BACTERIAL ISOPRENOID BIOSYNTHESIS

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CHAPTER 1 (Cont'd) Page No.

ABSTRACT

This thesis describes a possible alternative isoprenoid pathway in bacteria by considering some previously unpublished feeding studies in the context of the related background literature.

Three synthetic routes to 2,4-dihydroxy-4-methyltetrahydropyran (63) and three synthetic strategies towards the synthesis of 2-carboxy-2,4-dihydroxy-4-methyltetrahydropyran (66) are discussed. These compounds are considered as potential intermediates in the proposed alternative bacterial isoprenoid pathway. Labelled synthesis of (63) and structural analysis of (63) and 4-hydroxy-2-methoxy-4-methyltetrahydropyran (99) by proton nmr are also described.

Feeding studies including the ^{13}C isotopically labelled tetrahydropyrans (63) and (99) are described and a revised interpretation of all of the feeding studies considered.

HMGCoA synthase is assayed in *Rh. capsulata* after a description of its assay in bakers yeast.

To Granny & Grandad

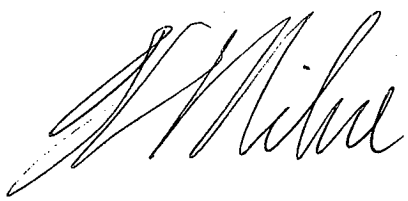
ACKNOWLEDGEMENTS

I would primarily like to thank Dr. R.L. Baxter for his enthusiasm and support throughout my Ph.D. In particular, I would like to thank him for helping me to become one of those people who ruin restaurant tablecloths and bar beer mats with hypothetical chemical schemes and mechanisms.

Thanks are due also to all of the analytical staff who helped me in the analysis of my compounds, especially Liz Stevenson and Alan Taylor in Mass Spectroscopy and John Millar, Heather Grant, Dr. Ian Sadler and Dr. David Reed in NMR Spectroscopy. I would like to acknowledge Dr. Andrew Ramsey and Dr. Deborah Moore for their guidance in the enzymological studies.

I would finally like to thank anyone else I have missed out, including all the people without whom Friday nights will never be the same.

This thesis is the original composition of the author's work, unless otherwise stated, and has not been submitted for any other degree.



POSTGRADUATE COURSES

The following is a statement of the postgraduate courses attended during the period of this work:-

Organic Research Seminars (3 years attendance).

Medicinal Chemistry, Professor P.G. Sammes (5 lectures).

Business Management, various lectures (3 lectures).

Radiation Course, various lectures (8 lectures).

Recent Advances in Organic Chemistry, various lecturers (4 lectures)

Modern NMR Techniques, Dr. I.H. Sadler (5 lectures).

Current Topics in Organic Chemistry, various lecturers (5 lectures).

2D Nuclear Magnetic Resonance, Drs. R.L. Baxter, I.H. Sadler and B. Birdsall (5 lectures).

I have also fulfilled the Departmental requirement of passing the German Translation Test.

LIST OF ABBREVIATIONS

AcCoA	acetyl coenzyme A
ADH	alanine dehydrogenase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AX or ax	axial
b	broad
bm	broad multiplet
b.p.	boiling point
BSA	bovine serum albumen
CIMS	chemical ionisation mass spectroscopy
cm	centimetre(s)
CoASH	coenzyme A
d	doublet
dd	doublet of doublets
DABCO	1,4-diazabicyclo[2,2,2]octane
DCPIP	2,6-dichlorophenolindophenol
DEPT	distortionless enhancement by polarisation transfer
DMAPP	dimethylallylpyrophosphate
DMF	dimethylformamide
DTT	dithiothreitol
δ_C	^{13}C NMR chemical shifts
δ_H	^{1}H NMR chemical shifts

EDP	Entner-Doudoroff pathway
EIMS	electron impact mass spectroscopy
EMP	Embden-Meyerhof pathway
Enz	enzyme
EQ or eq	equatorial
FABMS	fast atom bombardment mass spectroscopy
FPP	farnesyl pyrophosphate
ν_{max}	frequency of maxima
g	gram(s) or force of gravity
GPP	geranyl pyrophosphate
HCl	hydrochloric acid
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	high pressure liquid chromatography
hr	hour(s)
INADEQUATE	incredible natural abundance double quantum excitation
IPP	isopentenyl pyrophosphate
IR	infra red (spectroscopy)
J	spin-spin coupling constant
KDO	3-deoxy-D-manno-2-octulosonic acid
l	litre(s)
lit.	literature value
M	molar
m	multiplet or metre(s)

mg	milligram
MHz	megahertz
μl	microlitre(s)
μM	micromolar
min	minutes
ml	millilitre(s)
mmol	millimole(s)
m.p.	melting point
MS	mass spectroscopy
m/z	mass to charge ratio
N	normal
NADH	nicotinamide adenine dinucleotide (reduced form)
NBS	N-bromo succinimide
nm	nanometre(s)
NMR	nuclear magnetic resonance (spectroscopy)
NOE	nuclear Overhauser effect
P_i	orthophosphate
pH	concentration of H^+
PMS	phenazine methosulphate
ppm	parts per million
pro R	pro rectus
p.s.i.	pounds per square inch
q	quartet
re,re	rectus, rectus

R_f	relative retention
Rh.	rhodopseudomonas
RT	room temperature
Rubisco	ribulose bisphosphate carboxylase
s	singlet
S_N	nucleophilic substitution
Soln.	solution
t	triplet
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
TMS	tetramethylsilane
tris	[Tris(hydroxymethyl)aminomethane hydrochloride]
UV	ultraviolet
W	watts

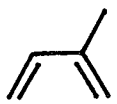
INTRODUCTION

1. TERPENOIDS

Terpenoids are the most taxonomically widespread and structurally diverse group of natural products known[1-4]. They are characterised by a carbon framework consisting of multiples of the regular five carbon (C_5) 2-methylbuta-1,3-diene (1) or "isoprene" fragment which is often obtained on pyrolysis of C_{10} compounds. The "structural isoprene rule" of Wallach and Robinson[3] originally defined isoprenoids as $(C_5)_n$ compounds produced by the successive linear head to tail condensation of isoprene units. Ruzicka[5] later amended this to the "biogenetic isoprene rule" to take into account isoprenoids derived from $(C_5)_n$ compounds in a mechanistically reasonable way, i.e. cyclisation or rearrangement.

Classification of isoprenoids is based on the term terpene, which was originally introduced to describe compounds containing ten carbons. This classification divides isoprenoids into: hemiterpenes (C_5); monoterpenes (C_{10}), e.g. camphor (2); menthol (3); sesquiterpenes (C_{15}), e.g. γ-bisabolene (4); diterpenes (C_{20}), e.g. vitamin A (5); gibberellin A_3 (6); sesterterpenes (C_{25}); triterpenes (C_{30}), e.g. cholesterol (7); carotenoids (C_{40}), e.g. β-carotene (8) and molecules of mixed biogenesis e.g. ubiquinone (9) and quinine (10) which are

familiar to us as natural fragrances, human and plant hormones and drugs and flavourings.



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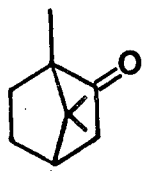

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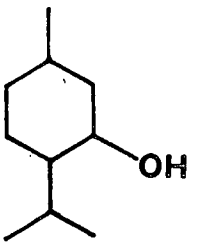

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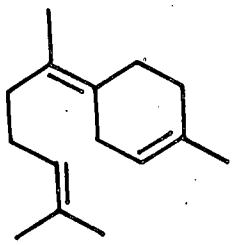

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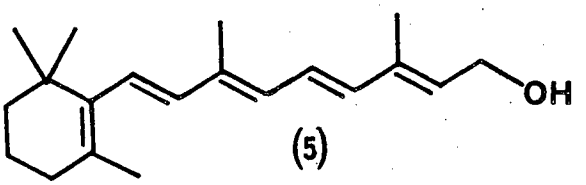

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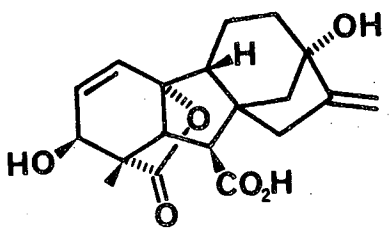

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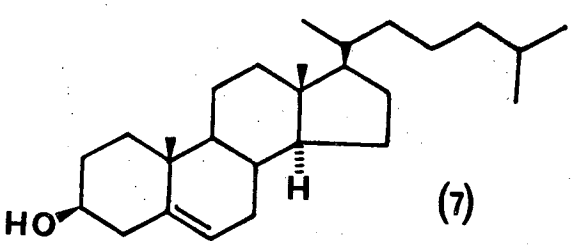

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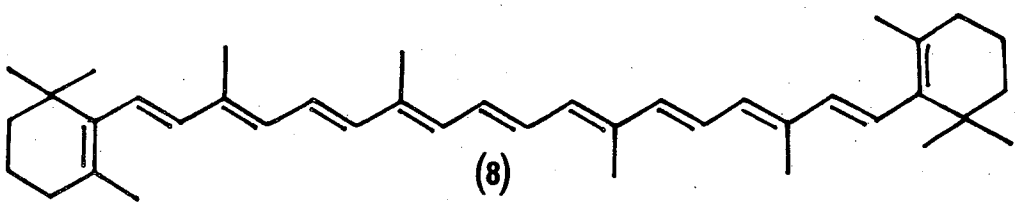

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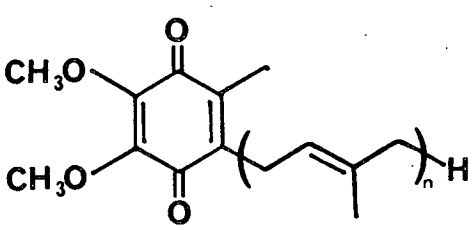

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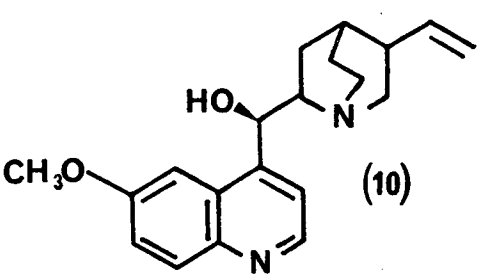

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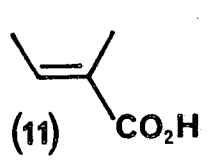
2. EUKARYOTIC ISOPRENOID BIOSYNTHESIS

For many years a number of C_5 acids and aldehydes (e.g. tiglic acid (11) and angelic acid (12)) were considered as possible precursors of the isoprenoids as isoprene (1) is not a naturally occurring compound. The first major advance came when Folkers[6-8] (1956) discovered that mevalonic acid (16) is a naturally occurring growth factor of certain strains of *lactobacilli*, and Tavormina[9,10] found that it is an extremely efficient precursor of cholesterol (7). Since then the biosynthesis of mevalonic acid (16), its conversion into the biological C_5 unit and elaboration into the isoprenoids has been completely elucidated due mainly to the pioneering work of Bloch[11-13], Lynen[14-16], Cornforth[17-20] and Popjak[21].



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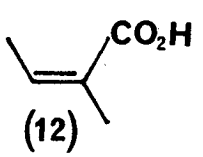

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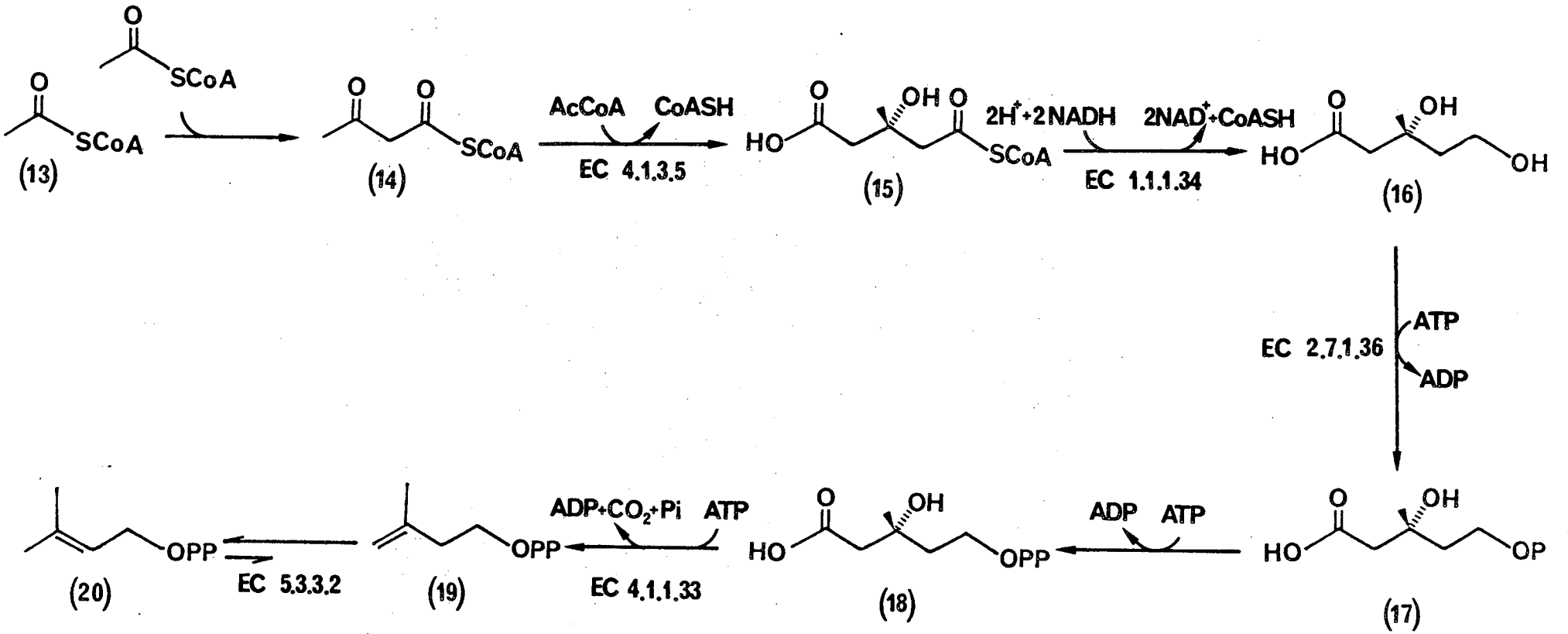
ACETOACETATE PATHWAY



SCHEME 1

The sequence begins (Scheme 1) with a condensation of two molecules of acetyl-CoA (13) catalysed by the enzyme thiolase. In the next step, β-hydroxy-β-methylglutaryl-CoA (HMGCoA) synthase[22-25] catalyses the condensation of a third acetyl-CoA (13) with acetoacetyl-CoA (14) to yield HMGCoA (15). This product is reduced to mevalonic acid (16) by HMGCoA reductase[26-28]. Mevalonic kinase[29] and phosphomevalonate kinase then sequentially catalyse the double phosphorylation of mevalonic acid (16) to give 5-pyrophosphomevalonate (18). Phosphorylation of the 3-hydroxyl group with ATP followed by pyrophosphomevalonate decarboxylase[30] catalysed *trans* elimination of carbon dioxide and phosphate gives isopentenyl pyrophosphate (IPP) (19). This is then isomerised to dimethylallyl pyrophosphate (DMAPP) (20) in the presence of isopentenyl pyrophosphate isomerase[31-33]. Addition of a proton from the medium onto the re,re face of IPP is concerted with the abstraction of the pro (R) hydrogen from C-2 (Figure 1).

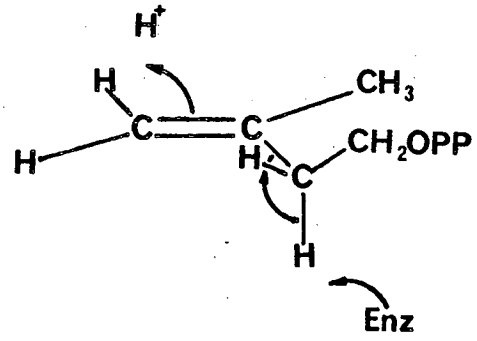


FIGURE 1

These two activated C_5 units (IPP and DMAPP) are the biological equivalent of isoprene and condense in the presence of transferases in a head to tail manner (Figure 2) to produce geranyl pyrophosphate (GPP) (C_{10}), farnesyl pyrophosphate (FPP) (C_{15}) and the other $(C_5)_n$ homologues. It is not certain whether this overall *syn* process is an S_N1 or S_N2 type mechanism. A consecutive *anti*-1,2-addition *anti*-1,2-elimination sequence and a carbocation intermediate have both been suggested[1].

All of the enzymes mentioned have been purified and characterised from yeasts and mammalian livers. Of particular importance is HMGCoA reductase as it has been found to be the major regulator of cholesterol biosynthesis[4]. The phosphorylation scheme for its regulation is shown in Figure 3.

HMGCoA reductase kinase kinase catalyses the phosphorylation and activation of HMGCoA reductase kinase. This latter kinase phosphorylates HMGCoA reductase which results in its inactivation. The effects are reversed by the action of HMGCoA reductase kinase phosphatase and HMGCoA reductase phosphatase[4].

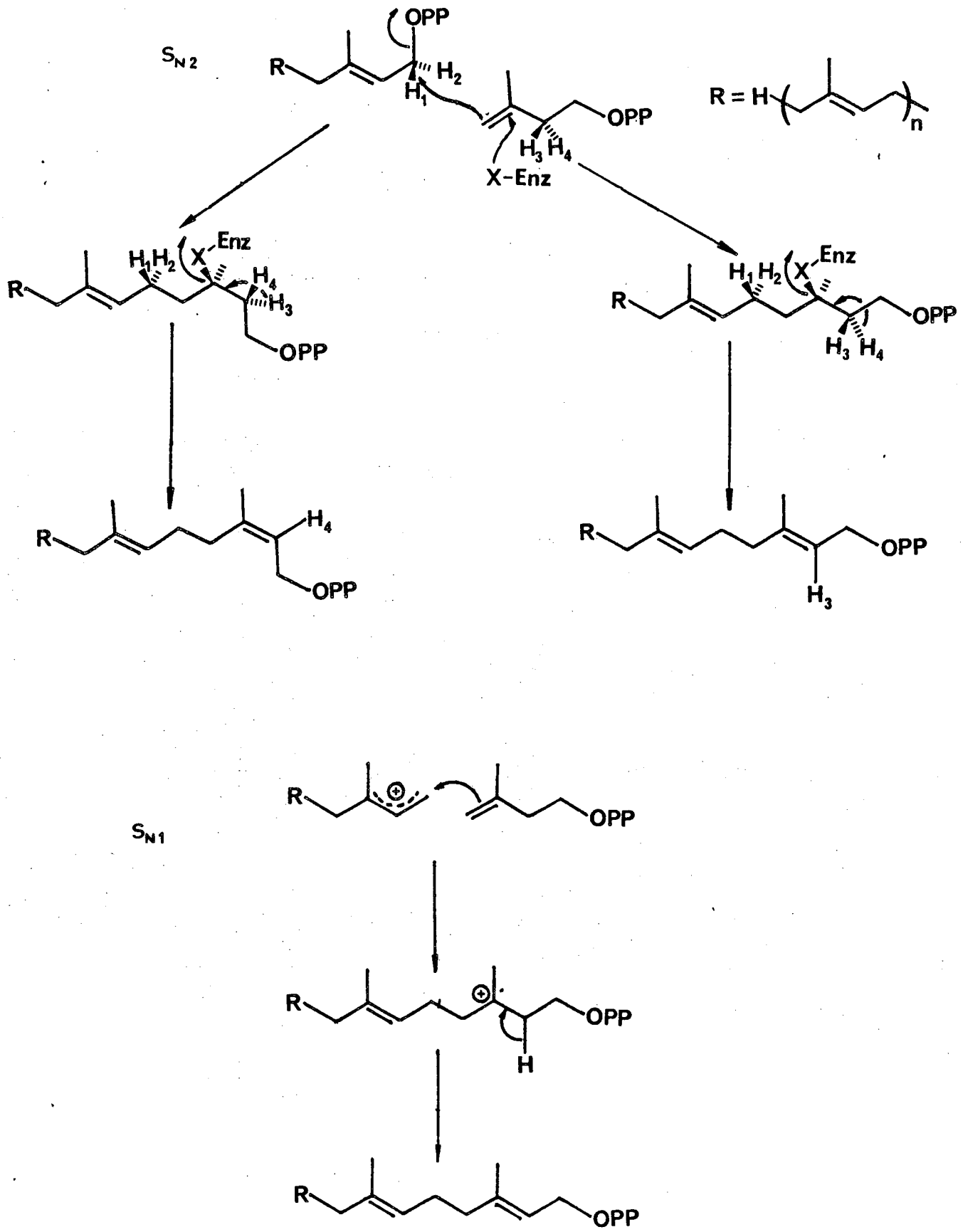

S_N2
OPP
R
H2
H1
OPP
H3 H4
X-Enz
R = H
n
H1 H2
X
Enz
H4
H3
R
OPP
H1 H2
X
Enz
R
OPP
H3 H4
R
H4
OPP
R
OPP
H3
S_N1
R
OPP
R
OPP
H
R
OPP

