BIOSYNTHETIC STUDIES OF NATURAL PLANT PRODUCTS

The Biosynthesis of Petasin

in <u>Petasites hybridus</u> (L) Compositae

A Thesis submitted to the University of Glasgow

for the Degree of Ph.D.

by

Robert Anthony Butler Keates

September 1970

ProQuest Number: 11011945

ProQuest 11011945

ACKNOWLEDGEMENTS

Department of Chemistry, University of Glasgow,

September 1970

CONTENTS

4   BIOCHEMISTRY AND METABOLISM OF MEVALONIC ACID IN
P. HYBRIDUS

CONVENTIONS AND NOMENCLATURE

In the text that follows, numbers appearing as a superscript, followed by a single bracket thus $^{103)}$ denote references, while those written in line with the text, between paired brackets thus (34) indicate drawings of chemical formulae, and may be referred to in the text as 'structure (34)'. In drawings of structure, stereochemistry is not implied unless specifically indicated. A thickened or broken bond denotes a substituent located respectively above or below the plane of the paper. A wavy bond indicates an epimeric mixture. A structure previously drawn without stereochemical detail, e.g. (34), is written with a suffix (34A) when stereochemistry is assigned. Isotopic labelling patterns in a structure are similarly distinguished from the unlabelled compound; thus 3R-mevalonic acid (6), $(2-^{14}C-3R)$ mevalonic acid (6A) and $(2-^{14}C-3R,4R-4-^{3}H)$ mevalonic acid (6B).

A conflicting system of identification appears, largely in the second chapter, where peaks on a chromatogram have been tabulated 1-40: these appear in the text as, for example 'peak 18'. Following characterisation by GC-MS and chromatographic isolation, these are then referred to as 'compound 18', etc.

1.1   GENERAL INTRODUCTION AND SUMMARY OF THE THESIS

In recent years, the study of natural products has undergone a shift in emphasis from the classical, purely structural type of chemical investigation to consideration of the relationship of such compounds with the organism of origin. The widespread application of modern chromatographic and spectroscopic techniques has considerably simplified the isolation and characterisation of these compounds, permitting increasing attention to turn to wider biological issues as well as purely chemical aspects. Among these are the examination of biosynthetic processes and pathways, and the solution of mechanistic problems.[1,2] The investigation of biosynthesis has led to a greater understanding of the metabolic significance of many natural products, and to consideration of their biological function[3]. In addition, the application of chemical characters to taxonomic[4] and phylogenetic[5] systematics has provided a valuable new source of evidence for the relationships between taxa.

This thesis describes an investigation into the biosynthesis of the sesquiterpenoid, petasin (1), isolated from the plant _Petasites hybridus_ (L) Compositae. the structure of which was originally determined by Aebi and

(1)

(2)

13
11
12
6 15 14
7 4
B 5 A 3
8 2
9 10 1

(3)

(3A)

OH

(4)

(5)

OPP

OPP = Pyrophosphate

(6)

OH
HOCH₂ CO₂H

● = ¹⁴C

(1A)

OAng
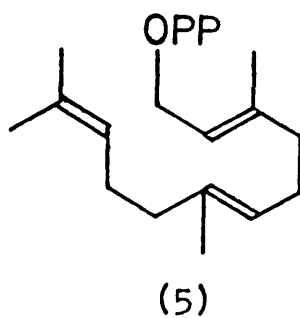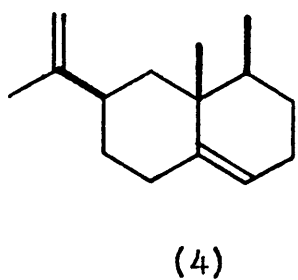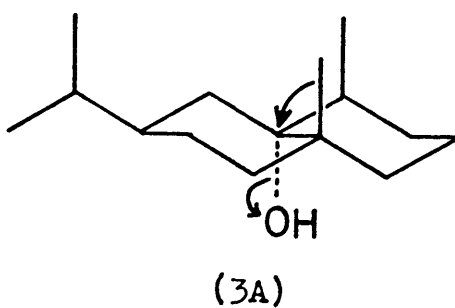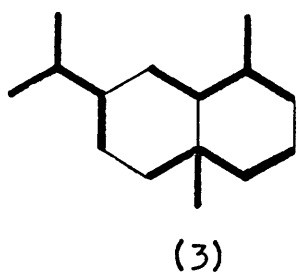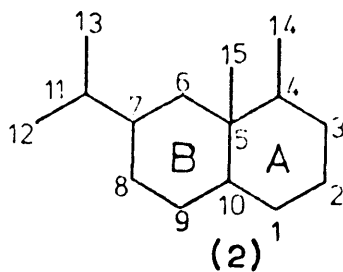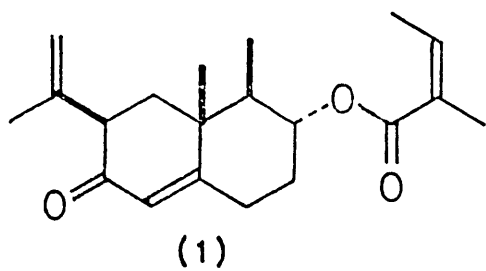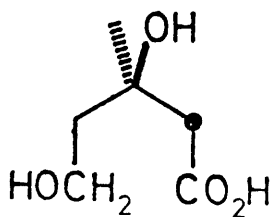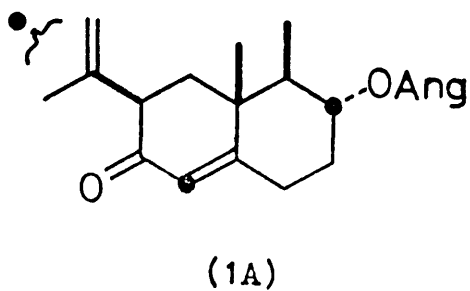
Waaler[6] . The work was initiated as part of a long-term project by Dr. G.H. Draffan and Dr. J.A. Zabkiewicz, formerly of this department. Their preliminary findings have already been described[7] .

Petasin has been chosen for this study because it possesses the eremophilane (2) carbon skeleton, which cannot be directly derived by the Biogenetic Isoprene Rule proposed by Ruzicka[8] . It has been suggested[9,10] that eremophilanes are derived from the eudesmane (3) skeleton by a methyl shift across the ring junction (3A). Another factor influencing the choice of petasin has been the easy availability of P.hybridus in the wild state. Finally, the chemotaxonomic studies of the Czech group of Šorm[11,12] into various Petasites species have provided valuable background information on the chemistry of the sesquiterpenoids of this genus.

Chemical studies of P. hybridus leaves from a number of sources thoughout Britain have demonstrated the consistent nature of the sesquiterpenoid constituents, the petasin esters being the principal components in each case. A more detailed examination, using the highly sensitive techniques of gas-liquid chromatography (GLC) and gas chromatography coupled to mass spectrometry (GC-MS), has revealed a number of other, non-esterified sesquiterpenoids, in particular, the hydrocarbon eremophilene (4)[13,14] .

Many of these compounds may be significant as intermediates
in the biosynthetic pathway. Using the available evidence of
the sesquiterpenoid constituents, coupled with chemotaxonomic
information, a rationalisation has been made of the probable
biosynthetic pathway leading to petasin. This places
eremophilene as the first fully cyclised component of the
sequence. Stereoelectronic considerations of the cyclisation
of trans,trans-farnesyl pyrophosphate (5) to eremophilene
have led to a detailed proposition for the mechanism of
these reactions, and for the mode of action of the enzymes
responsible. This approach may also be applied to the
biosynthesis of other classes of terpenoids, and is consistent
with currently available experimental results. A series of
conventional biochemical reactions is then postulated to
derive the oxidised products, including petasin.

Radioactive tracer methods have been employed in
this investigation of petasin biosynthesis. $(2-^{14}C)$Mevalonic
acid (6A) was fed to growing leaves of P.hybridus via a
cotton wick inserted through the petiole. Radioactive petasin
was isolated, and selective chemical degradation was carried
out to determine the location of the labelled carbon atoms.
Radioactivity was found in petasin as shown in structure (1A)
confirming the origin of the eremophilane skeleton from

mevalonic acid in the manner originally proposed by Robinson[9].
However, ^{14}C-labelling on its own gives no information as to
the mechanism of cyclisation, and the crucial point concerning
the origin of the methyl group at C-15 of eremophilane remains
unanswered.

The main difficulty encountered in this work
concerns the ineffective penetration of externally fed
mevalonic acid to the site of synthesis in the plant. The
level of incorporation (0.003-0.03% of total radioactivity)
might appear to imply that mevalonic acid was not directly
involved in the biosynthesis, but for the specific location
of the label in the molecule of petasin. This type of result
is consistent with work covering other isoprenoid classes
from higher plants[15,16]. Only the phytosterols show
significant levels of incorporation. Experiments with
P. hybridus have shown that labelled mevalonic acid passes
from the point of application up the petiole to the leaf.
Distribution is apparently effective within the leaf, with
rapid incorporation into sterols and water-soluble compounds.
Failure of the labelled mevalonate to reach the site of
petasin biosynthesis is apparently an intracellular effect,
possibly due to compartmentation, similar to the proposal
of Rogers, Shah and Goodwin[17]. $^{14}CO_2$ is readily and rapidly
incorporated into petasin-type esters, implying that synthesis

of these compounds is actually taking place under the
conditions of the investigation.

The final section of this thesis deals with the
application of liquid-gel chromatography to the analysis
and separation of mixtures of lipophilic compounds. This
aspect of the work was undertaken to fulfil a need for highly
effective separation and purification procedures, in view
of the low level of incorporation of radioactivity from
labelled mevalonic acid into petasin esters. This follows
the development by Ellingboe, Nyström and Sjövall[18] of a
chemical modification of 'Sephadex' dextran gel by covalent
attachment of a long chain alkyl group. The modified gel,
unlike the commercially available product, is suitable for
'use in non-polar organic solvents. An examination in detail
of the chromatographic behaviour of model compounds has
been made, covering both 'straight-phase' and 'reversed-
phase' partition, and gel filtration (where polarity
differences in sample molecules do not contribute to the
separation and elution is in order of decreasing molecular
size). The practical application of these techniques to
the separation of terpenoids in plant extracts is described.
Experimental details cover the preparation of gels for
maximum efficiency, the regeneration of columns for repeated

use, and the use of the Haahti-Sjövall flame ionisation
detector for monitoring column effluents.

(1) R =   -OAng
(Angelate)

(7A) R =

(7B) R =
CH₃
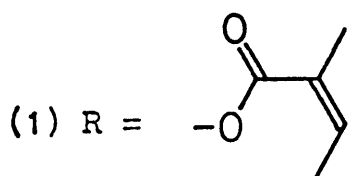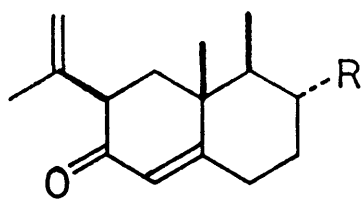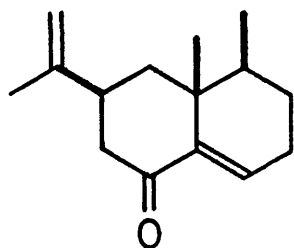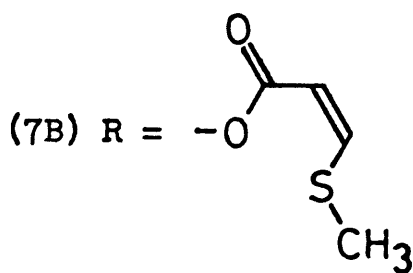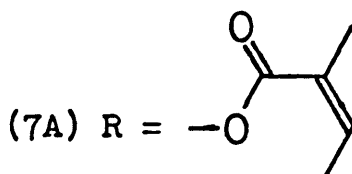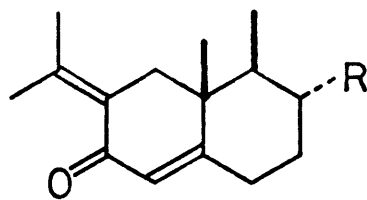
(8)
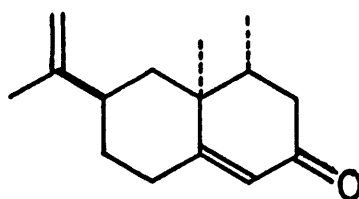
(9)

2   CHEMICAL STUDIES OF P.HYBRIDUS CONSTITUENTS

2.1.1   Introduction and Historical Background

The sesquiterpenoid ester, petasin (1), was first
isolated in the course of pharmacological investigations
in the laboratory of Professor Büchi[19] in Zurich. The
structure of petasin was determined by Aebi and Waaler[6]
and absolute configuration assigned by Aebi and Djerassi[20,21].
These workers found that petasin co-occurred in the plant
Petasites hybridus with isopetasin (7A) and S-isopetasin (7B).
Isomerisation of petasin to isopetasin was found to be very
facile, traces of acid or base catalysing an allylic
rearrangement, with migration of a double bond into
conjugation. Petasin was found to possess anti-convulsant
properties[19] : however, pharmacological activity was lost
on isomerisation to isopetasin, and the difficulty encountered
in storage of petasin diminished its potential for drug use.

Petasites hybridus was the second species found
to contain an eremophilane-type sesquiterpenoid, following
the original discovery of eremophilone (8) and related
compounds in Eremophila Mitchelli (Myoporaceae) by Penfold
and Simonsen[22,23]. Subsequently, a large number of other
compounds having this carbon skeleton, either in the normal
stereochemistry of eremophilone (8), or the partly antipodal
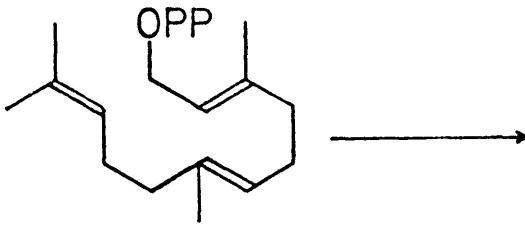
OPP = Pyrophosphate
(5A)

(10)

(11)

(14)

(2)

(12)

(15)

(13)

(16)

(17)
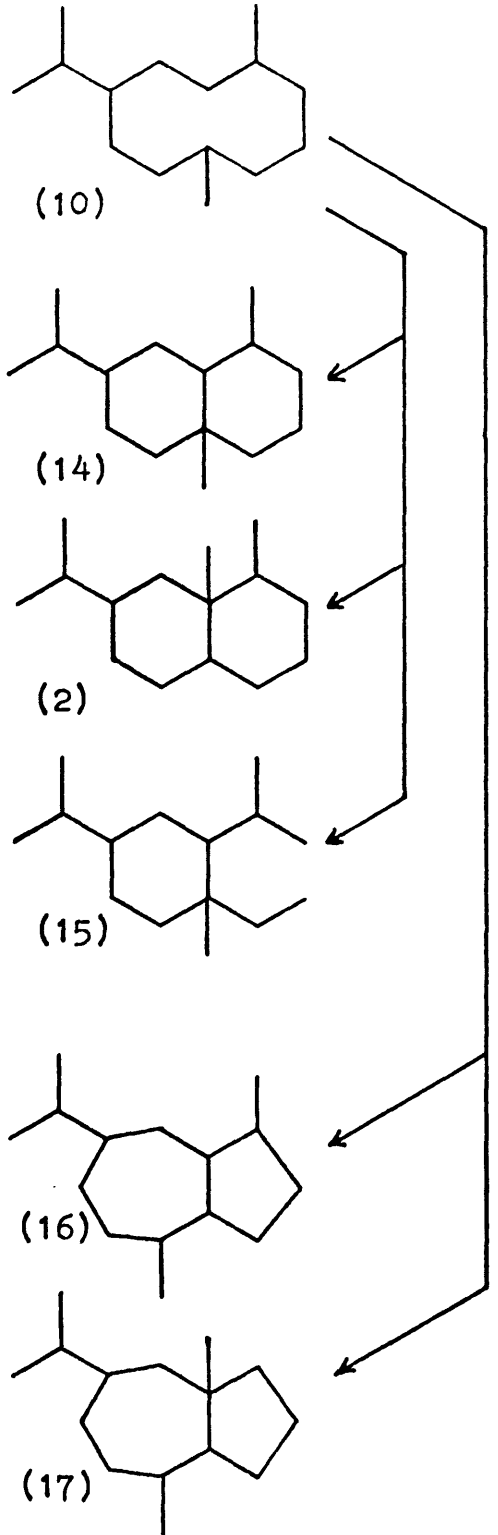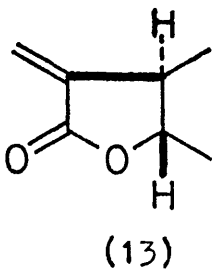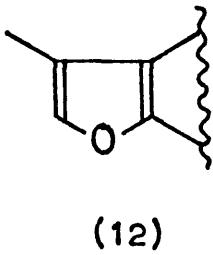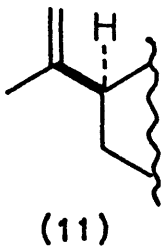
form represented by nootkatone (9), have been isolated from plant species of a variety of families. This topic has been covered recently in reviews by Pinder[24].

2.1.2   Chemotaxonomic Aspects

The investigations of Novotný, Herout and Šorm have demonstrated that eremophilane-type sesquiterpenoids occur, not only among members of the Petasites genus[12] but also in other members of the tribe of the Senecioneae. These compounds serve as a chemotaxonomic character that distinguishes this tribe from others of the Compositae family[11], and fully supports the tribal classification originally based on morphological grounds.

In general, the sesquiterpenoids of the Compositae may be formally derived by cyclisation of trans,trans-farnesyl pyrophosphate (5A)[25] to give a germacrane (10) type intermediate. Sesquiterpenoids from Compositae species are characterised by certain structural features, including the β-stereochemistry of the isopropenyl group (11), and products of enzymic oxidation involving this group containing furan (12) or γ-lactone (13) type rings. Tribal differences within the Compositae are expressed by transannular reactions modifying the basic germacrane skeleton: unchanged germacranes (10), eudesmanes (14), eremophilanes (2),

(18)

(19)

(20)

(21)

(1)

(22)

(23A) R = -CO.C(CH₃)=CH(CH₃) cis-

(23B) R = -CO.CH=CH.S(CH₃) cis-

(4)
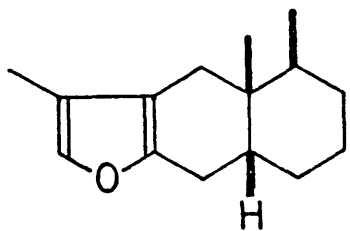
(18)
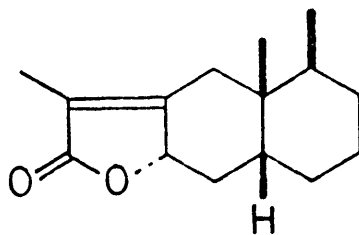
(19)
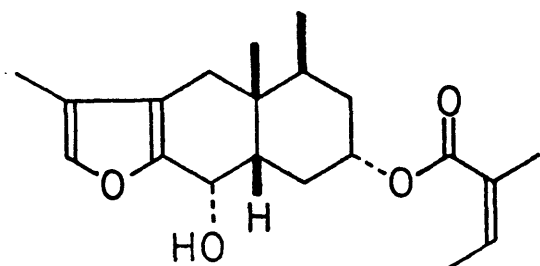
(20)
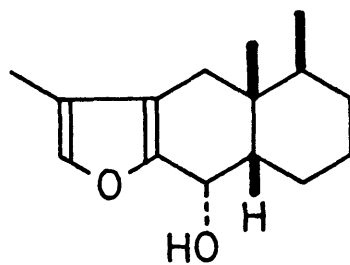
(21)
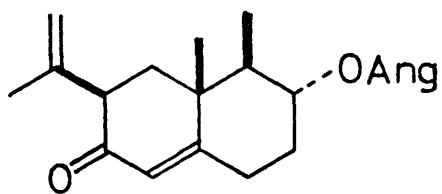
(1)
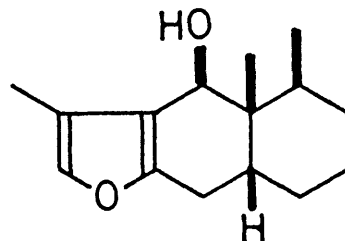
(22)
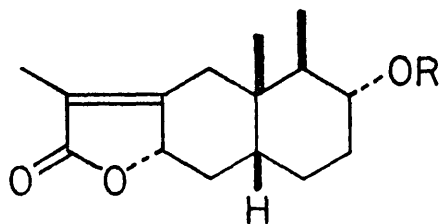
(4)

(23A) R = $-CO.C(CH_3)=CH(CH_3)$ cis-

(23B) R = $-CO.CH=CH.S(CH_3)$ cis-

elemanes (15), guaianes (16) and ambrosanes (pseudoguaianes) (17) have been shown to occur [11,26]. Differences in oxidation patterns, and more rarely, further slight skeletal changes, distinguish species, and in some cases, individual phenotypes.

The Petasites genus has been the subject of particularly detailed examination because of its taxonomic interest. In contrast to the results of Aebi and Waaler[19] the Czech group under Šorm first isolated compounds derived from furanoeremophilane (18) and eremophilenolide (19)[27,28]. A variety of Petasites hybridus was found to contain the angelate ester furanopetasin (20)[29] and other furano-eremophilanes oxygenated at C-9 (21), but not petasin (1). P. albus was also found to contain furanoids, but these were oxygenated at C-6 as in petasalbin (22)[30]. A comparison of a number of samples from widespread localities throughout Europe has shown that P.hybridus may exist in two chemically distinct forms, either containing petasin or furanopetasin[12]. It may be seen that there is a clear distinction in the oxygenation of these two compounds; the closure of a furanoid ring does not convert petasin into furanopetasin, and it seems likely that the two compounds have an independent biosynthetic pathway, though possibly from a common hydrocarbon precursor. P.albus was also found to occur in both furanoid and non-furanoid varieties[12].

A series of lactones, the petasitolides (23), analogous

to the petasin esters, has been isolated from Petasites

officinalis [27] ; it is generally accepted that this is not

a distinct species, but identical to P.hybridus [31] . The

petasitolides co-occur with furanoeremophilanes, but neither

type has been isolated from plants producing petasin. There

is some morphological variability within this species, so

it is not surprising to find that intraspecific chemical

characters also display differences in the genotype.

The finding of greatest significance from the

biosynthetic point of view has been the distribution of the

hydrocarbon eremophilene (4) [13,32] throughout the Petasites

genus [12] , and also in Adenostyles alliariae, another species

within the Senecioneae from which eremophilane-type sesqui-

terpenoids have been isolated [33] . This evidence supports the

proposal,discussed in a later chapter, that eremophilene

may act as a common biogenetic parent of the oxygenated

eremophilane sesquiterpenoids in all these species.

Table 1: Origin of *P. hybridus* Samples

Sample	Location	Flower
1	Disley, Cheshire	-
2	Near Disley, Derbyshire	-
3	Hathersage, Derbyshire	Male
4	Scotch Corner, N. Riding	-
5	Carlisle, Cumberland	-
6	Balmaha, Stirlingshire	-
7	Dumbarton, Dumbartonshire	Male
8	Bridge of Allan, Stirlingshire	-
9	Chee Dale, Derbyshire	Male
10	Marlborough, Wiltshire	-
11	Near Merthyr Tydfil, Breconshire	-
12	Newtown, Glamorganshire	-
13	Dovedale, N. Staffordshire	Male
14	Sheffield, West Riding	Male
15	Motherwell, Lanarkshire	Male
16	Garscube Estate#	Male

\# Propagated from rhizome stock taken from Motherwell Site.

2.2   RESULTS AND DISCUSSION

2.2.1   Preliminary Examination of Samples from Different
        Localities

Leaf samples of wild growing Petasites hybridus
were collected from a number of sites throughout Britain.
These plants agreed morphologically with the description
of P. hybridus [35], and in some cases this identification
was confirmed by subsequent examination of flowers (Table I).

Plants from the Motherwell site (No.15) had already
been investigated in this laboratory, and the petasin esters
isolated and characterised chemically [7]. This sample was
used as a standard for correlation with the remaining
specimens by thin-layer chromatography (TLC) of the benzene
extracts. Individual compounds were identified by comparison
with authentic standards and by characteristic behaviour
with spray reagents.

All samples were qualitatively identical by TLC,
affording three bands with quenching of fluorescence under
UV light, characteristic of the petasin esters. A further
band of high R_f was also found in all samples and has
subsequently been identified as eremophilene. No furanoids
were apparent, nor was there any evidence for petasitolide-
type lactonic esters. Other non-sesquiterpenoid compounds

Table 2: TLC Examination of Benzene Extracts of

Petasites hybridus and *Petasites albus*

Silicagel-G; 0.25 mm layer, 30 cm run.
15% ethyl acetate: 85% petroleum ether 60°-80°
Double development.

R_f	P.hybridus	P.albus	Compound or class	Spray reaction
0.96	+++	+++	eremophilene	H_2SO_4 - purple
0.94	+++	+++	β-carotene	H_2SO_4 - blue
0.90	+++	+++	sterol esters	H_2SO_4 - red
0.88	+	+	autoxidised β-carotene	H_2SO_4 - blue
0.85	++	++	terpenoid quinone (?)	H_2SO_4 - buff
0.73	+	+	terpenoid quinone (?)	H_2SO_4 - buff
0.62	-	+++	furanosesquiterpenoid	Stahl-Müller[35]
0.56	+	-	lactone 18	H_2SO_4 - blue
0.52	+	-	sesquiterpenoid 17c	UV quenching
0.48	++	++	4,4-dimethylsterol	H_2SO_4 - buff
0.46	-	+	furanosesquiterpenoid	Stahl-Müller
0.41	+++	-	isopetasin	UV quenching
0.38	+	+	4α-methylsterol	H_2SO_4 - buff
0.36	+++	-	petasin	UV quenching
0.27	+++	+++	phytosterol	H_2SO_4 - red
0.21	+++	+++	pheophytin a	H_2SO_4 - green
0.16	++	-	S-isopetasin	UV quenching
0.12	-	+	furanosesquiterpenoid	Stahl-Müller
0.08	++	++	pheophytin b	H_2SO_4 - green

were also tentatively identified (Table 2). A sample of
P. albus was analysed at the same time, and compared with
P. hybridus. Four major sesquiterpenoids were located,
giving positive reactions for furanoids with Stahl-Müller
reagent [27,35].

It may be concluded that these plants, which were
also morphologically identical in the leaf, are identical
in their genotype. The widespread occurrence of a single
genotype may be attributed to the habit of P.hybridus of
propagation through the rhizome. The flowers are unisexual,
and within the British Isles, the male is known to
predominate, the female occurring in isolation and only in
a few known locations. In the absence of sexual reproduction,
exchange of characters will not take place.

2.2.2 · GLC Examination of Leaf Extract

An examination by gas-liquid chromatography (GLC)
of a typical leaf extract was undertaken in order to
identify or characterise minor terpenoid constituents. It
was hoped that components might be isolated that acted as
intermediates in the biosynthesis of petasin. Combined
gas chromatography - mass spectrometry (GC-MS) has been
applied in this study to determine the chemical nature
of individual peaks in the chromatogram.

The Need for Preliminary Group Separation: It was necessary first to establish a method for the group separation of the sesquiterpenoids from the total extract. The total isopropanol extract of plant tissues contains products of a very diverse chemical nature. Compounds such as the condensed tannins, pigments, glycosides and the phospholipids are involatile, and thermally unstable. If injected into a gas chromatographic column at high temperature, these compounds give rise to pyrolysis products that interfere with the chromatographic record and cause rapid deterioration of the column. A preliminary group separation ideally removes the undesired compounds, leaving the components under investigation intact, both in quantity and in chemical nature. This is necessary if biosynthetic or chemotaxonomic deductions are to be made from the results.

Methods based solely on the polarity of the sample are only partly effective, because sesquiterpenoids vary greatly in polarity, and may be interspersed among a variety of interfering compounds, including carotenoids, chlorophylls and sterol metabolites. Distillation, either directly, or in steam, is not considered appropriate to this type of investigation because of the high risk of thermally-induced rearrangement.

(1) → silicagel / alumina / DEAE cellulose → (7A)

(25) → 100°C or silicagel/C_6H_6 25°C → (24)
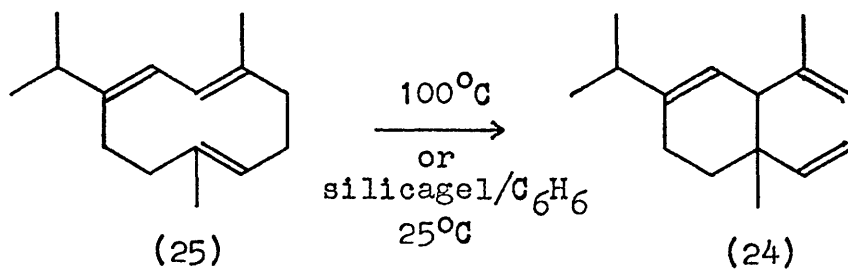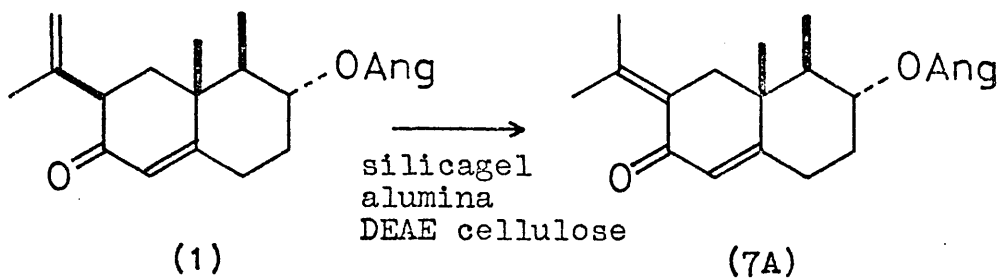
An early approach to this problem involved the use
of silver nitrate-impregnated silica-gel for TLC[36]. The
highly unsaturated molecule of β-carotene formed π-complexes
with the silver ions, and was retained on the baseline.
Pheophytins were also removed by this method. More detailed
GLC examination of extracts recovered from TLC plates
showed that some sesquiterpenoids, including S-isopetasin
were lost by this procedure. As there was, in addition, the
risk of oxidation of samples in the presence of Ag^I, the
technique was not adopted for routine use. Simple TLC
separation was also investigated, but still gave poor
recovery, coupled with rearrangement processes that increased
the complexity of subsequent GLC analysis results. (The
formation of isopetasin (7) from petasin (1)[6], or of
δ-elemene (24) from germacrene-C (25)[37] have been reported
to occur when (1) and (25) are left in contact with various
chromatographic materials.) Changes, possibly due to allylic
rearrangements, were noted among the sesquiterpenoid alcohol
constituents.

Application of Gel Chromatography: Most of the interfering
compounds have high molecular weights (>600), compared with
a range of 200 to 400 for sesquiterpenoids and their
derivatives. This difference has been utilised for separation

Fig. 1: Preliminary Group Extraction Procedure
for P. hybridus Extract

TOTAL ISOPROPANOL
LEAF EXTRACT

Gel-Filtration

A

HIGH MOLECULAR WEIGHT
INVOLATILE
M.W. 500

BC

LOW MOLECULAR WEIGHT
M.W. 500

Straight-Phase
Partition

B

GLC VOLATILE
FRACTION

C

POLAR FRACTION
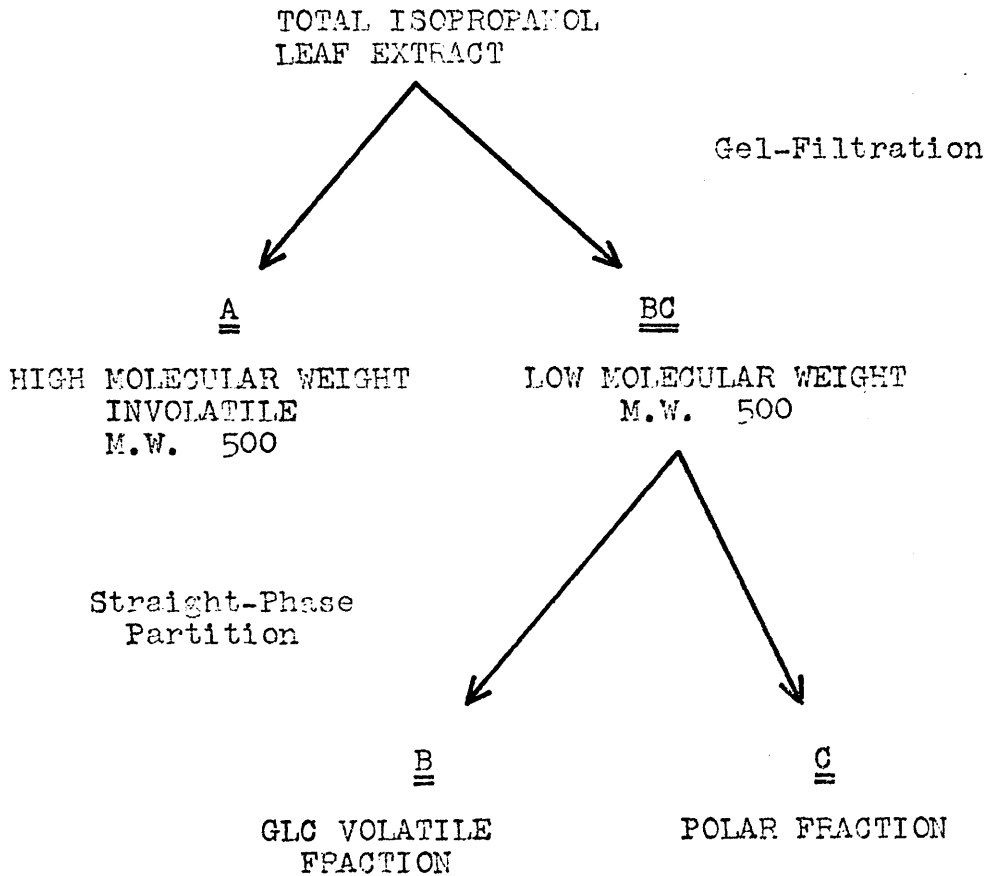
by gel-filtration, where compounds are eluted in order of decreasing molecular size. Under the correct conditions*, sample polarity has little effect on the sequence of elution.

A preliminary separation by gel-filtration removed compounds of high molecular weight (>500-600; Fraction A) and a subsequent simple straight-phase separation eliminated excessively polar components (e.g. free acids and glycosides; Fraction C). The remaining extract (Fraction B) consisted almost entirely of compounds suitable for GLC analysis, and at the same time contained all the neutral and non-conjugated sesquiterpenoids (Fig.1). GLC analysis of total extract, Fraction BC and Fraction B gave a chromatogram that was both qualitatively and quantitatively the same at each stage. The requirements originally stated (p.13) have therefore been met: the sample under investigation was unchanged by the group separation process.

The chromatogram obtained by GLC on the stationary phase 1% OV-1 is shown in Fig. 2. Temperature programming

* The establishment of correct conditions for gel-filtration and liquid-gel partition chromatography are described in detail in Part II of this thesis. Differences in polarity between gel and solvent give rise to separation by partition processes: 'straight-phase' where the gel is the more polar component, and 'reversed-phase' when it is less polar than the eluting solvent.

Fig. 2

GLC of Fraction B of P. hybridus Extract:

10 1% OV-1; 50 ml/mm N$_2$

Temperature programmed 100°-250° at 2.5° per minute

⟵ minutes
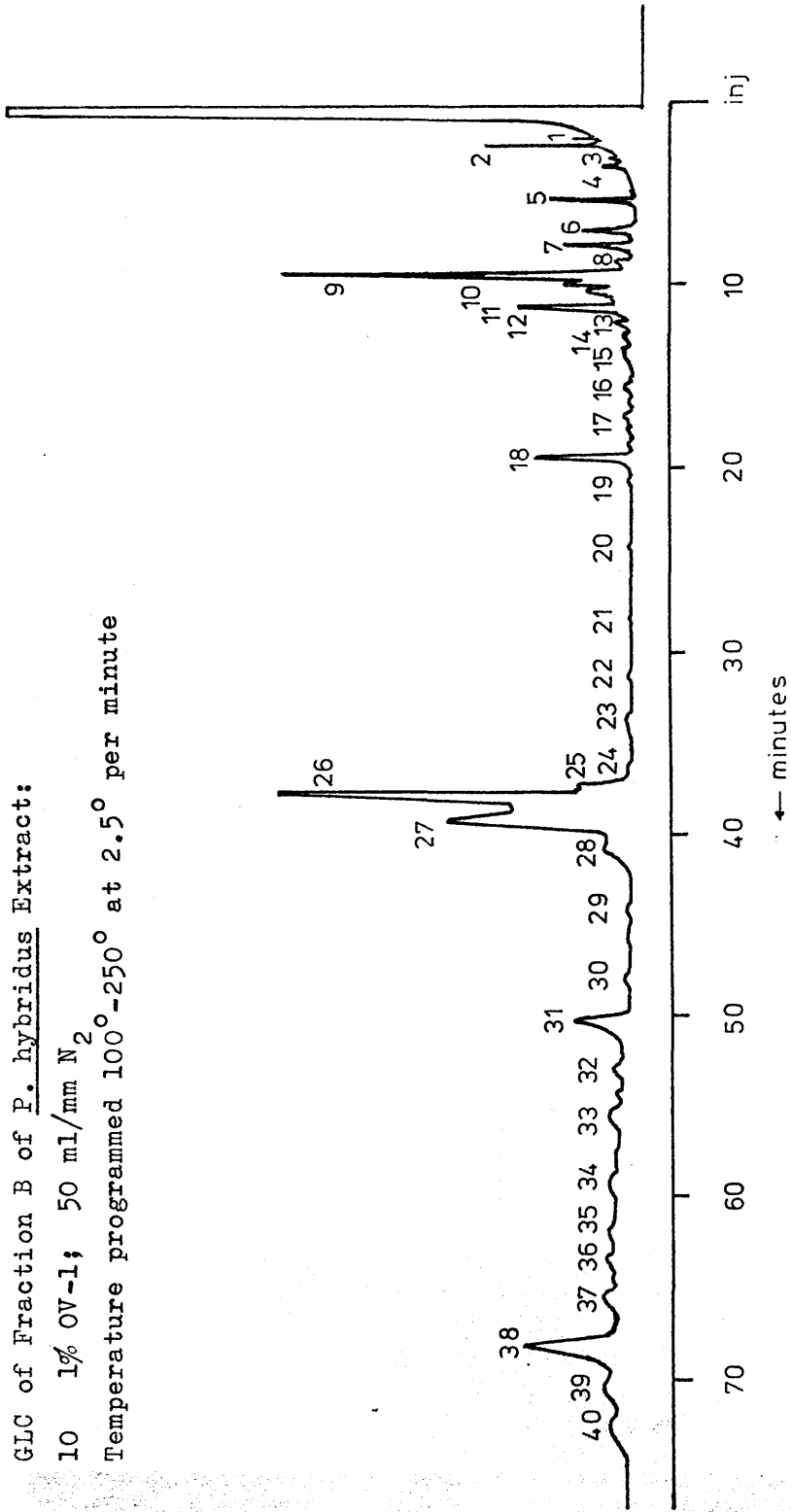
Table 3: Preliminary GC-MS Identification of Peaks in Fig. 2

Peak	Compound or type	Retention Index[*]	Molecular Ion m/e
1,2,4,5	n-alkanes C_{10} - C_{13}	1000-1300	
6. - 12	sesquiterpene hydrocarbons	1350-1540	204
13 - 17	sesquiterpenoid alcohols	1550-1750	220,222
18	sesquiterpenoid lactone	1770	234
19 - 22 & 24	n-alkanes C_{18} - C_{22}	1800-2200	
23	C_4 esters of petasol	2170	304
25	saturated C_5 ester of petasol	2280	318
26 - 28	petasin esters	2300-2400	316
29	n-$C_{25}H_{52}$	2500	352
30	n-$C_{24}H_{49}OH$	2600	354
31	S-isopetasin	2700	334
32	n-$C_{26}H_{53}OH$ & squalene	2800	382,410
33	n-$C_{29}H_{60}$	2900	408
35	n-$C_{31}H_{64}$	3100	436
36	campesterol	3150	400
37	stigmasterol	3180	412
38	β-sitosterol	3220	414
39	24-methylenecycloartenol	3280	440
40	citrostadienol	3310	440

[*] GLC on 1% OV-1; approximate values taken from temperature programmed run.

was necessary to cover fully the range of compound isolated. Forty peaks were counted on the original GLC trace, and the numbering of peaks 1-40 was based on this result. Subsequent work, involving further separation into groups and recording at higher sensitivity, has shown over 120 individual compounds. Re-numbering was made by adding a suffix a,b,c etc to identify each peak.

2.2.3   Identification by GC-MS of General Leaf Constituents

The peaks in Fig.2 were characterised by GC-MS, and most compounds other than sesquiterpenoids were identified by this means. Table 3 shows the results of this investigation. Two groups of peaks, 1-5 and 19-24, were found to be n-alkanes, probably arising from contamination, either in the laboratory, or of the plant itself from insecticide sprays. These peaks have not been observed in subsequent extractions of leaf material, and the lower alkanes are not considered to be part of the normal constituents of P. hybridus. High molecular weight alkanes, in particular n-nonacosane and n-hentriacontane, were constituents of the leaf wax. These naturally occurring wax alkanes are commonly found in plant material, and are predominantly saturated straight chain hydrocarbons having an odd carbon number[38]. The artefacts located above comprised both odd and even numbered homologues,

consistent with an accidental origin.

Compounds 6-18 were identified as sesquiterpenoids,
and could be characterised as hydrocarbons, alcohols,
ketones or lactones by molecular ion determination and
examination of the fragmentation pattern. Compounds 23-28
were found to give the common fragment of m/e 216,
characteristic of petasin esters, by loss of the acyl moiety.
A base peak of m/e 148 indicated petasol as the alcohol
moiety of esters 23, 25 and 26, while 27 and 28 gave a base
peak of m/e 161, associated with isopetasol [7]. Similarly,
peak 31 was found to be S-isopetasin. Of the remaining peaks,
29-35 were identified as leaf wax constituents comprising
odd-numbered n-alkanes and even numbered n-alcohols. 36-40
consisted of the phytosterols, the major peak being
β-sitosterol. (Acknowledgments are due here for the kind
assistance of Dr. B.A.Knights in the provision of reference
spectra of the phytosterols.)

2.2.4   Further Subdivision of the Extract into Groups

Before detailed examination of the various classes of
sesquiterpenoids, it was advantageous first to separate these
classes from each other, and from other non-sesquiterpenoids.
Reversed-phase chromatography was found to be effective in

FRACTION BC - Low molecular weight compounds

Reversed-Phase Separation

BI
Oxygenated
Sesquiterpenoids

BII
Sesquiterpene
Hydrocarbons

BIII
Wax Constituents
and Phytosterols
Traces of Carotenes
and Chlorophylls

Straight-Phase Separation

BII/1

BII/2
Sesquiterpene
Hydrocarbons

BII/3
Traces of Sterols
and Carotenoids

Straight-Phase Separation

BI/1
a) Petasin Esters
b) Ketones and
   Lactone

BI/2
Sesquiterpenoid
Alcohols

BI/3
Petasol and
Isopetasol

BI/4
Traces of
Sterol
Glycosides
Free Fatty
Acids.

subdividing fraction BC into three groups, found to contain
primarily oxygenated sesquiterpenoids (BI, Fig. 3),
sesquiterpene hydrocarbons (BII), and the sterols and wax
constituents (BIII). The separation was carried out by
column chromatography with alkylated 'Sephadex' dextran
gels [18]. Straight-phase separations were then used as shown
in Fig. 3 to fractionate the oxygenated sesquiterpenoids
according to polarity. This step also effected further
purification of both oxygenated sesquiterpenoids and the
hydrocarbons. The fractions obtained by this procedure were
substantially free of contaminants, and contained compounds
of similar chemical character. Each fraction was suitable
for examination by GLC using isothermal operation.

The use of modified dextran gels for the above
separations offered a number of advantages. Comparison of
peak areas in chromatograms from the isolated fractions with
those of the original extract, run under identical
conditions of concentration, indicated that compounds
were recovered qualitatively unchanged. Slight quantitative
losses of the more volatile compounds could be attributed to
evaporation in the course of removal of solvents, and could
be minimised by taking appropriate precautions. The elution
of samples from the columns was rapid, and elution volumes
and sample volumes were considerably smaller than is normally
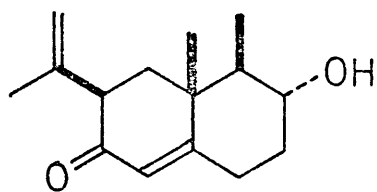
(26)
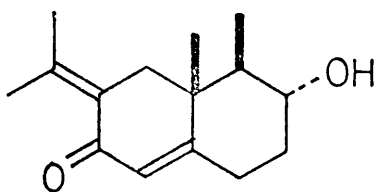
(27)
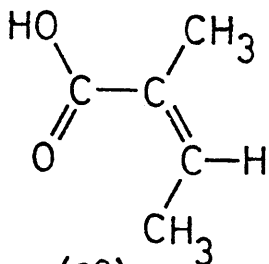
(28)
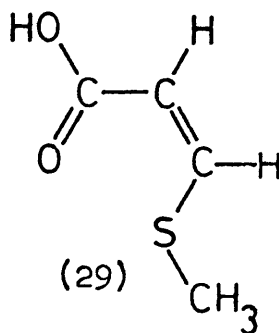
(29)
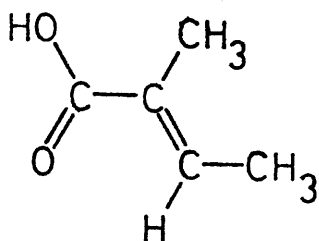
(30)
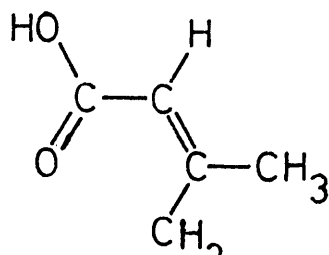
(31)
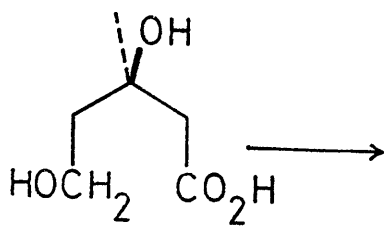
(6)

● = ^{14}C
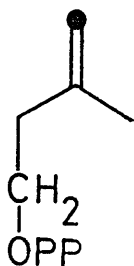
(32)
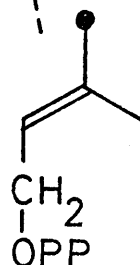
(33)

OPP = Pyrophosphate

the case with silicic acid or alumina. There was thus
less risk of contamination by solvent residues.

2.2.5 Characterisation and Identification of Major and Minor Sesquiterpenoids

Fraction BI/1a; Petasin esters[*]:

The fraction, eluted in the range SEV 51-60[#] from a
column operating under straight-phase conditions, contained
the petasin esters. These are esters of the sesquiterpenoid
ketoalcohols petasol (26) and isopetasol (27) with a variety
of acids. Mass spectrometry (Appendix I) distinguished the
two major esters by their fragmentation patterns. Petasin
gave a base peak of m/e 148, ascribed to loss of isoprene
from the fragment of m/e 216 obtained by de-acylation.
Isopetasin underwent cleavage in ring A, giving a base peak
of m/e 161 [39] (Fig.4). The molecular ion of the ester indicated
the molecular weight of the acyl moiety. Mass spectrometry
gave no further evidence for the structure of the acid,
because the acyl moiety did not give rise to any
recognisable fragment ions. Esters of saturated C_4 and
C_5 acids were found in addition to those of angelic acid (28)
and β-methylthioacrylic acid (29) which Aebi has

[*] The term 'petasin esters' is used to describe the
group of compounds consisting of esters of the alcohols
petasol and isopetasol with a variety of carboxylic
acids, which have been found to co-occur in P.hybridus.

[#] For a definition of SEV, see p.186 of this thesis.

GLC of Fraction BI/1a: Petasin Esters

6' 1% OV-1; 200°C (isothermal)
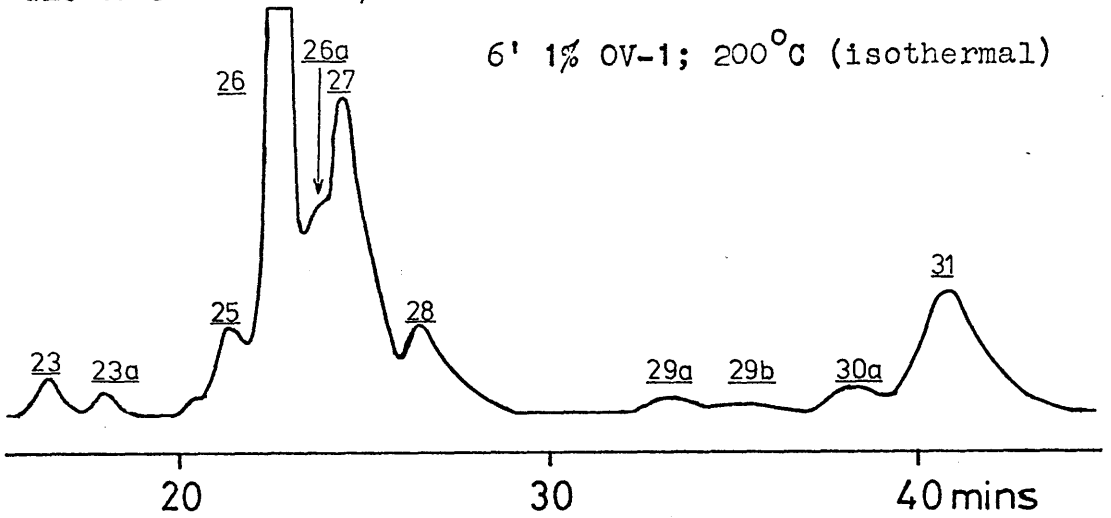
GC-MS/AVA Scan

——— m/e 148 – Petasin Type Esters

------ m/e 161 – Isopetasin Type Esters
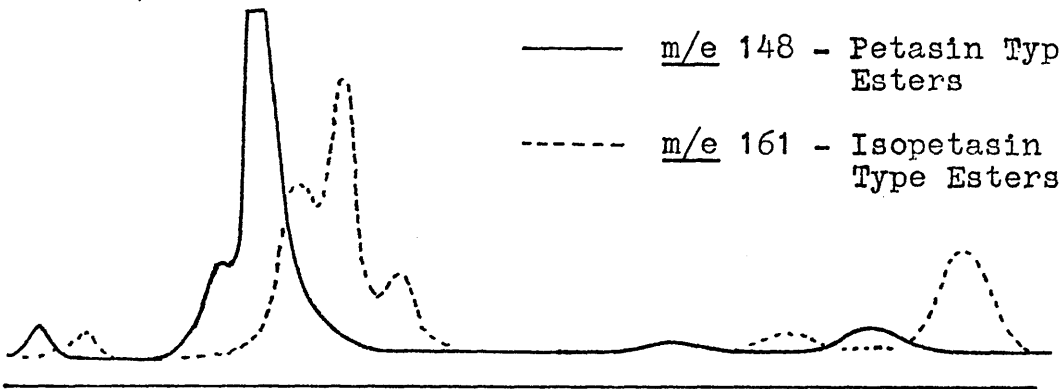
GC-MS/AVA Scan

—·—·—· m/e 304

——— m/e 316

------ m/e 318

·············· m/e 334
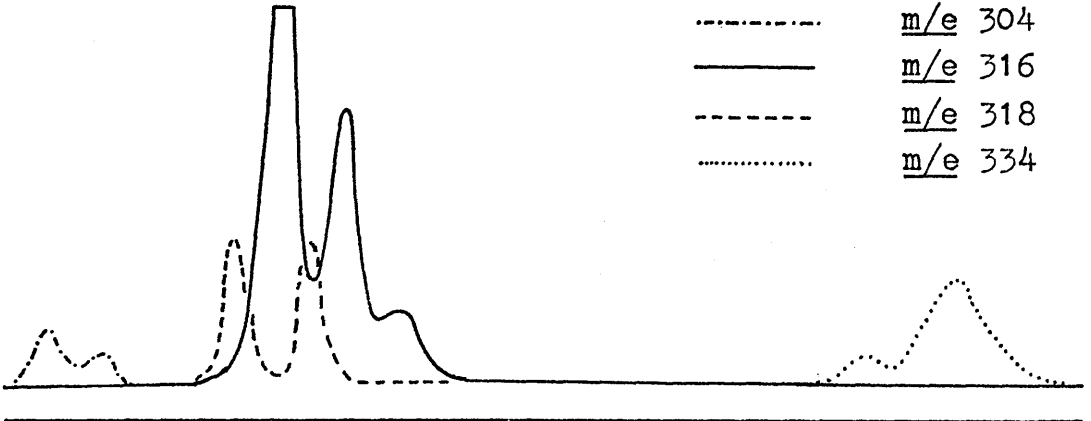
Fig. 4: Formal Derivation of the Base Peaks in Mass Spectra
of Esters of Petasol and Isopetasol
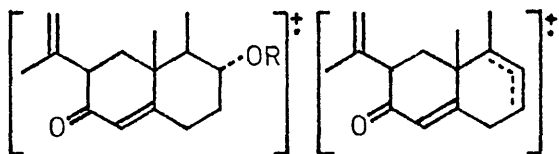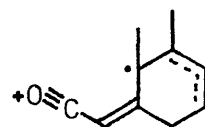
$M^+ = (233 + R)$ m/e 216   m/e 161   m/e 148
                                            base peak of   base peak
                                            isopetasol   of petasol
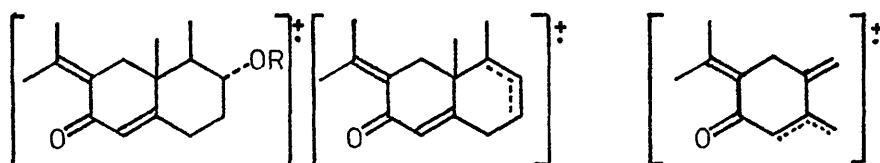
Table 4: AVA Analysis of the Petasin Esters

Compound	Alcohol	Acid	$I_{OV-1}^{226°}$	M^+	Trivial Name
23	petasol	C_4 sat.	2170	304	
23a	isopetasol	C_4 sat.	2210	304	
25	petasol	C_5 sat.	2285	318	
26	petasol	angelic	2305	316	Petasin
26a	isopetasol	C_5 sat.	2330	318	
27 ·	isopetasol	angelic	2350	316	Isopetasin
28	isopetasol	C_5 unsat.	2400	316	
29a	petasol	?	2555	?	
29b	isopetasol	?	2600	?	
30a	petasol	-methylthio-			S-petasin
31	isopetasol	acrylic	2705	334	S-isopetasin

already reported[6], and a further ester, isomeric with the
angelate, which might involve tiglic (30) or dimethylacrylic
(31) acid. This last structure would be more consistent with
the observation that one ester, not petasin or isopetasin,
incorporated radioactivity from $(2-^{14}C)$ mevalonic acid into
the acyl moiety very readily. This might occur via isopentenyl
pyrophosphate (32) and dimethylallyl pyrophosphate (33),
non-specific enzymic hydrolysis and oxidation giving rise to
dimethylacrylic acid.

Gas-liquid chromatography of these compounds, on the
stationary phase OV-1 (1%), gave a complex unresolved grouping
of esters of both satured and unsaturated C_5 acids. A change
of stationary phase to OV-22, XE-60 or Carbowax 20M altered
the pattern, but did not lead to resolution of the peaks.
In order to define more clearly the individual components,
GC-MS was used in conjunction with the Accelerating Voltage
Alternator (AVA). This system operated the mass spectrometer
at a fixed magnetic field, and acted as a selective detector
for GLC which recorded only those compounds giving certain
predetermined fragment ions. The AVA alternated the
accelerating voltage between three preset levels, so that
in combination with the fixed magnetic field, the intensities
of up to three fragment ions were detected in rapid succession
and recorded independently on the chromatogram[40].

This procedure was carried out with petasin esters, in the first instance to distinguish petasin types from isopetasin, the selected fragment ions being the base peaks of these two isomers. A second scan for ions of $\underline{m/e}$ 304, 316 and 318 resolved the peaks assigned to the different acyl moieties.

Fraction BI/1b; Lactones and Ketones:

This fraction, eluted between SEV 60 and 70 on the straight-phase column, was found to contain four compounds - one in particularly low concentration, which was insufficient for further characterisation, even by mass spectrometry. The four compounds, numbered 17b, 17c, 17e and 18 according to the system described (p.16) were separated by high-resolution reversed-phase chromatography. Details of chromatographic characterisation are given in Table 5.

The mass spectral fragmentation of compounds 17b and 17c suggested a ketonic oxygen function (Appendix I). Deuterium labelling was carried out 'on column' by gas-liquid chromatography[41] on the stationary phase 1% APL/0.5% Ba(OH)$_2$, activated by pre-injection with deuteriomethanol, CH$_3$OD[42]. These conditions were known to result in deuterium exchange with enolisable protons in the sample molecule.

Table 5: Components of Fraction BI/1b

Compound	$I_{OV-1}^{147°}$	SEV[a)	M⁺	Formula[b)	Recovery[c)
17b	1720	116	220	$C_{15}H_{24}O$	100 μg
17c	1725	90	218	$C_{15}H_{22}O$	600 μg
17e	1760	-	-	-	< 10 μg
18	1770	103	234	$C_{15}H_{22}O_2$	8 mg

a) N1114-50%-LH20/methanol
b) Derived from mass spectrometric fragmentations
c) From 150 g fresh weight of leaf tissue;
   estimated from GLC peak areas.

Fig. 5: Deuteriation Patterns of α,β-Unsaturated Ketones

i) d_{10}

ii) d_8

iii) d_8

iv) d_8

v) d_6

vi) d_4
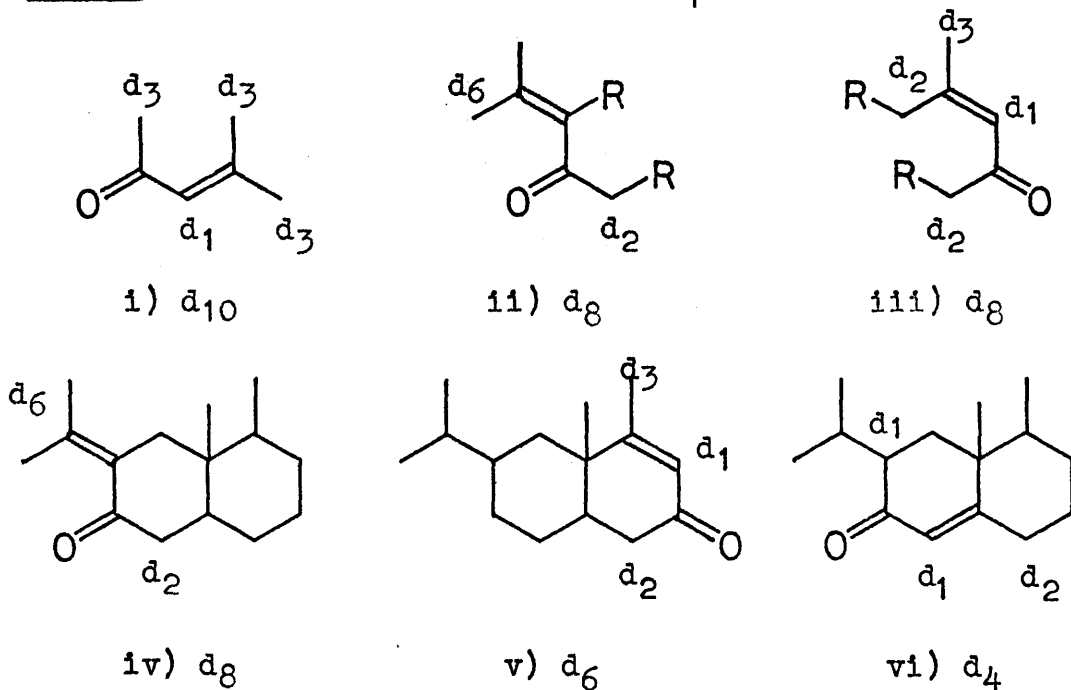
The molecular ion of compound __17b__ was increased
by eight mass units. The high level of deuteriation could
only be obtained if the number of enolisable positions was
increased by α,β-unsaturation of the ketone. α,β-Unsaturated
ketones were known to isomerise in the presence of barium
hydroxide in the stationary phase[42]. The correlation of
simple and deuteriated mass spectra in this case indicated
that isomerisation had not occurred. The ultra-violet (UV)
spectrum of compound __17b__ indicated conjugation of an
α,β-unsaturated ketone.

Fig. 5 shows the maximum level of deuteriation
obtainable for a singly unsaturated ketone. Up to ten
exchangeable protons are available in the structure depicted,
considered as an entire molecule (Fig. 5,i). If this system
is incorporated as a part structure in a ring system, at
least two enolisable positions are lost as ring residues,
in either of two possible arrangements (Fig. 5,ii&iii).

The abundance of fragments that gave rise to d_6
or d_2 substitution (Table 6) and the comparative absence of
d_3, d_4 and d_5 deuteriated fragments strongly suggested the
first of these two part structures for compound __17b__, where
the isopropylidene side chain was likely to behave as one
unit in the prominent fragmentations. The consistent nature
of the deuterium substitutions in the fragment ions,

(34)

(34A)
H
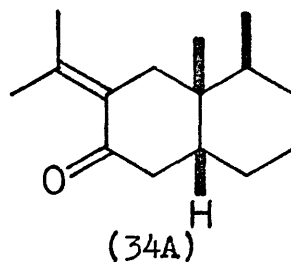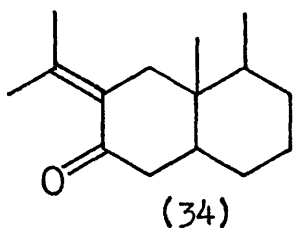
Table 6: Tabulations of Mass Spectra Taken at 20eV of Compound 17b, with and without Deuterium Exchange Labelling

Fragment m/e	Relative Intensity	Deuterium Substitution					
		d_8	d_6	d_5	d_2	d_1	d_0
220	78	78	–	–	–	–	–
205	8.4	4	–	4	–	–	–
177	6.8	3	–	3	1	–	–
152	6.0	–	–	–	3	3	–
149	12.4	–	9	–	4	–	–
123	12.4	–	–	–	6	6	–
111	22	12	–	–	–	–	9
110	41	17	–	–	–	–	24
109	100	–	–	–	–	–	100
108	22	–	–	–	–	–	22
96	44	–	44	–	–	–	–
95	13.4	–	6	–	–	–	7
83	5.7	–	6	–	–	–	–
82	7.9	–	8	–	–	–	–
81	7.5	–	–	–	–	–	7.5
68	27.6	–	28	–	–	–	–

Adjusted to 100% saturation by deuterium

apparently free from the effects of hydrogen rearrangements commonly observed in mass spectrometry, may be ascribed to the low ionisation energy employed (20eV). Initial ionisation under these conditions would be more likely to be controlled by the functional groups, with reduced potential for charge rearrangements that could give rise to transfer of deuterium atoms to different sites in the molecular ion and subsequent fragments.

The structure (Fig.5,iv) was the only arrangement that permitted the full d_8 substitution when fitted to the eremophilane skeleton, and other possibilities having the structures shown (Fig.5,v & vi) possessed a reduced number of enolisable positions.

The structure (34) may therefore be assigned tentatively to compound 17b. A known compound, fukinone (34A), having cis- stereochemistry at the ring junction, has been isolated from Petasites japonicus Maxim. by a Japanese group[43]. Unfortunately, these authors did not publish a mass spectrum, but merely quoted the molecular ion and base peak, m/e 220 and 109. These were consistent with our own results, as was the UV absorption maximum (found, λ_{max}249nm; ε_{max}8000: quoted[43], λ_{max}251nm; ε_{max}6800).

The major fragmentations are tabulated in Table 6, and their respective shifts on deuteriation are indicated.

Fig. 6a Mass Spectral Fragmentation of Compound 17b (34)

[m/e values quoted for (34) without deuteriation]

(34)

M^+ 220; d_8

m^* 68.7

m^* 41.8

m^* 54.0

m/e 152; d_1/d_2

m/e 123; d_1/d_2

m/e 109; d_0
Base Peak

m/e 96; d_6
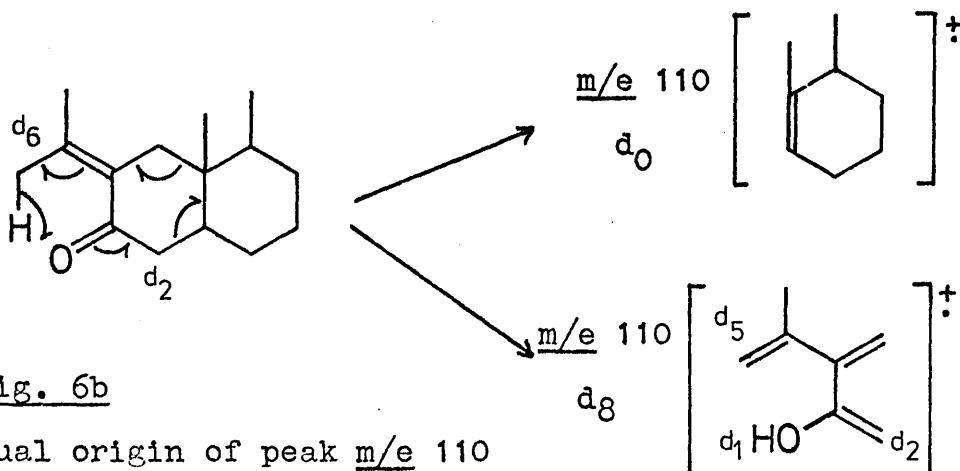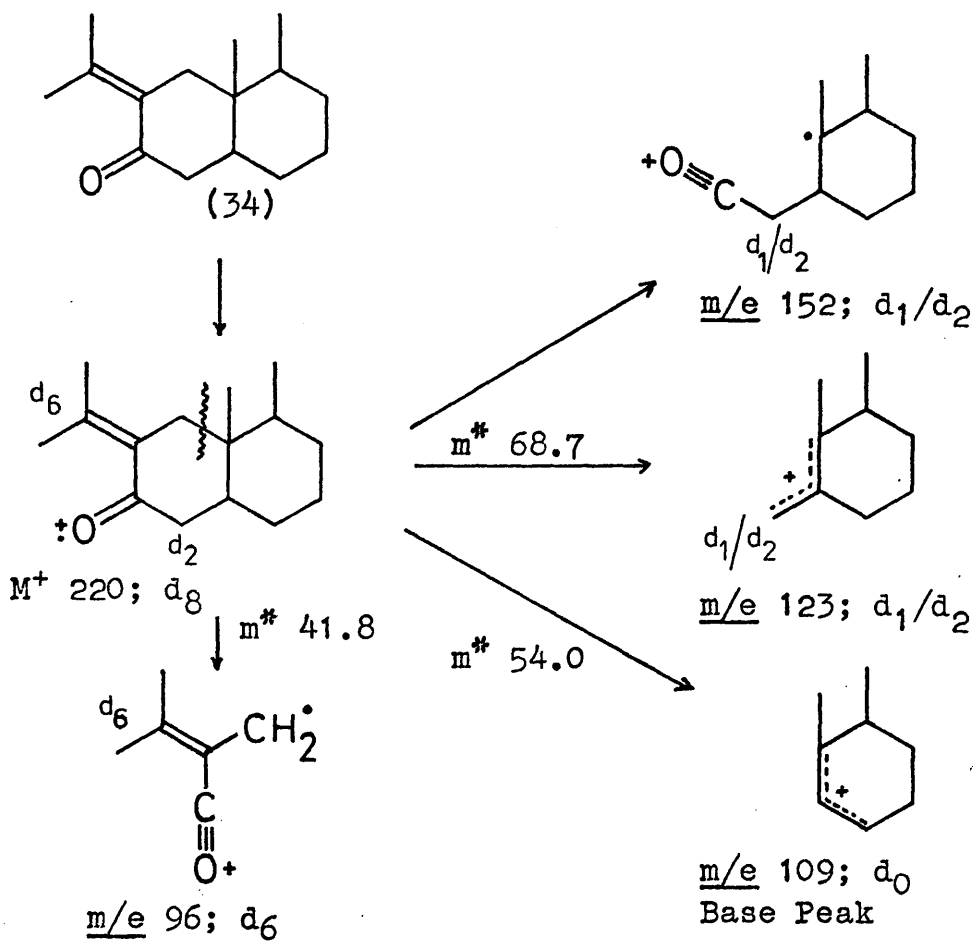
Fig. 6b

Dual origin of peak m/e 110

m/e 110
d_0

m/e 110
d_8

The deuterium substitution of the molecular ion indicated an abundance of protium of 5% in the enolisable positions: contributions to the molecular ion were: d_8, 51%; d_7, 41%; d_6, 8%. Assuming protium to be evenly distributed in all eight positions, the expected contributions to a true d_6 fragment were calculated: d_6, 66%; d_5, 30%; d_4, 4%. Observed d_8 and d_6 fragments having this distribution of satellite ions were reported as d_8 and d_6 respectively, the relative abundance of the peak being adjusted by summation of the contributions due to the satellite ions. True d_5 fragments in the presence of d_6 would be indicated by abnormally high d_4 intensity.

The lack of incorporation of deuterium into the base peak, m/e 109, indicated that this was the hydrocarbon fragment from ring A depicted in Fig. 6a. Fragmentation appeared to be directed by the cleavage of the bond between C-5 and C-6 as shown, this being β with respect to the double bond and γ to the carbonyl function. Further bond fissions led to four of the abundant ions of the mass spectrum. Ions having m/e 110 were coincidentally precisely half the mass of the molecular ion, m/e 220. Deuteriation demonstrated that both hydrocarbon and carbonyl containing fragments contributed to this peak. A rearrangement of the McLafferty type, but with an enlarged ten-membered transition state might account for
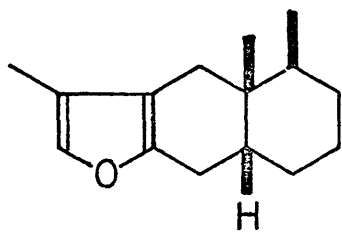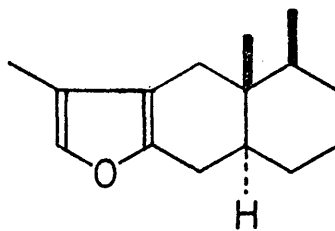
(35A)   (35B)

Fig. 7: Stereochemical Control of Mass Spectral Fragmentation
Evidence for the Identity of Compound 17b
as Fukinone (34A)
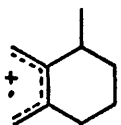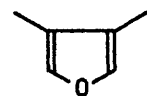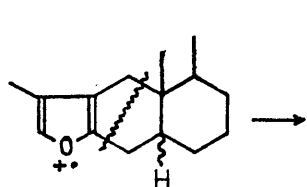
(Data from G.H. Draffan,
Ph. D. Thesis, 1967)

m/e 122   Relative Abundance
          36% from (35A) cis-
          4% from (35B) trans-

m/e 96   Relative Abundance
         From Compound 17b and
         Fukinone (34A) 44%
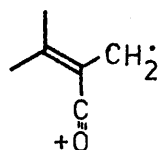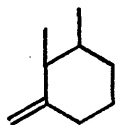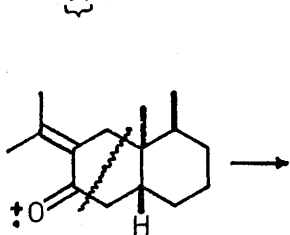
(A sample of Fukinone was kindly supplied by Professor
K. Naya of Kwansei University, Japan.)

the formation of these radical ions (Fig.6b).

One detail of the structure of compound 17b is lacking in the above discussion: this concerns the stereochemistry of the ring junction, determined for fukinone by optical rotatory dispersion. Lack of sample prevented correlation by measurement of optical rotation. However, while mass spectrometry does not easily discriminate stereochemical detail of this nature, there may be some analogy between the fragmentation of this compound and that of furanoeremophilane (35). Mass spectrometry of the cis- (35A) and trans- (35B) isomers of furanoeremophilane demonstrated a mode of bond fission where C-9 was retained by the hydrocarbon fragment derived from ring A. Ions of this type were observed in significant abundance from the cis-isomer, but were negligible in the mass spectrum of the trans- form. [7] (Fig.7). In compound 17b, hydrocarbon ions of the type described were not particularly abundant, but the complementary carbonyl-containing ion, m/e 96 was prominent, and a metastable peak, m/e 41.8, indicated its direct formation from the molecular ion (Fig.6a). This contrast may be considered to arise from the difference in charge directing properties of the furan and carbonyl functionalities, while retaining the proposition that the stereochemistry of the ring junction controls this particular mode of bond fission.

(36)   (37)
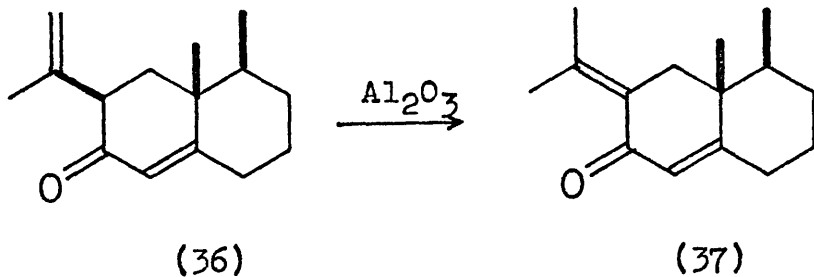
Fig. 8: Proposed Scheme of Mass Spectral Fragmentation
For Compound <u>17c</u> (36) and Base-Isomerised
Product (37)
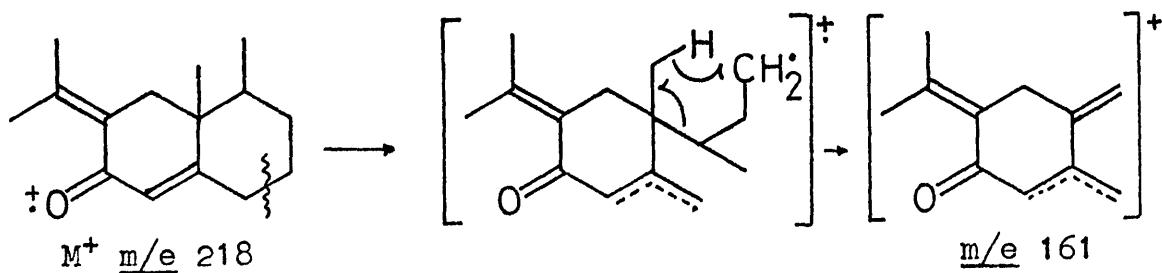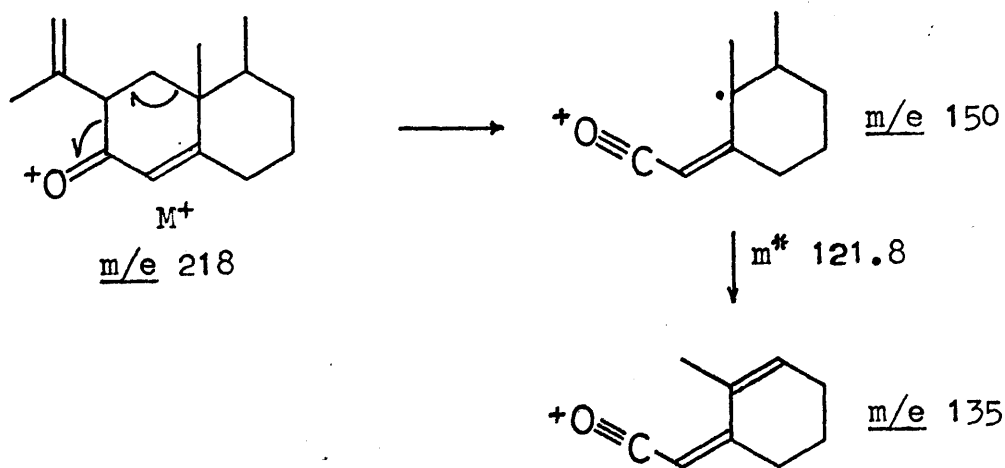
In the cis- isomers of both (34) and (35), the bond that
is required to break is parallel to the C-10 - H bond.
Any rearrangement process that involves the removal of the
hydrogen from C-10 would then favour fission of the 8(9)
bond in the cis-, but not the trans- isomer (Fig. 7).

