene [119] as the major fraction (presumably formed by an acid-catalysed addition of two moles of ethanol to the ketone) as well as an unidentified product as a minor fraction. In a second attempt to form acetal [117] the same procedure was repeated except that after the overnight reflux traces of acid were removed by washing with bicarbonate solution rather than neutralising with triethylamine. This modified procedure gave, after distillation, a single compound in reasonable yield (58.7%) consistent in its spectral properties with those expected for the desired compound [117]. This acetal was sealed in a Carius tube, together with a trace of orthophosphoric acid, and heated at 150°C for 30 minutes. After work-up the mixture was purified by distillation at reduced pressure to give three fractions, containing the desired aldehyde ([114], 13.8% yield),

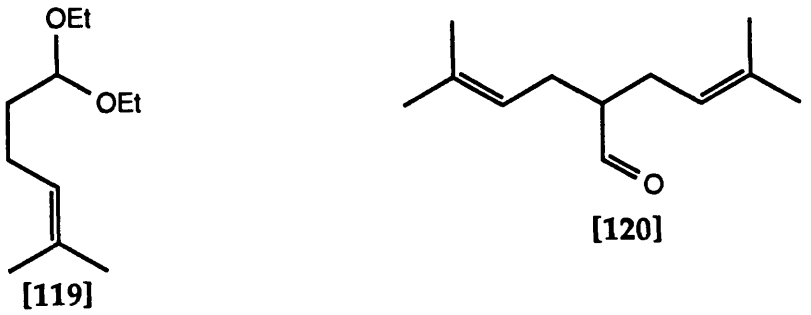
1,1-diethoxy-5-methyl-hex-4-ene [119] and 5-formyl-2,8-dimethyl-non-2,7-diene [120].

A Grignard reaction between vinyl magnesium bromide and aldehyde [114] produced 7-methyl-1,6-octadien-3-ol [118] which was oxidised without further purification using Jones' reagent to give, after purification, the desired ketone ([109], 23.4% yield based on the aldehyde). HPLC analysis indicated this ketone was approximately 95% pure.

Section 7.2 Preparation of Linalool by a Grignard reaction

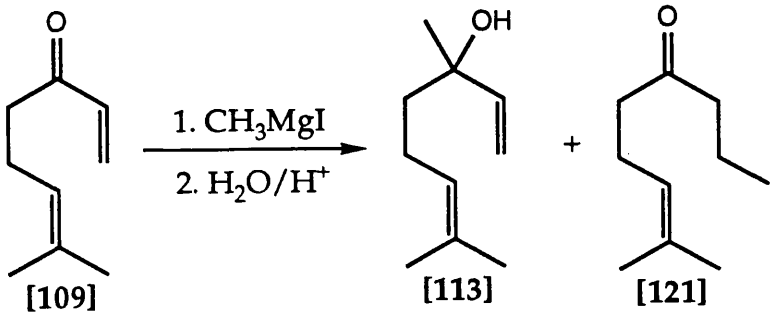
7-Methyl-1,6-octadien-3-one [109] being an α,β-unsaturated ketone can react with a Grignard reagent in two ways to give either the "normal" 1,2- addition product [113] or the conjugate (Michael) 1,4-addition product [121] (see figure 7.4). Several factors affect the ratio of products formed,[159,160] for instance, the quality of the magnesium used.[161]

A Grignard reaction between ketone [109], iodomethane and magnesium (1 : 1 : 1 molar ratio) was performed using the same vacuum apparatus and general procedure as described elsewhere for the synthesis of α-terpineol (see section 6.1.2.1(a)). The mixture was refluxed gently for 90 minutes and after cooling the Grignard complex was decomposed and the reaction mixture worked-up to give an oil. This was analysed by TLC and GLC and found to consist mainly of linalool [113] with a lesser amount of an unknown compound later identified as the 1,4-addition product ([121], ratio of 1,2- : 1,4-; 4.5 : 1 by GLC areas). The mixture was purified by column chromatography to give linalool ([113], 52% yield) and the 1,4-addition product ([121], 8.6% yield). The major product was identified as linalool [113] by comparison of its chromatographic and spectral properties with those



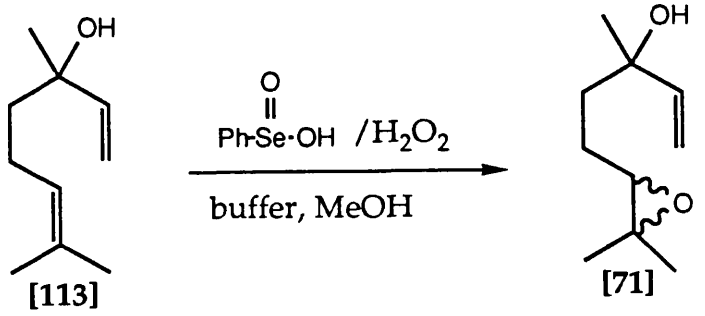
[119] [120]

Figure 7.4



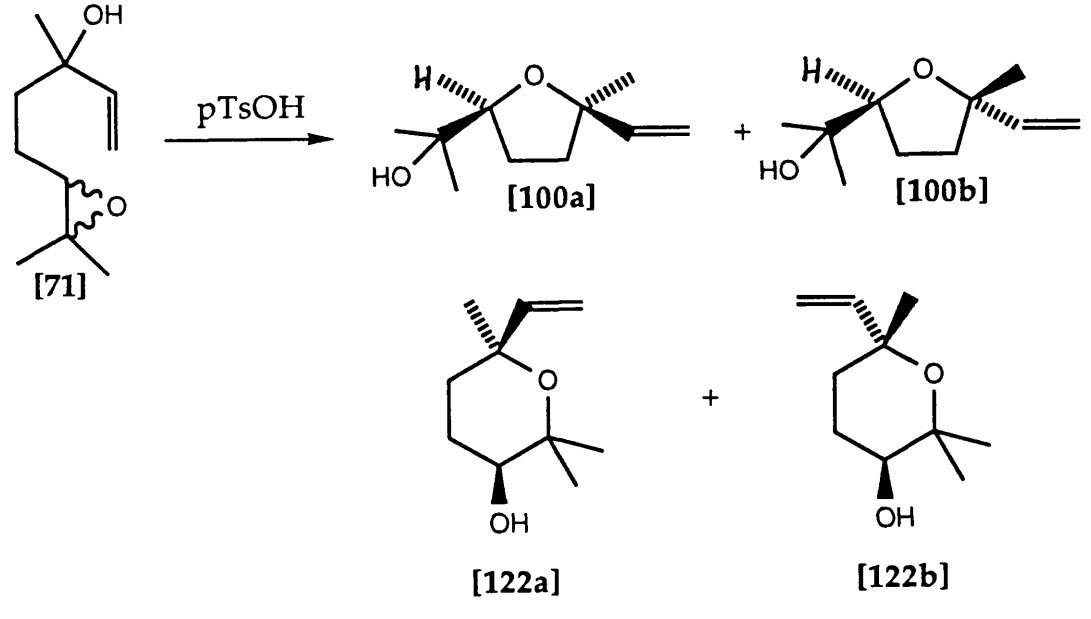
[109] [113] [121]

Figure 7.5



[113] [71]

Figure 7.6



[71] [100a] [100b]

[122a] [122b]

of an authentic sample. The minor product was identified as 8-methyl-7-nonene-4-one [121] by comparison of its spectral data with published values.[162] Although it may have been possible to increase the proportion of 1,2-adduct by the use of organolithium or other organoalkali metals the yield obtained using magnesium (52% isolated yield) was considered acceptable for the purposes of this study.

Section 7.3 Epoxidation of linalool

Linalool [113] was epoxidised using m-chloroperoxybenzoic acid (mCPBA) as described by Winterhalter *et al.*[113] After 2 hours the reaction mixture was worked-up and purified to give an oil in 38% yield. The NMR spectrum of this oil did not correspond to published values for 6,7-epoxylinalool [71],[163,164] but rather to those given for the *cis* and *trans* furanoid isomers of linalool oxide ([100a] and [100b] respectively).[165] Indeed this oil was identical in its chromatographic and spectroscopic properties with an authentic mixture of these furanoid isomers (ex. BBA Ltd, Walthamstow).

The furanoid isomers of linalool oxide (and the pyranoid isomers [122a] and [122b]) are known to be formed by the acid-catalysed rearrangement of 6,7-epoxylinalool [71],[163] and hence the reaction was repeated using a dichloromethane-aqueous sodium bicarbonate biphasic solvent system which is reported to reduce such acid-catalysed rearrangements.[132] GLC analysis of the crude reaction products obtained by this modified procedure revealed two unknown compounds had been formed as the major products (small amounts of [100a], [100b], [122a] and [122b] were also detected). Comparison of the ^{1}H NMR spectrum of this crude mixture with published values for 6,7-epoxylinalool [71] showed the two major products to be the two diastereoisomers of 6,7-epoxylinalool [71]. However, during the course

of separation by silica gel column chromatography these diastereoisomers rearranged to give the 4 linalool oxides. Several unsuccessful attempts were made to purify further samples of crude [71] using different grades of silica gel and also flash chromatography (which has been used by others to separate acid-sensitive compounds). Fringuelli *et al*[164] have recently reported that they too found column chromatography to be an unsuitable method for the purification of these two diastereoisomers.

An alternative published preparation of epoxide [71] is by the epoxidation of linalool [113] with benzeneseleninic acid and hydrogen peroxide (figure 7.5).[166] This method is reported to give the desired product in 63% yield, although when the method was followed in the present work a mixture was obtained which consisted almost entirely of the 4 cyclic linalool oxides.

Although crude ^{14}C-labelled [71] could be obtained by epoxidation of ^{14}C-labelled [113] with mCPBA, this could not be used for radioactive feeding experiments as labelled compounds of high purity are required. The use of reduced pressure distillation (which has been used to purify large quantities of [71])[163] would not be feasible in this case as a synthesis of ^{14}C-labelled [71] would necessarily be carried out on a small scale. Insufficient time was available to establish whether purification by preparative-GLC could be accomplished and thus a synthesis of ^{14}C-labelled [71] was not attempted.

Section 7.4 Preparation of cis and trans linalool oxides[163]

The *cis* and *trans* linalool oxides ([100a] and [100b] respectively) were formed as the major products (40.1 and 39.1% of total GLC area respectively) by treatment of crude 6,7-epoxylinalool

[71] with p-toluenesulphonic acid (figure 7.6). Hence a route to radiolabelled [100a] and [100b] had been established and radioactive material could be prepared in future using this method.

Section 7.5 Summary and further work

In this chapter a number of synthetic routes leading to 6,7-epoxylinalool [71] were investigated and a route to the two furanoid isomers ([100a] and [100b]) of [71] developed. Whilst 6,7-epoxylinalool [71] was detected in crude reaction mixtures, this product decomposed during purification by silica gel column chromatography to give a mixture of the furanoid ([100a] and [100b]) and pyranoid ([122a] and [122b]) isomers of [71]. Insufficient time was available to attempt purification using alternative methods, e.g. preparative-GLC. The ease with which the cyclisation of 6,7-epoxylinalool [71] occurs must draw into question the reported[112-114] natural occurrence of this compound. It seems reasonable to suggest that 6,7-epoxylinalool [71] exists *in vivo* in a phosphorylated and/or glycosidic form.

If greater time had been available then efforts would have been directed towards the preparation of ^{14}C-labelled phosphorylated and glycosidic derivatives of 6,7-epoxylinalool [71], the *cis* and *trans* furanoid forms of 6,7-epoxylinalool [71] as well as 6,7-epoxylinalool [71] itself. These compounds could then be fed to tissue cultures, plant material and/or cell-free extracts of *Pelargonium fragrans* and the metabolism of these compounds followed. Such studies, when combined with a more extensive investigation into the biological role of limonene-1,2-epoxide [75], should provide considerable insight into the metabolism of the monoterpene epoxides and their derivatives in higher plants and tissue cultures.

CHAPTER 3

Experimental 3.1

Culture conditions

Callus cultures were initiated from stem segments of seedlings on Murashige and Skoog medium, supplemented with sucrose (3% w/v), NAA (0.5mg/l), kinetin (0.25mg/l), IBA (2.5mg/l), GA_3 (2mg/l) and agar (1% w/v). Cultures were maintained on a medium of the same composition as the initiation medium and were subcultured at 3-weekly intervals. Throughout this study, cultures were illuminated continuously (600 lux, Philips "Warm White", λ_{max} = 580nm) and the temperature of the cabinets containing the cultures were regulated at 24°C.