FIGURE 2

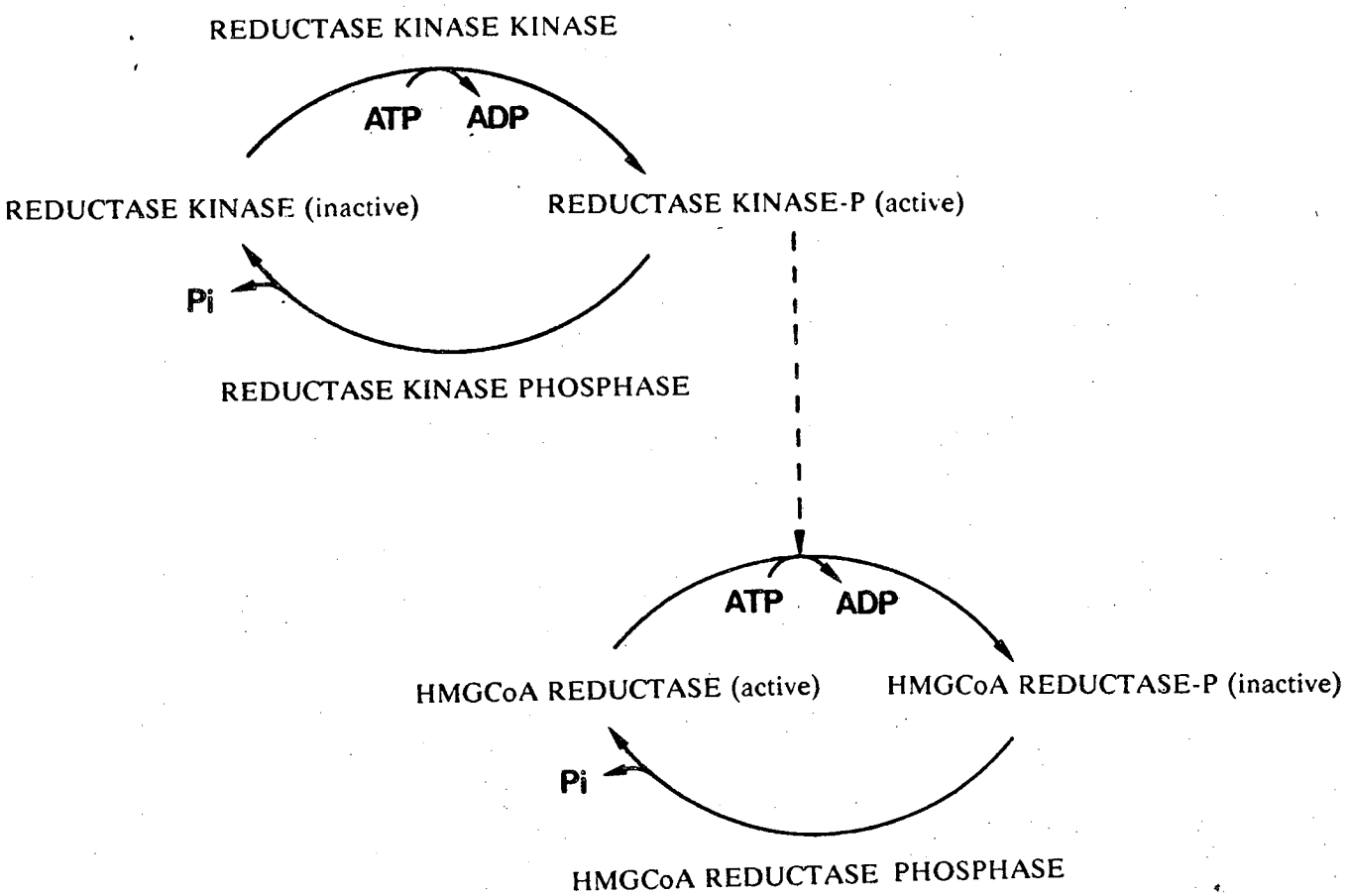
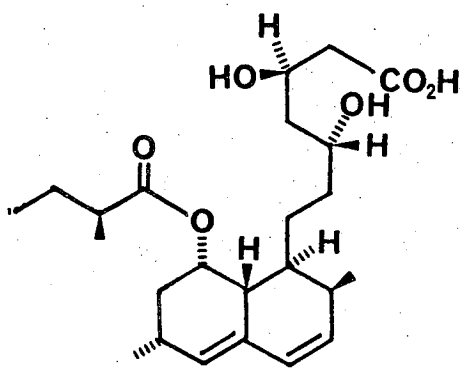

REDUCTASE KINASE KINASE
ATP
ADP
REDUCTASE KINASE (inactive)
REDUCTASE KINASE-P (active)
Pi
REDUCTASE KINASE PHOSPHASE
ATP
ADP
HMGCoA REDUCTASE (active)
HMGCoA REDUCTASE-P (inactive)
Pi
HMGCoA REDUCTASE PHOSPHASE

FIGURE 3

More important is the clinical control of HMGCoA reductase. It has long been recognised that a correlation exists between high serum cholesterol (7) levels and cardiovascular disease and that most of the serum cholesterol (7) is made in the liver[4]. Thus, since HMGCoA reductase catalyses the key regulatory step in its synthesis it seems logical to search for a drug that would inactivate it. One such drug mevinolinic acid (21) has been found to inhibit HMGCoA reductase and reduce plasma cholesterol (7) levels by up to 30 percent[4].

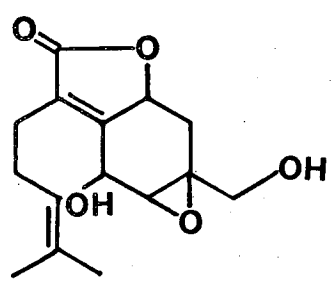


(21)

3. ALTERNATIVE EUKARYOTIC PATHWAYS

Despite the generally accepted ubiquity of the acetoacetate pathway (Scheme 1), a number of anomalous incorporations of isotopically enriched precursors into eukaryotic isoprenoids have been reported[34-38].

It has been known for many years that HMGCoA (15) is also formed in the degradation of leucine (22) (Scheme 2) and because of this it has long been considered that leucine (22) might be directly involved in isoprenoid biosynthesis[20]. However, although radioactivity from ^{14}C leucine has been incorporated into various terpenoids in animals, plants and micro organisms it has been shown recently that leucine is not directly involved in the biosynthesis of paniculide B (32) in the plant *Andrographis paniculata*[34].

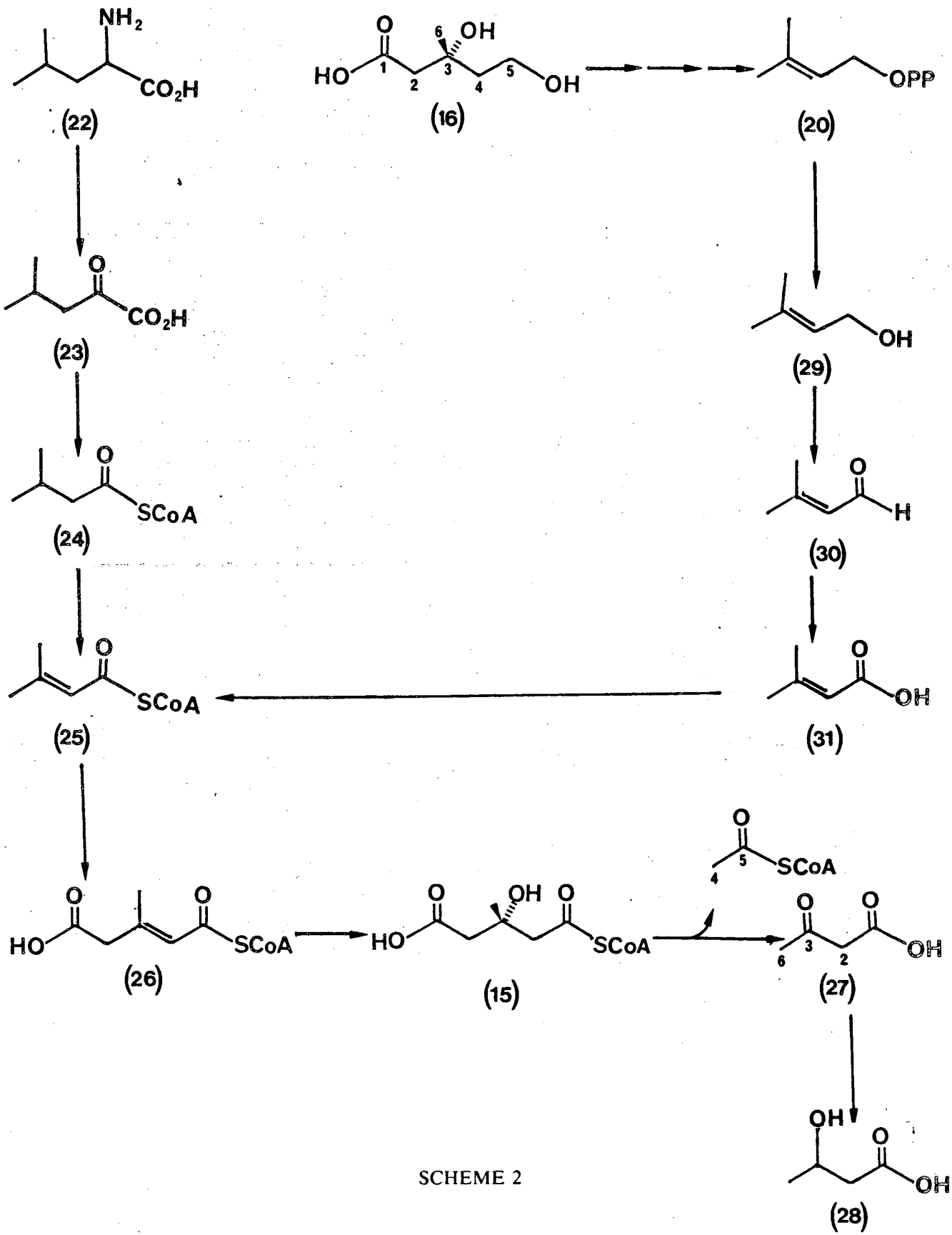


(32)

Incorporation patterns from [2-^{13}C] and [3-^{13}C] leucine feeding studies indicate that leucine is broken down to acetyl-CoA (13) before incorporation.

LEUCINE CATABOLISM

SHUNT PATHWAY



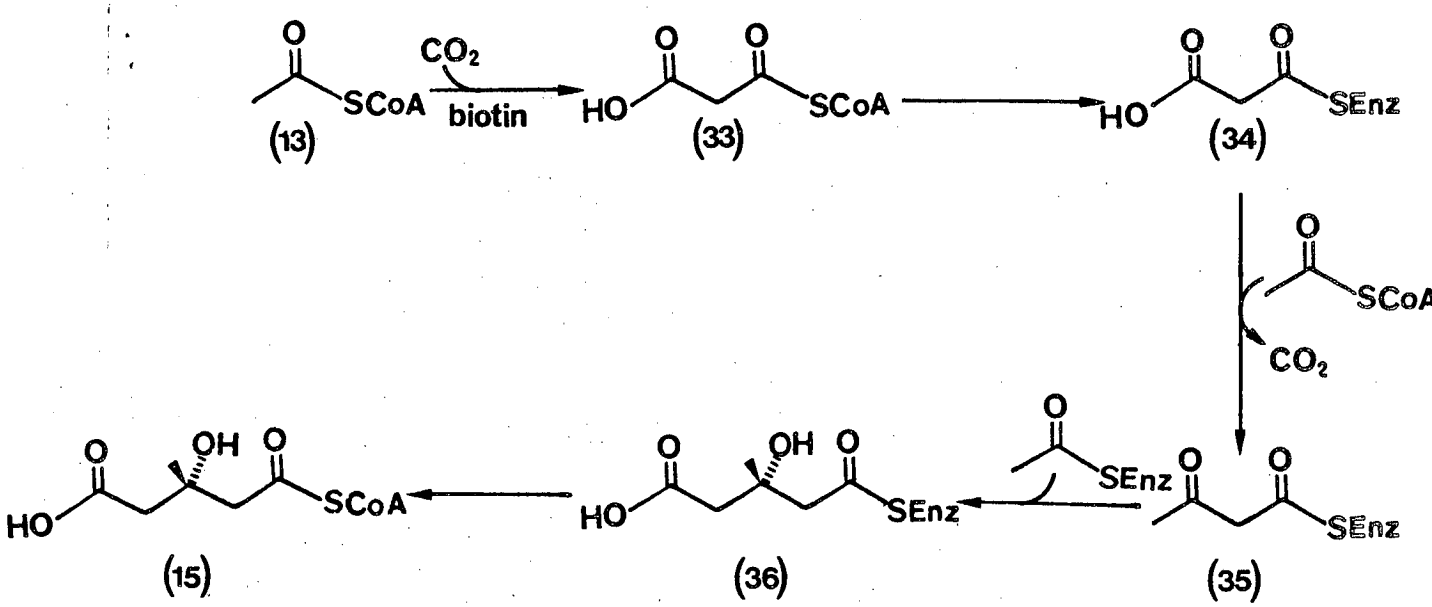
SCHEME 2

Leucine catabolism has also been invoked for the "shunt pathway" of mevalonate metabolism[35] (Scheme 2). The existence of this pathway is based on identification of ^{14}C labelled prenoates (e.g. 3,3-dimethylacrylate (31)) in tissues from rats injected with ^{14}C labelled mevalonate (16). Further support came from the distributions of ^{14}C in 3-hydroxybutyrates (28) formed by diabetic rats injected with various ^{14}C labelled mevalonates[35].

The labelling patterns show that:- C-1 of mevalonate (16) is lost in the formation of acetoacetate (27), C-2, 3 and 6 are present in acetoacetate (27) and C-5 becomes C-1 and 3 of acetoacetate (27) in keeping with the condensation of acetyl-CoA (13) units formed from mevalonate (16). It is argued that this "mevalonate recycling" occurs in the mitochondria and that it is the extramitochondrial pool of HMGCoA (15) that is the precursor of mevalonate (16)[35].

The role of malonate[36] in isoprenoid biosynthesis has been the subject of experimentation for many years and there is evidence to support an alternative pathway to HMGCoA (15) *via* malonyl CoA (33) (Scheme 3). The key difference between this alternative pathway and the "acetoacetate" pathway (Scheme 1) is the way in which the acetoacetyl thioester is formed. In the "acetoacetate" pathway, acetoacetyl-CoA (14) is formed by the condensation of two molecules of acetyl-CoA (13).

MALONATE PATHWAY

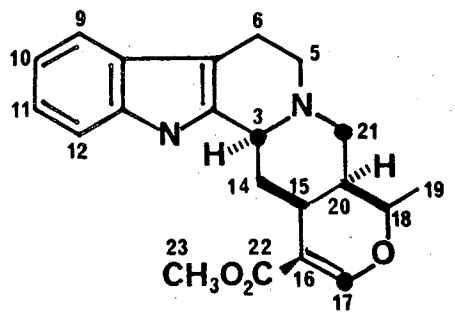


SCHEME 3

This reaction, which has an equilibrium constant (1.6×10^{-5} at pH 7.0) that is clearly in favour of thiolysis, is used to produce acetyl-CoA (13) in the oxidation of fatty acids. In the malonate pathway the acetoacetyl moiety is formed bound to protein, from a protein bound acetate molecule with the concomitant evolution of carbon dioxide. This reaction is already known as a stage of fatty acid biosynthesis and at first would appear to be the most likely pathway for the biosynthesis of acetoacetate. However, it has been shown[36] that incorporation of acetyl-CoA (13) into isoprenoids in cell free preparations

of rat liver was not inhibited by avidin and was not stimulated by bicarbonate. Avidin is a protein which binds very strongly to biotin (the prosthetic group of acetyl-CoA carboxylase) (step 1, Scheme 3). Thus lack of inhibition of isoprenoid biosynthesis by its presence suggests that carboxylation of acetyl-CoA and the malonate pathway are not involved in the synthesis of isoprenoids. Moreover, the labelling patterns of HMG-CoA (15) formed from [2-^{14}C] and [1,3-$^{14}C_2$] malonyl-CoA (33) in rat and pigeon liver preparations were those that would be expected if malonyl-CoA underwent decarboxylation to acetyl-CoA (13) before incorporation[36].

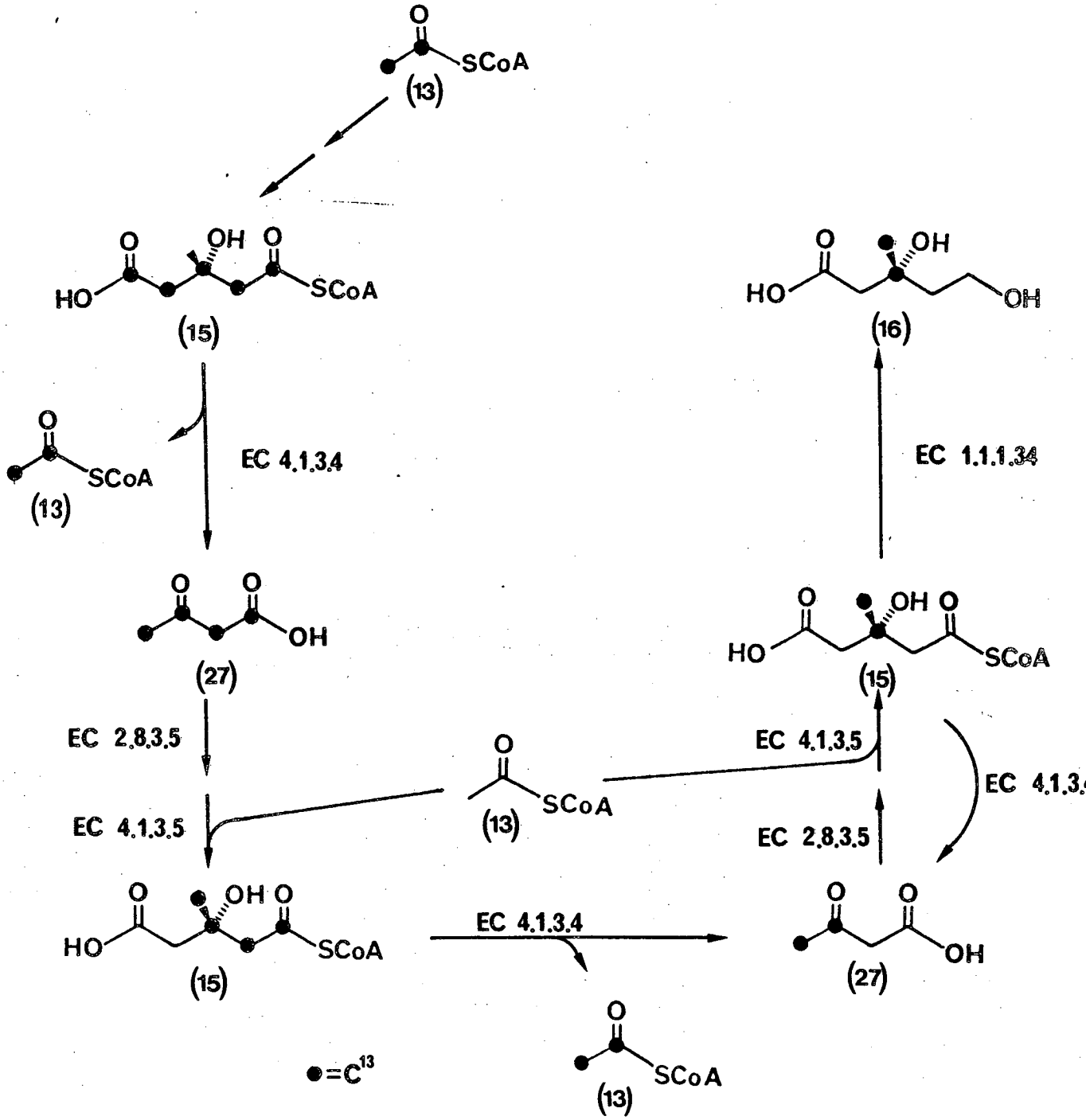
The incorporation of ^{13}C labelled acetates into the monoterpenoid derived C_{10} unit of indole alkaloids such as ajmalicine (37) from tissue cultures of the plant *Catharanthus roseus* have also been found to be at variance with the pattern predicted by the acetoacetate pathway[37].