Other instances of stereochemical control of this
type in mass spectrometry have been reported in the steroid
field. Egger has found that 5α- and 5β- isomers of Δ^1-3-oxo-
steroids show significant differences in the abundance of the
M-42 peaks arising from ketene elimination[44a]. A second
example may be seen in the formation preferentially from
3-oxo-5β-steroids of M-70 peaks due to loss of ring A by
retro-Diels-Alder rearrangement of the enol form[44b].

On the basis of the above evidence, and by
chemotaxonomic correlation with other Petasites types, which
are known to contain predominantly A/B cis- isomers of the
eremophilane skeleton, it may be tentatively proposed that
compound 17b has the structure of fukinone (34A)[43].

The mass spectrum of compound 17c demonstrated
fragmentation strikingly similar to that of petasin. A major
peak, m/e 150 appeared to be derived by the same cleavage
that gave the base peak of petasin, m/e 148, the two additional
mass units arising from the fully saturated ring A, which
remained intact in the fragment ion (Fig. 8; Appendix I).

Table 7: Ultraviolet Absorption Data for Compound 17c

Compound	λ_{max}(EtOH)	ε_{max}	λ_{calc} [45]
17c (36)	243 nm 283 nm	6000 700	244 nm
17c Isomer (37)	251 nm 280 nm	8000 4800	254 nm
Isopetasol (27)	250 nm 281 nm	10000 6000	254 nm

Ring A is lost in the formation of the ion m/e 161 in petasin, and this ion occurred unchanged in the mass spectrum of compound 17c. Deuteriation of compound 17c by GLC was only a partial success: isomerisation apparently occurred on the column in the presence of barium hydroxide. The relative abundance of the ion m/e 161 was dramatically increased, while those at m/e 135 and 150 virtually disappeared. This was analogous to the relationship between the mass spectra of petasin and isopetasin, where petasin favoured the formation of m/e 148 ions, and isopetasin gave the m/e 161 ion preferentially (p.19). Both isomerisation and exchange were relatively slow processes, and while isotopic saturation of the ring positions appeared satisfactory, there was incomplete exchange of deuterium into the isopropylidene side chain, as this could only occur after isomerisation. These results were of little value for structure elucidation.

A small sample of compound 17c was isomerised by leaving a solution in benzene over basic alumina for 24 hours. Ultraviolet spectra of the unchanged and isomerised samples showed differences in absorption maxima, which corresponded to those calculated by the Woodward-Fieser rules [45] (Table 7). The low value for the extinction coefficient for compound 17c may be due to diaxial interaction between the isopropenyl side chain and the angular methyl group, leading to a twist conformation of ring B with loss of co-planarity of the
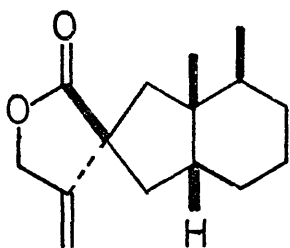
(38)
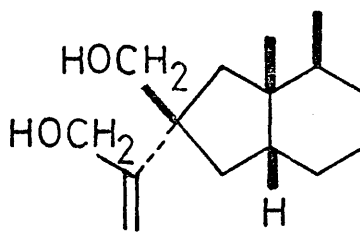
(39)

Table 8: Characterisation of Compound 18

	Compound 18	Bakkenolide-A [47]
m.p.	80° mixed m.p. 80°	80°–81°
$\alpha_D^{25°}$	+17°	
analysis	found C 76.94% H 9.21% $C_{15}H_{22}O_2$ requires C 76.88% H 9.40%	analysed as $C_{15}H_{22}O_2$
Mass Spectrum	m/e 234 12% of base peak m/e 124	m/e 234 10% of base peak m/e 124
max KBr Disc	3090 cm^{-1}(w) 1765 cm^{-1}(s) 1664 cm^{-1}(m) 895 cm^{-1}(m)	3090 cm^{-1}(w) 1767 cm^{-1}(s) 1668 cm^{-1}(m) 895 cm^{-1}(m)

An authentic sample of Bakkenolide-A was kindly supplied
Professor Y. Kitahara, of Tohoku University, Sendai, Japan.

chromophore. GC-MS of the alumina-isomerised sample of compound 17c indicated changes in the mass spectrum corresponding to those observed in the attempted deuteriation. The structure, eremophila-9,11-dien-8-one (36) may be tentatively assigned to compound 17c, and the product of isomerisation may be written as eremophila-7(11),9-dien-8-one (37). The GLC retention index on the stationary phase OV-1 of the compound 17e, which was insufficiently abundant for characterisation, suggested that this might be the isomerised form of compound 17c.

Compound 18 was a prominent sesquiterpenoid component of P. hybridus leaves, and was first recognised in the initial TLC experiment as a compound giving a pale blue colour with spray reagents containing sulphuric acid (p.11). Extraction of this band after preparative TLC, and examination by GLC, gave a peak that was later correlated with compound 18 by GC-MS.

The mass spectrum of compound 18 (Appendix I) showed a molecular ion at $\underline{m/e}$ 234, suggesting the formula $C_{15}H_{22}O_2$, subsequently confirmed by analysis. It was otherwise uninformative, having a prominent base peak, $\underline{m/e}$ 124, and an ion, $\underline{m/e}$ 109, produced by loss of methyl from the base peak, but little other fragmentation of diagnostic value. The infra-red spectrum was characterised

100 MHz NMR (C_6H_6 solution) of Bakkenolide-A
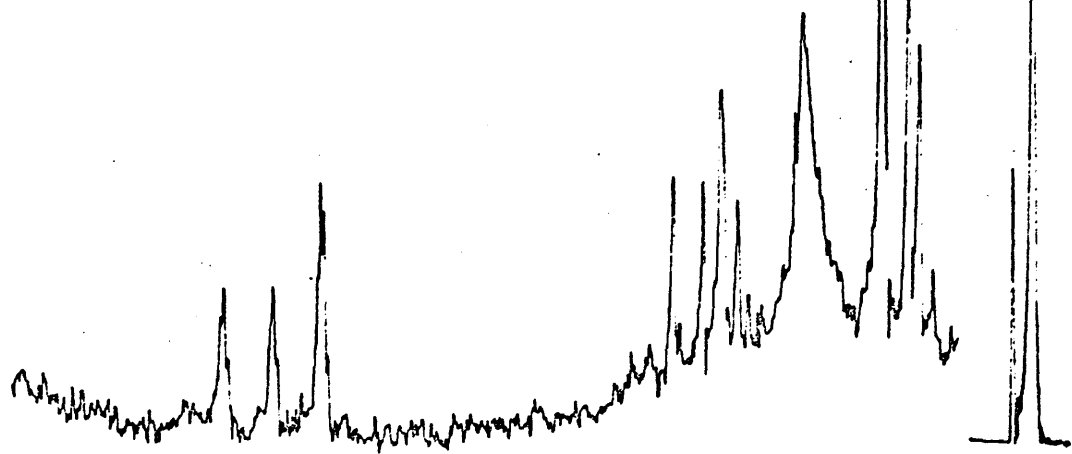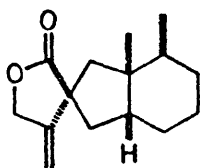
τ values

100 MHz NMR ($CDCl_3$ solution) of

Bakkenolide-A-Diol

$HOCH_2$
$HOCH_2$
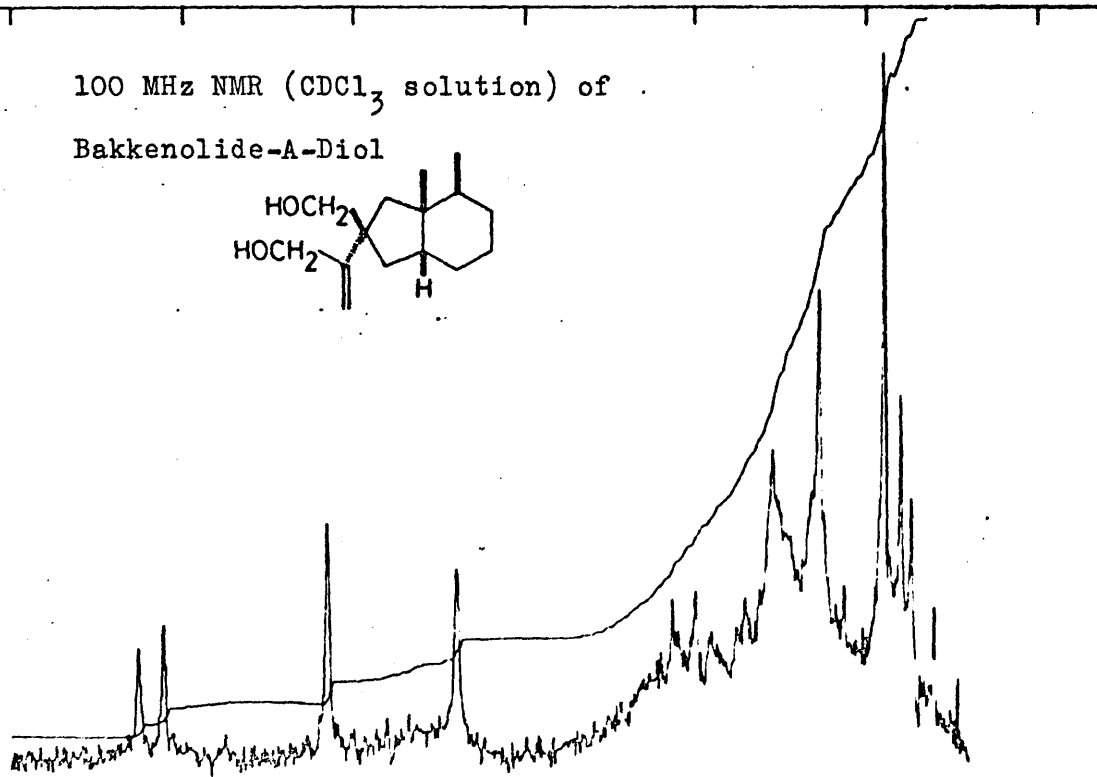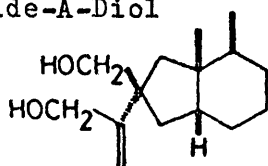
τ values

by a prominent band at 1765cm^{-1}, assigned to a γ-lactone; weaker bands at 3090,1664 and 895 cm^{-1} suggested a terminal methylene. There was no significant UV absorption at wavelengths above 210nm, indicating an unconjugated lactone, which was in any case implied by the carbonyl absorption band in the infra-red.

Nuclear magnetic resonance (NMR) of a solution in CDCl$_3$ provided most of the useful evidence for the assignment of structure. Four low field protons confirmed the presence of a terminal methylene (2H, τ = 4.90,4.98) and an esterified primary alcohol (2H, broad singlet, τ = 5.25). A methyl singlet could be accounted for by an angular methyl group (τ = 8.99), and a methyl doublet occurred at slightly higher field (τ = 9.14). This suggested, but was not conclusive evidence for, an eremophilane-type structure in ring A. A slightly broad signal (3H, τ = 8.02) added some confusion, indicating, in conjunction with the methylene protons, an unsubstituted isopropenyl side chain. These data could not be fitted satisfactorily to a single structure.

When the solvent was changed to benzene, there were quite significant shifts of many signals. The apparent 'singlet' at τ = 8.02 disappeared, and was evidently produced by accidental equivalence of some sharp methylene signals in this region. A part structure could now be drawn

for the lactone moiety, which suggested a spiro-γ-lactone
of the bakkenolide type, produced by contraction of ring B
of an eremophilane skeleton. Bakkenolide-A (38) was first
isolated by Abe in Japan, from <u>Petasites japonicus</u> subsp.
<u>giganteus</u>[46]. Comparison of our own data revealed that
infra-red, 100 MHz NMR and mass spectra were identical with
those reported for bakkenolide-A[47]. Compound <u>18</u> was reduced
to the diol with lithium aluminium hydride, and 100 MHz NMR
of the product was identical to that reported for
bakkenolide-A-diol (39). We consider the correlation of data
(Table 8) good evidence for the identification of compound <u>18</u>
as bakkenolide-A. Bakkenolide-A has also been isolated by
Czech workers from the closely related species <u>Homogyne alpina</u>
(Senecioneae) of European origin[48]. Authentic samples have
subsequently confirmed the above assignments for <u>17b</u> and <u>18</u>

Fraction BI/2; Alcohols:

The systematic survey of retention behaviour
of model compounds in gel chromatography[49], reported in
a later chapter, provides evidence for a general rule
that compounds eluted after SEV 75 in the straight-phase
system, N1114-50%-LH20 with benzene as the mobile phase,
have hydroxylic functions. In accord with this hypothesis,
the fraction eluting in the range SEV 72-115 from such a
straight-phase system was found to consist entirely of
sesquiterpenoid alcohols. GLC of the complex mixture showed

Table 9: Chromatographic Characterisation of the Alcohols of Fraction BI/2

Compound	$I_{OV-1}^{139°}$	SEV a)	M^+ b)
11a	1510	83	222
13	1560	77	222
13a	1570	76	222
14	1590	106	222
14a	1595	88	222
15a	1610	86	222
15b	1620	86	222
15c	1635	88	222
15d	1645	78	220
16	1660	93	220
16a	1665	100	222
17	1685	109	220
17a	1695	123	220
17d	1730	-	220

a) N1114-50%-LH20/benzene
b) Values assigned from AVA scans of (M-18) peaks

eight major components and a number of minor constituents
(Table 9). The average content of these alcohols was
similar to that of the ketones 17b and 17c. One reason
for the large number of components of this type is that they
may in some cases arise by direct cyclisation of farnesyl
pyrophosphate: individual constituents do not necessarily
possess the eremophilane skeleton. The constancy of the
proportions of these compounds from different extractions
suggested enzymic regulation of their production.

It was the primary intention of this investigation
to isolate potential intermediates in the biosynthetic
pathway between farnesyl pyrophosphate and petasin. The
determination of structures irrelevant to this problem was
considered a diversion, and some criterion was required
for the selection of specific components for further
examination.

GC-MS of the total alcohol fraction showed that
there were some alcohols of molecular weight 220 among a
majority having molecular weight 222, but individual
spectra were inhomogeneous because of the complexity of
the mixture and poor resolution of some of the components.
The AVA system on the GC-MS instrument was tuned to detect
ions of m/e 204, 202 and 200, derived by loss of water
from the molecular ion of the alcohols, molecular weight
222, 220 and 218 respectively. This procedure demonstrated

Fig. 9: GLC of Fraction BI/2: Sesquiterpenoid Alcohols

10' 1% OV-1
139°C isothermal
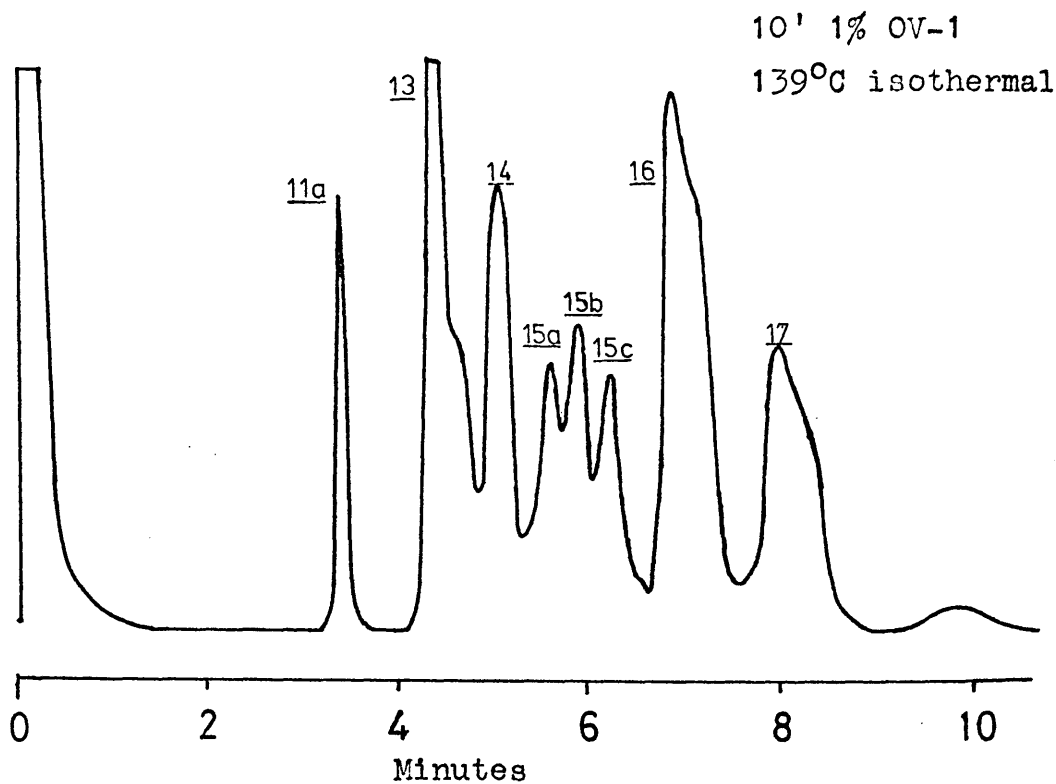
GC-MS/AVA Scan of Alcohol Fraction*

—— m/e 204
······ m/e 202
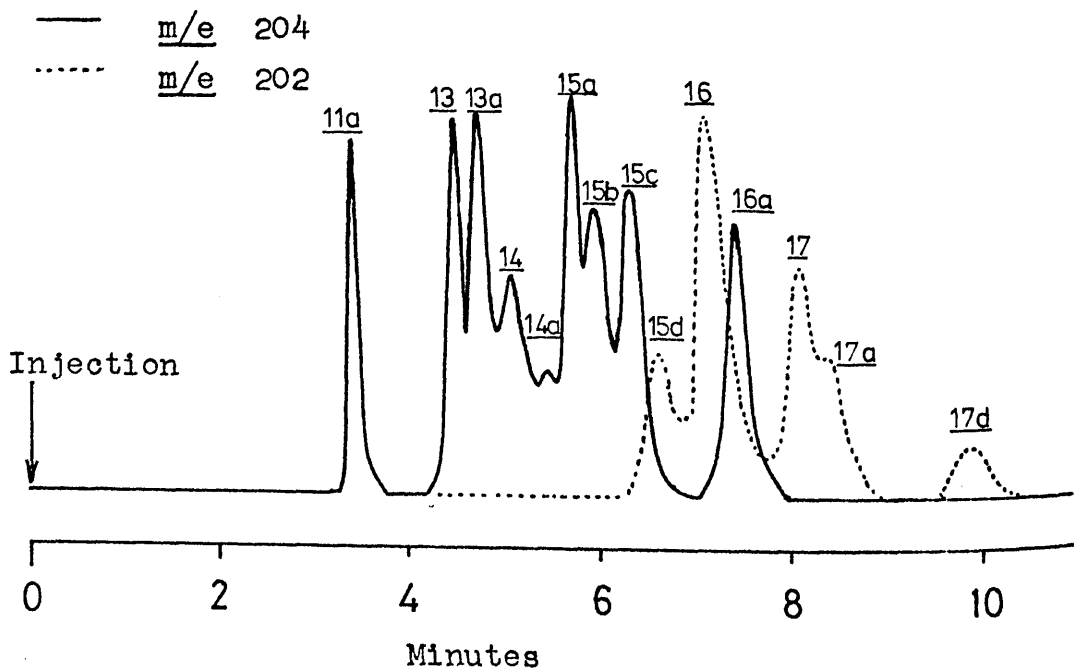
* Peak heights depend on relative abundance of ion scanned
in addition to concentration of individual components.

the fourteen components listed in Table 9, showing clearly
the molecular weight of each, despite difficulties with the
resolution (Fig.9).

The formal derivation of petasin from farnesyl
pyrophosphate requires three oxidative steps in the transition
from the oxidation level of farnesol to that of petasol.
Compounds on the direct biosynthetic pathway are likely
to have intermediate levels of oxidation. Thus the five
alcohols of molecular weight 220, one oxidation level
above farnesol, were likely to be of interest as potential
intermediates. Of these, only compounds 16 and 17 were
present in sufficient quantity for further studies.

Partial separation of the alcohols was carried out
by high-resolution chromatography on the straight-phase
system, with results as indicated in Table 9. Unfortunately,
quantities were insufficient for NMR spectrometry, and the
complex fragmentations of cyclic sesquiterpenoids reduce the
value of mass spectrometry for diagnostic purposes.
Comparison with authentic samples demonstrated the absence
of farnesol and nerolidol, and none of the spectra could be
correlated with sesquiterpenoid alcohols listed in mass
spectral collections. Attempts to oxidise compounds 16 and
17 to the corresponding ketones have so far been unsuccessful;
the small samples available suffered over-oxidation to

indeterminate products. Nevertheless, this technique, suitably refined, in conjunction with isomerisation to conjugated ketones, deuteriation by isotope exchange GLC and mass spectrometry, is a potential practical approach to the elucidation of the structures of these minor constituents.

BI/3 and Later Fractions:

The third group fraction taken from straight-phase chromatography of Fraction BI was specifically designed to isolate petasol (26) and isopetasol (27). The fraction covered the range SEV 115-130, which was the region in which isopetasol, obtained by hydrolysis of petasin esters, was known to be eluted. GLC and GC-MS demonstrated the presence of naturally-occurring free petasol and isopetasol in minute quantities. The ratio of the two isomeric ketoalcohols was similar to that observed for their esters, petasin and isopetasin, and the difference in GLC retention index was also the same. Petasol was otherwise inaccessible, any attempt at hydrolysis of petasin giving rise to isopetasol by isomerisation. The fraction obtained, on standing in contact with alumina overnight, gave isopetasol as the sole constituent.

The mass spectra (Appendix I) of petasol and isopetasol are of interest, because in contrast to
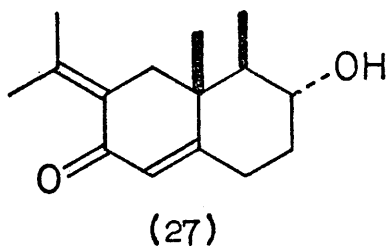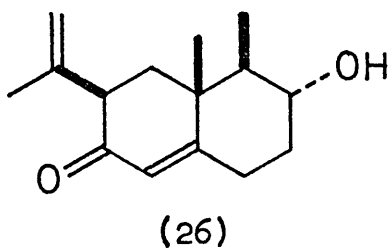
Fig. 10: Proposed Scheme for Mass Spectral Fragmentation of Petasol (26) and Isopetasol (27)

m* 116.7

M+   m/e 234

m/e 166
Base Peak

m* 89.6

m/e 122

m* 72.4

m/e 94

M+   m/e 234
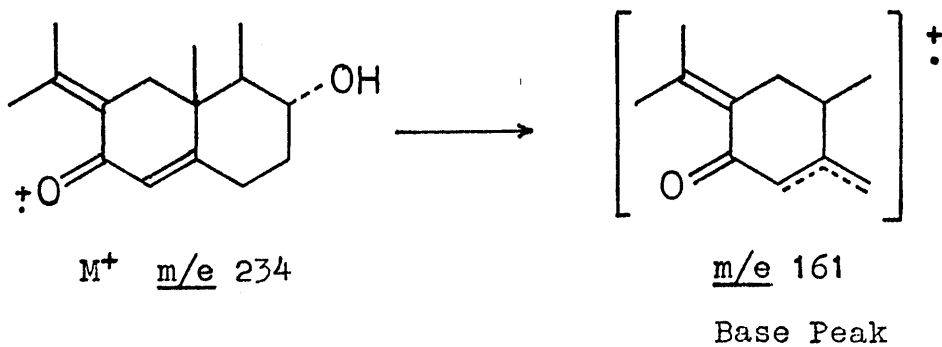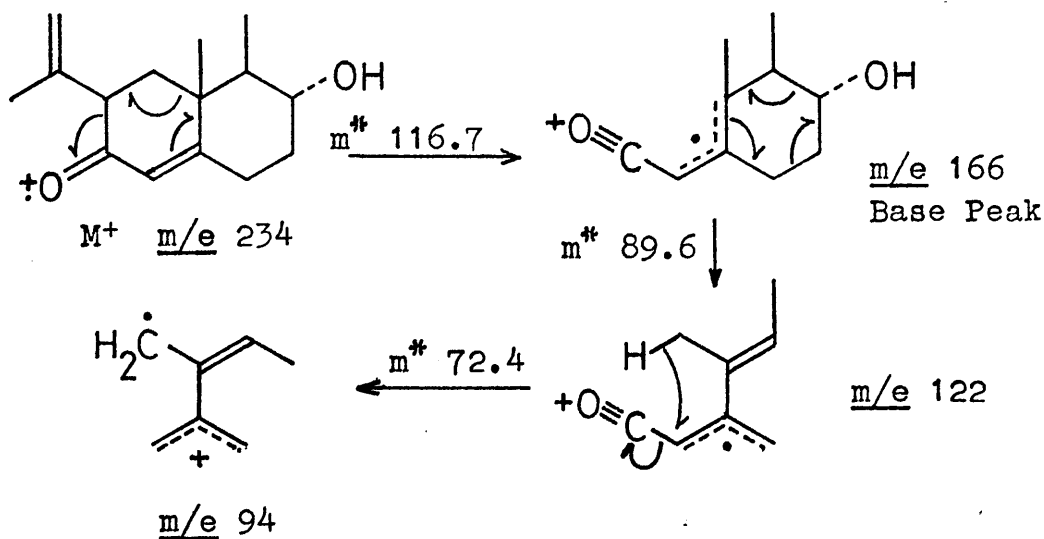
m/e 161

Base Peak

de-acylation of the petasin esters, dehydration was not a major fragmentation process. The base peak of isopetasol, m/e 161, was unchanged, because C-3, the hydroxyl-bearing carbon atom was lost in this fragmentation; that of petasol was shifted from m/e 148, as in petasin, to m/e 166, the loss of isoprene apparently being more facile than dehydration. A retro-Diels-Alder rearrangement resulting in loss of acetaldehyde might account for an ion at m/e 122, and expulsion of carbon monoxide permits derivation of a hydrocarbon fragment corresponding to the ion observed at m/e 94. Metastable peaks were observed for each of these transitions.

Collection from the straight-phase column was continued up to SEV 500, but GLC of the fractions gave no further peaks in the expected region for sesquiterpenoid ketoalcohols or diols. The column was then purged with an isopropanol-benzene mixture (1:3 v/v), which was known to be effective in eluting rapidly samples that were retained by the stationary phase when benzene alone was used as the eluant [49]. This final fraction was further separated by ion-exchange chromatography using triethylaminoethyl-cellulose (TEAE-cellulose), which gave neutral, 'weak acid' and 'strong acid' fractions.

TLC of the neutral compounds gave two major spots with a pink colouring when sprayed with Liebermann-Burchard reagent and heated briefly[35]. Comparison with literature R_f values suggested sterol glycoside as the spot of lower R_f[50], and esterified sterol glycoside for the less polar spot[51]. This reaction with spray reagent suggested Δ^5-type sterols[50], and acid hydrolysis of a sample of the neutral fraction gave sterols, mainly β-sitosterol, identified by chromatographic behaviour. No sesquiterpenoid was found as a result of the acid hydrolysis.

GLC of the 'weak acid' fraction gave only indefinite peaks, as expected for free carboxylic acids. Treatment with ethereal diazomethane, and repetition of the GLC showed a number of sharp peaks that clearly formed a homologous series. Comparison of GLC retention behaviour, on the stationary phases 1% OV-1 and 0.5% XE-60, with authentic standards, demonstrated the identity of the peaks as methyl esters of even-numbered, saturated, straight chain fatty acids. Two maxima of abundance were observed corresponding to the C_{16} and C_{18} acids of intracellular lipids, and the C_{24} and C_{26} acids of cuticular wax. No sesquiterpenoids were apparent.

The 'strong acid' fraction was treated with dilute hydrochloric acid to promote hydrolysis of pyrophosphate

Table 10: Chromatographic Characterisation of
Hydrocarbons of Fraction BII/2

Compound	$I_{OV-1}^{114°}$	SEV [a]	M^+
6	1375	217	204
6a	1380	204	204
7	1410	196	204
8	1445	195	204
9	1465	192	204
9a	1470	219	204
10	1485	161	204
10b	1495	161	204
11	1505	235	204
12	1525	203	204

a) N1114-50%-LH20/methanol

esters. The fraction was then extracted with ethyl acetate
and the extract examined by GLC. No sesquiterpenoids were
apparent before or after hydrolysis.

Fraction BII/2; Hydrocarbons:

Whereas oxygenated sesquiterpenoids were eluted
relatively early from reversed-phase columns, sesquiterpene
hydrocarbons were retained to a significantly greater extent,
permitting their isolation as a separate fraction at this
stage. The hydrocarbon fraction, BII, from reversed-phase
chromatography, was purified further by straight-phase
methods (Fig.3; opposite p.18). A narrow fraction,
SEV 62-75, contained all the sesquiterpene components,
compounds of higher molecular weight being effectively
removed at this stage.

GLC of the total hydrocarbon fraction showed
the presence of at least ten compounds. AVA scanning for
ions having m/e 204, 202 and 200 indicated that all these
compounds had a molecular weight of 204, and unresolved
components of higher oxidation level were absent (Table 10).
This molecular weight is expected for a sesquiterpene
derived by direct cyclisation of farnesyl pyrophosphate.

The complex mixture was then separated by
high-resolution reversed-phase chromatography (Table 10).

(4)
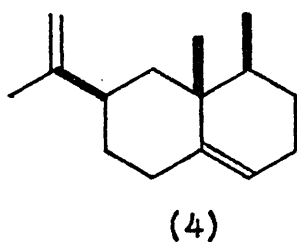
Fig. 11a: Proposed Scheme of Mass Spectral Fragmentation
for Eremophilene (4)

M⁺ m/e 204

m/e 161

m* 88.0

m/e 119

m/e 109

Base Peak
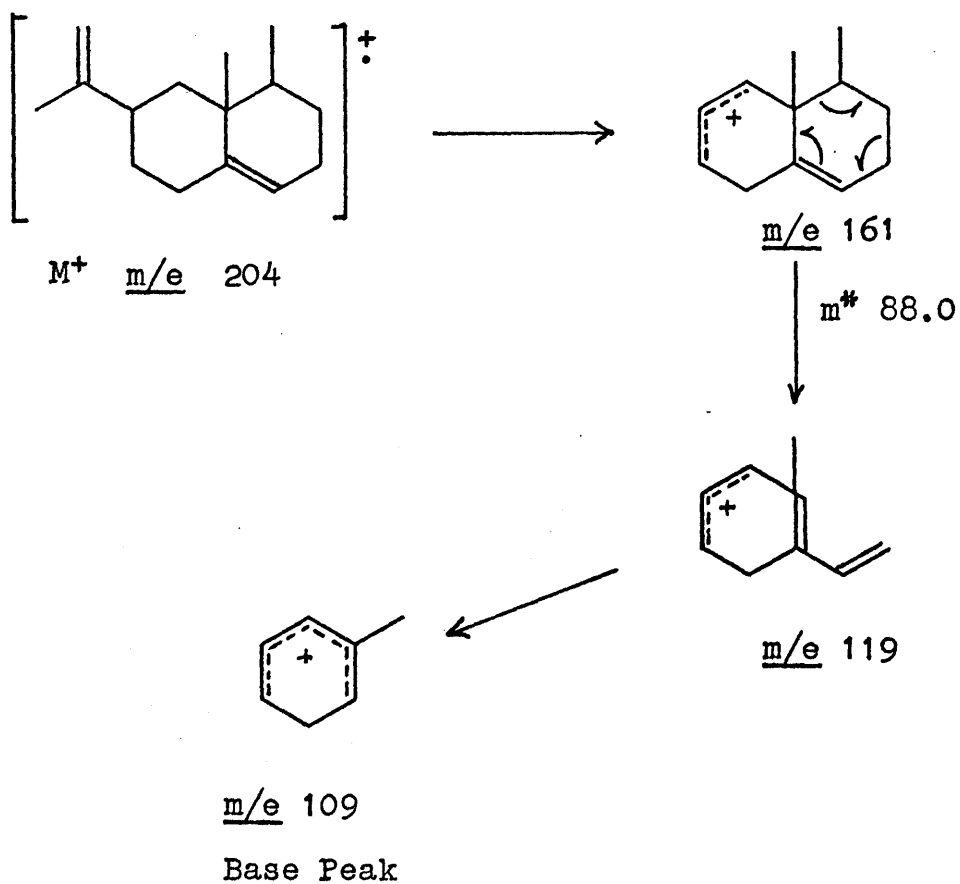
100 MHz NMR (CDCl$_3$) of Eremophilene (4)
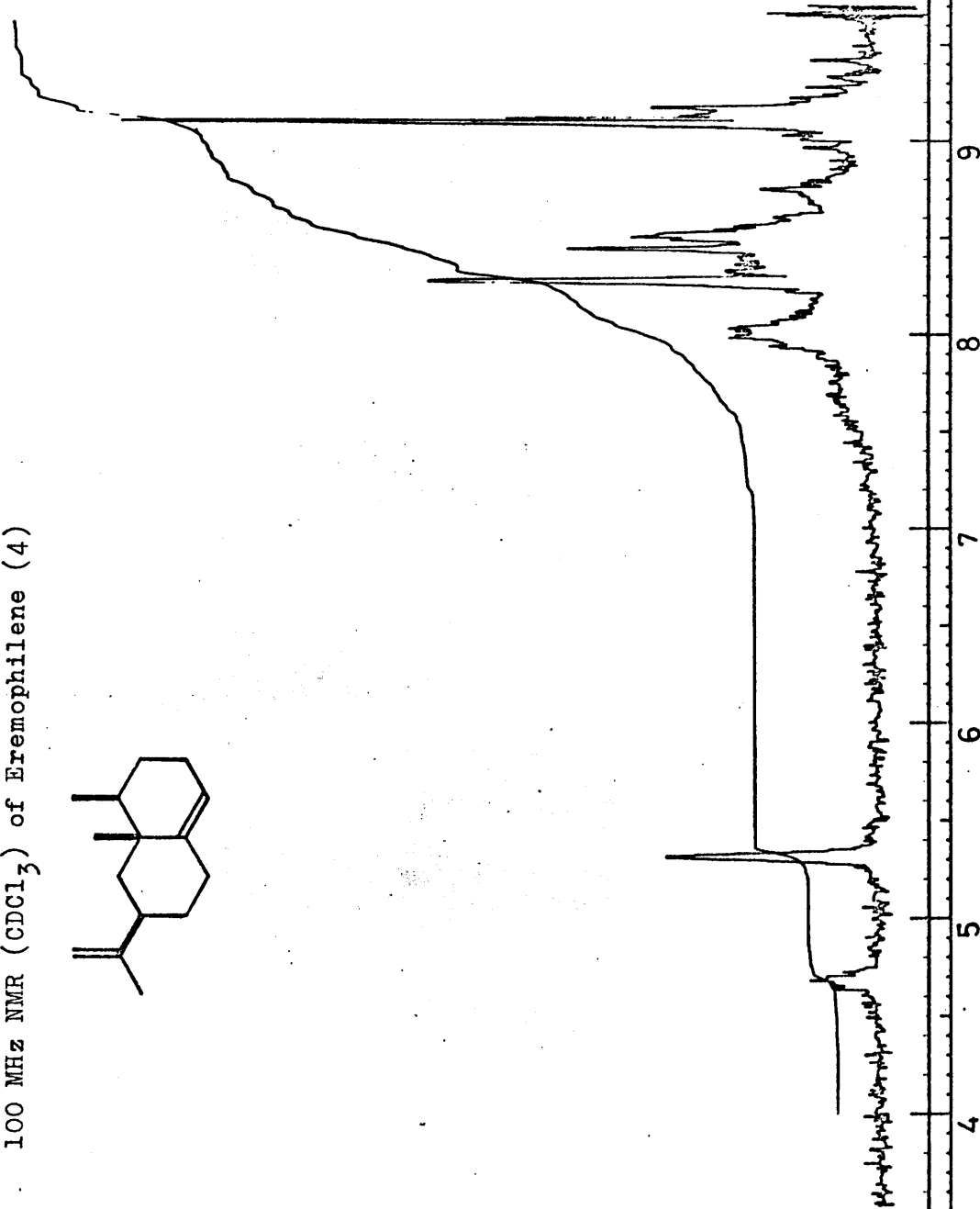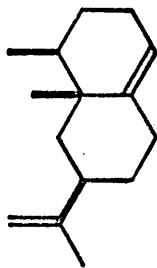
τ values

Pure samples of compounds 9, 9a, 11 and 12 were obtained,
and NMR spectrometry permitted structures to be assigned
to three of these. Compound 9a was found to be eremophilene (4)[14]
by comparison of the 100 MHz NMR spectrum with a spectrum
of authentic material generously supplied by Dr. L. Novotný
of the Czechoslovak Academy of Science. Comparison of
infra-red spectra[32] supported this assignment. A sample
of eremophilene, also supplied by Dr. Novotný, was found to
contain equal quantities of compounds 9 and 9a. Presumably,
the sample had been collected by preparative GLC, and
collection of a mixture had arisen because of the similarity
of retention characteristics of these two compounds on many
stationary phases. Compound 9 was clearly not eremophilene
(as discussed below). The component of the sample
corresponding to compound 9a in GLC retention indices
was found also to give a mass spectrum identical to
compound 9a. The fragmentation pattern was of little
diagnostic value (Fig.11a;Appendix I).

The 100 MHz NMR spectrum of compound 9 showed
five protons at low field. A sharp doublet (1H; τ = 4.21)
coupled with a quartet (1H; τ = 4.77; J = 16 c/s) was
indicative of a trans- disubstituted double bond[52] (Fig.11b),
confirmed by an infra-red band at $980cm^{-1}$. Of known
sesquiterpenes, only the humulenes have a trans-

$J_{ab} = 16 \text{ c/s}$

$J_{bc} = 10 \text{ c/s}$

$J_{bd} = 0$

$\phi = 90°$
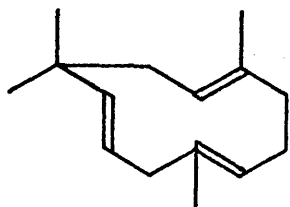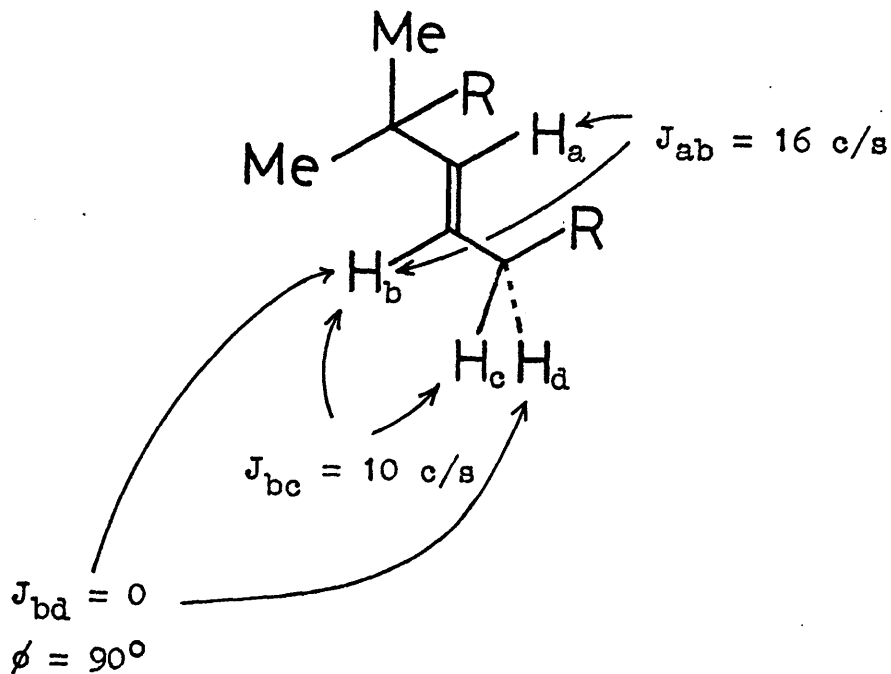
(40)
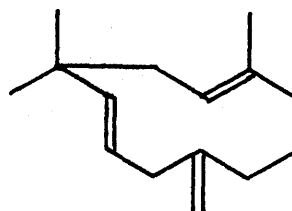
(41)
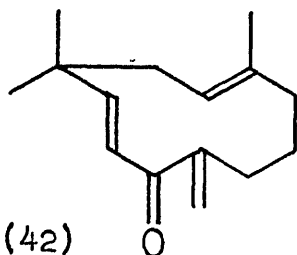
(42)

100 MHz NMR (CDCl$_3$) of β-Humulene

16 c/s →

10c/s
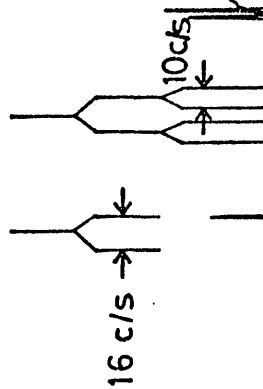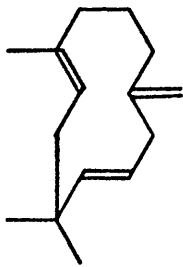
τ values

disubstituted double bond in a cyclic structure[53].

Comparison of the infra-red spectrum of compound $\underline{9}$ with those published by Šorm[54] for α- and β- humulenes (40,41) demonstrated a striking similarity with that of β-humulene (41). The exomethylene group that distinguished the β- isomer was apparent from a strong band in the infra-red at 890 cm^{-1} and a characteristic AB system in the NMR spectrum[52] (2H; τ_A = 5.22, τ_B = 5.27; J = 2 c/s). The absence of a weak band at 830 cm^{-1} in the infra-red spectrum of compound $\underline{9}$ could be attributed to the freedom from contamination by α-humulene. Šorm claimed only 83% purity of the β-isomer in his investigation[54] and the 830 cm^{-1} band is prominent in the spectrum of α-humulene. The high-field region of the NMR spectrum was consistent with the structure of β-humulene, and indicated stability on the NMR time scale of two conformers at room temperature. This situation has previously been noted for zerumbone (42), but α-humulene required reduced temperatures to give any sign of slow conformational interconversion[55].

It appears highly likely that compound $\underline{9}$ has the structure of β-humulene (41).

The NMR spectrum of compound $\underline{12}$ lacked signals above τ 8.4, which restricted the structure of this sesquiterpene to one having all methyl groups allylic, excluding most bicyclic
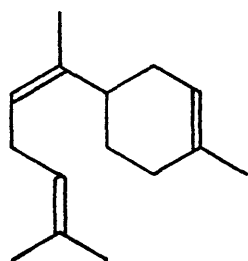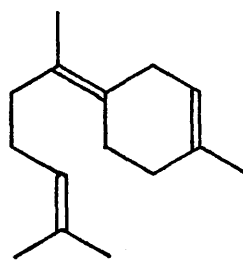
(43)   (44)

Fig. 12: Correlation of Compound 12 with the Structure
         of α-Bisabolene (43) by NMR and Mass Spectral
         Data.

J = 7 c/s

τ = 7.31

J = 7 c/s

M⁺   m/e 204

-H₂

m/e 109
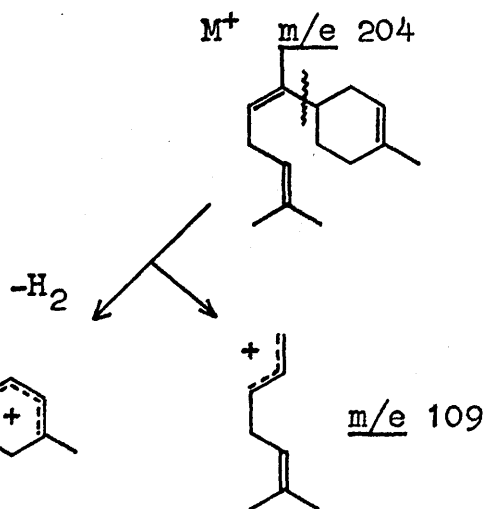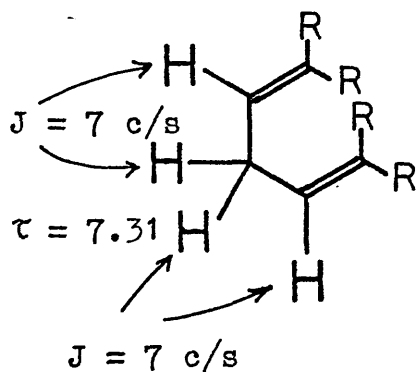
m/e 93

Base Peak

100 MHz NMR (CDCl$_3$) of α-Bisabolene (43)

7 c/s

7 c/s
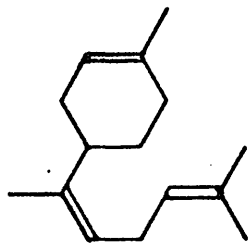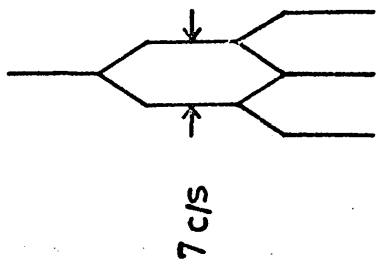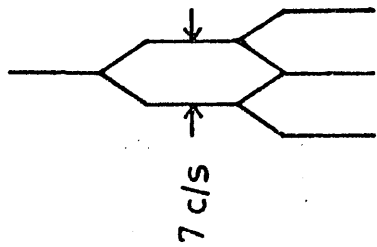
τ values

skeletons. Peaks corresponding to four allylic methyl groups, (3H, $\tau = 8.29$; 9H, $\tau = 8.36$) occurred as broad singlets. A doubly allylic methylene triplet (2H, $\tau = 7.31$, J = 7c/s) suggested a part structure fitting the molecules of α-bisabolene (43) or γ-bisabolene (44)[23, 53] (Fig.12). The triplet fine structure of this signal was better explained by a symmetrical environment as in α-bisabolene (43). The part structure was confirmed by the occurrence of two low-field signals due to vinylic protons (1H, $\tau = 4.61$; 2H, broad triplet, $\tau = 4.88$, J = 7c/s), thereby excluding structure (44) with only two vinylic protons.

The mass spectrum gave a base peak m/e 93, favouring the α- rather than the γ- isomer of bisabolene, and a prominent ion m/e 109 was probably the fragment complementary (in terms of carbon skeleton) to that of the base peak (Fig. 12). A radical-ion, m/e 136, might arise from retro-Diels-Alder cleavage of the ring, and though this is a comparatively minor fragment, further cleavage or rearrangement may result in ions m/e 109 and 93. On the basis of the above evidence, compound 12 may be tentatively assigned the structure of α-bisabolene (43).

No structure could be assigned to compound 11 on the basis of spectroscopic evidence alone.

(5A)

OPP = Pyrophosphate

(45)

Postulated Involvement of
β-Germacrene (45) in the
Biosynthesis of Eremophilene

(4)
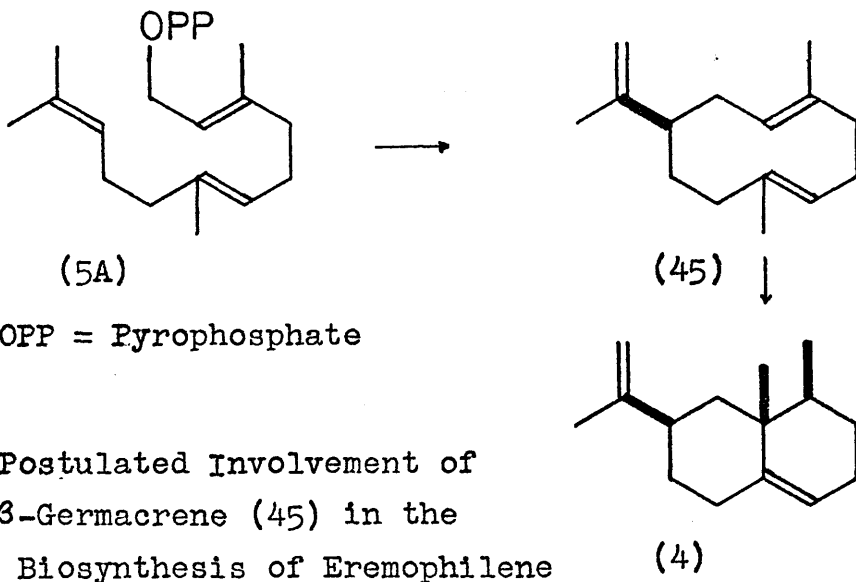
Fig. 13: Correlation of the Postulated Fragmentation of
β-Germacrene with the Observed Mass Spectral
Fragmentation of Compound 7

M^+ m/e 204

$[C_3H_5]^+$

Base Peak

m/e 161
42%

m/e 133
70%

m/e 93
96%

m/e 69
97%
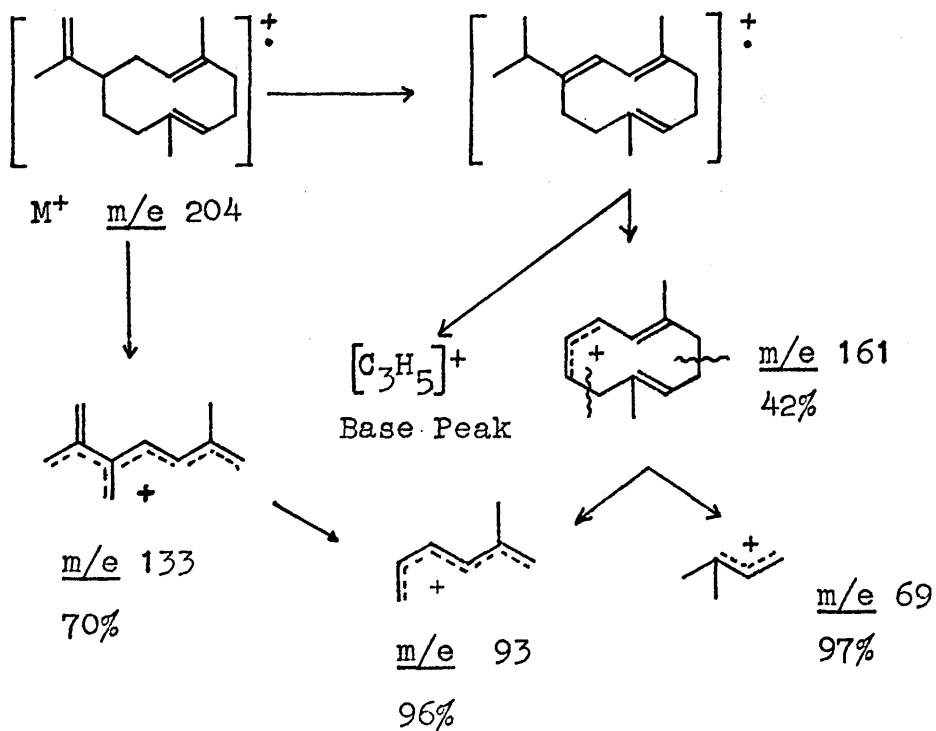
The last major hydrocarbon component of the
sesquiterpenoid fraction was compound 7 which could not be
isolated free from β-humulene by chromatographic methods,
making spectroscopic study impossible except by GC-MS.
A highly speculative allocation of structure may be obtained
from the mass spectrum (Appendix I), β-germacrene (45)
being well fitted to the fragmentation pattern observed.
The migration of the isopropenyl double bond into the
ring system must be assumed to account for the ion m/e 161,
and this fragment was seen also in the mass spectrum of
eremophilene and most other sesquiterpenes of this degree
of unsaturation [56]. The major peaks of the mass spectrum
could then be rationalised by postulating the fission of
homoallylic bonds (Fig.13). The peaks m/e 69 and 133
might arise by similar processes involving the molecular
ion. β-Germacrene (45) is important as a postulated
intermediate of cyclisation of farnesyl pyrophosphate (5)
to eremophilene (4) and hence may be extremely important
in the biosynthesis of petasin (1). (For a discussion,
see sections 3.2.1 and 5.1.)

In the original investigation of sesquiterpene
hydrocarbons of P. hybridus, Novotný and Herout reported
the presence of eremophilene (4), the still unelucidated
tricyclic petasitene, α- and β- humulenes (40,41),

(4)

(46)

(40)

(41)

(47)

(44)

(20)   H

(25)

(24)
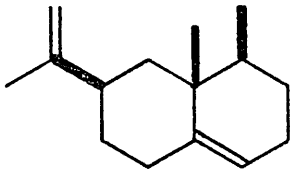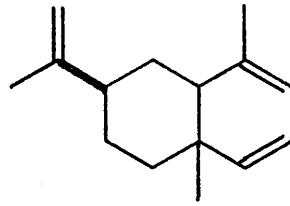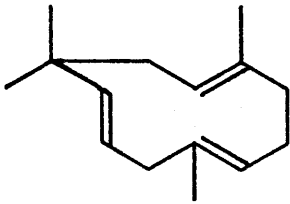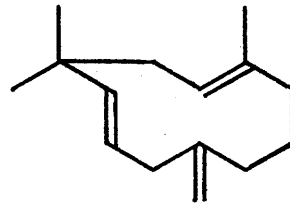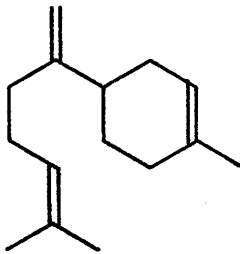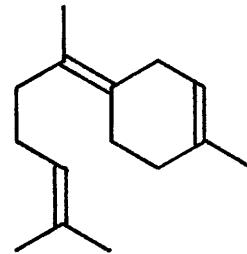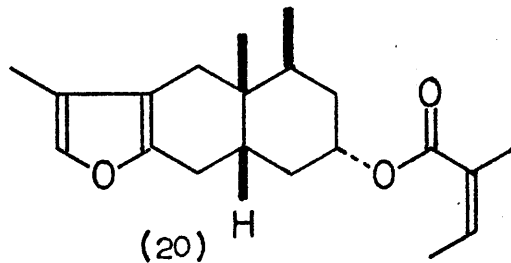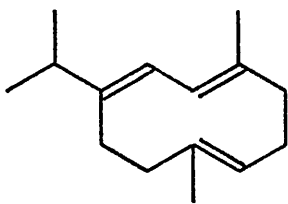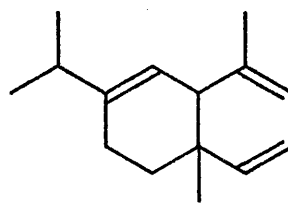
β-elemene (46) and β- and γ- bisabolenes (47,44)[32].

Although their specimens of P. hybridus were of the variety

producing furanopetasin (20)[12], they subsequently

demonstrated that this series of hydrocarbons was

generally distributed among Petasites species[11,57].

Similar compounds may thus be expected to occur in

P. hybridus of the petasin-producing type. However, their

isolation procedure involved steam distillation, and

rearrangement from the isomers reported in this thesis

to those identified by the Czech workers might have

occurred in this process. Certainly the facile rearrangement

of germacrene-C (25) to δ-elemene (24) is known[37].

In this light, our assignments agree in part with the

findings of Novotný, Herout and co-workers.

2.2.6   Quantitative Estimation of Leaf Sesquiterpenoids

The observation that different specimens of

P.hybridus leaves gave substantially the same proportions

of sesquiterpenoid constituents suggested strongly that

these are genetically regulated. The asexual mode of

propagation of P. hybridus would account for this

observation. In contrast, other workers have found

substantial variation from sample to sample, but in species

where pollination and seeding is the normal reproductive

Table 11: Quantitative GLC Estimation of Leaf Sesquiterpenoids of _Petasites hybridus_

Compound	Assigned Structure or Formula	Concentration mg/100g fresh wt	GLC Standard & Isothermal Temp.
6 & 6a	$C_{15}H_{24}$	1.0	
7	$C_{15}H_{24}$	2.0	
8	$C_{15}H_{24}$	0.3	
9	β-humulene	16.0	β-humulene
9a	eremophilene	4.0	110°
10	$C_{15}H_{24}$	1.5	
10b	$C_{15}H_{24}$	1.2	
11	$C_{15}H_{24}$	1.2	
12	α-bisabolene	2.5	
11a	$C_{15}H_{26}O$	1.0	
13	$C_{15}H_{26}O$	3.7	
14	$C_{15}H_{26}O$	1.7	
15a	$C_{15}H_{26}O$	0.6	
15b	$C_{15}H_{26}O$	0.8	drimenol
15c	$C_{15}H_{26}O$	0.7	135°
16	$C_{15}H_{24}O$	1.3	
16a	$C_{15}H_{26}O$	0.8	
17	$C_{15}H_{24}O$	1.0	
17a	$C_{15}H_{24}O$	0.4	
17b	fukinone	0.05	
17c	eremophiladienone	0.4	bakkenolide-A
18	bakkenolide-A	6.0	145°
20a	petasol	0.02	isopetasol
20b	isopetasol	0.01	162°
23	C_4 ester of petasol	1.7	
25	C_5 ester of petasol	9.3	
26	petasin	41.6	isopetasin
27	isopetasin	29.1	225°
28	isomer of isopetasin	2.7	
31	S-isopetasin	11.7	

GLC on 10' 1% OV-1, 50 ml/min N_2

habit[58,59]. Zabkiewicz has found in <u>Pinus radiata</u> that terpene composition was identical in trees of the same clone obtained by grafting of stock, but showed dramatic variations between trees of different genetic origin[60].

This prompted an effort to obtain a quantitative record of sesquiterpenoid content. Such results are more likely to have significance in <u>P. hybridus</u> than in plants where individuals are likely to determine the proportions of terpenoid constituents according to their genetic origin. Furthermore, sesquiterpenoids are sufficiently involatile not to be affected greatly by changes in climatic conditions, unlike monoterpenoids, where simple evaporation may account for sudden changes in levels after a hot day[15].

It should be stressed that the results quoted in Table 11 refer to leaf material of <u>P. hybridus</u> growing in the wild state, and that significant variation was found in other tissues, and in laboratory grown plants where long-term environmental changes may have interfered with normal regulatory mechanisms.

2.2.7.   <u>Comparison of Flower, Rhizome and Leaf Sesquiterpenoids</u>

The studies reported in this chapter have concerned leaf material exclusively, for the simple reason that at the time of these experiments, incorporation of

Fig. 14: Comparison by GLC of Extracts from Different Tissues of P. hybridus

10' 1% OV-1; Temperature Programmed 100°– 250° 4°/min

a) Mature Leaf

b) Rhizome (Winter)

c) Flower Bud
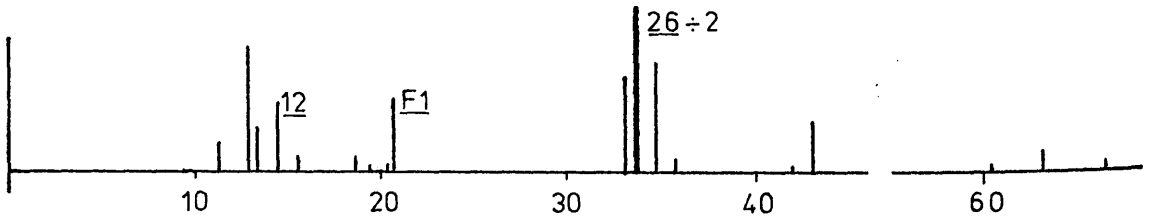
d) Opened Flower

e) Emerging Leaf

f) 'Precursor' Leaf

Numbering of Peaks as for Table 3
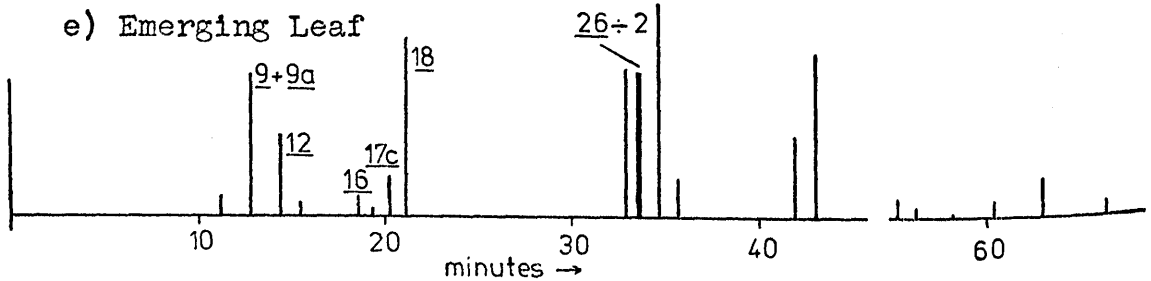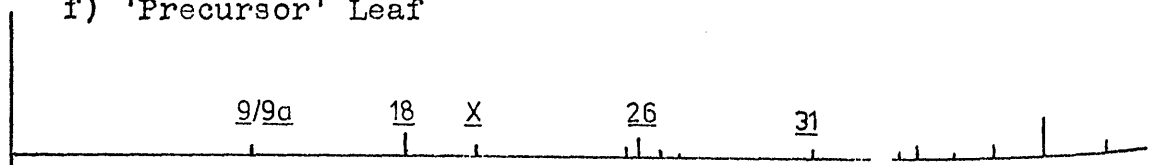
radioactivity from fed precursors into sesquiterpenoids
was only known to occur in the leaf[7]. This finding did
not imply that synthesis did not take place in other
tissues, but merely that translocation of precursors was
directed to the leaves under the conditions of the experiment.
(c.f. Chapter 4.) Subsequently, better understanding of
the conditions for growth have allowed investigation of
flowers, both for their chemical content and for tracer
labelling experiments, when incorporation was found to
be superior to that obtained in leaves (section 3.2.3).

Preliminary separations as discussed in section
2.3.4 were carried out on extracts of rhizome, flowers
and young emergent leaves. GLC of these samples
demonstrated that petasin was the dominant sesquiterpenoid
in all tissues. Simple comparative estimation indicated
that the petasin ester content of rhizome and flower
exceeded that in leaves by a factor of two. There were
considerable differences in the content of other constituents.

The gas chromatogram from the rhizome extract
(Fig.14b) indicated an additional major component, R5, and
five other new peaks in appreciable quantities, R1-6.
R5 had a retention index slightly less than petasin and
gave a molecular ion, m/e 332 (GC-MS). Bakkenolide-A,
compound 18, appeared to be virtually absent, a peak in

this region of the chromatogram having the retention index of compound 17c rather than compound 18 of the leaf extract (Fig.14a). There were substantial changes in the distribution of the alcohols and hydrocarbons, and clear correlation between leaf and rhizome constituents was only possible for the hydrocarbons. The high molecular weight region of the chromatogram was dominated by a compound R6 of higher retention index than β-sitosterol. This sample of rhizome consisted of fully vernalised tissues gathered in mid-winter, about two months before the normal flowering season.

Flowers varied in content according to the phase of development. Immature buds were similar in some respects to the rhizome, but the components peculiar to the rhizome R1-6 appeared to be virtually absent (Fig.14c). Bakkenolide-A was again absent, being replaced by another new compound F-1 which could not be correlated with any leaf component on the basis of GLC retention data. Flowers at a later stage of growth, with many florets opened, were notable for the virtual disappearance of non-esterified sesquiterpenoids (Fig.14d).

Normal emergent leaves (Fig.14e) were similar in content to mature leaves (Fig.14a), with slight enhancement of some components, in particular, those having the retention indices of compounds 16 and 17c.

It was generally observed that the first leaf
of the growing season possessed unusual morphology, and
failed to mature, the maximum growth, 3-5cm span, being
rapidly exceeded by second and subsequent leaves. GLC
of a sample of these 'precursor' leaves showed the virtual
absence of sesquiterpenoids, sterols being the major
components of the GLC-volatile fraction (Fig.14f).
The actual sterol concentration was comparable to that
of normal leaf extracts: these differences represent a
drastic change in the leaf chemistry. Plants grown in the
laboratory, and kept without vernalisation for two seasons,
showed similar defects, both in morphology and terpenoid
content. These results stressed the importance of obtaining
fresh plant stock from wild sources each winter.

These observations suggest a number of
speculations as to the metabolic status of sesquiterpenoids
of P. hybridus. Synthesis of petasin esters would appear
to occur in leaves, in proportion to their growth, if the
generally constant concentration of these compounds
isolated from leaf samples is to be explained. In the
flower, synthesis was apparently limited to a short period
in the early 'compact' phase of the developing inflorescence.
This conclusion assumes that the disappearance of the free
sesquiterpenoids in mature flowers resulted from the

cessation of synthesis and gradual loss by evaporation
of all but the comparatively involatile petasin esters.
The implications for the feeding of precursors are of
great importance. There would appear to be no particular
optimum for leaf feedings, as this process is apparently
continuous. In the flower, potentially higher rates of
synthesis appear to be concentrated in the early phases
of development. Feeding to immature flower buds might thus
result in considerably enhanced incorporation of mevalonic
acid into petasin esters.

2.3   EXPERIMENTAL

2.3.1   General Chromatographic and Spectroscopic
        Procedures[*]

Thin-layer chromatography (TLC) was carried out
on plates prepared from Merck 'Kieselgel-G' using 0.25mm
layers for analytical purposes, and 1.0mm layers for
preparative use. Silver nitrate-impregnated layers were
prepared by including 5% by weight of silver nitrate
(relative to the weight of the silica-gel) in the slurry
used to prepare the plates[61]. The following reagents were
used for the detection of bands:-

3-hydroxy-pyrene-5,8,10-trisulphonic acid trisodium salt,
used as a non-destructive fluorescent dye for visualisation
by quenching or fluorescence of bands under ultra-violet
illumination;

50% sulphuric acid, as a general purpose detection
reagent;

Stahl-Müller reagent[35] for the detection of
furanosesquiterpenoids[12];

Liebermann-Burchard reagent[35] for the detection of
sterols and oxygenated sesquiterpenoids.

[*] These details are generally applicable throughout
    this thesis.

Table 12A: GLC Columns for Aerograph Model 204 Gas Chromatograph

1	10'	1% OV-1	Gas Chrom-P
2	7'	0.5% XE-60	Gas Chrom-P
3	7'	5% Carbowax 20M Gas Chrom-Q
4	5'	3% OV-22	Gas Chrom-P

Table 12B: Columns for Preparative Gel Chromatography

Column	i.d. cm	Length cm	Bed Volume ml	Packing	Mobile Phase
I	3.2	30	260	N1114-50%-LH20	Benzene:Isopropanol 3:1 (v/v)
II	2.0	14	49	N1114-50%-LH20	Methanol
III	2.5	30	123	N1114-50%-LH20 24-32μ	Benzene
IV	1.0	100	76.0	N1114-50%-LH20 17-23μ	Benzene
V	1.0	100	72.8	N1114-50%-LH20 24-32μ	Benzene
VI	1.0	30	25.5	N1114-50%-LH20 17-23μ	Benzene

Gas-liquid chromatography was accomplished
using the Varian Aerograph Model 204 dual column
gas chromatograph fitted with hydrogen flame-ionisation
detectors. Modifications permitted the use of glass
columns, with direct injection of samples onto the
packing, avoiding contact with the metal walls of the
flash heater in the conventional assembly. Packings were
prepared by the methods of Horning[62]. Acid-washed and
silanised Gas Chrom P (Applied Science Laboratories Inc.),
sieved to 100-120 mesh size, was used as the solid support.
The following stationary phases were employed:-

OV-1 methyl siloxane polymer;

OV-17 and OV-22 phenyl/methyl siloxane polymer;

XE-60 cyanoethyl/methyl siloxane polymer;

Carbowax 20M polyalkylene glycol (M.W. ca. 20,000).

Columns were made up from Pyrex glass tubing, 3mm i.d.,
and were 5, 7 or 10 foot in length (Table 12a). Normal
operating conditions used nitrogen as carrier gas, at a
flow rate of 50 ml/min.

Columns for preparative liquid chromatography
were packed with the modified Sephadex gel[18] N1114-50%-LH20
using the methods for preparation and packing detailed
in section 6.3.1. Ordinary columns used a preparation of

gel from commercial Sephadex LH-20 (Pharmacia Fine Chemicals).
Where especially high resolution was required, preparations
were made from Sephadex G-25 (Superfine grade), with
further fractionation to ranges of uniform particle size,
17-23μ and 24-32μ (diameter of dry beads). Six columns
were used routinely for the separations described, and
their characteristics have been listed in Table 12b.
Fractions were collected from the ordinary columns by
estimation of the required elution volume from known
retention data. Normally, the volume of solvent supplied
to the column was measured, thereby avoiding the necessity
of collecting the eluant in graduated containers.
An automatic fraction collector (BTL Chromafrac; or Central
Fraction Collector) was used to collect samples from
high resolution columns. Fraction changing was made on
a timed basis, the interval being adjusted so that each
component would be distributed over three to five fractions.

Ultra-violet spectra were measured on the
Unicam Model SP 800 automatic recording spectrophotometer.
Infra-red spectra were obtained using the Unicam Model
SP 200 spectrophotometer for routine spectra, or the
Perkin-Elmer Model 257 spectrophotometer for solution
spectra. Mass spectra were recorded with the LKB 9000
combined gas chromatograph - mass spectrometer when the
GLC sample introduction system was required, or with the

AEI MS-12 mass spectrometer when samples were obtained unsuited to GLC. NMR spectra were determined at 100MHz with the Varian Model HA-100 spectrometer.

Melting points were determined on a Kofler micro hot stage. Optical rotation was measured with a Hilger-Watts 'Micro-optic' polarimeter using a sodium lamp as light source.

Thanks are due for the services of Mr. J.M.L. Cameron and his staff for microanalysis, Mrs. F. Lawrie (Infra-red laboratory), Miss J. Johnston (GC-MS) and Mr. J. Gall (NMR).

2.3.2   Plant Material

Plant material was taken from localities where
it was found growing wild, and identified according to
the description given in "Flora of the British Isles"[34].
The particular feature that distinguished leaf samples
of P. hybridus ('Butterbur') from P. albus, also readily
found growing wild, and from the morphologically similar
Tussilago ('Coltsfoot') species, was the presence of a
basal leaf vein.

Rhizomes from a site near Motherwell[7] were
lifted after vernalisation in the early winter, after
several frosts had caused the old season's leaves to
wither. This was carried out in the seasons of 1967-68
and 1968-69, and the rhizomes gathered were propagated
at the Botany Department annexe in the Garscube Estate.
In the winter of 1969-70, rhizomes were taken from a new
source near Dumbarton, after the demise of the Motherwell
plants as a result of housing development. GLC examination
of extracts of samples from each site indicated that the
sesquiterpenoid contents were comparable.

The chemical studies reported in this chapter
were carried out on leaf, flower and rhizome specimens
from plants growing wild. Plants for labelling studies
described in following chapters were grown from vernalised
rhizomes gathered and replanted in mid-winter, in pots

7" in diameter, or in polythene bags equivalent in capacity to 12" pots. The increased capacity for root development in the latter cases proved to be an advantage. Growth could be induced in these plants before the normal season by transfer to warmer greenhouse conditions, flower and leaf development following about a month after the change in environment. Plants from a previous season could be kept in the greenhouse over the winter: however, without vernalisation, these were found to become vegetative, and did not develop flowers. Both growth of leaves and sesquiterpenoid production were impaired when plants were kept in this manner, and the conditions were considered unsuitable for experimental purposes.

Our thanks are due to Dr. A.M.M.Berrie of the Botany Department for providing these facilities, and for advice on botanical aspects, and for supervising the maintenance of our plant stock; also to the gardeners and ground staff at the Garscube Estate for most of the work involved in keeping the plants.

2.3.3   Extraction of Plant Tissues

Originally, extraction was carried out by maceration of leaf or rhizome samples in benzene [7] and steeping for 24 hours. Investigations showed that benzene

alone was an inefficient extracting solvent, and that water present in tissues tended to separate to form gels, trapping substantial amounts of organic matter in tissue residues. Acetone, chloroform-methanol mixtures and isopropanol proved to be highly effective in extractions, judged by exhaustive re-extraction of residues. In the case of isopropanol, subsequent evaporation of the solvent also removed co-extracted water as an azeotrope, and there was no need for further drying of extracts.

Normal procedures involved immersing fresh tissue cut into small pieces in ten times the volume (w/v) of cold isopropanol. Preliminary shredding, in the Townson and Mercer 'Top Drive' Macerator for 30 seconds, reduced the sample to fragments of diameter approximately 1-3mm, and destroyed the fibrous structure. This step was omitted for samples of less than 30g fresh weight of tissue. Homogenisation for one minute at maximum speed, using the Polytron Ultrasonic extractor, was followed by filtration of the suspension, and re-extraction twice more in the same manner.

The combined extracts were then evaporated to small volume ($\sim$ 25ml/100g fresh weight) in the Büchi rotary evaporator, and an equal volume of benzene was added. The mixture was allowed to stand for 3-6 hours,

and a white powdery precipitate formed, composed of non-lipids extracted by the large bulk of the isopropanol. The extract was cleared by filtration through Celite 535. The volume was further reduced to 5ml/100g fresh weight on the rotary evaporator, and an equal volume of benzene added. Centrifugation (15 minutes at 1800x$\underline{g}$) sedimented a hard orange gum of further non-lipid material, leaving a dark blue-green (from leaves) or brown (from rhizome) supernatant that was ready for chromatography.

2.3.4. <u>Separation Procedures</u>

High molecular weight compounds were removed by gel-filtration on Column I [N1114-50%-LH20/ benzene:isopropanol (3:1 v/v)]. With the bed volume measured as 260ml, two fractions were taken:-
A, SEV 30-46, collection being started when 80ml of solvent had eluted, and stopped when 120ml had passed; Collection of BC, SEV 46-77, was then started, and stopped when a total of 200ml had passed through the column. The complete passage of the green chlorophyll band through the column served as a visual indication of the boundary between the two fractions, the collection being changed when the effluent first became distinctly orange (due to carotenoids). Fraction A was found to be free from

sesquiterpenoids, and all compounds giving GLC peaks over the range 80°-250° in the total extract were found in fraction BC. The extract from up to 80g fresh weight of tissue could be processed in a single run of the column, larger quantities requiring repetitive operations. The factor limiting the effective capacity of the column was the presence of high molecular weight condensed tannins in the extract, increasing the viscosity of the sample. Most of these compounds were eluted in the void volume of the column, and were effectively removed from the fraction BC.

Excessively polar materials could be separated from fraction BC by simple straight-phase chromatography. Using a column specially prepared for the purpose (diameter 3.2cm, volume 80ml; N1114-50%-LH20/benzene), the fraction A', SEV 30-50, was collected starting when 24ml, and stopping when 40ml of benzene had been eluted. This was combined with fraction A above. The collection of fraction B, SEV 50-500, was then completed when a total of 400ml of benzene had been eluted. This fraction contained all compounds giving GLC peaks as above. Fraction C consisted of what remained on the column, displaced by addition of 25% isopropanol (v/v) to the mobile phase (Fig.1).

Table 13a: Reversed-Phase Chromatography of Fraction BC
Column II
Stationary Phase: N1114-50%-LH20
Mobile Phase: Methanol (49 ml bed volume)

Fraction	SEV	Volume Eluted	GLC Analysis
BO	30-50	15-25 ml	No GLC peaks
BI	50-130	25-64 ml	Oxygenated sesquiterpenoids
BII	130-250	64-123 ml	Sesquiterpene hydrocarbons
BIII	250+	123 ml +	Sterols and waxes

Table 13b: Straight-Phase Chromatography of Fraction BI
Column III
Stationary Phase: N1114-50%-LH20 (24-32)
Mobile Phase: Benzene (123 ml bed volume)

Fraction	SEV	Volume Eluted	GLC Analysis
BI/O	30-51	37-62.5 ml	No GLC peaks
BI/1a	51-60	62.5-73.5 ml	Petasin esters
BI/1b	60-70	73.5-86 ml	Ketones and Lactone
BI/2	70-114	86-140 ml	Alcohols 11a - 17a
BI/3	114-130	140-160 ml	Petasol & isopetasol
BI/4	130-500	160-615 ml	No major peaks
BI/5	500+	615 ml +	No major peaks

Table 13c: Straight-Phase Chromatography of Fraction BII
Column VI
Stationary Phase: N1114-50%-LH20 (17-23)
Mobile Phase: Benzene (25.5 ml bed volume)

Fraction	SEV	Volume Eluted	GLC Analysis
BII/1	30-62	7.7 -15.8 ml	No GLC peaks
BII/2	62-74	15.8-19.0 ml	Sesquiterpene hydrocarbons
BII/3	74+	19 ml +	Sterol & wax alcohols

The procedures described above were employed when it was merely necessary to obtain a comprehensive GLC-volatile fraction relatively free from compounds liable to decomposition at high temperature. Examples include the preliminary GC-MS survey of the extract, section 2.2.3, and the comparative GLC studies of tissue extracts other than the leaf, section 2.2.7.