After several passages on the above initiation medium, callus material (of known mass) was transferred onto one of four different maintenance media containing different amounts of sucrose (3, 6, 9 and 12% w/v) but with other supplements remaining as detailed above. After three weeks the callus material was weighed, enabling growth rates to be calculated as follows:

$$\% \text{ growth rate} = \frac{\text{increase in fresh mass of callus cells over culture period}}{\text{initial mass of callus cells at start of culture period}}$$

Five culture flasks were used for each experiment (i.e. sucrose level) with the individual growth rates measured for each of these five being averaged together to give a mean growth rate.

Analysis of cultures for total anthraquinone content: At the end of the culture period and after weighing of the callus material, the pigments were extracted exhaustively with 80% aqueous ethanol. The extract was made up to a convenient volume by the addition of 80%

ethanol and a UV-VIS spectrum of the extract recorded directly or after appropriate dilution (O. D. 0.05-0.5) in the range 400-600nm. Generally $O.D._{max}$ was found at around 410nm. The amount of anthraquinones (both glycosides and aglycones will be recorded) present in these cell cultures was then determined and expressed in μmole per gram (of fresh weight) of culture cells based on the molar extinction coefficient of alizarin (ϵ = 5,500$dm^3mol^{-1}cm^{-1}$ at 434nm in 80% ethanol). Results were then averaged to give a mean pigment concentration. This procedure was repeated for each of the experiments allowing a comparison of variation in anthraquinone accumulation with sucrose level to be made.

Experimental 3.2.1

Initiation and maintenance of callus: Excised stems of *Galium verum* * (5cm in length, 5mm in diameter and with leaves and auxiliary buds removed) were sterilised with sodium hypochlorite (3% v/v) solution for five minutes, followed with five successive washes with fresh, sterile distilled water. The washed stems were cut into sections (1.5cm long), each of which was divided longitudinally and placed on an agar medium with the cut section in contact with the agar. This medium consisted of a solution of Murashige and Skoog salt mixture (ex. Flow laboratories Ltd, Irvine, Scotland), containing sucrose (3% w/v), agar (1% w/v) and supplemented with the appropriate hormones as detailed in table 3.1, prepared as recommended by Flow laboratories Ltd: the pH of the medium containing all components except the agar was adjusted to 5.5 whilst at 80% of the final volume, agar was then added and the volume made up to total with distilled water. The mixture was shaken thoroughly and transferred into conical flasks

* I should like to thank Chris Brierley of UCL botanic garden for providing seedlings of the plant.

(100ml) with 30ml of medium being added to each flask. The flasks were plugged with non-absorbent cotton wool, sealed with aluminum foil and autoclaved (120°C at 15 torr for twenty minutes). Incubation was carried out at 26°C under either continuous light (600 lux, Philips "Warm White", λ_{max} = 580nm) or photoperiod (16 hours light and 8 hours dark) conditions. Typically callus appeared within seven days and after four weeks this was transferred onto fresh media. Thereafter the cultures were subcultured at three week intervals.

Attempted extraction of pigment secreted into agar medium: The coloured portion of the spent agar medium from ten flasks (ca. 40ml) was heated on a steam bath until all the medium had liquefied. The mixture was allowed to cool to 40°C and then washed successively with petroleum spirit and chloroform, although the colouring matter remained in the aqueous layer. In an attempt to discharge the colouring matter, the aqueous layer was firstly acidified (pH 2) using dilute hydrochloric acid and then made alkaline (pH 10) using dilute sodium hydroxide solution.

Extraction of callus material: Typically, callus (20g), acid-washed sand (1g) and warm methanol (50ml) were ground together in a pestle and mortar. The methanolic extract was filtered and the residue re-extracted (2×50ml) with methanol, after which the remaining tissue was almost white. The combined methanolic extracts were then washed with hexane (2×30ml) to remove lipids and the solvent removed on the rotary evaporator.

Experimental 3.2.2

The following mobile-phase combinations were used to analyse the crude extracts using silica gel, cellulose and PEI-cellulose as stationary phases:

Toluene	Methanol	Acetic acid
100	0	0
0	100	0
100	0	2
100	0	5
90	10	0
90	10	0.5
90	10	1
85	15	2
80	20	5
75	25	0

Ethyl acetate	Pet. spirit	Acetic acid
100	0	0
50	50	0
45	55	0
40	60	0
35	65	0
35	65	1
35	65	2
35	65	5
30	70	0
30	70	1

Ethyl acetate	Methanol	Water
100	16.5	13.5
90	16.5	13.5

Toluene	Ethyl acetate	Acetic acid
75	25	1
70	30	1

Experimental 3.2.3

Acid hydrolysis of extracts: Typically, the extract (100mg) was hydrolysed with hydrochloric acid (1M, 50ml) at 85-90°C for two and a half hours. After cooling, the pigments were extracted with diethyl ether (3×50ml), the ether layer then dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* using a rotary evaporator.

Experimental 3.2.3.2

Extracts were taken up in HPLC grade methanol and filtered through a 0.2μm Millipore membrane. The solvent was then removed *in vacuo* and the dried extracts weighed accurately. The extracts were taken up in a known volume of methanol and a measured amount of this solution (typically 5μl) was then injected into the analytical reverse-phase HPLC system described in section 2.1.3. Compounds present in the extracts were eluted using a solvent gradient of 0 to

100% B over one hour, where A = water + 0.1% TFA and B = acetonitrile + 5% water + 0.1% TFA.

Experimental 3.2.5.1

Analytical TLC: Fractions obtained from any of the separation methods used were routinely analysed using the following system: toluene/ methanol/ acetic acid (90 : 10 : 0.5 v/v).

Sephadex LH-20: The combined acid-hydrolysed methanolic extract was eluted using methanol through a column packed with Sephadex LH-20 (50g) pre-swollen by placing in a beaker with methanol (250ml) overnight. The coloured bands were collected, the solvent concentrated using a rotary evaporator and the fractions reapplied to give a total of seven fractions.

Silica gel column chromatography: The mixture from the fifth Sephadex LH-20 fraction (made up in the eluent) was applied to the top of a column (250mm × 27mm (i.d.)) packed with pre-solvated silica gel (70g). The mixture was eluted using toluene/ methanol/ glacial acetic acid (90 : 10 : 0.5 v/v) at a flow rate of one drop every two seconds. Two compounds were obtained in reasonable purity (approximately 75% pure), but five remained unresolved. Unsuccessful attempts were made to separate these using the same procedure but with different solvent mixtures: toluene/ acetonitrile/ glacial acetic acid (50 : 50 : 0.5 v/v); a gradient system: toluene (200ml), toluene/ acetonitrile/ glacial acetic acid, (50 : 50 : 0.5 v/v, (200ml)); and an alternative gradient of toluene/ methanol/ glacial acetic acid, (90 : 10 : 0.5 v/v, (200ml)), toluene/ methanol/ glacial acetic acid (80 : 20 : 0.5 v/v, (200ml)). Various other solvent mixtures were used to equilibrate the column before use: toluene (100%), toluene/ acetonitrile/ glacial acetic acid (50 : 50 : 0.5 v/v).

Preparative TLC: Two solvent systems were used: (i) toluene/ methanol/ glacial acetic acid (90 : 10 : 0.5 v/v), (ii) toluene/ acetonitrile/ glacial acetic acid (50 : 50 : 0.5 v/v). Polar components (i.e. those with $R_f < 0.4$ in system (i)) were rechromatographed using this second solvent system.

Experimental 3.2.5.2

The overall purification scheme is shown in figure E1, in addition to which the following should be noted‡:

(i) Often during a preparative separation injections were made before all compounds from previous injections had eluted. Thus the resulting fractions obtained contained late running impurities which were easily removed by reinjection of the fraction in one or two injections using the same solvent system. This procedure saved considerable time.

(ii) To avoid loss of peak resolution it had been necessary to inject the sample in small amounts. Thus a typical separation required twenty to thirty injections. Such a method, where small amounts of the desired compound are collected in a large solvent volume often results in the isolation of impurities, namely plasticisers from the mobile phase and octadecylsilane from the column packing. The level of these impurities was minimised by a final purification whereby concentrated solutions of the compounds were injected (i.e. one to two injections).

(iii) The success of any separation was established by referral to a standard analytical system, namely, a gradient run of system two (0 to 100% acetonitrile over one hour).

(iv) Attempts to further purify compound VIII were halted when sample losses, due to (it was suspected) either decomposition or volatilisation were observed.

‡Points (i) and (ii) involved extra preparative separations which are not, for reasons of simplicity, detailed in the flowchart.

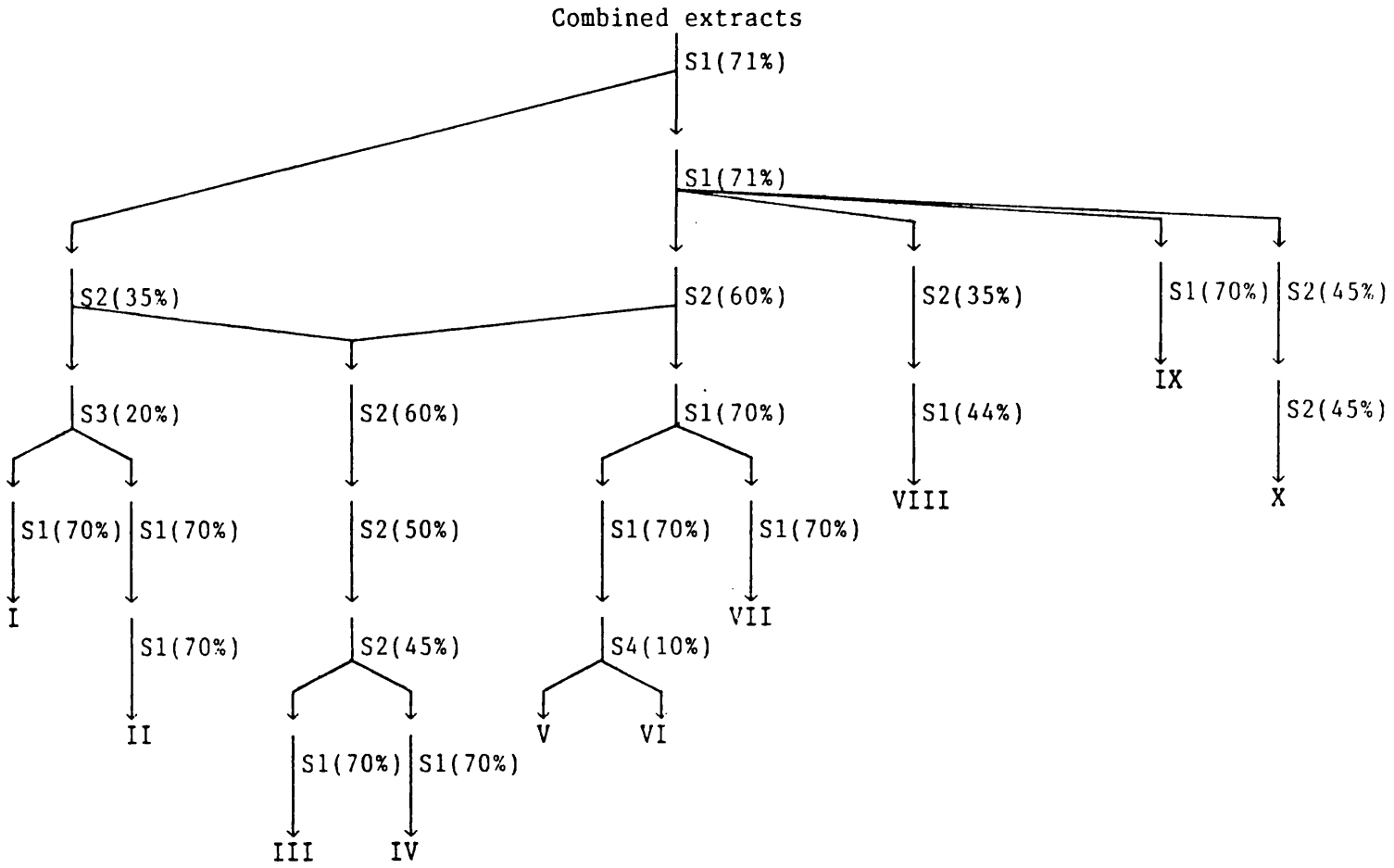


Figure E.1 Flowchart showing the HPLC purification method used

(see footnotes for details of notation used)

Footnotes to figure E1:

(a) S1, S2, S3 and S4 are solvent systems one, two, three and four respectively and are defined as follows: (i) System one (S1): solvent A = water + 0.1% (v/v) trifluoroacetic acid (TFA), solvent B = methanol + 0.1% (v/v) TFA; (ii) System two (S2): solvent A = water + 0.1% (v/v) TFA, solvent B = 95% acetonitrile + 5% water + 0.1% (v/v) TFA; (iii) System three (S3): Solvent A = hexane + 0.1% (v/v) TFA, solvent B = 75% hexane + 25% isopropyl alcohol + 0.1% (v/v) TFA; and (iv) System four (S4): Solvent A = hexane + 0.1% (v/v) TFA, solvent B = ethyl acetate + 0.1% (v/v) TFA.