(37)

predicted (1,2-$^{13}C_2$) acetate inc. pattern via acetoacetate pathway

The ^{13}C NMR spectrum of ajmalicine (37) derived from [2-^{13}C] acetate showed enrichment at C-16, 17, 18, 19 and 20 positions, [1-^{13}C] acetate derived ajmalicine (37) showed no enhancement of signal intensities over natural abundance and [1,2-$^{13}C_2$] acetate derived ajmalicine (37) showed the same pattern as [2-^{13}C] acetate but no multiplet signals corresponding to the incorporation of intact acetate units. The loss of acetate ^{13}C carboxy enrichment and the redistribution of ^{13}C labelling from the methyl group into two adjacent positions (which are formally derived from the same acetate molecule) indicates that extensive randomisation of acetate ^{13}C methyl enrichment *via* the Krebs cycle (see Appendix) occurs to afford a mixture of [2-^{13}C] and [1-^{13}C] acetate before incorporation to mevalonic acid (16). The regiospecificity of incorporation at the carbon atoms derived from the methyl and quaternary carbons suggests that HMGCoA (15) may be undergoing metabolic cycling (Scheme 4). The existence of such a futile cycle in the acetoacetate mevalonate pathway permits rationalisation of the variable enrichments obtained in earlier studies of terpenoid biosynthesis in higher plants[38].

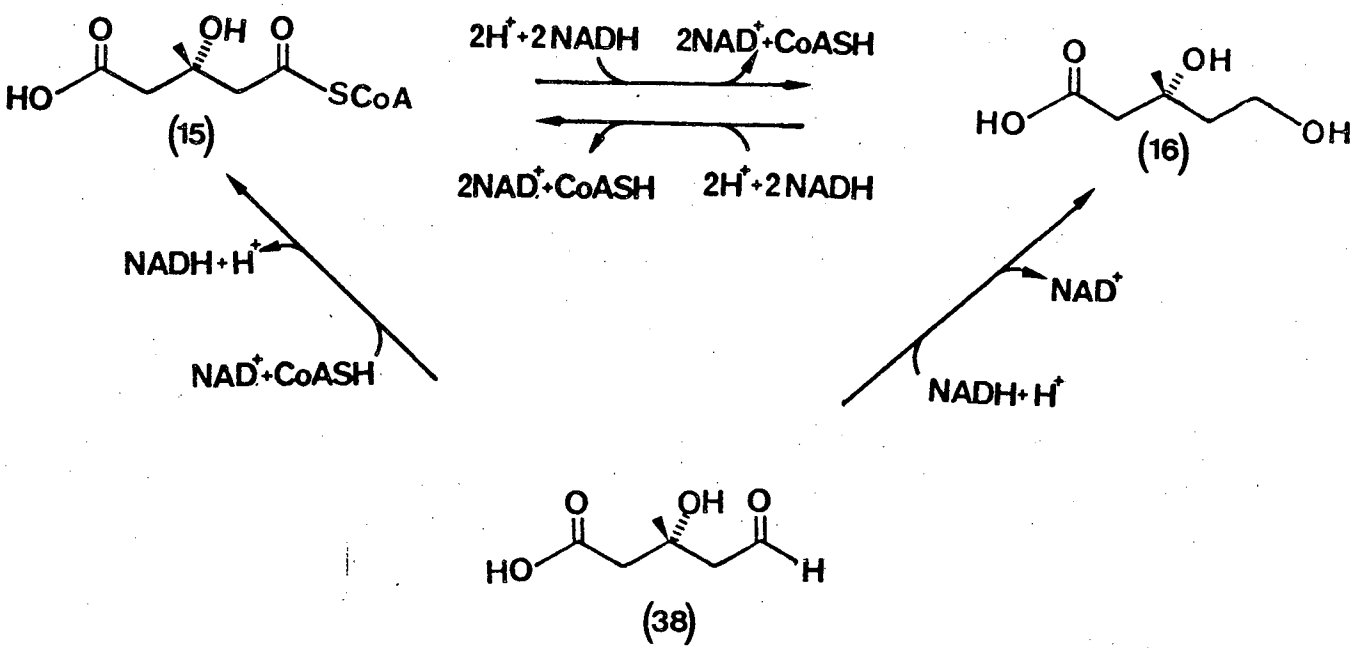

O
SCoA
(13)
OH
HO
(15)
EC 4.1.3.4
(16)
EC 1.1.1.34
(27)
EC 2.8.3.5
EC 4.1.3.5
EC 4.1.3.4
EC 4.1.3.5
EC 2.8.3.5
EC 4.1.3.4
●=C13

SCHEME 4

4. PROKARYOTIC ISOPRENOID BIOSYNTHESIS

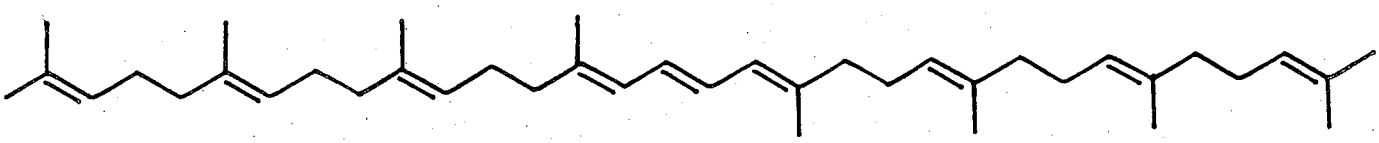
The acetoacetate pathway (Scheme 1), considered to be proven for eukaryotes, has been generally assumed to be the route utilised by prokaryotes. There is indeed some evidence to support this view[39-50].

A few of the acetoacetate pathway enzymes have been separated from bacteria and their properties investigated. HMGCoA reductase has been purified from *Pseudomonas*[39] to over 95% homogeneity and 38.9 μmol/mg min^{-1} specific activity by gel electrophoresis. It is unusual in that it appears to be a multifunctional enzyme which catalyses all of the reactions in Scheme 5. Mammalian liver and yeast



SCHEME 5

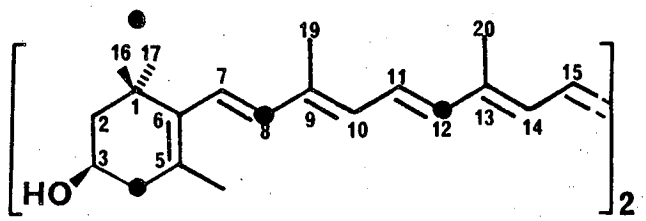
HMGCoA reductases are reported only to catalyse reductive deacylation of HMGCoA (15) to mevalonate (16) essentially irreversibly[39]. Mevalonate kinase has been found in a cell free extract from a phytoene (39) accumulating *Staphylococcus*[40,41] mutant and shown to convert mevalonic acid (16) into mevalonate-5-phosphate (17). Isopentenyl pyrophosphate isomerase has only recently been separated chromatographically from an extract of *Escherichia coli*[42] and assayed in a cell free extract of *Streptomyces arenae*[43]. In contrast to yeast and mammalian systems there is little information available on the enzymes of the acetoacetate pathway in bacteria. To date there have been no reports of bacterial HMGCoA synthase, mevalonate-5-phosphate kinase or mevalonate pyrophosphate decarboxylase.



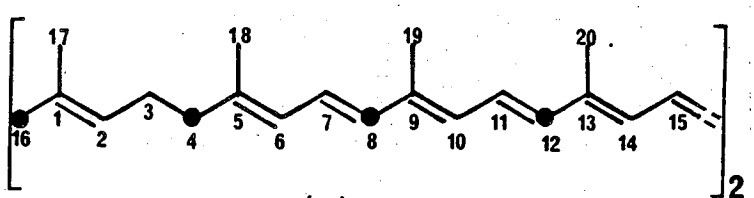
(39)

Some isotopic tracers have been incorporated into bacterial isoprenoids in a way consistent with the isoprenoid pathway. In studies on the mechanism of β-ring cyclisation in carotenoids, [2-^{13}C] mevalonate was incorporated into zeaxanthin (40) and its acyclic

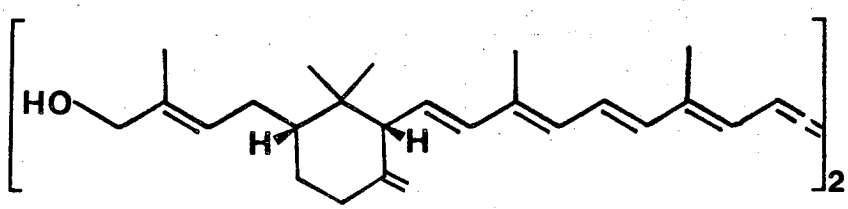
precursor lycopene (41) in *Flavobacterium*[44]. ^{13}C NMR analysis showed ^{13}C enrichment in both compounds at C-4, 8, 12, 16, 4′, 8′, 12′ and 16′ as would be expected *via* the acetoacetate pathway. Cell free systems from *Corynebacterium poinsettiae* and *Micrococcus luteus*[45] incorporated various ^{14}C and ^{3}H labelled mevalonic acids into acyclic C_{40} and cyclic C_{50} carotenoids, e.g. sarcinaxanthin (42). The $^{14}C/^{3}H$ ratios obtained were used to investigate the stereochemistry of β-, γ- and ε-ring formation.



(40) ● = C^{13}



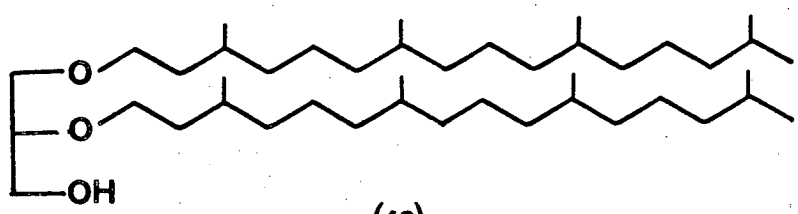
(41)



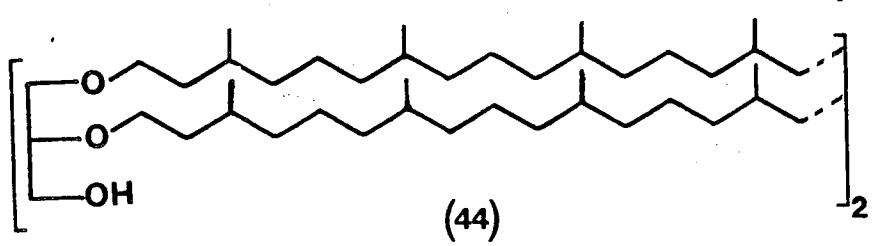
(42)

During whole cell studies on the elucidation of the chemical structure of unsaponifiable lipids, [2-^{14}C] mevalonic acid was incorporated into undecaprenol (C_{55}) in *Lactobacillus caesi*[46]. The conversion of [2-^{14}C] mevalonic acid into unsaponifiable lipids has also been demonstrated with lysates of *Lactobacillus plantarum*[47].

The total lipids of the archaebacterium *Methanaospirilium hungates*[48] were analysed by ^{13}C NMR after growth on ^{13}C labelled acetates and ^{13}C labelled carbon dioxide. In common with other archaebacteria 94% of the lipids were derivatives of diphantanyl glycerol (43) and bidiphantanyl glycerol (44). The labelling pattern, with each phytanyl C_5 chain fragment originating from one molecule of mevalonate, was consistent with the typical condensation of three acetate units to form one mevalonate (16) molecule.

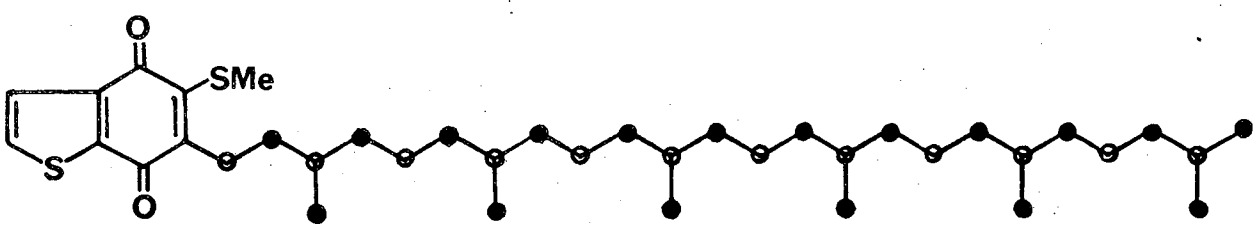


(43)



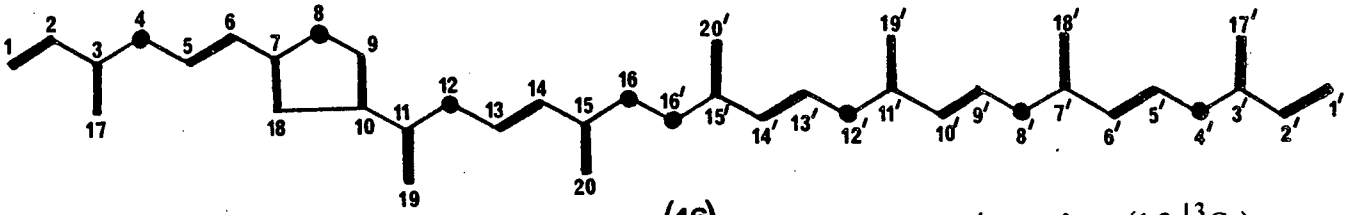
(44)

The same ^{13}C acetate labelling patterns were observed in the lipids of the extreme thermophilic archaebacterium *Caldariella acidophila*[49]. Structure (45) shows the mixed biogenesis metabolite caldariellaquinone[49] after [1-^{13}C] and [2-^{13}C] acetate incorporations. Moreover, the ^{13}C NMR spectra of its hydrocarbons[50] (46)-(49) after incorporation of [1,2-$^{13}C_2$] acetate showed C-4, 4', 8, 8', 12, 12', 16 and 16' as singlets of enhanced intensity and the rest as multiplets with satellite pairs due to ^{13}C-^{13}C coupling ($^{1}J_{CC}$≈35 Hz). This indicated incorporation of two intact acetate residues per C_5 unit as would be expected *via* the acetoacetate pathway.

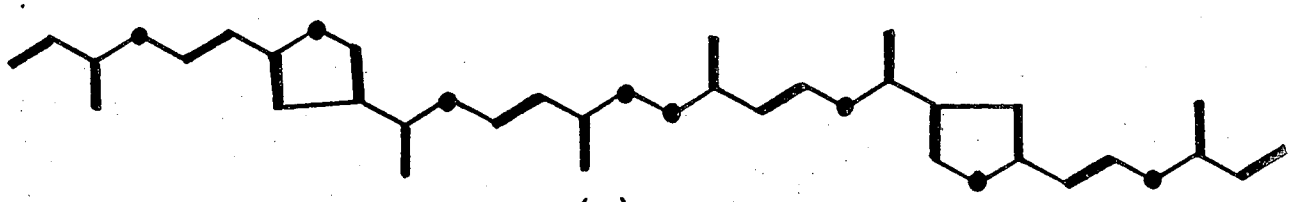


(45)

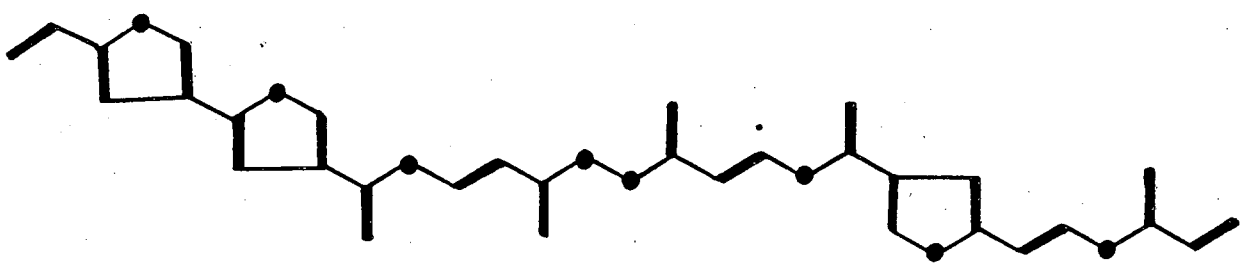
○ from (1-^{13}C) acetate ● from (2-^{13}C) acetate



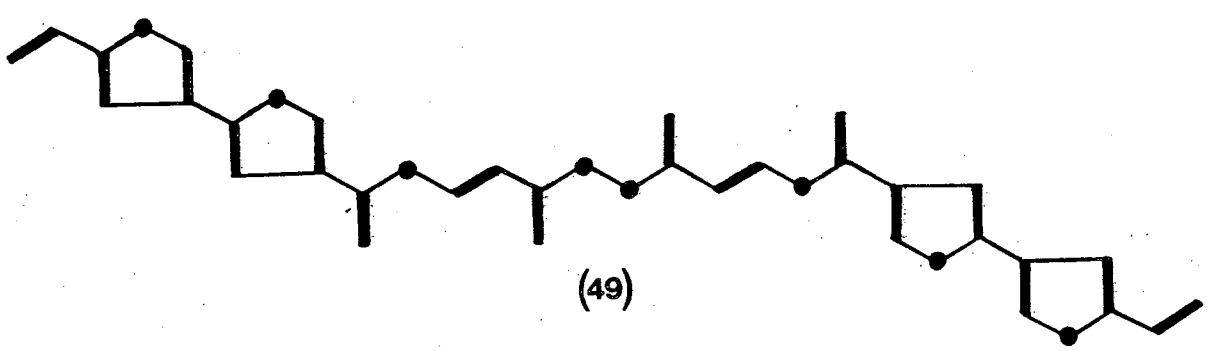
(46) ▬ and ● from (1,2-$^{13}C_2$) acetate



(47)



(48)

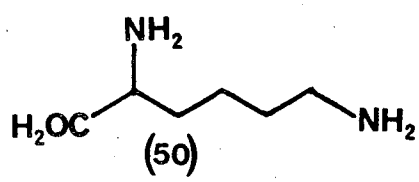


(49)

5. ALTERNATIVE PROKARYOTIC PATHWAYS

As with eukaryotes, a number of analogous incorporations of isotopically enriched precursors have been described and alternative pathways proposed[51,52].

It has been reported recently that mevalonic acid is not synthesised from three acetyl-CoA units in the aerobic archaebacteria *Halobacterium curtirubrum* and *Halobacterium halobium*[51]. ^{13}C Labelling studies have confirmed the incorporation of acetate into phytanyl chains (43) and (44), presumably *via* acetyl-CoA, but revealed an unexpected lack of label transfer from acetate, glycerol or alanine to the branch methyl and methine carbon atoms. Thus, it appears that of the three two carbon units normally used as precursors of mevalonate (16) only two originate from acetate. ^{14}C Labelled amino acids, especially leucine, were found to label the lipids but the most significant incorporation was found with lysine (50).



(50)

This is quite surprising as there is no knowledge of any metabolic route for converting lysine to the phytanyl chains of lipids. A general scheme for the utilisation of lysine is shown (Figure 4).