A more elaborate scheme was used for group separation of sesquiterpenoids according to polarity and type. The initial gel-filtration step was followed by a reversed-phase separation into the four fractions BO, BI, BII and BIII (Table 13a). Straight-phase chromatography of each of the sesquiterpenoid containing fractions BI and BII (Tables 13b & 13c) completed the group separation procedure. GLC analysis of the fractions obtained located five groups, each containing sesquiterpenoids of similar chemical character.

Separation of the groups obtained above, into individual components, was completed using the 100cm high-resolution columns. Reversed-phase chromatography was employed for the separation of the lactone and ketone fraction BI/1b and the hydrocarbon fraction BII/2, whereas straight-phase methods were preferred for the alcohols BI/2. The automatic fraction-collector was used to collect small

fractions, 0.5-3ml, as accurate retention charateristics were not known in most cases. Fractions were examined by GLC, and individual compounds recovered by evaporation of solvent. When GLC indicated cross contamination of two components, separation was attempted by further high-resolution chromatography, using a column having opposite polarity characteristics. Thus a further straight-phase separation was required to obtain compound 17c free from petasin esters, and also for the preparation of an analytical sample of compound 18.

2.3.5   Chemical Techniques

Isomerisation of Eremophila-9,11-dien-8-one and Petasol:

A small sample of eremophila-9,11-dien-8-one, compound 17c (100μg estimated), was dissolved in 0.25ml benzene, and 100mg basic alumina (Woelm grade I) added. After standing for 24 hours, the alumina was filtered, and washed with three 1ml portions of ethyl acetate. After evaporation of solvent, 100μl ethanol was added, and the product estimated by GLC by comparison of peak areas with a standard sample of compound 18. 110μg of the isomer of compound 17c was found ($I_{OV-1}^{162^{o}}$ = 1765). The solution in ethanol, made up to 2.5ml, was transferred

to a cuvette for ultraviolet spectroscopy.

A similar technique was applied to the sample of petasol (30µg; estimated by comparison with an isopetasol standard). GLC of the product indicated that the petasol peak had disappeared, and that the isopetasol peak was increased. Recovery was not fully quantitative, which may be ascribed to losses by irreversible adsorption on the alumina.

Lithium Aluminium Hydride Reduction of Compound 18

Lithium aluminium hydride (2mg) was stirred with 0.25ml of ether, freshly dried by filtration through Woelm grade I basic alumina, and 5mg of compound 18 in 0.25ml dry ether added dropwise. Sample tube and pipette were rinsed with 0.1ml dry ether, and the washings added to the reaction mixture. Stirring was continued for 2 hours when a few drops of ethyl acetate were added to destroy excess reagent. When the reaction had subsided, 2.5ml ethyl acetate was added with 0.5ml water, and after agitation and centrifugation, the organic layer was removed. The aqueous layer was re-extracted with a further 2.5ml ethyl acetate, and after evaporation of solvent, 4.5mg of a colourless viscous oil was obtained. GLC indicated two peaks, the first ($I_{OV-1}^{162°}$ = 1770),

corresponding to unchanged lactone was less than 5% of
the area of the major product ($I_{OV-1}^{162^0}$ = 1875). GC-MS gave
a molecular ion m/e 238, 0.8% of the base peak, m/e 109,
with the M-18 peak at 11.5% and M-36 at 3.5% relative
abundance (Appendix I). Reversed phase separation
(N1114-50%-LH2O/methanol; SEV 50-80), permitted recovery
of the pure diol (4mg): ν_{max}: 3400cm^{-1}, 1640cm^{-1}, 1070cm^{-1},
1040cm^{-1}, 910cm^{-1}; 100 MHz NMR (CDCl$_3$), τ: 9.12 (3H),
9.26 (3H doublet, J = 6c/s), 6.62 (2H), 5.58 (2H),
4.90 (1H), 4.76 (1H).

These results were in good agreement with published data
for bakkenolide-A-diol[47].

2.3.6   Quantitative Estimation of Sesquiterpenoids

The estimation of sesquiterpenoids in leaf
samples was made by quantitative GLC analysis of standard
preparations. 43g fresh weight of tissue was extracted
and preliminary separation gave fractions BI and BII by
the procedures described. The fractions were made up in
2ml of solvent, and these standard solutions used for
GLC.

Quantitative estimates (to ± 5% accuracy) were
obtained by comparison of GLC peak areas with reference
standards, from the mean of three determinations.
Measurements were made using the Pye Model 104 Gas

Chromatograph. A 9' column, packed with the stationary phase 1% OV-1 prepared from acid washed and silanised Gas Chrom-P, was used at a flow rate of 45ml/min of nitrogen.

Though no further separations were made of fractions BI and BII, each group of compounds of a given type was determined independently, operating isothermally at an appropriate temperature. Reference standards were made up from readily available compounds, matched in chemical type to the particular group of compounds under investigation. This necessarily involved making estimates of samples in unresolved mixtures. In such cases, an arbitrary boundary between adjacent peaks was taken at the point of minimum detector response. This was considered sufficiently accurate for these determinations, where results were not required for any critical calculation. These methods were not effective in estimating compound 17b, petasol and isopetasol, which were determined later, after more complete separation procedures.

3   RADIOACTIVE TRACER LABELLING STUDIES IN THE
     INVESTIGATION OF PETASIN BIOSYNTHESIS

3.1   INTRODUCTION AND HISTORICAL BACKGROUND

3.1.1   Common Pathways of Terpenoid Biosynthesis

The problems of terpenoid biosynthesis are essentially the problems of the assembly of a complex carbon skeleton, and the correlation of the diverse structures observed. A distinctive feature of all terpenoids is the apparent involvement of a five-carbon fragment as the common sub-unit of structure. The normal terpenoids contain this sub-unit in simple multiples, and this frequently accounts for the entire carbon content of the molecule. Early workers noted that the location of functional groups in the skeleton strongly suggested isoprene, or a molecule having a similar distribution of functionality, as this basic five-carbon unit. These ideas were rationalised by Ruzicka, in his original expression of the Biogenetic Isoprene Rule[8a]. He noted that assembly appeared to be organised so that isoprene units were linked in a head-to-tail manner, and that this implied an acyclic, linear oligomer of 'active isoprene' as a precursor of cyclic terpenoids.

Experimental evidence for the biogenetic origin
of terpenoids had already been obtained by Bloch[63] who
found acetate to be an effective precursor of cholesterol
in the rat. Cholesterol may be regarded as a triterpenoid
that has lost three methyl groups. This was proved when
it was found that the acyclic triterpenoid squalene was
converted <u>in vivo</u> into cholesterol[64]. At the same time,
it was found that radioactivity from acetate labelled
in the methyl group was incorporated into eighteen
specific positions in squalene. Label from carboxyl-
labelled acetate was incorporated into the twelve
complementary positions. This ratio of 3:2 implied the
assembly of groups of three acetate units, and loss of
one carboxyl-derived carbon atom from each group.

Early attempts to identify the C_5 unit gave
largely inconclusive results. While there was incorporation
into cholesterol from hypothetical precursors such as
dimethylacrylic acid and 3-hydroxy-3-methylbutyric acid,
there was no satisfactory evidence that these compounds
lay on the direct biosynthetic pathway between acetate
and cholesterol. The discovery of mevalonic acid,
(3,5-dihydroxy-3-methylpentanoic acid) (6)[65], which
acted as a precursor of cholesterol superior to acetate[66]
provided the key to this problem. The condensation of three

(48)

(6)
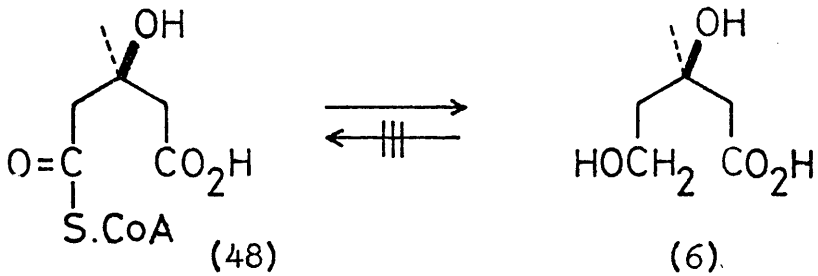
Fig. 15: Biogenesis of Mevalonic Acid

$CH_3 CO.SCoA$ $\longrightarrow$ HSCoA

$CH_3COCH_2CO_2H$

$CH_3CO.SCoA$

$CH_3COCH_2CO.SCoA$

$CH_3CO.SCoA$

HSCoA

NADH

HSCoA, NAD^+

OH

$O = C$ CO_2H

S.CoA

HMGCoA

OH

OHC CO_2H

$O = C$ CO_2H

S.CoA

NADH

Biotin

NAD^+

Biotin-CO_2^-

OH

$HOCH_2$ CO_2H

MVA

$O = C$

S.CoA

DMACoA
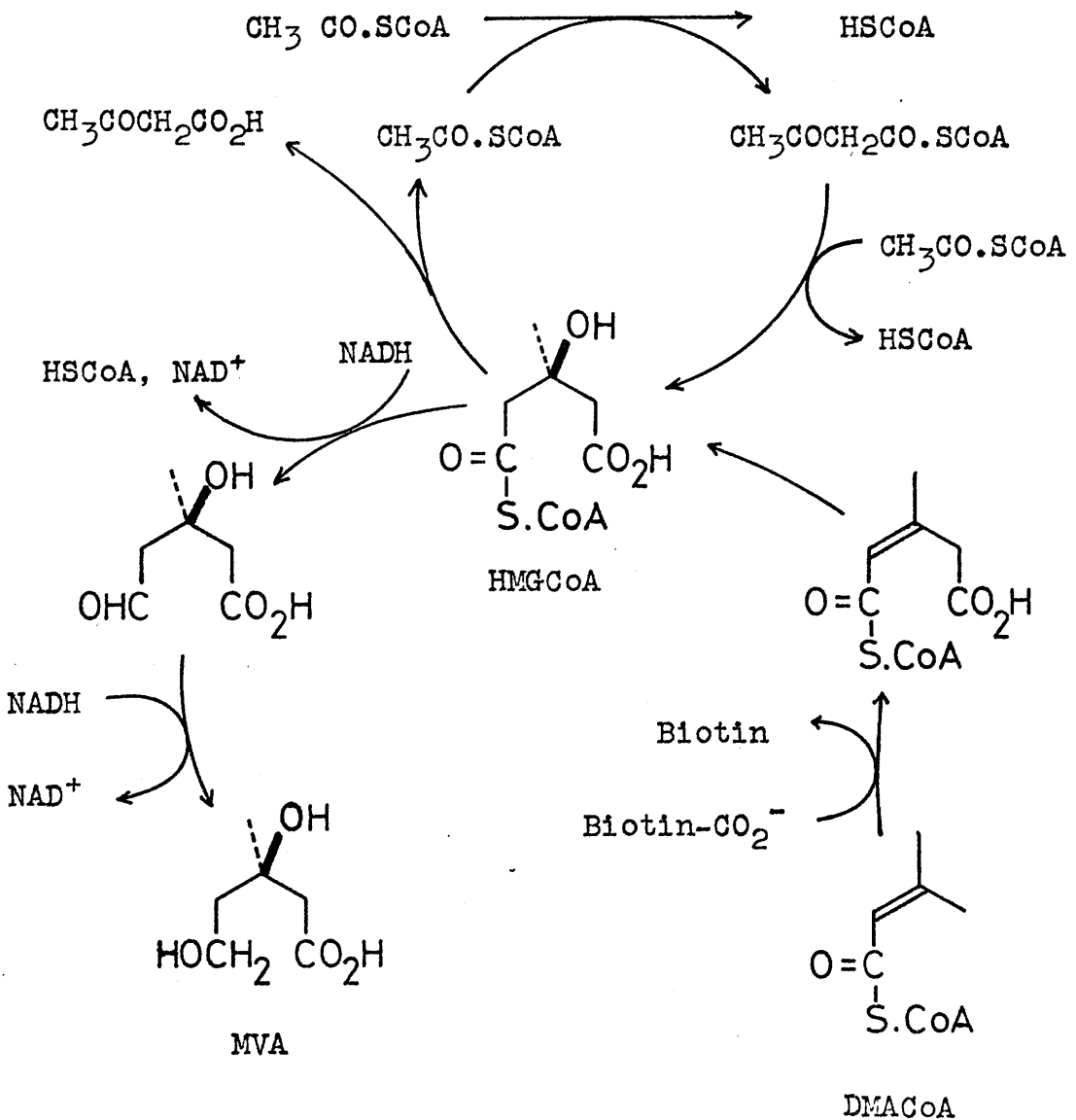
acetate units to give mevalonic acid was elucidated as outlined in Fig.15[67]. Birch and others have demonstrated[68] that enzymic reduction of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMGCoA; 48) produced 3R-mevalonic acid (6) in an irreversible step, whereas other postulated intermediates such as dimethylacrylic acid appeared to undergo degradation to acetate before incorporation[69]. The role of dimethylacrylic acid was clarified when it was found that biotin deficiency prevents its incorporation into sterols, by failure of a carboxylation step[70] (Fig.15). This pathway is utilised by some plants and micro-organisms, permitting direct conversion of leucine to mevalonic acid[71].

The utilisation of mevalonic acid (MVA) in biosynthesis follows a unique pathway. Apart from the reversible action of mevaldate reductase, an equilibrium that strongly favours mevalonic acid production, the only known specific enzymic reaction of mevalonic acid is phosphorylation, to give 5-phosphomevalonate[68b]. A second phosphorylation, followed by decarboxylation, gives isopentenyl pyrophosphate (IPP), in a sequence clearly demonstrated by comparison of the fates of $(2-^{14}C)$MVA and $(1-^{14}C)$MVA. The latter gives only two labelled phosphorylated products, and the radioactivity gradually disappears from the system. $(2-^{14}C)$MVA results in accumulation of label in a third product, identified

Fig. 16: Common Pathways of Terpenoid Biosynthesis

ATP   ADP

HO_2C —— OH —— CH_2OH

MVA
Mevalonic Acid

HO_2C —— OH —— CH_2O (P)

MVAP

ATP

ADP

OH

HO_2C —— CH_2O (P)(P)

MVAPP   ATP

ADP, P_i

CO_2

CH_2O (P)(P)

DMAPP

CH_2O (P)(P)

IPP

(P) = Phosphate ester
P_i = Inorganic
     Phosphate

(P)(P)
O

CH_2O (P)(P)

PP_i

CH_2O (P)(P)

+

H

$-H^+$

Monoterpenoids

CH_2O (P)(P)

Geranyl pyrophosphate

IPP

PP_i

Sesquiterpenoids
Triterpenoids
Steroids

CH_2O (P)(P)

Farnesyl pyrophosphate

IPP

PP_i **Higher Prenyl**
       pyrophosphates
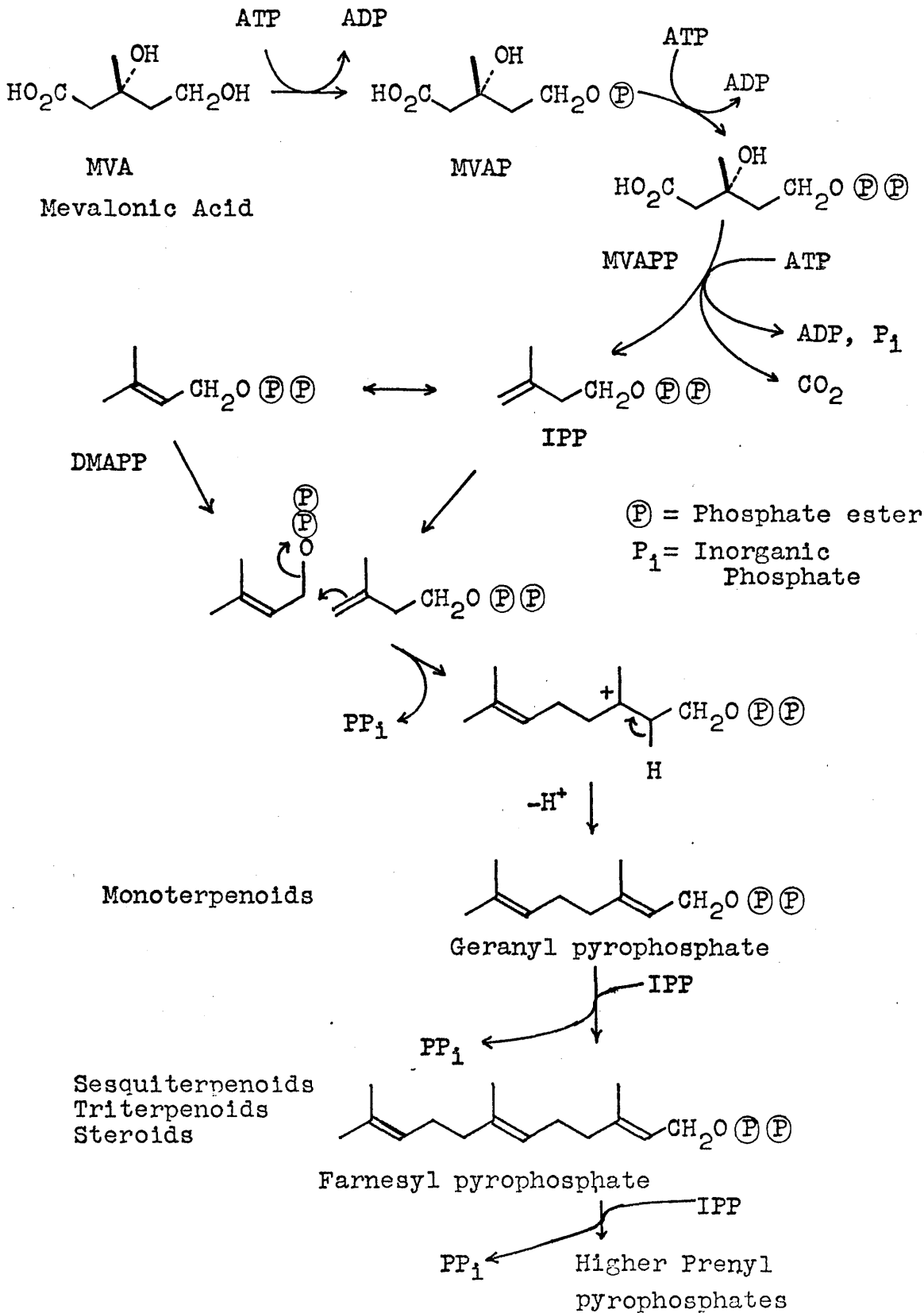
as isopentenyl pyrophosphate[72] (IPP; Fig.16). This compound is generally regarded as having the properties of the 'active isoprene' required by Ruzicka's hypothesis[8a].

Enzymes that are responsible for the assembly of the C_5 sub-units were first isolated from yeast autolysates by Lynen and co-workers[73] and have been found in liver extracts by a number of workers including Popják[74]. The assembly is initiated by isomerisation of one molecule of isopentenyl pyrophosphate to give dimethylallyl pyrophosphate (DMAPP). Coupling successively to two further molecules of isopentenyl pyrophosphate then follows, with elimination of inorganic pyrophosphate at each step (Fig.16). Popják has found that a single enzyme was responsible for both coupling reactions, and that geranyl pyrophosphate was not isolable as an intermediate in the synthesis of farnesyl pyrophosphate. It seems likely that other closely related enzymes terminate the reaction at the appropriate stage to produce geranyl-, geranylgeranyl- or higher prenyl pyrophosphates. Such enzymes would be expected to occur in systems specifically involved in the biosynthesis of mono-, di- and tetraterpenoids, or the assembly of terpenoid quinones having a polyprenyl side chain.

3.1.2   Farnesyl Pyrophosphate as the Common Precursor

of Cyclic Sesquiterpenoids

An early proposal cited farnesol as the presumed

common progenitor of the cyclic sesquiterpenoids, derived

on purely structural grounds [25]. When the involvement of

farnesyl pyrophosphate (5) in sterol biosynthesis became

known [73], this molecule became the preferred hypothetical

precursor for the cyclic sesquiterpenoids, the pyrophosphate

moiety being a vastly superior leaving group to the simple

hydroxyl function of farnesol [10].

The common biosynthetic pathway leading to farnesyl

pyrophosphate has never been examined in the same detail for

higher plants as for liver systems, because of the peculiar

difficulties of working with isolated enzymes from higher

plants. Bonner has obtained a cell-free system from pea

seedlings that gave the phosphorylated intermediates of

Fig. 16 when incubated with mevalonic acid [75]. Mevalonic

acid is a good precursor for phytosterols in vivo, and

while incorporation into other terpenoids may be poor, it

is not considered that a novel pathway may be involved.

A variety of other evidence suggests that there are

difficulties of permeability, preventing the exogenous

precursor reaching the site of synthesis in the plant [17].

Fig. 17a: Stereochemical Determination of Product in the
Cyclisation of Farnesyl Pyrophosphate

trans,trans- (5A)

Humulenes
Germacranes
(and related
bicyclic compounds)

cis,trans- (5B)

Bisabolenes and
related bicyclic
structures
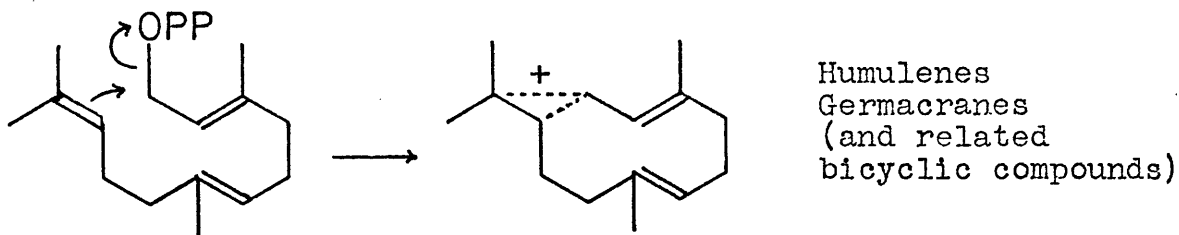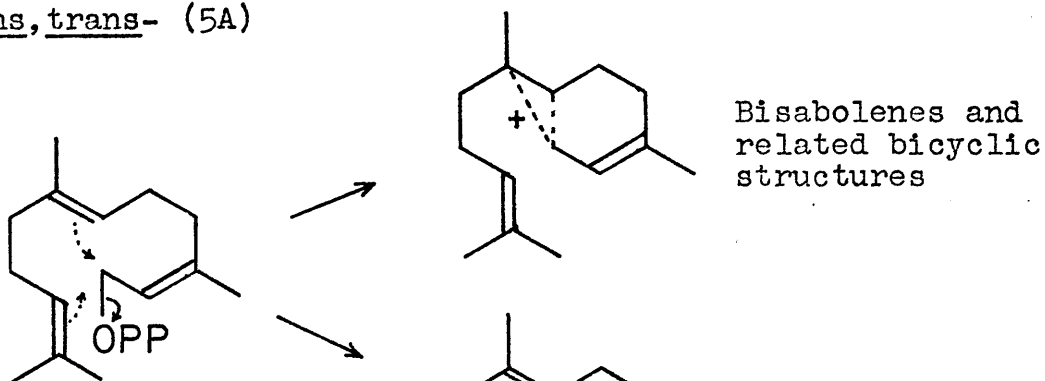
Derived bicyclic
structures
cis-Humulenes

cis,trans- (5B)

Carotanes
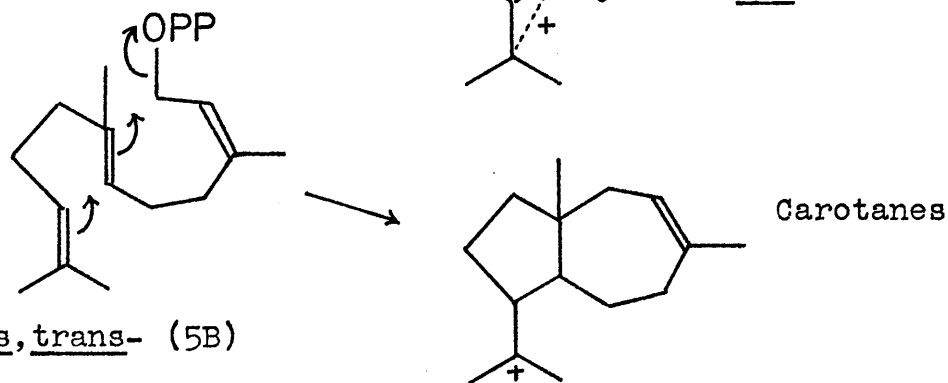
Fig. 17b: Derivation of Bicyclic Structures by Proton
Initiated Transannular Reaction

Eudesmanes
Eremophilanes etc.
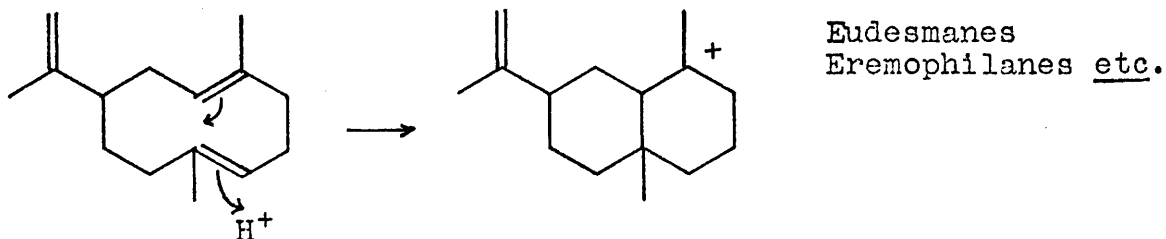
In any case, higher plants lie between the mammals and fungi in the scheme of evolution, and the pathway has been proven in each of the latter phyla. There are thus good reasons for accepting this hypothesis for sesquiterpenoid biosynthesis in higher plants.

Cyclic sesquiterpenoids are postulated to arise from farnesyl pyrophosphate by elimination of inorganic pyrophosphate. The mode of folding of the carbon chain is considered to determine the nature of the product[8]. This may arise by the participation of double bonds brought into proximity with the primary carbonium ion generated by the departing pyrophosphate anion. Deprotonation or solvent attack then gives a neutral species having a cyclic structure[25]. To some extent, folding is predetermined by the stereochemistry of the double bonds in farnesyl pyrophosphate. Some sesquiterpenoids require cis- stereochemistry at one or both of the double bonds (5B,5C) for generation of their carbon skeletons, instead of the trans,trans-farnesyl pyrophosphate (5A) produced by enzymes of the sterol pathway (Fig.17a). When this first cyclisation process produces ten- or eleven-membered rings, transannular reactions may reasonably be invoked to give rise to the bicyclic and tricyclic structures commonly found among sesquiterpenoid

skeletons [10,76] (Fig.17b). The anomalous structure of the

eremophilanes and ambrosanes is ascribed to methyl

migrations that may occur from a classical isoprenoid

precursor [9,25] .

Fig. 18: Modes of Cyclisation of Farnesyl Pyrophosphate
in Petasin Biosynthesis.

Patterns of Labelling in Petasin from $(2-{}^{14}C)$MVA

Derived by Hypothetical 'Clockwise' and

'Anti-Clockwise' Cyclisations

(6A)

● = ^{14}C

trans, trans (5A)

(1A)

trans, cis (5D)

(1B)

(2)
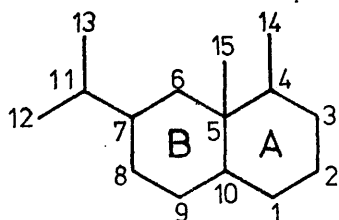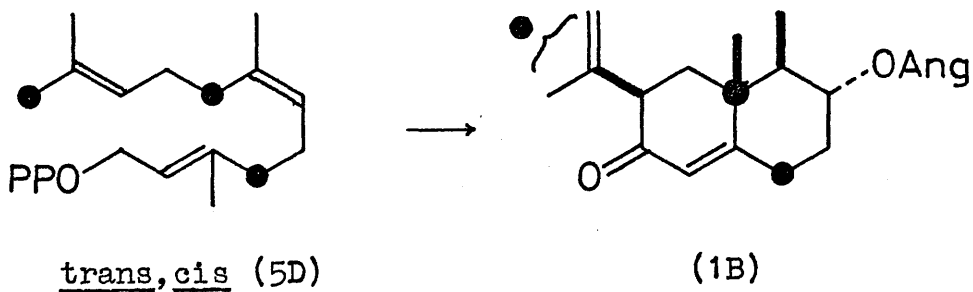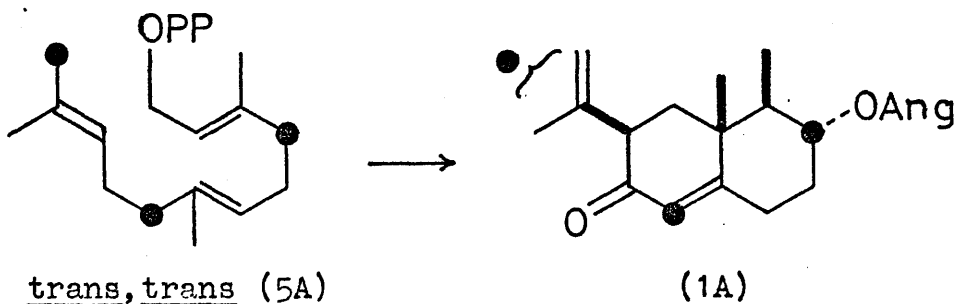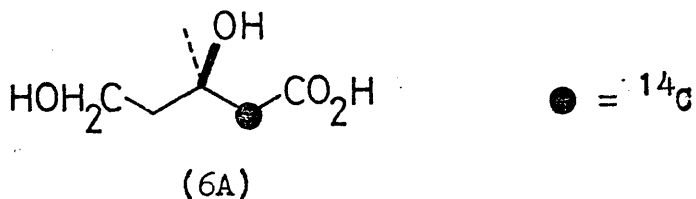
3.2   RESULTS AND DISCUSSION

3.2.1   General Plan of Labelling Experiments

These experiments were intended to investigate
the mechanism by which the eremophilane skeleton (2)
is generated in the biosynthesis of petasin, by studies
of the incorporation of radioactivity from specifically
labelled mevalonic acid. It was considered that there
were three points of significance that could be readily
investigated. This chapter describes the satisfactory
elucidation of the first of these.

i)   The direction of the primary cyclisation
may be anti-clockwise, involving a trans,cis-farnesyl
precursor (5D) or clockwise, as expected from
trans,trans-farnesyl pyrophosphate (5A). The latter
case appears the more probable, since the double bonds
are more favourably aligned for the transannular reaction,
and the expected product of such a reaction would correspond
to eremophilene. Label from $(2-^{14}C)$ mevalonic acid (6A)
would be incorporated in petasin (1A) at C-3, C-9 and one
further position - either C-12 or C-13. If the anti-clockwise
cyclisation occurred, label in the nucleus would be
expected at C-1 and C-5 of petasin (1B).

ii)   The mevalonoid origin of the angular methyl
group may be established by incorporation of label from

$(6-{}^{14}C)$ mevalonic acid. The alternative process of demethylation from C-10 followed by remethylation at C-5 by S-adenosylmethionine appears unlikely in view of the results of Nes, who found no incorporation from $(methyl-{}^{14}C)$ methionine into the anomalously positioned methyl group of α-amyrin[77].

iii)   Double labelling with $(2-{}^{14}C-3\underline{R},4\underline{R}-4-{}^{3}H)$ mevalonic acid (6B) results in farnesyl pyrophosphate (5E) with complete retention of the tritium label[78]. The distribution of tritium in petasin so derived would give strong evidence for the extent to which the transannular reaction and the methyl migration are concerted. A totally unconcerted mechanism, involving a neutral eudesmanoid intermediate (Scheme I) should result in loss of tritium at C-5, which becomes a quaternary centre in petasin (1C).   Loss of tritium from C-1 may also occur as detailed below, if tritium becomes the 1α-(axial) substituent in the above intermediate.

It seems more probable that the transannular reaction and the methyl migration may be linked. Transannular reaction of a <u>chair-</u> folded precursor may be initiated by equatorial proton attack at C-1, giving rise to a eudesmanoid intermediate as a carbonium ion (Scheme II; for clarity, details of ring B are omitted). If normal

$T = {}^3H$

$\bullet = {}^{14}C$

HOH_2C ... OH ... CO_2H

(6B) ${}^3H/{}^{14}C = 1:1$

$\longrightarrow$

OPP

(5E)
${}^3H/{}^{14}C = 3:3$

Scheme I

Scheme II

—OAng

O

T

(1C) ${}^3H/{}^{14}C = 2:3$

—OAng

O

T

(1D) ${}^3H/{}^{14}C = 2:3$

Scheme I:

R
R
T
H^+

$\downarrow$

R
R
T
H^+

$\downarrow$

R
R
$+$
T

Scheme II:

4 3 2
5 10 1
T T
H^+

$\downarrow$

$+$
5 3 2
1 H
T T
X^-

$\downarrow$

R
T
5 4 10 3 2
1 H
T
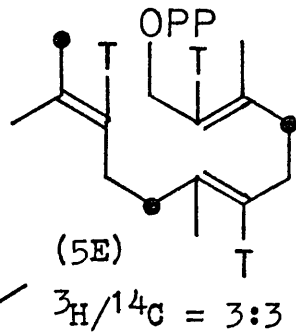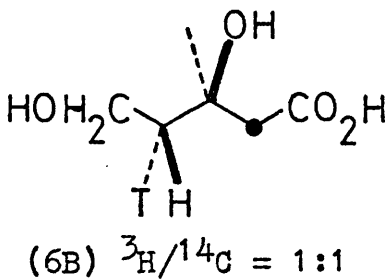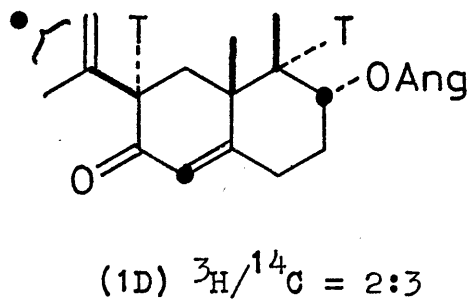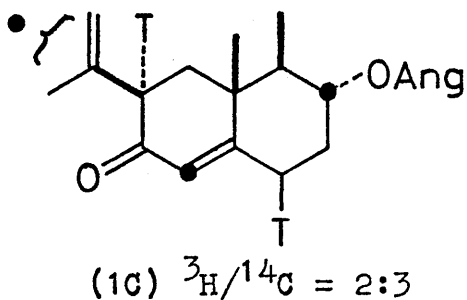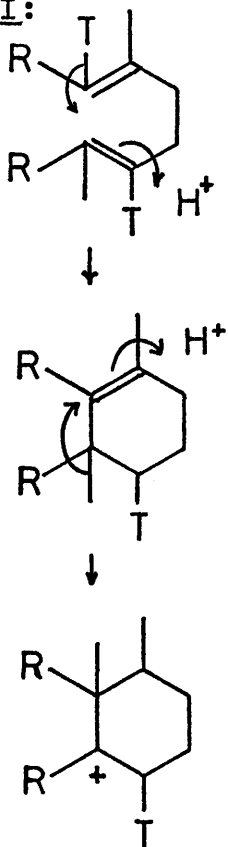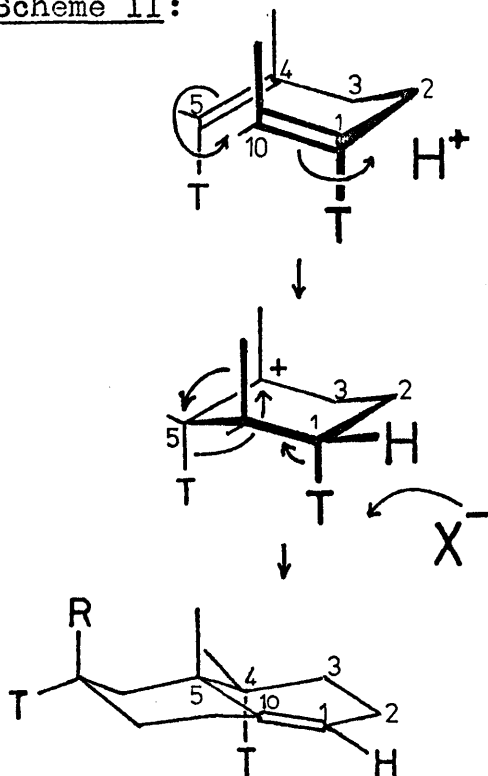
petasin stereochemistry is to be attained, folding of the
precursor on the enzyme surface must locate the methyl
groups on the β-face as shown, and the incoming proton
becomes the 1β- (equatorial) substituent. When ring A is
in the <u>chair</u> conformation, tritium at C-5 and the angular
methyl group at C-10 are both axial. If neutralisation
of the charged transition state takes place by a specific
deprotonation from C-1α or C-9α (axial substituents),
as may occur in an enzyme, there could follow a series
of 1,2-shifts, with migration of the tritium from C-5α
to C-4α and of the angular methyl group from C-10β to
C-5β. Tritium may thus be expected to be retained at
C-4 of petasin. Such a process would be accompanied
by ring inversion (Scheme II).

Deprotonation from C-1α, which leads directly
to the structure of eremophilene (4A), where the double
bond is located between C-1 and C-10, would be expected
to remove tritium from this position, as the mechanism
invokes stereospecific attack of a proton onto C-1β.
The expected labelling pattern of petasin derived by
this mechanism will be as in (1D). If, however, eremophilene
is not an obligatory intermediate in petasin biosynthesis,
and deprotonation from C-9α is permitted to give the
9(10)- double bond directly (49), then all tritium atoms

T = ^{3}H

● = ^{14}C

− T-1α

(4A)

(1D)

^{3}H/^{14}C = 2:3

−OAng

− H-9α

(49)

(1E)

^{3}H/^{14}C = 3:3

−OAng

^{3}H/^{14}C = 3:3

H

H $^{19}_{}$ CH$_2$

HO

8

9

14

13

17

20

R
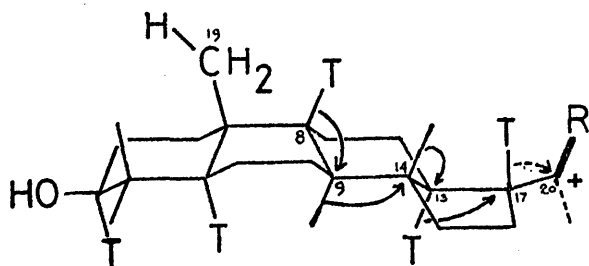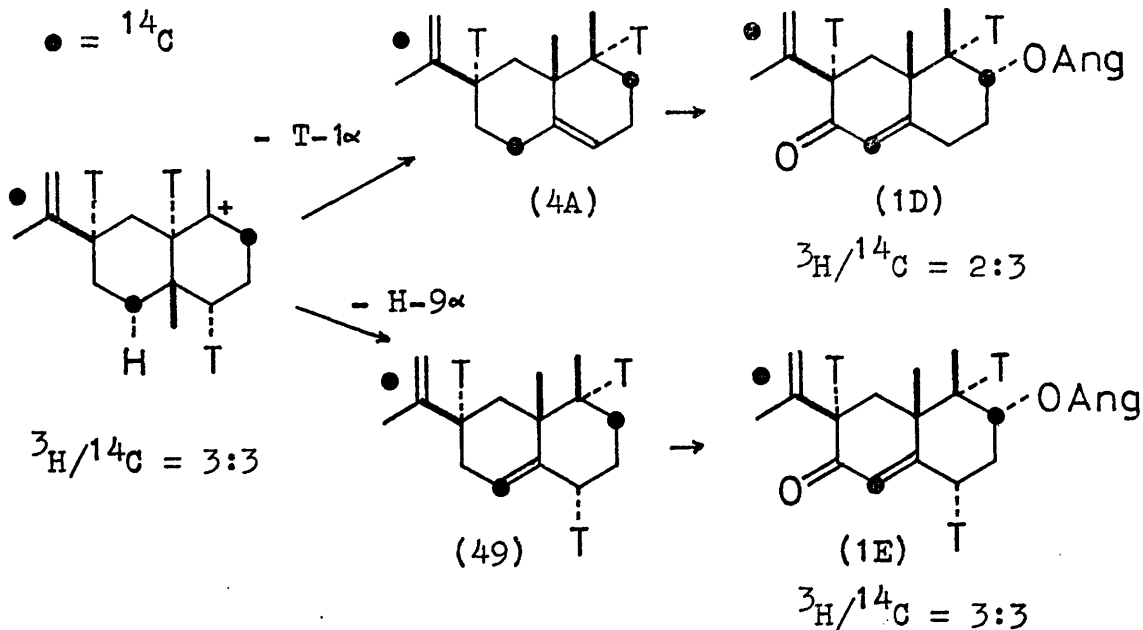
Scheme III

Sterol
Biosynthesis
from
(3R,4R-4-^{3}H)
MVA

− T-8β

Lanosterol

− H-19

HO

(50)

HO

(51)
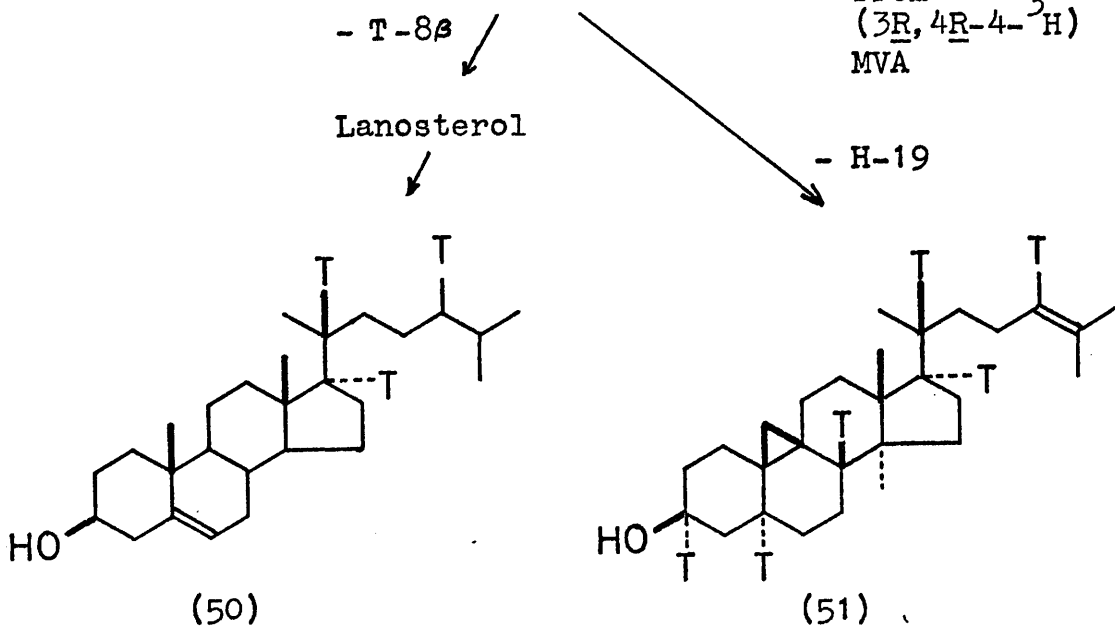
may be retained, and the petasin may be labelled as in (1E).

Extended sequences of 1,2- shifts have previously been invoked to account for certain aspects of triterpenoid biosynthesis[8b], and experimental evidence has confirmed such a mechanism in sterol biosynthesis (Scheme III). Bloch and Cornforth demonstrated independently that methyl migration occurred as two 1,2- shifts rather than a single 1,3- shift[79,80], and Caspi has found that tritium from ($3R,4R$-4-^{3}H) mevalonic acid was transferred to C-17α and C-20β of cholesterol (50)[81a]. Rees obtained a similar result in cycloartenol (51), and in this case, tritium was also found to have been transferred from C-8 to C-9β[81b], whereas this atom was lost in the formation of the 8(9)-double bond en route to cholesterol.

Availability of labelled precursors: While specifically labelled mevalonic acid is commercially available for the first and third of these experiments, (6-^{14}C) labelled precursor would require special synthesis. A possible route starts with methyl-labelled acetic acid, in an esterified form. A Grignard reaction with two molar equivalents of allyl magnesium bromide gives the tertiary alcohol (52), and cleavage of the double bonds affords the hydroxydialdehyde (53)[82]. Reduction to the triol (54) and oxidation with Fétizon's reagent[83] completes the

Proposed Route For Synthesis of $(3\underline{RS}\text{-}6\text{-}^{14}C)$ MVA
Based on the Method of Fétizon[83]

$(2\text{-}^{14}C)$ Acetate
Tetrahydropyranyl ester $\longrightarrow$

Allyl Magnesium Bromide

(52)

$\downarrow$ $OsO_4/NaIO_4$

(53)

$\xleftarrow{\text{NaBH}_4}$

(54)

$\downarrow$ Ag_2CO_3

(55)

$\xrightarrow{OH^-}$

(60)

$\bullet = {}^{14}C$
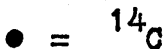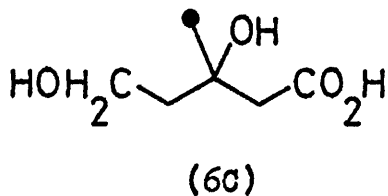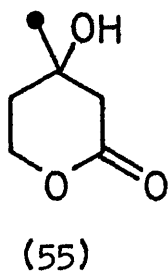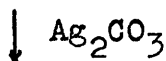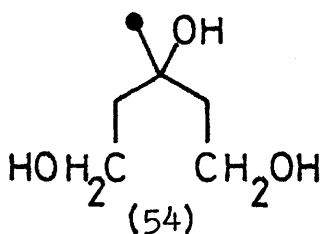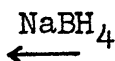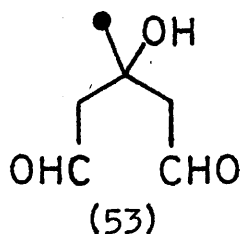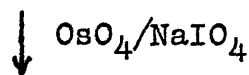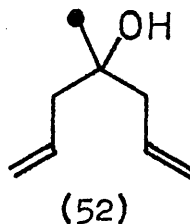
synthesis of mevalonolactone (55), opening to give
($3\underline{RS}$-6-^{14}C) mevalonic acid (60) being optional. This
sequence was attempted under the writer's supervision by
an undergraduate student, Mr. E. Cuthbertson. Thin-layer
chromatography indicated mevalonolactone as a major
component of his product, but yield was not estimated.
Further development of procedures would be required before
this synthesis could be undertaken on a sub-millimolar
scale with radioactive starting materials. For these
reasons, no attempt has been made to elucidate the problem
discussed in paragraph ii) above.

3.2.2   Feeding of Labelled Mevalonolactone to

P. hybridus Plants

Preliminary results reported elsewhere[7] indicated
that (2-^{14}C) mevalonic acid, fed either as the salt or in
the lactone form, is incorporated rather poorly into
petasin esters in the leaf (incorporation of the order
0.003-0.03% of total radioactivity) and not at all in the
rhizome. Further exploratory experiments are reported in
the following chapter.

On the basis of these experiments, 90μCi of
(2-^{14}C-$3\underline{RS}$) mevalonolactone was fed to actively growing
leaves of P. hybridus, and the plants were allowed 48 hours

to metabolise the precursor. After extraction of the
petasin esters according to the established procedures,
isopetasol was obtained as the common product of hydrolysis.
Repeated chromatographic purification gave radiochemically
homogeneous material. The specific activity of the sample
was correlated with the total quantity of petasin esters
obtained, and incorporation of radioactivity into the
alcohol moiety of the esters determined as 10,500dpm,
or 0.005% of the total radioactivity of the racemic
precursor.

Further experiments were prompted by a report of
exceptionally good incorporation of mevalonic acid into
rose petal monoterpenoids[84]. Francis considers that
flower feedings in general lead to better incorporation
from mevalonic acid[85], and to increased probability of
obtaining symmetrical labelling of terpenoids. In contrast,
leaf feeding experiments have commonly given poor
incorporations[15,16] and occasionally, asymmetric labelling
patterns[16a,86,87]. (Labelling of terpenoids is termed
asymmetric if the radioactivity is not distributed equally
among all the isoprene units. Asymmetric labelling may
frequently invalidate experiments involving dual labelling
by changing isotopic ratios in an inconsistent manner.)

The first part of this proposition would seem
entirely reasonable, considering that the inflorescence
is not photosynthetically active, but obtains a significant
proportion of carbon compounds through the transpiration
stream, rather than by carbon dioxide fixation as occurs
in the leaf. Provided that terpenoid synthesis is active
at the time of feeding, it appears highly likely that
exogenous mevalonate should be more effectively incorporated
in the flower than in the leaf. One may conclude from the
findings noted in section 2.2.7 that feeding should be
made at the earliest opportunity, and that still better
results might possibly be obtained in P. hybridus by
unearthing and feeding of the immature flower while it is
still below ground level.

In the first test of this hypothesis, three
developing flowers were fed with a total of 15 μCi of
$(2-^{14}C-3\underline{RS})$ mevalonolactone. Feeding was commenced after
the appearance of the flower above ground level, but well
before the florets had opened; 24-30 hours were allowed for
utilisation of the precursor. Petasin was extracted in the
normal manner and isopetasol recovered after hydrolysis
and purification to radiochemical homogeneity. The
incorporation of radioactivity into the sesquiterpenoid
moiety of the petasin esters was estimated to be 6000dpm,

or 0.018% of the total radioactivity fed (as the racemic precursor).

The final experiment involved feeding of $(2-{}^{14}C-3R,4R-4-{}^{3}H)$ mevalonolactone (and its enantiomer), 25 μCi ${}^{14}C$ and 125 μCi ${}^{3}H$, to five developing flowers. In this case, the flowers had partly expanded (although the florets had still not opened), and it was possible that the optimum time for administration of the precursor had passed. It appeared likely that synthesis would still be occurring, but at a reduced rate: 48 hours were allowed for utilisation of the precursor. Petasin esters were isolated in the normal manner, but no further results have been obtained from this experiment at the time of writing. It was impossible to obtain an estimate of incorporation by radioassay of the petasin esters, because of the greater specific activity of the acyl moiety. Hydrolysis to isopetasol was inapplicable to this sample because of the risk of loss of tritium from enolic positions in petasin.

3.2.3   Location of Label from (2-^{14}C) Mevalonic Acid

         in the Molecule of Petasin

         General Approaches to Isolation

Three carbon atoms, one in each isoprene unit

of petasin (1A), were expected to become labelled by

incorporation of (2-^{14}C-3RS) mevalonic acid, if the preferred

mode of cyclisation discussed in section 3.2.1 was operative.

It had been assumed that the 3S-enantiomer of mevalonic acid

was inert, and no reference to the fate of this molecule

was found in the literature.

The purpose of this part of the investigation

concerned the isolation of carbon C-3, C-9, C-12 and C-13

of petasin, and the determination of radioactivity in each

position.

The label from (2-^{14}C) MVA may be situated either

at C-12 (methyl) or C-13 (terminal methylene) in petasin (1A),

but the lability of this compound with respect to isomerisation

made its isolation difficult: the distinction between C-12

and C-13 was too easily lost. Conversion by hydrolysis to

isopetasol (27A) rendered these two positions equivalent

in biogenetic origin. This step was, however, a convenient

method of removing the radioactivity in the acyl moiety, and

no attempt was made at the independent isolation of these

two carbon atoms. Instead the retro-aldol reaction,

(1A)

OH⁻ →

(27A)

O₂/OH⁻

OH⁻/H₂O

(59)

(56)

+

CH₃–CO–CH₃
(57)

IO⁻

CHI₃
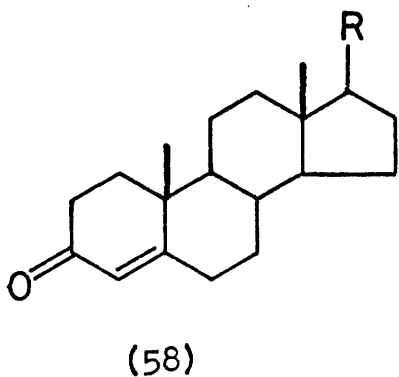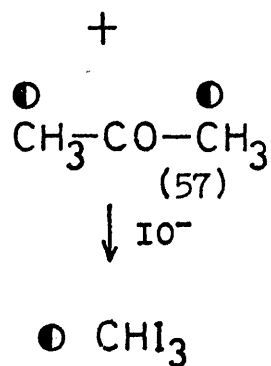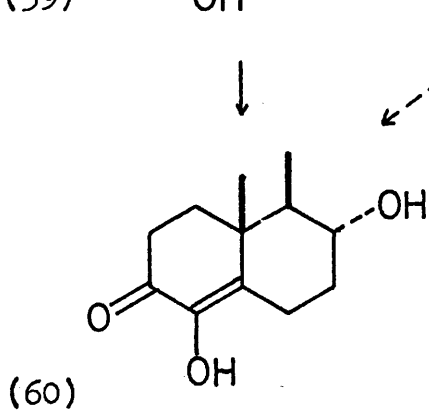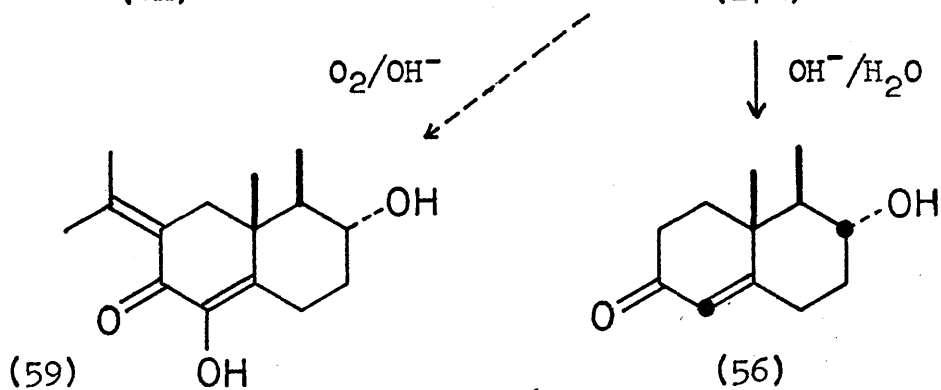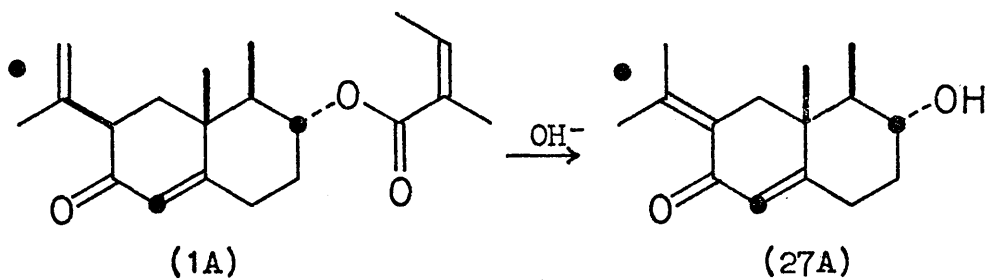
50% of Label at
C-12 (or C-13)
of Petasin

(60)

(58)

reported by Aebi[6] to give desisopropylideneisopetasol (56), was explored. C-12 and C-13 could then be recovered as the methyl groups of acetone (57). The iodoform reaction would permit the isolation of one of these methyl groups, and the average radioactivity at C-12 and C-13 could thus be determined.

Desisopropylideneisopetasol was considered an ideal molecule for further degradative processes to isolate C-3 and C-9. It appeared likely that many reactions of Δ^4-3-oxo-steroids (58) might be applicable to such a system.

Apart from indicating that the reaction was carried out under basic conditions in a nitrogen atmosphere, Aebi reported few experimental details for the cleavage. Early attempts at the retro-aldol degradation gave irreproducible results, but it soon became clear, as might have been expected, that water participated in the reaction, and its concentration determined the rate of cleavage. Failure to allow sufficient volume to dissolve the isopetasol, which was sparingly soluble in the 50% aqueous methanol used, resulted in reduced yields, by decomposition of undissolved isopetasol to give tarry products. Autoxidation was a serious problem as a result of the prolonged reaction times, and the use of nitrogen was

obligatory if good yields were to be obtained. The reaction mixture rapidly became deep red, presumably due to the formation of diosphenols, e.g. (59) and (60), the colour probably arising from a variety of condensation products of the diosphenols. A final refinement of the method involved de-aeration of solvents before isopetasol was added; yields as high as 95% were then obtained, and the reaction mixture remained uncoloured throughout. Distillation of organic solvents from the mixture at the end of the reaction gave a methanolic solution of acetone, which was converted to iodoform. This iodoform originated solely from C-12 and C-13 of isopetasol.

Further degradations to isolate C-3 and C-9 of desisopropylideneisopetasol were then completed by two parallel pathways, each leading to the removal of one of the remaining labelled atoms from the molecule. This procedure was preferred to a sequential degradation, where both atoms would be isolated successively from the same sample. The small quantity of radioactive material available made the second method especially susceptible to poor yields, and left little room for error or failure of a reaction step in the sequence.

Favorskii
Rearrangement

Beckmann
Rearrangement

Baeyer-Villiger
Oxidation

Hunsdiecker-type
Decarboxylation

Schmidt Reaction
or Curtius
Rearrangement

Barbier-Wieland
Degradation

3.2.4   Isolation of C-3 of Desisopropylideneisopetasol

    C-3 of desisopropylideneisopetasol (56) carried the hydroxyl function of this ketoalcohol. Isolation of the atom was therefore feasible by any of the methods readily applicable to a carbonyl function. These included Favorskii rearrangement to a ring-contracted acid, Beckmann rearrangement, or Baeyer-Villiger oxidation to a lactone (Fig.19). Simple chromium trioxide ketone cleavage and ring contraction was not suited to this problem, as it was unlikely to discriminate between C-2 and C-3. The above methods selectively convert the desired carbon atom to a carboxyl group, from which it could be isolated by several processes, e.g. the Schmidt reaction or Curtius rearrangement to give isocyanate, a modified Hunsdiecker decarboxylation, or Barbier-Wieland degradation to give benzophenone. This last method had the advantage of easy recovery of both fragments of the cleavage (Fig.19).

    Preliminary experiments indicated the need to remove or protect functional groups in ring B, in order to prevent competition in the degradative reactions aimed at ring A. One method, planned for a Favorskii reaction at a later stage, involved hydrogenation to the saturated ketoalcohol (61) followed by Grignard reaction to give the tertiary alcohols (62). GC-MS of the crude product showed two peaks, both giving weak molecular ions m/e 226,

$\text{(56)} \xrightarrow{\text{H}_2/\text{Pd}} \text{(61)} \xrightarrow{\text{EtMgBr}} \text{(62)} \xrightarrow{\text{Cr}^{VI}} \text{(63)}$

(64) $\xleftarrow{\text{SO}_2\text{Cl}_2}$ (63)

(64) $\xrightarrow{\text{NaOMe}}$ (65)

(63) → (66) → (67)

(65) → (68)
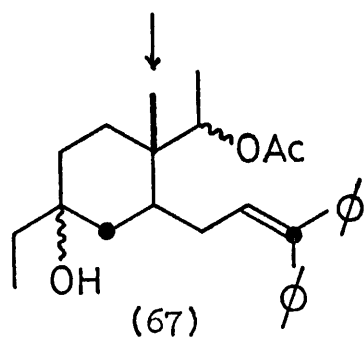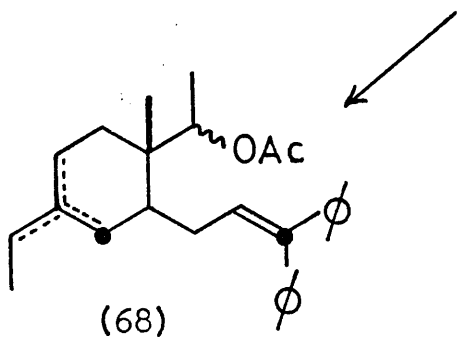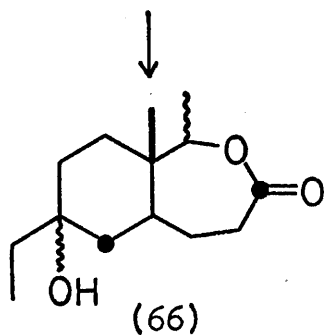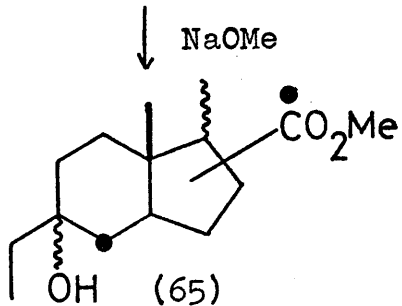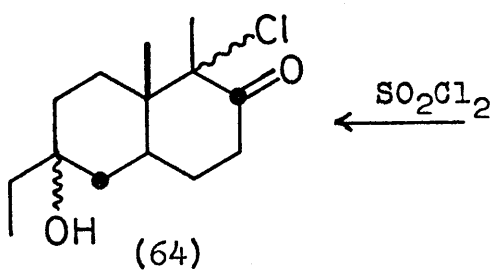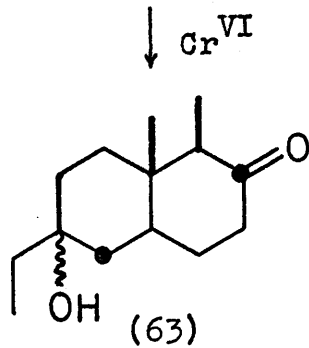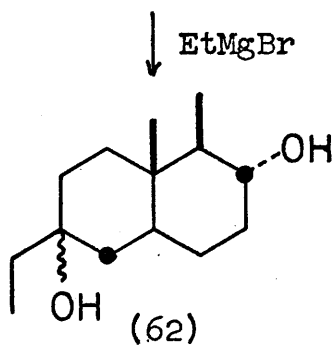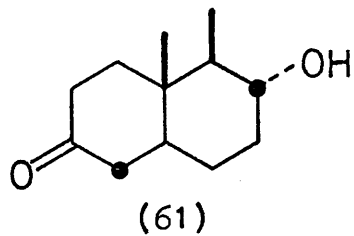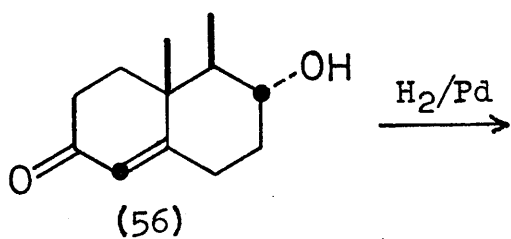
corresponding to a pair of epimers.

Jones oxidation gave the ketoalcohol (63) which
was chlorinated at the tertiary position at C-4 using one
molar quantity of sulphuryl chloride[88]. NMR of the product
showed the methyl doublet due to C-14 was shifted from
$\tau = 8.9$ to $\tau = 8.3$, indicating selective halogenation, but
the methyl signals, due to C-14 and C-15, which should have
been sharp singlets, now appeared as doublets, suggesting
epimerisation at C-4. Treatment of (64) with sodium methoxide
in refluxing ether[89] gave a mixture of products, including
a large proportion which could be hydrolysed to acidic
material. It appeared that the reaction was not
stereospecific. Though the production of an acid was
probably indicative of the success of the Favorskii
rearrangement, the variety of products obtained of general
formula (65) would have made characterisation and
purification excessively difficult. The scheme was
abandoned.

Attention turned to Baeyer-Villiger oxidation,
to be followed by Barbier-Wieland degradation. The
ketoalcohol (63), obtained as above, was treated with
m-chloroperbenzoic acid. No significant reaction was observed
after 24 hours at room temperature, but refluxing for
8 hours with an excess of the per-acid gave a mixture

of ketone (63) with about an equal amount of lactone (66),
judged by the intensity of their respective IR bands.
Without further separation, this mixture was treated with
an excess of phenyl magnesium bromide, and the product
dehydrated and acetylated in the normal manner for Barbier-
Wieland degradation[90]. GLC data suggested that the
intended product(67) had dehydrated at C-8, and that the
major product was that represented by structure (68). The
presence of the extra double bond in ring B would have
interfered with the cleavage of the diphenylethylene (68).

Despite apparently poor results, the scheme
appeared potentially effective. Two steps needed revision:
first, it was necessary to remove all interfering functional
groups in ring B, and secondly, the yield of lactone from
the Baeyer-Villiger oxidation required drastic improvement.

The procedure described below was successful in
two successive trials with unlabelled material, and was
then completed with a sample of labelled desisopropylidene-
isopetasol derived from the experiments with $(2-^{14}C)$
mevalonolactone.

Hydrogenation of (56) gave the saturated
ketoalcohol (61) as before. The carbonyl function was
then reduced through to methylene via the tosylhydrazone[91]
to give the monofunctional saturated alcohol (69), and

(56) → (61)

(61) TsNHNH$_2$/NaBH$_4$ →

(69)

(70) ← CrO$_3$ (69)

(70) CF$_3$CO$_3$H ↓

(71) PhMgBr → (72)

(72) 1) AcOH 2) Ac$_2$O/pyr ↓

(73)

(75) ← RuO$_4$/NaIO$_4$ (73)

(74)

(75) CH$_2$N$_2$ ↓

(76) → (77)
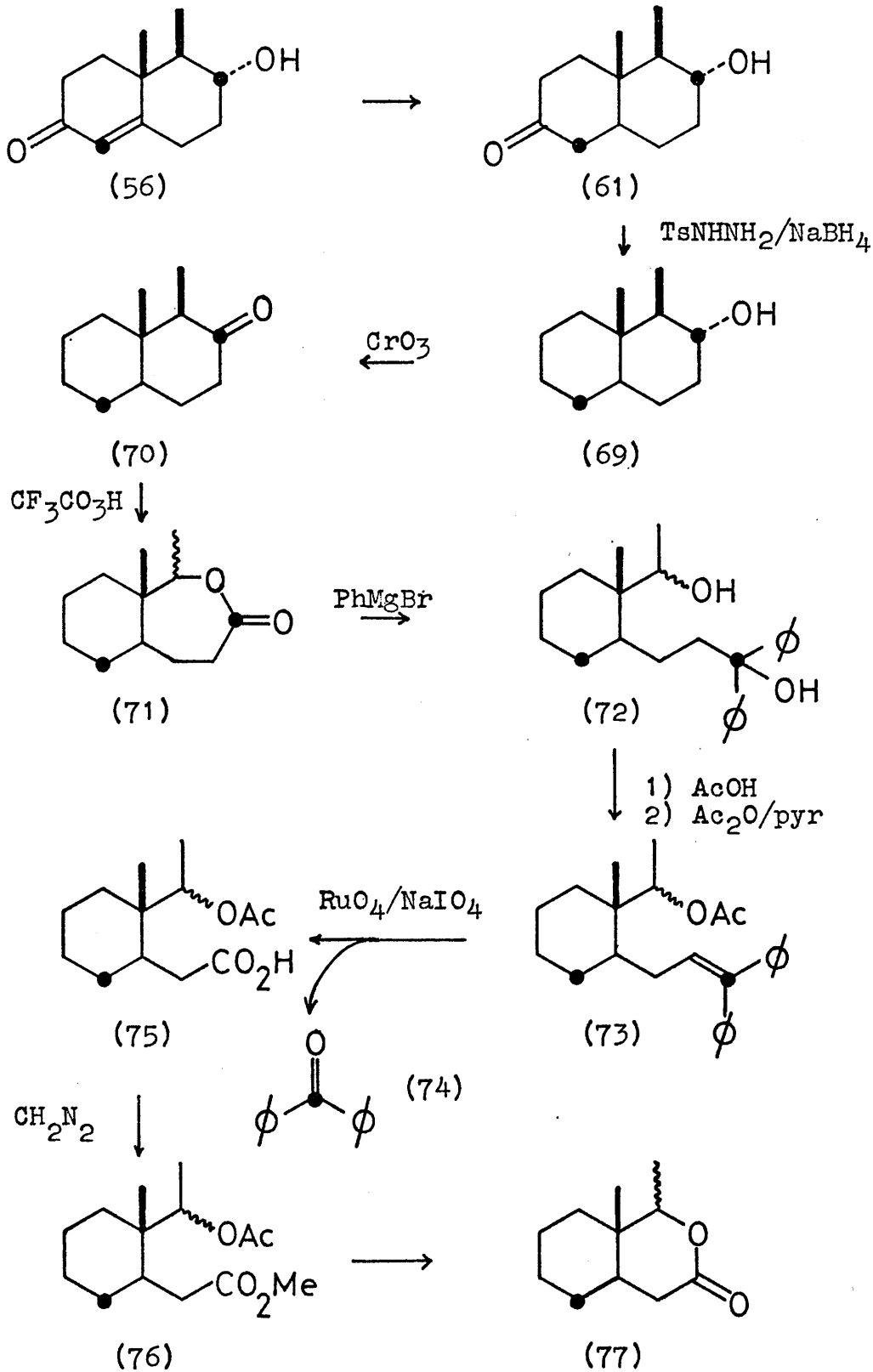
Jones oxidation of this gave the ketone (70). Overall
yield at this stage was about 70% in each of the trials,
but for reasons unknown, only 35% of labelled ketone (70)
was obtained. GLC indicated the complete disappearance of
ketoalcohol (61) in the formation of tosylhydrazone, so it
appears that either the reduction or the solvolysis of the
alkyltosylhydrazine intermediate was incomplete.

Baeyer-Villiger oxidation was completed with great
success using peroxytrifluoroacetic acid [92]. The yield of
lactone (71) was almost quantitative, the production
of ring-opened hydroxyacid being minimised by buffering
the reaction mixture with dry disodium hydrogen phosphate [92,93].
Treatment with excess phenyl magnesium bromide then gave the
diphenylcarbinol (72) and dehydration and acetylation the
acetoxydiphenylethylene (73),in about 55% yield based on (70).

The acetoxydiphenylethylene (73) was treated with
ruthenium tetroxide and sodium periodate, a method for
cleavage of the double bond in the Barbier-Wieland
degradation, which many workers have found more effective
than traditional reagents such as chromium trioxide, ozone
or osmium tetroxide [94,95]. The neutral fraction was found
to contain benzophenone (74), and after treatment of the
acidic fraction with ethereal diazomethane, the methyl
ester (76) was found almost pure, in 35% yield overall

Fig. 20: Literature Methods Considered for Application
to the Isolation of C-9 of Desisopropylidene-
isopetasol (56)

a) Hartmann, Tomasewski and Dreiding[96]

1) O$_3$
2) H$_2$O$_2$
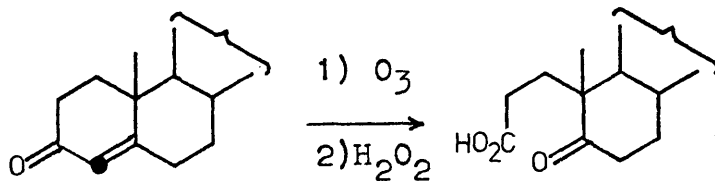
b) Caspi and Balasubrahananyan[97]

H$_2$O$_2$/SeO$_2$
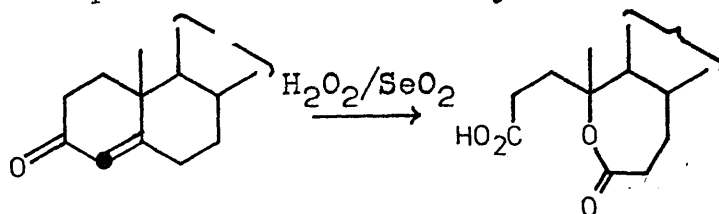
c) Ryerson, Wasson and House[98]

BF$_3$
70%

NaOH

30%
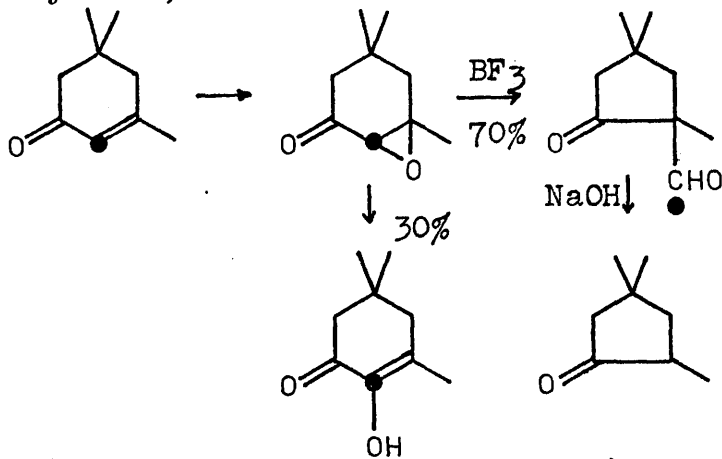
d) Tanabe, Crowe, Dehn and Dene[101]

TsNHNH$_2$

TFA
HgO
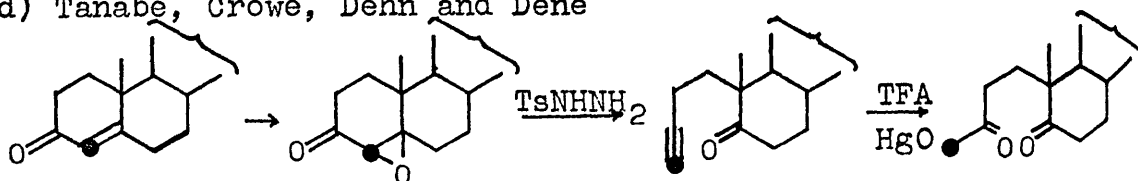
from (70). A final check on the structure of (76) was made
by hydrolysis of the ester functions, and acidification
of the hydroxyacid formed to give the δ-lactone (77) in
good yield. The δ-lactone (77) was correlated by GLC and
mass spectrometry as the nor-homologue of ε-lactone (71).