Solvent mixtures of S1 and S2 were used in conjunction with reverse-phase columns, whilst those of S3 and S4 were used in conjunction with normal-phase columns.

(b) the figures appearing in brackets after the solvent system type are the proportion of the organic modifier (i.e. solvent B above). For example, "S1 (71%)" represents a solvent system comprising of 71% methanol, 29% water and 0.1% TFA.

(c) I, II, and III etc. are the purified compounds.

Experimental 3.2.6

Experimental 3.2.6.1

CI mass spectrum: m/z (relative intensity) 285 (M^+ + 1, 100), 270 (M^+ + NH_4 - MeOH, 45).

EI mass spectrum: m/z (relative intensity) 284 (M^+, 7), 269 (M^+ - CH_3, 5), 255 (M^+ - CHO, 4), 254 (M^+ - CH_2O, 27), 253 (M^+ - OCH_3, 22), 252 (M^+ - CH_3OH, 100), 224 (252 - CO, 7), 196 (224 - CO, 21), 168 (196 - CO, 9), 139 (168 - CHO, 13).

accurate mass: 284.0692 ($C_{16}H_{12}O_5$ requires 284.0685).

^{13}C NMR: ($CDCl_3$) "semi-quantitative" spectra, D1 = 6sec, decoupler

mode = NNY (i.e on during data acquisition only) δ 186.87 (C-9), 182.21 (C-10), 164.04 (C-1 or C-3), 161.84 (C-1 or C-3), 134.12 (C-6), 134.12 (C-7), 134.03 (C-4a), 133.50 (C-8a or C-10a), 133.46 (C-8a or C-10a), 127.33 (C-5 or C-8), 126.69 (C-5 or C-8), 114.32 (C-2), 109.75 (C-4), 109.60 (C-9a), 68.92 ($\underline{C}H_2OCH_3$), 59.36 ($CH_2O\underline{C}H_3$).

^{1}H NMR: ($CDCl_3$) δ 13.27 (1H, s, exchangeable with D_2O), 9.37 (1H, s, exchangeable with D_2O), 8.22-8.28 (2H, m), 7.72-7.80 (2H, m), 7.28 (1H, s), 4.91 (2H, s), 3.55 (3H, s); (C_6D_6) 13.71 (1H, s, exchangeable with D_2O), 9.37 (1H, s, exchangeable with D_2O), 8.21-8.22 (2H, m), 7.68 (1H, s), 6.95-7.01 (2H, m), 4.50 (2H, s), 2.70 (3H, s).

IR ν_{max}^{KBr}: 3180 (OH), 1670 (C=O), 1625 (C=O, chelated), 1595cm^{-1}

UV-VIS: λ_{max}^{EtOH}: 242 (sh), 246, 282, 334, 415nm; $\lambda_{max}^{EtOH/OH^-}$: 249, 268, 299 (sh), 313, 330 (sh), 502nm.

Experimental 3.2.6.2

CI mass spectrum: m/z (relative intensity) 257 (4), 256 (14), 255 (M^++ 1, 100), 241 (8).

EI mass spectrum: m/z (relative intensity) 254 (M^+, 40), 237 (M - OH, 8), 236 (M - H_2O, 37), 225 (M - CHO, 13), 211 (M - $COCH_3$, 12), 209 (237 - CO, 17), 208 (236 - CO, 100), 183 (211 - CO, 15), 180 (208 - CO, 10), 168 (13), 155 (183 - CO, 9), 152 (180 - CO, 19), 139 (25), 127 (155 - CO, 23).

accurate mass: 254.0584 ($C_{15}H_{10}O_4$ requires 254.0579).

^{1}H NMR: ($CDCl_3$) δ 8.22-8.28 (2H, m, H-8 and H-5), 8.13 (1H, d, J = 8.5Hz, H-4), 7.72-7.78 (2H, m, H-6 and H-7), 7.34 (1H, d, J = 8.5Hz, H-3), 6.65 (1H, broad singlet exchangeable with D_2O), 4.02 (3H, s, OCH_3).

UV-VIS: λ_{max}^{EtOH}: 247, 270, 284 (sh), 330, 381nm; $\lambda_{max}^{EtOH/OH^-}$: 249, 268 (sh), 315, 498nm.

Experimental 3.2.6.3

CI mass spectrum: m/z (relative intensity) 285 (M^+ + 1, 100), 255 (27%, impurity).

EI mass spectrum: m/z (relative intensity) 284 (M^+, 77), 269 (M^+ - Me, 17), 267 (M^+ - OH, 26), 266 (M^+ - H_2O, 100), 265 (57), 255 (14), 254 (19), 249 (17), 241 (53), 238 (23), 237 (73), 236 (26), 223 (16), 220 (11), 211 (21), 208 (24), 183 (18), 181 (13), 170 (58), 155 (11), 142 (15), 139 (14), 126 (23), 114 (32), 113 (29).

accurate mass: 284.0679 ($C_{16}H_{12}O_5$ requires 284.0685).

^{1}H NMR: ($CDCl_3$) δ 8.20-8.27 (2H, m), 7.71-7.78 (2H, m), 7.70 (1H, s), 4.08 (3H, s), 3.99 (3H, s); (CD_3OD) 8.15-8.23 (2H, m), 7.75-7.83 (2H, m), 7.70 (1H, s), 4.05 (3H, s), 3.91 (3H, s); (C_6D_6) 8.24-8.27 (2H, m), 7.61 (1H, s), 7.12 (2H, m), 5.80 (1H, s, which disappeared on shaking with D_2O), 3.75 (3H, s), 3.00 (3H, s).

UV-VIS: λ_{max}^{EtOH}: 226, 282, 318nm; $\lambda_{max}^{EtOH/OH^-}$: 230, 260, 319, 519nm.

Experimental 3.2.6.4

CI mass spectrum: m/z (relative intensity) 315 (M^+ + 1, 37), 300 (M^+ + NH_4 - MeOH, 42), 285 (100).

EI mass spectrum: m/z (relative intensity) 314 (M^+, 7), 299 (M^+ - CH_3, 6), 285 (M^+ - CHO, 9), 284 (M^+ - CH_2O, 67), 283 (M^+ - CH_3O, 25), 282 (M^+ - CH_3OH, 100), 256 (284 - CO, 3), 255 (283 - CO, 5), 254 (282 - CO, 6), 226 (254 - CO, 8), 198 (226 - CO, 2).

^{1}H NMR: ($CDCl_3$) δ 13.39 (1H, sharp s, exchangeable with D_2O), 9.30 (1H, broad s, exchangeable with D_2O), 8.20 (1H, d, J = 8.6Hz), 7.68 (1H, d, J = 2.7Hz), 7.28 (1H, s), 7.23 (1H, dd, J = 8.6, 2.7Hz), 4.91 (2H, s), 3.96 (3H, s), 3.55 (3H, s); (CD_3OD) δ 8.15 (1H, d, J = 8.3Hz), 7.59 (1H, d, J = 2.6Hz), 7.28 (1H, dd, J = 2.6, 8.3Hz), 7.18

(1H, s) - other signals were obscured by the intense signals of the residual methanol; (C_6D_6) δ 13.96 (1H, sharp s, exchangeable with D_2O), 9.34 (1H, sharp s, exchangeable with D_2O), 8.14 (1H, d, J = 8.6Hz), 7.75 (1H, s), 7.67 (1H, d, J = 2.7Hz), 6.77 (1H, dd, J = 2.7, 8.6Hz), 4.52 (2H, s), 3.10 (3H, s), 2.69 (3H, s).

UV-VIS: λ_{max}^{EtOH}: 275, 280 (sh), 308 (sh), 340, 430nm; $\lambda_{max}^{EtOH/OH^-}$: 262, 303 (sh), 327, 505nm.

Experimental 3.2.6.5

EI mass spectrum: m/z (relative intensity) 320 (6), 305 (17), 279 (27), 278 (100), 277 (15), 253 (6), 165 (1), 139 (2).

^{1}H NMR: ($CDCl_3$) δ 13.20 (1H, s), 8.22-8.30 (4H, m), 7.76-7.84 (4H, m), 7.35 (2H, s), 5.45 (2H, s).

UV-VIS: λ_{max}^{EtOH}: 246, 278, 335 (sh), 410nm. $\lambda_{max}^{EtOH/OH^-}$: 222, 260 (sh), 312, 348 (sh), 502nm.

Experimental 3.2.6.6

CI mass spectrum: m/z (relative intensity) 255 (M^+ + 1, 100).

EI mass spectrum: m/z (relative intensity) 254 (M^+, 100), 236 (M^+ - H_2O, 5), 226 (M^+ - CO, 10), 225 (M^+ - CHO, 10), 208 (236 - CO, 4), 197 (225 - CO, 9), 180 (208 - CO, 5), 169 (197 - CO, 4), 152, 141, 115, 105.

accurate mass: 254.0583 ($C_{15}H_{10}O_4$ requires 254.0479).

^{1}H NMR: ($CDCl_3$) δ 13.19 (1H, sharp s, exchangeable with D_2O, chelated OH), 8.22-8.30 (2H, m, H-5 and H-8), 7.74-7.80 (2H, m, H-6 and H-7), 7.27 (1H, s, H-4), 2.23 (3H, s, Me).

UV-VIS: λ_{max}^{EtOH}: 242 (sh), 245, 279, 310, 414nm; $\lambda_{max}^{EtOH/OH^-}$: 225, 300 (sh), 312, 495nm.

Experimental 3.2.6.7

CI mass spectrum: m/z (relative intensity) 255 (M^+ + 1, 100).

EI mass spectrum: m/z (relative intensity) 254 (M^+, 100), 253 (5), 252 (5), 237 (1), 226 (5), 225 (7), 198 (2), 197 (10), 181 (2), 169 (3), 152 (2), 151 (1), 149 (1), 147 (1), 141 (3), 139 (3), 127 (3), 121 (3).

accurate mass: 254.0569 ($C_{15}H_{10}O_4$ requires 254.0579).

^{1}H NMR: ($CDCl_3$) δ 13.06 (1H, s, disappeared on shaking with D_2O), 8.24 (1H, d, J = 8.4Hz), 7.71 (1H, d, J = 7.6Hz), 7.63 (1H, d, J = 2.7Hz), 7.49 (1H, broad doublet, J = 7.6Hz), 7.20 (1H, dd, J = 2.7, 8.4Hz), 2.36 (3H, s).

UV-VIS: λ_{max}^{EtOH}: 220, 247, 270, 283 (sh), 294 (sh), 388 (sh), 412, 433 (sh); $\lambda_{max}^{EtOH/OH^-}$: 216, 310, 345 (sh), 501nm.

Experimental 3.2.6.8

CI mass spectrum: m/z (relative intensity) 299 (M^+ + 1, 100), 284 (M^++ NH_4 - MeOH, 12).

EI mass spectrum: m/z (relative intensity) 298 (M^+, 21), 266 (M^+ - CH_3OH, 100), 238 (266 - CO, 33), 210 (238 - CO, 5), 182 (210 - CO, 7), 154 (182 - CO, 7), 126 (154 - CO, 20).

accurate mass: 298.0489 ($C_{16}O_{10}O_6$ requires 298.0477).