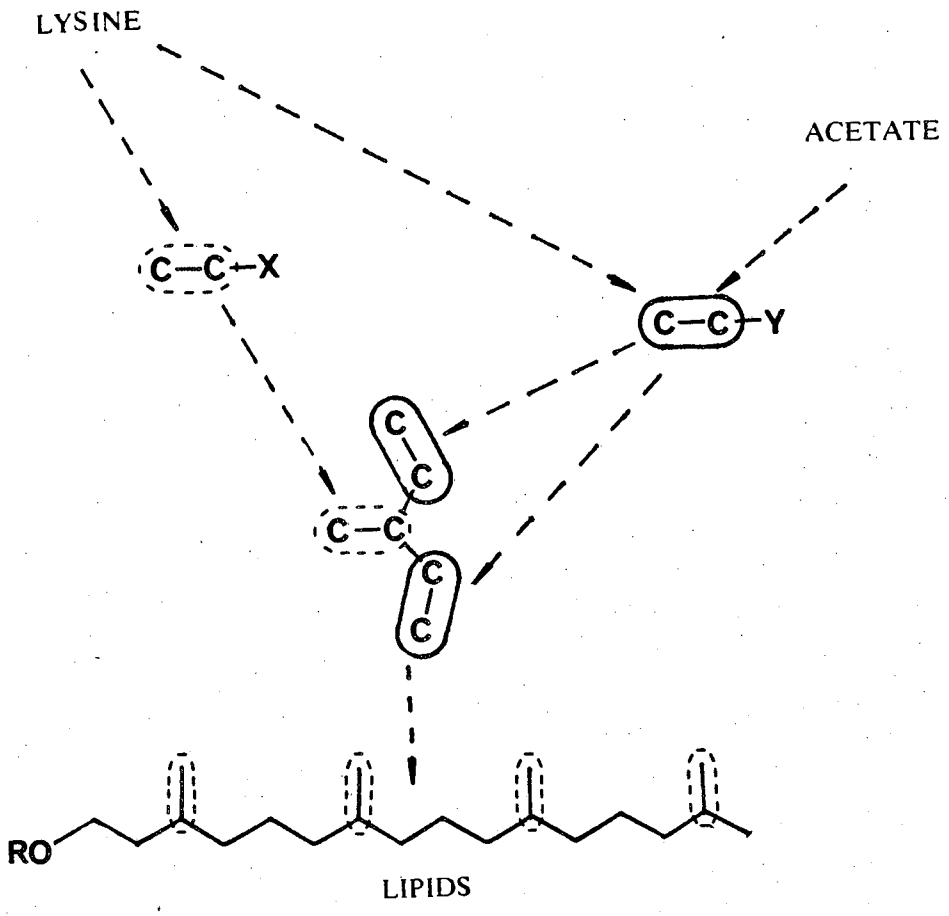
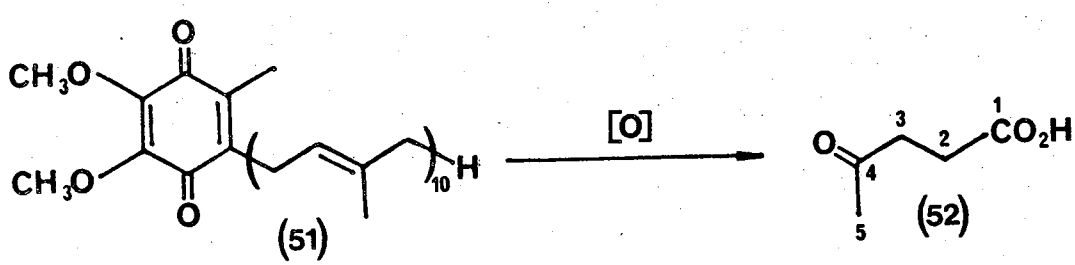


FIGURE 4.

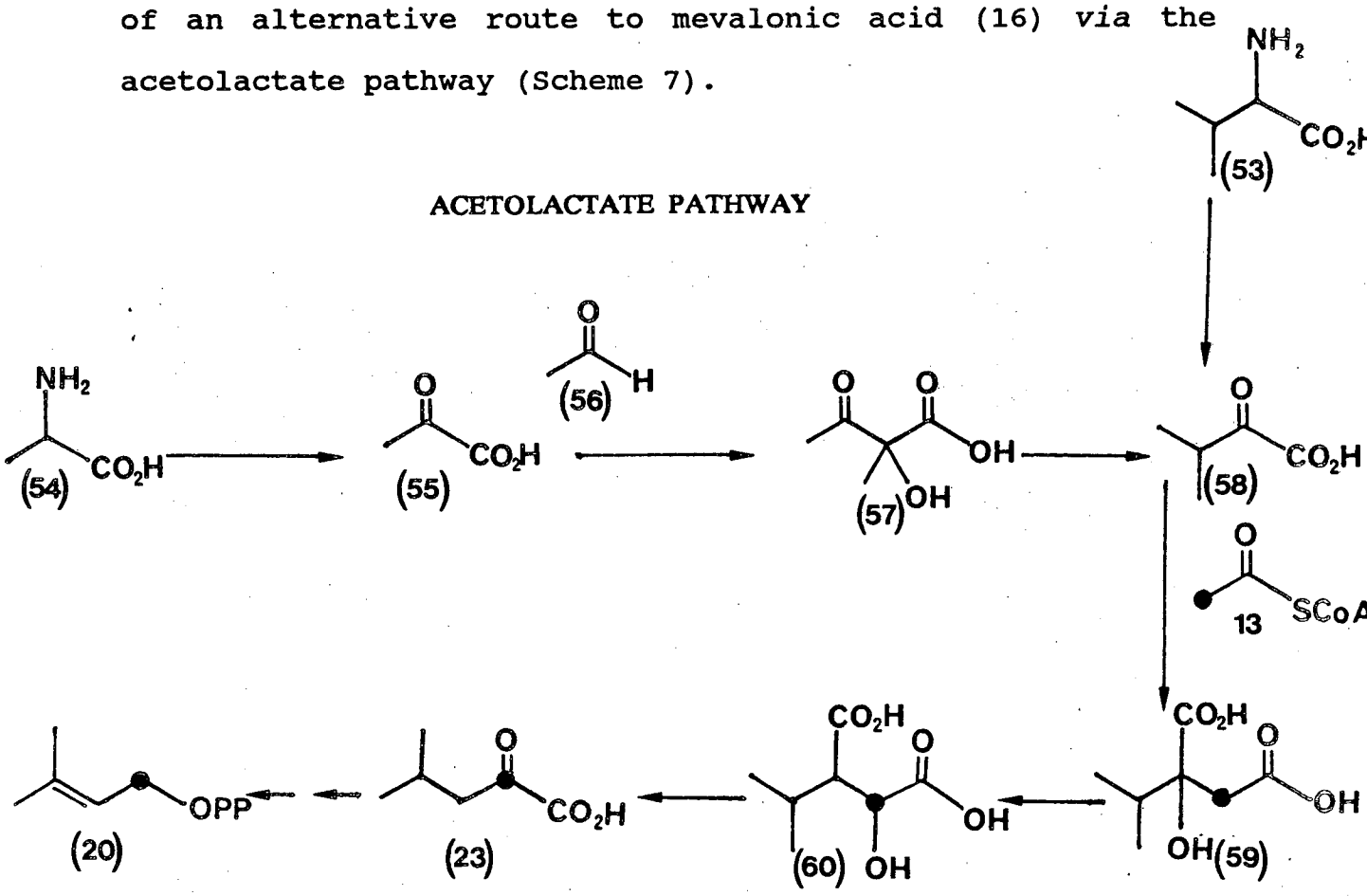
It is possible that the general scheme of the biosynthesis is the same as in higher organisms proceeding *via* acetyl-CoA and the basic difference lies in the enzymes used by halobacteria. Operation of a mixed thiolase, which could use acetyl-CoA derived from acetate and acetyl-X derived from degraded amino acids, would explain the labelling pattern of the phytanyl chains observed in halobacteria. The malonate pathway (Scheme 3) has also been invoked as a possible explanation for these anomalous results[51].

The pattern of incorporation of radioactivity from [1-^{14}C] acetate and [2-^{14}C] acetate into the polyprenyl side chain of ubiquinone-10 (51) in *Rhodopseudomonus capsulata*[52] has also been studied. Labels from both ^{14}C labelled acetates were found to be incorporated exclusively into the C-2 position of laevulinic acid (52) (a degradation product of the polyprenyl side chain) suggesting that the acetoacetate pathway was not operative in this bacterium.



SCHEME 6

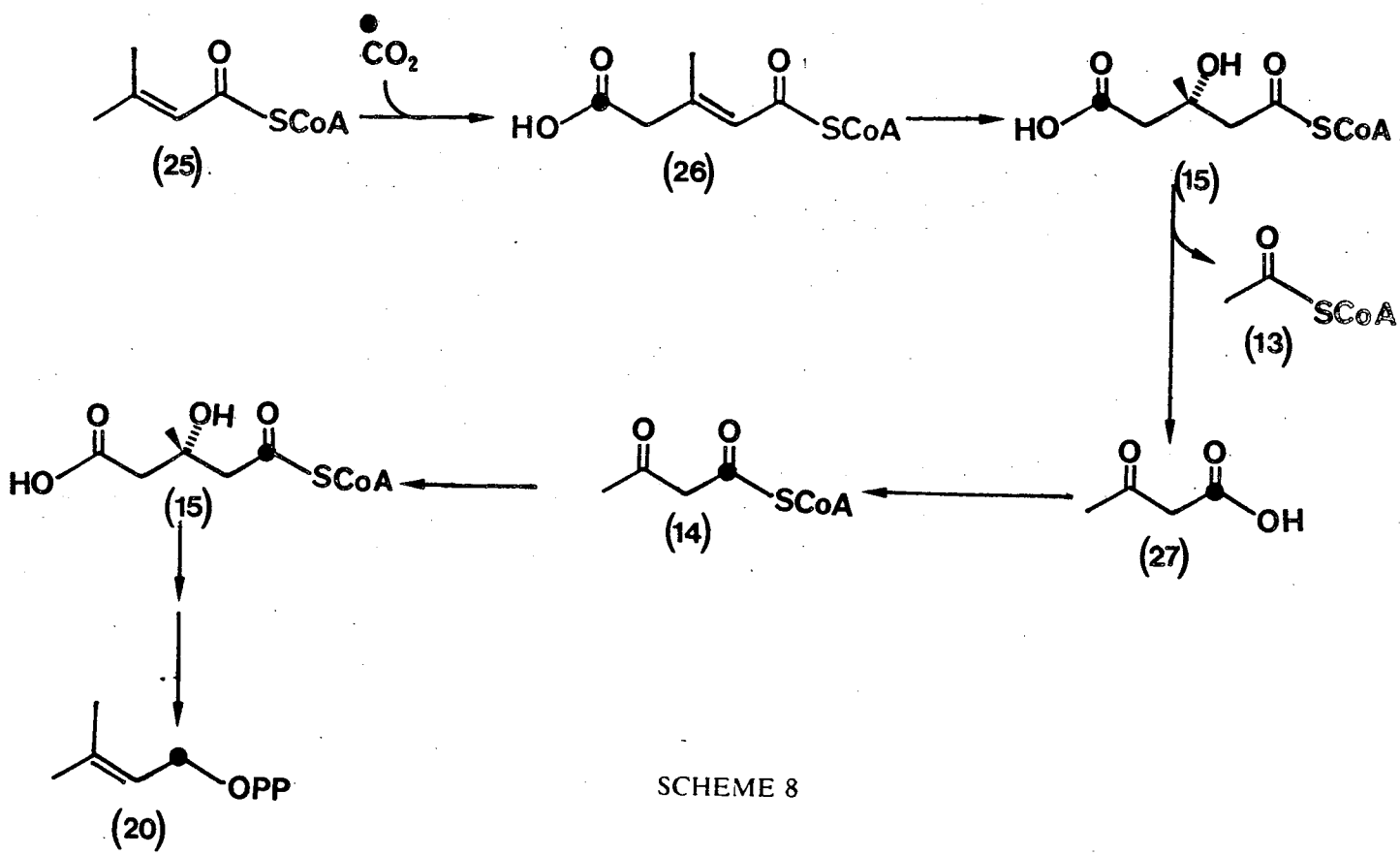
Furthermore, the patterns of incorporation of radioactivity from [U-^{14}C] valine (53), [U-^{14}C] alanine (54) and [^{14}C] sodium bicarbonate into the side chain of ubiquinone-10 (51) in *Rh. capsulata* were used as evidence of an alternative route to mevalonic acid (16) *via* the acetolactate pathway (Scheme 7).



SCHEME 7

The sequence begins with the condensation of pyruvate (55) (a degradation product of alanine (54)) and acetaldehyde (56) to give acetolactate (57) which rearranges to give 2-oxoisovalerate (58) (a degradation product of valine (53)). A 1,2 shift of the tertiary alcohol (59) followed by oxidation gives 2-oxo-4-methylpentanoate (23) which is then said to elaborate isoprenoids *via* leucine catabolism

(see Scheme 2). Radioactivity from [U-^{14}C] alanine and [U-^{14}C] valine was distributed evenly among the C-1, 3, 4 and 5 carbons of laevulinic acid (52) with negligible radioactivity in C-2. Radioactivity from [^{14}C] sodium bicarbonate was incorporated exclusively into the C-2 carbon of laevulinic acid (52). It was proposed that incorporation of the labelled C-2 carbon of acetate was also *via* carbon dioxide. A mechanism for the incorporation of carbon dioxide is shown in Scheme 8. All of these results are fully in accordance with biosynthesis via the acetolactate pathway and although it cannot be argued that all bacteria synthesise isoprenoids through the acetolactate pathway preliminary evidence has also been claimed for its existence in *Lactobacillus caesi*[52].

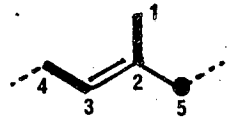
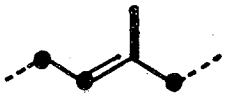
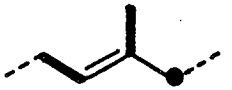
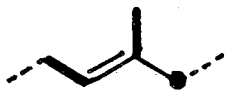
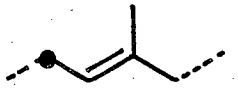
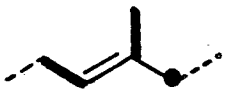
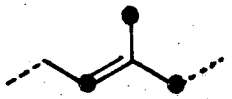
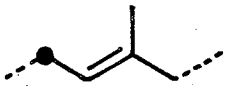
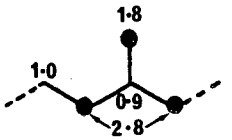
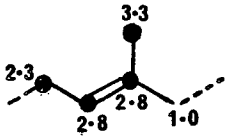
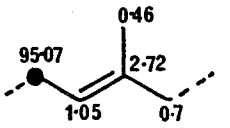
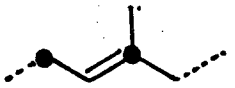
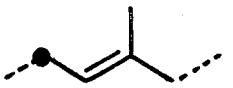
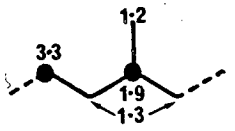
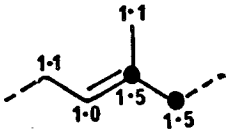
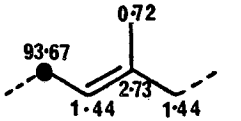
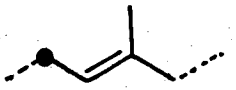
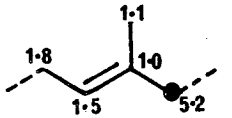
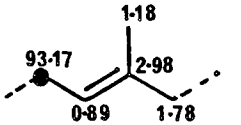
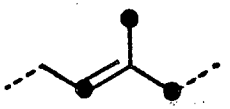
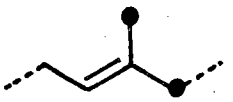
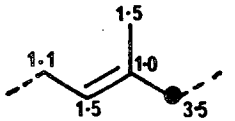
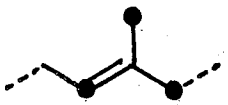
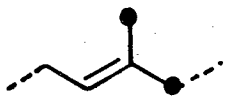
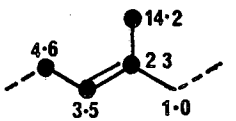
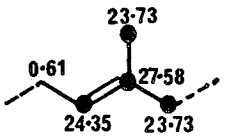
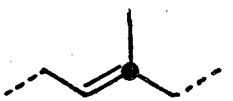
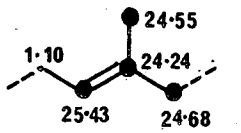


SCHEME 8

6. PRELIMINARY FEEDING STUDIES

In an earlier study in this laboratory[53] a series of stable isotope precursor feeding experiments were carried out in *Rh. capsulata* to elucidate whether the acetolactate pathway was indeed involved in the generation of the isoprenoid side chain of ubiquinone-10 (51). The results of these earlier studies are shown in Table 1 together with:- the anticipated labelling patterns which would arise from the acetolactate and acetoacetate routes, the observed incorporations of radioactive precursors leading to the proposed acetolactate pathway and the stable isotope incorporation patterns in the polyprenyl side chain of caldariellaquinone (45) from *Caldariella acidophila*[49].

In general terms the stable isotope studies in *Rh. capsulata*[53] and *Cald. acidophila*[49] are at variance with the radioisotope study in *Rh. capsulata*[52] although relatively consistent with each other. In particular, the stable isotope studies show much less specific incorporation of ^{13}C labelled acetates into the isoprene C-4 carbon (laevulinic C-2) and there was no incorporation of [3-^{13}C] LD-valine into ubiquinone-10 (51) in *Rh. capsulata* as would be predicted by the acetolactate pathway.

PRECURSOR	PREDICTED INCORPORATIONS: ACETOACETATE	PREDICTED INCORPORATIONS: ACETOLACTATE	OBSERVED INCORPORATIONS: CALD. ACIDOPHILA CALDARIELLAQUINONE	OBSERVED INCORPORATIONS: * RH.CAPSULATA UBIQUINONE 10	OBSERVED INCORPORATIONS: ** RH. CAPSULATA UBIQUINONE 10
(U-$^{13}C_6$) glucose					
(1,2-$^{13}C_2$) acetate					
(2-^{13}C) acetate					
(1-^{13}C) acetate					
$NaH^{13}CO_3$	none				
(3,4-^{13}C) glucose					
(3-^{13}C) DL-alanine					
(3-^{13}C) DL-valine	none			none	

* Obtained from preliminary feeding studies done in this laboratory, expressed as a multiple of ^{13}C NMR natural abundance intensities.

** Expressed as a % of total incorporation

TABLE 1

Stable isotope [$^{13}C_6$] glucose and [$^{13}C_2$] acetate studies in *Rh. capsulata* showed the expected acetoacetate pathway incorporation patterns, with ^{13}C-^{13}C couplings indicating incorporation of two intact acetate molecules per isoprene unit as observed with the hydrocarbons (46)-(49) of *Cald. acidophila*[50]. Singly labelled stable isotope [^{13}C] acetates however, did not show the expected acetoacetate or acetolactate incorporation patterns, both [1-^{13}C] acetate and [2-^{13}C] acetate studies show scrambling but more conspicuously, anomalous incorporations in the isoprene C-5 carbon.

No comparison with the ^{13}C incorporation patterns of the unsaponifiable lipids of *Cald. acidophila* could be made at this point. In the ^{13}C NMR spectrum of caldariellaquinone[49] (45) no distinction could be made between the isoprene C-5 and C-3 carbons and no information on the intensities of incorporations of ^{13}C labelled acetates into the hydrocarbons (46)-(49) has been reported.

In *Rh. capsulata* [1-^{13}C] acetate gave significantly higher incorporation into the isoprene C-5 carbon than predicted by either the acetoacetate or acetolactate pathways. It is conjectured that [1-^{13}C] acetate is losing its ^{13}C enrichment in the form of carbon dioxide while being metabolised in the Kreb's cycle (see Appendix). This carbon dioxide is then reincorporated *via* the Calvin cycle (see Appendix) into glucose-6-phosphate

and ultimately ubiquinone-10 (51). In support of this view [^{13}C] sodium bicarbonate was incorporated specifically at the isoprene C-5 position.