3.2.5   Isolation of C-9 of Desisopropylideneisopetasol

The obvious analogy between desisopropylidene-
isopetasol (56) and Δ^4-3-oxo-steroids did not prove to be
advantageous in the isolation of C-9, in terms of readily
applicable methods of degradation in the literature. C-4
of Δ^4-3-oxo-steroid is commonly isolated by ozonolysis
and oxidative cleavage[96] (Fig.20a), but this procedure
did not seem attractive on a small scale. Two further
methods were investigated briefly, but without success.
Caspi has described a reaction of hydrogen peroxide,
catalysed by selenium dioxide, where C-4 of the steroid
was lost in a single reaction that presumably involved
a succession of Baeyer-Villiger oxidations, epoxidation
and oxidative cleavage[97] (Fig.20b). Attempts to reproduce
this reaction with a sesquiterpenoid substrate met with
repeated failure. House has investigated a method for
ring contraction of cyclic conjugated ketones such as
isophorone, involving rearrangement with boron trifluoride
of the derived α,β-epoxyketone[98] (Fig.20c). This too was

H_2O_2/Na_2CO_3
r.t. 5 hrs

(79)

(56): R = H

(78): R = Ac

$TsNHNH_2/EtOH$

$50°$ 1 hr

TFA/HgO
r.t. 1 hr

(81)

(80)

$NaBH_4$

(82)

(83)
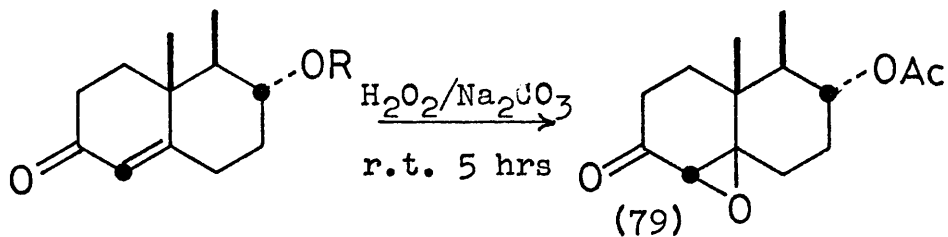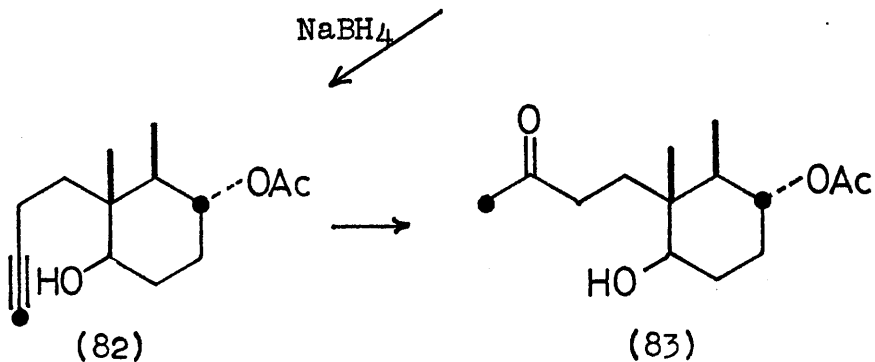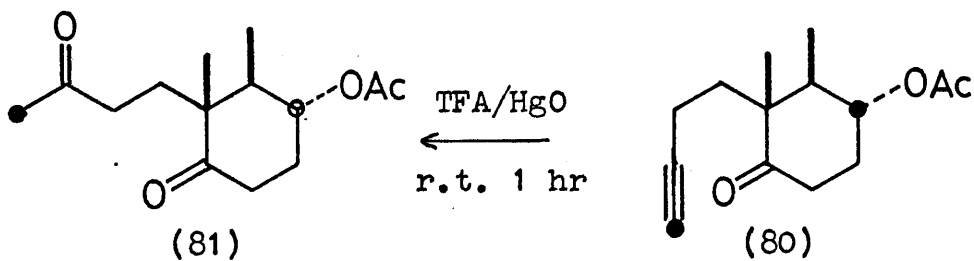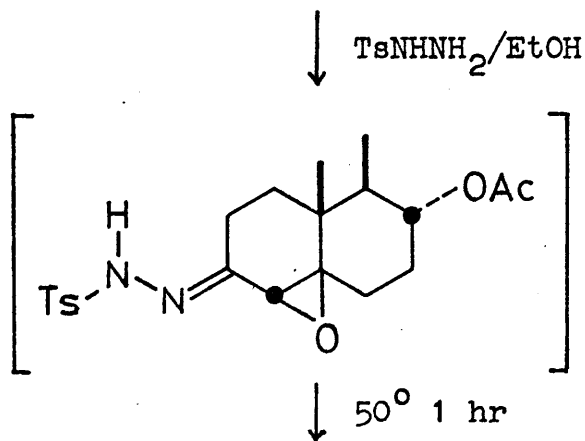
● = ^{14}C

unsuccessful, the apparent product being the diosphenol
rather than the 9-nor-ketone. Collins has found that
cholest-4-en-3-one produced mainly the diosterol rather
than 4-nor-cholestan-3-one [99]. This depended on the
configuration of the epoxide intermediate, the readily
obtainable 4β,5β-epoxide giving preferentially the
diosterol. Epoxidation of desisopropylideneisopetasol
produced the α- and β- epoxides in a ratio of one to three.

Slightly greater success was obtained from a
fragmentation reaction of an α,β-epoxyketone by solvolysis
of the tosylhydrazone [100], as applied to testosterone
by Tanabe [101] (Fig. 20d). The hydroxyl group of
desisopropylideneisopetasol (56) was first protected as
the acetate (78), and epoxidation gave a mixture of
α- and β- epoxides (79). Formation and solvolysis of the
tosylhydrazone was performed in a single operation, and the
acetylenic ketone (80) was obtained in excellent yield.
Hydration of the triple bond gave the diketone (81) in
60-65% overall yield from (56). The success of the ring-
opening reaction was marred by the unexpected failure,
despite repeated attempts, of cleavage of the methyl
ketone of (81). The iodoform reaction was unsuccessful,
but the diketone (81) was generally recoverable from these
attempts. The more stable potassium hypobromite was

found to be effective in the cleavage of the side chains of progesterone and pregnenolone [102], as models for the system under investigation. When this reagent was employed with the diketone (81), starting material was rapidly consumed, and the sample was recovered from an acidic fraction. However, attempts to characterise the product, which was an indeterminate mixture giving many spots by TLC, were unsuccessful. Mass spectrometry gave a molecular ion, m/e 446, with satellites at m/e 444 and 448, having a ratio of abundance suggesting a doubly brominated species. There was no evidence that this was the sole component in the sample, which had been introduced on the probe, but merely that this compound gave the ion of highest mass.

Evidently, the diketone structure was highly unstable under the conditions of the bromoform reaction, possibly as a result of a condensation reaction. Attempts to convert the acetylenic ketone (80) to an acetylenic alcohol (82) were partly effective: yields were poor, and no greater success was apparent in the subsequent bromoform reaction of ketoalcohol (83) than for the diketone (81).
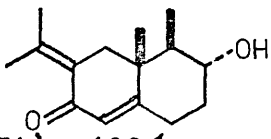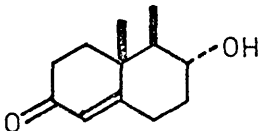
The final attempt at isolation of C-9 of desisopropylideneisopetasol was made by osmylation and

(86) → (87) → (88)

(78) —NaBH₄→ (84)

(85)

(84) —OsO₄/NaIO₄→ (89)

(90)

(90) —NaBH₄→ (91) → (92) R = H

(93) R = Ac
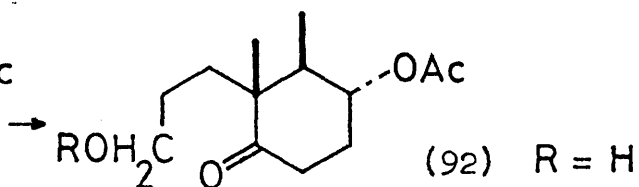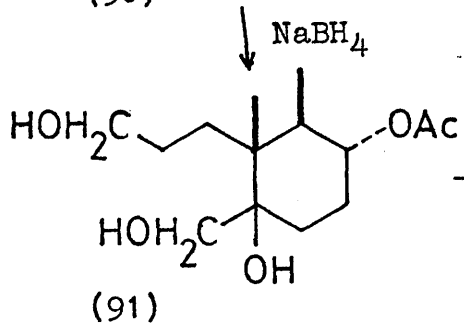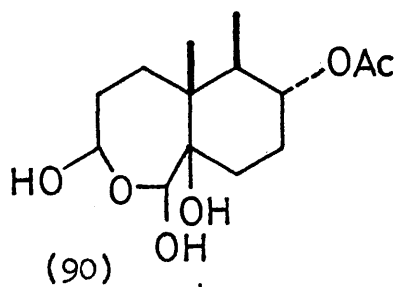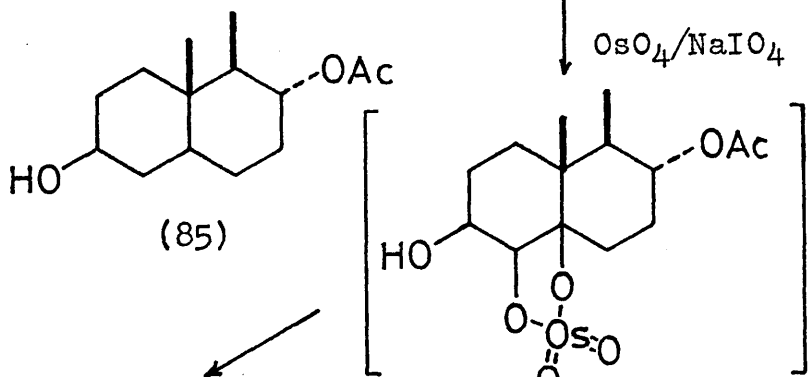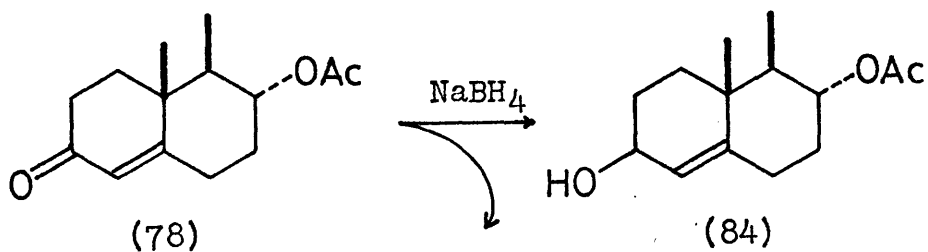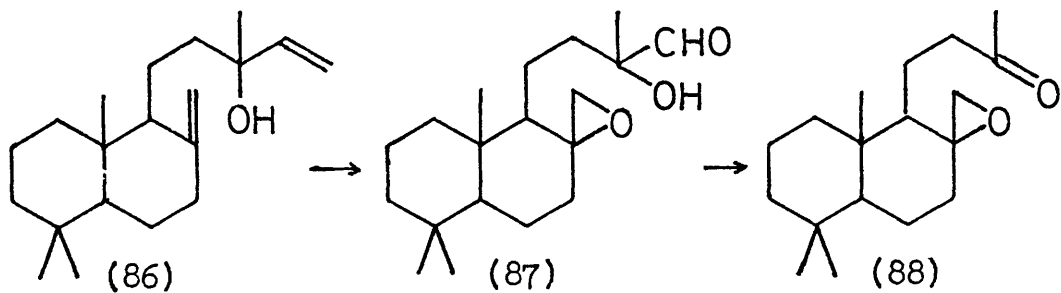
oxidative diol cleavage. Desisopropylideneisopetasyl
acetate (78) was reduced with sodium borohydride to give
the allylic alcohol (84) as the major product, but some
saturated alcohol (85) was also evident by GC-MS. The
allylic alcohol was treated with sodium periodate in the
presence of catalytic quantities of osmium tetroxide[103].
The allylic alcohol, manool (86), has been reported to give
initially the α-hydroxyaldehyde (87), which cleaved further
with loss of a C_1 unit to the ketone (88)[104]. No report
has been found of attempted cleavage of a cyclic system
as in (84).

The expected product of the reaction was the
acetoxyketoaldehyde (89) but yield was lower than had
been anticipated. Nevertheless the compound was readily
isolated, though characterisation was rendered more
difficult by lack of sample. The major product of the
reaction was characterised as the unexpected acetoxy-
bis-hemiacetal (90), which resisted further attempts at
oxidative cleavage by periodic acid or sodium bismuthate[105].
Reduction with borohydride gave the triol (91) in good
yield, albeit very slowly. Periodic acid treatment of the
triol then gave the acetoxyketoalcohol (92), acetylated
to give (93). Both ketoaldehyde (89) and the bis-acetoxyketone
(93) were isolated and used separately for radioassay.

Table 14: Distribution of Radioactivity in
Isopropylidene Side Chain of Isopetasol (27A)
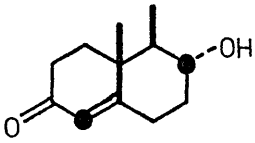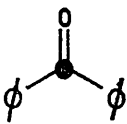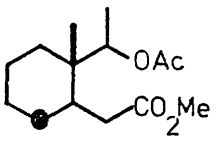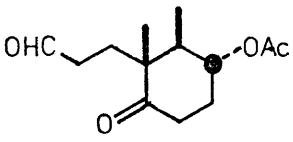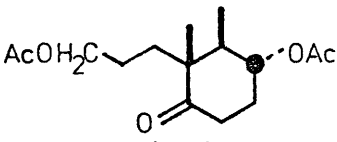
Sample and Ideal Distribution of Radioactivity	Leaf Experiment dpm/μmol	Flower Experiment dpm/μmol
(27A); 100%	27.8 ± 0.4 100%	37.2 ± 0.6 100%
(56); 67%	17.9 ± 0.3 64.5% of (27A)	24.4 ± 0.4 65.5% of (27A)
CHI_3 17%	5.1 ± 0.2 18.5% of (27A)	6.4 ± 0.3 17% of (27A)

3.2.6   Distribution of Radioactivity in Isopetasol

Degradation of isopetasol from the leaf and flower feedings was completed separately as far as desisopropylidene-isopetasol (56), and iodoform was recovered in each case. The results from the leaf feeding indicated that one third[*] of the radioactivity was located in the C-12 or C-13 methyl groups of isopetasol (27A), the remainder being in the ring system of (56). Similar figures were obtained from the sample derived from P. hybridus flowers. There was thus a strong suggestion at this stage that labelling had proceeded in a specific manner, without degradation of mevalonate to smaller molecules and subsequent reincorporation of the radioactivity. Furthermore, it appeared likely that labelling of each isoprene unit was substantially symmetrical, the deviation from the expected value of 33% being of the order of 1-2%, within the range of experimental and statistical errors. It was also possible that traces of contaminating material of high specific activity could have accounted for the anomalous 1-2% difference between observed and ideal results, this contaminant being removed selectively at the retro-aldol stage. The results in general, however, indicated that the effect of any such contaminant was

[*] The specific activity of the iodoform was one sixth of that of isopetasol because it contained equal contributions from both labelled and unlabelled methyl groups.

Table 15: Distribution of Radioactivity in the

Desisopropylideneisopetasol Nucleus

Compound Isolated	Atoms Present	Radioactivity dpm/μmol	Idealised Distribution
(56)	C_{12} a)	10.2 $\pm$ 0.2 b) 65% of (27A)	67%
(74)	C-3	5.0 $\pm$ 0.1 32% of (27A)	33%
(76)	C_{12} minus C-3	5.2 $\pm$ 0.1 33% of (27A)	33%
(89)	C_{12} minus C-9	5.25 $\pm$ 0.15 33.5% of (27A)	33%
(93)	C_{12} minus C-9	5.3 $\pm$ 0.15 34% of (27A)	33%

a) C_{12} represents the 12 carbon atoms of
   desisopropylideneisopetasol.

b) Specific activity after combination of leaf and flower
   samples, and dilution with unlabelled material.

Fig. 21: Distribution of Radioactivity in the Eremophilane Skeleton of Petasin, after Feeding (2-^{14}C) Mevalonolactone.
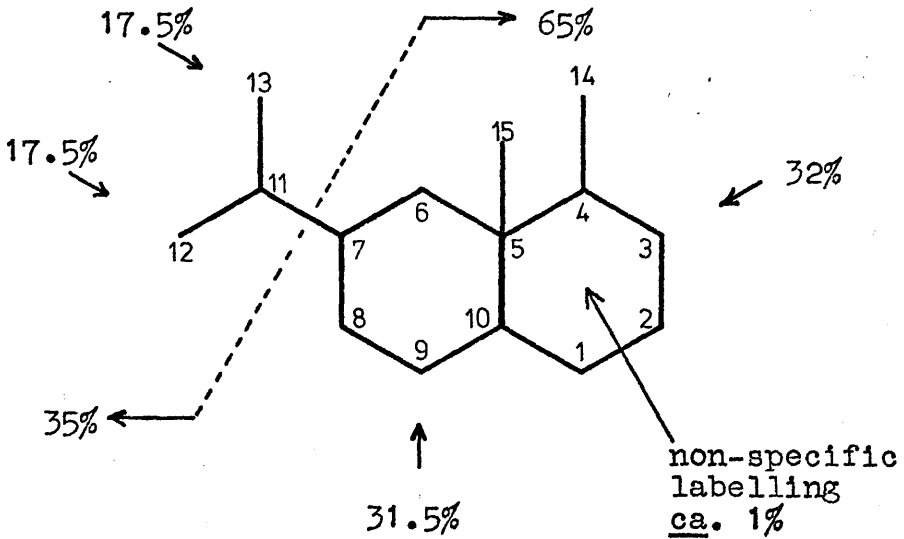
trivial, and did not have a significant bearing on the outcome of the experiments.

It was considered that the consistent nature of the results obtained above permitted combination of the two samples from leaf and flower feedings. At the same time, the radioactivity of the sample of desisopropylideneisopetasol (56) was diluted with an equal quantity of the unlabelled compound. These two operations simplified later stages in the degradation, increasing both mass and radioactivity of the samples available for the various reaction schemes involved in the isolation of the remaining two labelled positions.

The distribution of radioactivity in desisopropylideneisopetasol (56) was estimated by isolation of benzophenone (74) containing C-3 of isopetasol exclusively, and the remainder of the molecule of desisopropylideneisopetasol was isolated as the methyl ester (76). Half the radioactivity of (56) was shown to be located at C-3 (Table 15). C-9 was not recovered as such, but the remaining eleven carbon atoms were isolated in two separate forms, as the acetoxyketoaldehyde (89) and as the bis-acetoxyketone (93). Again, half the radioactivity was lost by excision of C-9.

The overall pattern of labelling of the eremophilane skeleton is depicted in Fig. 21, taking mean values of the separate results for leaf and flower feedings for labelling in the side-chain. The equal distribution of radioactivity between C-12 and C-13 was an artefact of isolation of the atoms, and does not imply randomisation between these two positions in the molecule of petasin. Results in Table 15 indicated about 1.5% of non-specific labelling of atoms other than C-3 and C-9 in the ring system.

These results are in full support of the proposed biosynthetic scheme for eremophilane sesquiterpenoids involving cyclisation of trans,trans-farnesyl pyrophosphate.

3.2.7 Proposed Isolation of Tritium Label in Petasin from $(2-^{14}C-3R,4R-4-^{3}H)$ Mevalonic Acid

The approach to degradation of tritium labelled molecules is simplified by the introduction of a second isotope, such as ^{14}C, which acts as an internal standard for estimation of the sample used in radioassay. The change in isotopic ratio is a convenient and an accurate indication of the removal of tritium atoms from the molecule. A disadvantage of tritium labelling in general is the increased lability of the tracer, as a result of hydrogen rearrangement that may occur in biosynthetic processes,

(6B)

(1E)

(27B)

(94)

(96)

(95)

(97)

$$T = {}^{3}H$$

$$\bullet = {}^{14}C$$

(98)

(99)
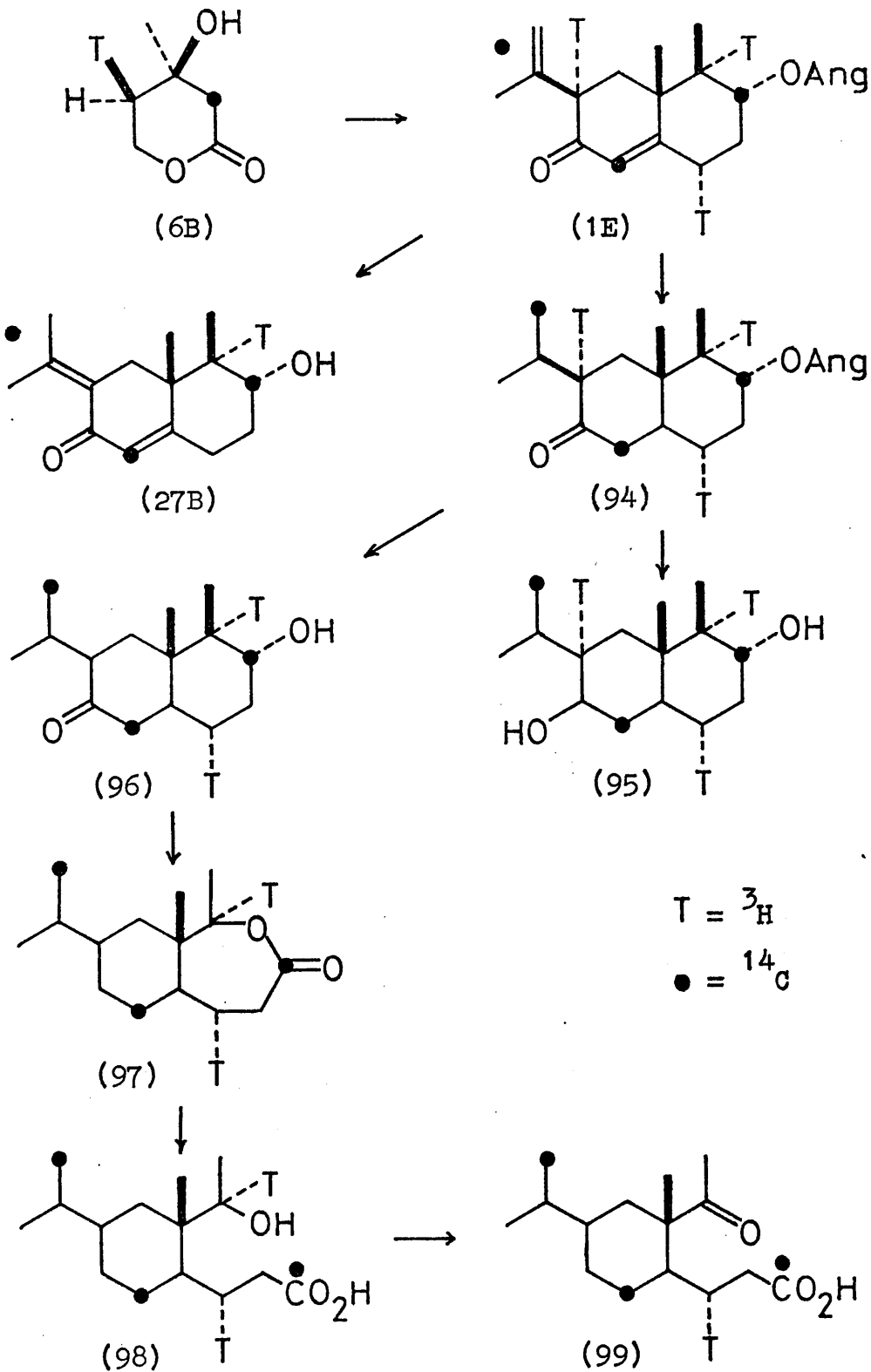
for example, the N.I.H. shift[106], and the possibility
of losses in work up by exchange processes[107].

It is the second of these problems that is
encountered in the isolation of tritium label from petasin,
with the possibility of enolisation occurring in two out of
three potential sites (1E) for the location of tritium from
$(2-^{14}C-3\underline{R},4\underline{R}-4-^{3}H)$ mevalonic acid (6B). The successful
isolation of tritium from C-7α would be an indication that,
if tritium was absent at C-1, this resulted from biosynthetic
processes, and not from exchange in the course of degradation.
The risk in this case is that of isomerisation to isopetasin,
rather than simple enolisation, and thus retention of tritium
at C-7α is an indication that the molecule has not been
exposed to conditions where enolisation may have occurred.

This risk may be removed by hydrogenation, a rapid
exposure to catalysts such as palladised charcoal being
unlikely to induce exchange of tritium[107]. An aliquot of
hexahydropetasin (94), could then be reduced with lithium
aluminium hydride to the saturated diol (95) which would
remove, at the same time, the acyl moiety, which is likely
to have higher specific activity than the sesquiterpenoid.
This route appears more likely to lead to isolation of
a derivative of petasin with its tritium content intact
than other methods that have been considered.

Alkaline hydrolysis of hexahydropetasin (94) should give tetrahydropetasol (96) with tritium specifically removed from C-7α. Hydrolysis of petasin would give isopetasol (27B) for comparison with (96), any tritium at C-1 being removed by enolisation. Proof of location of tritium at C-4α might be achieved by the formation of the ϵ-lactone (97) from tetrahydropetasol (96) by the same procedures that led to the lactone (71) in the isolation of C-3 of isopetasol. Hydrolysis to the hydroxyacid (98) would permit oxidation to the ketoacid (99), with specific removal of tritium from C-4.

Lack of time prevented the completion of this sequence before preparation of this thesis.

3.3   EXPERIMENTAL PROCEDURES

3.3.1   Feeding of Mevalonolactone

$(2-^{14}C-3\underline{RS})$ Mevalonolactone and $(3\underline{R},4R-4-^{3}H+3\underline{S},4S-4-^{3}H)$
mevalonolactone were obtained from the Radiochemical Centre,
Amersham, at various specific activities.

Plants were grown as detailed in section 2.3.2.
For the leaf feeding, six plants were used, and the precursor
was fed to fourteen actively growing leaves via a cotton
wick, threaded through the petiole 5-7 cm below the base of
the leaf. The ends of the wick dipped into a small vial
(capacity 0.5 ml) attached to the petiole by 'sellotape'.
100 μCi of $(2-^{14}C-3\underline{RS})$ mevalonolactone (specific activity
5.4 μCi/μmol) was made up in 4ml sterile water, and 3.6 ml
of this solution (90 μCi) was used in the feeding. The
solution was distributed, 0.3 ml to each of the eight
larger leaves, and 0.2 ml to the six smaller leaves. Uptake
of solution from the vials was complete after 6-8 hours,
and a further 42 hours was allowed for translocation and
metabolism.

Three flowers were fed by a similar procedure, the
wick being inserted into the flower stem below the head of
florets. In properly immature flowers (as in this case)
this space was extremely constricted. Each flower was fed
0.25 ml of a solution containing 50 μCi $(2-^{14}C-3\underline{RS})$

mevalonolactone (specific activity 6.4 μCi/μmol) in 2.5 ml sterile water. Uptake was complete in 3-4 hours, and a further 20-25 hour period was allowed for metabolism.

Five flowers were fed in a similar manner with 0.25 ml each of a solution containing, in 2.5 ml, 50 μCi (2-^{14}C-3RS) mevalonolactone (specific activity 6.4 μCi/μmol) and 250 μCi (3R,4R-4-^{3}H + 3S,4S-4-^{3}H) mevalonolactone (specific activity 116 μCi/μmol). In this case, the stem had begun to extend, and the head of florets was well separated from ground level. A period of 48 hours was allowed for metabolism.

3.3.2   Isolation of Isopetasol from (2-^{14}C-3RS) Mevalonolactone Feedings

The fourteen leaves, 180 g fresh weight, were cropped 48 hours after the initial administration of 90 μCi of (2-^{14}C-3RS) mevalonolactone. Extraction was completed without further delay, using the procedures described in sections 2.3.3-4 to isolate the petasin esters as a pale yellow oil (120 mg). TLC of a small portion, followed by scanning of the chromatoplate for radioactivity (using the Panax Model RTLC Radio-Chromatogram Scanner) indicated that radioactivity was associated with an ester of the petasin type, having R_f = 0.38, between that of petasin (R_f = 0.34)

and isopetasin (R_f = 0.44) in this system (0.25 mm layer, Silica-gel-G; developed twice in 15% ethyl acetate- 85% petroleum ether 60°-80°). It was considered that most of the radioactivity was associated with the acyl moiety, possibly involving dimethylacrylic acid, which might have arisen by oxidation of monoprenyl alcohols (see also section 2.2.4, petasin esters). No attempts were made to isolate this acid as it was of little importance in relation to the biosynthesis of the sesquiterpenoid moiety.

The crude petasin esters were saponified to their common hydrolysis product, isopetasol, 55 mg of crude yellow crystalline solid being obtained. Radio-scanning of a thin-layer chromatogram of a sample of this isopetasol failed to detect any radioactivity, because the specific activity of the isopetasol was below the lower sensitivity limit of the apparatus. Purification to radiochemical homogeneity was then completed by repeated chromatography.

The specific activity of the recovered product was determined after each step by liquid scintillation counting of a small aliquot, using the Philips Model PW 4530 Liquid Scintillation Analyser. The liquid scintillator consisted of a solution in toluene of 3 g/litre of 2,5-diphenyloxazole (PPO) and 0.1 g/litre of 1,4-bis-2'(4'-methyl-5'-phenyloxazolyl)-benzene (diMePOPOP),

TABLE 16

Purification of isopetasol from leaf feeding experiment

	Step	Recovery		Molar Specific Activity
1	Hydrolysis of petasin esters	Isopetasol	55 mg	-
2	N.1114-50%-LH20/Benzene,SEV 115-120	Isopetasol	44.9 mg	35.2 dpm/µmol
3	Acetylation	Isopetasyl acetate	52 mg	-
4	N.1114-50%-LH20/Benzene,SEV 58-61	Isopetasyl acetate	49.2 mg	31.1 dpm/µmol
5	N.1114-50%-LH20/Methanol,SEV 68-70	Isopetasyl acetate	47.3 mg	29.3 dpm/µmol
6	Hydrolysis	Isopetasol	42.5 mg	-
7	N.1114-50%-LH20/Methanol,SEV 64-66	Isopetasol	37.5 mg	26.3 dpm/µmol
8	Silica gel/Diethyl ether	Isopetasol	33.0 mg	27.2 dpm/µmol
9	N.1114-50%-LH20/Benzene,SEV 115-120	Isopetasol	30.0 mg	27.8 dpm/µmol ± 3% S.E.M.

TABLE 17

Purification of isopetasol from flower feeding experiment

Step	Recovery		Molar Specific Activity
1 Hydrolysis of petasin esters	Isopetasol	36.6 mg	-
2 N.1114-50%-LH20/Benzene, SEV 115-120	Isopetasol	25.8 mg	53.9 dpm/μmol
3 Acetylation	Isopetasyl acetate	30.3 mg	-
4 N.1114-50%-LH20/Methanol, SEV 68-70	Isopetasyl acetate	29.2 mg	38.1 dpm/μmol
5 Hydrolysis	Isopetasol	23.2 mg	-
6 N.1114-50%-LH20/Methanol, SEV 64-66	Isopetasol	22.2 mg	37.9 dpm/μmol
7 N.1114-50%-LH20/Benzene, SEV 115-120	Isopetasol	21.1 mg	37.4 dpm/μmol

$\pm$ 3% S.E.M.

(Packard Instruments Inc.). Samples were counted in 10ml of this solution; for ^{14}C, efficiency was 90.6% and background 27.5 cpm.

The specific activity recorded for the samples recovered at each step in the purification demonstrated rapid convergence of specific activity over the first three stages, and effectively constant specific activity over the last three steps (Table 16). As each stage involved either a different chromatographic process, or a change in characteristics of the sample, this was considered an effective criterion of radiochemical purity. The final product, 30.0 mg of isopetasol, had a specific activity of 27.8 dpm/ mol. Extrapolated back to the original yield of 120 mg of petasin esters, the total incorporation was estimated as 10,500 dpm, or 0.005% of the total radioactivity administered $[200 \times 10^6$ dpm (90 μCi) of (2-^{14}C-3RS) mevalonolactone].

A similar process gave 51.1 mg of petasin esters from 26.1 g (fresh weight) of flowers. Hydrolysis to isopetasol and purification in four steps (Table 17) gave 21.1 mg of pure isopetasol, specific activity 37.4 dpm/ mol. This indicated an incorporation of 0.018% of total radioactivity fed $[33 \times 10^6$ dpm (15 μCi) of (2-^{14}C-3RS) mevalonolactone].

Hydrolysis of Petasin Esters:

Petasin esters (120 mg) were dissolved in ethanol (4 ml), and 3.5M aqueous potassium hydroxide (0.5 ml) added. The mixture was refluxed for 30 minutes, and the volume of ethanol reduced to 2 ml in a stream of nitrogen. The mixture was extracted three times with benzene (10 ml), water (1 ml) being added to separate out the potassium hydroxide. Removal of solvent gave crude isopetasol (55 mg) as a yellow crystalline solid. GLC indicated the identity of the isopetasol by comparison with an authentic sample[7], using two stationary phases ($I_{OV-1}^{162^{O}} = 1965$; $I_{XE-60}^{162^{O}} = 2600$), and this was further confirmed by GC-MS (molecular ion, m/e 234, 73% of base peak, m/e 161).

Acetylation of Isopetasol

To the purified isopetasol (45 mg) was added pyridine (0.25 ml) and acetic anhydride (0.5 ml): the reaction was left in a stoppered tube overnight at room temperature. Methanol (2 ml) was added, and the solvent removed in a stream of nitrogen. Pyridine was removed azeotropically with ethanol. A clear, odourless oil (52 mg) was recovered. GLC and GC-MS indicated its identity as isopetasyl acetate by comparison with an authentic sample[7] ($I_{OV-1}^{180^{O}} = 2075$; $I_{XE-60}^{162^{O}} = 2625$; molecular ion m/e 276, 30% of base peak m/e 161).

Hydrolysis of Isopetasyl Acetate

Isopetasyl acetate (47 mg) was dissolved in ethanol (2 ml) and 3.5 M aqueous potassium hydroxide (0.2 ml) was added. The mixture was refluxed for 20 minutes and benzene (10 ml) and water (1 ml) were added. The organic layer was removed, and the aqueous layer re-extracted twice more with benzene (10 ml). After evaporation of solvents, crude pale yellow crystalline isopetasol (42.5 mg) was recovered and characterised as above.

3.3.3   Retro-Aldol Reaction and Isolation of Iodoform and Desisopropylideneisopetasol

Isopetasol from the leaf feeding (29 mg) was dissolved in methanol (3 ml), and 3.5 M aqueous potassium hydroxide (2.8 ml) was added. The mixture was refluxed for 18 hours, under a nitrogen atmosphere. The flask was fitted for distillation, and methanol and acetone were distilled over, and were collected in a graduated centrifuge tube immersed in ice-salt freezing mixture ($-15^{\circ}C$). When 2.5 ml of organic solvent had been collected, and oily drops began to separate from the reaction mixture, the distillation was stopped, and the aqueous base immediately extracted with a mixture of benzene and ethyl acetate (5 ml of each). Re-extraction twice more with the same solvent mixture

and evaporation to dryness gave 23.3 mg of pale yellow
oil. Chromatography on the 100 cm high-resolution column
[N1114-50%-LH20(24-32μ)/Benzene; SEV 122-130] gave pure
desisopropylideneisopetasol (18.2 mg; 77% theoretical yield):
a trace of unchanged isopetasol eluted in the range
SEV 115-120. GLC and GC-MS confirmed the identity of the
product by comparison with a sample of the properly
characterised compound ($I_{OV-1}^{150^{O}}$ = 1770; $I_{XE-60}^{150^{O}}$ = 2430;
molecular ion m/e 194, 37% of base peak m/e 176).

Similar procedures led to the isolation of
desisopropylideneisopetasol (12.9 mg) in 75% yield from
isopetasol (20.7 mg) obtained from the flower feeding.

Isolation of Iodoform

To the chilled solution of acetone in methanol
(obtained above from the leaf experiment) was added
1 M potassium hydroxide (4 ml), and iodine crystals (50 mg)
were then dissolved in the mixture with shaking. A dense
yellow precipitate of iodoform separated immediately, and
on warming and recooling, distinct platelike crystals were
formed. The iodoform was centrifuged down when all the
iodine had dissolved. The supernatant was tested with one
further crystal of iodine, but no more precipitate formed.
The mass of yellow crystals was resuspended twice in
distilled water, and separated again by centrifugation.

Finally, the sample was recrystallised from 80% aqueous ethanol, and air-dried overnight. After brief exposure to vacuum, 4.6 mg of iodoform was transferred to a counting vial for radioassay.

By similar procedures, 2.1 mg of iodoform was recovered from the retro-aldol degradation of isopetasol from the flower feeding experiment.

Characterisation of Desisopropylideneisopetasol

Isopetasol (450 mg) was dissolved in methanol (50 ml). The mixture was de-aerated by shaking vigorously in vacuo, pure nitrogen being readmitted to the flask. This process was repeated twice more, and 3.5 M aqueous potassium hydroxide (50 ml) was prepared in the same manner, and added to the solution of isopetasol. The mixture was refluxed for 20 hours in a nitrogen atmosphere, and at the end of the reaction was virtually uncoloured by autoxidation products. Extraction of the reaction mixture gave on evaporation a colourless oil (370 mg), and GLC analysis indicated complete conversion of isopetasol into a single product ($I_{OV-1}^{150^{\circ}}$ = 1770). GC-MS gave a molecular ion $\underline{m/e}$ 194 ($C_{12}H_{18}O_2$ requires 194) but the mass spectrum was of little diagnostic value (Appendix I). Further purification by straight-phase chromatography gave a colourless crystalline solid (350 mg) [Column III, 30 cm x 2.5 cm i.d.; N1114-50%-LH20 (24-32μ)/

100 MHz NMR (CDCl$_3$) of Isopetasol (27)

τ values
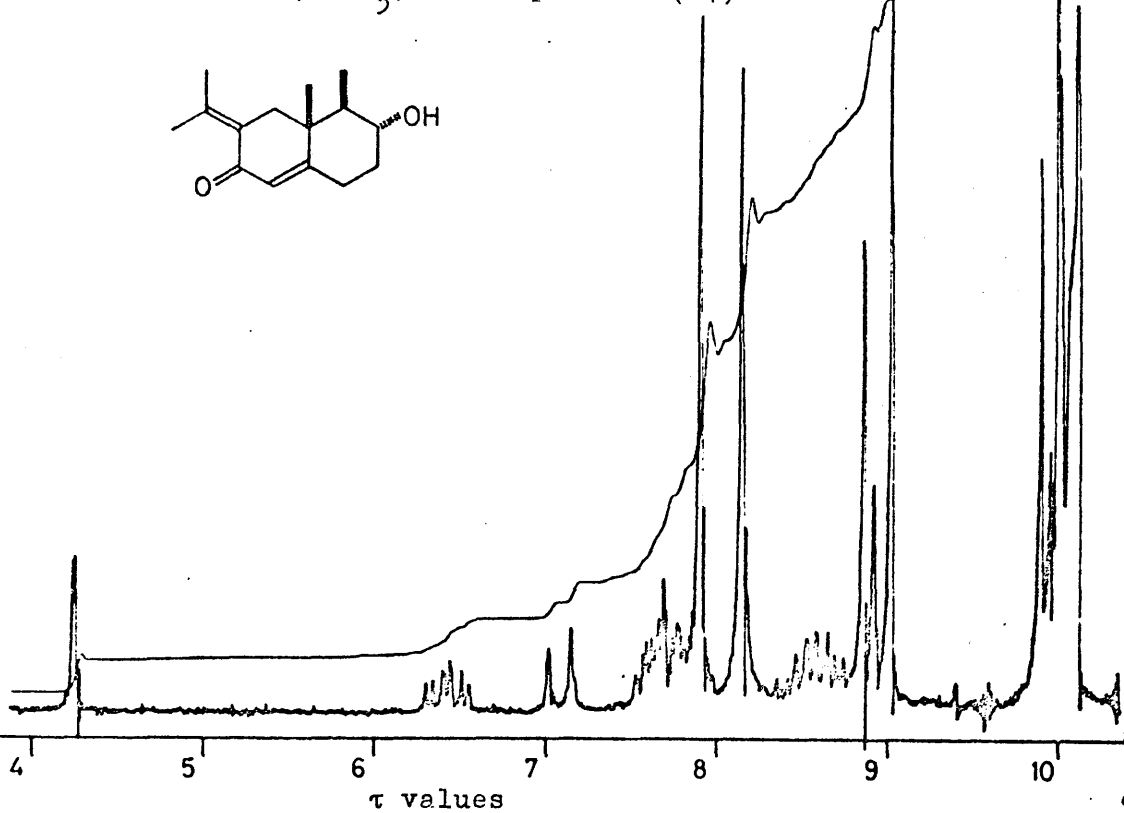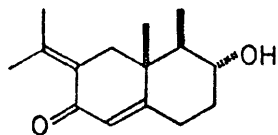
100 MHz NMR (CDCl$_3$) of
Desisopropylideneisopetasol (56)

τ values
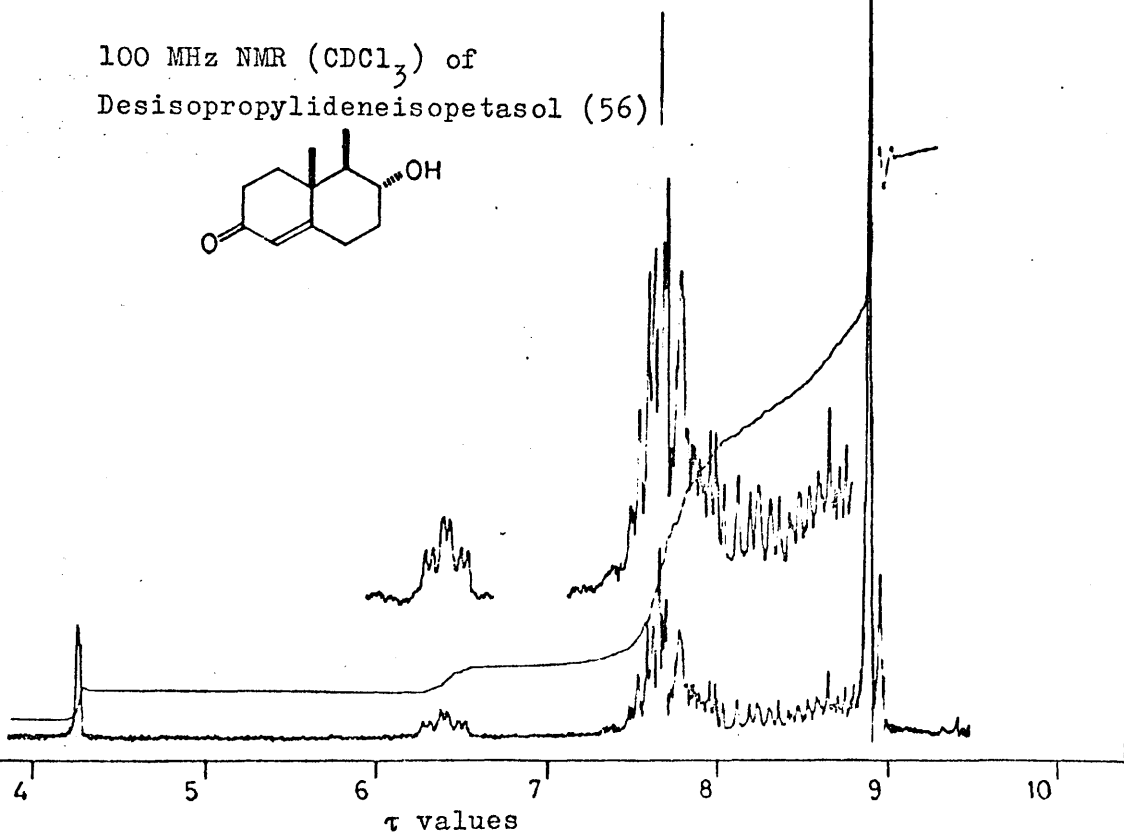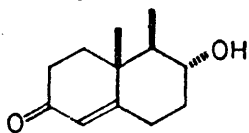
Fig. 22: NMR Assignments for Isopetasol (27) and
Desisopropylideneisopetasol (56)
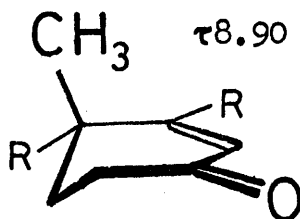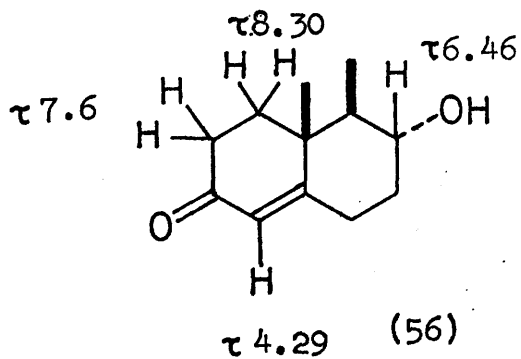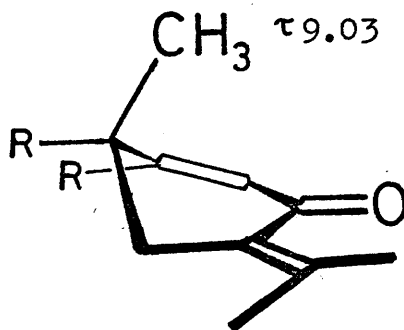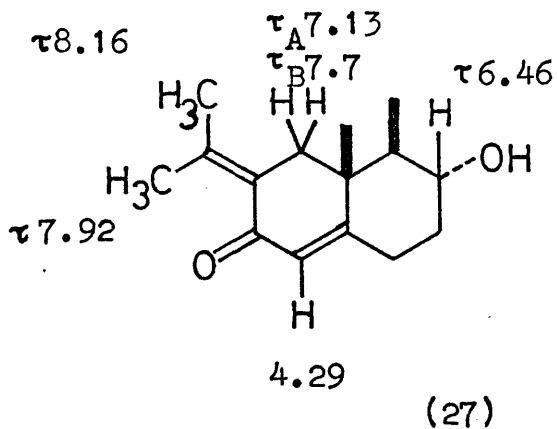
benzene; SEV 120-130]. The sample was run in two batches to avoid overloading the column; the automatic fraction collector was used to recover the samples. Further purification of a smaller sample by reversed-phase chromatography (N1114-50%-LH2O/methanol; SEV 58-62) gave a crystalline product (30 mg), m.p. 103°; ν_{max} (Nujol) 3500 cm^{-1}(s), 1680 cm^{-1}(s) and 1620 cm^{-1}(w); λ_{max} 238 nm (ε_{max} 11,000). (Found: C, 74.03%; H, 9.58%: $C_{12}H_{18}O_2$ requires C, 74.19%; H, 9.34%.) Aebi reported[6] m.p. 102-103°; λ_{max} 238 nm (ε_{max} 17,500).

In the absence of an authentic sample, confirmation of the structure (56) was made by spectroscopic examination. IR and UV confirmed the continued presence of hydroxyl and α,β-unsaturated ketone functions. Comparison of 100 MHz NMR spectra of isopetasol and desisopropylideneisopetasol showed the disappearance of signals due to the isopropylidene side-chain, a shift to higher field of methylene protons at C-6 (allylic in isopetasol), and a slight deshielding of the angular methyl group that indicated the expected conformational change in ring B. (Isopetasol, τ = 9.03; desisopropylideneisopetasol, τ = 8.90). A Dreiding model of desisopropylideneisopetasol favoured a conformation in which the angular methyl group lay close to the axis of the carbonyl bond; in isopetasol, the angular methyl group was

(56) $\xrightarrow{\text{H}_2/\text{Pd-C}}$ (61)
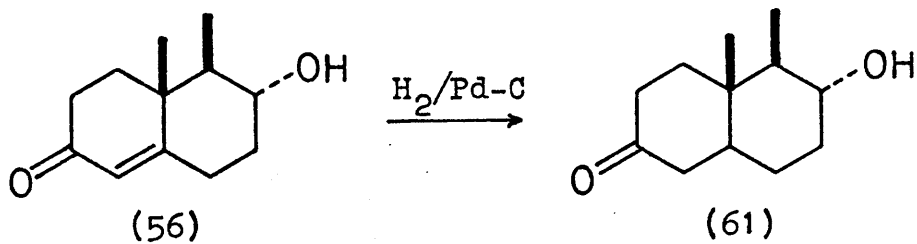
1) TsNHNH$_2$
2) NaBH$_4$
3) MeOH

(69)   (100)

CrO$_3$

(70) $\xrightarrow{\text{CF}_3\text{CO}_3\text{H}}$ (71)
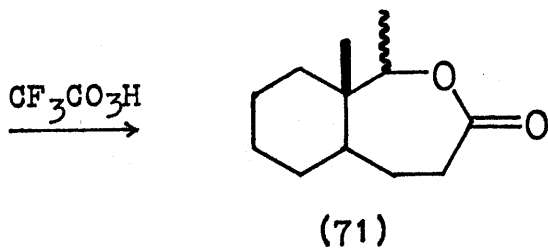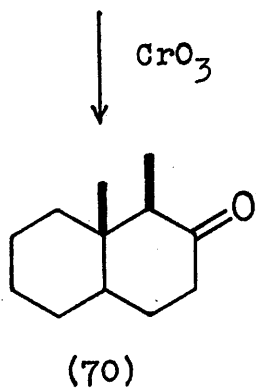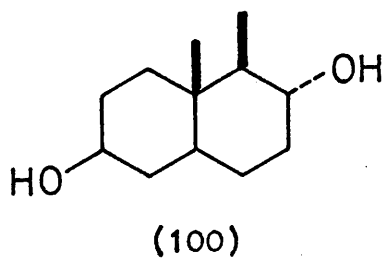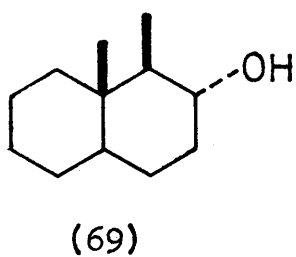
at 45° to the axis. Other signals in the NMR spectra were
substantially unchanged (Fig.22).

3.3.4   Isolation of C-3 of Isopetasol

         Hydrogenation of Desisopropylideneisopetasol (56)

         Desisopropylideneisopetasol (31.8 mg) was
hydrogenated at room temperature and pressure in ethyl
acetate (2.5 ml) in the presence of 10% palladium on charcoal
(7 mg) as catalyst. After 30 minutes, uptake of hydrogen had
ceased. The reaction mixture was filtered (porosity 4 glass
sinter) and the residue washed with ethyl acetate (2 ml).
The product (32.0 mg) was recovered as a colourless
crystalline solid by evaporation of solvent (100% yield).
GLC indicated complete conversion into the saturated
ketoalcohol (61), $I_{OV-1}^{146°} = 1690$; $M^+ = 196$ ($C_{12}H_{20}O_2$
requires 196); ν_{max} (Nujol) 3450 cm^{-1}(s), 1710 cm^{-1}(s)
and 1045 cm^{-1}(m).

Reduction of (61) to alcohol (69)[91]

         The saturated ketoalcohol [(61); 32 mg] was treated
with p-toluenesulphonylhydrazine (32 mg; 1.05 equivalents)
and refluxed in methanol (2 ml) for 2 hours, when GLC
indicated the complete disappearance of (61) by formation
of the tosylhydrazone. On cooling, sodium borohydride
(40 mg) was added batchwise over 90 minutes, to reduce

the tosylhydrazone to a substituted tosylhydrazine. After addition was complete, the mixture was refluxed again for five hours to decompose the tosylhydrazine. Water was added (1 ml), and the alkalinity (pH 9) indicated that no additional base was required to ensure the removal of the p-toluenesulphinic acid formed. The reaction mixture was extracted twice with ethyl acetate-benzene mixture (5 ml; 4:1 v/v). On evaporating to dryness, a colourless oil (30 mg) was obtained. GLC indicated a single peak ($I_{OV-1}^{119^{o}}$ = 1450) in the first two trials with unlabelled material, but in the case of the radioactive sample, there was a second peak ($I_{OV-1}^{140^{o}}$ = 1720). The pure samples of the saturated alcohol (69) gave M^{+} = 182 ($C_{12}H_{22}O$ requires 182); ν_{max}(liquid film) 3450 cm^{-1}(s), 1045 cm^{-1}(m) and saturated C-H and C-C modes only.

GC-MS of the second peak gave a weak molecular ion, M^{+} = 198 ($C_{12}H_{22}O_{2}$ requires 198): stronger peaks were obtained for M-18 and M-36 ions, consistent with the structure of the diol (100). No explanation was apparent for this isolated result, which seriously reduced the yield of (69).

Jones Oxidation of Saturated Alcohol (69)

The product from the previous step was taken without further purification, and a solution in acetone (1 ml)

was oxidised with Jones' reagent [108] (75 μl; 250 μmol).
After 2-3 minutes, water (0.5 ml) was added, and the mixture
extracted with benzene (2 x 5 ml). Evaporation of solvent
gave a colourless oil with a pronounced odour of mint.
GLC showed complete conversion of (69) into a new compound
($I_{OV-1}^{119°}$ = 1430). The product was purified by reversed-phase
chromatography (N1114-50%-LH20/methanol; SEV 90-110), to
give the saturated ketone [(70); 9 mg], in 35% overall yield
from desisopropylideneisopetasol (56), in the case of the
labelled sample. The two trial samples gave 60-70% overall
yields of (70) at this stage. The purified ketone (70) gave
M^+ = 180 ($C_{12}H_{20}O$ requires 180); ν_{max}(liquid film) 1710cm^{-1}.

Baeyer-Villiger Oxidation of Saturated Ketone (70)

Peroxytrifluoroacetic acid was prepared immediately
before use by adding, dropwise, trifluoroacetic anhydride
(250 μl) to a suspension of 90% hydrogen peroxide (40 μl)
in methylene chloride (250 μl; 0°C) [92]. 100 μl of this
solution oxidised 200 μmol of cholestanone (77 mg) to give
in quantitative yield the derived ε-lactone (80 mg).

A solution of the saturated ketone [(70); 9 mg]
in methylene chloride (250 μl) was stirred with freshly
dried disodium hydrogen phosphate (35 mg) [93]. The solution
of peroxytrifluoroacetic acid (30 μl) was added over
20 minutes, with vigorous stirring to prevent coagulation

of the solid phosphate buffer, which changed to a gummy paste in the course of the reaction. Stirring was continued for 1 hour, when the reaction mixture was diluted with methylene chloride (2 ml), and the buffer sedimented by centrifugation. The buffer was re-extracted by washing with water (1 ml) and ether (3 ml). The organic extracts were combined to give by evaporation ε-lactone (71) as a colourless oil 9.6 mg; 98% yield from (70) . GLC showed a closely spaced double peak ($I_{OV-1}^{140^o}$ = 1670, 1675), and GC-MS was consistent with the formation of a pair of epimeric structures, the two components giving identical mass spectra. C-4 was assigned as the centre of epimerisation. The normal mode of bond migration in the Baeyer-Villiger reaction[92,109] would lead to the product formulated in structure (71).

The ε-lactone (71) gave M^+ = 196 ($C_{12}H_{20}O_2$ requires 196); ν_{max}(liquid film) 1735 cm^{-1}(s).

Mass Spectral Evidence for the Structure (71)

The mass spectrum of the ε-lactone (71) gave a series of radical-ions, the proposed sequence of fragmentation being substantiated by metastable peaks (Appendix I). A sequence involving loss of acetaldehyde to give the fragment $[C_{10}H_{16}O]^{\ddagger}$ of m/e 152 was implicit in its subsequent loss of eighteen mass units (H_2O)

Fig. 23a: Proposed Scheme of Mass Spectral Fragmentation
for ε-Lactone (71)

m/e: 196   152   134   110   96   81

m* 118.1
m* 79.6
m* 68.4
m* 60.6

M⁺ m/e 196

m/e 152

-H₂O

m/e 81   m/e 96   m/e 110   m/e 134
Base Peak
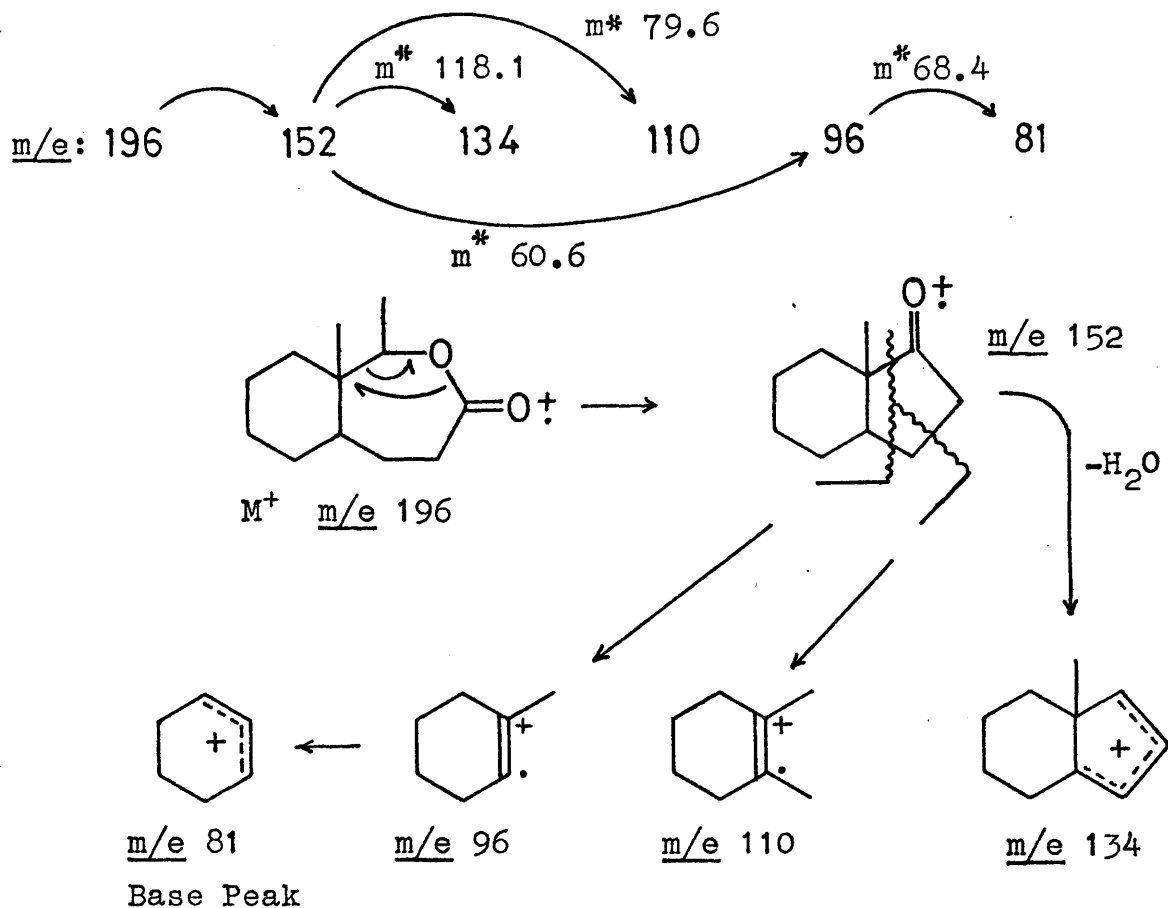
Fig. 23b: Proposed Fragmentation of Some ω-Substituted
Lactones[110,111]

(101)   R = H   M - 44 present
         R = CH₃   M - 44 present

(102)   R = H   M - 44 present
         R = CH₃   M - 44 present
         R = C₂H₅   M - 58 present; M - 44 absent

$$[O \diagdown O \diagdown R]^{\ddot{+}} \rightarrow {}^{+}\!O \diagup \square + RCHO$$
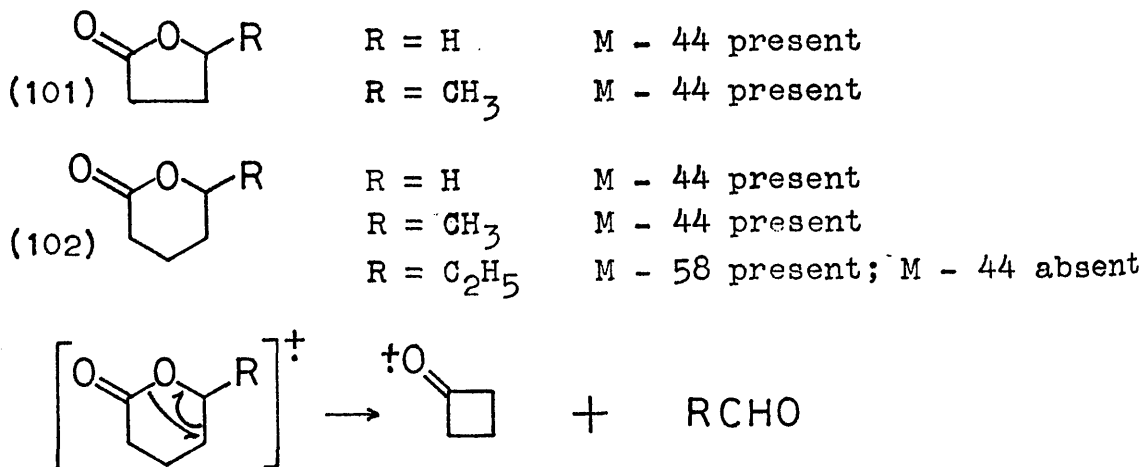
to give the ion $\underline{m/e}$ 134 $[C_{10}H_{14}]^{\ddagger}$, indicated by the metastable peak $\underline{m/e}$ 118.1, and by lack of other possibilities (Fig. 23a). This was considered strong evidence for a part-structure containing an ε-lactone with a terminal methyl substituent.

The analogous γ- and δ-lactones (101,102) having terminal methyl substituents (R = Me) have been found to give strong M-44 peaks, as did the unsubstituted lactones (R = H)[110]. It was proposed that this arose by loss of CO_2 in each case, though no high-resolution mass measurements were made to confirm this fragmentation. Subsequently, it has been found that (102) gave the M-58 peak rather than M-44 when R was ethyl[111], but the two results were not apparently correlated. It seems highly likely that aldehyde loss (M-RCHO) is the process actually involved in the fragmentation of the substituted δ-lactones [(102);Fig.23b], in support of the assignment made for the fragmentation of ε-lactone (71).

Barbier-Wieland Degradation[90,94,95] of ε-Lactone (71)

The lactone [(71); 9.6 mg, 49 μmol] was treated with an ethereal solution containing phenyl magnesium bromide (0.6 mmol), and stirred under reflux overnight. The adduct was treated with ice-water (5 ml) containing 2 M sulphuric acid (1 ml), and the organic product

extracted with ether (2 x 15 ml). Evaporation of solvent gave an oil (30 mg) with a strong aromatic odour. The oil was warmed _in vacuo_ until the volatile products (mainly biphenyl) were removed. Without further examination, the product was refluxed for 8 hours in acetic acid (1 ml) containing water (0.2 ml). Aqueous base was then added to pH 5.5-6, and the organic product extracted in benzene (3 x 5 ml). Evaporation yielded an oil, which was treated with dry pyridine (0.1 ml) and acetic anhydride (0.3 ml), and left in a stoppered tube for 12 hours at room temperature. The reagents were removed by evaporation of the azeotrope with ethanol. An orange crystalline material (20 mg) was obtained. The product was purified by straight-phase chromatography (N1114-50%-LH20/benzene; SEV 50-56) and gave the acetoxydiphenylethylene [(73); 10.2 mg], in 55% yield based on (70): M^+ = 376 ($C_{26}H_{32}O_2$ requires 376); ν_{max}(liquid film) 3040 cm^{-1}(w), 1730 cm^{-1}(s), 1600 cm^{-1}(m), 1500 cm^{-1}(m), 1255 cm^{-1}(s), 880 cm^{-1}(m) and 815 cm^{-1}(s); λ_{max}^{EtOH} 251 nm (ε_{max} 11,500). [1,1-diphenylethylene has [112] λ_{max}^{EtOH} 250 nm (ε_{max} 11,000).] An epimeric mixture (1:1) was indicated by GLC ($I_{OV-1}^{226^o}$ = 2675,2685) and by 100 MHz NMR (CDCl$_3$) τ 9.29 ($\frac{1}{2}$ 3H), 9.08 ($\frac{1}{2}$ 3H), 9.03 (3H doublet, J = 6 c/s), 8.40 ($\frac{1}{2}$ 3H), 8.10 ($\frac{1}{2}$ 3H), 5.1 (1H multiplet), 4.1 (1H broad) and 2.7-3.0 (10H complex).

Ruthenium Tetroxide Cleavage of Acetoxydiphenylethylene (73)

Ruthenium tetroxide was prepared by stirring a suspension of ruthenium dioxide (9.6 mg) in water (1 ml) containing sodium metaperiodate (50 mg) for 30 minutes, when the black particles of the dioxide had completely dissolved.

50 μl of this solution (ca. 600 μg RuO_4) was added to a solution of acetoxydiphenylethylene [(73); 10.2 mg] in acetone (1 ml), and finely powdered sodium metaperiodate (32 mg) was added in portions during 4 hours with continuous stirring. Water (0.2 ml) was added over the same period. After stirring for a further ½ hour, isopropanol (0.5 ml) was added to destroy excess reagent, and stirring was continued for 20 minutes. Acetone (5 ml) was added to the reaction mixture, and sodium iodate and ruthenium dioxide were removed by centrifugation. The precipitate was washed twice more with acetone (5 ml) and the extracts were combined and evaporated to 0.5 ml. 0.5 M sodium carbonate (1.5 ml) was added, and the neutral fraction isolated by extracting with benzene (2 x 5 ml). Evaporation of solvents gave the neutral products (8 mg). Analytical liquid chromatography[*] (N1114-50%-LH20/benzene) showed the presence of three peaks, SEV 53, 58 and 64. Authentic benzophenone gave a single peak, SEV 64, on the same column. The sample

[*] Using the Haahti-Sjövall circulating chain flame-ionisation detector to monitor column effluents.

was purified by straight-phase chromatography (N1114-50%-LH20/ benzene; SEV 61-67) and collected in three fractions. The middle fraction gave 1.435 mg benzophenone, identical by GC-MS to authentic benzophenone, which was used for radioassay. The first and last fractions were combined to give benzophenone (1.2 mg), spectroscopically identical with an authentic sample; $I_{OV-1}^{140^{\circ}} = 1600$; $M^{+} = 182$ ($C_{13}H_{10}O$ requires 182); ν_{max}(liquid film) 3080 cm^{-1}(w), 1655 cm^{-1}(s), 1600 cm^{-1}(m), 1580 cm^{-1}(w), 1450 cm^{-1}(m), 1320 cm^{-1}(m), 1280 cm^{-1}(s) and 815 cm^{-1}(s); λ_{max}^{EtOH} 253 nm (ε_{max} 16,500).

2 M sulphuric acid was added dropwise to the aqueous sodium carbonate layer obtained above, to pH 2.5. The acidic products of the cleavage reaction were extracted with ethyl acetate (2 x 5 ml) and the combined extracts were evaporated to give almost pure acetoxy acid [(75); 3.4 mg], in 55% yield based on (73). The acid was treated with ethereal diazomethane to give the methyl ester (76). Analytical liquid chromatography showed a single peak (N1114-50%-LH20/benzene; SEV 57) and (76) was further purified by the same procedure on a preparative-scale column. The middle fraction of three gave 1.990 mg of the methyl ester (76) which was used for radioassay after GC-MS had established the identity of the product. The remainder of the sample, contained in the first and third fractions

gave a further 1.4 mg of product, identical by GC-MS to methyl ester (76) obtained in trials with unlabelled material.

Methyl ester [(76); 10 mg] gave (as a mixture of epimers 1:1) $I_{OV-1}^{140°}$ = 1655, 1675; M^+ absent, M-60 = 196 ($C_{13}H_{22}O_4$ requires M = 256); ν_{max}(liquid film) 1735 cm^{-1}(s), 1255 cm^{-1}(s); 100 MHz NMR (CDCl$_3$) τ 9.20 ($\frac{1}{2}$ 3H), 9.00 ($\frac{1}{2}$ 3H) 8.92 ($\frac{1}{2}$ 3H doublet, J = 6.5c/s), 8.86 ($\frac{1}{2}$ 3H doublet, J = 6.5c/s) 8.01 (3H), 7.62 (1H multiplet), 6.39 (3H), 5.06 (1H quartet J = 6.5c/s).

Hydrolysis of Methyl Ester (76) and Formation of δ-Lactone (77)

Methyl ester [(76); 5 mg], from unlabelled material used in preliminary trials, was hydrolysed for 40 minutes in ethanol (1 ml) containing 3.5 M aqueous potassium hydroxide (0.1 ml). The reaction mixture was extracted with ethyl acetate, which gave negligible quantities of product on evaporation. The aqueous layer was acidified with sulphuric acid to pH 3, and re-extracted with ethyl acetate to give a colourless oil (3.5). The δ-lactone (77) was identified by GLC and GC-MS as the nor-homologue of ε-lactone (71): $I_{OV-1}^{140°}$ = 1570, 1580; M^+ = 182 ($C_{11}H_{18}O_2$ requires 182); ν_{max}(liquid film) 1735 cm^{-1}(s). (Traces of acetoxy acid (75) were apparent in the IR spectrum.)

The mass spectrum of δ-lactone (77) showed a

Fig. 24: Fragmentation of δ-Lactone (77)

m/e: 182 → 140
      182 → 138
      182 ⇢ 96
      96 → 81   m* 68.4
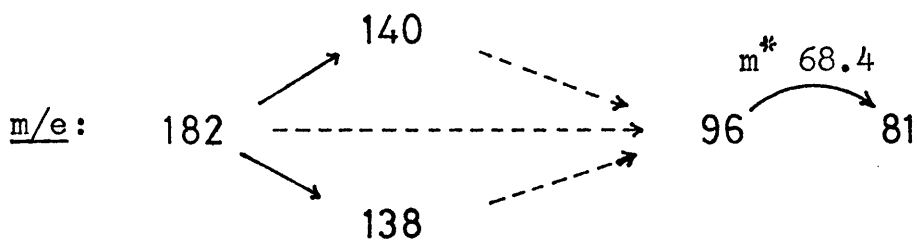
Formal Derivation of Ions m/e 140, 138 & 96

M⁺   m/e 182

m/e 138

m/e 140

m/e 96
Base Peak

m/e 81
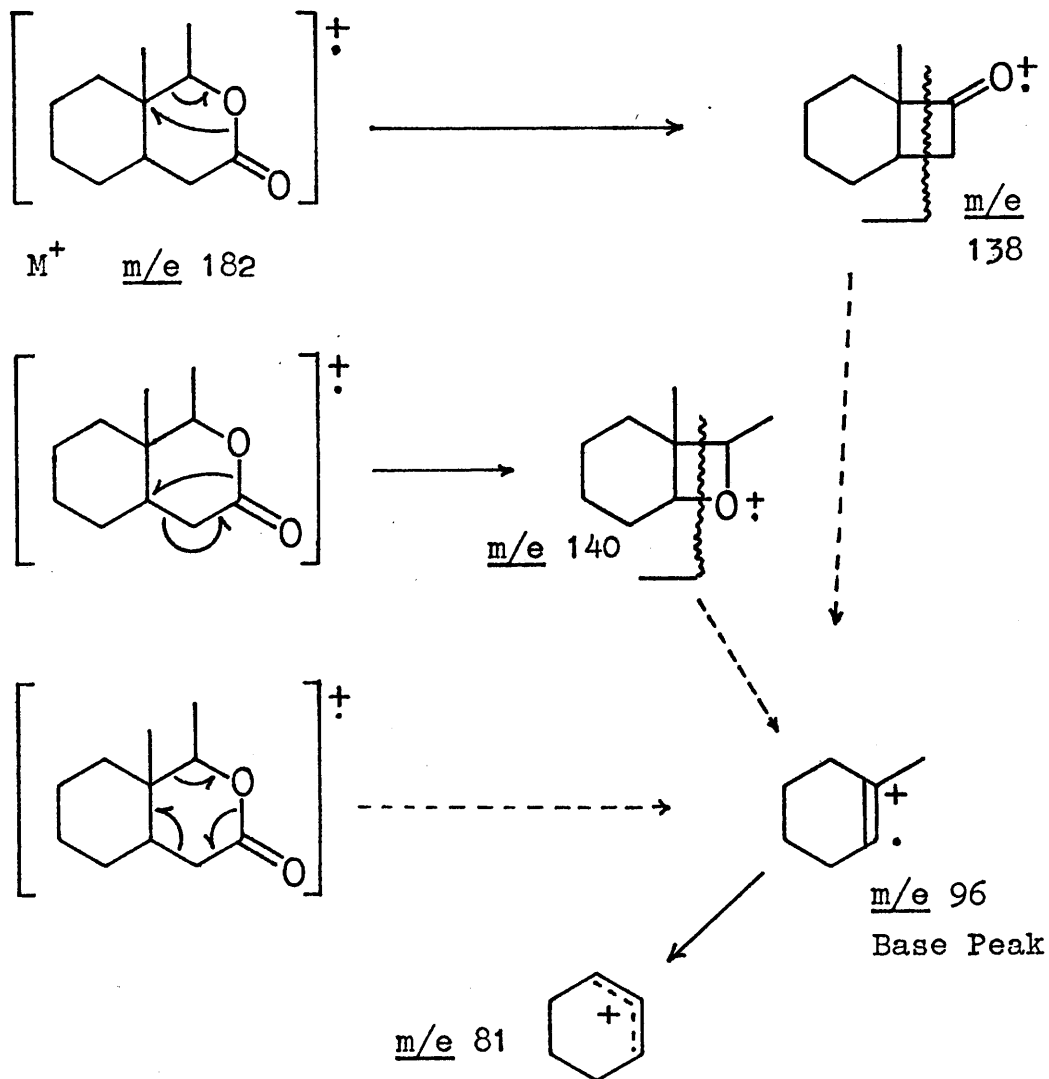
sequence of radical-ions similar to that found for ε-lactone (71), suggesting a relationship between the two structures (Fig. 24 and Appendix I) and providing confirmatory evidence for the specific removal of C-3 of the eremophilane skeleton.

3.3.5 Isolation of C-8 of Isopetasol

Acetylation of Desisopropylideneisopetasol (56)

Desisopropylideneisopetasol [(56); 8.4 mg] was treated with dry pyridine (0.1 ml) and acetic anhydride (0.3 ml) and left overnight in a stoppered tube. The reagents were removed by evaporation of the azeotrope with ethanol. The product, desisopropylideneisopetasyl acetate (78) was obtained as a colourless oil (10.4 mg): $I_{OV-1}^{162^{O}} = 1850$; $M^+ = 236$ ($C_{14}H_{20}O_3$ requires 236); ν_{max}(liquid film) 1735 cm^{-1}(s) 1675 cm^{-1}(s), 1625 cm^{-1}(m), 1250 cm^{-1}(s),1045 cm^{-1}(m).

Borohydride Reduction of (78)

A solution of desisopropylideneisopetasyl acetate [(78); 10.4 mg] in ethanol (1 ml) was treated with sodium borohydride (3 mg), and stirred for 1 hour. Water (1 ml) was added, and the product extracted twice with a mixture of ethyl acetate (3 ml) and benzene (1 ml). On evaporation, a colourless oil (10.4 mg) was obtained: GLC indicated at least two peaks, $I_{OV-1}^{162^{O}} = 1800$ (75%), 1810 (25%). GC-MS characterised the earlier peak as the allylic alcohol (84);

M^+ = 238 ($C_{14}H_{22}O_3$ requires 238), and the second peak as
the saturated alcohol (85); M^+ = 240 ($C_{14}H_{24}O_3$ requires 240).
The infra-red spectrum of the mixture gave ν_{max}(liquid film)
3450 cm^{-1}(s), 1730 cm^{-1}(s), 1655 cm^{-1}(w), 1250 cm^{-1}(s),
1040 cm^{-1}(s).

Cleavage of (84) with Lemieux-Johnson Reagent[103,104]

The mixture of saturated (85) and allylic (84)
alcohols was dissolved in a mixture of dioxan (0.3 ml)
and water (0.1 ml). Osmium tetroxide (2.2 mg) was added,
and the mixture stirred until the crystals had dissolved,
with the formation of a brown suspension after 1-2 hours.
Sodium metaperiodate (4 mg) was added and the mixture was
decolorised. Stirring was continued, and further portions
of sodium metaperiodate were added (to a total of 32 mg)
whenever the brown coloration returned. After 30 hours,
water (0.1 ml) was added, and after 48 hours, the brown
colour ceased to form. A small aliquot was worked up, and
GLC indicated the disappearance of the allylic alcohol (84),
while the saturated alcohol (85) remained unchanged.
Water (2 ml) was added, and the organic products extracted
twice with a mixture of ethyl acetate (3 ml) and benzene (1 ml).
Evaporation gave a brownish oil (12 mg). Analytical liquid
chromatography (N1114-50%-LH20/benzene) gave peaks
SEV 58, 100, 105, 142 and 148. The desired product,

the acetoxyketoaldehyde (89), unlike most other structures
that might have arisen from the reaction, had no hydroxyl
function, and could thus be correlated with the peak at
SEV 58[49]. Preparative separation (N1114-50%-LH20/benzene;
SEV 55-61) afforded this product (1.2 mg). No peak was
obtainable by GLC of this fraction. The sample gave
ν_{max} (CCl$_4$) 2820 cm^{-1}(w), 2710 cm^{-1}(w), 1745 cm^{-1}(shoulder),
1735 cm^{-1}(s), 1710 cm^{-1}(s), 1228 cm^{-1}(s) and 1018 cm^{-1}(m);
the infra-red spectrum obtained was consistent with the
structure of the acetoxyketoaldehyde (89). A sample (0.970 mg)
was transferred to a counting vial for radioassay,
structure (89) being assumed for the purposes of calculations.