^{1}H NMR: ($CDCl_3$) δ 14.81 (1H, sharp s exchangeable with D_2O, OH), 12.64 (1H, sharp s exchangeable with D_2O, OH), 8.30-8.33 (1H, m, H-5 or H-8), 8.24-8.27 (1H, m, H-5 or H-8), 7.76-7.84 (2H, m, H-6 and H-7), 7.38 (1H, s, H-4), 4.06 (3H, s, OCH_3).

spin simulation experiment: Coupling system type = AA'BB'. Region of spectrum simulated = 3100 to 3400Hz. Estimated chemical shift values, δ = 3326.0, 3304.0, 3125.0 and 3112.0Hz (for A, A', B and B' respectively). Linewidth = 0.7Hz. Coupling constants, $J_{AA'}$ = 0.35, J_{AB}

= 7.74, $J_{AB'}$ = 1.35, $J_{A'B}$ = 1.56, $J_{A'B'}$ = 7.56 and $J_{BB'}$ = 7.58Hz.

UV-VIS: λ_{max}^{EtOH}: 246, 285, 335, 414nm; $\lambda_{max}^{EtOH/OH^-}$: 221, 273, 312 (sh), 350 (sh), 510nm.

Experimental 3.2.6.9

Isolation of caffeic acid methyl ester: This compound (retention time = 24.5 minutes in table 3.5) was isolated in one step from the acid-hydrolysed extract of the flowers of *Galium verum* by reverse phase HPLC: semi-preparative column; solvent system: S2 (25%B); flow rate: 4.9 mls/min; retention time: 12.4 minutes (see section 3.2.5.2 for explanations).

Characterisation of caffeic acid methyl ester:

The spectroscopic data for this sample were identical to those of an authentic specimen of caffeic acid methyl ester.

CI mass spectrum: m/z (relative intensity) 212 (M^+ + 18, 100), 195 (M^+ + 1, 83).

EI mass spectrum: m/z (relative intensity) 194 (M^+, 85), 163 (M^+ - OMe, 100), 145 (7), 135 (26), 134 (24).

accurate mass: 194.0564 ($C_{10}H_{10}O_4$ requires 194.0579).

^{1}H NMR: ($CDCl_3$) *trans* isomer δ 7.56 (1H, d, J = 16.0Hz), 7.06 (1H, d, J = 2.0Hz), 6.99 (1H, dd, J = 8.2, 2Hz), 6.85 (1H, d, J = 8.2Hz), 6.24 (1H, d, J = 16Hz), 3.77 (3H, s, OMe); *cis* isomer δ 7.93 (1H, d, J = 8.3Hz), 6.84 (1H, d, J = 8.3Hz), 3.86 (3H, s).

UV-VIS: λ_{max}^{EtOH}: 215 (sh), 249, 299 (sh), 330nm. $\lambda_{max}^{EtOH/OH^-}$: 221, 296, 389nm.

Characterisation of [VIII]

CI mass spectrum: m/z (relative intensity) 226 (M^+ + 18, 53), 209 (M^+ + 1, 100).

EI mass spectrum: m/z (relative intensity) 208 (M^+, 100), 193 (M^+ -

Me, 3), 177 (M^+ - OMe, 69), 149 (12), 145 (27), 134 (11), 133 (14), 117 (13).

accurate mass: 208.0723 ($C_{11}H_{12}O_4$ requires 208.0736).

^{1}H NMR: ($CDCl_3$) δ 7.60 (1H, d, J = 15.9Hz), 7.06 (1H, dd, J = 8.2, 1.9Hz), 7.01 (1H, d, J = 1.9Hz), 6.90 (1H, d, J = 8.2Hz), 6.27 (1H, d, J = 15.9Hz), 3.91 (3H, s), 3.78 (3H, s).

UV-VIS: λ_{max}^{EtOH}: 234, 299 (sh), 324nm. $\lambda_{max}^{EtOH/OH^-}$: 250, 300, 309, 379nm.

Experimental 3.2.6.10

CI mass spectrum: m/z (relative intensity) 269 (16), 255 (M^+ + 1, 90), 254 (M^+, 49), 239 (100), 225 (18).

EI mass spectrum: m/z (relative intensity) 254 (M^+, 89), 236 (M^+ - H_2O, 17), 226 (254 - CO, 22), 225 (254 - CHO, 100), 208 (236 - CO, 19), 207 (80), 197 (226 - CHO, 7), 180 (208 - CO, 14), 152 (180 - CO, 39), 151 (180 - CHO, 22), 139 (14).

accurate mass: 254.0582 ($C_{15}H_{10}O_4$ requires 254.0579).

^{1}H NMR: ($CDCl_3$) δ 13.03 (1H, s, chelated OH, disappeared on shaking with D_2O), 8.28-8.32 (2H, m, H-5 and H-8), 7.74-7.86 (4H, m, H-3, H-4, H-6 and H-7), 5.40 (1H, broad s, $CH_2\underline{OH}$, disappeared on shaking with D_2O), 4.85 (2H, s, $C\underline{H}_2OH$); (C_6D_6) δ 13.19 (1H, s), 8.16-8.19 (1H, m), 8.03-8.06 (1H, m), 7.83 (1H, d, J = 7.8Hz), 7.36 (1H, d, J = 7.8Hz), 6.99-7.03 (2H, m), 4.54 (2H, s).

UV-VIS: λ_{max}^{EtOH}: 224, 245 (sh), 254, 280 (sh), 326, 407nm; $\lambda_{max}^{EtOH/OH^-}$: 222, 250, 272 (sh), 311, 504nm.

Chapter 6

Experimental 6.1.1.1 (a)

Isoprene (25g, 0.37mol) and methyl vinyl ketone (24g, 0.34mol) were placed in a dry Carius tube together with hydroquinone (1g). The tube was sealed under nitrogen and placed in a furnace at 140°C for 3 hours. The resulting adduct was fractionally distilled at reduced pressure to give one major fraction (40.3g, 85.8% yield; 68-70°C at 5mmHg).

Analysis of distillate:

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) 1 spot R_f= 0.61

GLC: (i) (see table 5.1(i)): 1 peak R_t= 5.15 minutes (> 95% of total area); (ii) (see table 5.1(v)): R_t= 16.96 (74.7% of total area), 18.72 minutes (25.3%).

^{1}H NMR: ($CDCl_3$) major component: δ 5.39 (1H, s), 2.53 (1H, m), 2.18 (3H, s), 2.16-1.92 (4H, m), 1.66 (3H, s), 1.6 (2H, m). minor component: δ 5.39 (1H, s,), 2.61 (1H, m), 2.19 (3H, s,), 2.16-1.92 (4H, m), 1.68 (3H, s), 1.49 (2H, m).

^{13}C NMR (APT): ($CDCl_3$) δ 211.93 (C=O), 211.73 (C=O), 133.81 (quaternary carbon), 132.39 (quaternary carbon), 120.59 (CH), 119.21 (CH), 47.82 (CH), 47.20 (CH), 31.37 (CH_2), 29.46 (CH_2), 27.99 (CH_3), 27.98 (CH_3), 27.01 (CH_2), 24.86 (CH_2), 24.78 (CH_2), 24.45 (CH_2), 23.57 (CH_3), 23.39 (CH_3).

IR ν_{max}^{liq}: 3400 (w, C=O overtone), 3010 (m, alkene C-H str), 1710 (s, C=O str), 1675 (w, C=C str), 1440 (m, CH_3CO group), 1375 (m, CH_3CO group), 1355 (m, CH_3CO group), 1165 $cm.^{-1}$

EI mass spectrum: m/z (relative intensity) 138 (M^+, 32), 123 (M^+ - CH_3, 23), 105 (7), 95 (M^+ - CH_3CO, 100), 79 (22), 77 (14), 67 (41), 55 (25), 53 (15), 43 (CH_3CO^+, 96).

accurate mass: 138.1039 ($C_9H_{14}O$ requires 138.1044)

microanalysis: Calculated for $C_9H_{14}O$: C, 78.21%; H, 10.21%. Found: C, 78.12%; H, 9.54%.

Experimental 6.1.1.1 (b)

(+)-Limonene oxide (11.22g, 73.7mmol) in dry dichloromethane (400ml) was ozonised as described elsewhere for β-pinene (see 7.1.1). Owing to time limitations the reaction had to be halted prematurely. The solution containing the ozonide was carefully reduced to about 50ml in volume (rotary evaporator, room temperature) and then added dropwise with stirring to a cooled suspension of zinc (29.8g, 455.9mmol), anhydrous sodium acetate (6.8g, 82.8mmol) and sodium iodide (20.4g, 136.1mmol) in glacial acetic acid (75ml). This mixture was stirred overnight (17 hours), filtered to remove zinc residues and these residues washed with acetic acid and dichloromethane. The combined filtrates were washed successively with water, dilute sodium hydroxide solution and brine, dried (anhydrous $MgSO_4$), filtered and the solvent removed to give an oil (7.90g).

Purification of Oil: Column chromatography (400×40mm, silica gel 63-200μm, eluent: 90% pet. spirit (60-80°C)/ 10% ethyl acetate) yielded two major fractions: (i) 2.29g (limonene) (ii) the desired ketone [101](3.11g, 39.5% yield based on reacted epoxide).

Analysis of fraction (ii):

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) R_f= 0.58 (1 spot).

GLC: (i) (see table 5.1(v)): one peak, R_t= 16.96 minutes (>95% by area); (ii) (see table 5.1(i)): one peak, R_t= 5.12 minutes.

^{1}H NMR: ($CDCl_3$) δ 5.39 (1H, s), 2.53 (1H, m), 2.18 (3H, s), 2.16-1.92 (4H, m), 1.66 (3H, s), 1.6 (2H, m).

^{13}C (APT) NMR: ($CDCl_3$) δ 211.92 (C=O), 133.81 (quaternary carbon), 119.22 (CH), 47.21 (CH), 29.47 (CH_2), 27.98 (CH_3), 27.02 (CH_2), 24.87

(CH_2), 23.40 (CH_3).

EI mass spectrum: m/z (relative intensity) 138 (M^+, 69), 123 (M^+ - CH_3, 50), 105 (17), 95 (M^+ - CH_3CO, 90), 93 (22), 79 (32), 77 (21), 67 (58), 55 (39), 53 (21), 43 (CH_3CO^+, 100).

accurate mass: 138.1048 ($C_9H_{14}O$ requires 138.1044).

Experimental 6.1.1.2

Preparation of methyltriphenylphosphonium iodide: Methyl iodide (3.36g, 23.6mmol) in sodium-dried benzene (5ml) was added at room temperature to a solution of triphenylphosphine (4.00g, 15.2mmol) in dry benzene (20ml) contained in a dry ground glass conical flask (50ml) over 30 minutes. The solution turned cloudy and a white precipitate formed. The reaction vessel was stoppered and left overnight in a desiccator containing phosphorus pentoxide. The phosphonium salt liberated was washed thoroughly with dry benzene and left in an oven at 100°C for 2 days to yield a white solid (4.48g, 73% based on triphenylphosphine, m.p. 189°C).

Wittig reaction: (All apparatus was thoroughly dried prior to use either in an oven or desiccator). A 3-necked flask (10ml, fitted with a serum cap, central stopper and containing a magnetic stirrer) and a reflux condenser were assembled and connected via a T-piece to a supply of dry nitrogen (dried by bubbling through conc. H_2SO_4, and then through a tube containing self-indicating silica gel) and a bubbler. The apparatus was flushed through with dry nitrogen for 30 minutes, after which sodium hydride (0.10g, 4.16mmol) was introduced via the central neck. The apparatus was flushed through with dry nitrogen for a further 20 minutes with the central neck being open and the nitrogen supply being directed away from the bubbler. After the central stopper had been replaced and the nitrogen flow redirected,

dry DMSO (2ml, dried by standing overnight over calcium sulphate, followed by distillation at reduced atmosphere from calcium hydride) was injected via the serum cap. The mixture was stirred at 70°C until all the sodium hydride had dissolved (about 1 hour). The solution was cooled in ice-cold water whilst methyltriphenylphosphonium iodide (0.67g, 1.66mmol) dissolved in dry DMSO (2ml) was added dropwise with constant stirring. After stirring at room temperature for a further 20 minutes, 4-acetyl-1-methylcyclohex-1-ene (0.23g, 1.67mmol) in dry THF (1ml) was added to the reaction mixture over a period of 10 minutes. This mixture was stirred for 3 hours at 55°C ± 5°C, after which the mixture was cooled, poured onto crushed ice (15g) and extracted with petroleum (40-60°C, 4x10ml). The combined extracts were dried over anhydrous magnesium sulphate and the solvent removed by rotary evaporation to give a yellow oil (0.19g).