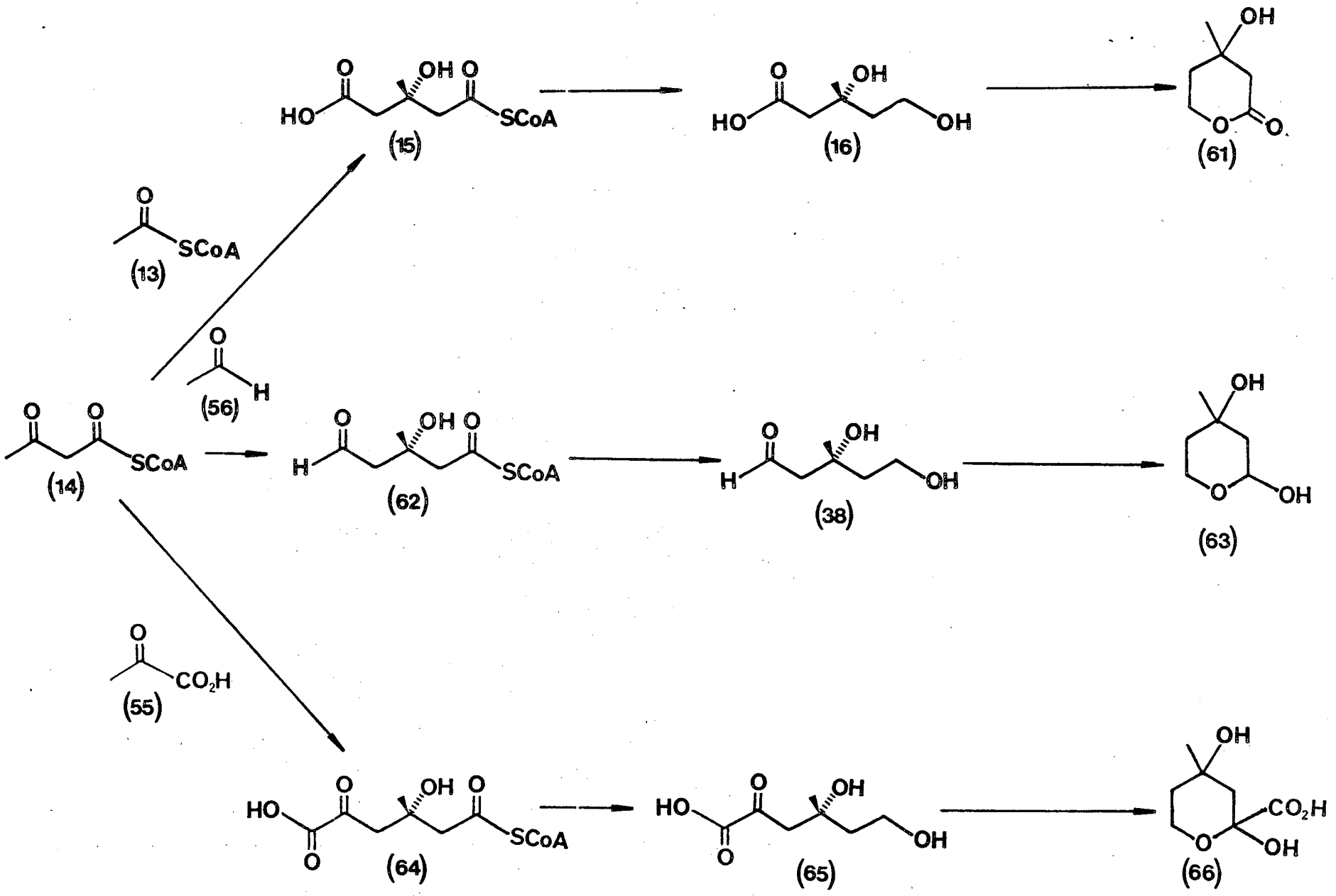
Absence of ^{13}C enrichment at the C-5 isoprene carbon after [2-^{13}C] acetate incorporation in *Rh. capsulata* together with the [1-^{13}C] acetate incorporation pattern suggested that a molecular species other than a third acetate may be condensing with acetoacetyl-CoA (14) in the biosynthesis of isoprenoids. It was subsequently proposed that either pyruvate (55) or its biological equivalent acetaldehyde (56) might be involved. This view was supported by the incorporation of [3,4-$^{13}C_2$] glucose specifically into the isoprene C-5 carbon. [3,4-$^{13}C_2$] Glucose is metabolised *via* the Entner-Doudoroff pathway (see Appendix) to [3-^{13}C] pyruvate and [1-^{13}C] glyceraldehyde-3-phosphate. In the event of an analogous pyruvate-acetoacetyl-CoA condensation to that of acetyl-CoA (13) and acetoacetyl-CoA (14), [3-^{13}C] pyruvate would enrich the isoprene C-5 carbon as was observed.

The condensation of pyruvate (55) and acetoacetyl-CoA (14) would also explain the [^{13}C] bicarbonate and [1-^{13}C] acetate results. [^{13}C] Carbon dioxide would give [3-^{13}C] glucose-6-phosphate after incorporation *via* the Calvin cycle, subsequent metabolism *via* the Entner-Doudoroff cycle would give [3-^{13}C] pyruvate and condensation with acetoacetyl-CoA would enrich the isoprene C-5 carbon as was observed.

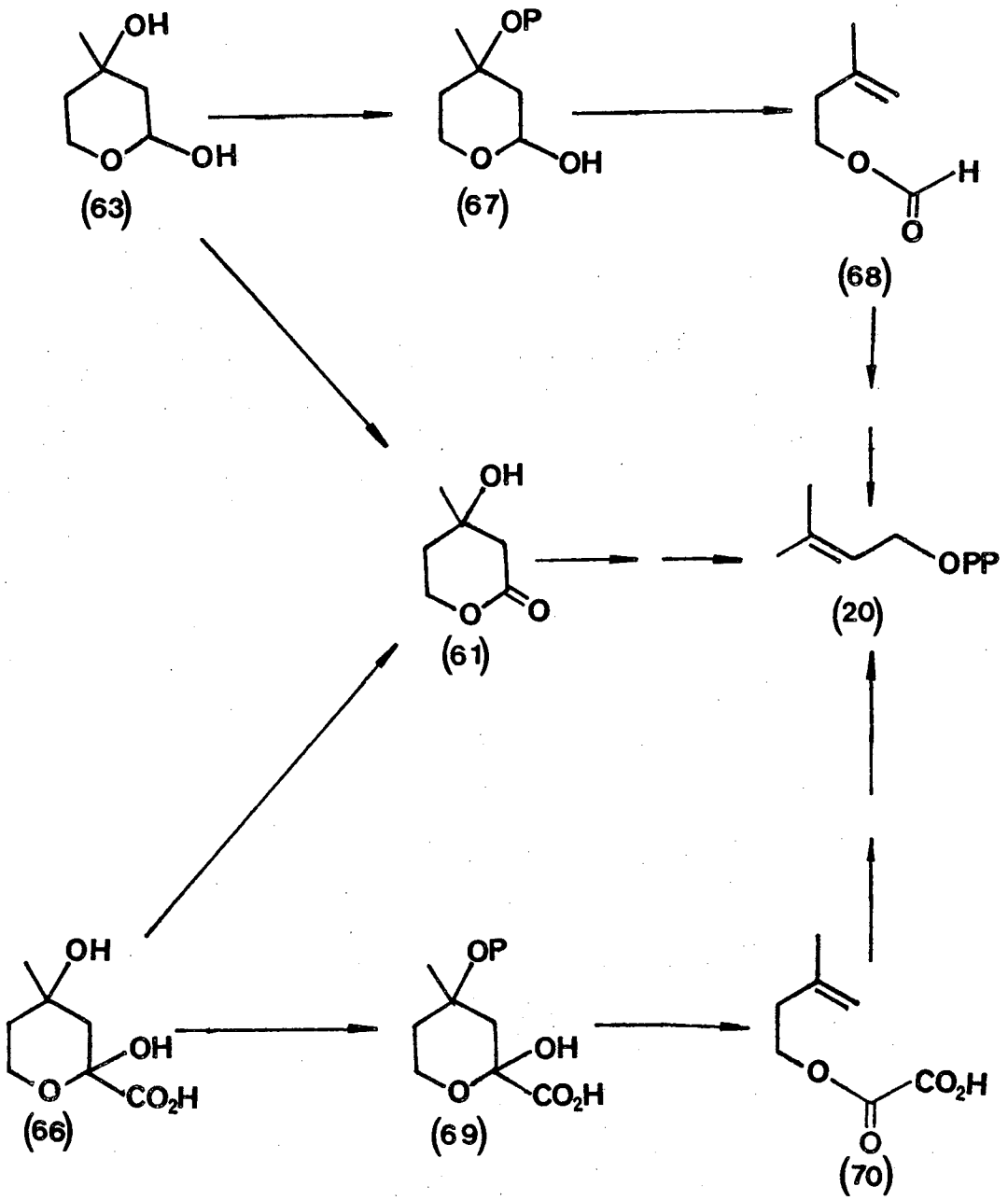
The result of pyruvate (55) and acetaldehyde (56) condensations with acetoacetyl-CoA are shown in Scheme 9. Reduction of the HMGCoA analogues (62) and (64) in an analogous way to that of HMGCoA (15) would give the mevalonic acid analogues (38) and (65). Subsequent cyclisation would give 2,4-dihydroxy-4-methyltetrahydropyran (63) and 2-carboxy-2,4-dihydroxy-4-methyltetrahydropyran (66). Isopentenyl pyrophosphate formation from these tetrahydropyrans could be envisaged *via* the mechanisms shown (Scheme 10).

In order to test these possibilities it was necessary to synthesise the tetrahydropyrans (63) and (66) in a labelled form. Specific incorporation of these advanced precursors would then definitively prove the existence of this alternative pathway to isoprenoids in *Rh. capsulata*.

The remainder of this thesis describes synthetic approaches towards these tetrahydropyrans, the results of advanced precursor feeding studies and the assay of HMGCoA synthase in *Rh. capsulata*.



SCHEME 9

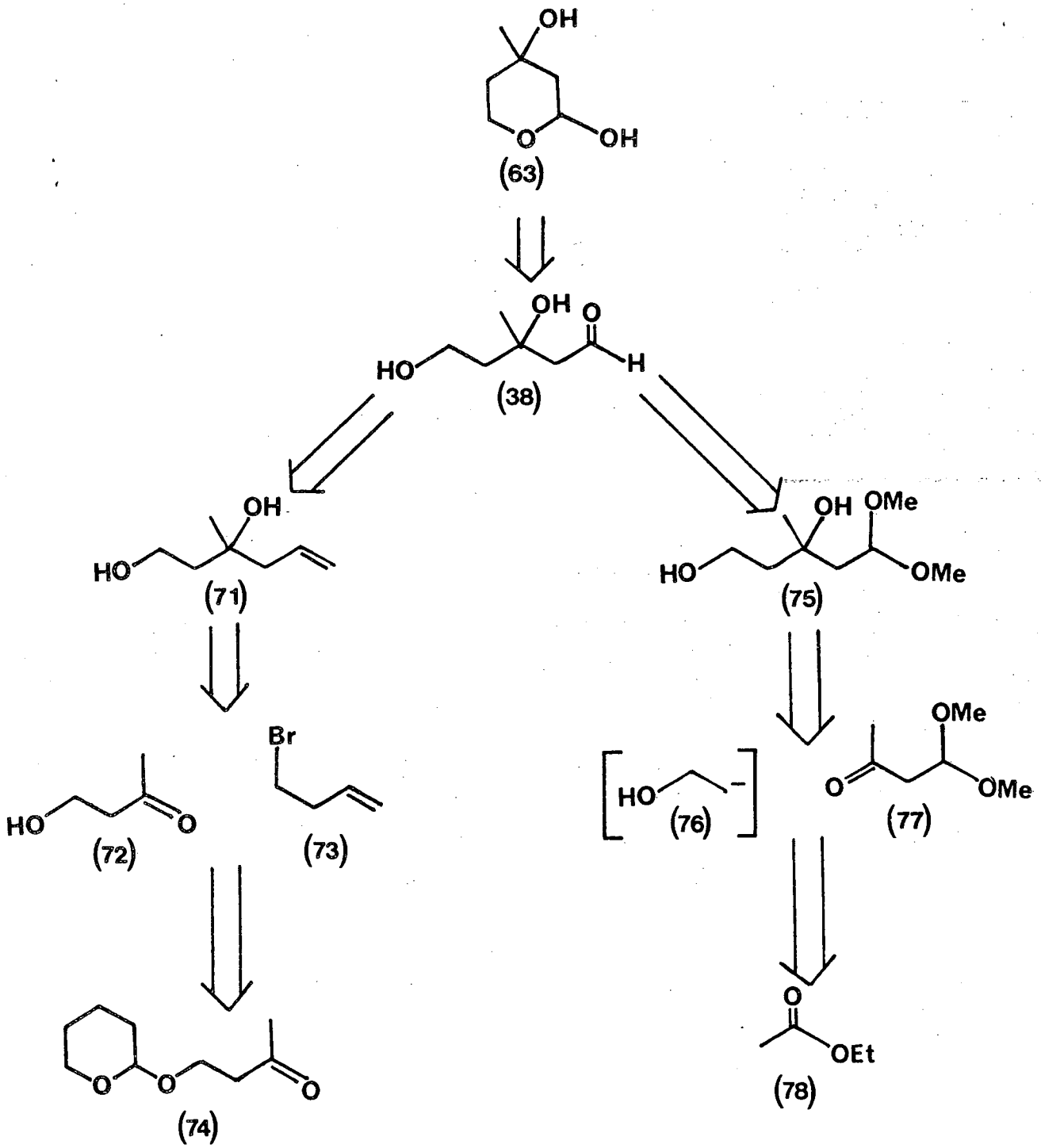

OH
OH
O
(63)
OP
OH
O
(67)
O
H
O
(68)
OH
O
O
(61)
OPP
(20)
OH
OH
CO2H
O
(66)
OP
OH
CO2H
O
(69)
O
CO2H
O
(70)

SCHEME 10

CHAPTER 1: SYNTHESIS OF 2,4-DIHYDROXY-4-METHYL-TETRAHYDROPYRAN (63)

1.1 RETROSYNTHETIC ANALYSIS

Retrosynthetic analysis[54,55,56] of the structure (63) was carried out and revealed many plausible routes. Outlined in Scheme 11 are two of the most promising.



SCHEME 11

Cleavage of the hemiacetal (63) *endo* C-O bond results in aldehyde and alcohol functionalities (38) which would be expected to spontaneously cyclise. The immediate synthetic precursor of (38) would therefore logically have to contain a protected aldehyde moiety. Two possible protection strategies involve either generation of the aldehyde from ozonolysis of the alkene (71) (Strategy 1) or protection of the aldehyde as an acetal (75) and subsequent deprotection by mild acid hydrolysis (Strategy 2).

In Strategy 1, synthesis of the alkene (71) could be effected by Grignard Reaction of allyl bromide (73) with 3-oxobutan-1-ol (72) although it would be more economic to protect the alcohol and use only one equivalent of Grignard reagent. Tetrahydropyranyl protection appeared to be a good choice in this case as the group is stable to Grignard conditions and easily removed by mild acid catalysis.

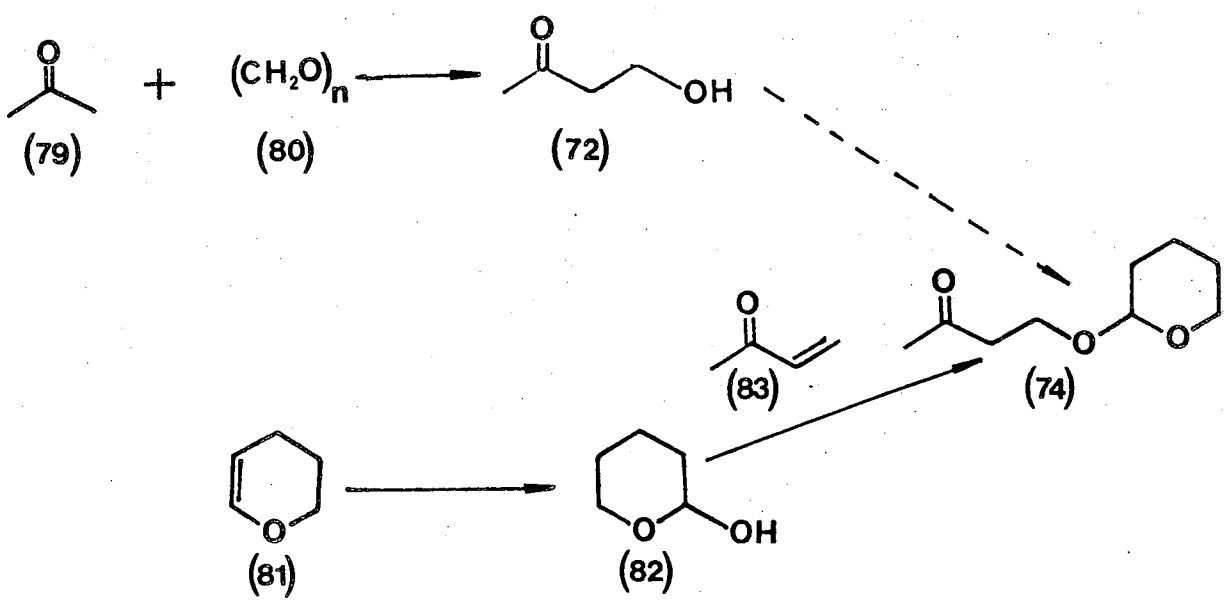
In Strategy 2, cleavage of the acetal (75) as shown reveals the ketoacetal (77) and a two carbon anion synthon (76), the synthetic equivalent of which is ethyl acetate (78). The C-C bond could thus be created by reaction of the lithium enolate of ethyl acetate (78) with the ketone of the ketoacetal (77). Subsequent lithium aluminium hydride reduction of the ester would give the primary alcohol moiety.

1.2 STRATEGY 1

1.2.1 Establishing a Route

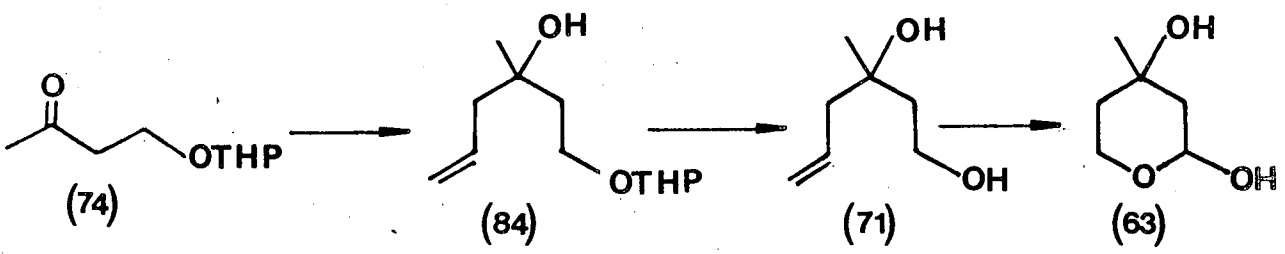
Initial attempts at synthesis of 3-oxobutan-1-ol (72) from acetone (79) and paraformaldehyde (80) using one equivalent of 3N methanolic potassium hydroxide as base gave low yields (reported as 27%)[57] of impure product. Attempts to purify the product by fractional distillation and chromatography were unsuccessful and further synthesis using the compound at the achievable level of purity led to intractable mixtures.

A more successful route invoked a Michael reaction. Hydration of dihydropyran[58] (81) using hydrochloric acid gave 2-hydroxytetrahydropyran (67%) (82) which, when reacted with methyl vinyl ketone (83) in the presence of a catalytic amount of 3N methanolic potassium hydroxide[59], gave a 67% yield of 1-(tetrahydropyranyl-2-oxy)-butan-3-one (74).



SCHEME 12

Grignard reaction of allyl bromide (73) with the ketone (74) gave a mixture of the monoprotected diol (50%) (84) and the unprotected diol (22%) (71). Acid catalysed hydrolysis of the tetrahydropyranyl protection[60] from (84) gave the diol (71) which was subjected to ozonolysis[61] at -78°C to give a high yield (90%) of the dihydroxytetrahydropyran (63) on reductive work up with triphenylphosphine. The structure of the tetrahydropyran (63) is discussed in Part 1.5.