A sample of (89) prepared from unlabelled material
was found to have partly decomposed: ν_{max} 1755 cm^{-1}
appeared in the product, ν_{max} 1710 cm^{-1} being diminished
in the process of decomposition. Mass spectrometry of this
sample (AEI MS-12; introduced on the probe) gave M$^+$ = 240
(C$_{13}$H$_{20}$O$_4$ requires 240).

The products obtained from a fraction SEV 95-110
were found by GLC and GC-MS to be the saturated alcohols (85)
as an epimeric mixture (2.8 mg) $I_{OV-1}^{162^o}$ = 1795 (30%), 1810 (70%).

The products obtained from a fraction SEV 130-160
gave a crystalline solid (3.6 mg), GLC indicating an
epimeric mixture, $I_{OV-1}^{162^o}$ = 1905 (25%), 1915 (75%).

(103)

(104)

(84)

(105)

(107)

(106)

(90)

(108)

(109)

(110)
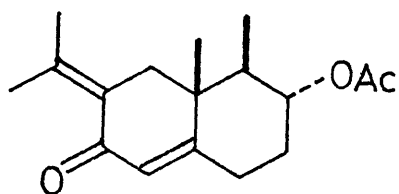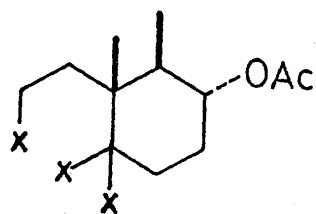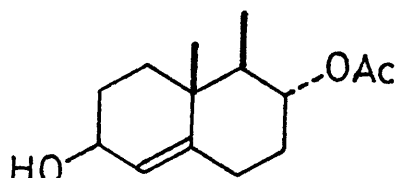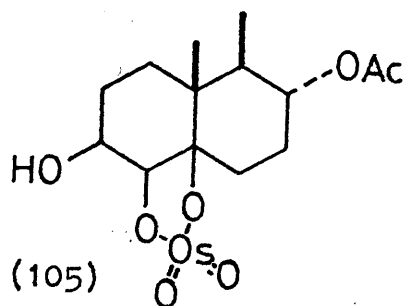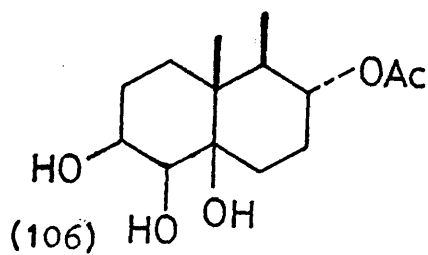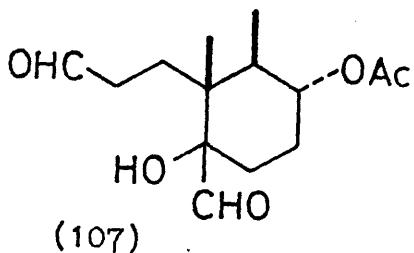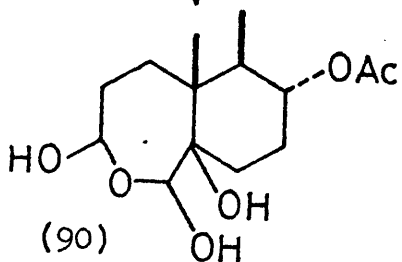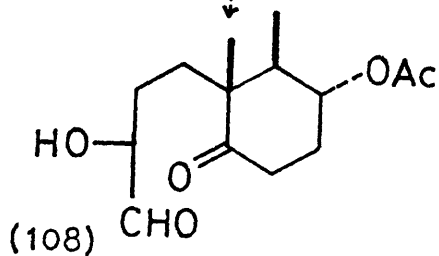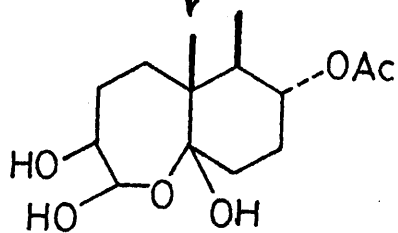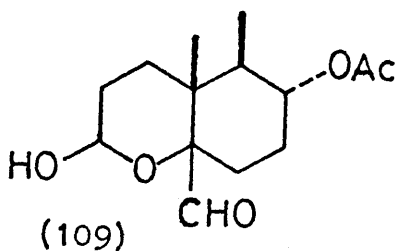
GC-MS gave no molecular ion, and the highest fragment,
m/e 224, could not be ascribed as M-60; a peak occurred
also at m/e 164, indicating that a process other than
de-acetylation was responsible for the first fragmentation.

Assignment of Structure (90) to the Major Product of

Lemieux-Johnson Cleavage of (84)

100 MHz NMR of a solution in $CDCl_3$ of the product
obtained above at SEV 130-160 gave signals τ:
9.14 (3H, doublet, J = 6c/s), 9.11 (3H), 8.2-8.6 (8H complex),
7.94 (3H), 7.73 (1H multiplet), 7.52 (1H doublet, J = 9c/s),
5.32 (1H, multiplet), 4.67 (1H, doublet, J = 9c/s),
4.35 (1H, triplet J = 1 c/s).

The high field region of the spectrum suggested
that the structure of ring A was intact. This was further
confirmed by spin-decoupling experiments, and correlation
of these results with data for isopetasyl acetate (103).
The four additional protons in the complex region ascribed
to ring methylene resonance suggested that a part of ring B
was also unchanged, as in part structure (104). This was
not unexpected, as the reaction of the Lemieux-Johnson
reagent[103] would be confined to C-8, C-9 and C-10 of
the allylic alcohol (84), producing as an intermediate,
via the osmate ester (105), the acetoxytriol (106).
Periodate cleavage would then give one of the products

100 MHz NMR (CDCl₃) of
acetoxy-bis-hemiacetal (90)
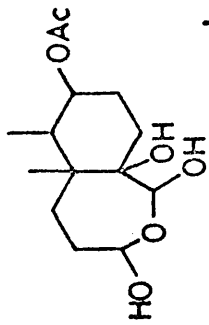
Irradiation at τ 8·5

D₂O exchange

←H₂O
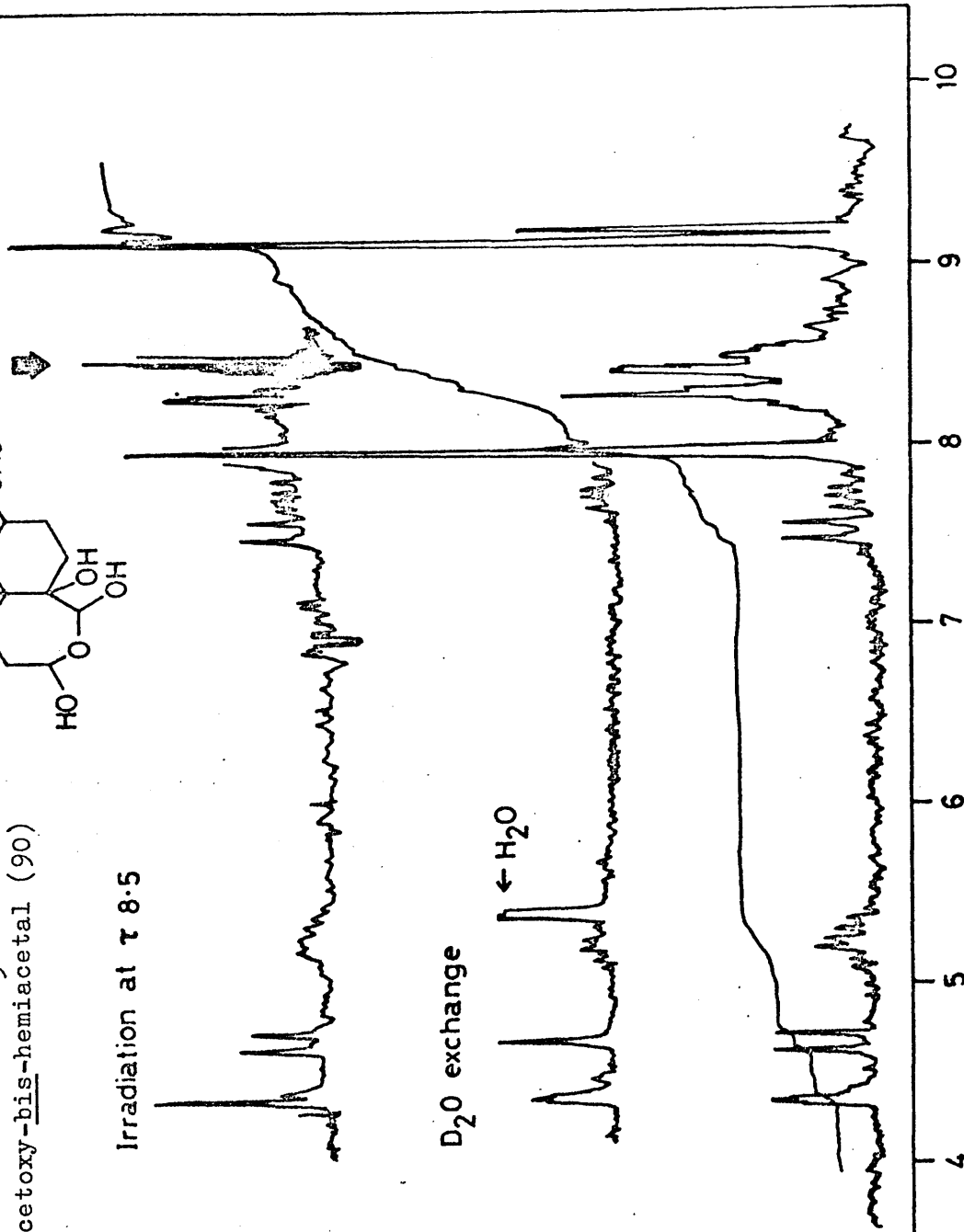
τ values

formulated as (107) or (108). The further cleavage of the
α-hydroxyaldehyde may be slower than the first step, and it
seemed highly likely that this was the stage at which a
side-reaction may have occurred.

The relative intensity of infra-red absorption
bands ν_{max} 1730 cm^{-1} and 1255 cm^{-1}, the lower frequency
band being the more intense, suggested strongly that these
derived solely from the acetate group, and that carbonyl
absorption from other sources was absent. This excluded the
possibility of an aldol-type condensation giving rise to
the product obtained. Hemiacetals, as in structure (109)
were also excluded by the physical data, not only by
infra-red absorption, but also by the lack of NMR signals
due to allylic protons, hydrogen α- to carbonyl groups,
or aldehyde protons (down to $\tau = -5.0$). Only the
bis-hemiacetals (90) and (110) were well fitted to the
spectroscopic evidence for the structure. The presence in
the infra-red spectrum of strong O-H stretching absorption
at 3400 cm^{-1}, and a complex series of bands in the range
1140-960 cm^{-1} assigned to C-O stretching modes, supported
this allocation.

Exchange with D_2O gave further evidence from the
NMR spectrum that prompted the assignment of structure (90).
After exchange, the signals at $\tau = 7.52$ disappeared, and

NMR Assignments for the Structure of the
Acetoxy-bis-hemiacetal (90), and Postulated
Derivation of Mass Spectral Ions

τ9.14
τ 9.11
Me Me
H τ7.73
OAc τ7.94
H τ5.32
τ 4.35 H
HO O OH
H OH

τ4.67

(Isolated Spin System)

M⁺ m/e 288; 0.02%

m/e 270

m/e 224

m/e 164
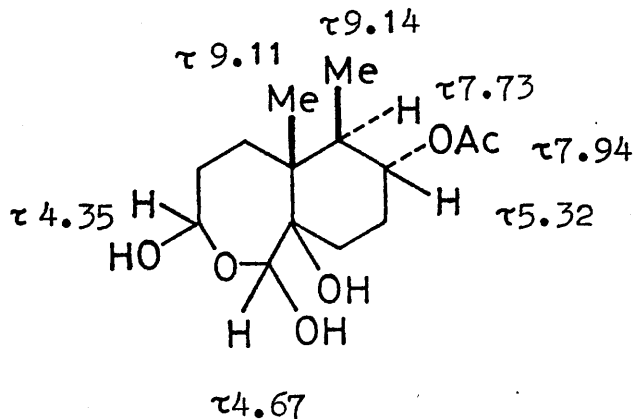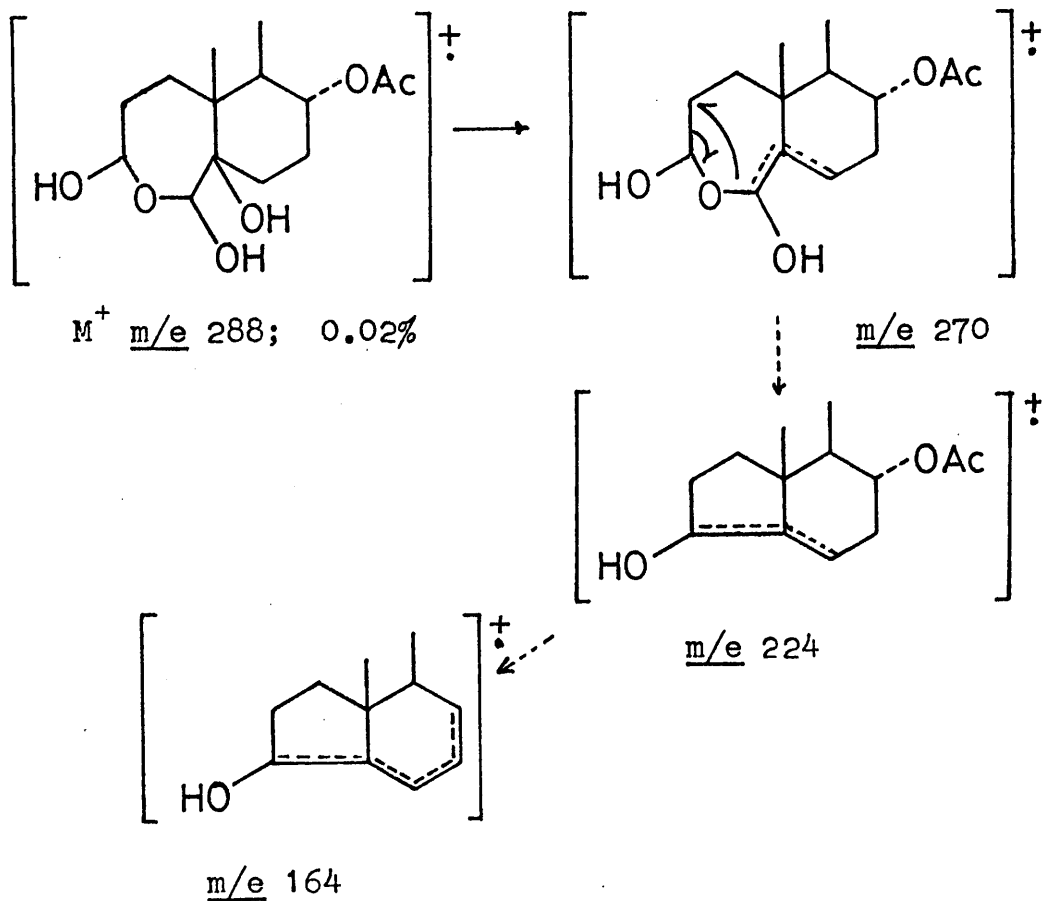
that observed at $\tau = 4.67$ collapsed to a sharp singlet, consistent with the isolated carbinol system as in (90). Irradiation at $\tau = 8.5$ sharpened the signal at $\tau = 4.35$, indicating the adjacency of this proton to ring methylene (Fig. 25). The low field of this signal was inconsistent with a simple carbinol as in (110), but better explained as a hemiacetal as in (90).

The mass spectrum (Appendix I) could be interpreted favourably in terms of structure (90). It was necessary to postulate loss of water as a primary fragmentation process, followed by other losses in the normal manner. The peak at m/e 224 may be formulated as M-18-46, ascribed to the loss of formic acid as a second fragmentation (Fig. 25). De-acetylation of this ion would then give the base peak, m/e 164. With foreknowledge of their expected location, the molecular ion, m/e 288, and M-18 peaks were just discernible.

There appeared to be no direct analogy for this reaction, but no reference was found to the use of this reagent with a cyclic allylic alcohol as in this instance. In the original report of the osmium tetroxide-catalysed periodate cleavage of double bonds, Johnson mentioned that cyclohexene gave poor yields of adipaldehyde when the reaction was carried out in dioxan[103]. He suggested

From Dauben et al[113]:

(111)   (112)

(90) $\xrightarrow[\substack{24 \text{ hrs} \\ \text{r.t.}}]{\text{NaBH}_4}$ (91)

$\downarrow$ NaIO$_4$/Et$_2$O
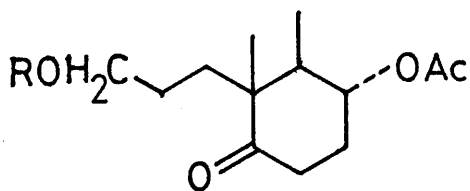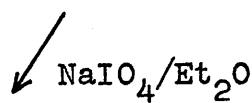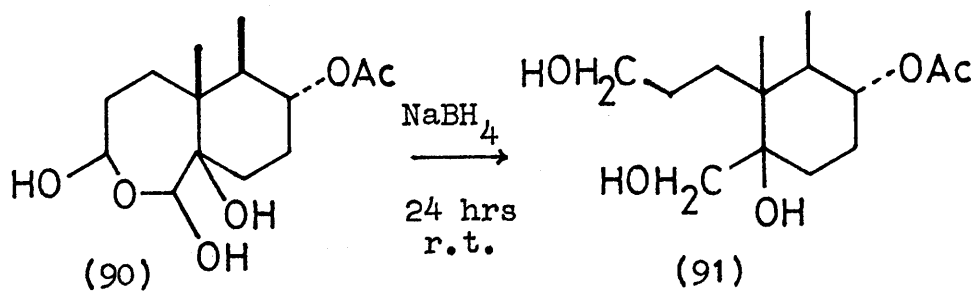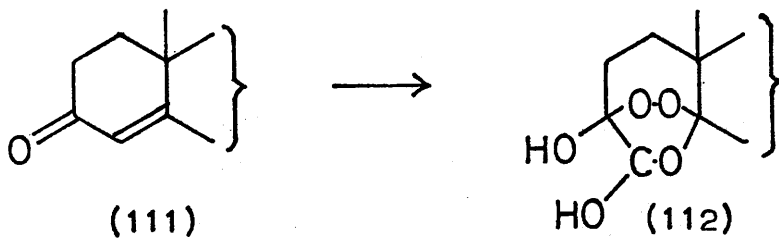
(92)   R = H

(93)   R = Ac

a cyclised by-product of the adipaldehyde as a cause of the loss in yield, but made no investigation as to its nature. Dauben and co-workers, investigating the ozonolysis of a cyclic enone system, e.g. (111), found the product (112) instead of the expected ozonide [113].

Further chemical evidence for the structure (90) was provided by the succeeding reactions.

Borohydride Reduction of Acetoxy-bis-Hemiacetal (90)

Attempted oxidative cleavage of acetoxy-bis-hemiacetal (90) was unsuccessful, neither periodic acid (in ether) nor sodium bismuthate (in acetic acid) [105] producing any change in the starting material, as judged by TLC of the reaction mixture, and IR of the recovered sample.

Reduction was carried out with sodium borohydride (2 mg) in ethanol (1 ml), the course of the reaction being followed by TLC. A spot at R_f 0.75 disappeared (Silicagel-G/ ethyl acetate-petrol 60°-80° 3:1), and material accumulated on the baseline. The reaction was complete after 24 hours; further sodium borohydride (2 mg) was added at 12 hours to replenish that lost by hydrolysis.

Simple extraction of the reaction mixture failed to afford any product, but after adding approximately 10 μl ethylene glycol to the aqueous mixture, a product could be extracted which showed R_f 0.2 (TLC system as above).

Evaporation of solvents gave a colourless oil (3.0 mg).
The behaviour described above was suggestive of the
formation of a borate-vic-diol ester complex, and added
support to the assignment of the structure of the
acetoxytriol (91) to the product of this reaction.

Periodate Cleavage of Acetoxytriol (91) [114]

A saturated solution of periodic acid was
prepared in ether, and this solution was added to the
product (91) obtained above, dissolved in ether (0.25 ml).
A white precipitate of iodic acid was formed with each drop
of reagent added, until this effect became obscured. Addition
dropwise was continued, until after 5 minutes standing,
the mixture retained oxidising activity to starch-iodide
paper. Excess periodate was destroyed with ethylene glycol,
as sodium metabisulphite had apparently promoted a side
reaction in an earlier trial. This measure precluded
recovery of labelled formaldehyde from the reaction mixture.

TLC of the product indicated two spots, R_f 0.8 and
0.9 (TLC system as above), the more polar being the major
product. Analytical liquid chromatography (N1114-50%-LH20/
benzene) gave three peaks, SEV 54, 79 and 109, the last
eluted being the major product. A preparative separation
(N1114-50%-LH20/benzene; SEV 100-120) gave this product
(1.2 mg), to which the structure (92) was assigned.

Fig. 26: Postulated Mass Spectral Fragmentation of bis-Acetoxyketone (93)

M^+ (0.05%) m/e 284

M-60   m/e 224

- 2 HOAc

m/e 164

m/e 124
Base Peak

(80) M^+ (0%) m/e 236

M-60
m/e 176

m/e 124   Base Peak

(81) M^+ (0.9%) m/e 254

M-60
m/e 194

m/e 124   Base Peak
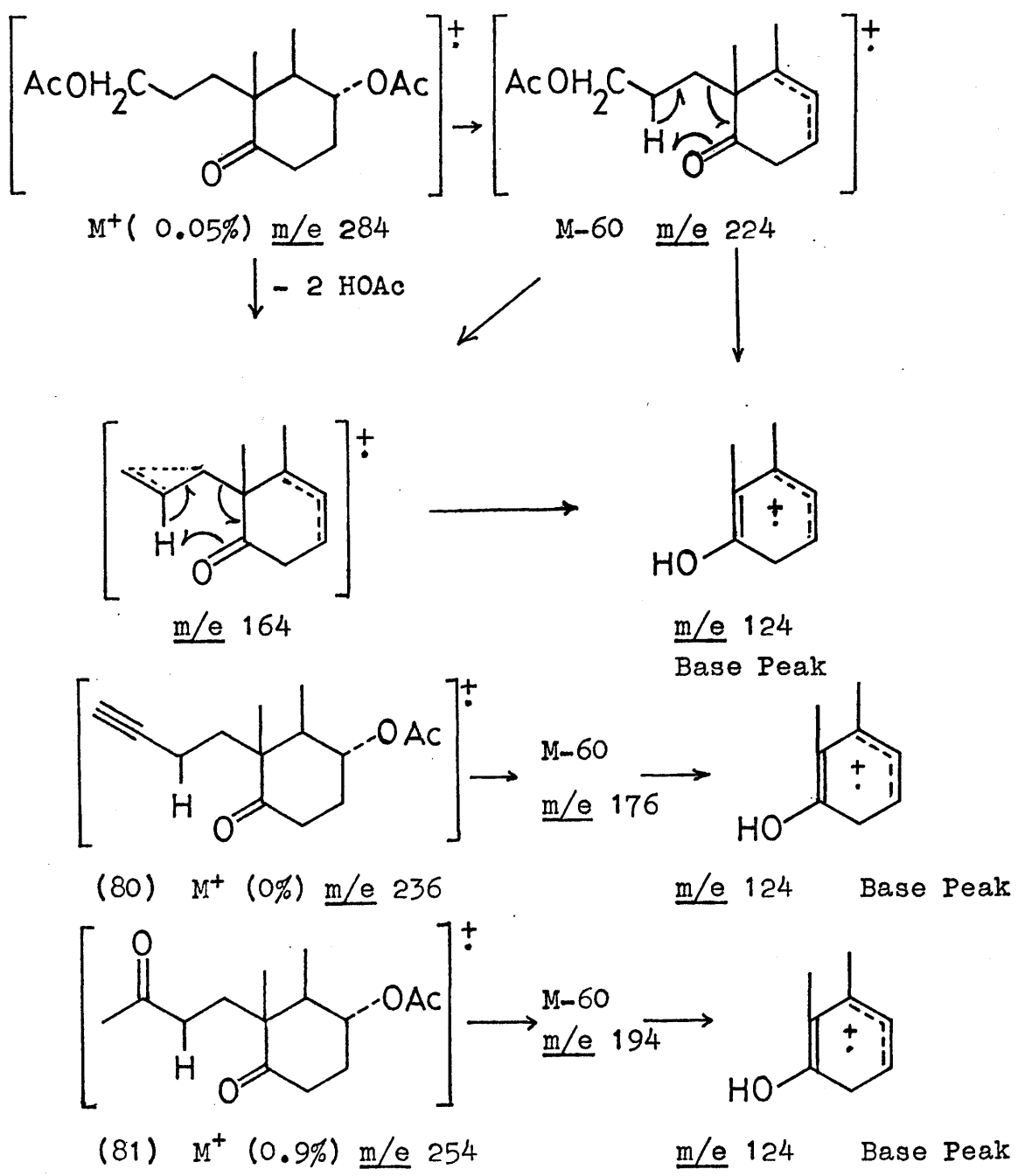
Acetylation of Acetoxyketol (92)

The acetoxyketol (92), $I_{OV-1}^{162^{O}} = 1760$, was acetylated by normal procedures (acetic anhydride-pyridine) to give bis-acetoxyketone (93), recovered as an oil (1.4 mg) from the reaction mixture, and purified by straight-phase chromatography (N1114-50%-LH20/benzene; SEV 50-58). The bulk of the recovered sample (0.880 mg) was used for radioassay, the remaining 0.2 mg being characterised as bis-acetoxyketone (93) by GLC and GC-MS. The sample gave one peak, $I_{OV-1}^{162^{O}} = 1900$; the molecular ion was absent from the mass spectrum (Appendix I), but abundant peaks m/e 224 and 164 could be postulated as M-60 and M-120 ions ($C_{15}H_{24}O_5$ requires $M^+ = 284$). The base peak, m/e 124, was observed for the analogous molecules of the acetylenic ketone (80), and the acetoxydiketone (81) and could be derived by McLafferty rearrangement of the molecular ions, followed by deacetylation of their common fragment, m/e 184. This mode of fragmentation served as further evidence for the structure (93).

TABLE 18

Radiochemical determinations and manipulations

a) Side-chain determination

Sample and Source		Radioactivity	Weight	Specific Activity
		dpm	mg	dpm/μmol
Isopetasol	- leaf	184.2 ± 1%	1.550	27.8
	- flower	73.6 ± 1%	0.460	37.4
Desisopropylidene-isopetasol	- leaf	48.1 ± 1%	0.520	17.9
	- flower	80.4 ± 1%	0.645	24.4

b) Iodoform determination

Source	Count	Background	Efficiency	d.p.m.	Weight	Specific Activity
	c/min	c/min			mg	dpm/μmol
Leaf	60.5	23.5	62%	59.9	4.6	5.13
Flower	56.1	29.9	74%	33.9	2.1	6.36

c) Combination and dilution of Desisopropylideneisopetasol (56)
    samples from leaf and flower feedings

	Weight	Radioactivity
	mg	dpm
Leaf	18.2	1680
Flower	12.9	1620
Unlabelled	30.6	-
Combined Sample	61.7	3300
		dpm estimated
Counting sample	1.44	75.8
		dpm measured

TABLE 18   (Contd)

d)   C-3 and C-9 determinations

	Radio-activity	Weight	Specific Activity
	dpm	mg	dpm/μmol
(56)	75.8	1.44	10.2
(74)	39.4 ±1%	1.435	5.0
(76)	40.5 ±1%	1.990	5.2
(89)	21.0 ±2%	0.97	5.25
(93)	16.4 ±2%	0.880	5.3

4   BIOCHEMISTRY AND METABOLISM OF MEVALONIC ACID

IN PETASITES HYBRIDUS

4.1.1   Introduction: The Metabolic Status of Mevalonic Acid

in Higher Plants

The study of terpenoid biosynthesis in vivo in

plants has demonstrated one generally consistent result:

the incorporation of mevalonic acid into the compounds under

investigation has rarely shown the success [15,16,115] that

has been obtained with mammalian systems [66].

Only the sterols, as a general class [17,116], and a

few isolated compounds such as gossypol (from cotton roots

and buds) [117] and geraniol-β-D-glucopyranoside (from rose

petals) [84,85], which appear to have some unusual metabolic

role in these plants, have shown incorporation in excess of

1%, a level considered by many workers to be a minimum

requirement for a significant result. It might appear from

such data that mevalonic acid was not the true precursor

of the terpenoids in higher plants. The successful

demonstration of a specific labelling pattern in terpenoids

in a number of examples [115] suggested that the above

conclusion was erroneous. Goodwin has found that an excess

of unlabelled mevalonic acid considerably diluted the

incorporation of $(2-{}^{14}C)$ sodium acetate into carotenoids [118].

He concluded, as a result of comparisons between CO_2, acetate

and mevalonate feedings, that poor incorporation of the
latter precursors was due to failure of the precursor to
penetrate to the site of synthesis, and not because of the
existence of an entirely novel biosynthetic pathway.

This observation stressed the fact that the plant is
an extremely complex organism. Higher plants are in general
autotrophic, and use carbon dioxide as sole carbon source:
this was one of the earliest conclusions of plant physiology [119].
In contrast to the liver cell, which has adapted to receive
assimilates from an external source, the cells of the green
leaf are primarily concerned with the export of products of
photosynthesis, mainly as sucrose [120,121]. 'Feeding' of a
precursor is an unnatural manipulation, and may require
reversal of normal processes of transport, not only within
the plant as a whole, but also from one location to another
within the cell. It should not come as a surprise to find
that some such reversals turn out to be unfavourable. As a
further complication, synthesis may be concentrated in a
specific phase of development [15,122] and incorporation may
then be observed only if feeding coincides with a period
of synthesis [84].

The poor incorporation probably led some early
workers to allow excessive periods for metabolism of the
precursor, on the assumption that utilisation of the tracer

was a slow process, and that gradual accumulation should

increase the overall radioactivity of the terpenoids.

However, Arigoni reported randomisation of label in cineole

from <u>Eucalyptus globulus</u> [123], while Birch, feeding over a

very much shorter period, obtained specific labelling [124].

The situation was clarified by Battu and Youngken, who found

in <u>Mentha piperita</u> that $(2-^{14}C)$ MVA was degraded to $^{14}CO_2$,

11% of the radioactivity being collected over fourteen days [125].

The randomly labelled monoterpenoids isolated had evidently

incorporated $^{14}CO_2$ to a greater extent, and direct

incorporation from mevalonate was obscured. Waller and

co-workers found that radioactivity from $(2-^{14}C)$ MVA

administered to <u>Nepeta cataria</u> was found in nepetalactone

after a few hours [126]. Little variation in incorporation

occurred over the first 5-6 days, but thereafter, the

radioactivity was increased. Again $^{14}CO_2$ evolution was

observed, and it seems likely that a first phase of specific

labelling was followed by random labelling (though this was

not the conclusion drawn by these authors). Loomis and

Burbott, also investigating <u>Mentha piperita</u> monoterpenoids,

used a variety of precursors, and found rapid incorporation

from glucose and CO_2 [15,127] but no incorporation from

mevalonate. This contradicted the idea that synthesis was

the slow process suggested by results obtained using

$(2\text{-}^{14}C)$ MVA. (Higher plants have a tendency to use fundamental, rather than specific, precursors: Steward and Bidwell found more ready utilisation of CO_2 and glucose than amino acids in protein synthesis [128].) Hefendehl and co-workers also compared the efficiency of precursors in Mentha piperita, and obtained increasing incorporation for the series $(1\text{-}^{14}C)$ acetate, $(2\text{-}^{14}C)$ acetate and $(2\text{-}^{14}C)$ MVA [129]. Sodium acetate, at a loading of $5\,\mu\text{mol/g}$ fresh weight, was found to give maximum incorporation after 96 hours. These workers also found that CO_2 was incorporated more effectively (by a factor of 2500) in light rather than dark growing conditions.

The original deduction by Goodwin, that low incorporation arose from failure of mevalonic acid to penetrate to the site of synthesis [118], has been elaborated as a mechanism for the regulation of terpenoid biosynthesis in higher plants [17,130]. This hypothesis, developed from observations with greening etiolated tissues, postulated the existence of a combination of enzyme segregation in organelles and substructures of the plant cell [131], and a comparative impermeability of the membranes to metabolites including MVA [17,132]. The overall effect would be the intracellular separation of synthetic pathways into 'chloroplastidic' and 'extra-chloroplastidic' compartments.

Synthesis of phytol, carotenoids and terpenoid quinones would occur only within the choloplast, from endogenous precursors, while production of sterols and ubiquinone, occurring outside the chloroplast, would be accessible to exogenous MVA. CO_2, in contrast, would penetrate to the chloroplast more effectively. A recent result reported by Hill, Shah and Rogers[133] has added support to the hypothesis; in ripened tomato fruit, the senescent chloroplasts appeared to lose their impermeability to some extent. Although glyoxalate incorporation decreased, indicative of a reduction in overall synthesis of chloroplast terpenoids[17] – an expected consequence of senescence – incorporation of mevalonate into these compounds was increased. This could only be interpreted as an increased penetration of the precursor to the site of synthesis.

While the intracellular location of carotenoids and terpenoid quinones in the chloroplast was demonstrated by fractionation of the organelles[130], no comparable study of lower terpenoids has been made[115]. Some success has been obtained from morphological studies, which indicated specialised multi-cellular structures, the glandular trichomes, as the location of essential oils[15,125,134]. However, it is not completely certain whether these structures serve as synthetic or storage centres.

Heinrich has reported methods for distinguishing volatile and non-volatile lipophilic constituents in electron microscopy[135]. In fruit of _Poncirus trifoliata_, he has found essential oil to be located solely in intact gland cells; this oil had a damaging effect when applied to other cells. Evidence was presented for the synthesis of the volatile oil in the plastids of the gland cells. These plastids had not developed as photosynthetically active chloroplasts, and appeared to be specialised for production of the lower terpenoids.

It may be possible that such cellular units rely mainly on sucrose as a carbon source, and thus neither $^{14}CO_2$ nor exogenous mevalonate effectively penetrate to the site of synthesis. Circumstantial evidence for such a view has been provided by Loomis, who found glucose to be the most effective precursor, of several that were examined (not including sucrose), for monoterpenoids in _Mentha piperita_[127] and _Humulus lupulus_[15]. Loomis also found that direct estimation of monoterpenoids of _M. piperita_ indicated that accumulation, and hence, synthesis, continued through the maturity of the leaf, whereas $^{14}CO_2$ incorporation only occurred in young leaves[15]. No label from mevalonic acid could be detected in the monoterpenoids[15].

One point that must be emphasised in the investigation
of terpenoid biosynthesis is the necessity for rigorous
purification of samples. The low incorporation, coupled
with the comparatively large quantities of essential oils,
may result in dilution of specific activity by a factor
of 10^6. Traces of impurities in a sample could thus account
for all the radioactivity, and radiochemical homogeneity
is satisfactorily proved only by the successful degradation
and isolation of the label. Thus it has been reported that
TLC of extracts of carrot slices gave highly radioactive
α- and β-carotene, but further purification finally yielded
completely unlabelled samples of these compounds [136].
A labelled impurity, present in such a sample, may be
selectively removed at a certain stage of a degradation
sequence, and a spurious result, indicating an asymmetric
labelling pattern, may thus be obtained.

4.2   RESULTS AND DISCUSSION

4.2.1   Preliminary Feedings and Plan of Experiments

An exploratory investigation of petasin biosynthesis
was carried out by Dr. G.H. Draffan and Dr. J.A. Zabkiewicz,
and the results were recorded in full in an earlier thesis[7].
It was found that both $(2-^{14}C)$ acetate and $(2-^{14}C)$ mevalonate,
wick-fed via the petiole with the allowance of periods of
4-14 days for metabolism, were incorporated to a very slight
extent in the leaf (0.003 - 0.010%), and not at all into the
rhizome and roots. This was consistent with the results of
other workers[115].

The object of this part of the investigation
concerned the possible reasons for the failure of the plant
to utilise exogenous mevalonate as a precursor in petasin
biosynthesis. It was considered that three factors may have
contributed to the results obtained above.

First the distribution of the tracer was investigated:
if tissues had failed to receive the precursor, no incorporation
could be expected. It was also useful to know how the rate of
distribution might affect the utilisation of the precursor.
Secondly, the intracellular uptake of label would be indicated
in the plant by the utilisation of mevalonate in pathways
other than that leading to petasin. The production of
phytosterols, which on the basis of published evidence[17,115]

would be expected to utilise efficiently the exogenous mevalonate, could exert a particularly strong competitive effect on the pool of labelled mevalonate available for petasin biosynthesis. The use of inhibitors of the sterol pathway [137,138] should to some extent counteract the effects of competition for precursor, assuming that these compounds would not also influence petasin biosynthesis adversely.

Finally the effects of compartmentation could be assessed by the use of more fundamental labelled precursors such as $^{14}CO_2$. In the first instance, this would confirm that synthesis of petasin occurred under the conditions of the experiments. An estimate of the rate of conversion from $^{14}CO_2$ to petasin could also be obtained by this method, and a comparative indication of the accessibility of $^{14}CO_2$ and its immediate metabolites to the site of petasin biosynthesis in P. hybridus could be assessed.

4.2.2   Distribution of $(2-^{14}C)$ Mevalonolactone, and Effect on Petasin Biosynthesis

It was already known that $(2-^{14}C)$ mevalonolactone (MVAL) was not incorporated into petasin in the rhizome. In the first experiment, a plant having two mature leaves and two emergent leaves was fed with $(2-^{14}C-3RS)$ MVAL, 6 μCi being administered by the wick method to each of the mature

Table 19: Distribution of Label After Administration of 12 μCi ($2-^{14}C-3RS$) MVAL to Mature Leaves of P. hybridus:

10 Days after Feeding

Fraction	Mature Leaf	Rhizome	Young Leaf	Total
Total Benzene Extract*	10.7%	0.069%	0.17%	10.9%
(Non-Saponifiable)	(10.0%)	(0.025%)	(0.02%)	
(Acyl Lipids)	(1.2%)	(0.053%)	(0.15%)	
Aqueous Extract*	59.4%	4.3%	0.70%	64.4%
Tissue Residues	10.7%	3.1%	0.064%	13.9%
Total Radioactivity	80.8%	7.5%	0.93%	89.2%

Radioactivity retained by wicks and vials   0.4%

$^{14}CO_2$ trapped   1.3%

Overall Recovery of Radioactivity   90.9%

* Uncorrected for quenching in toluene scintillator

leaves. The emergent leaves were not fed. After ten days,
when it was considered that the location of the tracer would
have become stabilised, the plant was cropped, and divided
into 'mature leaf', 'young leaf' and rhizome. These parts
were extracted separately, first with benzene, and then with
water, and each extract assayed for radioactivity. The dried
residues after aqueous extraction were assayed by pyrolysis
and collection of labelled CO_2. The results are shown in
Table 19.

Only the leaves that had been fed metabolised MVAL
to give benzene-soluble products. There was translocation
of label to other parts of the plant, but without conversion
into benzene-soluble terpenoids. This observation was
significant because it implied that tracer did not reach the
rhizome in the form of mevalonic acid. This was further
demonstrated by alkaline hydrolysis of benzene extracts.
Radioactivity due to terpenoids would be expected to remain
in the non-saponifiable fraction; non-terpenoid lipids would
be destroyed by this treatment, and radioactivity from this
source would be concentrated in the aqueous base-soluble
fraction of the hydrolysate. While the mature leaf extract
retained the bulk of its radioactivity in the non-saponifiable
fraction, the other two extracts contained a greater
proportion of radioactivity in acyl lipids (Table 19).

upper leaf surface
12 hours

upper leaf surface
24 hours

Fig. 27: Autoradiograms of P. hybridus Leaves 12 and 24 Hours after Feeding with $(2-^{14}C)$ Mevalonolactone

It would appear that translocation to the rhizome had occurred only after degradation of MVAL, though the nature of the products was not determined. Respiratory CO_2 was collected in the course of the experiment, and the net evolution over ten days is indicated in Table 19. Under the conditions of the experiment, partial re-incorporation of evolved CO_2 was likely to have taken place, and this may have accounted for the entire radioactivity in the 'young leaf' fraction.

Distribution of the label within the leaf was investigated in a further experiment by autoradiography of single leaf samples. Four leaves, each at approximately the same stage of development (semi-mature) were fed with $(2-{}^{14}C)$ MVAL, 1 μCi being administered by the wick method. The conditions were such that the tracer solution was taken up in 30-40 minutes. The leaves were permitted to grow for 3, 6, 12 and 24 hours, when autoradiograms were made of the frozen leaf specimens.

After 3 - 6 hours, the tracer was found to be concentrated in the petiole and the main veins of the leaf. At 12 hours, the radioactivity had transferred to minor leaf veins (Fig. 27). A dramatic change occurred after 24 hours, when the radioactivity had accumulated in the mesophyll. While the first twelve hours of assimilation

Table 20: Distribution of Radioactivity in Leaf Parts Dissected
After 6 Hours' Assimilation of Tracer Solution

Feeding Method and Conditions	Results	g (Fresh Weight) Radioactivity dpm x 10^{-3}			
	Upstream Petiole	Downstream Petiole	Veins	Proximal Leaf Zone	Distal Leaf Zone
Cut Stem 1 μCi MVAL in Water	- -	0.45 1215	0.65 231	0.65 471	1.05 117
Cut Stem 1 μCi MVAL 0.2 M Sucrose	- -	0.45 1410	0.60 321	0.65 314	0.95 142
Cotton Wick 1 μCi MVAL in Water	0.90 31	0.30 607	0.45 163	0.60 352	0.95 255
Cotton Wick 1 μCi MVAL 0.2 M Sucrose	0.45 42	0.25 472	0.25 139	0.45 396	0.75 444
Cotton Wick 1 μCi MVA 0.05 M tris pH 8.5	0.65 57	0.30 703	0.40 171	0.65 242	0.90 227

VEINS
(Nine excised
as depicted)

PROXIMAL
LEAF
ZONE

DISTAL
LEAF ZONE

'DOWNSTREAM'
PORTION OF PETIOLE

'UPSTREAM' PORTION
OF PETIOLE
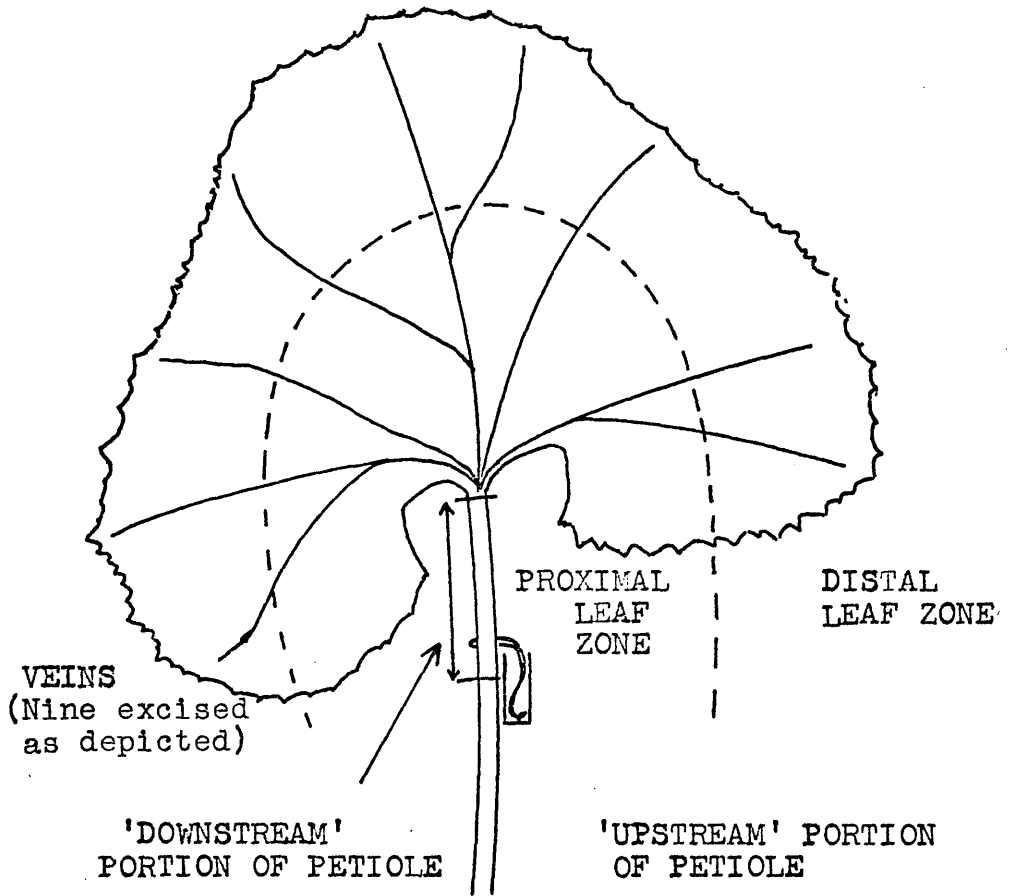
Fig. 28:Dissection of P. hybridus Leaf

    i) Upstream Portion of Petiole
   ii) Downstream Portion of Petiloe
  iii) Veins
   iv) Proximal Leaf Zone
    v) Distal Leaf Zone

had fallen in a period of illumination, only this last sample was allowed to continue distribution of tracer through a period of darkness. It was not determined whether the time factor alone, or the darkness, was responsible for the complete translocation of MVAL into the mesophyll.

The effect of certain other factors on the distribution of radioactivity was investigated by a third experiment. Five leaves were selected, and two were fed by dipping the cut end of the petiole into a solution containing $(2-^{14}C)$ MVAL, and the remaining leaves were fed by the wick method. Sucrose (0.2 M) was introduced into some tracer solutions to investigate the effect of osmotic properties on assimilation. After 6 hours, leaves were dissected according to the scheme detailed in Fig. 28, and each part extracted separately in isopropanol. (The 'vein' fraction contained specifically the nine major veins shown in the sketch.)

The results (Table 20) indicated that the tracer was most effectively moved to the leaf by wick feeding in the presence of sucrose, although some reservations may attach to a single result of this nature. It was considered that excessively hypotonic solutions (i.e. of precursor alone) might obstruct to some extent the normal transpiration by causing local swelling of the cells of the petiole. The concentration (0.2 M) of the sucrose was chosen so as not to

Fig. 29: Interconversion of Mevalonic Acid and Mevalonolactone in P. hybridus Leaf Tissue

(From Proximal Leaf Extracts, Table 20)

a) MVA Feeding

MVA

MVAL

Radioactivity

R_f 0   0·5   1·0

b) MVAL Feeding

MVAL

MVA

Radioactivity

R_f 0   0·5   1·0
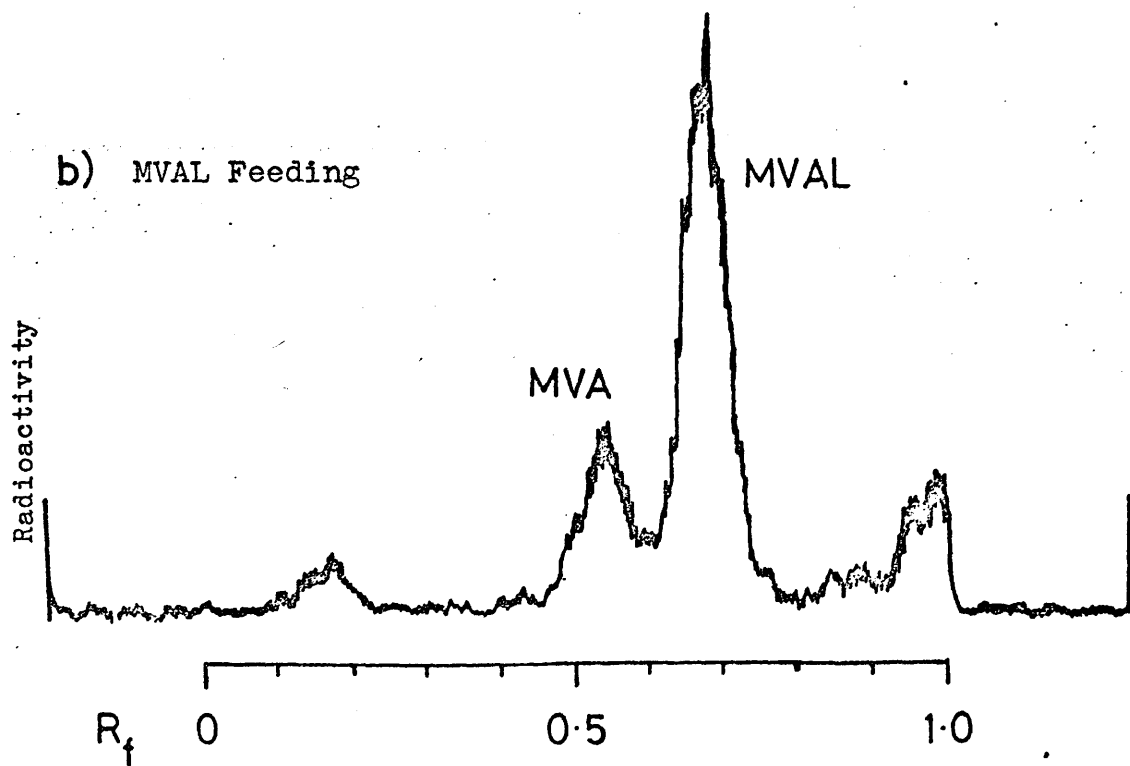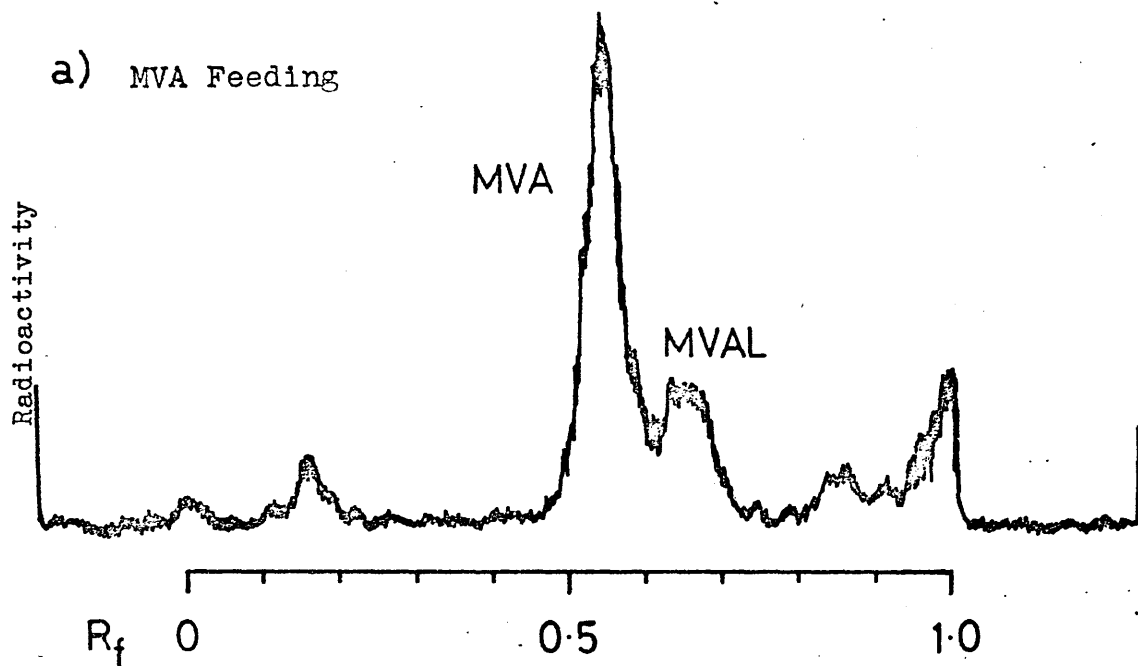
exceed the expected tonicity of the plant fluids.

A comparison was also made of the distribution of mevalonate
as the salt and in the lactone form. Equilibration of the
two forms in the plant was indicated by radio-scanning of
TLC plates of the extracts of the proximal leaf-zone. Free
acid was demonstrated from feedings of MVAL (Fig. 29), and
conversely, small amounts of lactone were formed from the
feeding of free mevalonic acid (MVA). The interconversion
may have been promoted by endogenous acids present in leaf
or petiole fluids.

Cut-stem feedings resulted in substantial wetting
of the outside of the petiole; it was doubtful whether all
the radioactivity found in the petiole (Table 20) could be
regarded as properly assimilated.

These results permit a number of general propositions
to be made with regard to wick-feeding of mevalonate. The
lack of incorporation of label from $(2-^{14}C)$ MVAL into petasin
esters in the rhizome would be easily explained by the poor
translocation in this direction. The apparently exclusive
transport of MVAL towards the leaf suggested that the tracer
entered the xylem transpiration stream [139]. The first
24 hours of a wick feeding appeared to be involved primarily
in transport rather than metabolism of the precursor. This
last result was of great importance for estimation of the

Table 21: Utilisation of $(2-^{14}C)$ Mevalonolactone in
P. hybridus Leaves

Feeding				12 hr	24 hr	48 hr
Isopropanol Extract				76.4%	56.8%	45.2%
Tissue Residues[a)				12.9%	18.8%	23.1%
Product:	R_f 1	2	3			
Squalene	0.80	0.96	0.99	0.1%	0.3%	0.2%
Sterol Ester	0.40	0.92	0.99	8.7%	13.0%	14.2%
2,3-Oxidosqualene	0.22	0.90	0.99	0.1%	0.3%	0.2%
Free Sterol	0	0.30	0.96	8.1%	7.7%	8.1%
Other Unidentified					0.1%	0.9%
" " b)				0.1%	0.3%	4.1%
" " c)					0.2%	2.3%
Mevalonolactone	0	0	0.68	47.8%	20.5%	11.2%
Mevalonic Acid	0	0	0.50	11.5%	14.5%	2.1%
Polar Compound	0	0	0.18	-	trace	1.8%

TLC Systems (Silicagel-G)
 1) 1% Ethyl Acetate/99% Petroleum Ether $60^{o}-80^{o}$
 2) 25% Ethyl Acetate/75% Petroleum Ether $60^{o}-80^{o}$
 3) Benzene-Chloroform-Methanol-Acetic Acid 4:8:3:1 (v/v)

Notes a) Results obtained by digestion of tissue residues in
   'Soluene' Scintillation Medium
  b) Associated with Esterified Sterol Glycoside band
  c) Associated with Sterol Glycoside band

time to be allowed for utilisation of precursor in feeding experiments.

4.2.3   Utilisation of Mevalonic Acid in P. hybridus

The experiments described in the previous section demonstrated the translocation of label into the leaf, and its distribution into the mesophyll. The actual uptake of the precursor into leaf cells should then be indicated by its utilisation in terpenoid biosynthesis, though not necessarily by sesquiterpenoid pathways. The TLC scans obtained in the previous experiment demonstrated some incorporation into products more mobile than MVAL, within 6 hours of feeding.

Utilisation of MVAL was investigated by feeding of the precursor to leaf specimens for 12 and 24 hour periods of assimilation. In addition, a sample of the extract was available from the 90 μCi bulk feeding, where 48 hours had been allowed for metabolism. The total isopropanol extract was assayed for radioactivity, and radioactivity in tissue residues was also measured (Table 21). Two points were apparent: the disappearance of radioactivity from the leaf (extract plus residue) and the increased fixation of tracer into a non-extractable form. (The results were not comparable with those of Table 19 because isopropanol was a more efficient extracting solvent than benzene.) Metabolites of

the tracer were investigated by TLC and scanning of extracts
for radioactivity.

Only two major radioactive zones could be
distinguished, other than unchanged MVA and MVAL; these
corresponded to free sterol and sterol ester bands on the
TLC plate (Table 21). The 12 hour feeding showed only
traces of radioactivity in other bands, but a number of
minor peaks were obtained after longer periods of metabolism.
Four of these peaks were tentatively identified as specific
precursors or metabolites of the sterols. Squalene and
2,3-oxidosqualene were identified by comparison of Rf values
in TLC with those of authentic samples.(We thank Dr. L.J. Goad
of the Department of Biochemistry, University of Liverpool,
for a sample of 2,3-oxidosqualene.) GLC also indicated the
presence of both these compounds in the extract, after
partial purification to remove wax constituents having
similar retention characteristics. Squalene was confirmed
by comparison with authentic squalene by GC-MS. Sterol
glycoside and esterified sterol glycoside were correlated
with two peaks of polarity intermediate between free sterol
and MVAL, and had already been identified in P. hybridus
extracts (section 2.2.5). Quite appreciable amounts of
radioactivity were associated with these latter peaks.

Three peaks remained unidentified: their behaviour

on a gel-filtration column suggested that the two less polar
peaks were due to high molecular weight compounds. The TLC
band (R_f 0.74; TLC system 'b', Table 21) corresponding to
one of these peaks was extracted. Only one component was
found by GLC ($I_{OV-1}^{250°}$ = 3900). GC-MS gave a molecular ion,
m/e 610, 1% of the base peak, m/e 383, but the mass spectrum
could not be correlated with a known compound. It was also
possible that the radioactivity could be associated with a
compound of still higher molecular weight, which was not
detectable by GLC. The polar peak (R_f 0.18; TLC system 'c',
Table 21) appeared to be comparable in molecular size to
MVA and MVAL. Non-specific enzymic oxidation of MVA, which
would not differentiate between the enantiomers, could have
given rise to 3-hydroxy-3-methylglutaric acid (HMG) from the
3S-mevalonic acid remaining after utilisation of the 3R-
isomer. The results in general suggested that degradation
of the unnatural enantiomer had occurred, as indicated by the
decrease in radioactivity measured in the MVA and MVAL bands.

In another experiment, the sterol components were
isolated from the saponified benzene leaf extract. Further
purification gave a sterol fraction which was assayed for
radioactivity. Total incorporation into sterols was 15.5%
after six days, and 12.9% after eleven days. The sterol
fraction was also examined by GC-MS, and its components were

Table 22: Acetylated Components of the Sterol Fraction
of P. hybridus Leaves: Identification by GC-MS[140])

Compound	$I_{OV-1}^{250^{o}}$	M^+	Base Peak
Campesteryl acetate	3290	442	382
Stigmasteryl acetate	3330	454	394
β-Sitosteryl acetate	3390	456	396
Cycloartenyl acetate	3420	468	69
24-Methylene cycloartenyl acetate	3475	482	43
Citrostadienyl acetate	3495	468	327

thus identified[140] (see also section 2.2.3). The three
components of the normal (unhydrolysed) sterol fraction
were present; in addition, a number of other components
were identified as 4α-methylsterol and 4,4-dimethylsterols,
which had apparently been present in the plant mainly in
the esterified form (Table 22).

The synthesis of sterols in P. hybridus leaves
indicated that MVAL had effectively penetrated to an
intracellular location. The incorporation of radioactivity
selectively into the sterols suggested that a compartmentation
effect operated on sesquiterpenoid biosynthesis, that could
be analogous to, but not necessarily identical with, that
proposed by Goodwin for chloroplast terpenoids[17,130]. This
would require confirmation, by the use of other tracers, to
demonstrate concurrent synthesis of both sterols and
sesquiterpenoids.

The results also indicated that mevalonolactone was
as satisfactory a precursor as the free acid, by virtue
of its easy conversion in plant tissues to the latter form.
There would appear to be little advantage in allowing
protracted periods for metabolism, as the labelled precursor
was found to be rapidly depleted.

4.2.4   Effects of Synthetic Inhibitors on Sesquiterpenoid

and Sterol Production

The observations reported in the previous section

indicated that radioactivity from (2-^{14}C-3RS) MVAL appeared

in sterols with good incorporation, but was only found in

small amounts in the sesquiterpenoid constituents. The

intention in this experiment was to reduce sterol synthesis

by specific inhibition, and so to observe the effect on

sesquiterpenoid incorporation created by relaxation of

competition for labelled precursor[141].

Synthetic inhibitors of sterol biosynthesis have

been investigated in vivo and in vitro in mammalian systems

by Holmes and DiTullio[137]. These workers reported, among

many other results, that SK&F 3301-A [2,2-diphenyl-1-

(β-dimethylaminoethoxy)-pentane hydrochloride] and SK&F 525-A

[β-diethylaminoethyldiphenylpropyl acetate hydrochloride]

caused inhibition of the isomerisation of isopentenyl

pyrophosphate to dimethylallyl pyrophosphate in rat liver

homogenates. With SK&F 7732-A$_3$ [tris-(2-dimethylaminoethyl)

phosphate trihydrochloride] and SK&F 7997-A$_3$ [tris-

(2-diethylaminoethyl) phosphate trihydrochloride] inhibition

occurred at a later stage and caused accumulation of a

triterpenoid, intermediate in polarity between squalene and

the 3-hydroxysterols. The subsequent discovery of the

involvement of 2,3-oxidosqualene in sterol biosynthesis[142]
would suggest that this was the intermediate accumulated.
Reid, investigating the effect of SK&F 7997-A$_3$ on the
incorporation of (2-^{14}C) MVAL in Nicotiana tabacum slices
found that a similar non-polar triterpenoid accumulated
radioactivity, while the synthesis of phytosterol was
inhibited[143]. In subsequent work, he has demonstrated
accumulation of label into squalene and 2,3-oxidosqualene
using the above system[144]. Bonner, Heftmann and Zeevaart
found that these inhibitors reduced incorporation from
(2-^{14}C) MVA into β-sitosterol and stigmasterol in plants[138].
There were also marked physiological effects in certain
species studied, notably the inhibition of floral induction.
This suggested that the hormone involved in the control of
flowering in these cases may have been a cyclic terpenoid
such as gibberellic acid.

The above results suggested that it might be possible
to inhibit the biosynthesis of sterols without necessarily
affecting the production of the lower terpenoids. Experiments
were undertaken on P. hybridus leaves, using SK&F 525-A and
7997-A$_3$. The inhibitor was administered by the wick-feeding
method, and a period of 24 hours was allowed for complete
uptake before the precursor was added. After 72 hours'

Table 23: Effect of Various Concentrations of SK&F 525-A and 7997-A$_3$ on the Incorporation of Mevalonolactone into Petasin Esters (Recovered as Isopetasyl Acetate)

Inhibitor	Fresh Weight of Leaves	Isopetasyl Acetate			
	grams	Weight mg	Radioactivity dpm	Specific Activity dpm/μmol	Incorporation
SK&F 525-A 25 μg	32.1	6.54	1785	75	0.0081%
50 μg	34.0	5.02	1452	79	0.0066%
100 μg	31.9	1.85	773	115	0.0035%
SK&F 7997-A$_3$ 25 μg	33.8	3.90	2071	144	0.0094%
50 μg	28.3	8.18	3254	135	0.0179%
100 μg	49.0	12.66	4939	130	0.0271%
Blank -	29.7	4.66	1041	61	0.0047%

Fig. 30: Inhibition of Sterol Biosynthesis by SK&F 7997 in P. hybridus Leaves: Evidence for its Action in Preventing Cyclisation of 2,3-Oxidosqualene
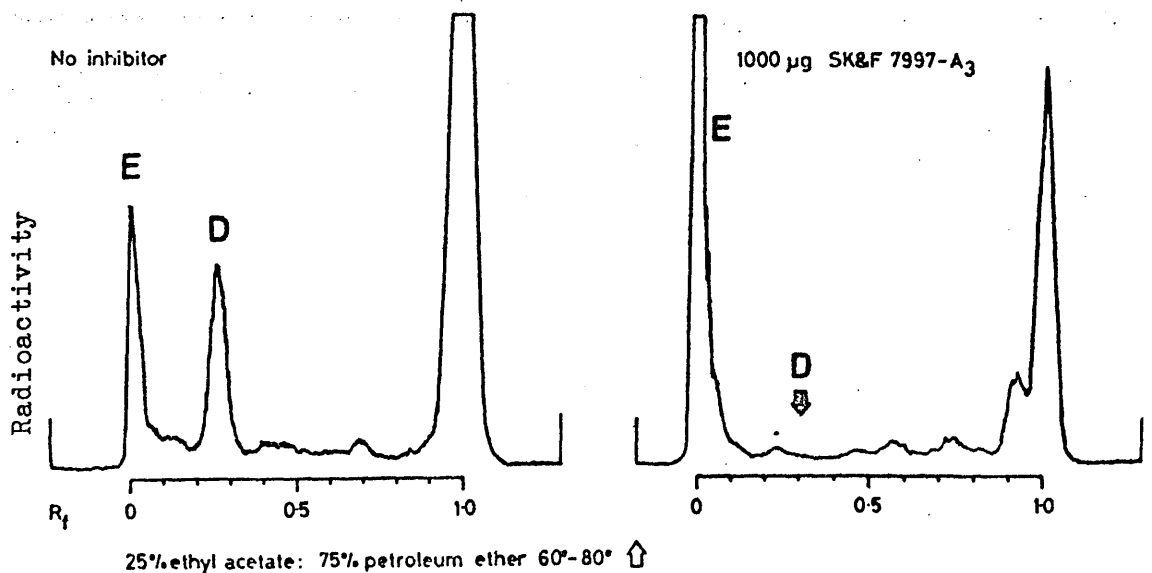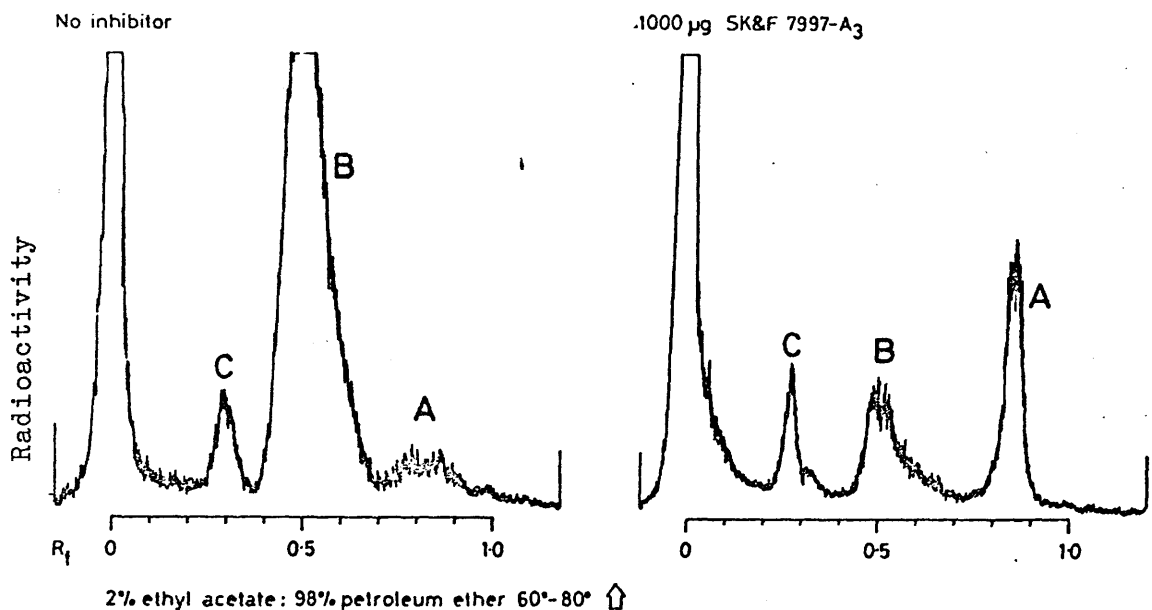
A – Squalene;   B – Sterol Esters;   C – 2,3-Oxidosqualene

D – Free Sterols (Mainly β-Sitosterol);   E – Mainly MVAL

inhibitor showed almost complete disappearance of the
labelled precursor in the same period of assimilation.
It was found that the plant was able to recover from the
effects of the inhibitor: 300 μg of SK&F 7997-A$_3$ per plant
was still effective after two days' metabolism, but not after
four days. 1000 μg of the inhibitor continued to block sterol
cyclisation after four days. Similar effects, arising from
isotope dilution, were observed by topical application of
squalene onto leaves before feeding.

These results indicated that SK&F 7997-A$_3$ induced
a slight enhancement of incorporation into sesquiterpenoids.
Degradation of the isopetasol obtained from these experiments
was not carried out, and there was no indication whether the
enhancement arose from specific incorporation of MVA, or by
degradation, and re-incorporation of a more fundamental
precursor.

The degree of enhancement of incorporation into
petasin esters was insufficient to correlate with the marked
inhibition of sterol biosynthesis, and the prolonged
accumulation of unchanged mevalonate. This evidence
suggested most strongly that the sesquiterpenoids and sterols
did not derive from the same metabolic pools of mevalonic
acid or farnesyl pyrophosphate. The competitive effect of
sterol biosynthesis would not appear to be the cause of poor

incorporation of MVAL into the sesquiterpenoids: it appeared, rather, that the site of synthesis of the sesquiterpenoids was inaccessible to the precursor. The observed incorporation of MVAL into the petasin esters could be accounted for by 'leakage' of precursor, the slight enhancement found in the above experiments possibly arising from increased availability of labelled precursor for 'leakage' to a location where sesquiterpenoid synthesis could have occurred.

4.2.5 Incorporation of $^{14}CO_2$ into Sesquiterpenoids and Sterols

All organic molecules in the plant derive ultimately from carbon dioxide, and this compound may be used as a precursor in order to determine whether there is active synthesis of a particular product in the plant. However, there are a number of different modes of carbon dioxide fixation, and different routes may contribute to the labelling of a particular product.

The primary course of CO_2 fixation in green tissues of temperate dicotyledons occurs by the photosynthetic carbon cycle of Calvin and Benson[145], leading to sugar phosphates, and thence to starch as the end product. Mobilisation of the starch, by the formation and translocation of sucrose, is a process which normally occurs only in the dark[146]. Thus

this pathway is of little concern in the biosynthesis of other compounds during a short period of continuous illumination. Degradation of Calvin cycle intermediates, for example by glycolysis, would not be expected to occur to any great extent, because of the organisation of the enzyme systems into separate locations. The alternative malate-producing photosynthetic pathway[147] appears on present evidence to be confined to tropical taxa, mainly from the monocotyledons.

Another important mode of CO_2 fixation produces glycollic acid, which may be a predominant early product of photosynthesis[148]. The origin of glycollic acid remains unelucidated; its investigation has been confused by apparently conflicting evidence from higher plants and algae. In higher plants, glycollic acid would appear to be independent of any Calvin cycle intermediates. Under certain conditions, glycollic acid has been obtained in tobacco leaf discs at a specific activity equal to that of $^{14}CO_2$ administered[149]. Furthermore, the two carbon atoms were symmetrically labelled. This evidence supported the de novo synthesis of glycollic acid from two molecules of carbon dioxide, without the intermediacy of sugar phosphates, as required by other biogenetic proposals[150]. However, no enzyme catalysing such a reaction has been isolated.