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) 1 spot R_f= 0.59

GLC: (see table 5.1(i)) 1 peak R_t= 5.12 minutes (> 97% of total area, starting material).

Preparation of methyltriphenylphosphonium bromide: Silver nitrate 1.28g, 7.53mmol) was dissolved in water (2ml) and added dropwise with constant shaking to a solution of methyltriphenylphosphonium iodide (2.82g. 6.98mmol) in methanol (25ml). A yellow precipitate of silver iodide had finished forming after 5 minutes of shaking. This mixture was filtered and the filtrate collected. This filtrate was then shaken with a solution of potassium bromide (0.89g, 7.48mmol) in water (1ml). The white precipitate of potassium nitrate was then removed by filtration and the filtrate taken to dryness on a rotary evaporator. The crude product was recrystallised from ethanol, with complete precipitation being achieved by addition of the product to diethyl ether (200ml). The product was filtered, washed with ether and dried

in an oven at 100°C for 2 days to give methyltriphenylphosphonium bromide (1.02g, 2.86mmol, 41% yield, m.p. 231°C, Lit. 232-233°C).

Wittig reaction using methyltriphenylphosphonium bromide: This reaction was carried out using the same techniques and apparatus as described above for the analogous iodide reagent, but using the following quantities: sodium hydride (0.08g, 3.33mmol), methyltriphenylphosphonium bromide (0.5g, 1.4mmol) and 4-acetyl-1-methylcyclohex-1-ene (0.24g, 1.74mmol). This gave 0.15g of a yellow oil.

Analysis of oil:

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) R_f= 1.0, 0.59.

GLC: (see table 5.1(i)) R_t= 3.50 (3% by area, limonene) and 5.23 minutes (96%, starting material).

Experimental 6.1.1.3

Solid *m*-CPBA (0.89g, 4.4mmol (based on the 85% purity of the commercial peracid)) was slowly added in small portions to a magnetically-stirred ice-cooled mixture of limonene (0.60g, 4.4mmol) in dichloromethane (20ml) and aqueous sodium bicarbonate (5% w/v, 10ml). The mixture was stirred at 0°C for 2 hours after which no starting material remained (as established by TLC). The two phases were separated, the organic phase washed successively with cold sodium hydroxide solution (10% w/v), brine, water and then dried (Na_2SO_4). The solvent was removed and the oil purified by column chromatography (silica gel, 95% pet. spirit 60-80°C/ 5% ethyl acetate) to give the epoxide as an oil (0.54g, 80.3% yield).

Analysis of oil:

TLC: (90% pet. spirit 60-80°C/ 10% ethyl acetate) one spot R_f= 0.57

GLC: (see table 5.1(iii)) R_t= 3.42 (50.2% of total area, *cis* isomer), 3.53 minutes (45.3%, *trans* isomer).

Experimental 6.1.2.1 (a)

Magnesium turnings (0.15g, 6.17mmol) were washed with diethyl ether to remove any grease and dried in an oven at 100°C. These turnings were then placed in a 10ml 3-necked flask, which had been fitted with a stopper, reflux condenser, septum inlet adaptor, septum and magnetic stirrer and which had been attached to the manifold as shown in figure E2. The apparatus was then flushed out with argon (taps C, E, G and H open, and all others closed) dried by passing through the calcium chloride column A. This procedure served primarily to remove moisture from between the column and the tap C. After 20 minutes the flow of dry argon was halted. The apparatus was then evacuated (to ≃ 0.02mmHg) with taps G, H and I open and all others closed. Methyl iodide (0.88g, 6.20mmol) in dry diethyl ether (3ml) was placed in a 10ml ground-glass test tube and attached to the manifold as shown. The test tube was then cooled in liquid nitrogen and when the contents had frozen tap F was opened. After a few minutes when an adequate vacuum had been reobtained (≃ 0.02mmHg) tap F was closed, the liquid nitrogen trap removed and the contents of the test tube liquefied by warming with a hot air blower. The solution was refrozen, tap F reopened and the apparatus evacuated again. This procedure was repeated until the solution had been degassed, then with tap I closed the liquid nitrogen trap was removed from its position cooling the contents of the test tube and placed around the bottom of the 3-necked flask. In this way the methyl iodide and diethyl ether were transferred from the test tube into the 3-necked flask. When transfer was complete (i.e. the Pirani vacuum gauge had returned to its previous value) an ice-cooled water flow to the condenser was started. Tap B was opened and, with a supply of dry argon maintained (as seen by observing the bubbler K), tap C (which had been scored with a

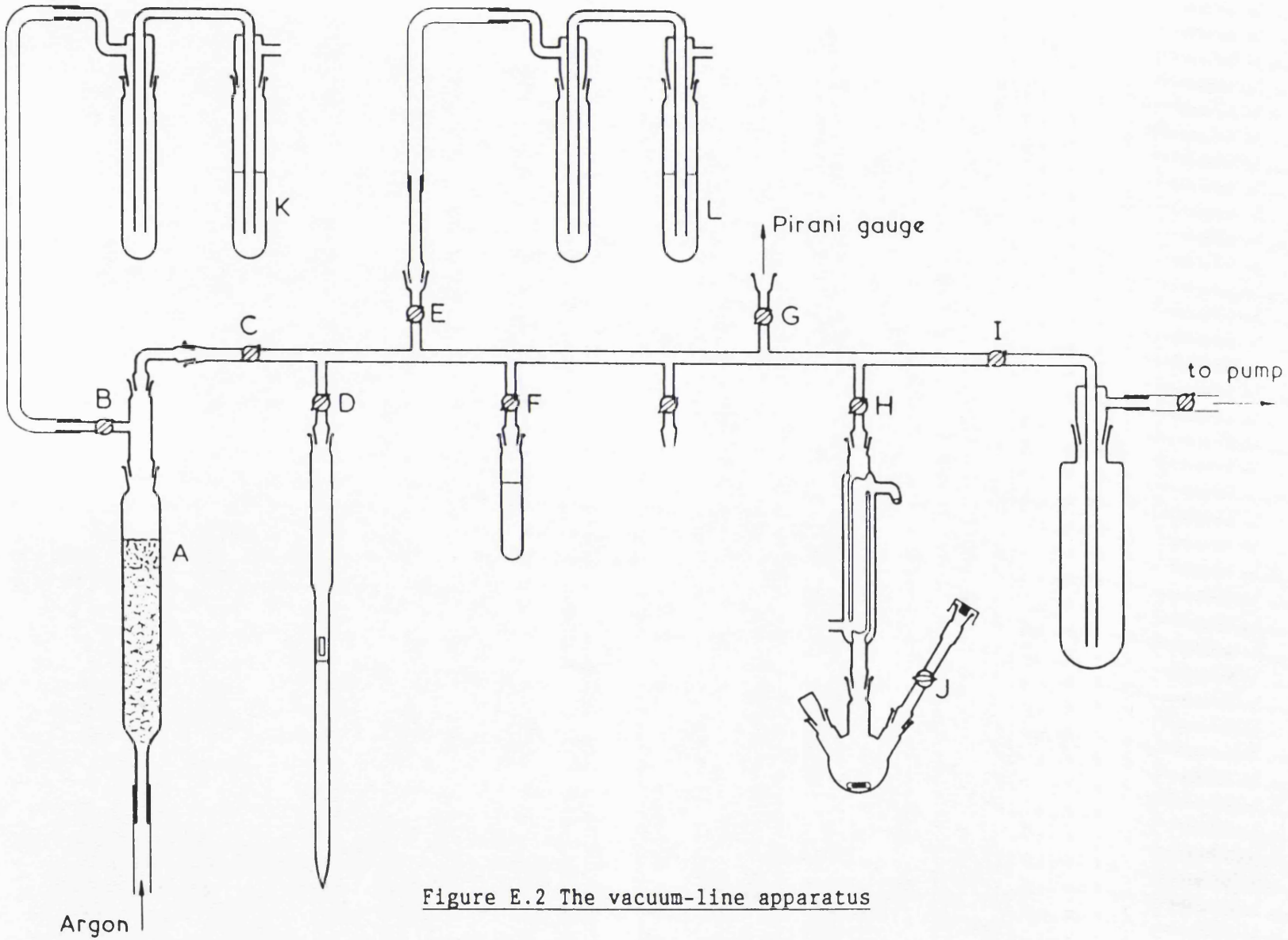


Figure E.2 The vacuum-line apparatus

glasscutter's knife) was turned slowly to admit dry argon into the vacuum apparatus. Tap E was then opened, tap B closed, and the flow of argon adjusted (as seen by observing the bubbler L) to maintain the apparatus under a small positive pressure of argon. The mixture in the 3-necked flask was then gently warmed by a water bath to initiate the formation of the Grignard reagent. The mixture was gently refluxed for 1 hour until all the magnesium had dissolved and then the solution was cooled in ice. Then, with tap J open, 4-acetyl-1-methylcyclohex-1-ene [101] (0.8559g, 6.2mmol) in dry diethyl ether (2ml) was slowly added by syringe, inserting the needle through the subaseal, past tap J, into the reaction vessel. After all of the ketone had been added, tap J was closed and the solution refluxed. After an hour, the solution was allowed to cool, and then poured into a 75ml separating funnel containing an ice-cold saturated solution of ammonium chloride. The 3-necked flask was rinsed with diethyl ether (2x5ml) and these washings added to the contents of the separating funnel. The mixture was then shaken well to decompose the Grignard complex. The organic layer was separated and the aqueous layer washed with more diethyl ether (2×10ml). The combined organic layers were washed once with 5% sodium bicarbonate solution (10ml), water (10ml) and dried (Na_2SO_4). The solution was filtered and the solvent removed to give an oil (0.7236g).

TLC: 90% pet. spirit 60-80°C/ 10% ethyl acetate) one spot R_f= 0.20

GLC: (see table 5.1(iv)) R_t= 2.76 (1.3% of total area), 9.09 (1.1%, starting material), 14.20 minutes (97.6%, α-terpineol).

Experimental 6.1.2.1 (b)

A trial Barbier synthesis of α-terpineol was made using the same set-up and apparatus as already described for the Grignard synthesis

(see section 6.1.2.1(a)), but with the exclusion of a magnetic follower. Magnesium (0.16g, 6.58mmol) and methyl iodide (0.93g, 6.55mmol) were placed in the 3-necked flask in the same way as for the Grignard reaction. The ketone [101](0.90g, 6.52mmol) was, however, added to the contents of the reaction vessel immediately after the admission of argon into the vacuum apparatus. When the diethyl ether and methyl iodide had melted, the contents of the reaction vessel were ultrasonically irradiated for 20 minutes using an ultrasonic cleaning bath (60W, 50KHz). After the usual work up and purification procedure (see section 6.1.2.1 (a)) α-terpineol was isolated as an oil (0.55g, 54.3% yield).

TLC: (90% pet. spirit 60-80°C/ 10% ethyl acetate) one spot R_f= 0.20

GLC: (see table 5.1(iv)) R_t= 14.24 minutes (98.6%, α-terpineol).

Experimental 6.1.2.2 (a)

(All apparatus was thoroughly dried prior to use either in an oven or desiccator). Magnesium (0.69g, 28.4mmol) and dry diethyl ether (3ml) were placed in a 3-necked flask (20ml, containing a magnetic follower, fitted with a dry-ice/acetone condenser and dropping funnel, and central stopper) via the central stopper. The reaction was carried out under nitrogen (dried by bubbling through sulphuric acid) supplied via a T-piece attached to the top of the condenser. The contents of the flask, cooled by immersion in an ice-bath, were stirred magnetically, whilst a solution of methyl iodide (3.56g, 25.1mmol) in dry diethyl ether (5ml) was added dropwise from the dropping funnel. The mixture was refluxed gently for one hour after which most of the magnesium had dissolved. To the cooled solution of this Grignard reagent α-terpineol (1.08g, 7.1mmol) in dry diethyl ether (5ml) was added. The resulting mixture was stirred at room temperature for four days and then

decomposed with dilute hydrochloric acid, added to ice and extracted with diethyl ether. This solution was dried (K_2CO_3) and the solvent removed to give the crude product (0.70g).

GLC: (see table 5.1(viii)) R_t= 9.0 (4% of total area), 43.2 minutes (95%, α-terpineol).