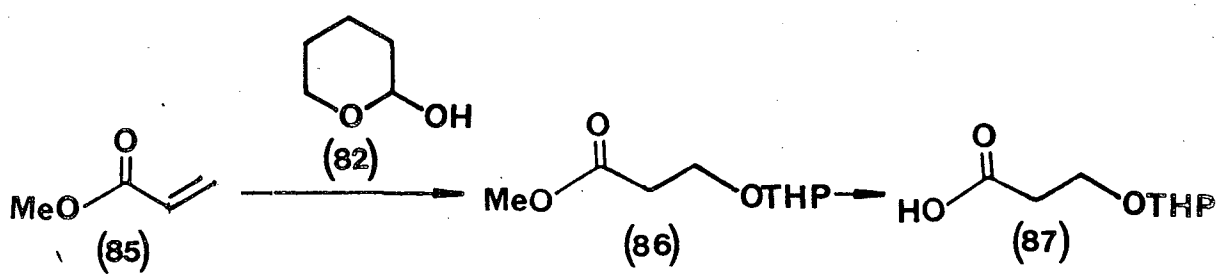
SCHEME 13

1.2.2 Labelled Synthesis of (63)

Analysis of Strategy 1 reveals 1-(tetrahydropyranyl-2-oxy)-butan-3-one (74) to be the most feasible target for inserting a stable carbon isotope as labelled allyl bromide (73) cannot be made cheaply. Labelling the methyl group of the ketone (74) (which becomes the 4-methyl in the dihydroxytetrahydropyran (63)) requires a synthesis of

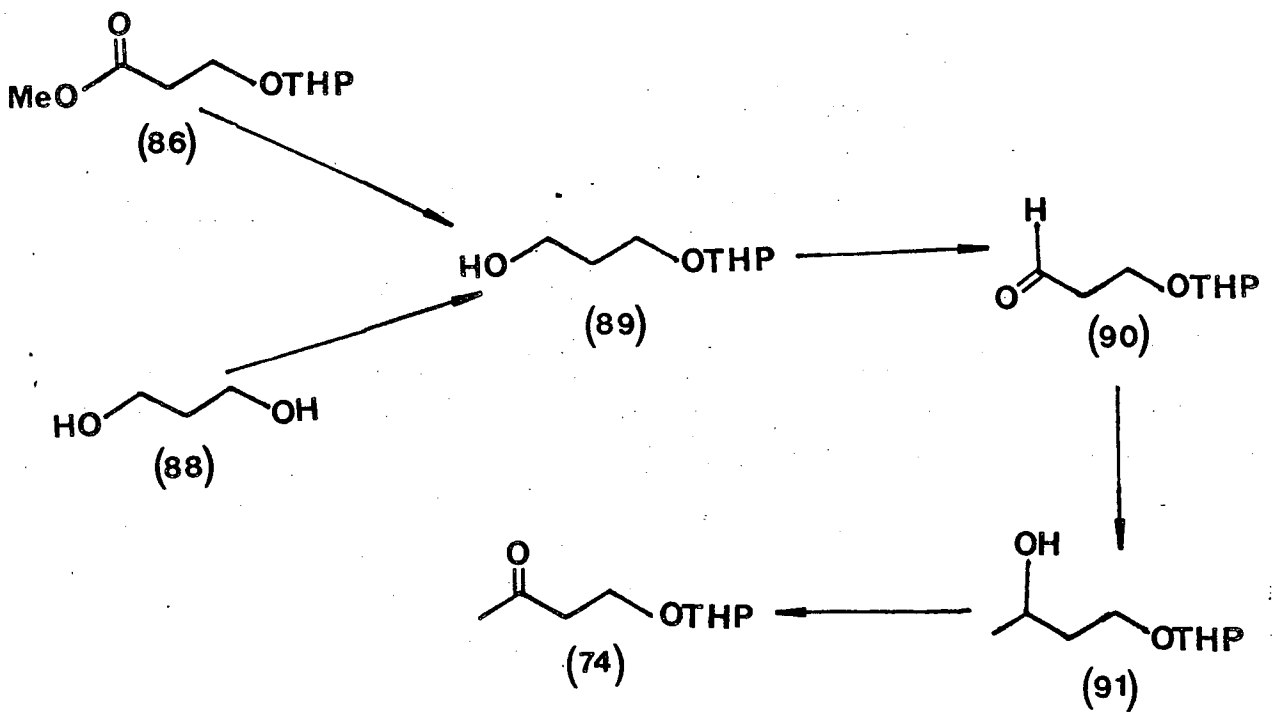
the ketone (74) in which introduction of this carbon could be made at a late stage. The standard routes for introduction of a methyl group include use of organocadmium reagents[62], Grignard reaction on nitriles[63], alkyl lithium reagents with lithium salts of carboxylic acids[64] and reaction of a methyl Grignard reagent with an aldehyde followed by oxidation of the secondary alcohol. Of these options the latter two were tried.

2-Hydroxytetrahydropyran (82) formed a tetrahydropyranyl protected β-hydroxyester (86) on reaction with methyl acrylate (85)[59] and methanolic potassium hydroxide in a Michael reaction analogous to that used in Scheme 12. Saponification of the ester (86) with 10% sodium hydroxide gave the free acid (87). Attempts to form the ketone (74) on reaction of the corresponding lithium salt of (87) with methyl lithium[65] were unsuccessful. While it is not certain why this reaction failed, the isolation of 2-hydroxytetrahydropyran (82) as a principal product suggested that degradation of the acid (87) possibly *via* a retro-Michael reaction (Scheme 14) had occurred.



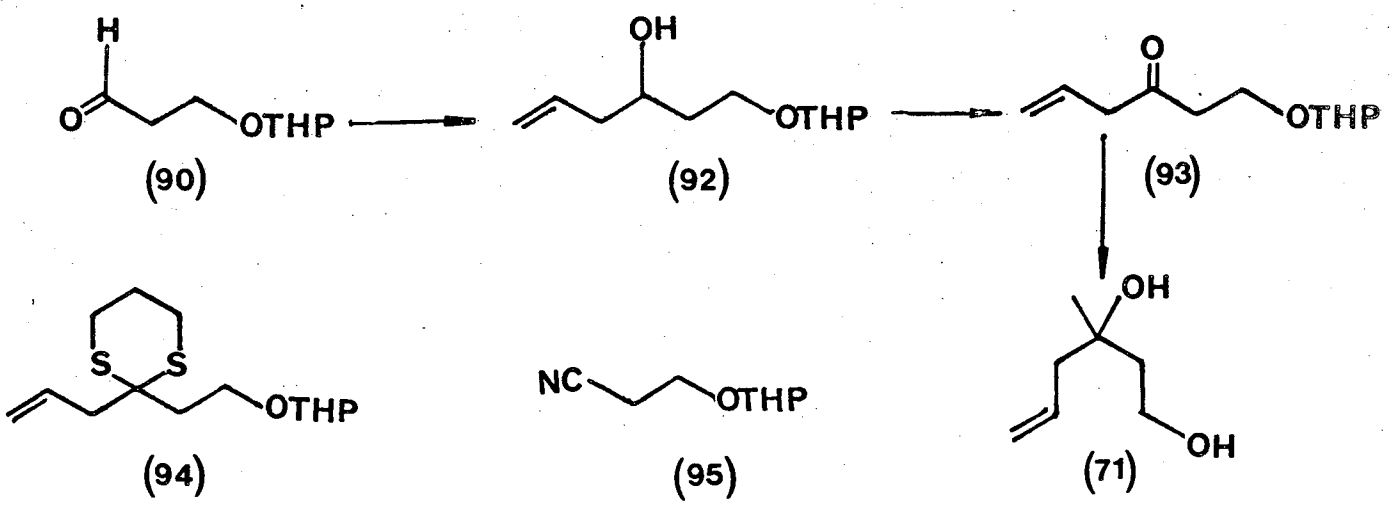
SCHEME 14

The second strategy outlined in Scheme 15 proved more useful. The monoprotected diol (89) was prepared for initial experiments from the β-hydroxyester (86) by reduction with lithium aluminium hydride[66]. Subsequently, it was found more convenient to prepare this compound (89) by reaction of propane-1,3-diol (88) with one equivalent of dihydropyran (81)[58] albeit in only 37% yield. This latter route was justified by the economy and ease of reaction. Oxidation of the alcohol (89) with pyridinium chlorochromate[67] to the aldehyde (90), followed by Grignard reaction[61] with methyl magnesium iodide and oxidation of the resulting secondary alcohol (91) with pyridinium chlorochromate gave the target ketone (74) in an overall 10% yield from the monoprotected diol (89).



SCHEME 15

Since the stable carbon isotope was to be introduced in the form of iodomethane, the overall yield of label incorporated into the hydroxytetrahydropyran (63) would be 9%, the worst step in the route being the Grignard reaction of methyl magnesium iodide with aldehyde (90) yield (33%). (This could possibly have been increased by using excess Grignard reagent although this would be impractical in terms of a labelled synthesis). It was hoped that changing the order of the Grignard reactions (Scheme 16), i.e. reacting allyl magnesium bromide with the aldehyde (90) and methyl magnesium iodide with the ketone (93), would permit introduction of the label at a later stage in the synthesis and lead to an improved yield. Reaction of the aldehyde (90) and allyl magnesium bromide however did not give the alcohol (92) possibly due to an ene reaction. Attempts to synthesise the ketone *via* deprotection of the dithiane[68,69,70] (94) and reaction of allyl magnesium bromide with the nitrile (95)[71] were also unsuccessful.

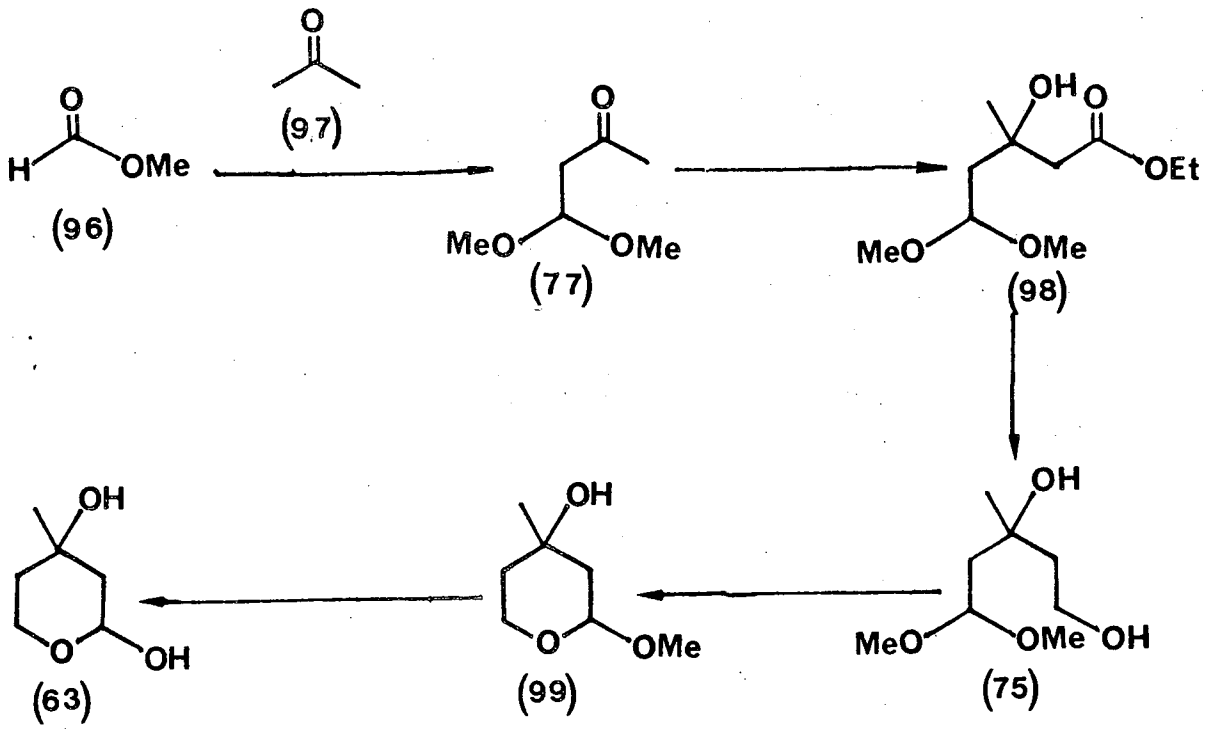


SCHEME 16

1.3 STRATEGY 2

1.3.1 Establishing a Route

The second strategy[72] turned out to be the most efficient in terms of label incorporation into the final product (63) (Scheme 17). Aldol reaction of acetone (97) and methyl formate (96) gave a 14% yield of the ketone (77). Reaction of the lithium enolate[73] of ethyl acetate with ketone (77) and lithium aluminium hydride reduction of the ester (98) gave the diol (75) which cyclised on stirring with acidic ion exchange resin in methanol to give a mixture of diastereomeric acetals (99). The structures of the acetals are discussed in Part 1.5. Hydrolysis of the acetal mixture (99) was effected using acidic ion exchange resin giving a mixture of hemiacetals (63) in an overall yield of 41% from ethyl acetate and the ketone (77).

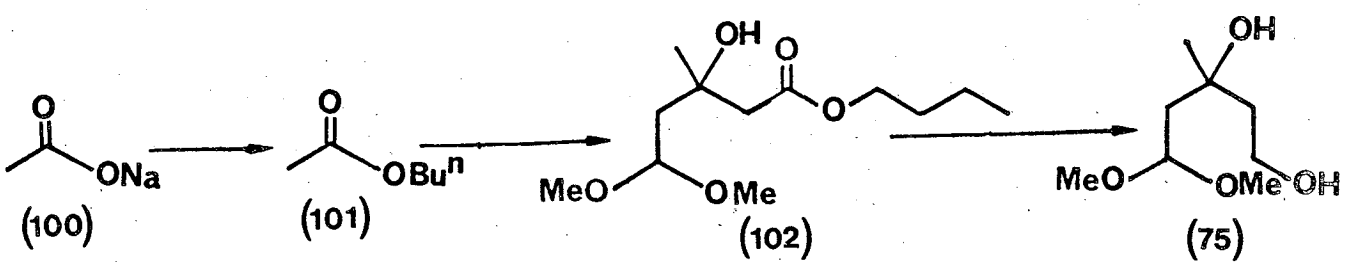


SCHEME 17

1.3.2 Labelled Synthesis of (63)

This strategy was then used in the labelled synthesis of the dihydroxytetrahydropyran (63) with some modifications to accommodate the use of sodium acetate (100) as a labelled starting material (Scheme 18).

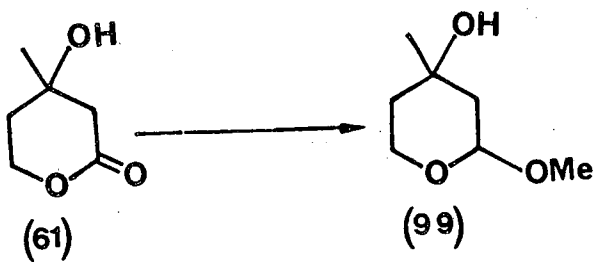
On reflux with tri-n-butylphosphate, sodium acetate (100) was esterified to its n-butyl ester (101) (which is less volatile than ethyl acetate and therefore more easily handled). The lithium enolate of the ester (101) was reacted with the ketone (77) in an analogous manner to that carried out with the lithium enolate of ethyl acetate in Scheme 17. Lithium aluminium hydride reduction of the ester (102) gave the diol (75). The rest of the synthesis was carried out as described previously. The route was used to synthesise both [5-^{13}C] and [5,6-$^{13}C_2$] dihydroxytetrahydropyrans (63) from [2-^{13}C] sodium acetate and [1,2-$^{13}C_2$] sodium acetate respectively. The overall yield for the five steps was 33%.



SCHEME 18

1.4 STRATEGY 3

It was later found that the acetal (63) could be synthesised by the diisobutylaluminium hydride reduction of mevalonic lactone (61) followed by an acidic methanol work up. The unoptimised overall yield of 50% makes this a viable two step route to the target dihydroxytetrahydropyran (63) as there are many established routes to labelled mevalonic lactone (61)[72,70,61] (Scheme 19).



SCHEME 19

1.5 STRUCTURES OF THE TETRAHYDROPYRANS (63) AND (99)

1.5.1 4-Hydroxy-4-methyl-2-methoxytetrahydropyran (99)

The title compound can be prepared either by acidic cyclisation of the acetal (75) or by reduction of mevalonic lactone (61). Both of these methods give a mixture of two diastereomeric pairs of enantiomers (Figure 5) which can be separated by flash column chromatography on silica. The diastereomers (99a) and (99b) were isolated in ratios of approximately 1:3. The major diastereomer (99b) was found to be the more polar with a boiling point of 46°C at 0.75 mm Hg as opposed to 60°C at

8 mm Hg for (99a).

Assignment of the relative configurations of the two diastereomeric acetals was based on ^1H NMR data. Accompanying the structures (99a) and (99b) in Figure 5 are the ^1H NMR. spectra of their respective C-1 hydrogens. The large coupling 7.8 Hz in Figure 5 enables assignment of the C-1 proton of (99b) as axial. The absence of such a large coupling in the spectrum of (99a) suggests that its C-1 proton is equatorial. Complete assignment of the ^1H-^1H coupling constants obtained for both diastereomers from 360 MHz ^1H NMR spectra are shown in Figure 5.

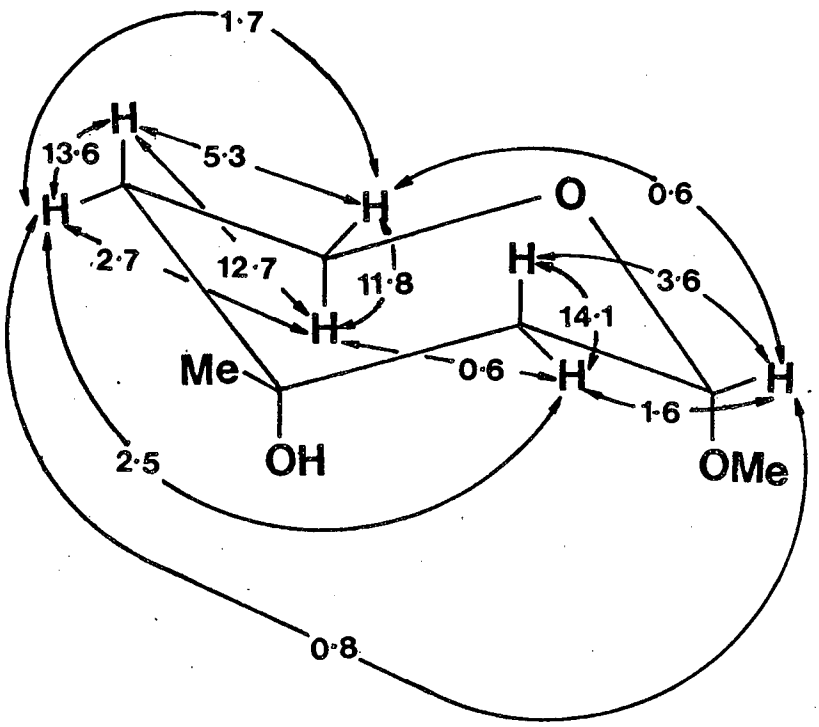
NOE difference spectroscopy (see Table 2 for NOE data) confirmed the assignment of the configuration of the C-3 position. Irradiation of the methyl group in each diastereomer did not increase the intensity of the C-1 or C-5 axial proton signals, thus the methyl group has to be equatorial in both cases. This was confirmed by the irradiation of the C-5 axial protons which showed no increase in the intensity of the methyl signal of either diastereomer. Irradiation of the C-5 axial protons also showed a 4% increase in the intensity of the C-1 proton signal of (99b) and no increase in the C-1 proton signal of (99a). This confirmed the assignment of the C-1 protons of (99a) and (99b) as equatorial and axial respectively.