Fig. 31: Fixation of $^{14}CO_2$ in Green Leaves:
The Role of the Glycollate-Glyoxalate Pathway
in the Formation of Acetyl-Coenzyme A

● = ^{14}C

CHLOROPLAST   CYTOPLASM

CO_2 CO_2

$CH_2OH.CO_2H$
glycollate   ← Exogenous glycollate

                  ← Exogenous glyoxalate

$CHO.CO_2H$
glyoxalate   ←   Endogenous
                                       acetyl CoA

$NH_2.CH_2.CO_2H$ $HO_2C.CH_2.CHOH.CO_2H$
glycine   malate

$CH_2OH.CH(NH_2).CO_2H$ $HO_2C.CH_2.CO.CO_2H$
L-serine   oxalacetate

$CH_3.CO.CO_2H$ ⟷ $CH_3.CO.CO_2H$
pyruvate   pyruvate

$CH_3.CO.SCoA$ $CH_3.CO.SCoA$
acetyl-CoA   acetyl-CoA

Chloroplastidic   Cytoplasmic pool
pool

Conversion of glycollic acid to acetyl-CoA probably proceeds by the route glycollate - glyoxalate - glycine - L-serine - pyruvate - acetyl-CoA (Fig. 31). The sequence from glycollate to L-serine has been well established to occur in green leaves[151], and the conversion of glycine to phytol has been shown to involve specifically the α-carbon atom[152]. Incorporation was considerably more efficient than was obtained directly from exogenously administered acetate. Rogers, Shah and Goodwin investigated the efficiency of each of these intermediates as precursors for terpenoid biosynthesis, and together with observations of the effects of isotope dilution, their results substantiated this pathway as the means of $^{14}CO_2$ incorporation into chloroplast terpenoids[17]. However, sterols appeared to incorporate glyoxalate by another route: isotope dilution by glycine of incorporation from glycollic acid or glyoxalic acid had little effect. Isotope dilution by pyruvate was rather more apparent, and this compound may be involved in the malate pathway[153] (Fig. 31) leading to sterols outside the chloroplast, and in the serine pathway leading to carotenoids and phytol within the chloroplast. The most probable location for the enzymes of the glyoxalate - malate pathway would be in the mitochondrion, as this system is metabolically related to the Krebs cycle[153].

i) $^{14}CO_2$ 3 hours

Radioactivity

R_f 0   0·5   1·0

ii) $^{14}CO_2$ 6 hours

Radioactivity

R_f 0   0·5   1·0

iii) MVAL 12 hours

Radioactivity

R_f 0   0·5   1·0
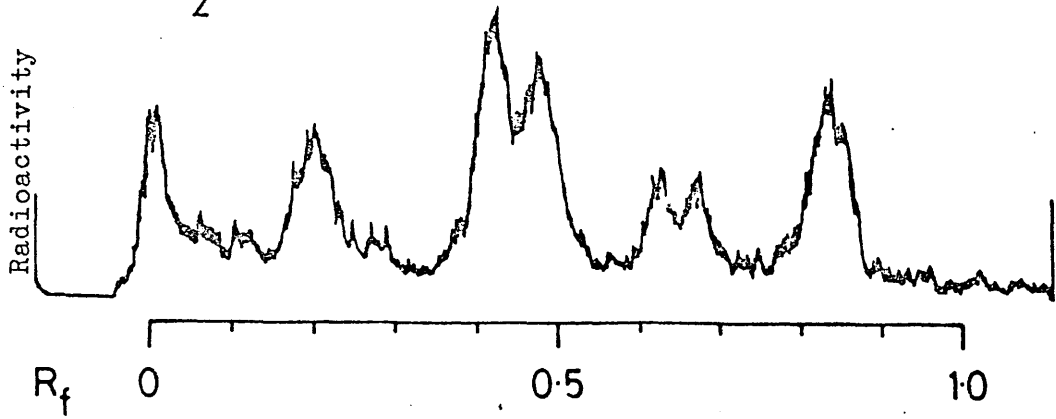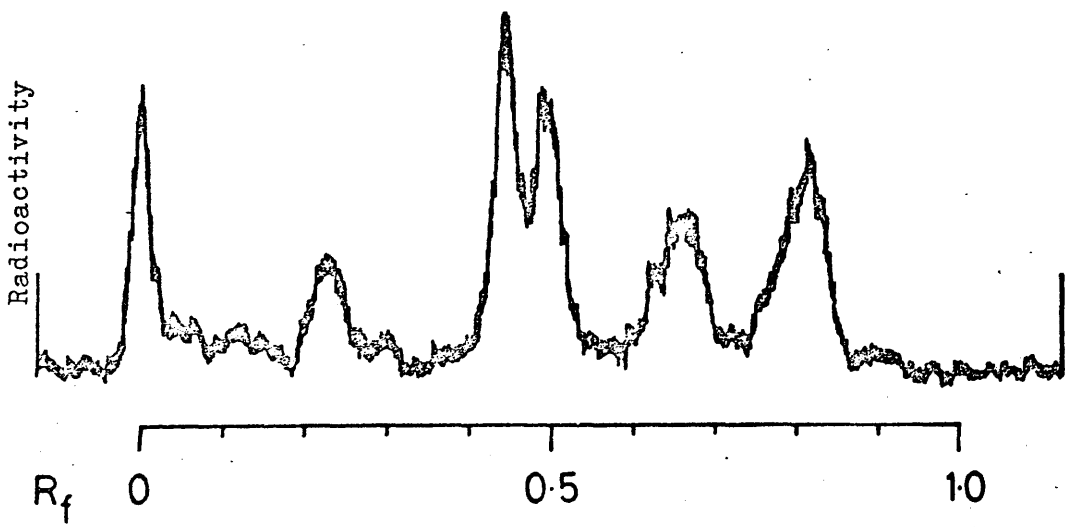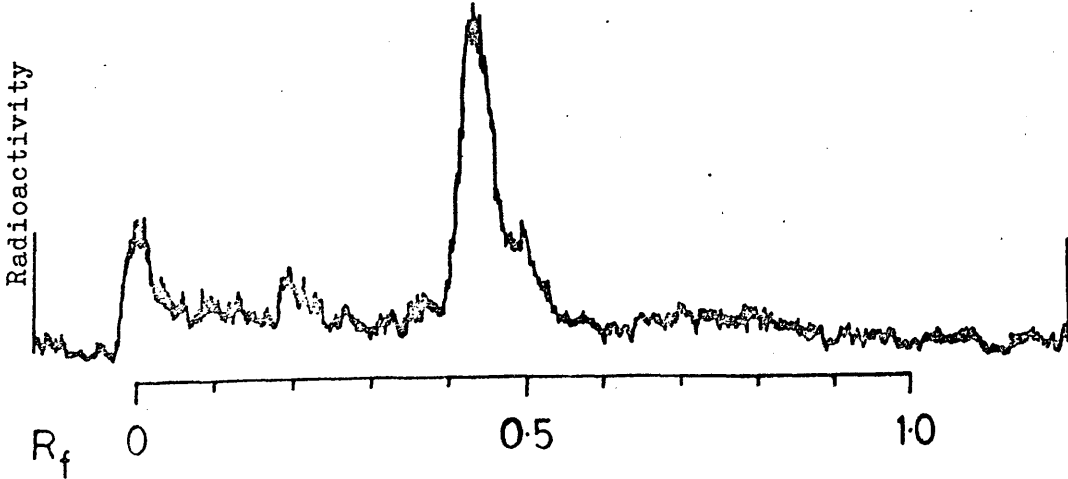
Fig. 32a (left): Radio-Chromatogram Scans of Extracts
from $^{14}CO_2$ and MVA Feedings

Fig. 32b (below): TLC of Authentic Standards

(Silicagel-G/25% Ethyl Acetate-
75% Petroleum Ether $60^{\circ}-80^{\circ}$)
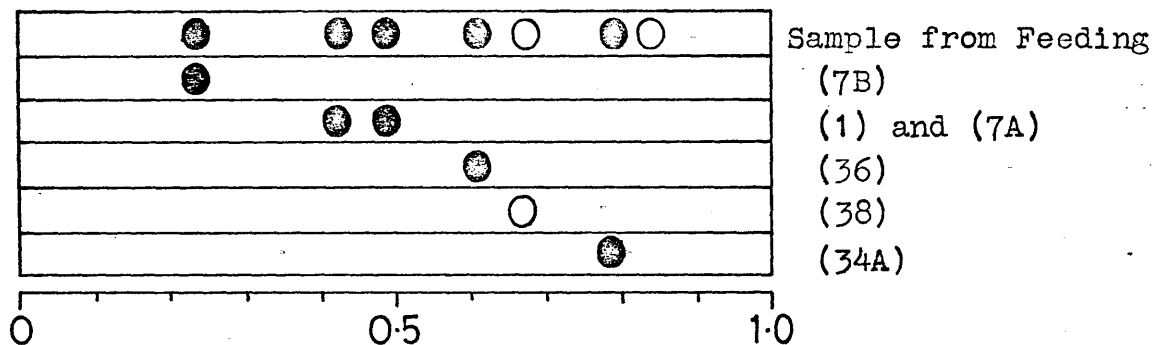
● UV Quenching   ○ Fluorescent after H_2SO_4 Spray

(1)

(36)

(38)

(7A) R =

(7B) R =
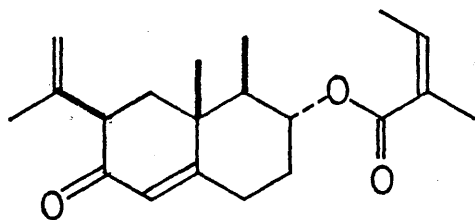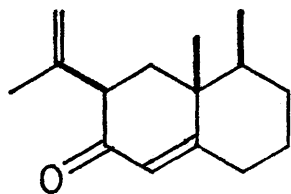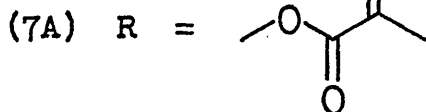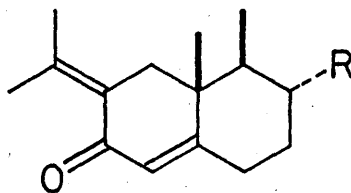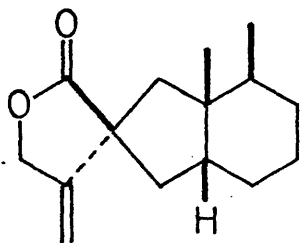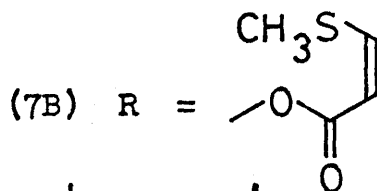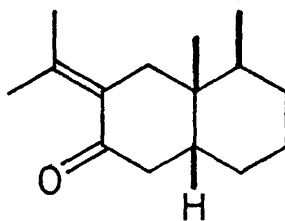
(34A)

Young leaves of <u>P. hybridus</u> were exposed to $^{14}CO_2$
for a short period, and were allowed to metabolise the
precursor in a normal atmosphere for various periods
following the exposure. An induction period of 2 hours after
the start of illumination was allowed for the commencement
of normal photosynthesis: thereafter, illumination was
continuous until cropping and extraction of the leaves.
A separate plant was fed with mevalonic acid for comparative
purposes.

The extracts were fractionated by column
chromatography with modified Sephadex gels. A sesquiterpenoid
fraction was obtained by methods similar to those of
section 2.2.4. In addition, sterol- and sterol ester-
containing fractions were isolated by these procedures.
The fractions were then examined by TLC and scanning for
radioactivity (Fig. 32a).

The components of the sesquiterpenoid fraction were
identified from their TLC and GLC behaviour, by comparison
of data with the compounds characterised in Chapter 2. One
compound (R_f 0.88; Fig. 32b) could not be so correlated with
a known structure. The distribution of radioactivity within
the sesquiterpenoids in indicated in Table 24.

Alkaline hydrolysis of this fraction gave free
isopetasol; the radioactivity recovered as isopetasol

Table 24: Distribution of Radioactivity[a] in Sesquiterpenoid Fraction from $^{14}CO_2$ Feedings

Feeding	Results dpm x 10^{-3}				
	$^{14}CO_2$ 3 hr	$^{14}CO_2$ 6 hr	$^{14}CO_2$ 9 hr	$^{14}CO_2$ 12 hr	$(2-^{14}C)$ MVA 12 hr
Radioactivity Administered (nominal)	225000	225000	410000	340000	11000
Incorporation:-					
Isopropanol Extract	8400	6300	11700	11600	6800
Petasin Esters (Total)	38.8	48.0	89.2	71.2	28.7
Petasin (1)	14.1	20.9	39.6	28.8	20.0
Isopetasin (7A)	15.1	18.8	34.9	29.3	6.1
S-Isopetasin (7B)	9.6	8.4	14.7	13.0	2.6
Isopetasol	30.1	41.7	78.2	56.7	0.9 b)
Compound 17c (36)	4.7	5.8	5.8	b)	b)
Bakkenolide-A (38)	4.5	10.5	7.8	b)	b)
Fukinone (34A)	13.8	5.0		b)	b)
Unknown (R_f 0.88)		15.2	3.2	b)	b)

a) Data obtained by quantitative TLC scanning

b) Insufficient radioactivity for detection by the Panax Chromatogram Scanner

indicated that the specific activity of the isopetasol was greater than that of the acyl moiety of the petasin esters, when $^{14}CO_2$ was used as a precursor. When MVA was used, the radioactivity was found almost entirely in the acyl moiety. [As there was no further attempt to purify the isopetasol obtained in this case, the figure for its radioactivity (900 dpm; Table 24) may still be slightly inflated.] The presence of bakkenolide-A was also confirmed by the hydrolysis. The lactone could be recovered as a neutral compound by acidification of the aqueous alkaline layer.

It was clear, particularly from the results of the 9 hour and 12 hour feedings, that the four compounds less polar than the petasin esters were involved in active turnover, while the petasin esters appeared to be end products, and retained their radioactivity. Compound <u>17c</u> (36) was considered, on structural grounds, to be a potential intermediate of petasin biosynthesis, and it was especially significant that this compound lost its radioactivity in the feedings of longer duration. Fukinone (34) may be considered as a potential precursor of the bakkenolide series [e.g. (38)] and has been found to co-occur with compounds of this structural type in <u>Petasites japonicus</u> [154]. Loss of radioactivity with increasing time was observed as expected for this compound. However, the loss of radioactivity after 12 hours from bakkenolide-A (38)

a) $^{14}CO_2$ 12 hours

← pheophytin

← Δ^5-sterol

4-methylsterols

Radioactivity

R_f 0   0.5   1.0

(silicagel-G/25% ethyl acetate-75% petroleum ether 60°-80°)

Fig. 33: Comparison of the Sterol Fraction from $^{14}CO_2$ and MVA Feedings

b) MVAL   12 hours

Radioactivity
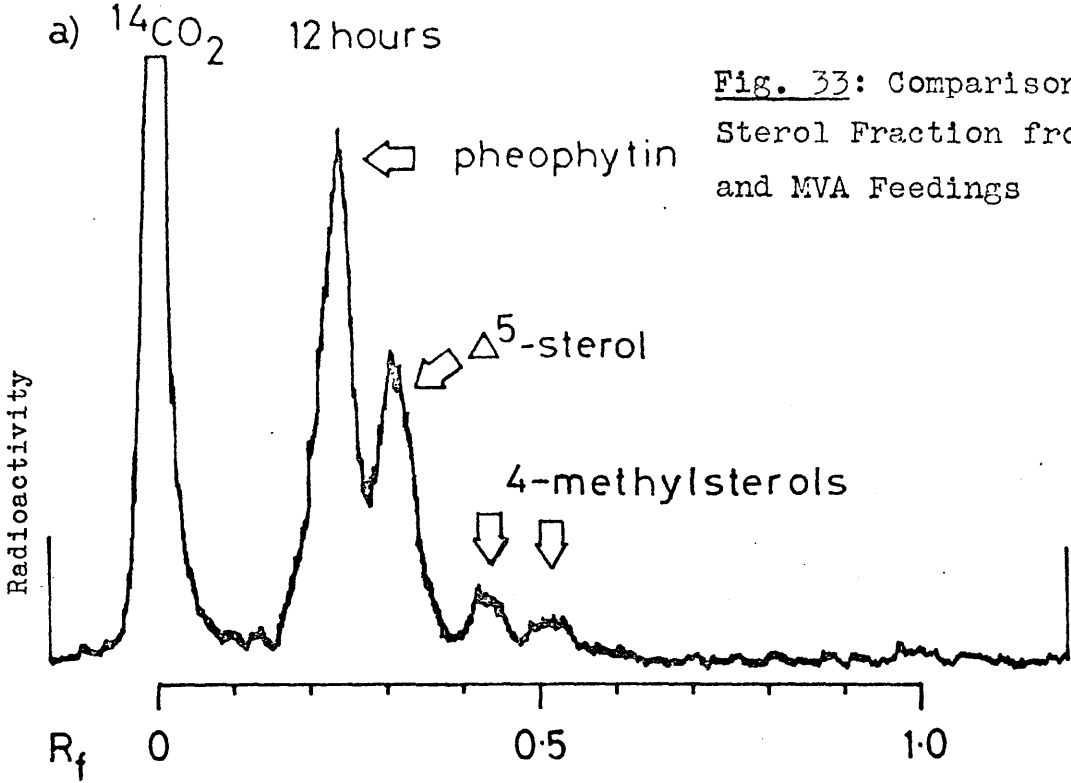
R_f 0   0.5   1.0
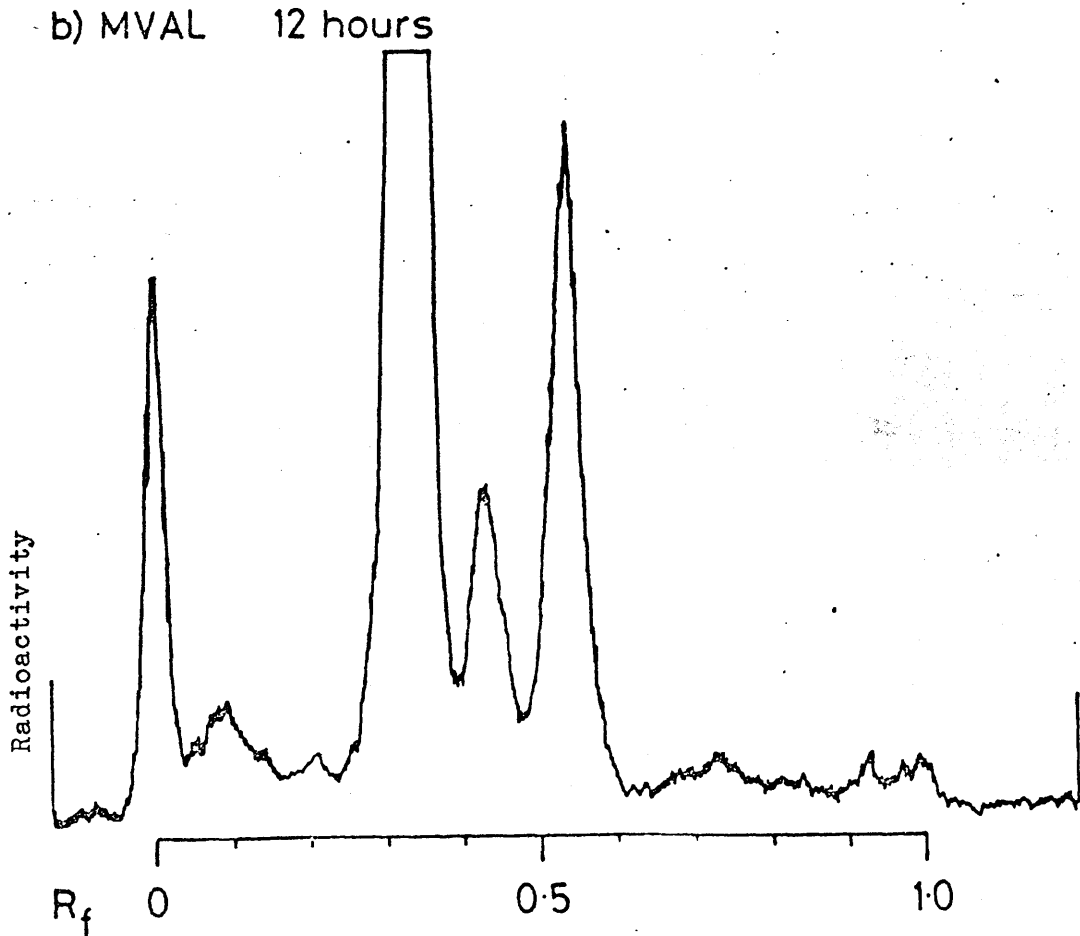
came as a surprise, as this compound appeared to be an end-product on structural grounds. A second skeletal rearrangement of bakkenolide-A, returning to the normal eremophilane skeleton, would seem to be highly improbable.

The massive incorporation of $^{14}CO_2$ into non-lipid-soluble products, presumably mainly as starch, obscured the relative enhancement of incorporation into isopetasol, compared with that observed with MVA as a precursor. When the radioactivity of the isopropanol-soluble fraction was taken as a basis for correlation, the enhancement was more apparent (Table 25).

TLC scanning of fractions other than the sesquiterpenoids indicated quite substantial differences in the distribution of radioactivity between $^{14}CO_2$ and $(2-^{14}C)$ MVA feedings. Radioactivity from $(2-^{14}C)$ MVA was found mainly in the sterols, as expected, or as unchanged MVA. The extracts from $^{14}CO_2$ feedings showed greater diversification of labelled products, and by comparison, the sterols were less prominent. It was presumed, from the compartmentation hypothesis[17,132] that the more highly labelled products were terpenoid quinones and other chloroplast constituents. The main radioactive band of the sterol fraction (Fig. 33) corresponded to pheophytin a (chlorophyll a with the magnesium removed), when $^{14}CO_2$ feedings were examined,

Table 25: Comparison of Sesquiterpenoid Incorporation from $^{14}CO_2$ with Total Lipid Extract and Sterol Fractions

Feeding	$^{14}CO_2$ 3 hr	$^{14}CO_2$ 6 hr	$^{14}CO_2$ 9 hr	$^{14}CO_2$ 12 hr	$(2-^{14}C)$ MVA 12 hr
Radioactivity Administered dpm x 10^{-6}	225	225	410	340	11
Total Isopropanol Extract dpm x 10^{-6}	8.4	6.3	11.7	11.6	6.8
Free Sterol dpm x 10^{-3}	-	76	126	136	851
Isopetasol dpm x 10^{-3}	30	42	78	57	1 (?)
Overall Incorporation	0.013%	0.019%	0.019%	0.017%	0.008%
Proportion of Total Lipid Radioactivity	0.36%	0.67%	0.67%	0.49%	0.015%
Isopetasol-Sterol Ratio of Radioactivity	-	0.55	0.62	0.42	0.0012

but this product was virtually unlabelled by $(2-^{14}C)$ MVA.

Two immediate conclusions could be drawn from this experiment. Petasin biosynthesis was shown to be a comparatively rapid overall process, with radioactivity from a short exposure to $^{14}CO_2$ reaching 75% of maximum accumulation within 3 hours (Table 25). Secondly, a comparison of incorporation into petasin esters and the phytosterols demonstrated synthetic activity for the sesquiterpenoids that was comparable to that found for the sterols. However, the intracellular location of carbon dioxide fixation could also determine the efficiency of labelling of a compound, according to the accessibilty of the initial products of CO_2 fixation to the site of synthesis of that compound. Thus the relative levels of incorporation of different classes of products did not necessarily correlate with their relative rates of synthesis.

The evidence that petasin was synthesised in the leaf, in proportion to growth (section 2.2.7) would predict a higher rate of synthesis of petasin esters than sterols. This evidence would suggest that petasin biosynthesis was isolated, not only from the point of entry of the exogenous mevalonate, but also from the major centres of CO_2 fixation. This deduction is in agreement with the conclusions of Loomis concerning monoterpenoid production [155], and also

adds support to the idea that the specialised glandular
structures could be the centres of terpenoid
biosynthesis [15,125,134,135,156].

4.2.6   An Approach to In Vitro Methods of Study

The difficulties encountered in the study of
terpenoid biosynthesis in vivo include the failure of
precursor to reach the site of synthesis, its utilisation
by competing pathways, and general lack of control over the
system under study. Most experiments have involved feeding
of a distant precursor, such as mevalonic acid, to investigate
the formation of a complex cyclised and functionalised
product. Feeding of precursors intermediate in the pathway
has resulted (in higher plants) in decreased rather than
increased incorporation [16c, 157], because of poor assimilation
of these compounds.

These difficulties are partly resolved by in vitro
methods of study, including tissue slice, tissue culture and
cell free systems. The advances made in the elucidation of
the processes of sterol biosynthesis have relied heavily on
the use of multienzyme, cell-free extracts from mammalian
liver [74], and similar systems derived from yeast [73].

Tissue slice or tissue culture methods are only
effective if the problems encountered in vivo have originated

mainly from ineffective distribution or translocation of the precursor at the extracellular level. Since the intracellular organisation organisation remains intact, the effects of competition by different pathways, or of compartmentation by membrane impermeability, are still operative. The growth of cells in culture also results in developmental changes which may appear as total or partial repression of terpenoid biosynthesis. Thus Henderson found that callus cultures of Pogostemon cablin failed to produce sesquiterpenoids [158] and Overton and co-workers found that suspension cultures of Androgaphis paniculata gave sesquiterpenoid lactones not representative of the whole plant [159].

The removal of the cell structure permits the easy introduction of substrate to cell-free systems. A further advantage is the possibility of closer control over the course of synthetic and competing reactions, by adjustments for optimum pH and co-factor requirements. The homogenates are also amenable to simple purification and concentration procedures, by centrifugation, precipitation and dialysis. Systems of this type may be used as multi-enzyme preparations, catalysing a sequence of reactions from a basic precursor, or for step-by-step examination of a complex pathway [74].

These methods have been routine in the study of intermediary metabolism in animal biochemistry, but have not

been widely applied to the investigation of terpenoid
biosynthesis in higher plants. One reason has been the
problem of endogenous tannins, and quinones formed after
cell rupture, which tend to precipitate and inhibit many
enzymes after isolation [160,161]. However, a variety of
methods now exist to protect enzyme preparations from the
action of these compounds. Some techniques rely on the use
of polyvinylpyrrolidone, and certain other polymers, to bind
the tannins [161,162]. Anderson considers that these polymers
are not always effective, and has investigated the use of
reducing agents, to prevent the formation of quinones, and to
convert any endogenous tannins into inactive phenols [160,163].
A number of successful applications of these procedures have
recently been described [164,165].

In a study of P. hybridus, young leaves were
homogenised in a medium containing reducing agents and
buffer. After filtration to remove cell debris, centrifugation
(1800 x g) sedimented the chloroplasts with cellulose, starch
and other cell fragments. The supernatant was obtained as a
cloudy liquid, not discoloured by tannins. These preparations
were incubated for two hours with mevalonic acid and ATP.
No radioactivity was found in the benzene extracts of the
incubates, but on acidification to hydrolyse pyrophosphate
esters, most of the radioactivity could be extracted in

Fig. 34: Incubation of Mevalonic Acid with Cell-Free
Extracts of P. hybridus Leaves
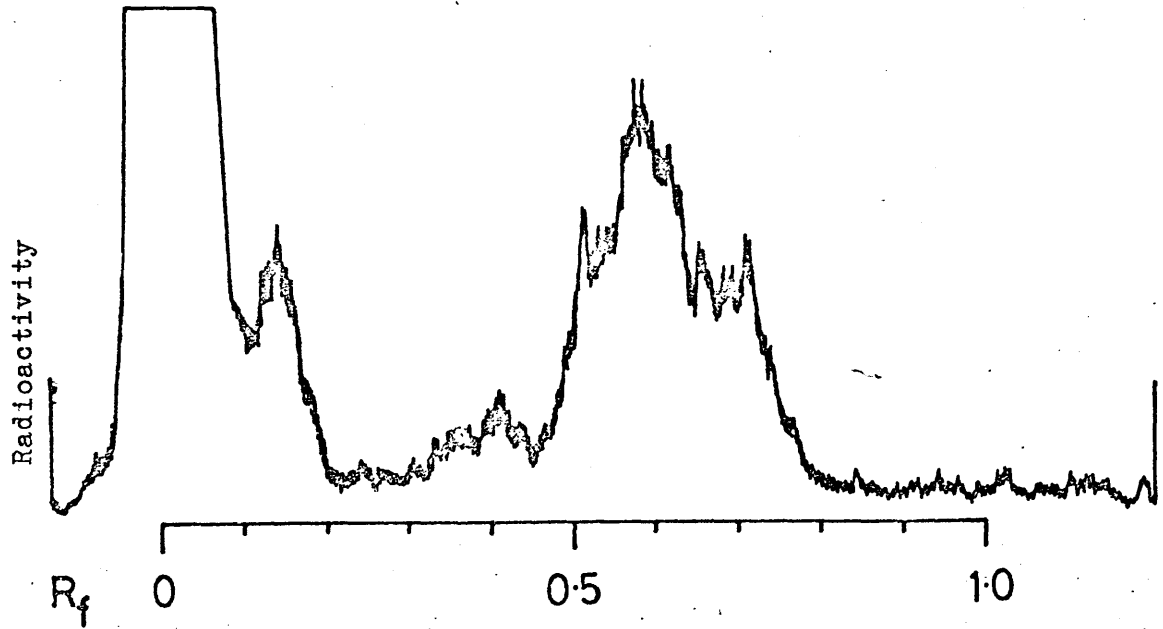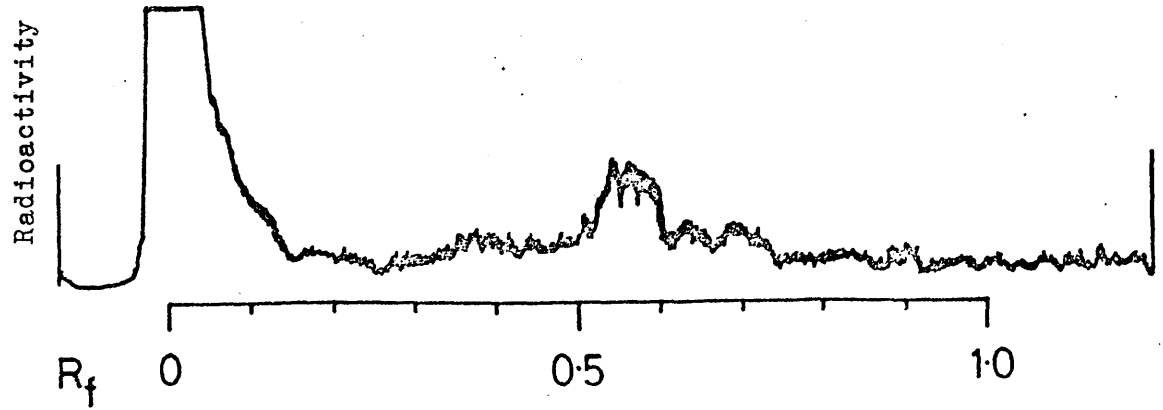
benzene. TLC plates of the extracts were scanned for radioactivity, and both the 'active' preparation and the boiled control were found to contain one major peak corresponding to MVAL. However, the active preparation also gave another composite series of peaks of polarity similar to C_{15} and C_{20} prenyl alcohols, and their acid rearrangement products (Fig. 34). About 0.5% of the total radioactivity was associated with these peaks. These results strongly suggested the accumulation of farnesyl (and possibly geranylgeranyl) pyrophosphate.

The small quantity of MVA used (ca. 0.2 nmol) suggested either that enzyme levels were very low, or that phosphatase was competing with the mevalonate activating enzymes. Fluoride had been included in the incubation to inhibit the phosphatases; this is known to enhance MVA phosphorylation in plant enzyme preparations[166]. The total quantity of protein in this preparation was very small. This contrasts with procedures described for liver enzymes, which give 30 - 50 mg of protein per ml of homogenate[74]; plants in general give 1 - 3 mg of protein[167,168], or even less[166], in the same volume. This renders such preparations susceptible to loss of activity by autolysis[169].

This point emphasises the difficulty of selecting an appropriate tissue for enzymatic study. Unlike animal

liver, there is no well-defined organ in a plant which is
known to be active in terpenoid biosynthesis, and which is
large enough for easy dissection. Where latex is produced,
this is sometimes found to be rich in enzymes, particularly
for rubber biosynthesis[170]. West has had particular success
with an enzyme system synthesising (-) kaurene from
geranylgeranyl pyrophosphate or MVA, isolated from the
liquid endosperm of immature seeds of Echinocystis
macrocarpa[168]. In both of these cases, the extracts required
minimal manipulation; only a brief centrifugation to remove
particulate material. Dilution of the preparation with a
homogenising medium was unnecessary, and since the procedure
did not involve drastic cellular disruption, proteases were
absent and autolysis did not result. The enzyme systems were
thus highly active (for plant preparations) and reasonably
stable for long-term storage. Bonner has also noted that
tapping of the latex of Hevea brasiliensis caused
de-repression of the genes for rubber synthesis, and a second
collection may be richer in enzymes than the initial sample[156].

This is not applicable to P. hybridus, which gives
neither latex nor (from the male flower) seeds. However, it
was noted that rhizome and flower buds were particularly
rich in sesquiterpenoids (section 2.2.7). Thus actively
growing tissues of these types may be a better source for

the enzymes of petasin biosynthesis (or at least, the
eremophilene cyclisation) than the leaf samples used above.
The report that biosynthesis of lower terpenoids occurred
in the plastids of gland cells[135] also opens possibilities
for the isolation of these organelles as a potentially
concentrated enzyme source.

4.3   EXPERIMENTAL PROCEDURES

4.3.1   General Techniques

Plants were grown as described in section 2.3.2 and extraction was completed by the procedures outlined in section 2.3.3. Benzene rather than isopropanol was used as solvent in the earlier experiments of this series. Unless otherwise specified, wick feeding was employed as described in section 3.3.1

4.3.2   Distribution Experiments

Whole Plant

The plant selected had two mature and two young leaves. Each mature leaf was fed with 0.5 ml of a solution containing $(2-{}^{14}C-3\underline{RS})$ mevalonolactone $(6 \ \mu Ci$; specific activity $4.8 \ \mu Ci/\mu mol)$; the young leaves were not fed. Metabolism was allowed to proceed for 10 days, with a 12 hour cycle of illumination. The plant was placed in a sealed system, and the atmosphere was changed every 12 hours, the effluent gases being passed through a CO_2-trapping system consisting of 3 Drechsel flasks in series, each containing 5 M aqueous potassium hydroxide (50 ml). Radioactivity was measured by liquid scintillation counting of an aliquot (0.1 ml) from each Drechsel flask. Toluene scintillator was used in conjunction with an acidic surfactant solubiliser

(Beckman Biosolv BBS-2; 0.5 ml), and a correction was made for quenching and ^{40}K content. (Efficiency for the quenched system was 79.9% in the ^{14}C channel.)

Aqueous extracts of plant tissues were counted using a neutral surfactant solubiliser (Beckman Biosolv BBS-3).

Pyrolysis of tissue residues was carried out in a silica tube packed with granules of cupric oxide, at $700^{o}C$, in a stream of oxygen. The effluent gases were passed through a CO_2 trapping system as above, and radioactivity determined as above.

Autoradiography

Four leaves, transverse span 10 - 12 cm, were selected and fed 25 μl of a solution containing (2-^{14}C-3RS) mevalonolactone (1 μCi; specific activity 5.4 μCi/μmol). Feeding was started at the beginning of the light period of the normal growth cycle (12 hour day). After the desired period of uptake, the leaf was placed between 2 sheets of X-ray film (Ilford Red Seal) which had been pre-chilled in the light-tight envelope. The leaf was then deep-frozen to prevent further movement of tracer during the exposure. After 10 days, the films were removed, and processed in the normal manner.

Dissection Experiments

The dissected parts of the leaf (Table 20) were extracted in ethanol (10 ml): an aliquot (10 μl) of each extract was counted in toluene scintillator. A further aliquot (0.1 ml) of the proximal leaf fraction was examined by TLC (silicagel-G/benzene - chloroform - methanol - acetic acid 4:8:3:1 v/v) and the plates were scanned for radioactivity using the Panax RTLC Radiochromatogram Scanner.

4.3.3   Utilisation of Labelled Precursor

12 Hour, 24 Hour and 48 Hour Feedings

Two plants were fed with o.5 ml of a solution containing (2-^{14}C-3RS) mevalonolactone (7 μCi; specific activity 6.4 μCi/μmol) and metabolism was allowed to continue for periods of 12 hours and 24 hours respectively. The leaves (ca. 7 g fresh weight each) were extracted with isopropanol, and the extract was concentrated to 10 ml. A sample (ca. 2%) was also obtained from the 48 hour feeding made under identical conditions (Section 3.3.1). The extracts were assayed by liquid scintillation counting. Tissue residues were assayed by digestion in 'Soluene' tissue solubiliser (Packard Instruments Inc.). A sample of dried tissue residue (25 mg) was treated with 'Soluene' (1 ml) and warmed (60°C) for 2-3 hours. Toluene scintillator solution (15 ml) was

added, and the resulting suspension was stabilised with
0.4% Cab-O-Sil (Packard) before counting. Quenching factors
for this system were determined using unlabelled tissue
residues and reference standard ^{14}C-hexadecane, and were
correlated using the Automatic External Standard (Nuclear
Chicago Liquid Scintillation System). The method gave
adequate results for the purposes of this experiment, but
was not regarded as satisfactory for more critical use.

The extracts were examined by TLC using a variety
of solvent systems, and were scanned for radioactivity using
the Panax Radio-Chromatogram Scanner. A sample equivalent
to 0.1 μCi of administered MVAL was used for each TLC run.
The data in Table 21 were obtained from TLC scans by
estimation of peak areas.

6 Day and 11 Day Feedings

Four plants were each fed with (2-^{14}C-3RS)
mevalonolactone (10 μCi; specific activity 5.4 μCi/μmol),
2.5 μCi being administered to each leaf. The plants were
cropped, two after 6 days, and the remaining two after
11 days. The leaves (10-12 g fresh weight per plant) were
extracted with benzene, and the concentrated benzene extracts
were hydrolysed by refluxing in ethanolic potassium hydroxide
(ethanol, 1.8 ml; 4 M potassium hydroxide, 0.2 ml). The
sterols were isolated by preparative TLC (3 bands,

R_f 0.75, 0.80, 0.84; 50% ethyl acetate: 50% petroleum ether 60°-80°). The combined sterol bands were acetylated, and purified further by preparative TLC (R_f 0.5, 0.56, 0.64; 5% ethyl acetate: 95% petroleum ether 60°-80°). The samples recovered were used for radioassay and GC-MS.

4.3.4 Inhibitor Feedings

Plants were selected having a minimum of three mature leaves. Growth was standardised for four days before feeding (8 hours illumination at 20°).

Each plant was fed a total of 1 ml of an aqueous solution containing 25, 50 or 100 μg of SK&F 525-A or 7997-A$_3$. One plant was fed with water containing no inhibitor. Each of two separate leaves on every plant was fed via a wick dipping into a vial containing 0.5 ml of the solution. Later, after complete assimilation, 0.5 ml of a solution containing (2-^{14}C-3RS) MVAL (5 μCi, specific activity 4.8 μCi/μmol) was fed in a similar way to each leaf, followed 24 hours later by 0.5 ml of water. The plants were left for a further 48 hours before cropping and extraction.

The benzene extracts were concentrated and hydrolysed by refluxing for 2 hours in ethanolic potassium hydroxide (ethanol, 1.8 ml; 4 M potassium hydroxide, 0.2 ml). Isopetasol was recovered by preparative TLC (R_f 0.38;

50% ethyl acetate: 50% petroleum ether $60^{\circ}-80^{\circ}$). The crystalline isopetasol (slightly discoloured by impurities) was acetylated (acetic anhydride/pyridine) and isopetasyl acetate was further purified by preparative TLC (R_f 0.32; 15% ethyl acetate: 85% petroleum ether $60^{\circ}-80^{\circ}$; double development).

Isopetasyl acetate was estimated by quantitative GLC. A calibration curve was constructed for measurements based on peak heights, using standard solutions of isopetasyl acetate (0.1-1.0 mg/ml). Radioactivity was measured by liquid scintillation counting in toluene scintillator on the Packard Tri-Carb Scintillation Spectrometer. Counting efficiency was 82.4% in the ^{14}C channel, and background was 27 cpm.

Further feedings were made using solutions containing 100, 300 and 1000 μg/ml of SK&F 7997-A$_3$. (2-^{14}C-3RS) MVAL (5 μCi; specific activity 5.4 μCi/μmol) was fed over periods of 2,4 or 6 days. Benzene extracts from these experiments were examined by TLC and scanning for radioactivity. An aliquot (1%) was used for each TLC run.

4.3.5 $^{14}CO_2$ Feedings

Two plants were used, each having two actively growing young leaves. Growth was standardised (10 hour day) in the growth cabinet for several days before the experiment.

Table 26: Administration of $^{14}CO_2$ to P. hybridus Leaves

EXPOSURE	10 Minutes		20 Minutes	
$Ba^{14}CO_3$ used	1.973 mg 1110 x 10^6 dpm		4.96 mg 2800 x 10^6 dpm	
$^{14}CO_2$ recovered	480 x 10^6 dpm		2000 x 10^6 dpm	
$H^{14}CO_3^-$ remaining in generating vessel	180 x 10^6 dpm		50 x 10^6 dpm	
Net $^{14}CO_2$ Uptake	450 x 10^6 dpm		750 x 10^6 dpm	
Fresh Weight of Leaves	1.25 g	1.25 g	1.16 g	0.96 g
Time allowed for Metabolism of $^{14}CO_2$	3 hrs	6 hrs	9 hrs	12 hrs
Nominal uptake of Radioactivity	225 x 10^6 dpm	225 x 10^6 dpm	410 x 10^6 dpm	340 x 10^6 dpm

Two hours after the start of the normal illumination cycle, the plants were sealed in a perspex box (capacity 4.2 l) and ventilated with CO_2-free air. $^{14}CO_2$ was generated by the action of dilute hydrochloric acid on $Ba^{14}CO_3$ (specific activity 50.2 $\mu Ci/\mu mol$; Radiochemical Centre, Amersham). Warming of the solution was required to ensure complete evolution of gas. After the exposure, the box was ventilated with normal $^{12}CO_2$-containing air, and the effluent gases were passed through a CO_2-trapping system. CO_2 uptake was estimated from the quantity of barium carbonate used, deducting the radioactivity found in the CO_2 traps, and also in the $^{14}CO_2$-generating vessel (Table 26). No compensation was made for adsorption of $^{14}CO_2$ and uptake by soil bacteria. The distribution of label between the two leaves was presumed to be determined by leaf fresh-weight. Plants were then removed from the box, and kept under continuous illumination until the leaves were cropped and extracted.

A third plant was fed with ($2-^{14}C$-RS) mevalonic acid (solution in 0.05 M tris buffer pH 8.5), 2.5 μCi being administered to each of two leaves (2.6 g fresh weight); a period of 12 hours was allowed for the utilisation of the precursor.

Leaves were extracted immediately in isopropanol, and the concentrated extracts were fractionated by reversed-

phase chromatography (N1114-50%-LH20/methanol:heptane 9:1 v/v).
A sesquiterpenoid-containing fraction (SEV 55-105) was further
purified by straight-phase chromatography (N1114-50%-LH20/
benzene; SEV 51-68). The fraction was examined by TLC and
scanning for radioactivity. Peak areas were used to determine
the relative proportions of the total radioactivity associated
with each band, and absolute values were obtained by liquid
scintillation counting.

An aliquot of this fraction was hydrolysed with
ethanolic potassium hydroxide (ethanol, 2 ml; 4 M potassium
hydroxide, 0.2 ml). Isopetasol was recovered from the organic
layer by extraction with benzene (2 x 5 ml). The aqueous
alkaline layer was acidified to pH 3 extracted with benzene
(5 ml). The extract was washed with aqueous sodium carbonate
(1 ml) and the organic layer retained. Concentration (to 0.1 ml)
and examination by GLC ($I_{OV-1}^{162^{\circ}}$ = 1770) and TLC confirmed the
presence of bakkenolide-A.

Free sterols were isolated from the reversed-phase
column in the fraction SEV 210-320. The extract was examined
by TLC and scanning for radioactivity as above. This fraction
also contained chlorophyll a, which was converted to
pheophytin a by prolonged storage in solution in chloroform.

4.3.6   Cell-Free System from P. hybridus Leaves

Young leaves of P. hybridus (15 g fresh weight)
were homogenised in ice-cold medium (20 ml) containing
magnesium chloride (20 mM), sodium thioglycollate (25 mM),
sodium metabisulphite (5 mM) and phosphate buffer[171]   (50 mM;
pH 7). The extract was filtered through glass wool, and
centrifuged at 140 x g for 5 minutes. The dark green
supernatant was centrifuged at 1800 x g for 10 minutes, in
pre-chilled buckets. The pellet contained the chloroplasts,
nuclei, and other particulate debris. The supernatant (5 ml)
was clouded, but uncoloured by plastids or tannins. The
preparation was adjusted to pH 6.8 with a trace of maleic
acid, and 2 ml portions of supernatant and resuspended plastid
fractions were prepared in stoppered tubes. One sample of
each was boiled for 2 minutes to denature the enzymes.

A solution (0.25 ml) containing MVA (0.5 μCi;
80 nmol), magnesium chloride (1.0 μmol), manganous chloride
(1.0 μmol), cobaltous chloride (1.0 μmol), potassium fluoride
(10 μmol) and ATP (5 μmol) was added to each tube.

After 2 hours' incubation (25°C), the samples were
deactivated with acetone (1 ml) and extracted with benzene
(2 x 5 ml). 5 M hydrochloric acid (0.1 ml) was added to the
aqueous mixture, and the solution (pH 2) left overnight.
The acid-hydrolysed solutions were then extracted with

benzene (2 x 2.5 ml).

The extracts were examined by TLC and scanning
for radioactivity. Only the 'active' sample (supernatant,
unboiled) showed any peaks other than that due to MVAL.

Fig. 35: Evolutionary Scheme [Based on Chemotaxonomic Data[11)]]
for the Structural Features of Petasin and Related
Sesquiterpenoids

Chemical Character   Hierarchical

                                                                           Level

β-Germacrene type
     Cyclisation

Transannulation

Primitive Oxidative
     Elaboration

                          Asterales   Valerianales   ORDER
                                               (Valencene
                                                   types)

β-Germacrene types

C-8 Oxidation   Compositae   Myoporaceae   FAMILY
                                               (Eremophilone
                                                    types)

Lactone Formation

Complex Oxidation   Cynareae   TRIBE
     Patterns   Helentheae
Transannulation   Anthemidae
                                               + 9 others
Esterification
                                               (Germacranolides,
Eremophilene   eudesmanolides
     Skeleton   Senecioneae   guaianolides &
                                                ambrosanolides)
Furan Ring C

                          Petasites   GENUS

10β Stereochemistry

Bakkenolide
     Rearrangement

                          hybridus   japonicus   albus   SPECIES

                          PETASIN   (Japonicin) (Petasalbin)

Fixed Characters

Variable Characters

5   THE BIOSYNTHESIS OF PETASIN

GENERAL CONCLUSIONS

5.1   A Proposal for a Biosynthetic Sequence

Leading to Petasin

The Compositae family is one of the most highly evolved taxa of higher plants; the sesquiterpenoids found among members of the family reflect this by an unusually advanced level of oxidative elaboration. Within the Compositae, the chemotaxonomic relationships have been studied in great detail[11,26]. It is thus possible to construct a phylogenetic scheme for the evolution of sesquiterpenoid biosynthesis in this family, and the portion relevant to petasin appears in Fig. 35. The more fundamental characteristics at the family level include the widespread occurrence of compounds having structures derived from β-germacrene, modified by transannulation and the formation of lactones by oxidation of the isopropenyl side-chain (see diagram, facing p. 8). The β-germacrene structure appears to be even more primitive, and is found in many families closely related to the Compositae, in conjunction with rather more variable forms of transannulation and oxidation. The fundamental character of Compositae sesquiterpenoids, the γ-lactone ring, appears to have evolved in two steps. The first stage may be considered to have involved specialisation of primitive

oxidative enzymes to hydroxylate at C-8, adjacent to the isopropyl group. This is followed by completion of the lactone structure, by oxidation of the side chain and ring closure. The distribution of lactones in all tribes of the Compositae, and of furans mainly within the Senecioneae, suggests that the latter character is more advanced than the lactone, and only appeared at a stage in evolution after the tribal division.

Transannulation is regarded here as a primitive, but highly variable character, becoming fixed at about the same period as the tribes began to diverge. The Senecioneae, producing exclusively eremophilane types, may thus be regarded as a more advanced phylogenetic group.

'Fixation' refers here to the establishment of a structural feature as a significant taxonomic character. For chemical compounds, this may be considered to involve the specialisation of enzymes with regard to one particular biosynthetic pathway, and the establishment, in the population as a whole, of dominant genes for those enzymes[172]. The fixation of the eremophilane skeleton does not imply that members of the Senecioneae only produce this class of sesquiterpenoids, but that only this class has taxonomic importance, and furthermore, that only this class undergoes oxidative elaboration, because the latter enzymes have

specialised in this direction. In P. hybridus, this is
reflected by the variety of hydrocarbons and alcohols, which
occur at the same oxidation level as farnesol and may be
regarded as relics of genetic history. At higher oxidation
levels, all those compounds that have been identified either
possess the eremophilane skeleton, or have been derived from
it, for example, the bakkenolides.

In the molecule of petasin, the absence of the
characteristic furan or lactone structure is an apparent
contradiction of the general scheme. If petasin was the sole
product concerned, it would be reasonable to suppose it to be
an extremely primitive representative of the Compositae
sesquiterpenoids. However, more advanced furan types are
present, not only within the genus, but also in other
individuals of the same species [27]. This indicates that the
plant has reverted to the more primitive character, by the
deficiency of the lactone or furan-forming gene. This
illustrates another important genetic point, that, as Birch
has noted, the loss of existing biosynthetic steps is an
easier process than the evolution of new reactions [173]. The
status of taxonomic characters is thus not as absolute as the
terminology, 'fixed' and 'fundamental', might appear to imply [172b],
and even less so when dealing with the products of secondary
metabolism.

Fig. 36: Postulated Involvement of Acid Catalysis in Enzymic Mechanisms for Terpenoid Cyclisations
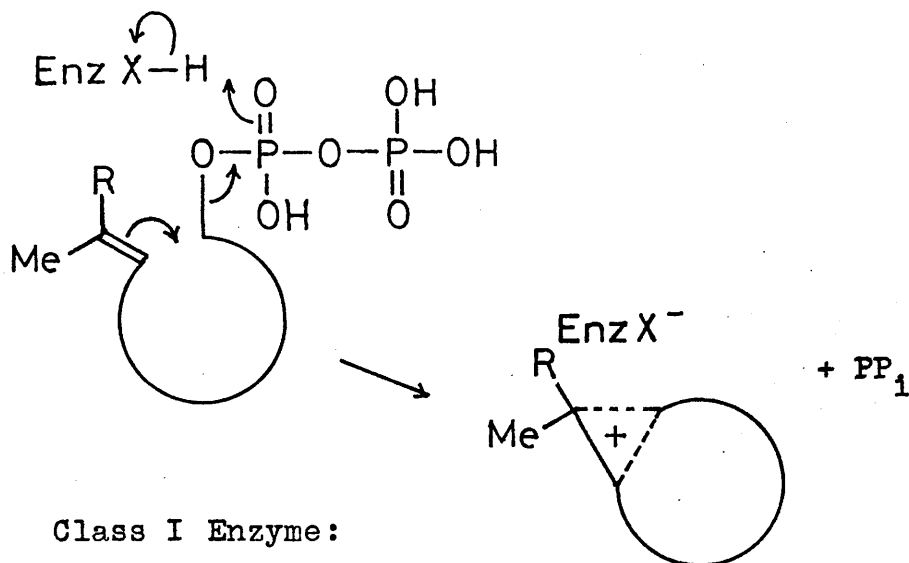
Class I Enzyme:

Pyrophosphate Elimination

(Monoterpenoids, Sesquiterpenoids, Ring C & D Formation in Diterpenoids)
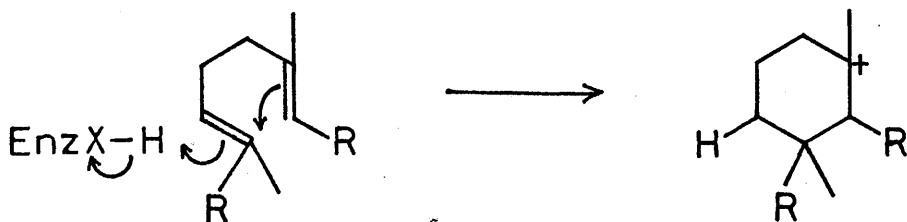
Class II Enzyme: Protonation Mechanism

(Sterol Cyclisation, Bicyclic Sesquiterpenoids, Carotenes, Ring A and B Formation in Diterpenoids)

The Cyclisation Reactions Leading to the Eremophilane Skeleton

The cyclisation reactions of terpenoids have previously been correlated in a hypothesis involving the formation and rearrangement of carbonium ion intermediates[8,10]. Within this general scheme, two classes of cyclisation may be distinguished, in which the carbonium ion arises either by loss of a pyrophosphate anion, or by protonation (Fig. 36). Both cases may be represented as involving acid catalysis by enzymes.

While many complex cyclisations, for example, of the pentacyclic triterpenoids, are well explained by a single process[8b], a great many others, including many bicyclic sesquiterpenoids, and tri- and tetracyclic diterpenoids, appear to involve both types of mechanism. This would suggest a two-enzyme process for the formation of such structures. The apparent separate evolutionary development of β-germacrene and transannulation cyclisation products adds support to this view. This division of the overall process into two steps was proposed for diterpenoid biosynthesis by Wenkert[174], and has recently been proved by West, who isolated not only the intermediate[175], but also the individual enzymes involved[176] (Fig. 37).

The formation of the eremophilane skeleton in P. hybridus was discussed in terms of stereochemistry in

Fig. 37: Specific Enzymic Control of Diterpenoid
Biosynthesis in Ricinus communis
(Robinson and West[176])

geranylgeranyl pyrophosphate

H$^+$

1 enzymic step

copalyl
pyrophosphate

Formation of each product
controlled by one specific enzyme

(+) Sandaraco-
pimaradiene

(+) beyerene

(−) Kaurene

(−) trachylobane
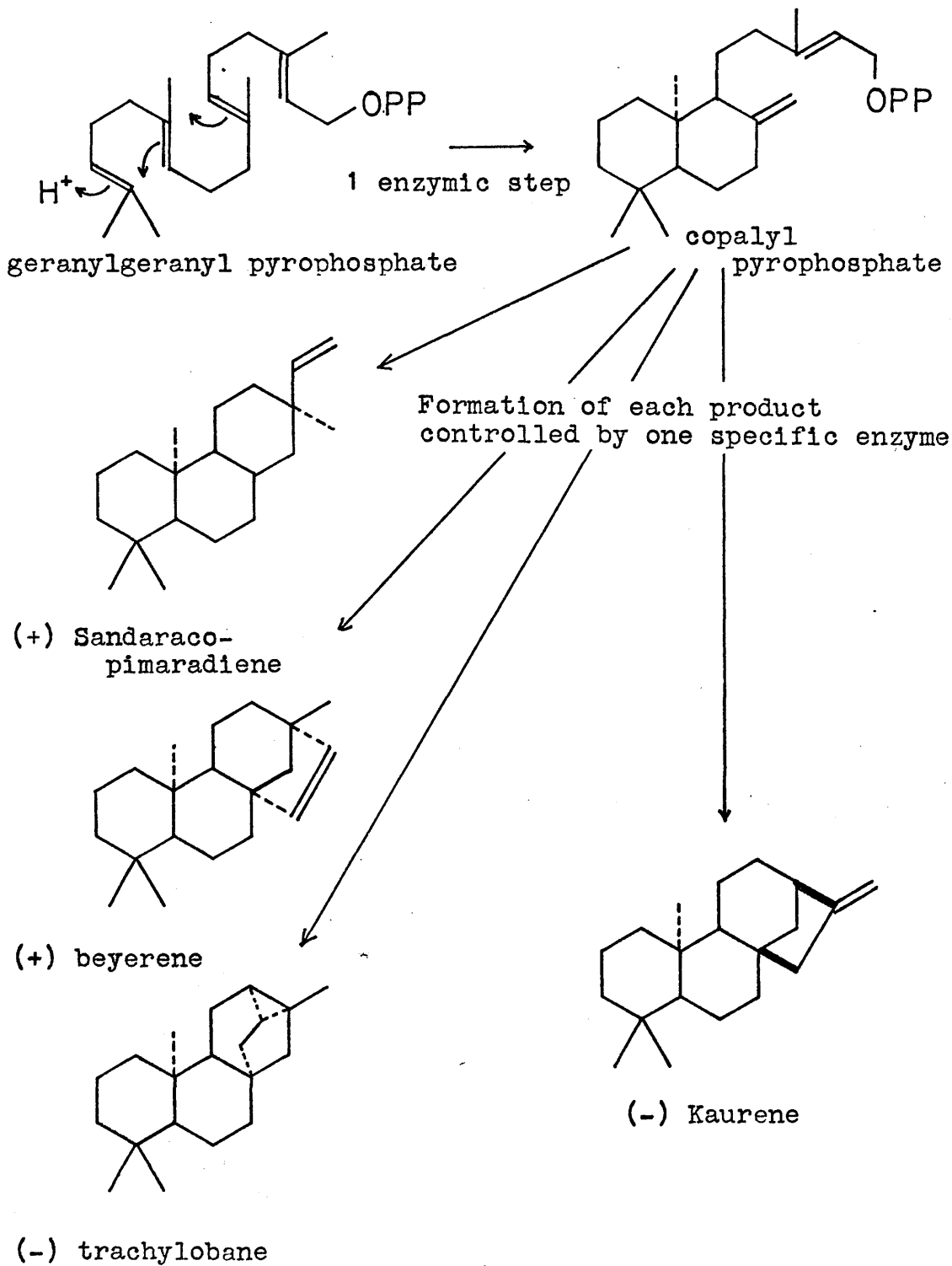
Chapter 3, making the assumption that the overall cyclisation was a two-step process, as suggested above. The evidence obtained from isotopic labelling studies was consistent with such a mechanism involving trans,trans-farnesyl pyrophosphate. Cyclisation would appear to occur by a first step involving enzymic elimination of the pyrophosphate anion. Specific deprotonation, presumably by a suitably located basic residue on the enzyme, is required for control of product formation from the carbonium ion intermediate, to give either β-germacrene or humulene (Fig. 36).

Modern understanding of the molecular basis of evolution postulates the origin of all enzymes from a common prototype[177]. In a family such as the Compositae, where sesquiterpenoid biosynthesis has specialised along particular lines, close relationships between the enzymes may thus be expected, both in protein structure, and in the mechanism of catalytic action. This idea of a common evolutionary origin has been used to formulate the mechanism for the transannular reaction of β-germacrene, to give eremophilene (described in stereochemical detail on pp. 69-71). Other cyclic sesquiterpenoids found among members of the Compositae have structures and stereochemistry that suggested a common origin from chair,chair-folded β-germacrene (Fig. 38), with the ring methyl groups located on the β-face of the molecule.

Fig. 38: Relationship of Enzymic Mechanisms for the
Transannular Reactions of Compositae
Sesquiterpenoids

chair,chair-

β-Germacrene

H$_\epsilon^+$ to C-1   H$_\epsilon^+$ to C-4

Charge stabilised
at C-10

Charge stabilised at
C-4

- H-1α

- H-9α

- H-14

- H-1β

Guaiane
type

Pseudoguaiane
type

Eremophilene   Δ-9
Isoeremophilene   β-Selinene   β-Elemene
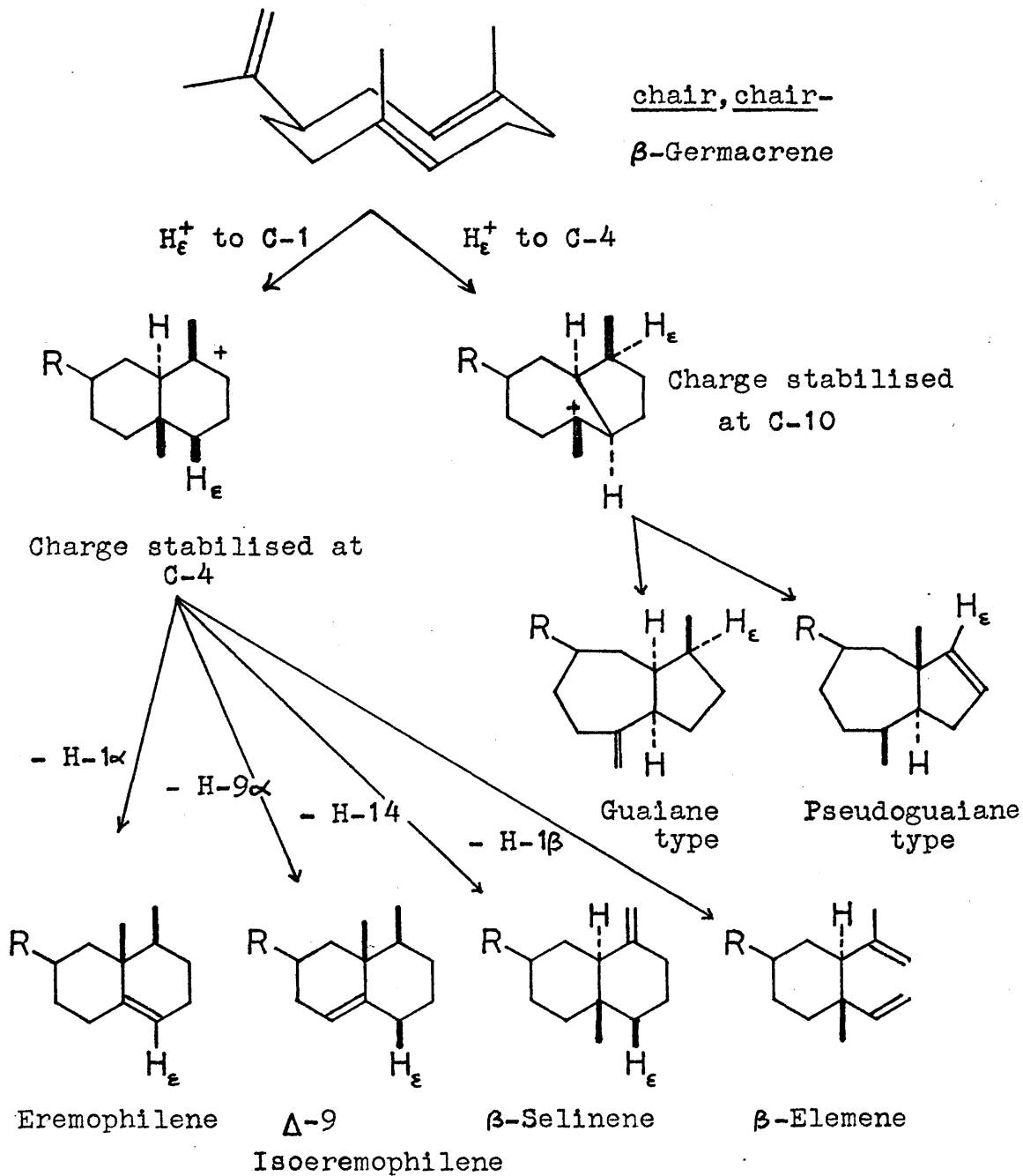
The proposal, that proton attack to give the guaianoid carbonium ion is directed in the opposite sense to that giving the eudesmanoid cation (contrary to the ideas of Hendrickson[10]), is based on the direction of migration of methyl groups in the derived pseudoguaiane and eremophilane skeletons. Wagner-Meerwein type 1,2- shifts would be expected to result in movement towards the site of positive charge.

The involvement of a cationic intermediate (as in Fig. 38) in terpenoid cyclisation mechanisms has been contested by a number of workers, including Robinson[178], who have pointed out the instability of a carbonium ion as an intermediate in aqueous solution. However, it has been emphasised by Perutz that enzymic reactions may take place, not on the surface of the protein, but in the hydrophobic interior, where a carbonium ion may be protected from solvent attack[179]. The reduced dielectric constant of such a medium would also have a stabilising effect on the cation, by electrostatic interaction with free carboxylate anions[180]. This type of consideration has recently led to the conclusion that a cationic intermediate may be involved in the hydrolytic mechanism of action of lysozyme[179,181]. An analogy in organic chemistry is shown by the effect of dielectric constant of the solvent on S_N1 reaction rates. Stabilisation of the salt form, in a non-polarisable solvent, leads eventually to

an end product by elimination, and not substitution[182].

The problem of specific product control in terpenoid cyclisation may be resolved by postulating selective proton abstraction as part of the mechanism of enzymic catalysis. The polarised species would then degenerate by the route most favoured by molecular conformation.

The general postulate for enzymic action in terpenoid cyclisation requires control at four stages:

i) The formation of a non-covalent enzyme-substrate complex with optimum substrate conformation;

ii) The initiation of reaction by specifically directed proton attack;

iii) The stabilisation of a cationic intermediate, possibly in a selective manner when two such intermediates are equally feasible;

iv) Specific proton abstraction.

The occurrence of this last process would explain the selectivity of product formation in the different species of Compositae: conformation alone does not provide the degree of control observed for the transannular reaction in P.hybridus. It is interesting to note that this scheme provides a biosynthetic origin for the elemane skeleton. Hitherto, this type of structure has been regarded with suspicion,because of

Fig. 39: Proposals for a Biosynthetic Pathway Leading to Petasin

Oxidation Level (Relative to Farnesane)
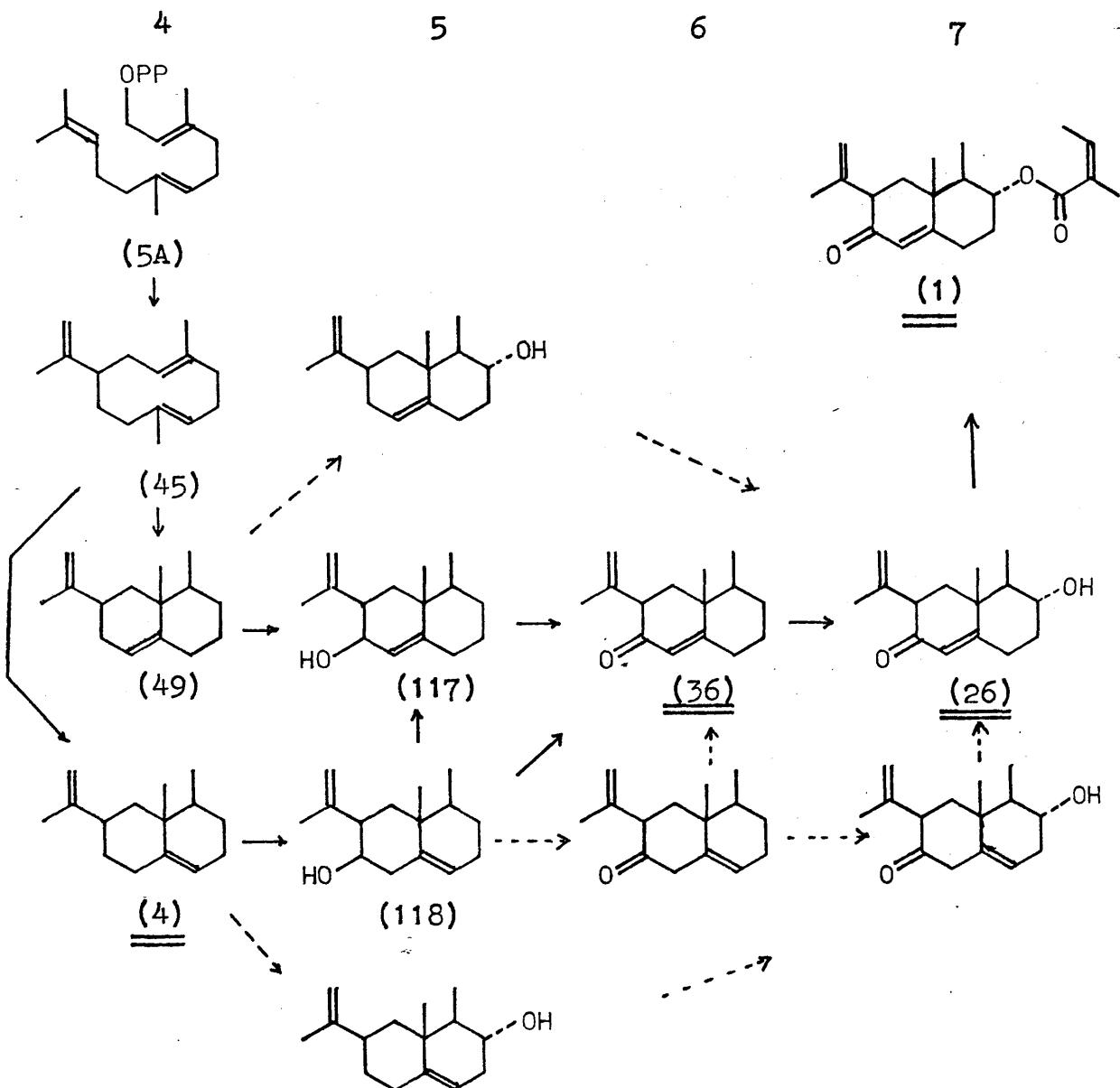
——————→ Probable Routes Leading to Petasin
- - - - → Possible Secondary Pathways

(4) etc. Components Identified in P. hybridus Extracts

the possibility of its formation by the thermally induced

Cope rearrangement, in the course of isolation. It is

difficult to see how such a process, involving a fully

concerted, electrocyclic mechanism, could have occurred in

the plant. The route via the cationic intermediate (Fig. 38)

permits the correlation of this structure with those of the

remaining Compositae sesquiterpenoids.

Biosynthetic Reactions Following Cyclisation

The consideration of the oxidation levels of these

compounds indicates that three oxidative steps are required

in the biosynthesis of petasin (1) from its potential precursors,

eremophilene (4) or Δ^9-isoeremophilene (49). For lack of

alternatives, the first stage must involve hydroxylation,

either at C-8 or C-3 (Fig. 39). The apparently primitive

origin of C-8 oxidation, as a chemotaxonomic character of the

Compositae, would favour initial hydroxylation at this

position, giving the hypothetical intermediates (117) and

(118). There is increasing evidence that terpenoid

hydroxylations occur by the action of mixed-function oxidases.

These enzymes, which act by reduction of molecular oxygen to

a thermodynamically active form, which is complexed with the

transition-metal containing enzyme, are known to be involved

in the biosynthesis of steroid hormones, and in the

demethylation of lanosterol [183]. A number of such systems

have been examined in detail <u>in vitro</u>, and have been shown to
include the haemoprotein, cytochrome P-450, as part of a
multiprotein enzyme system [184]. These enzymes are widespread
at all levels of evolution, and appear to perform a variety
of oxidative reactions, including hydroxylation, dehydrogenation
and epoxidation. A special feature of the mixed-function
oxidases is the ability to catalyse specific oxidations at
saturated carbon; in this respect, they differ from
peroxidases, which hydroxylate at allylic positions in
unsaturated compounds [183]. West has demonstrated that
cytochrome P-450 systems catalyse the first four oxidative
steps of gibberellin biosynthesis [185].

It would seem likely that enzyme systems of this
type could be responsible for both hydroxylations in the
biosynthesis of petasin (Fig. 39).

The occurrence of the compound, eremophila-9,11-
dien-8-one (36), was strongly indicative of its function as
a key intermediate in the biosynthesis of petasin. Supporting
evidence was provided by the $^{14}CO_2$ feeding experiment
(section 4.2.5), where this compound showed active turnover
of radioactivity. Eremophila-9,11-dien-8-one may be formed
by dehydrogenation of the postulated intermediate (117), or
with an isomerisation step[*], from (118). Hydroxylation of
eremophila-9,11-dien-8-one at C-3 would then give petasol (26),

[*] By analogy with steroid biosynthesis.

which could be esterified in a final biosynthetic step
to complete the structure of petasin (1).

5.2   Compartmentation of Sesquiterpenoid Biosynthesis

The failure of most terpenoids in higher plants to
incorporate radioactivity efficiently from mevalonic acid
(MVA) has been a common experimental observation[15,115].
Goodwin has suggested a scheme of intracellular segregation
of enzymes and their substrates, to account for his results
in labelling experiments with chloroplast terpenoids[17,130].
This satisfied most convincingly his findings, that glycollate
and glyoxalate were readily incorporated into β-carotene,
when exogenous MVA was not. The evidence for isolation of
the chloroplast from exogenous MVA was consistent with the view
that endogenous MVA acted as a precursor to the chloroplast
terpenoids.

In P. hybridus, there was evidence (sections 2.2.6
and 2.2.7) that accumulation of petasin continued through
maturity of the leaf, and that accumulation was correlated
with leaf growth, which also continued until senescence.
The rate of synthesis would thus be expected to lead to
equal utilisation of endogenous MVA by sterol and sesqui-
terpenoid pathways. This was not the case with exogenous MVA,
which was incorporated by sterols several thousand times more

Fig. 40: Sequence of Assimilation of Tracers by
Terpenoid Pathways in P. hybridus:
Evidence for Three Sites of Synthesis

^{14}C - MVA
(exogenous) $\longrightarrow$ Sterols

Chloroplast Terpenoids

Sesquiterpenoids

$^{14}CO_2$ $\longrightarrow$ Chloroplast Terpenoids

Sterols
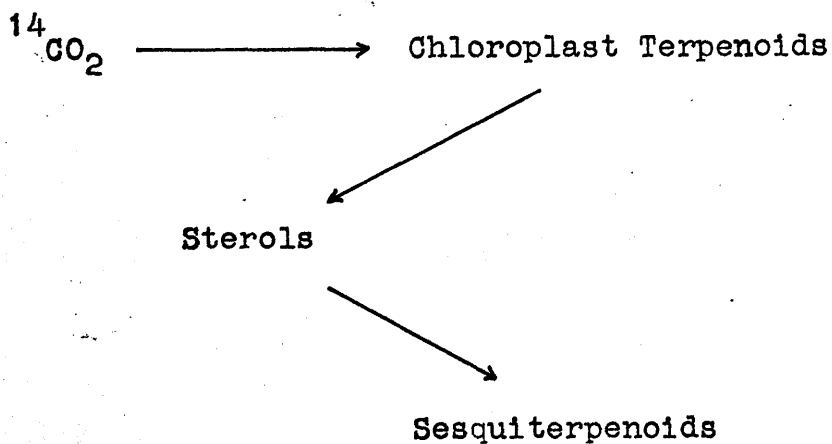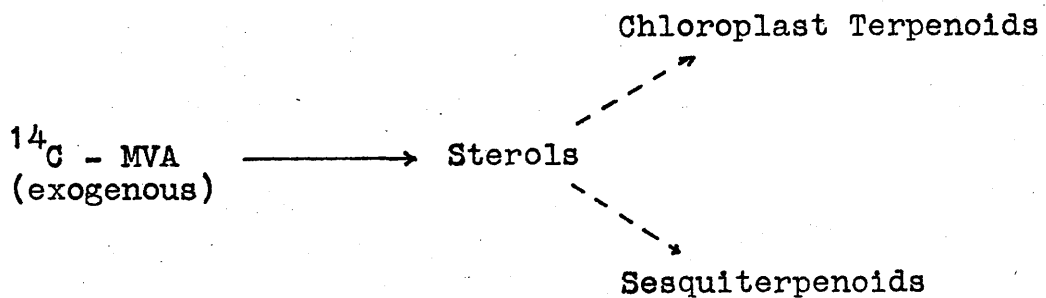
Sesquiterpenoids

efficiently. Selective inhibition of the sterol pathway (section 4.2.4) was only marginally effective in releasing more radioactivity for incorporation into sesquiterpenoids, by relaxation of competition for labelled precursor. The result provided evidence for the involvement of separate metabolic pools of precursor for each pathway (Fig. 40). In contrast, $^{14}CO_2$ feedings gave levels of incorporation for both sterols and sesquiterpenoids that were more nearly comparable with synthetic rates based on the evidence of accumulation. However, sesquiterpenoids incorporated less radioactivity than the sterols: this would suggest that the location for synthesis of the sesquiterpenoids was more remote than that of the sterols from the site of CO_2 fixation (Fig. 40).

A more detailed model for the compartmentation of terpenoid synthesis is depicted in Fig. 41. The evidence of section 4.2.2 would suggest that labelled MVA, when fed, entered the xylem transpiration stream. It would be more difficult to account for this polarisation of translocation in the phloem. Cells immediately adjacent to the vascular system would thus receive a disproportionate share of the radioactivity. This scheme supposes that the glandular structures are more remote from the vascular system than normal parenchyma tissue. If, as reported by Amelunxen for Mentha species, the glandular structures are surrounded by

Fig. 41: Model Proposed for the Compartmentation of
Terpenoid Biosynthesis in P. hybridus
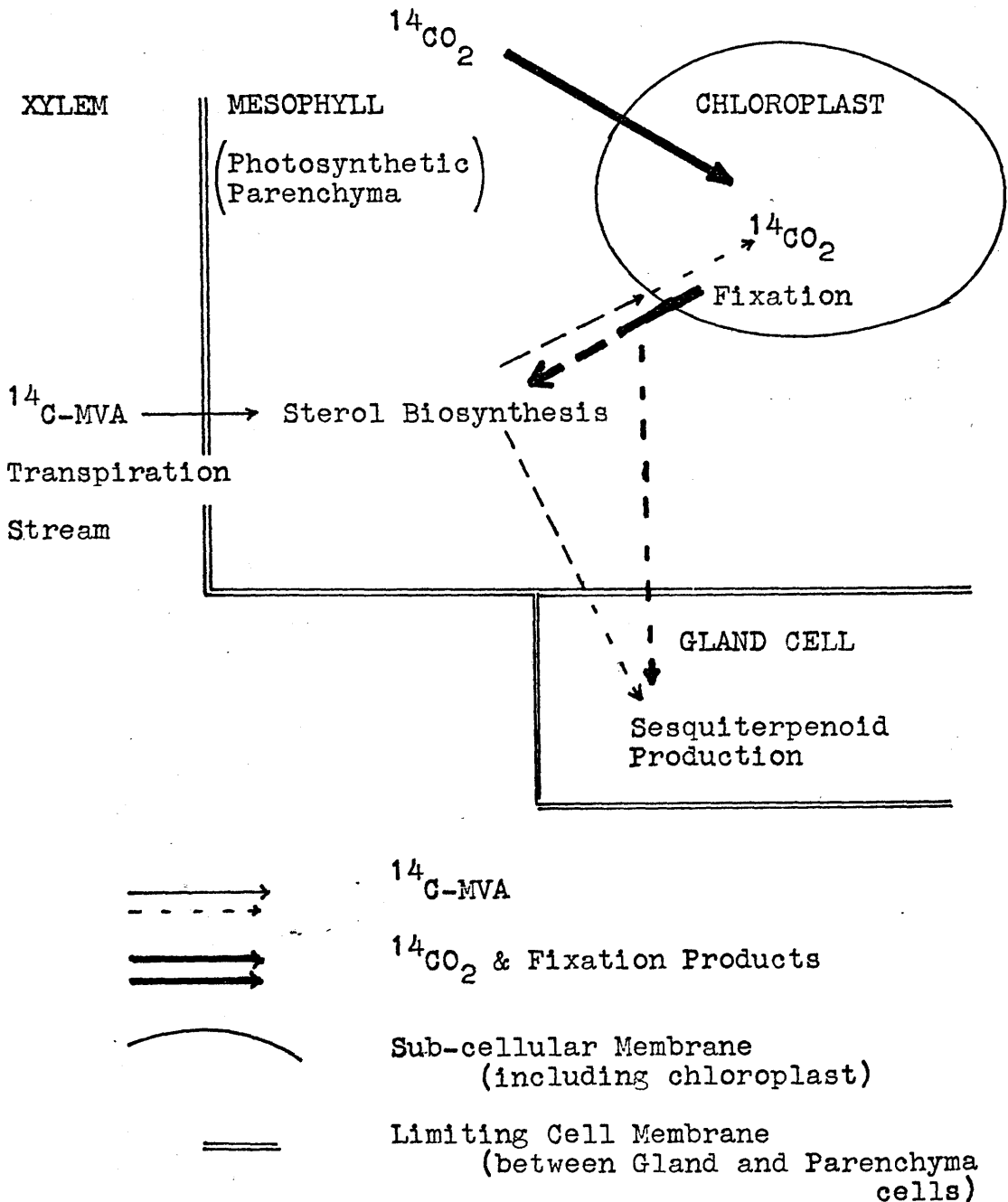
a heavy cuticular layer[186], and the plastids are not developed into photosynthetically active chloroplasts[135], then such cells would have a reduced level of transpiration, and a low demand for water.

$^{14}CO_2$ enters the plant by fixation in the chloroplasts of the photosynthetic parenchyma. Under the conditions of the experiment, most of the tracer would be fixed as starch[146]. The fraction entering as glycollic acid may be expected to act as the primary source of label for terpenoid biosynthesis. As indicated by Goodwin, glycollate or glyoxalate may enter the cytoplasm (the chloroplast membrane being permeable to these compounds[17]) and so be transferred to neighbouring gland cells for incorporation into sesquiterpenoids.