From 4-acetyl-1-methylcyclohex-1-ene: The apparatus and method used were the same as for the reaction using α-terpineol above. The following quantities were used: magnesium (0.71g, 29.2mmol), methyl iodide (4.29g, 30.2mmol) and 4-acetyl-1-methylcyclohex-1-ene (1.09g, 7.9mmol). After stirring for 4 days at room temperature, the mixture was worked-up as above to give the crude product (1.01g).

GLC: (see table 5.1(viii)) R_t= 43.2 minutes (> 95% of total area, α-terpineol)

Experimental 6.1.2.2 (b)

Neutral alumina (1.0g) was placed in a 2-neck 10ml flask. To this was added pyridine (0.08g) and α-terpineol (0.56g, 3.6mmol). After heating at 220°C (oil bath) for 6 hours the products were extracted with diethyl ether. This organic extract was washed with dilute hydrochloric acid, dilute sodium bicarbonate solution and water, dried (potassium carbonate) and the solvent removed to leave a brown residue (0.30g).

GLC: (see table 5.1(viii)) R_t= 7.14 (6% of total area, limonene), 9.83 (6%), 43.2 minutes (80%, α-terpineol).

Experimental 6.1.2.2 (c)

α-Terpineol (1.83g, 11.9mmol) was dissolved in dry pyridine (10ml) and placed in a 3-neck flask which had been fitted with a reflux condenser (protected by a calcium chloride drying tube),

stopper and serum cap. The mixture was cooled to 0°C (ice-bath) whilst thionyl chloride (3.0g, 25.2mmol) was added by syringe via the serum cap. The mixture was stirred at 0°C for 45 minutes after which small pieces of ice were added to decompose any excess of thionyl chloride. Diethyl ether was added and the organic layer extracted with dilute hydrochloric acid, sodium bicarbonate and water, dried (magnesium sulphate) and the solvent removed to give a brown residue (0.78g).

GLC: (see table 5.1(viii)) 7.14 (57% of total peak area, limonene), 9.38 minutes (43%, pyridine).

Experimental 6.1.3.1

Acetyl chloride/ pyridine method: α-Terpineol (0.69g, 4.5mmol) was dissolved in dry pyridine (5ml) and placed in a 10ml 3-necked flask fitted with a stopper, subaseal, double surface condenser and magnetic stirrer. Dry acetyl chloride (0.37g, 4.7mmol) was slowly added to this cooled/stirred solution via a syringe. This cooled mixture was stirred for a further 3 hours after which the resulting brown solution was transferred to a separating funnel containing ether (30ml). The organic phase was washed with dilute hydrochloric acid (0.2M, 3×10ml), water (1×10ml), and dried (Na_2SO_4). The solvent was removed to give a crude oil (0.45g, 51% yield).

Analysis of Oil:

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) R_f= 0.65 (terpinyl acetate), 0.21 (α-terpineol).

GLC: (see table 5.1(iii)) R_t= 6.80 (92.4% by area, α-terpineol), 6.95 minutes (terpinyl acetate, 6.3%).

Ac_2O / DMAP / TEA method: α-Terpineol (0.49g, 3.2mmol) was dissolved in dry TEA (3ml) and placed in a 3-necked (5ml) flask fitted with a stopper, subaseal, double surface condenser and magnetic stirrer. To

this cooled and stirred solution was added dry acetic anhydride (0.75g, 7.35mmol) dropwise, via a syringe, followed by DMAP (0.04g, 0.33mmol) dissolved in TEA (2ml). This mixture was stirred at 40°C for 24 hours. Methanol (5ml) was then added, and, after 30 minutes of stirring, the reaction mixture was concentrated at the rotary evaporator. The residue was taken up in ether (30ml), and this ether solution washed with dilute hydrochloric acid (2×10ml), dilute sodium bicarbonate (5%, 10ml), brine (10ml) and dried ($MgSO_4$) to give a light brown oil (0.53g, 84.5%).

Analysis of Oil:

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) 1 spot R_f= 0.65 (terpinyl acetate).

GLC: (see table 5.1(iii)) R_t= 6.80 (0.6% by area, α-terpineol), 6.97 minutes (93.4%, terpinyl acetate).

Experimental 6.1.3.2

Design of Pyrolysis column: The column consisted of a pyrex tube (18×180mm) fitted with B14 joints and containing glass helixes (3mm diameter) held in place by a glass wool plug. A chromel-alumel thermocouple was attached to the outer wall and connected directly to a multimeter giving a display in millivolts. Using conversion tables this was converted to a temperature reading. This set-up did not contain a reference junction and hence there was a small error in values thus obtained (not greater than 5°C). Nichrome wire was wrapped around the central 130mm long portion of the tube. This heating element was insulated with KAO® ceramic fibre the whole being encapsulated in a glass tube (150×50mm) sealed at either end with a temperature resilient resin. Note: It is important that the heating coil is not too close to the glass joints if a good seal is to be

maintained. A drawing of the apparatus is shown in figure E3.

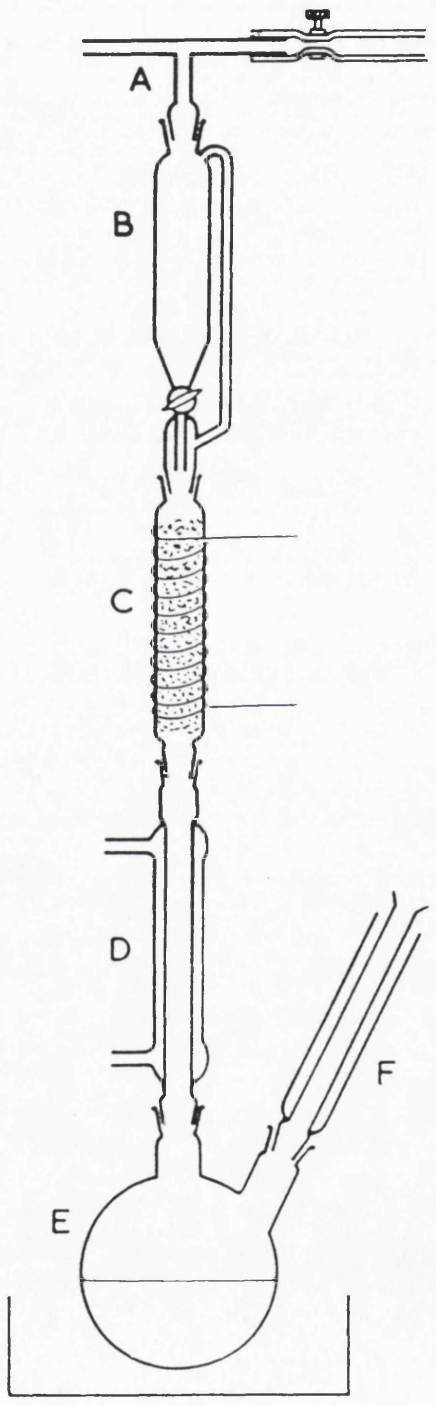
Exclusion of oxygen from apparatus: Within the operating temperature used the presence of oxygen might lead to undesired side reactions and hence the reaction was carried out under argon which had been passed through an alkaline solution of pyrogallol.

Typical procedure: α-Terpinyl acetate (0.398g, 2.03mmol) in hexane (5ml) was placed in the pressure-equalising separating funnel B. The whole apparatus was flushed through for 30 minutes with dry and oxygen-free argon supplied via the T-piece A. The pyrolysis column was then switched on and allowed to equilibrate to approximately 425°C with the stream of argon being maintained. The acetate solution was then added at the rate of one drop every 2 seconds, and swept through the column by the argon flow. The pyrolysate was trapped in flask E which was cooled in a dry-ice/acetone bath with the dry-ice/acetone condenser F, providing a secondary trap. TLC analysis indicated that after one pass through the heated column, no unreacted starting material was remaining. Hence, the column was allowed to cool and washed with a little hexane. The pyrolysate was then washed successively with water, sodium bicarbonate solution and again with water, dried over magnesium sulphate and the solvent removed. The oil obtained was purified by column chromatography to give a colourless oil (0.152g).

Purification of Oil: Column chromatography (90×20mm; silica gel, 63-200μm; eluent: 90% pet. spirit (60-80°C)/ 10% ethyl acetate) yielded the product as a mixture with terpinolene.

GLC: (see table 5.1(ii)) R_t= 4.04 (68.6% by area, limonene), 5.65 minutes (17%, terpinolene).

Figure E.3 The pyrolysis apparatus



Experimental 6.2

After the diethyl ether/ methyl iodide solution contained in the test tube had been degassed, the sealed ampoule containing the ^{14}C-methyl iodide was attached to the vacuum apparatus as shown in figure E2 with a small iron ball carefully positioned above the magnetic break-seal. The portion of the ampoule below this seal was then cooled in liquid nitrogen. Then, with taps D, G, H and I open, and all others closed, the apparatus was evacuated to a high vacuum (0.02mmHg). The seal was then broken using the iron ball and a magnet. Tap I was then closed, tap F opened and thus (with the assistance of an hot air blower) the contents of the test tube were transferred into the lower portion of the ampoule. The contents of the ampoule were then degassed using a similar procedure to that which had been used for the contents of the test tube. When this was complete, the liquid nitrogen trap was removed from its position cooling the contents of the ampoule into the bottom of the 3-necked flask. The rest of the procedure was then carried out as for the non-radioactive "dry run".

Purification of ^{14}C-limonene oxide: Column chromatography (90×20mm; silica gel, 5g, 70-200μm; eluent: 95% pet. spirit (60-80°C)/ 5% ethyl acetate) yielded the epoxide (103.7mg), which was purified further by passage through a second column (90×20mm; silica gel, 63-200μm; eluent: 97% pet. spirit (60-80°C)/ 3% ethyl acetate) to give an oil (66.9mg, 1.91μCi/mmol).

Experimental 6.3.1

Feeding of epoxide to cuttings of *Pelargonium fragrans*: Cuttings of *P. fragrans* were taken under water with a scalpel and shortened pipette tips placed over the stems. The function of the tips being to provide tight seals around the base of the stems. The stems were inverted,

removed from the water and supported by a clamp stand. In this way each stem contained a small reservoir of water. No reduction in the height of these reservoirs was noticed over ten minutes. The leaves were illuminated with a 60W lamp (at a distance of about 30cm) whilst a stream of air was blown across them. This did cause the levels to drop, indicating adequate flow of fluid through the xylem vessels. The epoxide (7μl, 85mCi) in methanol (40μl) was pipetted into the reservoirs and the forced transpiration continued. When the reservoirs were almost empty a little water was added. After a while the stems were righted, placed in a small water vessel and kept in a fume cupboard (continuous light) for 60 hours.

Extraction of plant material: After 60 hours the cuttings (4.17g) were ground into a powder with liquid nitrogen, placed in a conical flask (25ml) together with diethyl ether (10ml). This mixture was sonicated (10 minutes), the solution dried ($MgSO_4$) and filtered into a pear-shaped flask (10ml). This flask was immersed in an ice bath and the volume of liquid reduced to about 100μl using a stream of dry nitrogen.

Purification of extract: Prep TLC: silica gel 60Å, 10x20cm, 250μm layer thickness; eluent: 90% pet spirit (60-80°C fraction), 10% ethyl acetate. Temp. = 20°C. The extract was applied as a narrow band about 6cm long.

Counting of material separated on TLC plates: After elution, TLC plates were scraped in 4mm wide portions (the width of the spatula used) and each portion was transferred to a scintillation vial insert containing Ecoscint "O" scintillation fluid (4ml, ex. National Diagnostics, Aylesbury, Bucks). A background vial was prepared by scraping an equal portion of the plate at its edge. i.e. where solvent alone had run. Inserts were capped, placed in vials and then counted

for 50 minutes each on the Packard Tricarb model 3255 scintillation counter. The resulting sets of counts were entered into a computer program to give quench and background corrected values (see section 5.3.1). After exclusion of any abnormal values a mean count rate for each vial was determined.