	Me	$H4_{eq}$	$H2_{ax}$	$H4_{ax}$	$H2_{eq}$	MeO	$H5_{AX}$	$H5_{eq}$	Hl
99b	irr	<——— 1½% ———>							
						irr			6%
					3%	1%	2%		irr
		2%					irr	5%	4%
99a	irr	<——— 1½% ———>							
						irr	1½%		4½%
			1½%		1½%	1%			irr
		1½%					irr	14%	
		1%					13%	irr	

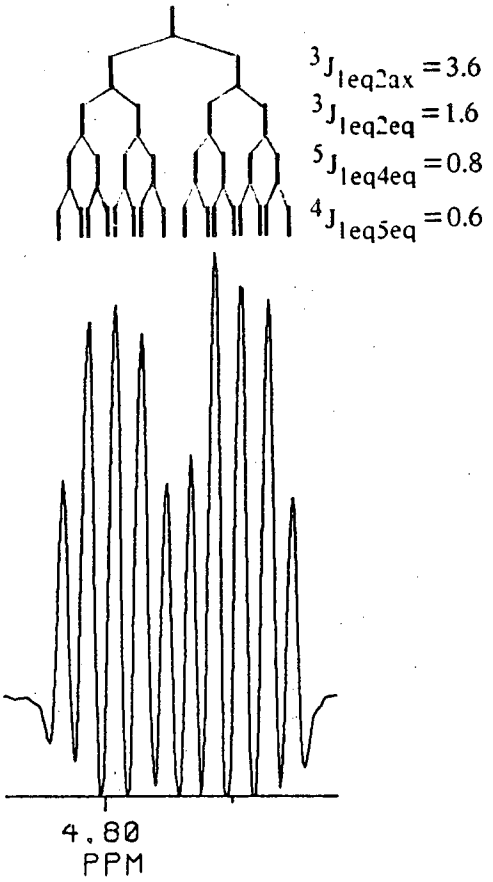
(irr = proton irradiated)

(% = increase in neighbouring protons signal)

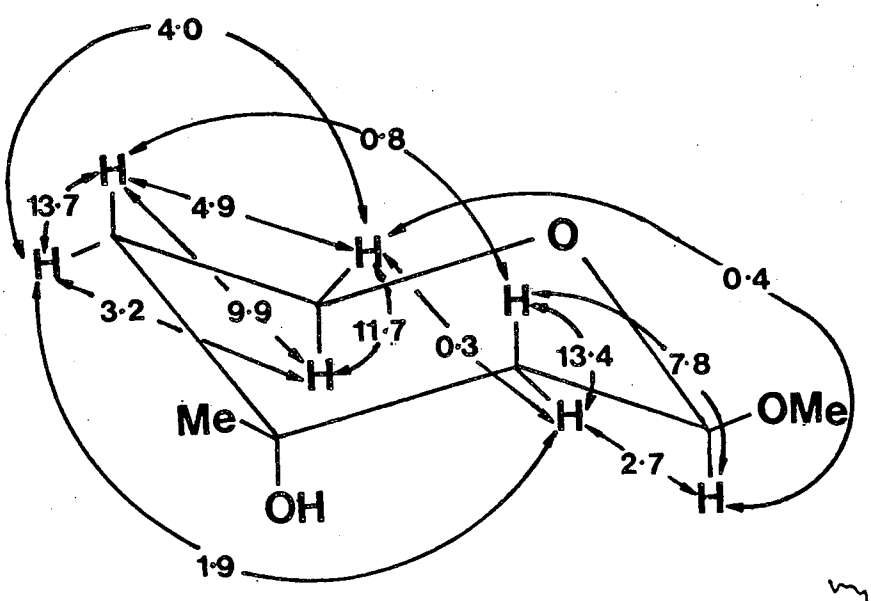
Table 2



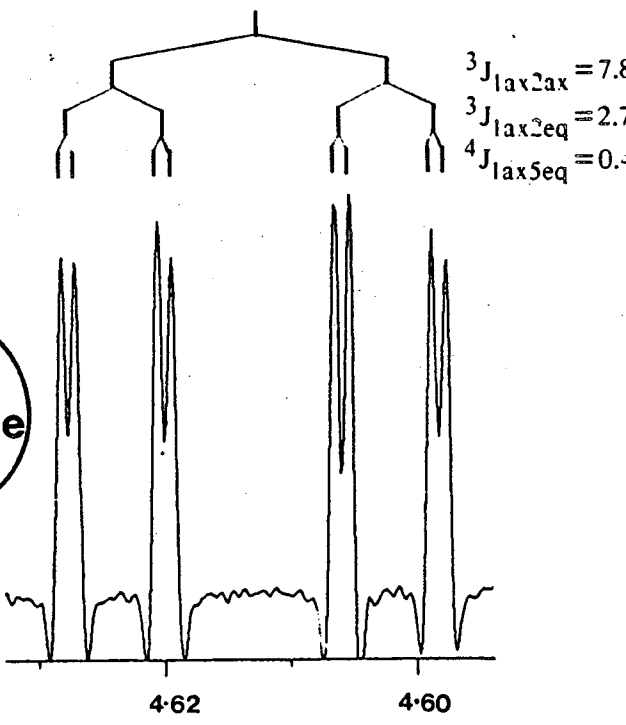
(99a)



H1eq



(99b)



H1ax

FIGURE 5

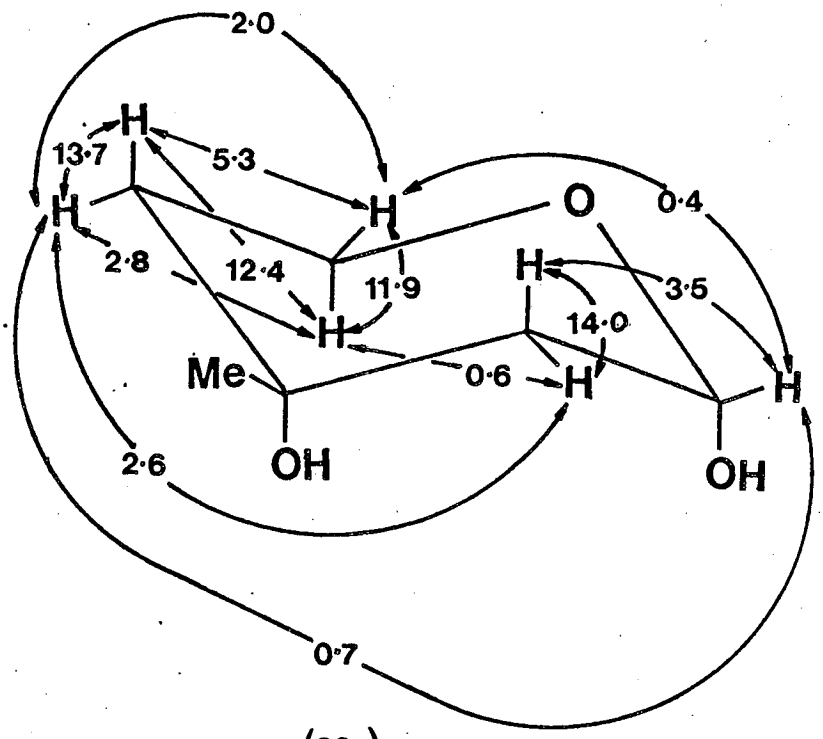
The more polar nature of (99b) exhibited in its t.l.c. and b.p. characteristics can be explained from these structural assignments. Intramolecular hydrogen bonding present only in (99a) makes it less capable of intermolecular hydrogen bonding and thus more volatile. The other diastereomer (99b) is incapable of intramolecular hydrogen bonding because of the distance between the hydroxy and methoxy groups.

1.5.2 2,4-Dihydroxy-4-methyltetrahydropyran (63)

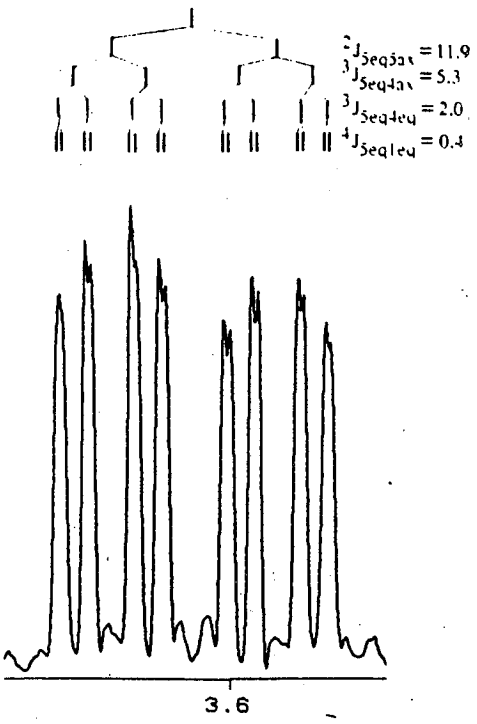
The title compound can be prepared either by ozonolysis of the alkene (71) or by hydrolysis of the acetal (99). Both methods gave a mixture of diastereomers which could only be separated with difficulty by flash column chromatography on silica. Although the ratio of the two diastereomeric pairs of enantiomers varied with the method of preparation, ^{13}C NMR spectra of the mixture typically showed an intensity ratio of around 3:10 for the methyl signals at δ 30.2 and 30.9 ppm.

The structure of the predominant diastereomer (63a) (Figure 6) was deduced by comparison of its ^{13}C NMR and ^{1}H NMR spectra with those of the acetals (99a) and (99b). The minor diastereomer (63b) was however never obtained in a pure enough form for rigorous NMR characterisation.

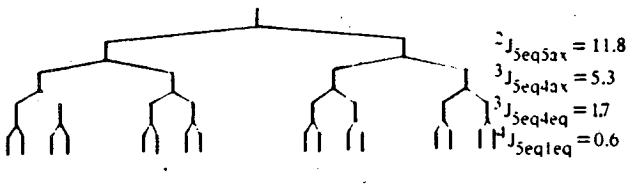
It can be seen from the ^{1}H NMR spectra of the H5eq, H5ax, H4ax and H2ax proton signals in Figures 6 and 7 that

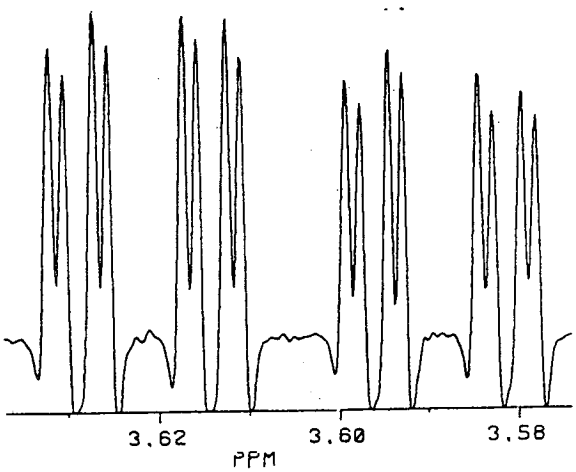


(63a)

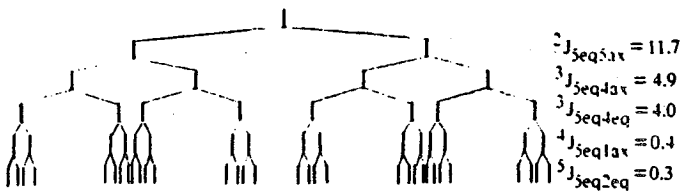


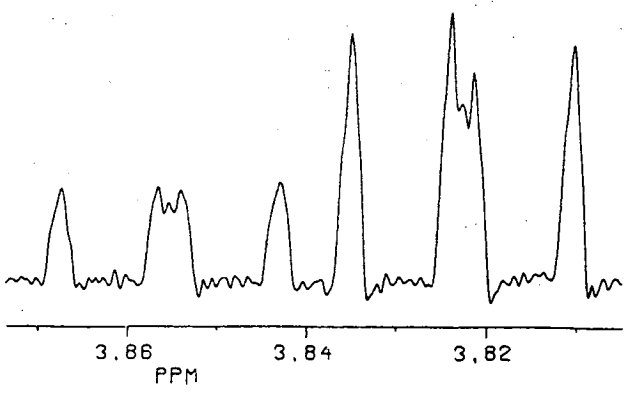
(63a) H5eq





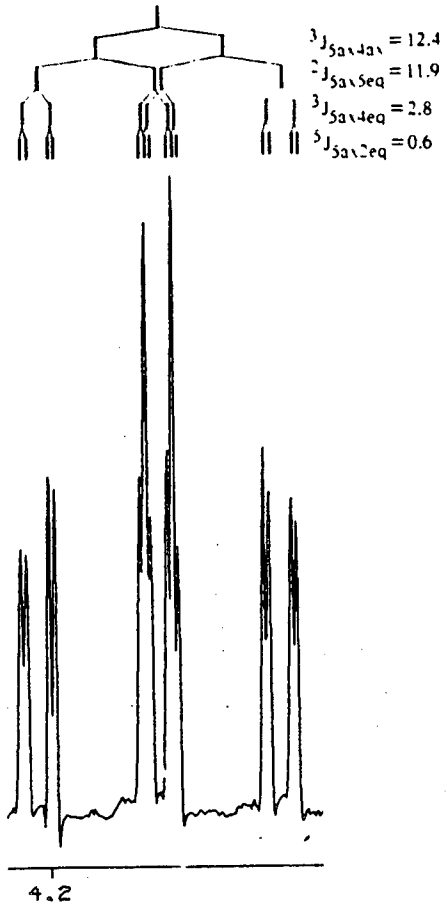
(99a) H5eq



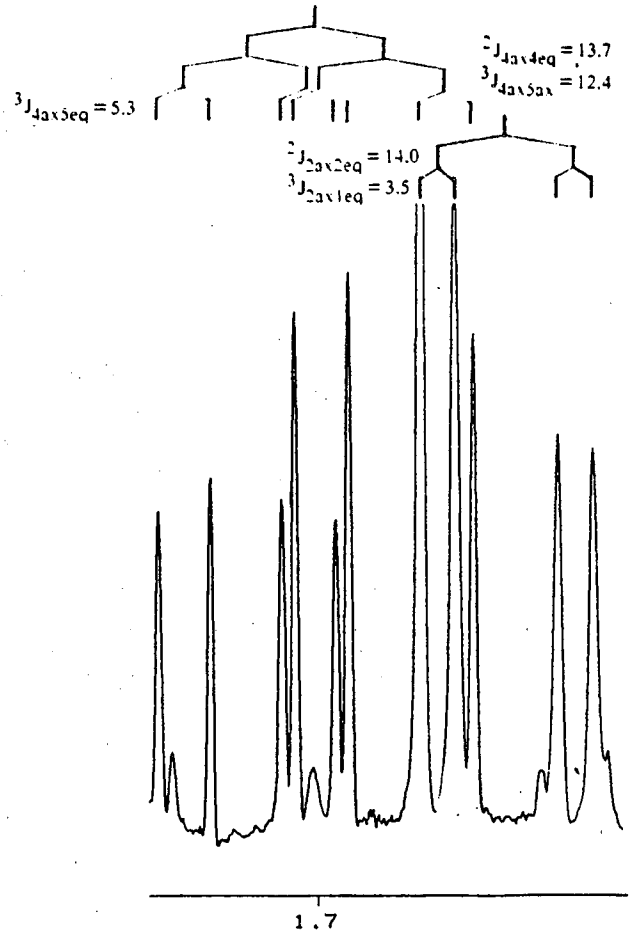


(99b) H5eq

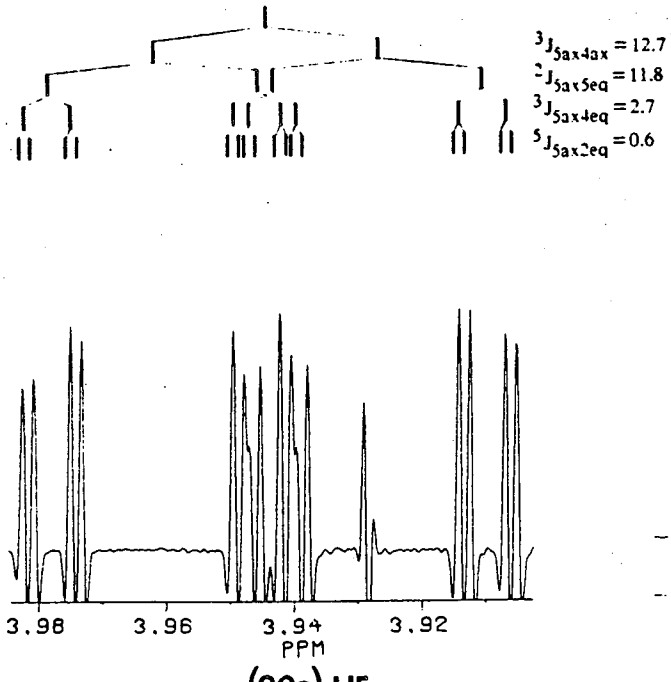
FIGURE 6



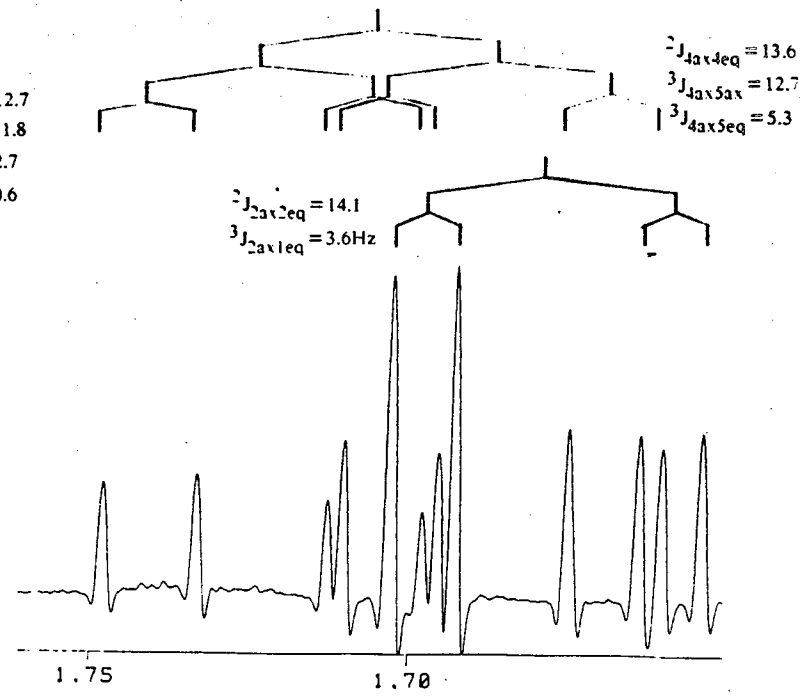
(63a)H5ax



(63a) H4ax,H2ax



(99a) H5ax



(99a) H4ax,H2ax

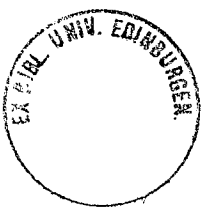
FIGURE 7

(63a) correlates very closely in both chemical shift and arrangement of couplings with (99a) (Figure 5). The H5eq proton for example (Figure 6) resonates at δ 3.6 ppm in both cases and has four couplings of 11.8, 5.3, 1.7 and 0.6 Hz in the acetal case (99a) as compared with 11.9, 5.3, 2.0 and 0.4 Hz for (63a). This contrasts with the H5eq proton of (99b) which resonates at δ 3.8 ppm and has five couplings of 11.7, 4.9, 4.0, 0.4 and 0.3 Hz (Figure 6). The observed couplings for (63a) are also shown in figure 6.

The ^{13}C NMR data supports the ^{1}H NMR structural assignment (Table 3). Four out of six of the ^{13}C n.m.r. resonances of (63a) are closer to those of (99a) than (99b) with four of them being within 0.7 ppm. The other diastereomer (63b) shows analogy with the acetal (99b) with four out of six ^{13}C NMR resonances closer to it than (99a), and three within 0.7 ppm.

	C1	C2	C3	C4	C5	Me
63a	92.1	41.0	68.0	37.9	55.6	30.2
63b	93.0	45.2	69.2	37.8	61.2	30.9
99a	98.8	40.9	66.5	37.9	54.9	29.8
99b	99.8	43.8	69.0	38.4	55.9	30.6

Table 3



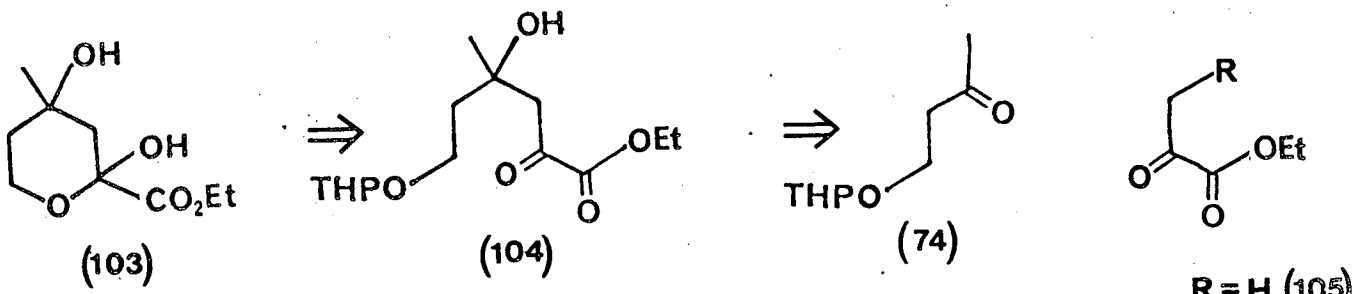
The preferred conformation of a 2-hydroxy tetrahydropyran is to have an axial hydroxy group, the anomeric effect stabilises this isomer by π-σ^* back bonding[74]. In accordance with this the major dihydroxytetrahydropyran (63a) has an axial 2-hydroxy substituent, the major acetal (99b) (56%) however is found to have an equatorial 2-methoxy. In the latter case the increased 1,3 diaxial interactions of the methoxy group are of sufficient destabilising nature to make it take up the equatorial conformation.

It is notable that some long range couplings are observed in these ring systems, $^5J_{2eq5ax}$ = 0.6 Hz in both (63a) and (99a), $^5J_{2eq5eq}$ = 0.3 Hz in (99b), $^5J_{1eq4eq}$ = 0.7 Hz in (63a), and $^5J_{1eq4eq}$ = 0.8 Hz in (99a). Large W couplings are also observed, $^4J_{2eq4eq}$ = 2.5 Hz in (99a) and $^4J_{2eq4eq}$ = 2.6 Hz in (63a) and a four bond diaxial coupling $^4J_{2ax4ax}$ = 0.8 Hz in (99b). The observation of the long range coupling is a consequence of the rigidity of the ring systems.

CHAPTER 2: TOWARDS THE SYNTHESIS OF 2-CARBOXY-2,4-DIHYDROXY-4-METHYLTETRAHYDROPYRAN (66)

2.1 STRATEGY 1

Before any chemical reactions were attempted the target molecule (103) Scheme (20) was retrosynthetically analysed[54,55,56]. Cleavage of the endo hemiketal bond reveals an α-ketoester (104), this functionality has few general methods of synthesis and therefore dominates strategic considerations.