In this hypothesis, there is no need to postulate actual impermeability of gland cells to mevalonic acid. If the glands are distant from the xylem vessels, uptake by the intervening cells may be expected to deplete the labelled precursor, allowing only a small proportion to reach the site of sesquiterpenoid biosynthesis. Photosynthetic cells may, however, be adjacent to the gland, and some may be expected to function specifically to supply the gland with organic nutrient. Thus $^{14}CO_2$ fixation products may be expected to penetrate more effectively.

One consequence of the compartmentation hypothesis is the existence of entirely distinct enzyme systems for the utilisation of MVA[132], with independent genes. This is required for the separate regulation of the two pathways. Evidence for this view has been provided by Goodwin, who demonstrated a different pH optimum for MVA kinase from isolated chloroplasts, from that of the enzyme occurring in the cytoplasm[17,187]. Thus the 'biosynthetic tree', commonly published as depicting terpenoid biosynthesis from a common pathway, appears to be incorrect from the point of view of the enzymology. A system of parallel pathways, using the same processes and intermediates, but isolated from each other, would probably present a more accurate picture of the organisation of terpenoid biosynthesis in higher plants.

Certain terpenoid cyclisations require the cis- prenol isomers, rather than the trans- forms produced by the sterol pathway[10]. Independent enzyme systems for these precursors would thus permit stereospecific synthesis, and would not require their production by isomerisation of the trans- isomers. A test of this scheme would be the retention of tritium from $(3R,4S-4-^3H)$ MVA in derived terpenoids; all the radioactivity would be lost if an all-trans- intermediate was formed. Polyprenols of mixed stereochemistry are already known to incorporate the label from this precursor exclusively into the cis- vinylic positions.

PART II

STUDIES IN THE APPLICATION OF GEL CHROMATOGRAPHY

6   <u>STUDIES OF GEL CHROMATOGRAPHIC SYSTEMS USING</u>

    <u>HYDROXYALKOXYPROPYL DERIVATIVES OF SEPHADEX</u>

6.1.1   <u>INTRODUCTION</u>

Gel chromatography[189] is a general term describing

all separation processes in which a solute is distributed

between a mobile liquid phase and a stationary gel phase.

The physical nature of the gel state differs from

that of solids and liquids: these differences confer the

distinctive properties and advantages of gel chromatographic

systems. Classical liquid chromatography[190] either employs

a solid directly for its surface properties, as in adsorption

chromatography, or indirectly, as a support for liquid phases

in partition chromatography. In either case, the effective

volume of stationary phase available for solutes to enter

is only a small fraction of the total volume of the system.

Distortion of the eluted zones at high solute concentrations

may thus result from non-linearity of the adsorption

isotherms, or, in the case of partition systems, from the

irregular behaviour of non-ideal solutions in the stationary

phase. In gel chromatography, the solutes may enter the

entire gel phase, which occupies a volume equivalent to that

of the mobile phase. The effective capacity of the system

is thus very much increased, and the elution of zones in

ideal Gaussian form will be maintained, even at

high solute concentration.

The first major advances in the field of gel chromatography concentrated on the development by Porath and Flodin of systems based on the bacterial polysaccharide, dextran, crosslinked by reaction with epichlorohydrin[191]. The rapid commercial development of these discoveries, in the form of Sephadex (AB Pharmacia), led to their widespread application in chromatographic systems. Similar media were later devised, based on the synthetic product, polyacrylamide (Bio-gel; Bio-Rad Laboratories, U.S.A.)[192], and agarose, a component of agar, extracted from red seaweed[193].

In aqueous solution, these products become swollen with solvent, and a solute may penetrate the gel to a degree dependent on its molecular size, and the porosity of the gel matrix[189]. The polarity of both aqueous liquid, and the resulting gel phase, is comparable, and thus this property is of little importance in determining the distribution of solutes between the two phases. When applied in a chromatographic system, these media separate mixtures primarily on the basis of differences in molecular size, and similar steric effects[194]. Selective effects, due to residual charged groups on the gel matrix, may be neutralised by the use of solvents of moderate ionic strength. This process was originally termed 'gel filtration' by Porath and

Flodin[191], although other expressions have been used by later workers, for example, gel permeation, molecular sieving, and steric chromatography.

The success of these methods in application to water-soluble systems soon led to attempts to extend their use to organic solvents. The existing gel constituents were found to be unsuitable by their failure to swell under conditions of lower polarity[194]. New materials were developed, including polystyrene crosslinked with divinylbenzene[195], and a copolymer of methyl methacrylate and ethylene glycol dimethacrylate[196]. A different approach was adopted by other workers, who started with the already well established Sephadex products, and reduced the polarity of the gel matrix by substitution of the free hydroxyl groups. Flodin reported that acetylation of Sephadex produced a lipophilic derivative, capable of swelling in solvents such as chloroform and toluene[194]. Later, it was considered that the stability of ether derivatives was preferable to that of esters, which would be more susceptible to hydrolysis. At Pharmacia, the β-hydroxypropyl ether of Sephadex was developed, and is now commercially available as Sephadex LH-20[197]. Nyström and Sjövall made extensive investigations of methylated derivatives, mainly of Sephadex G-25[198,199].

The original applications of gel media for

chromatography in organic solvents were directed toward the

gel filtration mechanism of separation. This probably arose

because of the association of the technique with the typical

aqueous systems then in use. One field where these methods

were found to be highly effective was for the analysis of

molecular weight distributions in polymer preparations[189].

In this application, the properties of the gels were well

suited to the requirements of the polymer chemist, who did

not need a highly selective separation, but preferred a

system dealing with a wide range of molecular size.

These limitations were less attractive to the

organic chemist, whose molecules show less variation in size,

and would thus be poorly separated by gel filtration. The

modified Sephadex gels[197,198] were slightly more effective

in their separating power, possibly because of closer control

over polymer size distribution and crosslinking in the process

of manufacture. Even so, their introduction did not provide

a method as effective as traditional methods of chromatography,

and their adoption for routine laboratory use has been slow.

When organic solvents are used in gel chromatography,

the variability of polarity of both solvent and gel matrix

is superimposed on the molecular sieving effect. This was

expressed in the equation derived by Martin and Synge[200]:

$$\alpha = \frac{A_m}{A_s} \left(\frac{1}{R_f} - 1 \right)$$

Sjövall has considered this equation in relation to gel chromatography, and has associated variability of the term A_m/A_s with the molecular sieving effects used in gel filtration[198], the term α being related to the partition coefficient of the solute between the mobile and stationary phases. The appreciation of the utility of partition processes for separation was an important factor in the development of gel chromatography by the Karolinska group.

Three cases may be distinguished in the methods of gel chromatography:

i)   Straight-phase partition, where the gel is more
     polar than the mobile phase, and samples are eluted
     in order of their polarity;

ii) Gel filtration, where the gel and solvent have the
     same polarity characteristics, and elution is in
     order of decreasing molecular size of the solutes;

iii) Reversed-phase partition, where the gel is less polar
     than the solvent, and samples are eluted in inverse
     order of their polarity.

It is important to be aware that the term 'polarity' covers a wide range of intermolecular forces, including

hydrogen bonding and dipolar interactions, that vary quite considerably in character. A broader definition of these forces should include lipophilic interactions, which are hydrophobic in nature, and hence under the older definition 'non-polar'. Nevertheless lipophilic forces may make a strong contribution to certain separations, in particular those involving reversed-phase partition.

The appropriate choice of substituent for the gel matrix, and the use of a complementary solvent would thus permit the selective involvement of specific interactions for liquid-gel partition chromatography. Investigations in this direction are likely to prove to be a fruitful area of study for the future.

An example of this type of development is shown by the recent preparation and application of a lipophilic-hydrophobic derivative of Sephadex involving the formation of a long-chain alkyl ether [18]. Earlier attempts to prepare such derivatives, using long-chain terminal epoxyalkanes, were frustrated by the difference in polarity between the gel and the reactant. In a non-polar reaction medium, the dextran gel failed to swell, and the epoxyalkane did not penetrate; under polar conditions, the epoxyalkane was insoluble, and failed to react because of poor dispersion. The answer to these problems was found by the preliminary preparation of

a derivative of Sephadex with intermediate polarity. Sephadex LH-20 was found to be a suitable intermediate[18], and reacted readily with epoxyalkanes to give products with degrees of substitution variable up to the theoretical maximum[201]. These new materials were found to be strongly hydrophobic, and were particularly applicable to reversed-phase methods of chromatography[18,201]. One advantage of this type of system was the modification of polarity by covalent attachment of the non-polar component to the gel matrix. The characteristics of the preparation could thus be maintained in long term operation, and this had beneficial effects on the reproducibility of separations and the working life of the columns. When column effluents were monitored using a continuous detection system (for example, the circulating chain flame ionisation detector), the stability of the stationary phase minimised the contribution of column 'bleed' to background noise[201].

6.2   RESULTS AND DISCUSSION

6.2.1   General Principles

The general scheme of this study was the
investigation, using model compounds, of the chromatographic
properties of certain lipophilic derivatives of Sephadex.
The information obtained from this survey could then be used
to gain some understanding of the systematic separation
processes operative under various conditions. In application
to biological extracts, this would provide a means for the
prediction of the elution behaviour of known samples, and for
structural correlation of unknown compounds on the basis of
chromatographic observations.

For this reason, the investigation has been directed
toward the detailed study of relatively few gel-solvent
combinations, rather than the more general type of survey
that had already been undertaken by Ellingboe, Nyström and
Sjövall[201]. The need for reproducibility has led to a
preference for simple solvent systems having only one or two
components. It might be possible to optimise separations by
the use of more complex, multi-component solvent systems,
but there was the risk that these would be rather specific
in application, and less suited for routine use.

Reporting of Data

     In any chromatographic process, the behaviour of samples may be recorded either in an absolute manner, which is frequently difficult to estimate, or by trivial measurements, which are valueless for correlation. A compromise between these two extremes must provide ease of measurement, and also permit comparison of data from different sources.

     For thin-layer and gas-liquid chromatography, this is provided respectively by R_f and Kováts' retention index [202]. In gel filtration, the terms K_{av} and K_d [194] have been used, but require the determination of several column parameters for their estimation. The only factor having a simple one-to-one relationship with the elution volume (V_e) of any sample is the total volume of the gel bed (V_t) contained in the column. Under comparable conditions of column preparation, a given sample should show a constant ratio between the two terms (V_e/V_t) that is independent of column dimensions.

     This quantity has been used by Sjövall and co-workers in the form of the percentage of the total column volume [198,201]. With the prospect of constant routine use of gel chromatographic data (as in chapters 2 and 3 of this thesis), an extension of this idea has been proposed. The term Standard Elution Volume has been defined as the

Fig. 42: Elution of n-Alkanes from the Straight-Phase System (Nl14-50%-LH20/Benzene) Dependence of Retention on Chain Length
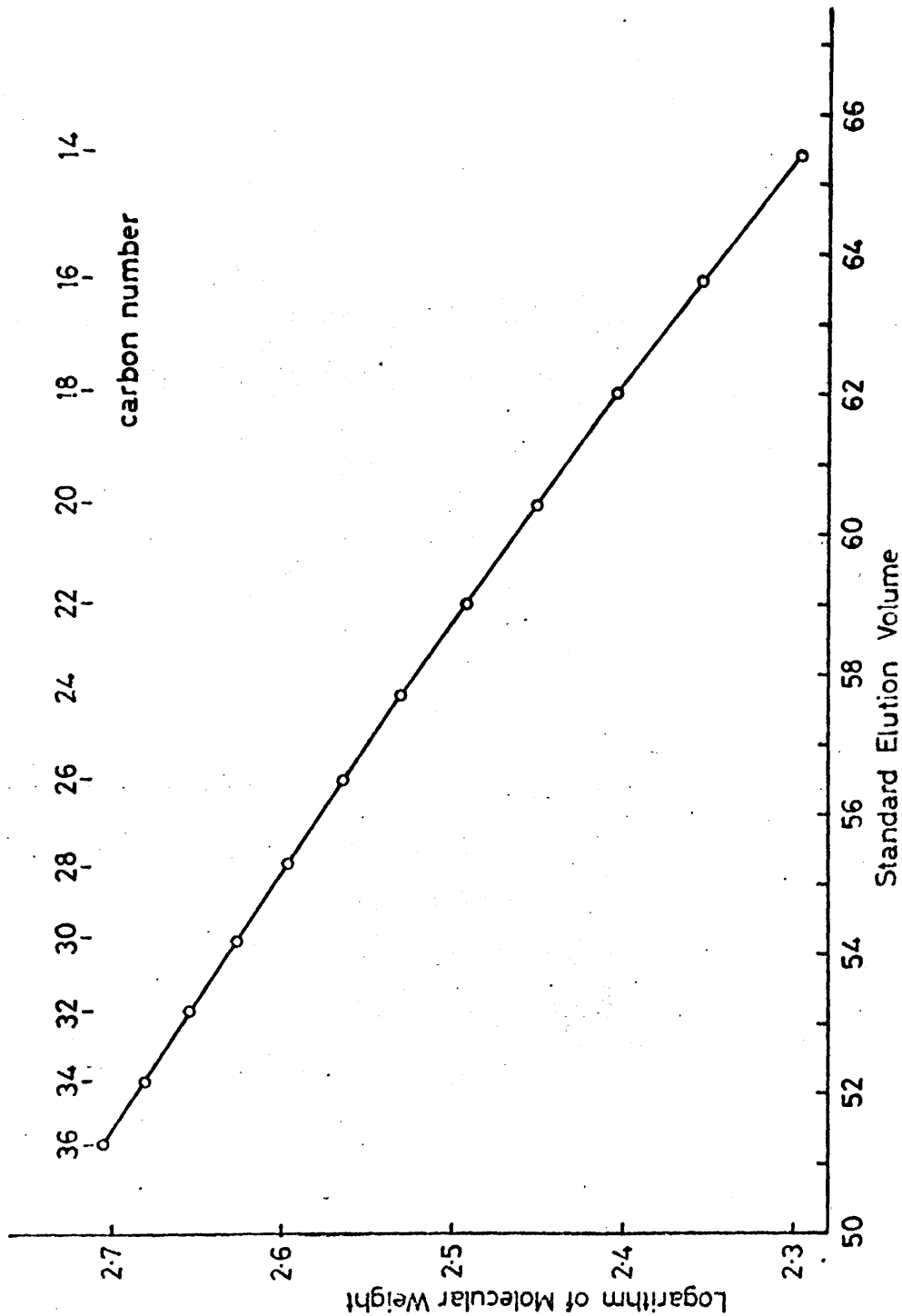
Table 27: SEV Data for Non-Hydroxylic Compounds on the Straight-
Phase System (N1114-50%-LH2O/Benzene)

Column 100 cm x 0.9 cm i.d. Flow 6 ml/hr

Compound	SEV	M.W.	Compound	SEV	M.W.
n-Hexatriacontane	51.3	506	α-Amyrin acetate	57.1	468
n-Tetradecane	65.4	198	Stigmasteryl acetate	55.2	454
Methyl lignocerate	53.4	382	Cholesteryl TMSi	55.9	458
Methyl behenate	54.5	354	Cholestan-3-one	60.0	386
Methyl arachidate	55.6	326	Cholest-4-en-3,6-dione	58.6	398
Methyl stearate	56.8	298	Cholesteryl chloride	59.7	404.5
Methyl myristate	59.5	242	Progesterone	61.3	314
Methyl laurate	60.8	214	5α-Androstan-17-one	64.3	274
Cholesteryl palmitate	49.0	624	Isopetasin	56.9	316
Cholesteryl butyrate	54.5	456	Isopetasyl acetate	59.1	276
Cholesteryl acetate	56.3	428	Isopetasone	61.3	232
Cholesteryl benzoate	54.0	490	S-Isopetasin	54.2	334
Cholestane	62.7	372	Convertifolin	66.2	234
Coprostane	62.6	372	Santonin	67.2	246
5α-Cholane	65.3	330	Tetralin	71.0	132
5α-Pregnane	67.7	288	Decalin	73.8	138
5α-Androstane	68.5	260	Naphthalene	73.2	128
Triolein	42.2	884			
Tripalmitin	43.2	848	β-Carotene	47.4	536
Squalene	52.0	410	Pheophytin a	45.0	866

hypothetical elution volume from a column having a bed volume
of 100 arbitary units [203]. In practice, this figure is
obtained from the ratio of the measured elution volume and
the total column volume, by multiplying by a factor of 100.

$$SEV = 100 \times \left(\frac{V_e}{V_t}\right)$$

Standard Elution Volume (SEV) thus determined is formally
a dimensionless quantity. Results quoted in this manner are
numerically equivalent to values recorded as the percentage
of the total column volume [198,201].

The system based on SEV has already been used to
report the collected data for straight-phase and gel
filtration chromatographic systems [49].

6.2.2 Examination of a Straight-Phase Partition System

The lipophilic dextran derivative [18,201] N1114-50%-LH20
(see experimental section 6.3.1 for details of this
nomenclature) has been investigated using benzene as the
mobile phase. Elution data for model compounds are presented
in Tables 27 and 28.

For non-hydroxylic compounds, separation appeared
to be controlled by molecular sieving processes (Table 27).
Members of a homologous series, for example, the n-alkanes,

Table 28: SEV Data for Hydroxylic Compounds on the Straight-
Phase System (N1114-50%-LH2O/Benzene)

Column 100 cm x 0.9 cm i.d. Flow 6 ml/hr

Compound	SEV	M.W.	Compound	SEV	M.W.
Ceryl alcohol	88.1	382	Phytol	93.5	296
Lignoceryl alcohol	91.3	354	trans,trans-Farnesol	97.7	222
Behenyl alcohol	94.6	326	cis,trans-Farnesol	93.5	222
Arachidyl alcohol	99.0	298	Nerolidol	77.7	222
Stearyl alcohol	103.3	270	Isopetasol	119.6	234
Cetyl alcohol	108.2	242	Drimenol	106.5	222
Myristyl alcohol	113.2	214	Cedrol	85.8	222
Cholesterol	110.2	386	Methyl deoxycholate	156	406
Lanosterol	82.3	426	Methyl hyodeoxycholate	236	406
Dihydrolanosterol	84.5	428	Methyl ursodeoxycholate	134.5	406
Cycloartenol	86.5	426			
Stigmasterol	105.1	412	Methyl chenodeoxycholate	156	406
β-Sitosterol	107.4	414			
Campesterol	109.2	400	2,2,4,4-Tetramethyl-epicholesterol	74.5	442
			3α-Methylcholestanol	88.5	402
Manool	76.8	290	Estrone	360	270
Sclareol	133	308	Chlorophyll a	1140	890
Totarol	236.5	286	Chlorophyll b	1600	904
			Lutein	115	568
			Neoxanthin	256	600

showed a progression in SEV values that was linearly related
to the logarithm of molecular weight (Fig. 42).

The behaviour of samples containing free hydroxyl
groups, which were markedly retarded on the gel, indicated
that the system was operating by straight-phase partition
(Table 28). Samples having other polar functional groups,
for example, ketonic compounds, were not retarded, and the
system was unable to distinguish the latter class of compounds
from hydrocarbons. In examples reported in Tables 27 and 28,
all compounds eluted after SEV 75 had hydroxyl functions,
and their respective degrees of retention were determined
primarily by the number and chemical environment of such
groups.

It was clear that the partition mechanism was highly
selective, with the gel acting exclusively as the basic
component in hydrogen-bonding interactions. The operation
of hydrogen-bonding only in one direction suggested that the
exposed acetal and ether oxygen atoms played a greater part
in these interactions than the relatively hindered hydroxyl
groups of the gel matrix.

Hydroxylic compounds in general displayed two forms
of regularity in behaviour. In the first instance, steric
and electronic effects played a major part in determining
the retardation of the sample. Steric hindrance, as in

Fig. 43: Systematic Behaviour of Various Classes of Compounds Eluted from the
Straight-Phase System (N114-50%-LH20/Benzene)
Influence of Molecular Size on Retention
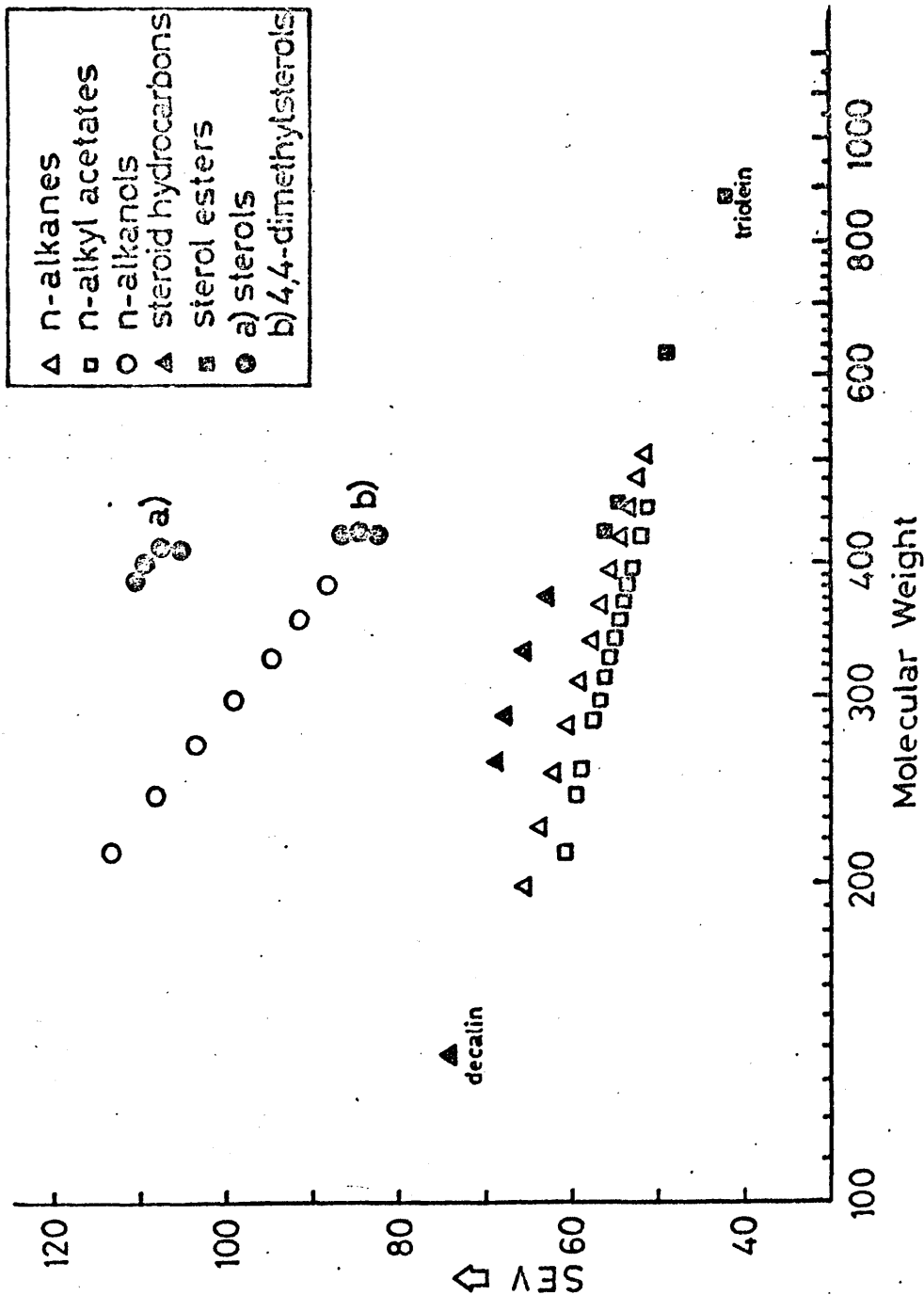
tertiary, or other alcohols surrounded by bulky groups, caused reduced retardation. Acidity of the hydroxyl function increased retardation, and phenols eluted more slowly than aliphatic alcohols, while carboxylic acids did not appear to emerge from the column, even after prolonged elution. It would seem likely that this retardation applied, not only to hydroxyl groups, but possibly also to amino and thiol functions, which would be active in similar hydrogen-bonding interactions. In a further situation, chlorophylls were found to be strongly retained by the gel, in contrast to the corresponding pheophytins, which differed only in the absence of the chelated magnesium ion. It would appear that the cationic complex of the intact chlorophyll was also capable of participating in the hydrogen-bonding type of interaction in a manner similar to a hydroxyl group.

A further regular pattern in behaviour was controlled by difference in molecular size. It appeared that molecular sieving was operative, not only on the non-hydroxylic compounds, but also as a secondary process affecting the elution of hydroxylic samples. The n-alkanol series showed a progressive change in SEV that could be related to carbon number in the same manner as for the n-alkanes (Fig. 43).

TABLE 29

SEV data for N.1114-50%-LH20/Benzene-isopropanol (3:1 v/v)

Column: 100 cm x 0.9 cm   Flow 6 ml/hr   HETP 0.17 mm

Compound	SEV	M.W.	Compound	SEV	M.W.
n-Hexatriacontane	50.3	506	5α-Cholan-24-ol	63.2	346
n-Tetradecane	64.0	198	5α-Cholan-3α,24-diol	59.3	362
Octacosyl acetate	48.1	452	5α-Cholan-3α,12α,	59.9	378
Myristyl acetate	55.2	256	24-triol		
Ceryl alcohol	53.9	382	5α-Cholan-3α,7α,12α,	59.2	394
Myristyl alcohol	62.2	214	24-tetrol		
Lignoceric acid	54.2	368			
Lauric acid	63.0	200	Lithocholic acid	58.6	376
			(3α-OH)		
Cholesteryl acetate	53.3	428	Deoxycholic acid	59.9	392
Cholesteryl	52.2	490	(3α,12α-OH)		
benzoate			Hyodeoxycholic acid	56.0	392
Cholestan-3-one	54.5	386	(3α,6α-OH)		
Cholesterol	60.8	386	Ursodeoxycholic acid	56.0	392
Cholestane	62.8	372	(3α,7β-OH)		
Coprostane	62.6	372	Chenodeoxycholic	59.6	392
5α-Cholane	65.9	330	acid (3α,7α-OH)		
5α-Pregnane	68.2	288	Cholic acid	59.4	408
5α-Androstane	69.3	260	(3α,7α,12α-OH)		
Stigmasteryl acetate	52.1	454	Gibberellin A_1	64.5	348
Stigmasterol	59.2	412	Gibberellin A_3	64.5	346
Lanosterol	58.5	426	Gibberellin A_4	61.6	332
Isopetasin	50.3	316	Gibberellin A_5	59.1	330
Isopetasyl acetate	51.1	276	Gibberellin A_7	61.6	330
Isopetasone	53.1	232	Gibberellin A_9	56.8	316
Isopetasol	58.2	234			
			Phosphatidyl Choline	36.8	-
Chlorophyll a	43	890	Phosphatidic Acid	43.8	-
Pheophytin a	44	866	Tripalmitin	39.8	806
β-Carotene	47	536	Dipalmitin	45.2	568
			Monopalmitin	56.5	330
			Galactosyl Diglyceride	41	-

Fig. 44: Influence of Alcohols in the Eluting Solvent on the Retention of Hydroxylic and Non-Hydroxylic Compounds
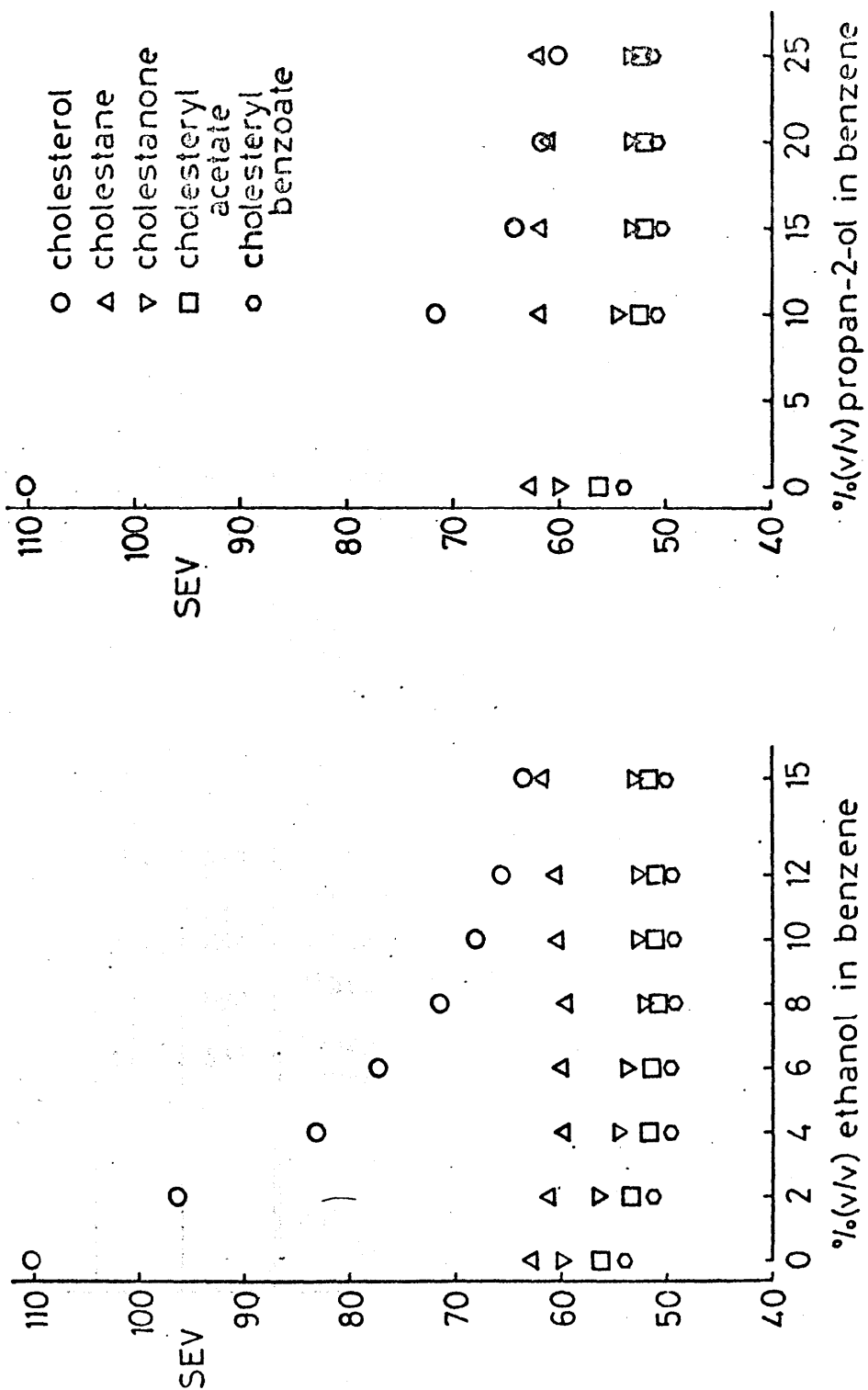
6.2.3   Examination of Some Gel Filtration Systems

On addition of an alcohol to the eluting solvent

for the gel system already described (N1114-50%-LH20/benzene),

hydroxylic compounds were selectively displaced from the gel,

presumably through competitive hydrogen bonding. Other

compounds were not affected to any great extent (Fig. 44).

At a level of 25% isopropanol in benzene, there was

no observable polarity effect for cholesterol, nor for any

of the more polar compounds studied. Higher levels of

alcohol gave rise to reversed-phase partition.

A chromatographic system, N1114-50%-LH20 with a

mobile phase containing the mixture benzene-isopropanol

(3:1 v/v), was tested with model compounds (Table 29).

Non-hydroxylic compounds behaved substantially as in the

system containing pure benzene (Table 27), but polar samples

no longer showed distinctive properties (Fig. 45). Free

carboxylic acids were similar in behaviour to the corresponding

alcohols. Molecular sieving was the major factor that

determined the elution of samples in this solvent system.

No compound was found to be retained beyond SEV 80.

This system had properties most comparable to the

ideal for gel filtration[*] of all those examined. The most

---

[*] In this thesis, the term 'molecular sieving' has been
     used to refer to the physical process, and 'gel
     filtration' to the chromatographic method, of separation
     of samples according to molecular size.

Fig. 45: Relationship Between Molecular Weight and SEV for the Gel Filtration System (N1114-50%-LH20/Benzene-Isopropanol 3:1 v/v)
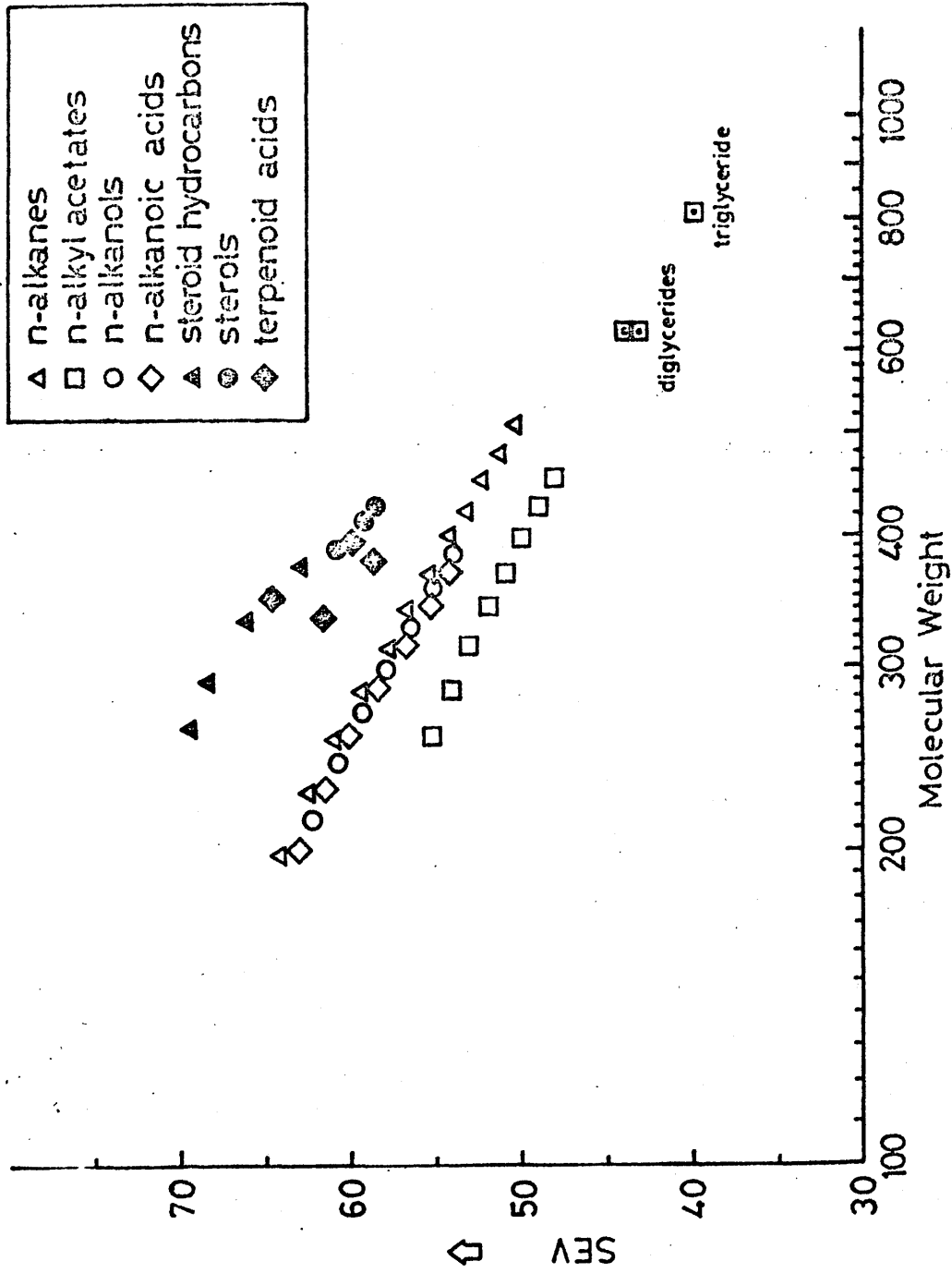
Table 30: SEV Data for the Gel Filtration System
(LH-20/Ethanol-Benzene 9:1 v/v)

Column   52 cm x 1.8 cm i.d.

Compound	SEV	M.W.	Compound	SEV	M.W.
Triolein	52.0	884	Cholesterol	70.0	386
Cholesteryl palmitate	55.5	624	β-Carotene	70.0	536
n-Pentatriacontane	58.2	492	Chlorophyll a	73.5	890
n-Tritriacontane	59.5	464	Cholesteryl benzoate	74.2	490
n-Hentriacontane	61.0	436	Petasin	76.5	316
n-Nonacosane	62.5	408	cis-Decalin	77.5	138
Cholesteryl butyrate	64.8	456	trans-Decalin	79.0	138
α-Amyrin acetate	67.2	468	S-Isopetasin	80.0	334
Cholesteryl acetate	68.0	428	Isopetasyl acetate	81.5	276
Lanosterol	68.5	426	Isopetasol	86.0	234
Stigmasterol	69.2	412	Tetralin	87.5	132
β-Sitosterol	69.2	414	Naphthalene	97.0	128
Chlorophyll b	70.0	904			

apparent abnormality was the elution of carbonyl-containing compounds, esters and ketones, earlier than might be expected on the basis of molecular weight. A similar effect had already been noted by Eneroth and Nyström for ketonic steroids on other lipophilic gels[204]. In this system, a carbonyl group decreased retention by an amount equivalent to the effect of about eight methylene units. Molecular shape also influenced elution, and acyclic molecules were less retarded than cyclised molecules of the same weight.

A further system was briefly investigated, that gave some regularity in behaviour approximating to gel filtration. This system employed commercial Sephadex LH-20, using the solvent mixture ethanol-benzene (9:1 v/v). Results have been recorded in Table 30. The system was found to be imperfect for gel filtration, because of the selective retention of samples according to aromaticity. This effect was superimposed on the order of elution of compounds as determined by molecular sieving alone.

These selective effects have been applied to the separation of polycyclic aromatic hydrocarbons[205], but are less useful for general chromatographic purposes.

Table 31: SEV Data for the Reversed-Phase System
(N1518-71%-LH20/Methanol-Heptane 9:1 v/v)
Column 28 cm x 1 cm i.d. Flow 8 ml/hr

Compound	SEV	Compound	SEV
Cholesterol	250	n-Decanol	58
Cholestanol	262	n-Dodecanol	65.5
Cholestanone	354	n-Tetradecanol	76
Cholesteryl acetate	541	n-Hexadecanol	92
Cholest-4-en-3-one	240·	n-Octadecanol	112
Cholest-5-en-3-one	353	n-Eicosanol	139
Cholest-4-en-3,6-dione	136	n-Docosanol	175
Campesterol	270	n-Tetracosanol	226
β-Sitosterol	294	n-Hexacosanol	296
Stigmasterol	256		
Ergosterol	232	n-Dodecanoic Acid	75
Lanosterol	242	n-Tetracosanoic Acid	87
24,25-Dihydrolanosterol	285	n-Hexacosanoic Acid	106
Pregnenolone	54	n-Octacosanoic Acid	147
Progesterone	54	n-Docosanoic Acid	227
Isopetasin	58.6	n-Tetracosanoic Acid	275
Isopetasyl acetate	49.8	n-Hexacosanoic Acid	382
Bakkenolide-A	70.7		
Drimenol	79.2		
Convertifolin	59.5		
Nerolidol	57.8		
Farnesol	59.6		
Phytol	86		

6.2.4   Examination of Reversed-Phase Partition Systems

The main part of this study concerned the Sephadex derivative, N1518-71%-LH20. Using the Nedox products (section 6.3.1), this was the maximum degree of substitution that could be obtained. It was considered that the high content of saturated hydrocarbon in the gel would favour reversed-phase partition.

Methanol was used as the mobile phase, with heptane added as a polarity moderator. Although methanol alone gave perfect reversed-phase partition properties, the insolubility of many samples in pure methanol, for example the sterols, required the addition of a less polar component to the solvent.

A systematic collection of data was made for the combination [N1518-71%-LH20/methanol-heptane (9:1 v/v)], with results as presented in Table 31. These served to illustrate the emphasis placed on the hydrocarbon region of the molecule in determining retention under reversed-phase conditions [201]. For the n-alkanols, separation was comparable to that obtained by GLC. This system thus offers a simple method for the separation of phytosterols, such as campesterol and β-sitosterol [201], where other chromatographic methods have been found less satisfactory. The effect of double bonds was also more pronounced, but in the opposite sense, i.e. decreasing,

Fig. 46: Effect of Degree of Alkylation of the Gel on the Retention of Polar and Non-Polar Compounds by the Reversed-Phase Systems

a) N1518-71%-LH2O/Methanol
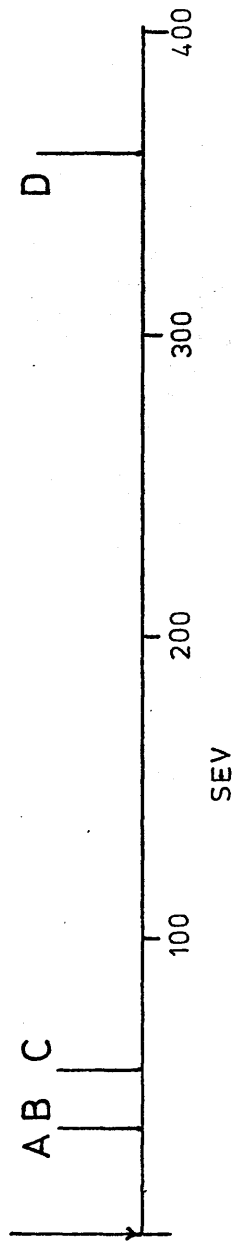
b) N1114-50%-LH2O/Methanol
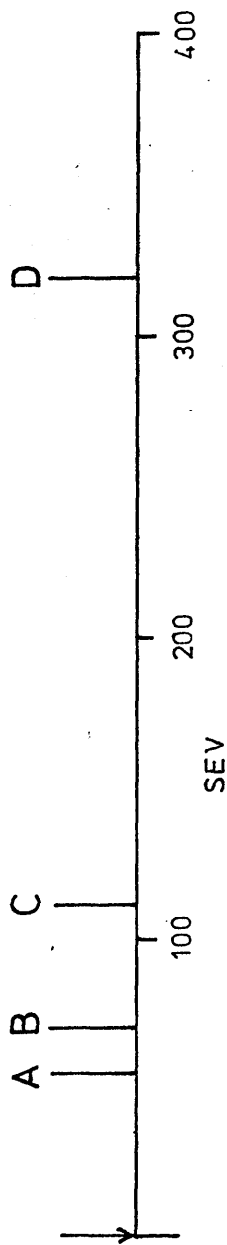
A — 5β-Cholan-3α,7α,12α,24-tetrol
B — 5β-Cholan-3α,12α,24-triol
C — 5β-Cholan-3α,24-diol
D — 5β-Cholan-24-ol

and not increasing, retention of the less saturated sample.
The additional double bond in the side chain of stigmasterol
cancelled the effect of the 24-ethyl group, and its retention
was closer to that of cholesterol than to β-sitosterol.
Reversed-phase chromatography on alkylated Sephadex
derivatives thus offers, in certain cases, a practical
alternative to the use of silver nitrate-impregnated silicagel
for separation on the basis of unsaturation. Ellingboe et al
have demonstrated its application to the separation of
methyl esters of unsaturated fatty acids[201].

Polar functions, by their presence in a sample
molecule, caused a large reduction in retention. However,
different classes of polar function were less distinctive
in their behaviour than was the case for straight-phase
partition. The presence of a carboxylic acid, for example,
was comparable, in the magnitude of its effect, to a hydroxyl
group.

The effect of the degree of alkyl substitution of
the gel was investigated briefly, and the system studied
above was compared with the less substituted gel, N1114-50%-LH20,
using the same solvent mixture in each case (Fig. 46). With
less polar samples, retention was increased with increasing
substitution. However, more polar compounds were poorly
separated in the system based on N1518-71%-LH20. When the

Fig. 47: Elution Profile of Cholesteryl Acetate from the Straight-Phase System
(N1114-50%-LH20/Benzene):

Effects of Higher Loading

Column 100cm x 0.9cm
N1114-50% - LH20
100% Benzene 6ml/hr.

0.5mg cholesteryl acetate

SEV Units →

Effects of Higher Loading and Examination of the
Degree of 'Tailing'

x100

10mg cholesteryl acetate
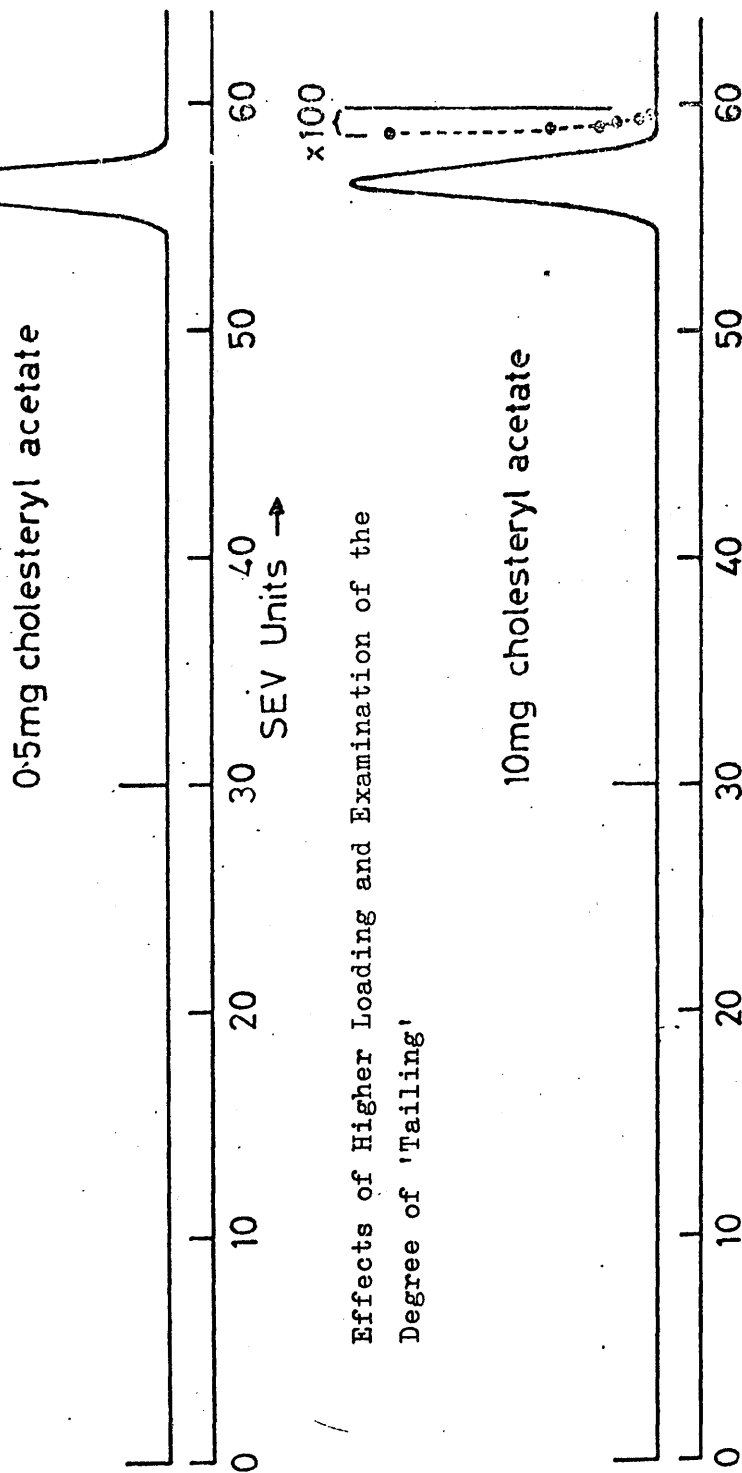
less substituted derivative, N1114-50%-LH20, was employed,
retention of these polar samples was increased, and separation
improved. These results would suggest that a lower degree of
substitution was more appropriate for the investigation of
polar samples.

A derivative of Sephadex, substituted with phenyl
groups, was also briefly investigated. Reversed-phase effects
were minimal for aliphatic compounds, when the system
PHE-50%-LH20/methanol was examined. There was, however, some
indication of selective reversed-phase retention for aromatic
molecules (in the sample tested, pheophytins). Insufficient
time prevented a systematic survey of this medium.

6.2.5   Performance of Gel Chromatographic Systems

The outstanding feature of the Sephadex derivatives
was the high efficiency obtained from columns employing these
media. Theoretical plate heights have been calculated from
the elution of cholesteryl acetate from the system
N1114-50%-LH20/benzene. Peaks appeared to possess a Gaussian
form, comparatively unaffected by higher loading (Fig. 47).
Other systems and samples gave comparable results, but were
not examined in the same detail as the above example.

As expected from the theoretical treatment of
column efficiency, best results were obtained when the

particle size distribution of the gel beads was minimised [206].
The fractionation of Sephadex G-25 (Superfine Grade) was
completed using the method of Hamilton [207] (section 6.3.1).
When the hydroxyalkoxypropyl derivative was prepared from
a sample of Sephadex G-25 having a narrow range of particle
diameters (24-32μ for the dry beads), theoretical plate
heights (HETP) were obtained in the range 0.07 - 0.10 mm.
The column (100 cm) gave an overall efficiency of 10,000 -
14,000 theoretical plates, with an analysis time of 15 hours
for a mixture containing cholesteryl acetate and cholesterol.

In comparison, low resolution columns, employing
derivatives prepared from unsieved Sephadex LH-20, gave HETP
of 0.5 mm, using the same sample and solvent system as above.

Re-use of Columns

In gel chromatography, the elution of a sample is
based on partition processes, and not adsorption: there
should thus be little tendency for the irreversible retention
of a sample in the gel phase. Samples that were comparatively
immobile in a certain solvent system could usually be
displaced from the column by adjustment of the polarity of
the mobile phase. Change of solvent from benzene, to benzene
containing 25% (v/v) of isopropanol, caused samples that
migrated slowly in the straight-phase system to become
concentrated in the rapidly moving interface between the

two solvents. The result of this operation was to purge the
column of all remaining solutes, leaving it uncontaminated
for further separations. This method was less easily applied
to reversed-phase columns, because the addition of non-polar
solvent to the methanol caused excessive expansion of the gel.
It was therefore necessary to limit the addition to about
25% of benzene, and to use a larger volume of this solvent
mixture to effect the decontamination.

The efficiency of these 'cleaning' operations could
be tested by the use of radioactive samples. Recovery of
radioactivity was found to be quantitative, and 'bleed' of
tracer diminished rapidly after the main sample had passed.
When $(24\text{-}^{14}C)$ cholic acid was tested (as the free acid) in
the system N1114-50%-LH20/benzene-isopropanol (3:1 v/v),
the sample (30 μCi; 200 μg) was recovered in the zone expected
for this compound. Radioactivity in the effluent was measured,
and had diminished to 0.1% of the total, 3 SEV units after the
main peak had passed. After a further 20 SEV units of solvent
had passed, the level of radioactivity had fallen to 0.001%.
One column volume of clean solvent was sufficient to remove
the last traces of radioactive bleed (Fig. 48).

Experience with columns in routine use with
radioactive samples has shown that straight-phase columns
were readily restored to a condition where background

radioactivity was negligible. With reversed-phase columns, the gradual accumulation of a permanent level of background radioactivity was observed in a column regularly employed for fractionation of labelled plant extracts. This level, 100-200 dpm per column volume, was not serious in use, but did imply that the method for purging the reversed-phase columns was less effective than that used for the straight-phase system.

The repeated re-use of these columns was a major factor contributing to the reproducibility of results, as there could be no variation introduced by slight differences in column preparation. This was a particular advantage for analytical applications of gel chromatography.

One source of error encountered in routine use derived from the degree of compression of the gel bed. The SEV system of reporting data was adopted for its simplicity in operation; unlike the more complex K_{av} system [194] it did not allow for such variations. When the gel became compressed, this could affect the mobile and stationary phases to a different extent. The ratio of the volumes of the two phases could thus be altered, and this would influence the elution of samples.

In the straight-phase system (N1114-50%-LH20/benzene), when the gel became compressed, a slight increase in SEV

Fig. 48: Elution of 30 μCi (24-^{14}C)
Cholic Acid from a Column
Employing the Gel Filtration
System (Nl114-50%-LH20/
Benzene-Isopropanol 3:1 v/v)

99% of the total radioactivity
was recovered in the shaded
zone. The logarithmic scale
(for radioactivity) permits
simultaneous comparison of
high and low levels of label
in the effluent.
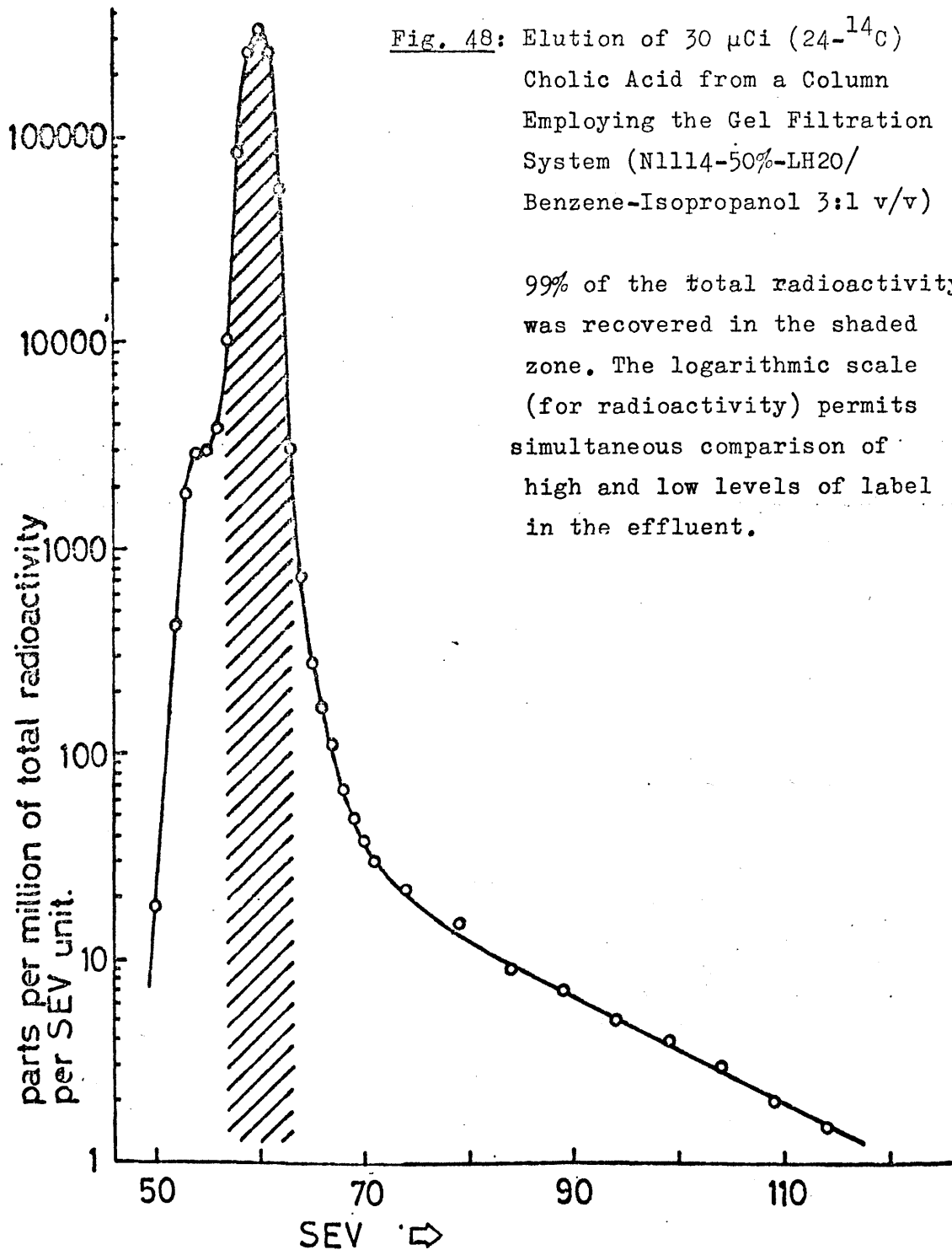
was observed for cholesterol and other polar samples. This could be interpreted as the result of selective compression of the mobile phase, arising from the distortion of the gel beads,which would reduce the interstitial space containing the mobile phase.

Variations of this kind could be avoided by restoring the column to its correct volume. This could be accomplished by reversing the flow of solvent, or in some cases, by the use of a solvent that caused slight expansion of the gel. When the original solvent was replaced, it was normally found that the gel returned to its true volume.

It was frequently found convenient to use the results of an analytical examination of a sample to determine the course of a subsequent preparative separation. Although reproducibility between different columns was never as good as obtained on a single column, the slight variations between different preparations of a gel derivative were systematic in nature. With experience of the characteristics of each column, it was possible to compensate for these variations in the calculation of the expected elution volume of each component in the sample.

The Sephadex derivatives are unusual among chromatographic media, possessing both the properties of high resolution, for analytical applications, and also a high sample capacity, as required for preparative separations.

Fig. 49: Hypothetical Two-Dimensional Distribution of Components of the P. hybridus Isopropanol Extract

Second Column: Straight-Phase (N1114–50%-LH20/Benzene)

First Column: Reversed-Phase (N1114–50%-LH20/ Methanol-Heptane 9:1 v/v)

Column Purge: Methanol-Benzene 3:1 v/v

	A (30, 51)	B1 (72)	2 (100)	3 (115)	4 (160)	C Purge (benzene-isopropanol)
I (30 SEV – 105 SEV)	Tannins, Quinones	Petasin Esters, Bakkenolide C_{15} Ketones	Nerolidol, Farnesol, Sesquiterpenoid Alcohols		Petasol, Violoxanthin	Galactosyl Glycerides, Phospholipids, Free Fatty Acid (to C_{14}), Sterol Glycosides, Polyfunctional Acids, Gibberellins
II (105 SEV – 220 SEV)		Humulene, Bisabolene, Eremophilene	Leaf Wax n-alcohols		Lutein	Free Fatty Acids (to C_{24}), Simple Triterpenoid Acids, Chlorophyll b
III (220 SEV – 400 SEV)	2,3-Oxidosqualene, Squalene			Sterols		Chlorophyll a
IV (400 SEV)	Pheophytin, Triglyceride, Sterol Esters, β-Carotene, Wax Alkanes					

6.2.6   Applications of Gel Chromatography

Examples of gel chromatography in application have
been provided in chapters 2 and 3 of this thesis.

In practice, two forms of application could be
distinguished: group separation, using low-resolution
techniques, and the selective isolation of individual
compounds, for which high-resolution columns were more
applicable. The variety of separation mechanisms available
made the Sephadex derivatives particularly applicable to
low-resolution group separation techniques. It has already
been noted that gel filtration could be used as a preliminary
purification for the removal of high molecular weight lipids,
and in the case of plant extracts, of chlorophylls and the
polymeric tannins (section 2.3.4).

The contrast between straight-phase partition,
where polar functional groups determined the retention of
samples, and reversed phase methods, in which differences
in the hydrocarbon regions of molecules became prominent,
permitted highly effective separations to be undertaken,
using both methods in combination. This has been illustrated
in Fig. 49 in the form of a hypothetical two-dimensional
distribution of the constituents of P. hybridus leaves.
(Using column chromatography, it was necessary to carry out
the separation in each system independently.) This scheme
provided the basis for the group separation of the

sesquiterpenoids (Fig. 3; facing p.18). The distribution of
compounds within the scheme was found to show many regularities,
which were less apparent when the separation in either system
was considered in isolation. It was frequently possible to
deduce the probable location of a particular compound or
class, and so to devise a separation for its isolation.
Conversely, the location of samples within the scheme
permitted the assignment of simple structural correlations
to samples of unknown constitution.

The separation of sterols provides an example of
this scheme in operation. In straight-phase chromatography,
retention would be controlled by the hydroxyl group: separation
into three classes would result, corresponding to normal
3β-hydroxysterol, 4α-methylsterol, and 4,4-dimethylsterol.
A subsequent reversed phase separation would then isolate
constituents according to alkylation of the side-chain, and
also the degree and location of unsaturation in the molecule.

The sequence of the two operations, reversed-phase
and straight phase, should be determined by the nature of
the sample. For the sesquiterpenoids, reversed phase methods
were first applied; in the above example, it was convenient
to employ the straight-phase method before the reversed-phase
separation.

6.3   EXPERIMENTAL PROCEDURES

6.3.1   Preparation of Gels

Dextran derivatives for use in these experiments were prepared in this laboratory, using Sephadex G-25 and LH-20 (Pharmacia AB, Uppsala) as starting materials. The methods of Ellingboe, Nyström and Sjövall were used in the preparation of these derivatives [18,201].

It would be expected from the theoretical discussions of Giddings and Mallik that higher column efficiency would be obtained using a packing having a narrow range of particle diameters [206]. In earlier work, this was achieved by sieving Sephadex LH-20 before the alkylation reaction [49]. Sjövall has reported particularly good results using a fraction of Sephadex [198] prepared by the continuous-flow differential sedimentation method of Hamilton [207]. This method permitted the isolation of finer particles than was possible by mechanical sieving. The use of such fine particles would result in more rapid equilibration of solutes in the gel phase, thereby allowing faster flow rates without loss of efficiency [206]. A practical limitation of this approach was the increased resistance to flow when excessively fine particles were employed.

The fractionation of Sephadex G-25 (Superfine Grade) was completed as described below. Sephadex G-25 (100 g) was equilibrated in water, containing 1% sodium azide as

Fig. 50: Apparatus for Fractionation of Sephadex Gel
Beads by the Continuous Flow Differential
Sedimentation Method of Hamilton[207]
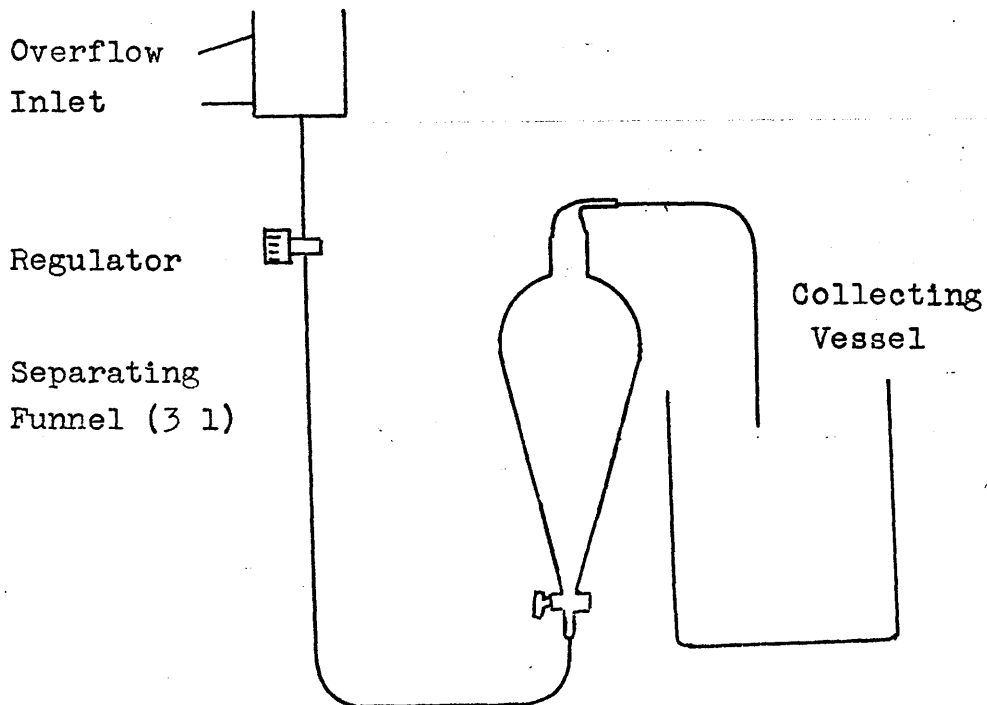
Table 32: Particle Size Distributions Obtained in Fractions
Derived from Sephadex G-25 (Superfine Grade)

Flow Rate ml/min	Particle Size Range (dry beads)	Recovery Dry Weight
0 - 40	Fines (discarded)	-
80	$8 - 16 \mu$	4 g
130	$17 - 23 \mu$	15 g
220	$24 - 32 \mu$	35 g
400	$33 - 45 \mu$	25 g

bacteriostat. The swollen gel was transferred to a conical

separating funnel with a narrow taper (capacity 3 1), as

described by Hamilton[207]. Water entered the lower end of the

separating funnel from a reservoir, adjusted to maintain a

constant hydrostatic pressure (Fig. 50). Flow was controlled

by a rotary valve (Quickfit Rotaflo), coarse adjustments

being made by altering the level of the reservoir. The flow

was first adjusted so that the rate of sedimentation of the

bulk of the gel beads just exceeded the upward movement of

the water at the widest part of the separating funnel. Under

these conditions, most of the gel was retained in the funnel,

and bead fragments, colloidal material and very fine particles

were removed in the effluent. After 24 hours, the opalescence

associated with this unwanted material had almost disappeared.

Flow was then increased in steps, and sufficient time was

allowed for the collection of 50-100 litres of effluent at

each stage. The sediments obtained from each collection

contained a different size of particles (Table 32).

After sedimentation, the supernatants were

decanted, washed with de-ionised water, washed again with

isopropanol, and dried as far as possible by suction in a

sintered glass funnel. The dried beads were examined by

microscope, and the particle size range was estimated using

a micron scale graticule. (Our thanks extend to Mr. T. Baird

for the loan of the graticule.) These preparations were far
superior to those obtained by sieving, where the tendency for
the dry beads to adhere to each other caused a variety of
fine particles to be retained in coarser fractions.

Preparation of Hydroxypropyl Sephadex

The fractionated Sephadex obtained above was
converted to the β-hydroxypropyl ether, using the method
described by Ellingboe et al [201]. After drying overnight
in vacuo at 80°C, the beads (35 g) were swollen in an excess
of 1 M aqueous sodium hydroxide. After 2-3 hours, the
supernatant was decanted, and 1,2-epoxypropane (700 ml) was
added. The mixture was stirred under reflux for 24 hours;
complete reaction was indicated when the initially pasty
Sephadex became evenly dispersed in the reaction mixture.
Reagents were then removed, and the product was washed with
distilled water until the effluent was neutral in pH. The gel
beads were dried by successive washing in isopropanol, acetone
and light petroleum. After drying in vacuo at 80°, the weight
of hydroxypropyl Sephadex recovered was found to be 72 g,
which corresponded to the theoretical maximum degree of
substitution by hydroxypropyl groups. This product had the
same properties as a commercial sample of Sephadex LH-20.

These methods were used in several separate
preparations of hydroxypropyl Sephadex.

Preparation of Hydroxyalkoxypropyl Sephadex
By Reaction with Nedox 1114

The LH-20-type derivative was converted to
hydroxyalkoxypropyl Sephadex by reaction with Nedox 1114[18].
This reagent (supplied by courtesy of the Ashland Chemical
Company, Columbus, Ohio) contained straight-chain terminal
epoxyalkanes, having 11 - 14 carbon atoms per molecule.

LH-20-type (hydroxypropyl) Sephadex (50 g) was
equilibrated with methylene chloride (170 ml) that had been
dried before use by filtration through basic alumina (Woelm;
activity grade I). The mixture was stirred gently using a
link type stirrer (magnetic stirrer bars tended to destroy
the bead structure by abrasion against the walls of the
reaction vessel[201]). Boron trifluoride etherate (20 ml) was
added. After continued stirring for 10 minutes, a mixture
containing Nedox 1114 (125 ml) and methylene chloride (125 ml)
was added over a period of 30 minutes. Stirring was continued
for a further 30 minutes. The product was then removed, and
washed successively with chloroform, methanol and acetone.
The gel was partly dried by suction in a sintered funnel,
and the last traces of solvent were removed in vacuo overnight
at room temperature. The dried product, unlike the starting
material, was hydrophobic and waxy. The bead structure was
intact. The weight of the dried gel was double that of the
starting material, i.e. the substituent hydroxyalkyl group

comprised 50% of the total weight of the dry gel. The
nomenclature used to describe this product, N1114-50%-LH20,
indicated the nature of the substituent (N1114), the degree
of substitution (50% by weight), and the nature of the
starting material (LH-20-type Sephadex). Where fractionated
Sephadex was employed as the starting material, the size
distribution of the fraction was frequently quoted also;
the figures given referred to the diameter of the dry beads
of the original Sephadex G-25.

By Reaction with Nedox 1518

Nedox 1518 consisted of 1,2-epoxyalkane, like Nedox
1114, but with longer carbon chains (C_{15} – C_{18}). Using the
procedures described above, Nedox 1518 (25 ml) reacted with
LH-20-type Sephadex (20 g) with boron trifluoride etherate
(7 ml) as catalyst. A product (30 g) was thus obtained,
having 33% by weight of hydroxyalkyl substituent.

This first product (N1518-33%-LH20; 22 g) was treated
again with Nedox 1518 (40 ml), in the presence of boron
trifluoride etherate (4.5 ml), and a second product (50 g)
was obtained, calculated as having 71% by weight hydroxyalkyl
substitution. This product, described as N1518-71%-LH20, was
used in the investigation of reversed-phase partition.

Preparation of Phenylhydroxyethoxypropyl Sephadex By

Reaction With Styrene Oxide

Sephadex LH-20 (5 g) was stirred with methylene

chloride (30 ml) and boron trifluoride etherate (1.5 ml)

was added. Styrene oxide was then allowed to enter slowly;

a large excess was used (35 ml; see Ellingboe et al[201]) for

proportions of Nedox used). After stirring for a further

2 hours the product was recovered and dried; the dry weight

(10 g) showed a degree of substitution (50% by weight) close

to the theoretical maximum for the phenylhydroxyethyl

group (52%). The product was described as PHE-50%-LH20.

6.3.2   Preparation of Columns

Three basically different types of column were

used in this investigation. Those employed for low-resolution

work were of the standard pattern used in normal methods of

column chromatography; these need no further discussion.

High-resolution glass columns were based on the

pattern in use at Karolinska. These consisted of three parts,

at the top, a solvent reservoir, connected to the middle part,

the chromatographic tube, and at the bottom, an end fitting.

The reservoir and chromatographic tube were constructed as

an integral unit; the assembly was thus self-contained, and

could be used without reliance on separate solvent sources.

Fig. 51: Features of Typical Column Design

Quickfit screw joint adaptor

Quickfit B19 socket

Mariotte Tube

Solvent Reservoir
Capacity 50 - 100 ml

End Fitting and Bed Support

glass column 12.5mm od
teflon nut
SBT O-ring
teflon fabric bed support
teflon collar

teflon connector Gienco No.3020 1/2" to 'B'
SBT O-ring
teflon nut
0.75mm id teflon tubing

Operating
Pressure
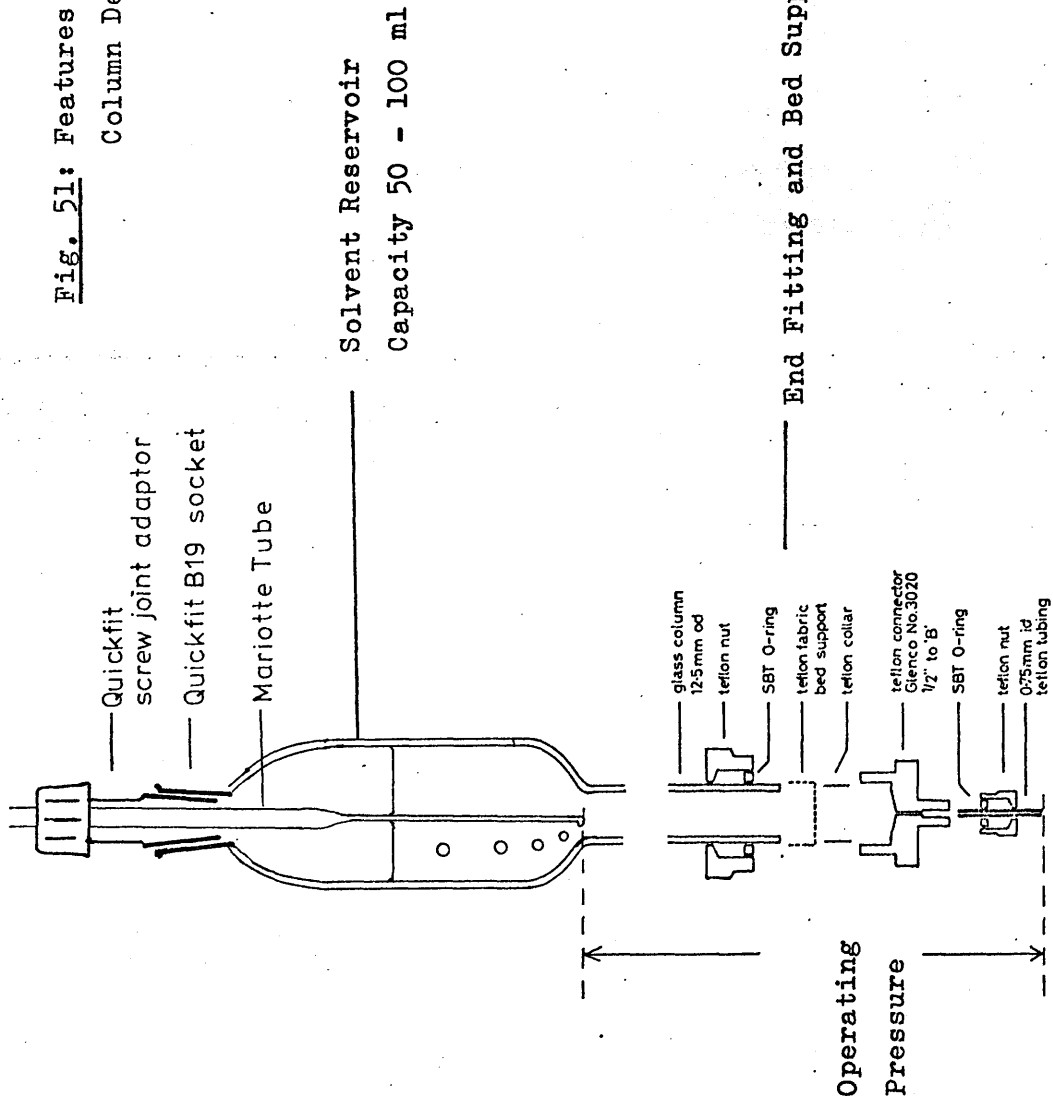
The gel was contained in the main part of the column, which consisted of a straight length of glass tubing, 1 cm bore for preparative columns, and 3 mm for analytical applications. An end fitting was employed that incorporated a standard Teflon tubing connector (Glenco No.3020; Glass Engineering Inc., Houston); one end admitted the chromatographic tube, and the other was connected to small bore (0.25 mm) Teflon tubing that linked the column to detector or fraction collector. The gel bed was supported at the lower end by a Teflon fabric disc (0659 SR 25/45 from Pharmacia AB). This could be formed into a cup by pressure, and cold flow of the Teflon, and was then inserted between the connector and the chromatographic tube. A good seal was ensured by selecting close-fitting tubing stock for construction of the columns. Otherwise it was necessary to use a Teflon collar (cut from wide bore tubing) to close the gap.

Capillary columns used narrow bore (1.5 mm) Teflon tubing (Chromatronix Inc.) to contain the gel bed. Construction was based on the methods of Nyström and Sjövall[208]. Solvent was dispensed under gas pressure, from a separate reservoir constructed from a 250 ml centrifuge bottle. A Teflon T-fitting (Chromatronix Inc.) was used to permit sample introduction by injection through a rubber septum into the solvent stream. This system was only briefly assessed; the methods appeared

to be especially suitable for regular and frequent use of
a particular column. When use was less frequent, there was
a tendency for columns to dry out, presumably because the
Teflon was sufficiently porous to permit diffusion of solvent
through the walls.

Packing of Columns

Before use, the columns were calibrated volumetrically,
to permit the estimation of bed volume in chromatographic
operations. The inside surfaces were treated with a solution
of dichlorodimethylsilane (5% in toluene).