Chapter 7

Experimental 7.1.1

Preparation of nopinone: The output from an ozoniser was bubbled through a cooled (-50°C, acetone/ dry ice) solution of β-pinene (19.99g, 147mmol) in dry dichloromethane (400ml) contained in a 3-neck flask. The outlet from this flask was bubbled into a solution of potassium iodide (5% in aqueous acetic acid). After 9 hours all of the β-pinene appeared to have reacted (the potassium iodide solution had turned brown and the solution in the reaction vessel had taken on a pale blue colour). The ozoniser was switched off, the solution purged with oxygen for 30 minutes and then allowed to warm to room temperature. The solution was reduced to about 50ml (rotary evaporator at room temperature) and added dropwise to a mechanically stirred cooled mixture of zinc (59.6g), sodium acetate (13.6g), sodium iodide (41g) in glacial acetic acid (150ml). This mixture was stirred overnight, filtered to remove zinc residues and these residues were washed with acetic acid and dichloromethane. The combined filtrates were washed with water, sodium hydroxide solution and brine, dried ($MgSO_4$) and the solvent removed (rotary evaporator) to give a yellow oil (22.09g) which was distilled at reduced pressure. The major distillation fraction (12.3g; 55°C, 2mmHg) was then purified by column chromatography (silica gel; eluent: 90% pet. spirit (60-80°C)/ 10% ethyl acetate) to give pure nopinone [110] (8.75g, 43% yield).

Analysis of [110]:

TLC: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) 1 spot, R_f= 0.49

^{1}H NMR: ($CDCl_3$) δ 2.6-1.8 (8H, m), 1.29 (3H, s), 0.81 (3H, s).

^{13}C NMR (APT): ($CDCl_3$) δ 215.0 (C=O), 57.9 (CH), 41.2 (quaternary carbon), 40.3 (CH), 32.8 (CH_2), 25.9 (CH_3), 25.2 (CH_2), 22.1 (CH_3), 21.4 (CH_2).

IR ν_{max}^{liq}: 1705 (s, C=O str), 1200 cm^{-1} (m).

Pyrolysis of Nopinone: This reaction used a similar apparatus to that for the pyrolysis of terpinyl acetate (see section 6.1.3.2). It did not, however, contain a built-in thermocouple but instead had to be calibrated somewhat unsatisfactorily using a digital thermometer probe.

Nopinone (3.50g) in hexane (20ml) was placed in the dropping funnel. The apparatus was flushed through with dry, oxygen-free, nitrogen for 30 minutes after which the solution was passed through the column (temperature 550°C) with the aid of the nitrogen gas in 45 minutes. The yellow liquid which had been collected in the trap was washed with water, dried ($MgSO_4$) and the solvent removed *in vacuo* to give a yellow liquid (2.40g).

TLC analysis of pyrolysate: (90% pet. spirit (60-80°C)/ 10% ethyl acetate) R_f= 0.85, 0.77, 0.69, 0.60, 0.52, 0.38 (major spot, unreacted nopinone).

Purification of pyrolysate: The crude reaction mixture was purified initially by column chromatography (silica gel; eluent: 90% pet. spirit (60-80°C)/ 10% ethyl acetate) to give: (i) a mixture containing the products (0.1g) and (ii) unreacted nopinone (1.73g).

Purification of products by HPLC: semi-preparative column; flow rate 4.9mls/min; eluent: 90% hexane/ 10% ethyl acetate.

Analysis of [109]:

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) 1 spot, R_f= 0.43

HPLC: (95% hexane/ 5% ethyl acetate) R_t= 6.39 (93.6% by area) 7.51 (2.4%), 9.08 minutes (2.3%).

^{1}H NMR: ($CDCl_3$) δ 6.35 (1H, ddd, J = 17.7, 10.5, 0.85Hz), 6.23 (1H, dd, J = 17.7, 1.1Hz), 5.82 (1H, dd, J = 10.5, 1.1Hz), 5.10 (1H, broad t, J ≃ 7Hz), 2.62 (2H, t, J = 7.6Hz), 2.30 (2H, m (apparent quartet), J ≃ 7.3Hz), 1.68 (3H, s), 1.62 (3H, s).

^{13}C (APT) NMR: ($CDCl_3$) δ 200.6 (C=O), 136.5 (CH), 132.7 (quaternary carbon), 128.0 (CH_2), 122.7 (CH), 39.6 (CH_2), 25.6 (CH_3), 22.6 (CH_2), 17.6 (CH_3).

EI mass spectrum: m/z (relative intensity) 138 (M^+, 14), 123 (M^+ - CH_3, 23), 95 (43), 83 (28), 82 (18), 70 (36), 69 (37), 68 (17), 67 (25), 55 (100).

Analysis of [112a]:

HPLC: (95% hexane/ 5% ethyl acetate) R_t= 4.49 (82.3% by area), 4.29 minutes (6.2%, *trans* isomer).

^{1}H NMR: ($CDCl_3$) δ 4.84 (1H, m), 4.68 (1H, m), 2.83 (1H, broad quartet, J = 7.5Hz), 2.5-1.8 (5H, m), 1.71 (3H, m), 0.89 (3H, d, J = 7.5Hz).

^{13}C (APT) NMR: ($CDCl_3$) δ 221.3 (quaternary carbon), 144.3 (quaternary carbon), 111.5 (CH_2), 47.0 (CH), 45.5 (CH), 37.0 (CH_2), 23.8 (CH_2), 22.6 (CH_3), 10.2 (CH_3).

Analysis of [112b]:

HPLC: (95% hexane/ 5% ethyl acetate) One major peak, R_t= 4.28 minutes (87.5% by area).

^{1}H NMR: ($CDCl_3$) δ 4.86 (1H, m), 4.82 (1H, m), 2.5-2.0 (5H, m), 1.76 (3H, t, J = 1.2Hz), 1.70 (1H, m), 1.04 (3H, d, J = 6.8Hz).

^{13}C (APT) NMR: ($CDCl_3$) δ 220.3 (quaternary carbon), 145.0 (quaternary carbon), 111.4 (CH_2), 52.3 (CH), 47.7 (CH), 37.3 (CH_2), 26.5 (CH_2),

19.3 (CH_3), 12.3 (CH_3).

Experimental 7.1.2

Preparation of acetal [117]: Orthophosphoric acid (88% solution, 3 drops) was added to a mixture of 3-methylbut-1-en-3-ol (49g, 0.57mol) and ethyl vinyl ether (85g, 1.18mol). The mixture was refluxed (double surface condenser) for 18 hours, cooled, diethyl ether (200ml) added, and this solution washed successively with dilute (5%) sodium bicarbonate solution (2×75ml), brine (2×50ml) and water (2×50ml). The solution was dried (anhydrous $MgSO_4$) and the solvent removed *in vacuo* to give a pale orange oil. This was distilled to give the acetal (52.9g, [117]) as a clear liquid (bp 55°C, 15mmHg).

Analysis of [117]:

TLC: (95% pet. spirit (60-80°C)/ 5% ethyl acetate) 1 spot, R_f= 0.60

HPLC: (95% hexane/ 5% ethyl acetate) 1 major peak, R_t= 7.35 minutes (92.5% of total area).

^{1}H NMR: ($CDCl_3$) δ 5.91 (1H, dd, J = 10.8, 17.6Hz), 5.14 (1H, dd, J = 1.2, 17.6Hz), 5.10 (1H, dd, J = 1.2, 10.8Hz), 4.74 (1H, q, J = 5.3Hz), 3.54 (1H, dq, J = 9.0, 7.1Hz), 3.44 (1H, dq, J = 9.0, 7.1Hz), 1.34 (3H, s), 1.30 (3H, s), 1.27 (3H, d, J = 5.3Hz), 1.17 (3H, t, J = 7.1Hz).

^{13}C (APT) NMR: ($CDCl_3$) δ 144.2 (CH), 113.4 (CH_2), 94.9 (CH), 75.6 (quaternary C), 59.3 (CH_2), 27.3 (CH_3), 26.3 (CH_3), 22.0 (CH_3), 15.4 (CH_3).

IR ν_{max}^{liq}: 3090 (m, alkene C-H str), 1640 (w, C=C str), 1260, 1215, 1150, 1120, 1085, 1060, 1030, 975, 925 cm^{-1}

EI mass spectrum: m/z (relative intensity) 143 (M^+- CH_3, 0.5), 99 (6), 73 (85), 71 (12), 70 (9), 69 (100).

accurate mass: 143.1072 ($C_8H_{15}O_2$ formed by loss of CH_3 from the

molecular ion requires 143.1071).

Preparation of 5-methyl-4-hexenal [114]: Orthophosphoric acid (88% solution, 3 drops) was added to the acetal, (52.1g, [117]) and the mixture heated in a sealed tube (sealed under argon) for 30 minutes at 150°C. The contents of the tube were then worked up (as for the acetal synthesis above) to yield an orange oil (22.78g) which was purified by reduced pressure distillation (4mmHg) to give 3 fractions (i) 5.10g [114], (ii) 4.00g [119] (iii) 6.65g [120].

Analysis of [114]:

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) 1 spot, R_f = 0.44

HPLC: (95% hexane/ 5% ethyl acetate) 1 major peak, R_t= 8.17 minutes (84% of total area).

^{1}H NMR: ($CDCl_3$) δ 9.76 (1H, t, J = 1.7Hz), 5.09 (1H, t of sept, J = 7.2, 1.4Hz), 2.46 (2H, td, J = 6.87, 1.7Hz), 2.32 (2H, m (apparent quartet), J ≃ 7Hz), 1.69 (3H, s), 1.63 (3H, s).

^{13}C (APT) NMR: ($CDCl_3$) δ 202.7 (C=O), 133.2 (quaternary), 122.0 (CH), 43.9 (CH_2), 25.6 (CH_3), 20.8 (CH_2), 17.6 (CH_3).

IR ν_{max}^{liq}: 3440 (w, C=O overtone), 2725 (m, C-H str from aldehydic proton), 1725 (s, C=O str), 1630 cm^{-1}(w, C=C str).

EI mass spectrum: m/z (relative intensity) 112 (M^+, 20), 97 ($M^+ - CH_3$, 31), 94 ($M^+ - H_2O$, 59), 69 (99), 67 (17), 57 (36), 56 (100), 55 (77), 53 (25).

Analysis of [119]:

TLC: (i) 1% ethyl acetate/ 99% pet. spirit (60-80°C): R_f= 0.14 (minor), 0.08 (major); (ii) 5% ethyl acetate/ 95% pet. spirit (60-80°C): R_f= 0.93 (minor), 0.83 (major).

GLC: (See table 5.1.(ii)) R_t= 8.25 (90.3% by area), 18.20 (0.9%), 20.06 (2.4%), 23.68 minutes (4.0%).

^{1}H NMR: ($CDCl_3$) δ 5.11 (1H, t of sept, J = 7.2, 1Hz), 4.47 (1H, t, J = 5.76Hz), 3.64 (2H, dq, J = 9.4, 7Hz), 3.49 (2H, dq, J = 9.4, 7Hz), 2.05 (2H, m (apparent quartet), J $\simeq$ 7.6Hz), 1.68 (3H, d, J $\simeq$ 1Hz), 1.64 (2H, dt, J = 7.8, 5.75Hz), 1.61 (3H, d, J $\simeq$ 1Hz), 1.2 (6H, t, J = 7.05Hz),

^{13}C (APT): ($CDCl_3$) δ 131.9 (quaternary C), 123.8 (CH), 102.5 (CH), 60.9 (CH_2), 33.6 (CH_2), 25.6 (CH_3), 23.4 (CH_2), 17.6 (CH_3), 15.3 (CH_3).

IR ν_{max}^{liq}: 1730 (w, C=C str), 1690 (w, C=C str), 1130, 1065, 980cm.$^{-1}$

EI mass spectrum: m/z (relative intensity) 186 (M^+, 0.4), 141 (15), 140 (27), 125 (17) 103 (27), 97 (14), 75 (42), 69 (100), 55 (22).

Analysis of [120]:

TLC: (98% pet. spirit (60-80°C)/ 2% ethyl acetate) 1 spot, R_f= 0.36

^{1}H NMR: ($CDCl_3$) δ 9.62 (1H, d, J = 2.0Hz), 5.07 (2H, t of sept, J = 7.2, 1.5Hz), 2.1-2.4 (5H, m), 1.69 (6H, broad s), 1.61 (6H, broad s).

^{13}C (APT) NMR: ($CDCl_3$) δ 205.3 (aldehydic carbon), 133.9 (quaternary carbon), 120.7 (CH) 52.7 (CH), 27.1 (CH_2), 25.8 (CH_3), 17.8 (CH_3)

IR ν_{max}^{liq}: 3430 (w, C=O overtone), 2730 (m, C-H str from aldehydic proton), 1725 (s, C=O str), 1675 cm^{-1} (m, C=C str).