The gel was preswollen in the intended solvent system
(ca. 10 ml per gram of dry gel) before being packed in the
column. A brief exposure to ultrasonic vibration speeded the
equilibration. The slurry was added to the column in a single
operation, and was allowed to settle under a gentle flow of
solvent. If the column was not perfectly vertical, uneven
settling of the packing could cause loss of efficiency by
irregular migration of zones. Rotation of the column about
its axis, at intervals during sedimentation of the gel, about
$\frac{1}{4} - \frac{1}{2}$ turn at a time, cancelled any unevenness, and improved
the efficiency of the packing.

The upper surface of the settled gel was protected
from mechanical disturbance by a filter paper. When the bed
volume changed, for examples as a result of solvent changes,

the filter paper moved freely with alterations in the level
of the bed surface.

Solvent changes were, in general, found to be quite
permissible. When very large changes in bed volume resulted,
it was advisable to undertake the change in steps, and to
allow equilibration at each stage. Two to three bed volumes
of the new solvent were usually sufficient for equilibration.

6.3.3   Sample Handling and Detection

Samples were applied to the top of the columns
without mixing by a standard procedure. Excess solvent was
allowed to drain into the bed, and the sample was applied to
the moist filter paper protecting the bed surface. The sample
was allowed to penetrate the bed surface, and was washed below
the surface by dropwise addition of solvent.

Samples were normally applied in the solvent to be
used for elution, in a volume not exceeding 1% of the total
column volume. It was sometimes necessary to use a co-solvent,
for example, to dissolve polar samples in benzene. With the
straight-phase system, the use of an alcohol as co-solvent
for sample introduction interfered with the hydrogen bonding
interaction and caused zone distortion and loss of column
efficiency. Dioxan or chloroform generally gave acceptable
results in this situation.

Earlier work had involved the use of a pump
(BTL Chromapump) for delivery of solvent at a constant rate [49].
Later, it was found more convenient to use a column design
incorporating a self-contained reservoir for the eluant.
Constant flow was obtained by the use of the Mariotte system
for maintaining a fixed hydrostatic pressure (Fig. 51). Flow
rate was measure by the timed collection of samples in the
course of operation. In preparative applications, where the
SEV data for the sample was known (by previous analytical
determination), it was found convenient to dispense the precise
volume of solvent to cause the zone to migrate to the end of
the column, but not to emerge. The zone could then be eluted
with a further small volume of solvent. This type of operation
permitted the use of the columns on the laboratory bench without
requiring a fraction collector.

Detection of Samples in Column Effluent

Column effluents that were collected as fractions
were examined by GLC or TLC of the fractions. Radioactivity
could be monitored rapidly, by spotting samples from each
fraction onto a TLC plate, and, without development of the
chromatoplate, scanning for radioactivity on the Panax
chromatogram scanner.

Elution profiles (e.g. Fig. 47) could be constructed
by quantitative estimation of the fractions.

Latterly, the acquisition of the Haahti-Sjövall circulating-chain flame ionisation detector [198,209] has considerably simplified these investigations. This apparatus permitted the continuous monitoring of column effluents, and results were displayed graphically as a trace made by a potentiometric recorder.

In this system, the column effluent was allowed to wet a circulating chain. The solvent evaporated as the chain passed through a heated zone, and involatile solutes remained behind. Solvents were specially redistilled to remove any undesirable involatile residues. The chain entered the detector cell through the jet at which the hydrogen flame burned. Organic samples, introduced on the chain, became ionised in the flame, and their presence was indicated by an increase in conductivity of the detector cell. This signal was then amplified and recorded on the chart paper (Kent Recorder; 1 mV full scale deflection).

The chains used by the detector became corroded, and eventually disintegrated. Continuous use was limited to 6 - 8 weeks. The short supply of these chains has been circumvented by the use of wire loops, which were found to act as an effective substitute for the chain. Single or double stranded wires were unsuitable, because of insufficient solvent capacity. The best results have been obtained using
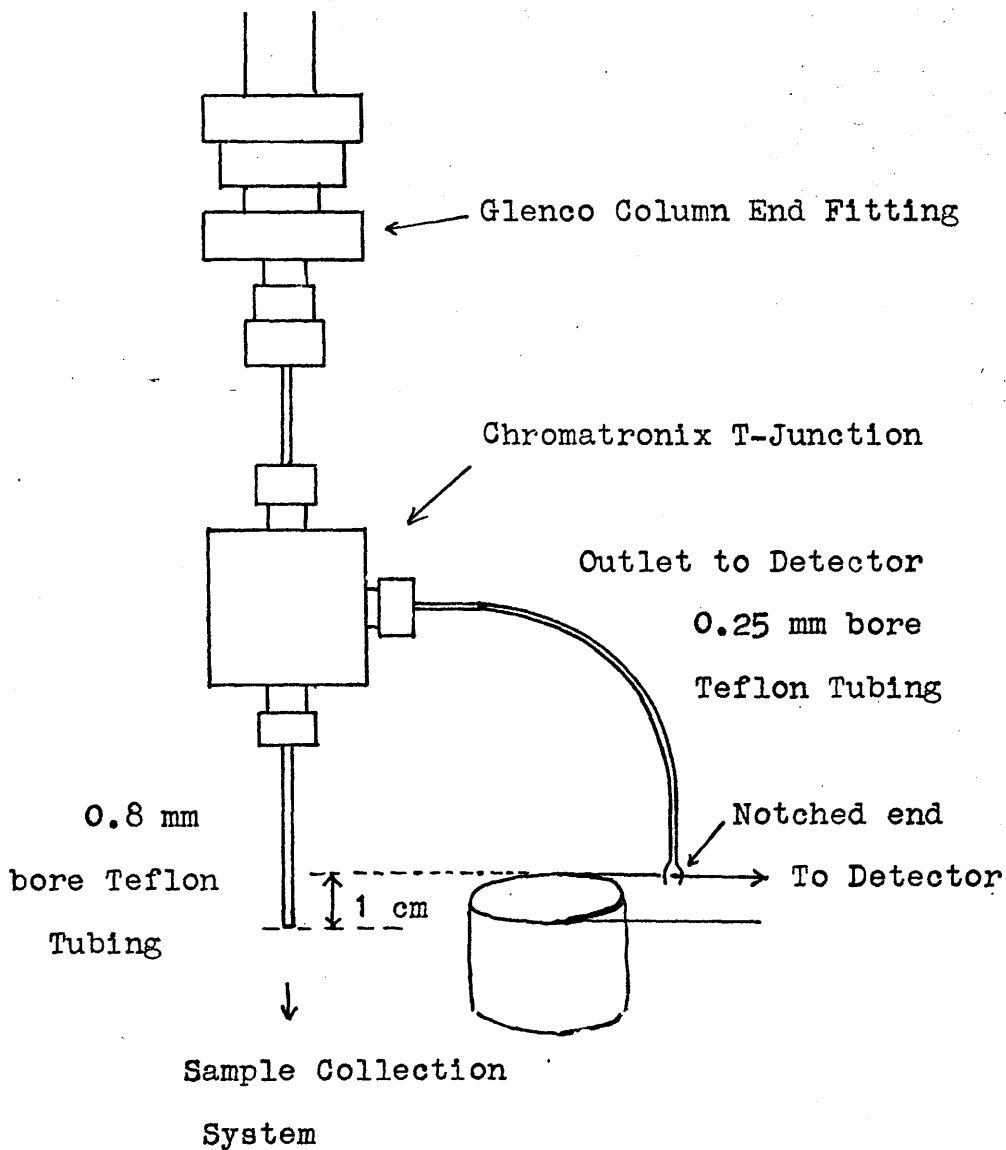
212.

a composite construction of four strands altogether.
'Vachrome' wire (0.15 mm diameter), a chrome-vanadium alloy
intended for use in electrical heating elements, was twisted
into a two-filament strand (ca. 8 turns/cm). Lengths (2 m) of
this strand were then doubled, and twisted together to form
the four-filament composite wire. The use of one length,
doubled over on itself, resulted in the formation of an eye
at one end, which was convenient for joining the wire into
a continuous loop in the detector. Careful closure of the
joint reduced the noise level in operation to that experienced
using chains in the conventional manner.

Best results were obtained when the two twisting
operations were performed in the same direction, although
the composite wire so constructed was less even in appearance.
A laboratory stirrer motor was adapted to carry out the
twisting of the strands.

Both chains and wires required rigorous cleaning
in the ultrasonic bath before use; after cleaning, handling
was avoided, and manipulations were performed using forceps.
The advantage of the wires was the cheapness and convenience
of preparation; this permitted frequent discarding of
the contaminated wires whenever noise levels became excessive.

Fig. 52: Stream Splitting System for Preparative Liquid
Chromatography in Conjunction with the
Circulating-Chain Flame Ionisation Detector

Glenco Column End Fitting

Chromatronix T-Junction

Outlet to Detector
0.25 mm bore
Teflon Tubing

0.8 mm
bore Teflon
Tubing

1 cm

Notched end

To Detector

Sample Collection
System

Use of the Detector in Preparative Applications

The combustion of the sample in the detector cell prevented its recovery; in preparative applications, it was necessary to divert only a small part of the effluent stream to the detector.

When the total solvent flow exceeded the capacity of the chain, drops formed, most of which could be collected from beneath the wire. However, overloading the wire with solvent occasionally resulted in drops becoming detached, and then entering the detector cell. When benzene was used as solvent, the detector would then require decontamination. A more satisfactory method involved the use of a separate stream splitter.

In gas chromatography, stream splitters operate by providing a different impedence to flow in each branch. This system is less satisfactory for liquid chromatography, because of interference by surface tension, and hydrostatic pressure differences at the outlets. The device described below employed the former effect for its operation.

At slow flow rates, where there is little resistance to flow in each branch, a system having two outlets at the same hydrostatic level would permit drops to form at each outlet. If the outlets were of different diameters, drops would only detach from the larger orifice, because of the effect of drop-weight and surface tension. Flow would
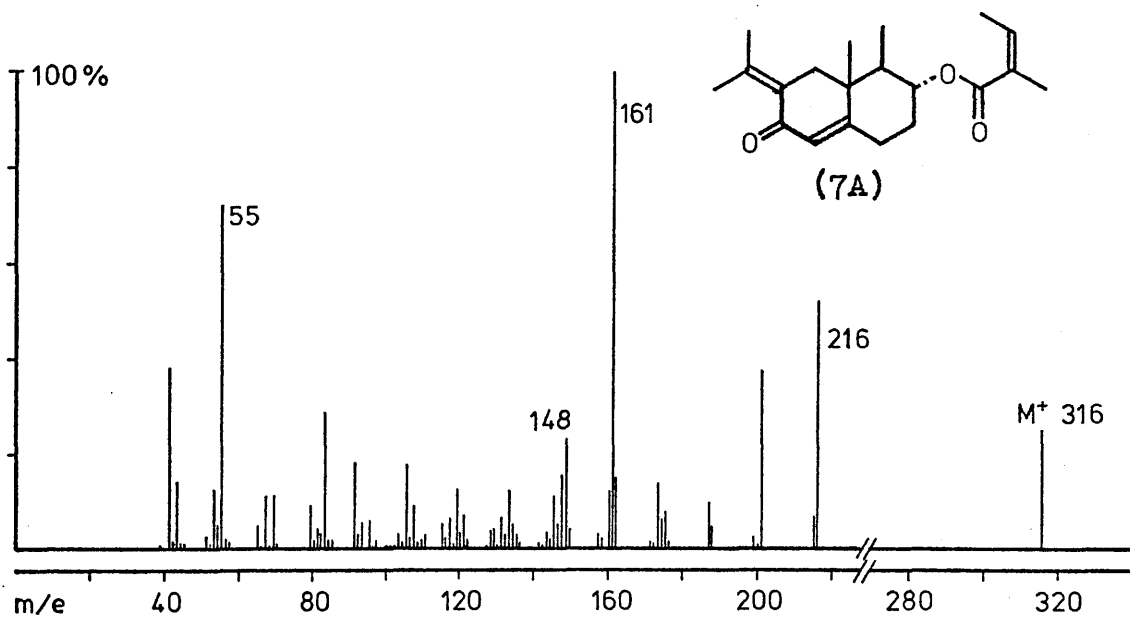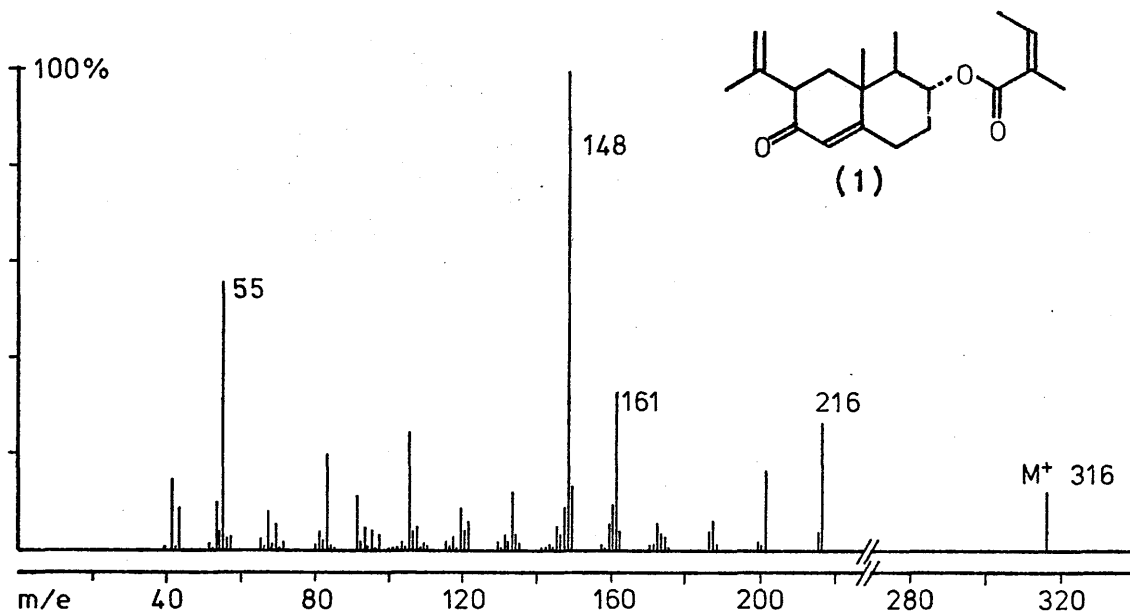
thus be limited in the smaller orifice to loss by evaporation. When this outlet was placed in contact with the circulating chain, solvent was removed by uptake on the chain, at a rate determined by the speed and capacity of the chain. Most of the eluate continued to flow through the larger outlet, where it could be recovered.

In actual operation, it was found convenient to split the end of the narrow bore tubing to form a notch, 3-4 mm deep, through which the chain passed. There was then less tendency for fluid contact to be lost between the outlet and the chain. Under these conditions, it was necessary to set the main outlet below the level of the chain. For benzene, a distance of 1 cm was effective. (Fig. 52).

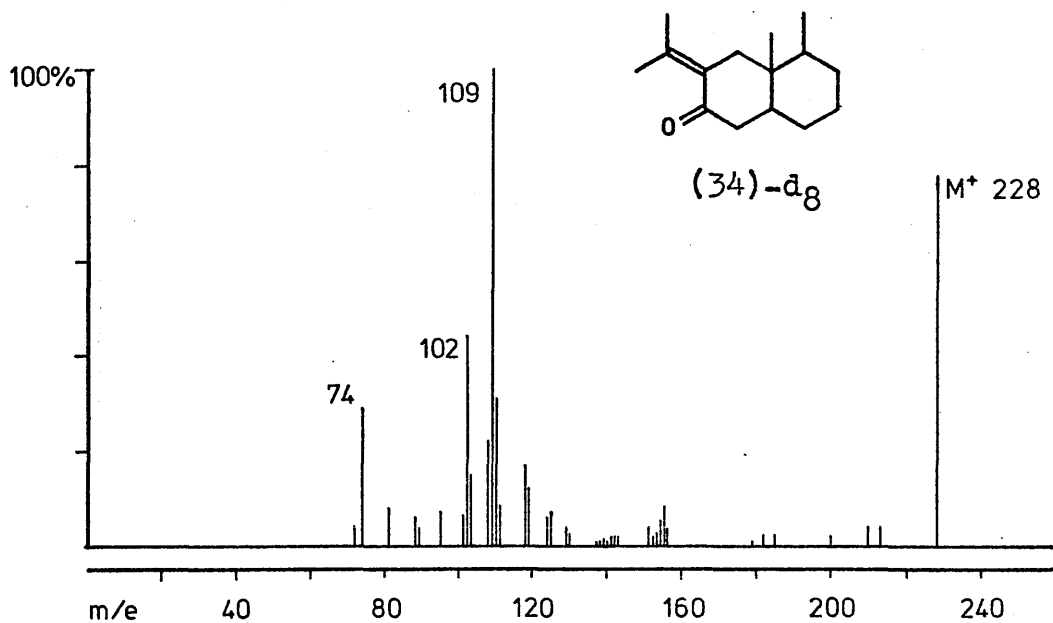A further source of failure in operation resulted from airlocks in the T-junction. However, these were usually present at the start, and rarely formed in the course of operation, after they had been properly eliminated.

APPENDIX I - MASS SPECTRA

Mass Spectra (70 eV) of Petasin (1) and Isopetasin (7A)
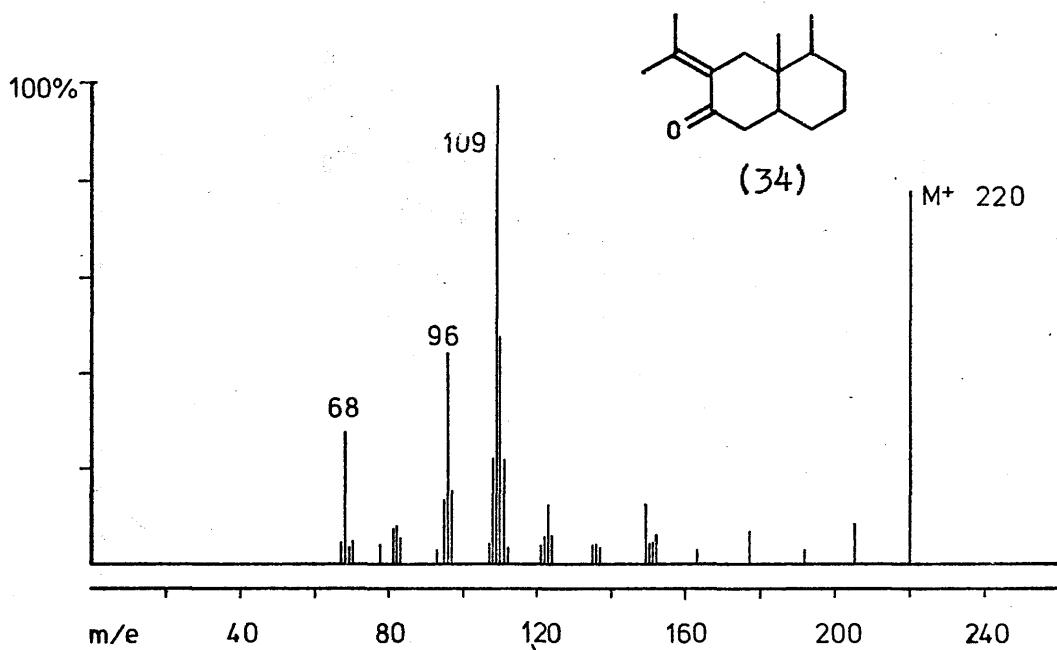
Mass Spectra (20 eV) of Compound 17b [Fukinone; (34)]
Normal and Deuterium-Substituted Samples
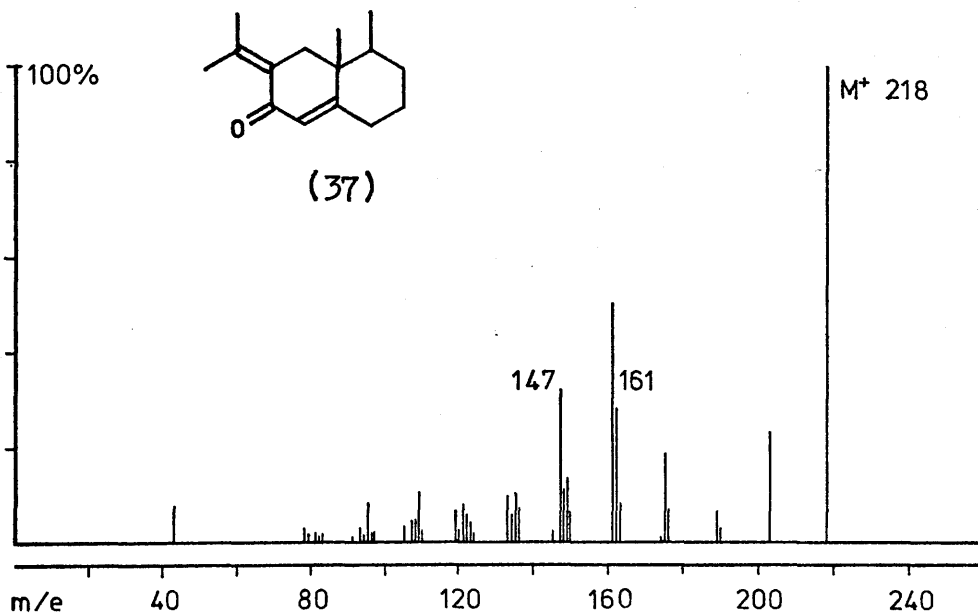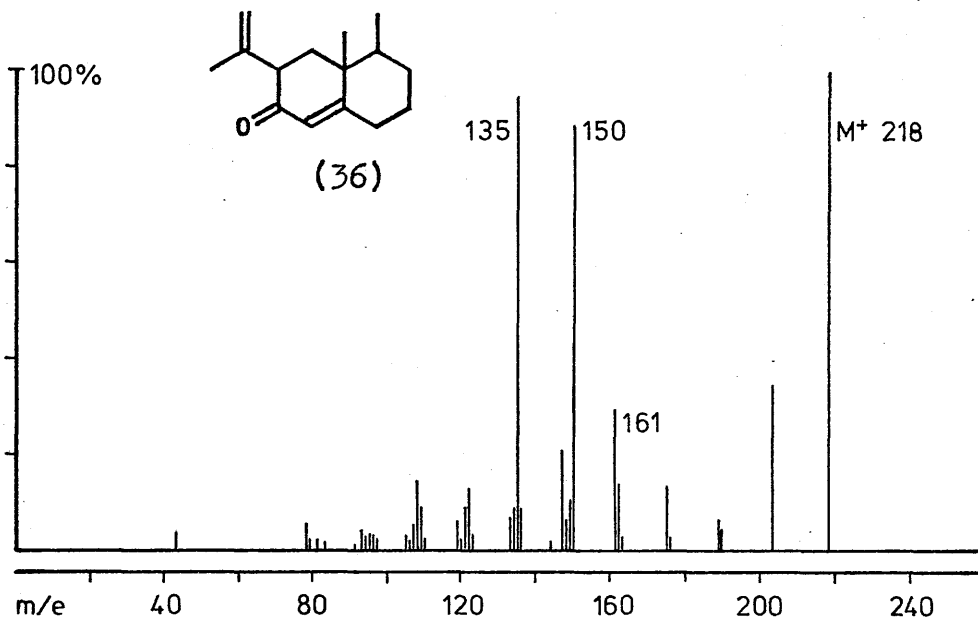
Mass Spectra (20 eV) of Compound 17c; Normal
[Eremophila-9,11-dien-8-one (36)] and Isomerised
[Eremophila-7(11),9-dien-8-one (37)] Samples
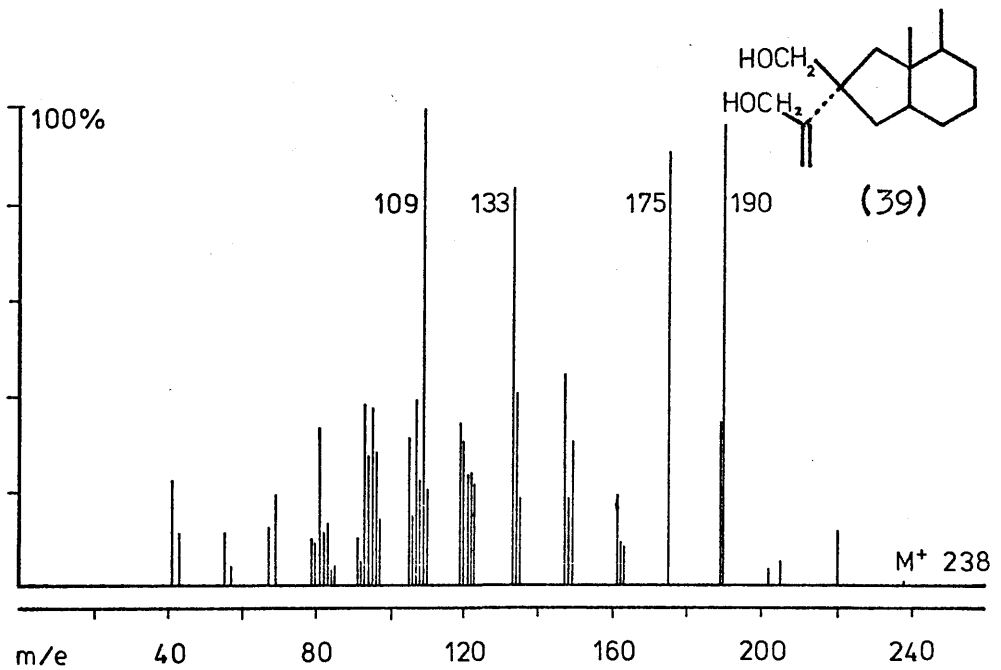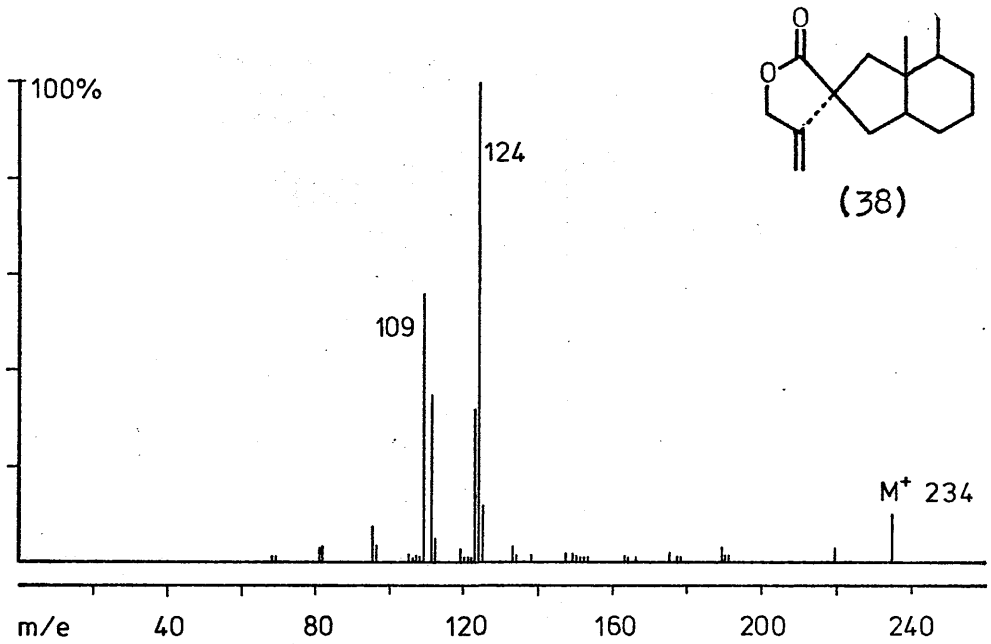
Mass Spectra (20 eV) of Bakkenolide-A (38) and
Bakkenolide-A-diol (39)
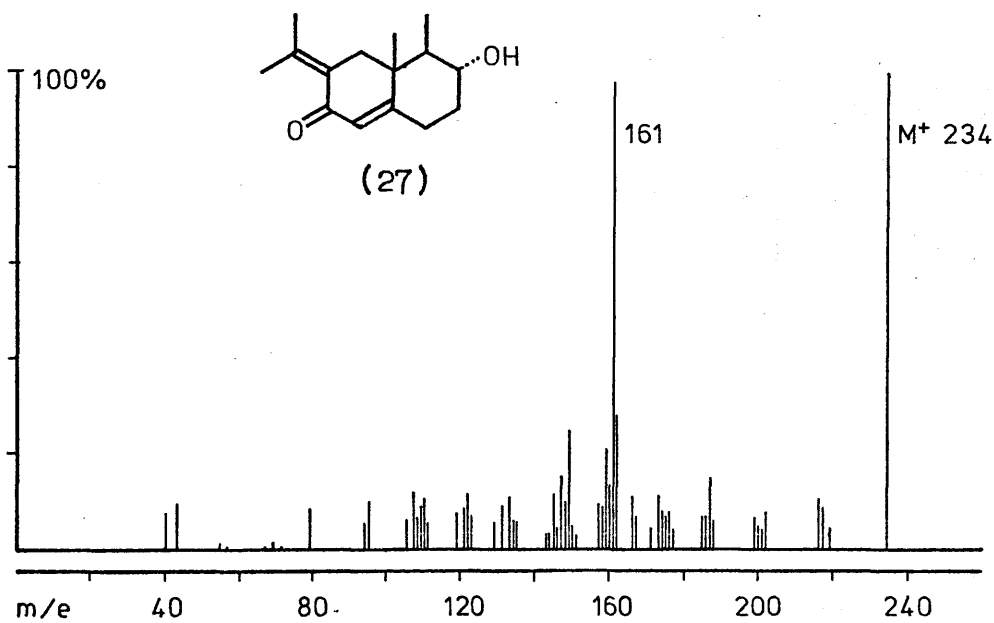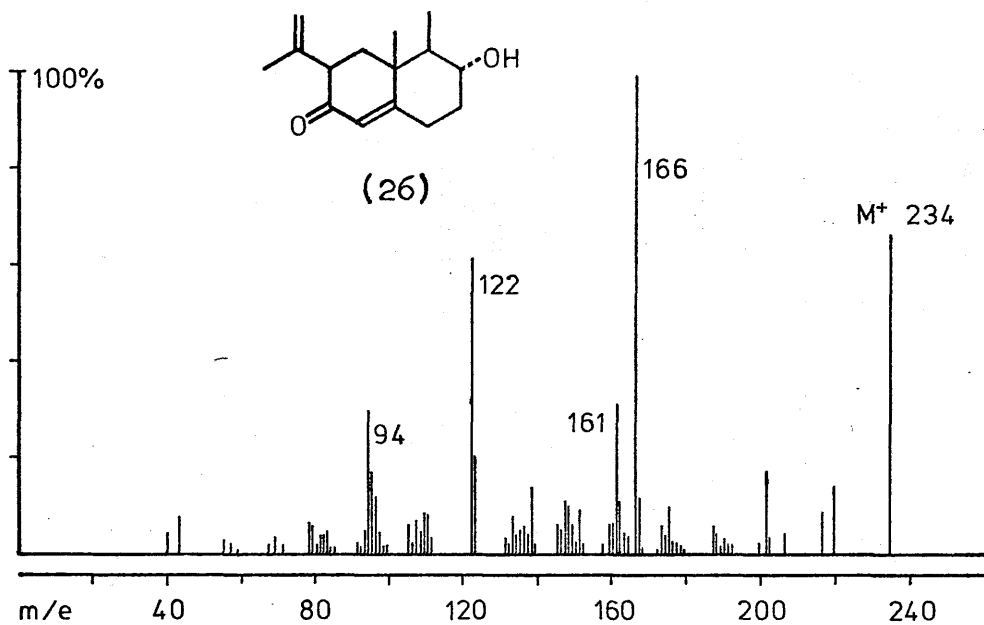
Mass Spectra (20 eV) of Petasol (26) and Isopetasol (27)
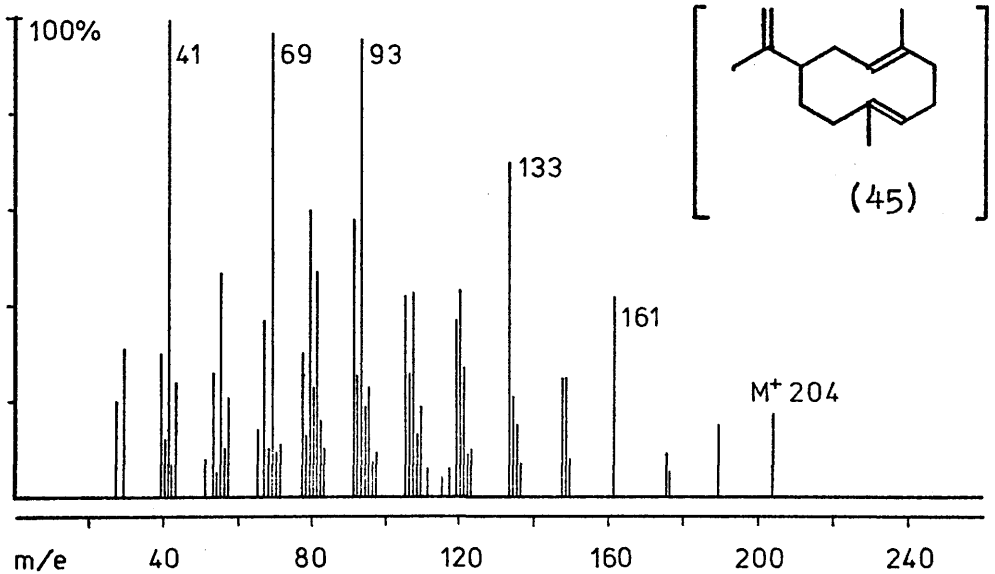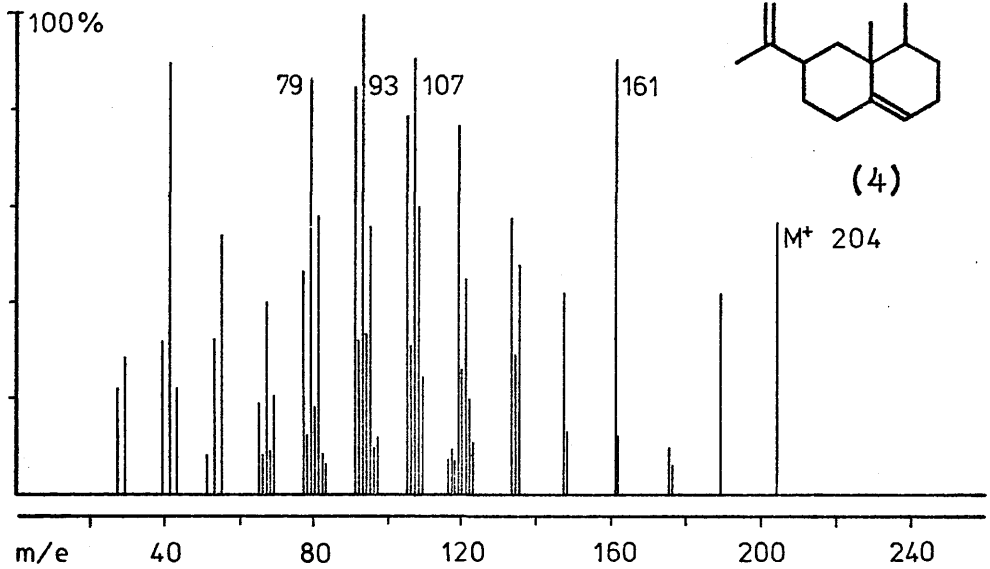
Mass Spectra (70 eV) of Eremophilene (4) and Compound 7 [possibly β-Germacrene (45)]
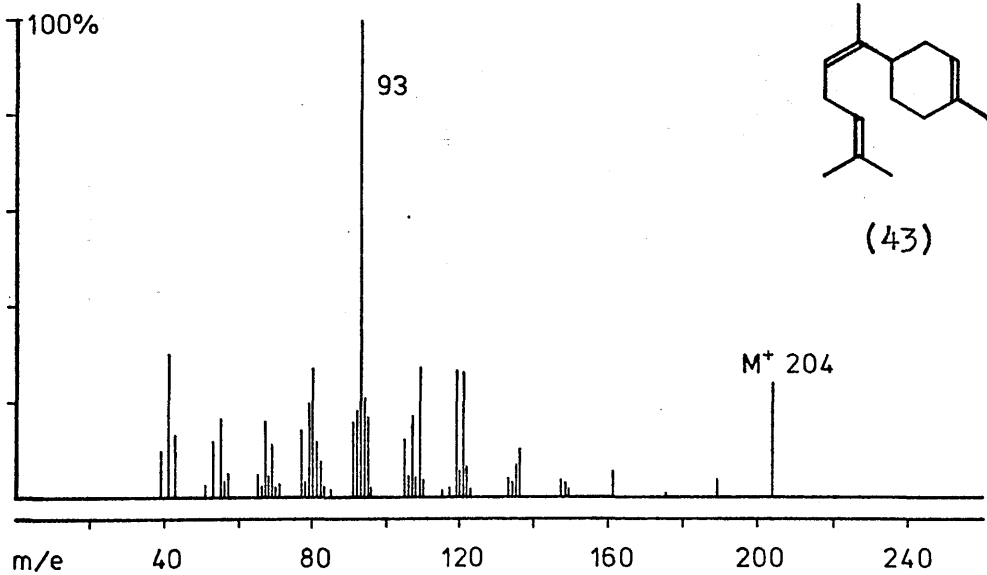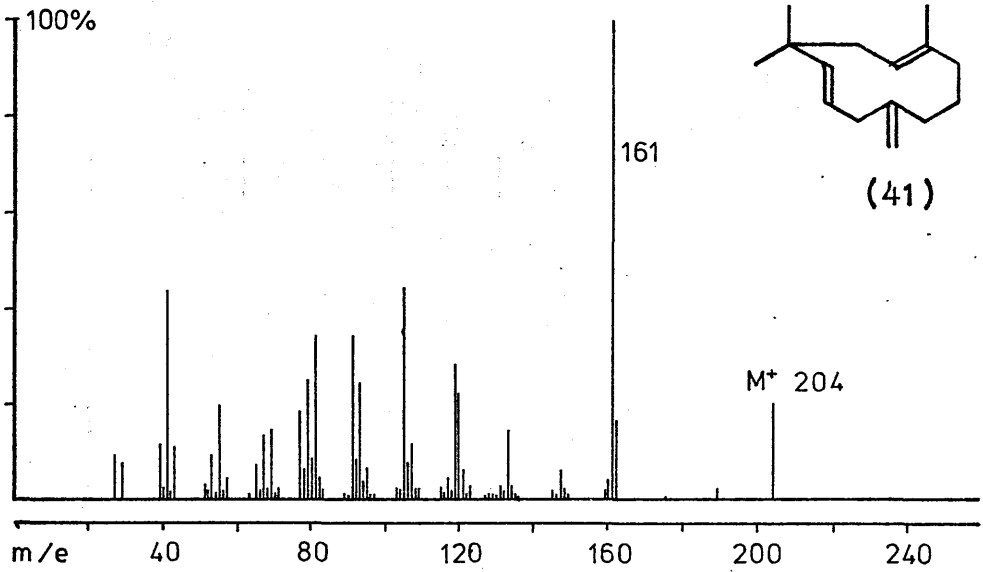
Mass Spectra (70 eV) of β-Humulene (41) and
α-Bisabolene (43)

A8
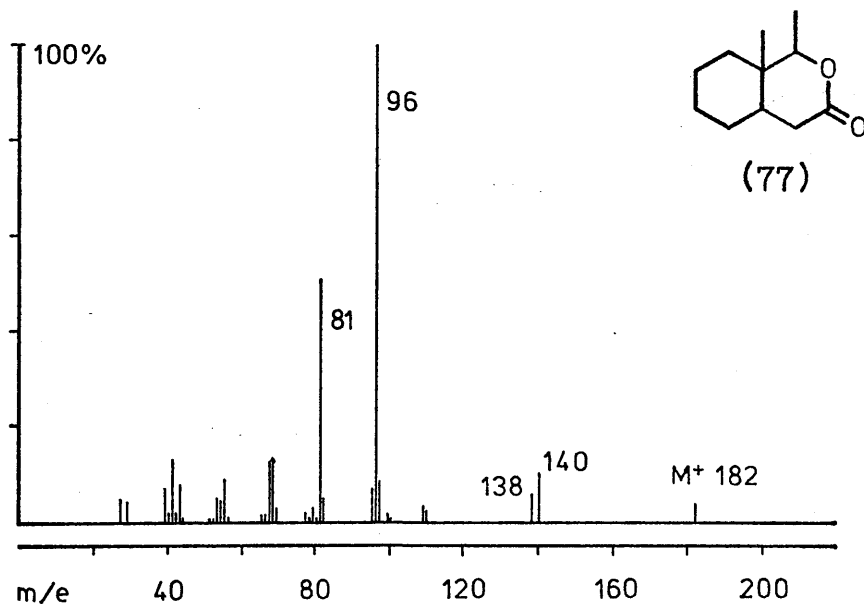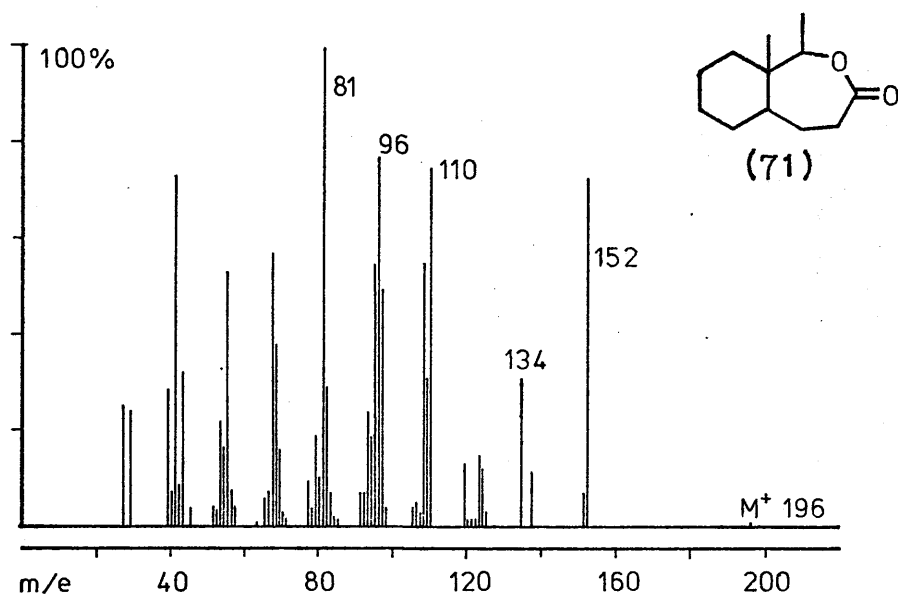
Mass Spectra (20 eV) of ε-Lactone (71) and
δ-Lactone (77)
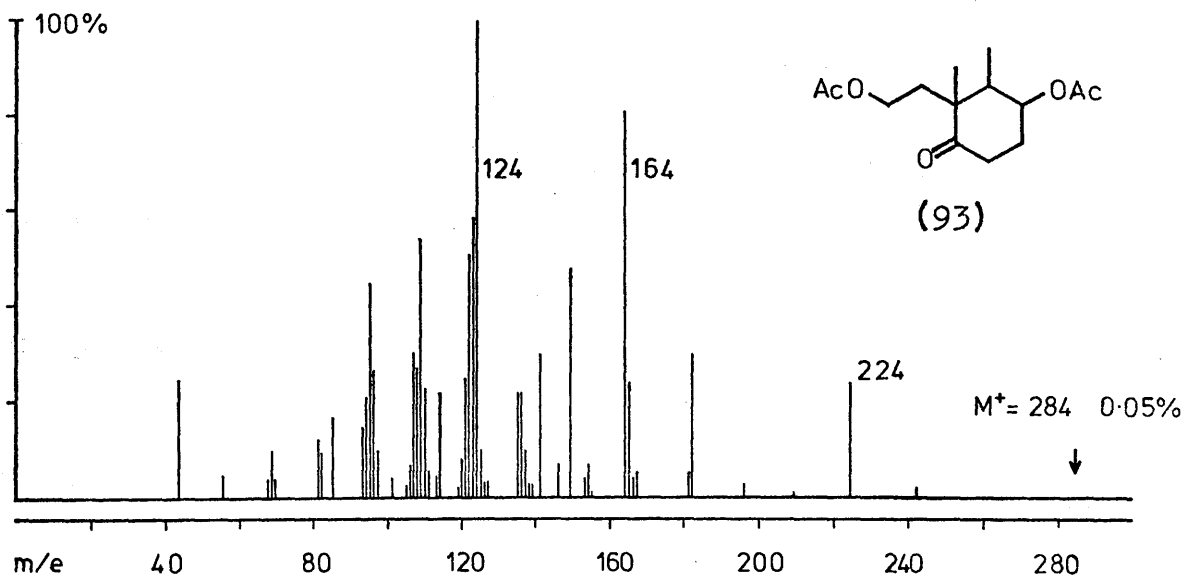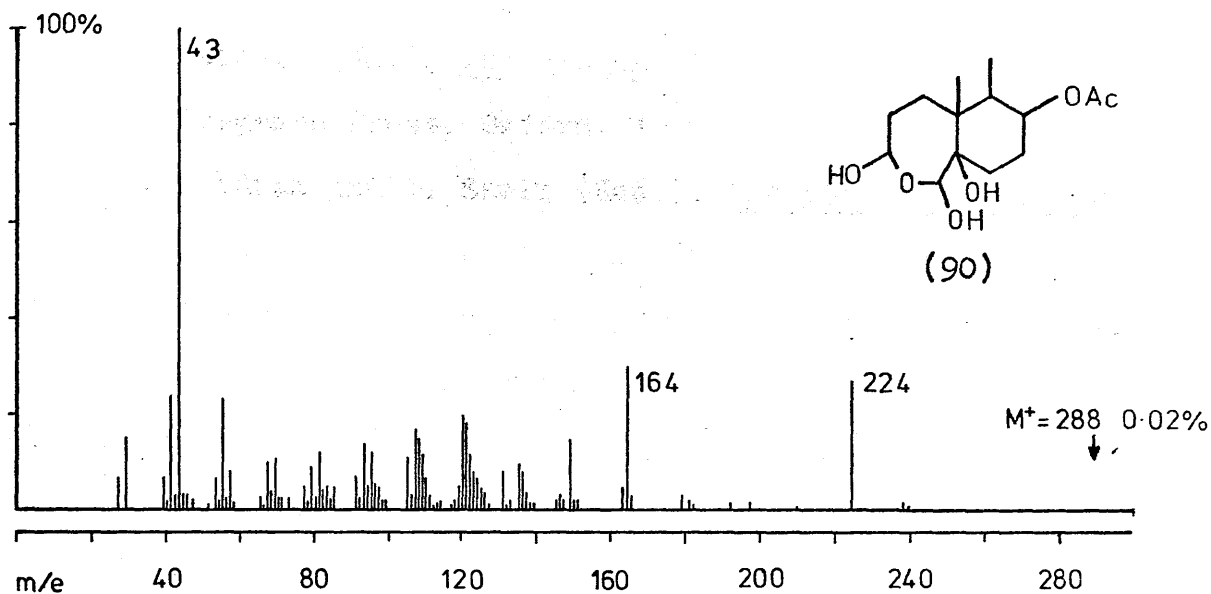
Mass Spectra (20 eV) of Acetoxy-<u>bis</u>-hemiacetal (90)
and <u>bis</u>-Acetoxyketone (93)

REFERENCES

1) P. Bernfeld (Ed.); The Biogenesis of Natural Compounds;
   Pergamon Press, Oxford, 1967.

2) J.B. Pridham and T. Swain (Eds.); Biosynthetic Pathways
   in Higher Plants; Academic Press, London, 1965.

3) T.W. Goodwin; in Terpenoids in Plants (Ed. J.B. Pridham);
   Academic Press, London, 1967 p.1.

4) R. Hegnauer; Chemotaxonomie der Pflanzen Bd. I-IV;
   Birkhauser Verlag, Basel, 1962.

5) J.B. Harborne (Ed.); Phytochemical Phylogeny;
   Academic Press, London, 1970.

6) A. Aebi and T. Waaler; Uber die Inhaltsstoffe von
   Petasites hybridus (L) Fl. Wett.;
   Verlag Helbing und Lichtenhahn, Basel, 1959.

7) G.H. Draffan; Ph.D. Thesis; University of Glasgow, 1967.

8) L. Ruzicka; Experientia 1953, 9, 357.
                Proc. Chem. Soc. 1959, 341.

9) R. Robinson; cited in A.R. Penfold and J.L. Simonsen;
   J. Chem. Soc. 1939, 87.

10) J.H. Richards and J.B. Hendrickson; The Biosynthesis of
    Steroids, Terpenes and Acetogenins;
    W.A. Benjamin, New York, 1964.

11) V. Herout and F. Šorm; in Perspectives in Phytochemistry
    (Ed. J.B. Harborne and T. Swain); Academic Press,
    London, 1969 p.139.

12) L. Novotný, J. Toman, F. Starý, A.D. Marquez, V. Herout
     and F. Šorm; Phytochemistry 1966, 5, 1281.

13) J. Hochmannová and V. Herout;
     Colln. Czech. Chem. Communs. 1964, 29, 2369.

14) J. Křepinsky, O. Motl, L. Dolejš, L. Novotný, V.Herout
     and R.B. Bates; Tetrahedron Letts. 1968, 3315

15) W.D. Loomis; in Terpenoids in Plants (Ed. J.B. Pridham);
     Academic Press, London, 1967, p.59.

16) M. Biollaz and D. Arigoni; Chem.Communs. 1969, 633:
    A. Corbella, P. Gariboldi, G. Jommi and C. Scolastico;
    ibid. 634.
    D.H.R. Barton, G.P. Moss and J.A. Whittle;
    J. Chem. Soc. C 1968, 1813.

17) L.J. Rogers, S.P.J. Shah and T.W. Goodwin;
    Photosynthetica 1968, 2, 184.

18) J. Ellingboe, E. Nyström and J. Sjövall;
    Biochim. Biophys. Acta 1968, 152, 803.

19) A. Aebi, J. Büchi, T. Waaler, E. Eichenberger and J.Schmutz;
    Pharm. Acta Helv. 1955, 29, 277.

20) A. Aebi and C. Djerassi; Helv. Chim. Acta 1959, 42, 1785.

21) D. Herbst and C. Djerassi;
    J. Amer. Chem. Soc. 1960, 82, 4337.

22) A.E. Bradfield, A.R. Penfold and J.L. Simonsen;
    J. Chem. Soc. 1932, 2744

23) J.L. Simonsen; in The Terpenes Vol. III;

    Cambridge University Press (2nd. Edition), 1961, p.212.

24) A.R. Pinder; Perfumery and Essential Oil Record

    1968, 59, 280: ibid. 645.

25) J.B. Hendrickson; Tetrahedron 1959, 7, 82.

26) C. Steelink and J.C. Spitzer; Phytochemistry 1966, 5, 357.

27) L. Novotný, J. Jizba, V. Herout and F. Šorm;

    Colln. Czech. Chem. Communs. 1962, 27, 1393.

28) L. Novotný, J. Jizba, V. Herout, F. Šorm, L.H. Zalkow,

    S. Hu and C. Djerassi; Tetrahedron 1963, 19, 1101.

29) L. Novotný, Ch. Tabačíková-Wlotzka, V. Herout and F. Šorm;

    Colln. Czech. Chem. Communs. 1964, 29, 1922.

30) L. Novotný, V. Herout and F. Šorm;

    Colln. Czech. Chem. Communs. 1962, 27, 1400.

31) G. Hegi; in Illustrierte Flora von Mittel-Europa

    Band VI:2Teil; Carl Hanser Verlag, Munchen,

    (Second Edition) 1954, p.680.

32) J. Hochmannová, L. Novotný and V. Herout;

    Colln. Czech. Chem. Communs. 1962, 27, 1870.

33) J. Harmatha, Z. Samek, L. Novotný, V. Herout and F. Šorm;

    Colln. Czech. Chem. Communs. 1969, 34, 1739.

34) A.R. Clapham, T.G. Tutin and E.F. Warburg; in

    Flora of the British Isles; Cambridge University

    Press (Second Edition), 1962, p.828.

35) D. Waldi; in Thin Layer Chromatography (Ed. E. Stahl);
        Springer Verlag, Berlin, 1965, p. 483.

36) J.A. Zabkiewicz, R.A.B. Keates and C.J.W. Brooks;
        Biochem J. 1968, 109, 929.

37) K. Morikawa and Y. Hirose; Tetrahedron Letts.1969, 1799.

38) G. Eglinton and R.J. Hamilton; in Chemical Plant Taxonomy
        (Ed. T. Swain); Academic Press, London, 1963, p.187.

39) A. McCormick; cited in G.H. Draffan, Ph.D. Thesis,
        University of Glasgow, 1967.

40) C.C. Sweeley, W.H. Elliott, I. Fries and R. Ryhage;
        Anal. Chem. 1966, 38, 1549.

41) H. Elias; in Advances in Chromatography Vol. 7
        (Eds. J.C. Giddings and R.A. Keller);
        Edward Arnold, London, 1968 p.243.

42) G.M. Anthony and C.J.W. Brooks; Chem. Communs. 1970, 200.

43) K. Naya, I. Tagaki, Y. Kawaguchi, Y. Asada, Y. Hirose and
        N. Shinoda; Tetrahedron 1968, 24, 5871.

44a) H. Egger; Monatsh. 1966, 97, 1201.

44b) H. Budzikiewicz and C. Djerassi;
        J. Amer. Chem. Soc. 1962, 84, 1430.
        P. Eneroth, B. Gordon, R. Ryhage and J. Sjovall;
        J. Lipid Res. 1966, 7, 571.

45) A.I. Scott; Interpretation of the Ultraviolet Spectra of
        Natural Products; Pergamon Press, Oxford, 1964.

46) A. Abe, R. Onoda, K. Shirata, T. Kato, M.C. Woods and
    Y. Kitahara; Tetrahedron Letts. 1968, 369.

47) K. Shirata, T. Kato and Y. Kitahara;
    Tetrahedron 1969, 25, 3179.

48) J. Harmatha, L. Novotný, Z. Samek and V. Herout;
    Colln. Czech.Chem. Communs. in preparation:
    cited in ref 11).

49) C.J.W. Brooks and R.A.B. Keates; J. Chromatog. 1969, 44, 509.

50) B.W. Nichols; in New Biochemical Separations
    (Eds. A.T. James and L.J. Morris); D. van Nostrand,
    London, 1964, p.321.

51) B.W. Nichols and A.T. James; in Progress in Phytochemistry
    Vol. 1 (Eds. L. Reinhold and Y. Liwschitz);
    John Wiley, London, 1968, p.1.
    L.J. Morris and B.W. Nichols; in Chromatography
    (Ed. E. Heftmann); Reinhold, New York, (2nd. Edition),
    1967, p.466.

52) R.H. Bible; Interpretation of NMR Spectra;
    Plenum Press, New York, 1965.

53) W. Cocker; Bull. Nat. Inst. Sci. India 1968, 37, 5.

54) V. Benešová, V. Herout and F. Šorm;
    Colln. Czech. Chem. Communs. 1961, 26, 1832.

55) S. Dev, J.E. Anderson, V. Cormier, N.P. Damodayan and
    J.D. Roberts; J. Amer.Chem. Soc. 1968, 90, 1246.

56) H. Budzikiewicz, C. Djerassi and D.H. Williams;

Mass Spectrometry of Organic Compounds;

Holden-Day, San Francisco, 1967.

57) J. Hochmannová, L. Novotný and V. Herout;

Colln. Czech. Chem. Communs. 1962, 27, 2711.

58) R.A. Appleton, R. McCrindle and K.H. Overton;

Phytochemistry 1968, 7, 135.

59) M.D. Sutherland and R.J. Park; in Terpenoids in Plants

(Ed. J.B. Pridham); Academic Press, London, 1967, p.147.

60) J.A. Zabkiewicz; personal communication, 1970.

61) L.J. Morris; J. Lipid Res. 1966, 7, 717.

62) E.C. Horning, W.J.A. VandenHeuvel and B.G. Creech;

Methods of Biochemical Analysis 1963, 11, 69.

63) K. Bloch and D. Rittenberg; J. Biol. Chem. 1944, 155, 657.

64) R.G. Langdon and K. Bloch; J. Biol. Chem. 1953, 200, 129.

J.W. Cornforth and G. Popják; Biochem. J. 1954, 58, 403.

65) D.E. Wolf, C.H. Hoffman, P.E. Aldrich, H.R. Skeggs,

L.D. Wright and K. Folkers;

J. Amer. Chem. Soc. 1956, 78, 4499.

66) P.A. Tavormina, M.A. Gibbs and J. Huff;

J. Amer. Chem.Soc. 1956, 78, 4498.

67) H. Rudney; in The Biosynthesis of Terpenes and Sterols,

CIBA Foundation Symposium; J.& A. Churchill,

London, 1959, p.75.

68a) A.J. Birch, R.J. English, R.A. Massy-Westropp and

  H. Smith; Proc. Chem. Soc. 1957, 233:

  J. Chem. Soc. 1958, 369.

68b) G. Popják and J.W. Cornforth; Advances in Enzymology

  1960, 22, 281.

69) A.J. Birch and H. Smith; in The Biosynthesis of Terpenes

  and Sterols, CIBA Foundation Symposium;

  J.& A. Churchill, London, 1959, p.245.

70) G.M. Jacobsen and R.C. Corley; Fed. Proc. 1957, 16, 200.

71) L. Ehrenberg and A.F. Daniel; Acta Chem. Scand.

  1962, 16, 1523.

72) K. Bloch; in The Biosynthesis of Terpenes and Sterols,

  CIBA Foundation Symposium; J.& A. Churchill,

  London, 1959, p.4.

73) B.W. Agranoff, H. Eggerer, U. Henning and F. Lynen;

  J. Biol. Chem. 1960, 235, 326.

  F. Lynen, B.W. Agranoff, H. Eggerer, U. Henning and

  E.M.Möslein; Angew. Chem. 1959, 71, 657.

74) G. Popják; in Methods in Enzymology Vol. XV, Terpenoids

  and Steroids (Ed. R.B. Clayton); Academic Press,

  London, 1969, p.393.

75) C.J. Pollard, J. Bonner, A.J. Haagen-Smit and C.C. Nimmo;

  Plant Physiol. 1966, 41, 66.

76) W. Parker, J.S. Roberts and R. Ramage; Quart. Rev.

  1967, 21, 331.

77) W.R. Nes and J.W. Cannon; Phytochemistry 1968, 7, 1321.

78) J.W. Cornforth, R.H. Cornforth, G. Popják and L. Yengoyan;
J. Biol. Chem. 1966, 241, 3970.

79) R.K. Maudgal, T.T. Tchen, and K. Bloch;
J. Amer. Chem.Soc. 1958, 80, 2589.

80) J.W. Cornforth, R.H. Cornforth, A. Pelter, M.G. Horning
and G. Popják; Proc. Chem. Soc. 1958, 112.

81a) E. Caspi and L.J. Mulheirn; Chem. Communs. 1969, 1423.
M. Jayme, P.C. Schaefer, and J.H. Richards;
J. Amer. Chem. Soc. 1970, 92, 2059.

81b) H.H. Rees, L.J. Goad and T.W. Goodwin; Biochem. J.
1968, 107, 417.

82) Y. Yura; Takamine Kenkyusho Nempo 1957, 9, 10.
Chemical Abstracts 1961, 55,14361.

83) M. Fétizon, M. Golfier and J-M. Louis; Chem. Communs.
1969, 1118.
M. Fétizon and M. Golfier; Compt. Rend. C 1968, 276, 900.

84) M.J.O. Francis and M. O'Connell; Phytochemistry
1969, 8, 1705.

85) M.J.O. Francis; Phytochemical Society Symposium,
"Biochemistry of the Terpenoids",Liverpool 1970;
Proceedings in preparation (Ed. T.W. Goodwin);
Academic Press, London.

86) D.V. Banthorpe and K.W. Turnbull; Chem.Communs. 1966, 177.

87) D.V. Banthorpe and D. Baxendale; Chem. Communs. 1968, 1553.

88) E.W. Warnhoff, D.G. Martin and W.S. Johnson; in

      Organic Syntheses Coll. Vol. 4 (Ed. N. Rabjohn);

      John Wiley, New York, 1963, p.162.

89) D.W. Goheen and W.R. Vaughan; *Organic Syntheses*

      Coll. Vol. 4 (Ed. N. Rabjohn); John Wiley,

      New York, 1963, p.594.

90) B. Riegel, R.B. Moffatt and A.V. McIntosh; *Organic Syntheses*

      Coll. Vol. 3 (Ed. E.C. Horning); John Wiley,

      New York, 1957, p.237.

91) L. Caglioti; *Tetrahedron* 1966, 22, 487.

92) W.D. Emmons and G.B. Lucas; *J. Amer. Chem. Soc.*

                  1955, 77, 2287.

93) W.F. Sager and A. Duckworth;

      J. Amer. Chem. Soc. 1955, 77, 188.

94a) S. Sarel and Y. Yanuka; *J. Org. Chem.* 1959, 24, 2018.

94b) F. Sondheimer, R. Mechoulam and M. Sprecher;

      Tetrahedron 1964, 20, 2473.

95) G. Stork, A. Meisels and J.E. Davies;

      J. Amer. Chem. Soc. 1963, 85, 3419.

96) J.A. Hartmann, A.J. Tomasewski and A.S. Dreiding;

      J. Amer. Chem. Soc. 1956, 78, 5662.

97) E. Caspi and S.N. Balasubrahamanyan; *J. Org. Chem.*

                  1963, 28, 3383.

98) G.D. Ryerson, R.L. Wasson and H.O. House; in
        Organic Syntheses Coll. Vol. 4 (Ed. N. Rabjohn);
        John Wiley, New York, 1963, p.957.

99) D.J. Collins; J. Chem. Soc. 1959, 3919.

100) A. Eschenmoser, D. Felix and G. Ohloff;
        Helv. Chim. Acta 1967, 50, 708.

101) M. Tanabe, D.F. Crowe, R.L. Dehn and G. Dene;
        Tetrahedron Letts. 1967, 3739.

102) J. Staunton and E.J. Eisenbraun; in Organic Syntheses
        Annu. Vol. 42 (Ed. V. Boekelheide); John Wiley,
        New York, 1962, p.4.

103) R. Pappo, D.S. Allen, R.U. Lemieux and W.S. Johnson;
        J. Org. Chem. 1956, 21, 478.

104) U. Scheidegger, K. Schaffner and O. Jeger;
        Helv. Chim. Acta 1962, 45, 400.

105) C.J.W. Brooks and J.K. Norymberski;
        Biochem. J. 1953, 55, 371.

106) G. Guroff, J.W. Daly, D.M. Jerina, J. Renson, B. Witkop
        and S. Udenfriend; Science 1967, 157, 1524.

107) E.A. Evans; Tritium and its Compounds;
        Butterworths, London, 1966.

108) C. Djerassi, R.R. Engle and A. Bowers;
        J. Org. Chem. 1956, 21, 1547.

109) W. von E. Doering and E. Dorfman;
        J. Amer. Chem. Soc. 1953, 75, 5595.

110) L. Friedman and F.R. Long; J. Amer.Chem. Soc.

1953, 75, 2832.

111) E. Honkanen, T. Moisio and P. Karvonen;

Acta Chem. Scand. 1965, 19, 370.

112) E.A. Braude and J.A. Coles; J. Chem. Soc. 1950, 2014.

113) W.G. Dauben, H.G. Wight and G.A. Boswell;

J. Org. Chem. 1958, 23, 1787.

114) E.L. Jackson; in Organic Reactions Vol.2 (Eds. R. Adams,

W.E. Bachmann, L.F. Fieser, J.R. Johnson and

H.R. Snyder); John Wiley, New York, 1944, p.341.

115) G.R. Waller; in Progress in the Chemistry of Fats and

Other Lipids Vol.X (Ed. R.T. Holman);

Pergamon Press, Oxford, 1969, p.151.

116) L.J. Goad; in Naturally Occurring Substances Formed

Biologically from Mevalonic Acid, Biochemical Society

Symposium No. 29 (Ed. T.W. Goodwin); Academic Press,

London,1969, p.45.

117) P.F. Henstein, F.H. Smith and S.B. Tove;

J. Biol. Chem. 1962, 237, 2643.

118) T.W. Goodwin; in The Biosynthesis of Terpenes and Sterols,

CIBA Foundation Symposium; J. & A. Churchill,

London, 1959, p.279.

119) J.B. van Helmont; Complexionium atque Mistionum

Elementalium Figmentum; Amsterdam, 1652.

J. Woodward; Phil. Trans. Roy. Soc. 1699, 21, 382.

120) J. Bonner and A.W. Galston; _Principles of Plant Physiology_; W.H. Freeman, San Francisco, 1952.

121) M. Zimmerman; in _Physiology of Plant Growth and Development_ (Ed. M.B. Wilkins); McGraw-Hill, London, 1969.

122) M.J.O. Francis and C. Allcock; _Phytochemistry_ 1969, __8__, 1339.

123) D. Arigoni; Terpenoids in Plants, Phytochemical Society Symposium, Aberystwyth 1966; unpublished contribution cited in Ref. 115).

124) A.J. Birch, D. Boulter, R.I. Fryer, P.J. Thomson and J.L. Willis; _Tetrahedron Letts._ 1959, No.3, p.1.

125) R.G. Battu and H.W. Youngken; _Lloydia_ 1966, __29__, 360.

126) F.E. Regnier, G.R. Waller, E.J. Eisenbraun and H. Auda; _Phytochemistry_ 1968, __7__, 221.

127) W.D. Loomis and A.J. Burbott; unpublished results cited in Ref. 115).

128) F.C. Steward and R.G.S. Bidwell; _J. Expt. Bot._ 1966, __17__, 726.

129) F.W. Hefendehl, E.W. Underhill and E. von Rudloff; _Phytochemistry_ 1967, __6__, 823.

130) T.W. Goodwin; in _Biosynthetic Pathways in Higher Plants_ (Eds. J.B. Pridham and T.W. Swain); Academic Press, London, 1965, p.57.

131) L.J. Rogers, S.P.J. Shah and T.W. Goodwin; in
    Biochemistry of Chloroplasts Vol. II (Ed. T.W. Goodwin);
    Academic Press, London, 1966, p.283.

132) D.R. Threlfall and W.T. Griffiths; in Biochemistry of
    Chloroplasts Vol. II (Ed. T.W. Goodwin);
    Academic Press, London, 1966, p.255.

133) H.M. Hill, S.P.J. Shah and L.J. Rogers;
    Phytochemistry 1970, 9, 1049.

134) W. Henderson, J.W. Hart, P. How and J. Judge;
    Phytochemistry 1970, 9,

135) G. Heinrich; Protoplasma 1970, 69, 15.

136) L.K. Lowry and C.O. Chichester; Phytochemistry
    1967, 6, 367.

137) W.L. Holmes and N.W. DiTullio;
    Amer. J. Clin. Nutr. 1963, 10, 310.

138) J. Bonner, E. Heftmann and J.A.D. Zeevaart;
    Plant Physiol. 1963, 38, 81.

139) A.S. Crafts; Translocation in Plants; Holt, Rinehart
    and Winston, New York, 1961.

140) B.A. Knights; J. Gas Chromatog. 1967, 5, 273.

141) J.A. Zabkiewicz, R.A.B. Keates and C.J.W. Brooks;
    Phytochemistry 1969, 8, 2087.

142) E.J. Corey, W.E. Russey and P.R. Ortiz de Montellano;
    J. Amer. Chem. Soc. 1966, 88, 4750.

    E.E. van Tamelen, J.D. Willet, R.B. Clayton and K.E. Lord;
    ibid. 4752.

143) W.W. Reid; Biochem.J. 1966, 100, 13P.

144) W.W. Reid; Phytochemistry 1968, 7, 451.

145) M. Calvin; J. Chem. Soc. 1956, 1895.

146) H.K. Porter; Ann. Revs. Plant. Physiol. 1962, 13, 303.

    H.K. Porter, R.V. Martin and I.F. Bird;

        J. Expt. Bot. 1959, 10, 264.

147) H.P. Kortschak, C.E. Hartt and G.O. Burr;

        Plant Physiol. 1965, 40, 209.

148) B. Kok; in Physiology of Plant Growth and Development

        (Ed. M.B. Wilkins); McGraw-Hill, London, 1969, p.335.

149) I. Zelitch; J. Biol. Chem. 1965, 240, 1869.

150) J.W. Bradbeer and C.M.A. Anderson; in Biochemistry of

      Chloroplasts Vol. II (Ed. T.W. Goodwin);

    Academic Press, London, 1966, p.175.

151) R.M. Krupka and G.H.N. Towers; Can. J. Bot. 1959, 37, 539.

152) D.W.A. Roberts and H. J. Perkins;

        Biochim. Biophys. Acta 1966, 127, 42.

153) H.L. Kornberg; in Essays in Biochemistry Vol. 2

        (Eds. P.N. Campbell and G.D. Greville)

        Academic Press, London, 1966, p.1.

154) K. Naya, I. Takagi, M. Hayashi, S. Nakamura, M. Kobagashi

        and S. Katsamura; Chem. and Ind. 1968, 318.

155) A.J. Burbott and W.D. Loomis; Plant Physiol.

                         1967, 42, 20.

156) J. Bonner; in Plant Biochemistry (Eds. J. Bonner and
        J.E. Varner); Academic Press, New York, 1965, p.665.

157) D.J. Baisted; Phytochemistry 1967, 6, 93.

158) W. Henderson; personal communication to Dr. C.J.W. Brooks.

159) A.J. Allison, D.N. Butcher, J.D. Connolly and K.H. Overton;
        Chem. Communs. 1968, 1493.

160) J.W. Anderson and K.S. Rowan;
        Phytochemistry 1967, 6, 1047.

161) A.M. Firenzuoli, P. Vanni and E. Mastronuzzi;
        Phytochemistry 1969, 8, 61.

162) R.A. Anderson and J.A. Sowers;
        Phytochemistry 1968, 7, 293.

163) J.W. Anderson; Phytochemistry 1968, 7, 1973.

164) J. Battaile, A.J. Burbott and W.D. Loomis;
        Phytochemistry 1968, 7, 1159.

165) J.E. Graebe; Science 1967, 157, 73:
                Phytochemistry 1968, 7, 2003.

166) V.H. Potty and J.H. Bruemmer; Phytochemistry 1970, 9, 100.

167) F.B. Jungalwala and J.W. Porter; in Methods in Enzymology
        Vol XV, Terpenoids and Steroids (Ed. R.B. Clayton);
        Academic Press, London, 1969, p.454.

168) J.E. Graebe, D.T. Dennis, C.D. Upper and C.A. West;
        J. Biol. Chem. 1965, 240, 1847:
    C.D. Upper and C.A. West; J. Biol. Chem. 1967, 242, 3285.

169) S.P. Colowick and N.O. Kaplan (Eds.); Methods in
        Enzymology Vol. I; Academic Press, London, 1955.

170) R. Bandurski and H.J. Teas; Plant Physiol. 1957, 32, 643.

171) R.M.C. Dawson, D.C. Elliott, W.H. Elliott and K.M. Jones;
        Data for Biochemical Research; Oxford University
        Press, (Second Edition) 1968.

172) R.E. Alston and B.L. Turner; in Biochemical Systematics;
        Prentice-Hall, Englewood Cliffs N.J., 1963, pp.41&231:

172b) R.E. Alston and B.L. Turner; ibid. p.31.

173) A.J. Birch; in Chemical Plant Taxonomy (Ed. T. Swain);
        Academic Press, London, 1963, p.141.

174) E. Wenkert; Chem. Ind. 1955, 282.

175) I. Schechter and C.A. West; J. Biol. Chem. 1969, 244, 3200.

176) D.R. Robinson and C.A. West; Biochemistry 1970, 9, 80.

177) G.H. Dixon; in Essays in Biochemistry Vol. 2,
        (Eds. P.N. Campbell and G.D. Greville); Academic Press,
        London, 1963, p.148.

178) Sir Robert Robinson; in discussions in The Biosynthesis
        of Terpenes and Sterols, CIBA Foundation Symposium;
        J.& A. Churchill, London, 1959, p.229.

179) M.F. Perutz; Proc. Roy. Soc. B 1967, 167, 448.

180) H. Gutfreund and J.R. Knowles; in Essays in Biochemistry
        Vol. 3, (Eds. P.N. Campbell and G.D. Greville);
        Academic Press, London, 1967, p.25.

181) C.C.F. Blake, L.N. Johnson, G.A. Mair, A.C.T. North,

        D.C. Phillips and V.R. Sarma;

        Proc. Roy. Soc. B 1967, 167, 378.

      B. Capon; Chem.Revs. 1969, 69, 407.

182) A.J. Parker; Quart. Revs. 1962, 16, 163.

183) O. Hayaishi; Annu. Revs. Biochem. 1969, 38, 21.

184) G.S. Boyd; Biochem. J. 1969, 115, 25P.

185) P. Murphy and C.A. West;

        Arch. Biochem. Biophys. 1969, 133, 395.

186) F. Amelunxen; Planta Med. 1964, 12, 121:

                  ibid. 1965, 13, 457.

187) L.J. Rogers, S.P.J. Shah and T.W. Goodwin;

        Biochem. J. 1966, 100, 14C.

188) F.W. Hemming; in Naturally Occurring Substances Formed

        Biologically from Mevalonic Acid, Biochemical Society

        Symposium No. 29 (Ed. T.W. Goodwin);

        Academic Press, London, 1969, p.105.

189) H. Determann; Gel Chromatography; Springer-Verlag,

        Berlin, 1968:

        and in Advances in Chromatography Vol. 8

        (Eds. J.C. Giddings and R.A. Keller);

        Edward Arnold, London, 1969, p.3.

190) E. Heftmann (Ed.); Chromatography; Reinhold, New York 1967.

191) J. Porath and P. Flodin; Nature 1959, 183, 1657.

192) D.J. Lea and A.H. Sehon; Canad. J. Chem. 1962, 40, 159.

S. Hjertén and R. Mosbach; Anal. Biochem. 1962, 3, 109.

193) S. Hjertén; Arch. Biochem. Biophys. 1962, 99, 466.

194) P. Flodin; Dextran Gels and Their Applications in Gel

Filtration (Dissertation);Uppsala, 1962.

195) M.F. Vaughan; Nature 1960, 188, 55.

196) H. Determann, G. Lüben and T. Wieland;

Makromol. Chem. 1964, 73, 168.

197) M. Joustra, B. Söderqvist and L. Fischer;

J. Chromatog. 1967, 28, 21.

198) J. Sjövall, E. Nyström and E. Haahti; in

Advances in Chromatography Vol. 6

(Eds. J.C. Giddings and R.A. Keller);

Edward Arnold, London, 1968, p.119.

199) E. Nyström; Chromatography on Lipophilic Sephadex

Derivatives (Dissertation); Stockholm, 1968.

200) A.J.P. Martin and R.L.M. Synge; Biochem. J. 1941, 35, 1358:

R. Consden, A.H. Gordon and A.J.P. Martin;

Biochem. J. 1944, 38, 224.

201) J. Ellingboe, E. Nyström and J. Sjövall;

J. Lipid Res. 1970, 11, 266.

202) E. Kováts; Helv. Chim. Acta 1958, 41, 1915.

Helv. Chim. Acta 1959, 42, 2709.

203) R.A.B. Keates and C.J.W. Brooks; Biochem. J. 1969,114, 16P.

204) P. Eneroth and E. Nyström;

   Biochim. Biophys. Acta 1967, 144, 149.

205) M. Wilk, J. Rochlitz and H. Bende;

   J. Chromatog. 1966, 24, 414.

206) J.C. Giddings and K.L. Mallik; Anal.Chem. 1958, 30, 914.

207) P.B. Hamilton; Anal. Chem. 1966, 38, 997.

208) E. Nyström and J. Sjövall; J. Chromatog. 1966, 24, 212:

                           Arkiv. Chem. 1966, 29, 107.

209) E. Haahti, T. Nikkari and J. Kärkkäinen;

   J. Gas Chromatog. 1966, 4, 12.