EI mass spectrum: m/z (relative intensity) 180 (M^+, 15), 165 (M^+ - CH_3, 15), 125 (22), 112 (39), 111 (76), 97 (40), 95 (59), 81 (73), 69 (94), 55 (69).

Preparation of 7-methyl-1,6-octadien-3-ol [118]: 5-methyl-4-hexen-1-al [114] (4.88g, 44mmol) in dry THF was added dropwise over 30 minutes to a stirred ice-cooled solution of vinyl magnesium bromide in dry THF (52ml of a 1M solution, i.e. 52mmol ex. Aldrich). This reaction mixture was stirred at room temperature overnight and then an ice-cold solution of ammonium chloride (25ml) was added slowly. The organic

layer was separated and the aqueous layer extracted with ether (3x50ml). The combined THF-ether extract was dried (anhydrous Na_2SO_4) and the solvent removed *in vacuo* to give the allylic alcohol (6.39g), [118], as a pale yellow oil. This alcohol was not purified further but used immediately in the oxidation reaction using Jones' reagent.

Analysis of [118]:

TLC: (i) 95% pet spirit (60-80°C)/ 5% ethyl acetate (R_f of [114] = 0.44): R_f= 0.55 (minor), 0.15 (major); (ii) 90% pet. spirit (60-80°C)/ 10% ethyl acetate: R_f= 0.84 (minor), 0.45 (major).

HPLC: (95% hexane/ 5% ethyl acetate) R_t= 6.03 (3.9% by area), 6.36 (1%), 6.87 (0.6%), 7.96 (0.5%), 12.03 (0.23%), 13.86 (10.4%), 21.64 (82.6%), 25.74 minutes (0.7%).

^{1}H NMR: ($CDCl_3$) δ 5.88 (1H, ddd, J $\simeq$ 16.9, 10.4, 6.2Hz), 5.23 (1H, dt, J $\simeq$ 17, 1.5Hz), 5.13 (1H, t of sept, J $\simeq$ 7.2, 1.5Hz), 5.11 (1H, dt, J $\simeq$ 10.3, 1.5Hz), 4.11 (1H, broad m), 2.08 (2H, m (apparent quartet), J $\simeq$ 7Hz), 1.69 (3H, s), 1.62 (3H, s), 1.54-1.59 (3H, m).

^{13}C NMR: ($CDCl_3$) δ 141.2, 132.2, 123.9, 114.6, 72.9, 37.0, 25.7, 24.0, 17.7

IR ν_{max}^{liq}: 3350 (s, broad O-H str), 3090 (w, alkene C-H str), 1675 (w, C=C str), 1645 (w, $CH=CH_2$ str), 1115 (m), 1055 (m, C-OH str), 990 and 920 (m, $CH=CH_2$), 835 cm^{-1} (w, triply substituted double bond).

EI mass spectrum: m/z (relative intensity) 125 (M^+ - CH_3, 3), 122 (M^+ - H_2O, 5), 107 (23), 83 (33), 79 (73), 70 (45), 69 (59), 67 (24), 57 (54), 55 (100).

accurate mass: 125.0949 ($C_8H_{13}O$ fragment formed by loss of methyl group from molecular ion requires 125.0966); 122.1089 (C_9H_{14} fragment formed by loss of H_2O from molecular ion requires 125.0966).

Oxidation of 7-methyl-1,6-octadien-3-ol [118]: Jones' reagent* (12.0ml) was added in small portions at a time to an ice-cold solution of the alcohol (6.24g) in acetone (50ml). The reaction mixture was stirred for 30 minutes, diluted with brine (50ml) and extracted with diethyl ether (2×25ml). The ethereal phase was washed with dilute (5%) sodium bicarbonate solution (25ml), dried (Na_2SO_4) and the solvent removed *in vacuo* to give a dark yellow oil (3.14g). This was purified by column chromatography (silica gel; eluent: 95% pet. spirit (60-80°C)/ 5% ethyl acetate) to give an oil (1.36g).

(*Jones' reagent was prepared as follows: Water (77ml) was diluted with concentrated sulphuric acid (23ml) and to this manually stirred solution was slowly added chromium trioxide (26.72g) to form a red coloured clear solution).

Analysis of oxidation product:

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) 1 spot, R_f= 0.43 (R_f of alcohol [118] = 0.15).

HPLC: (95% hexane/ 5% ethyl acetate) R_t= 7.69 (94.9% by area), 10.16 minutes (2.8%).

This product was identical in its chromatographic properties with compound [109] formed from the pyrolysis of nopinone (section 7.1.1).

Experimental 7.2

This reaction was performed using the same apparatus and procedure as already described in section 6.1.2. The following quantities were used: ketone [109] (0.300g, 2.17mmol), iodomethane (0.312g, 2.2mmol) and magnesium (0.053g, 2.2mmol). A yellow oil (0.296g) was obtained.

Analysis of oil:

GLC: (see table 5.1(ii)) R_t= 11.70 (13.66% of total area, 1,4-adduct),

15.08 (61.80%, linalool), 33.02 (3.17%), 38.06 (1.35%), 38.54 minutes (3.47%).

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) R_f= 0.72 (minor), 0.29 (major, linalool).

Purification of oil: The crude reaction mixture was purified by column chromatography (silica gel, 40-63μm; eluent: 97% pet. spirit (60-80°C)/ 3% ethyl acetate) to give two major fractions: (i) the 1,4-addition product (0.029g) and (ii) linalool [113](0.174g).

Analysis of [113]:

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) 1 spot, R_f= 0.27

GC/IR/MS: R_t= 23.94 minutes (94.3% by area, identical to an authentic sample of linalool).

Analysis of [121]:

TLC: (95% pet spirit (60-80°C)/ 5% ethyl acetate) 1 spot R_f = 0.70

GC/MS/IR: R_t= 26.89 minutes (58.4% of total area).

^{1}H NMR: ($CDCl_3$) δ 5.04 (1H, t of septet, J = 7.2, 1.4Hz), 2.39 (2H, t, J = 7.2Hz), 2.35 (2H, t, J = 7.4Hz), 2.22 (2H, m(apparent quartet), J = 7.2Hz), 1.65 (3H, s), 1.59 (3H, s), 1.57 (2H, m(apparent sextet), J = 7.3Hz), 0.89 (3H, t, J = 7.4Hz).

^{13}C (APT) NMR: ($CDCl_3$) δ 211.1 (quaternary carbon), 132.6 (quaternary carbon), 122.8 (CH), 44.8 (CH_2), 42.8 (CH_2), 25.7 (CH_3); 22.5 (CH_2), 17.6 (CH_3), 17.3 (CH_2), 13.8 (CH_3).

EI mass spectrum: m/z (relative intensity) 154 (M^+, 26), 139 (M^+ - CH_3, 14), 111 (39), 99 (12), 83 (15), 71 (79), 69 (100), 58 (33), 55 (38).

IR ν_{max}^{vap}: 2972 (s), 1727 (s, C=O str), 1446 (w), 1363 (w), 1274 (w), 1124 (w), 1070 (w), 1002 (w), 824 cm^{-1} (w) .

Experimental 7.3

Epoxidation using m-chloroperoxybenzoic acid

Method I: *m*-Chloroperoxybenzoic acid (*m*CPBA, 0.66g, 3.25mmol*) was added in small portions with cooling (-5°C) to a solution of linalool (0.50g, 3.24mmol) in dry diethyl ether (15ml). After stirring for 2 hours at 0°C, the reaction mixture was washed with water, 10% sodium hydroxide solution, saturated sodium chloride solution and dried (Na_2SO_4). The oil obtained was purified by column chromatography (silica gel; eluent: 70% pentane : 30% diethyl ether) to give a colourless oil (0.21g).

(*based on the 85% purity of the commercially available material).

Analysis of oil:

GLC: (see table 5.1(iii)) R_t= 3.29 (54.1% of total area), 3.54 minutes (42.1%).

This oil was identical in its chromatographic and spectroscopic properties with an authentic mixture of the *cis* and *trans* furanoid isomers of linalool oxide.

Method II: *m*CPBA (0.71g, 3.50mmol*) was slowly added in small portions to a magnetically stirred mixture of linalool (0.54g, 3.50mmol) in dichloromethane (30ml) and sodium bicarbonate solution (0.5M, 10ml). After stirring at room temperature for 2 hours, dichloromethane (30ml) was added to the mixture and the organic phase separated. This was washed with 1M sodium hydroxide solution, water, dried (Na_2SO_4) and the solvent removed to give an oil (0.42g).

(*based on the 85% purity of the commercially available material).

Analysis of oil:

TLC: (80% pet spirit (60-80°C)/ 20% ethyl acetate) R_f = 0.51, 0.41, 0.37, 0.23 (major spot).

GLC: (see table 5.1(iii)) R_t= 3.29 and 3.54 (4.97% and 6.01% of total area respectively, *cis* and *trans* furanoid isomers of linalool oxide), 4.18 (6.76%), 7.77 and 8.32 (1.39% and 1.55% respectively, *cis* and *trans* pyranoid isomers of linalool oxide), 9.23 (35.81%), 9.47 (31.70%), 11.95 minutes (8.54%).

^{1}H NMR: ($CDCl_3$) δ 5.89 (1H, dd, J = 10.7, 17.4Hz, $C_2H=C_1$ of one diastereoisomer), 5.87 (1H, dd, J = 10.8, 17.3Hz, $C_2H=C_1$ of other diastereoisomer), 5.22 (1H, dd, J = 1.3, 17.3Hz, $C_2=C_1H_{trans}$ of one diastereoisomer), 5.21 (1H, dd, J = 1.2, 17.4Hz, $C_2=C_1H_{trans}$ of other diastereoisomer), 5.06 (1H, dd, J $\simeq$ 1.4, 10.8Hz, $C_2=C_1H_{cis}$ of one diastereoisomer), 5.05 (1H, dd, J $\simeq$ 1.4, 10.7Hz, $C_2=C_1H_{cis}$ of other diastereoisomer), 2.74 (1H, broad t, CHO), 1.8-1.5 (4H, m, $2CH_2$), 1.31 (3H, s, CH_3), 1.30 (3H, s, CH_3), 1.27 (3H, s, CH_3).

Epoxidation using "benzeneperoxyseleninic acid"

A stirred solution of benzeneseleninic acid (454mg, 2.4mmol) in methanol (4ml) was treated with hydrogen peroxide (544μl of a 15% solution, 2.4mmol). After 5 minutes, phosphate buffer (1.5ml, pH7†) was added, followed by the addition of linalool (0.31g, 2.0mmol) in methanol (2ml). The reaction was quenched after 20 minutes by the addition of silica gel (10g). After 60 minutes the silica gel was washed with a mixture of diethyl ether and hexane (1 : 1, 80ml). The solvent was then removed to give a yellow oil (0.35g).

(†prepared as detailed in "Data for Biochemical Research", edited by R. M. C. Dawson, D. C. Elliot and K. M. Jones, Clarendon Press, (1969), second edition).

Analysis of oil:

GLC: (see table 5.1(iii)) R_t= 3.27 and 3.53 (36.45% and 32.13% of total area respectively, *cis* and *trans* furanoid isomers of linalool

oxide), 5.86 (1.2%), 7.75 and 8.31 (11.83% and 14.56% respectively, *cis* and *trans* pyranoid isomers of linalool oxide), 15.80 minutes (1.60%).

GC-MS analysis confirmed that this oil was composed almost essentially of the four isomers of linalool oxide (*cis* and *trans* furanoid and *cis* and *trans* pyranoid structures).

Experimental 7.4

Crude epoxidation product (0.19g, 1.12mmol, prepared as in method II above) in diethyl ether (20ml) was stirred together with p-toluenesulphonic acid (10mg) for 1 hour at room temperature, after which the reaction mixture was washed with saturated sodium bicarbonate solution, saturated sodium chloride solution, dried (Na_2SO_4) and the solvent removed to give a pale yellow oil (0.16g).

Analysis of oil:

GLC: (see table 5.1(iii)) R_t= 3.26 and 3.52 (40.4% and 39.1% of total area respectively, *cis* and *trans* furanoid isomers of linalool oxide), 5.84 (1.5%), 7.73 and 8.28 (8.4% and 5.1% respectively, *cis* and *trans* pyranoid isomers of linalool oxide), 13.83 minutes (2.60%).

GC-MS analysis confirmed that this oil was composed almost essentially of the four isomers of linalool oxide (*cis* and *trans* furanoid and *cis* and *trans* pyranoid structures).

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