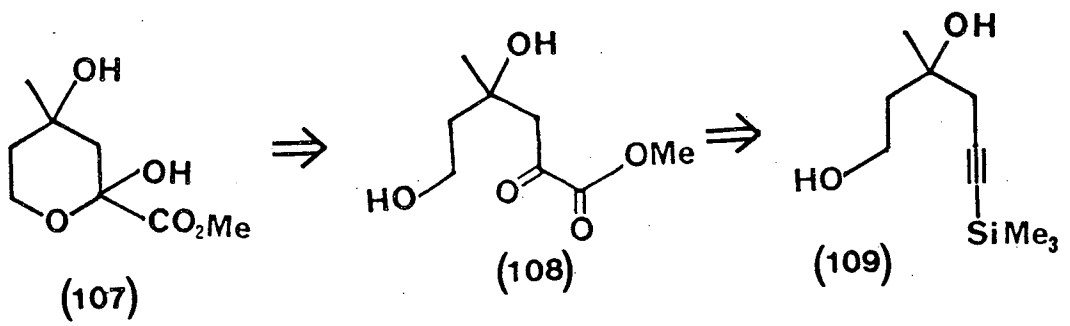
SCHEME 20

Using a similar strategy to that described in Scheme 17, the lithium enolate of ethyl pyruvate (105) or the Reformatsky reagent of bromoethylpyruvate (106) could be surmised to react with the ketone (74) (the synthesis of which was described in Chapter 1) and form the desired, protected α-ketoester (104). Removal of the THP protection and cyclisation would result in formation of the target molecule (103). However, both of the attempted carbon-carbon bond forming reactions gave complex mixtures

from which the only characterisable product was the starting material ketone (74). Attempts at reacting the lithium enolate of ethylpyruvate (105) with the more reactive carbonyl of benzaldehyde also failed possibly because the enolate reacts with itself.

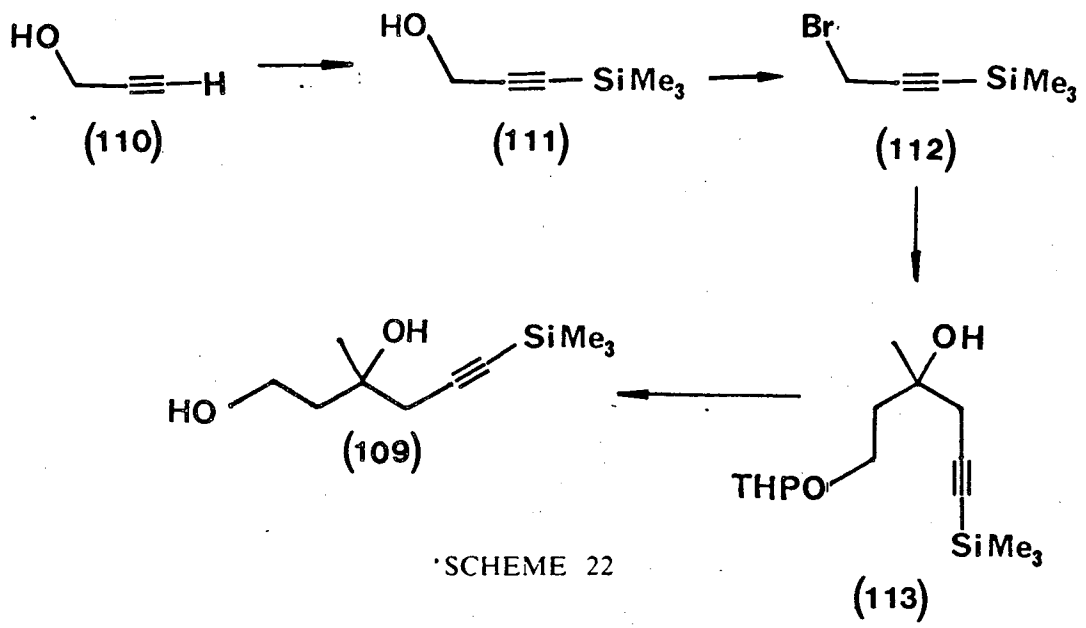
2.2 STRATEGY 2

It is well known that ruthenium tetroxide oxidises alkynes to α-diketones[75] and it has been shown more recently that osmium tetroxide oxidises 1-trimethylsilylalkynes to α-ketoesters[76]. Bromo alkynes have also been shown to be oxidised to α-ketoacids by ozonolysis[77]. It was therefore proposed that oxidation of the trimethylsilyl alkyne (109) (Scheme 21) would provide a route to the α-ketoester (108) and subsequent cyclisation of the α-ketoester (108) would give the target molecule (107).



SCHEME 21

Synthesis of the trimethylsilylalkyne (109) was relatively straightforward although low yielding (11% for the four steps) (Scheme 22). Reaction of propargyl alcohol (110) with trimethylsilylchloride, after activation with ethylmagnesiumbromide, gave the trimethylsilylalcohol (111)[78]. Reaction of the alcohol (111) with triphenylphosphine and bromine[78] gave the bromoalkyne (112) which formed the protected diol (113) on reaction of its Reformatsky reagent[79] with the ketone (74). Aqueous acid hydrolysis removed the THP protection quantitatively to give the target diol (109) (Scheme 22).



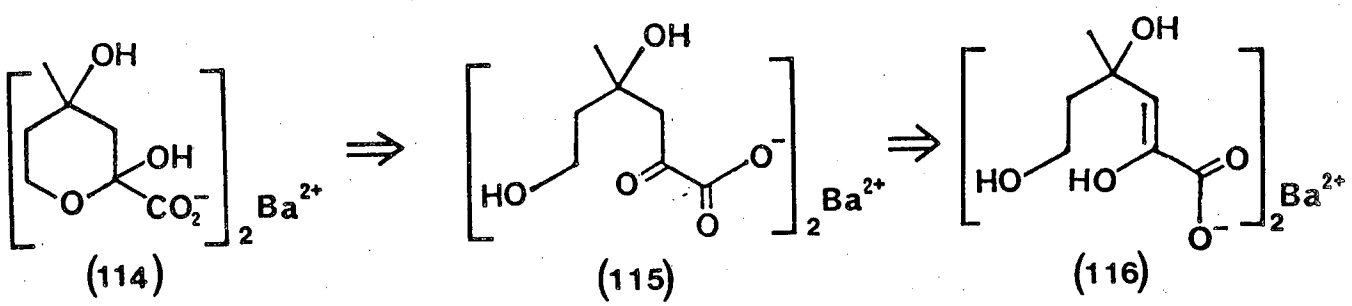
SCHEME 22

Attempted oxidation of the alkyne (109) using both catalytic and quantitative amounts of osmium tetroxide[76] resulted in formation of complex mixtures of products. The consumption of the starting material suggests that the reaction conditions were too harsh for the successful preparation of the target tetrahydropyran (107).

Ozonolysis of the alkyne (109) gave a more polar product which showed some of the expected target molecule characteristics. Although the FAB mass spectrum showed no molecular ion, two significant fragments at m/z 173 and m/z 155 were observed. These fragments suggest the presence of the ester (107) as they correspond to loss of a hydroxyl group and subsequent loss of a water molecule from a molecular ion of m/z 190. The ^{13}C NMR spectrum was also consistent with the proposed structure showing an anomeric carbon resonance at δ 95 ppm and seven other major carbon resonances. These resonances, in particular the anomeric carbon suggested the presence of a tetrahydropyran ring. The infra red spectrum showed carbonyl adsorptions at 1700-1770 cm^{-1} and hydroxyl adsorptions at 3500 cm^{-1} consistent with the proposed structure and the ^{1}H NMR spectrum, although complex showed resonances in the predicted regions. It is to be noted that on standing at room temperature over a few weeks the sample became contaminated with mevalonic lactone (61). Although the evidence strongly suggests the successful synthesis of the target molecule (107) the route is not viable as a labelled synthesis because of the low yield of label incorporation into the target molecule, approximately 4% from [^{13}C] methyliodide *via* the ketone (74) (Scheme 15).

2.3 STRATEGY 3

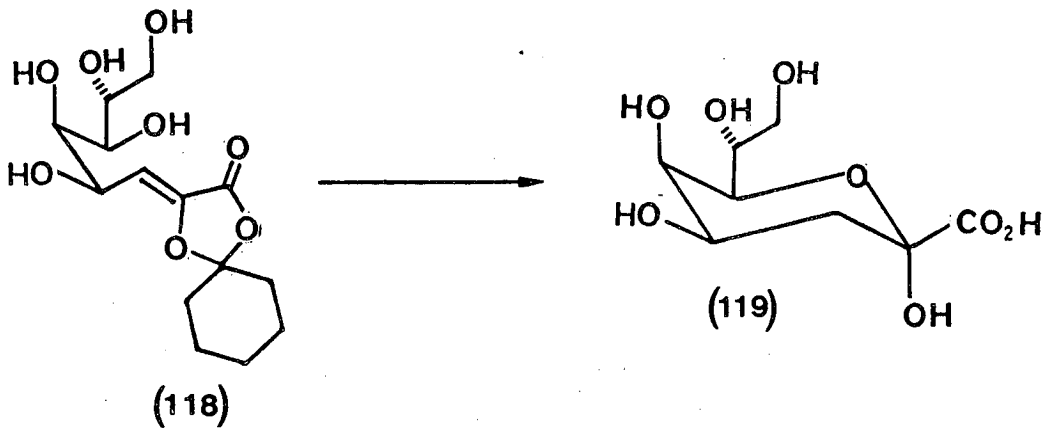
The third strategy to the tetrahydropyran (114) is based on the α-keto acid protecting "ylidene dioxolanone" methodology used recently in the synthesis of the lipopolysaccharide sugar 3-deoxy-D-manno-octulosonic acid (KDO)[80] (119). Analysing the strategy retrosynthetically (Scheme 23), tautomerism of the α-ketone in the α-keto acid (115) gives the 1,2 diol (116). This diol (116) could then be derived from the 5-ylidene-1,3-dioxolan-4-one (117) by cleavage of the acetal moiety.



(114) (115) (116) (117)

SCHEME 23

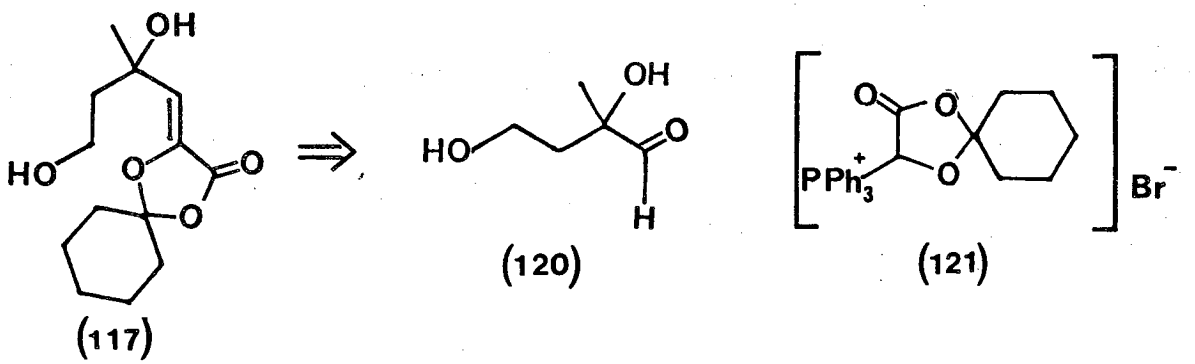
The 1,3-dioxolan-4-one (118) is stable to acid and can be cleaved under mild base hydrolysis conditions to give the α-keto acid (119) as its barium salt (Scheme 24)[80].



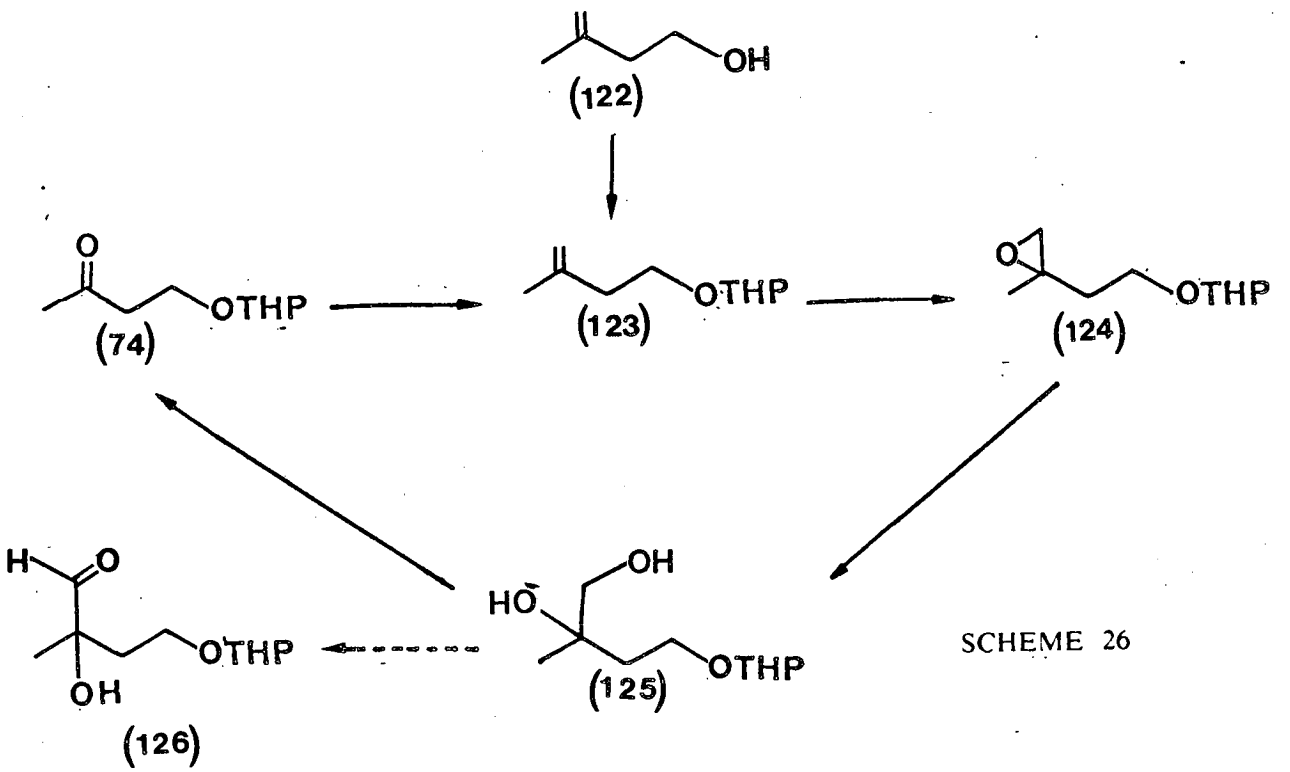
SCHEME 24

Thus hydrolysis of the 1,3-dioxolan-4-one (117) should give the barium salt of the α-keto acid (115) which should spontaneously cyclise to give (114).

The desired 1,3-dioxolan-4-one derivative (117) could be conjectured to be formed from the Wittig reaction of the aldehyde (120) and the Wittig reagent (121) (Scheme 25).



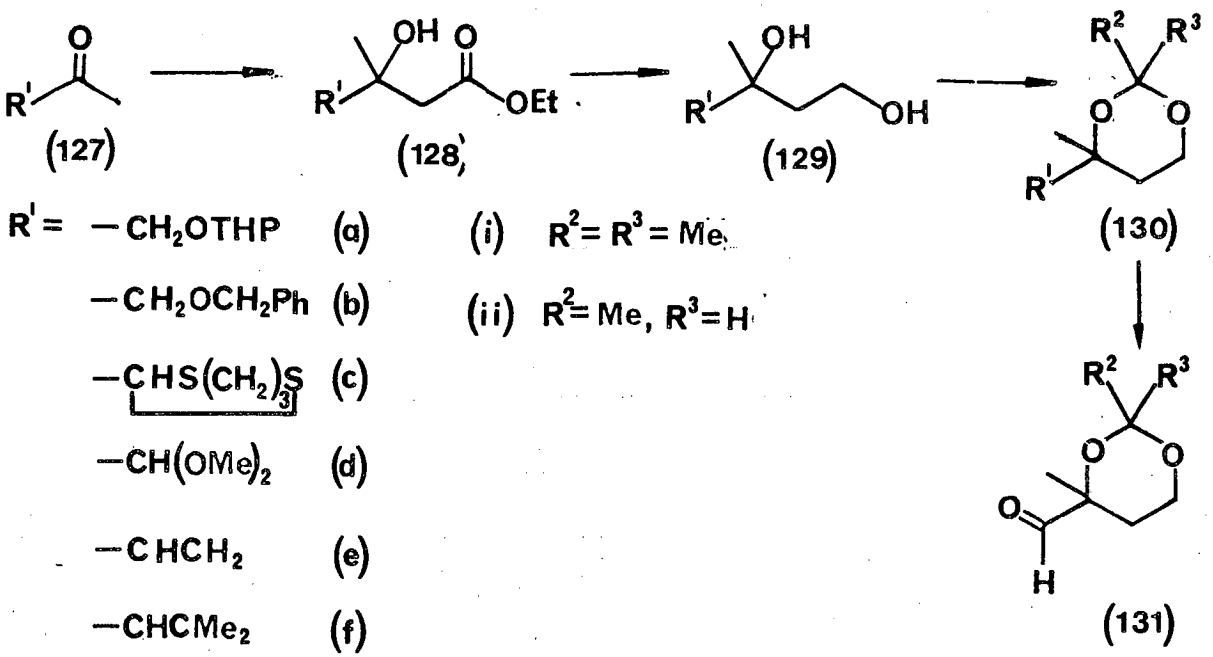
SCHEME 25



SCHEME 26

However the aldehyde (120) cannot be used in this case as it would be expected to spontaneously cyclise to a tetrahydrofuran. THP protection of the primary alcohol would stop this cyclisation.

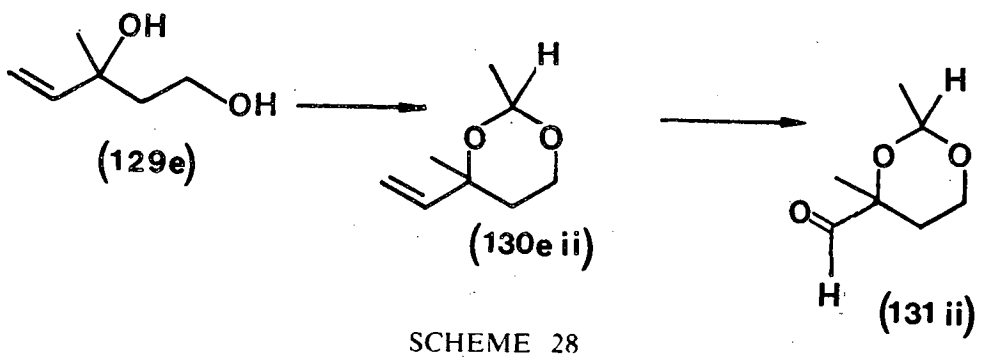
Attempted synthesis of the aldehyde (126) (Scheme 26) used the ketone (74) as a starting material. Reaction with the Wittig reagent methyltriphenylphosphonium iodide[81] gave the protected isopentenyl alcohol (123) which was also made by THP protection of the alcohol (122). Reaction of the alkene (123), with metachloroperbenzoic acid[82] gave the epoxide (124) which formed the diol (125) when refluxed overnight with 5N sodium hydroxide. Attempted oxidation of the diol (125) with pyridinium chlorochromate[67] resulted in a reaction giving the original ketone (74) (77% yield) instead of the desired aldehyde (126).



SCHEME 27

While it may have proven possible to generate the desired aldehyde (126) by other procedures - for example by ozonolysis of the alkene (129e) after THP protection of the primary alcohol (Scheme 27), an alternative strategy involving the construction of the diprotected synthon (131) (Scheme 27) was more attractive. Synthesis of the target skeleton (131) was attempted *via* an ethyl acetate lithium enolate strategy (cf Scheme 17) as ^{13}C label could easily be incorporated from ^{13}C labelled sodium acetate. A range of aldehyde (a-f) and diol ((i), (ii)) protection strategies were evaluated and are summarised in Scheme 27.

The principal problem encountered was the compatability of the aldehyde and diol protecting groups. It was found that the conditions required to perform the isopropylidene (i) protection of the diol, cleaved the dimethoxy (d) and THP (a) protecting groups and rearranged the alkenes (e) and (f). The more strenuous conditions needed to remove the benzyl (b) and dithiane (c) moieties tended to cleave the diol protection (i). Eventually it was found that acetaldehyde (56) and the diol (129e), in the presence of catalytic amounts of p-toluene sulfonic acid gave the 1,3-dioxan (130eii). Ozonolysis of this alkene (130eii) gave the aldehyde (131ii) (Scheme 28).



SCHEME 28

The ^{1}H NMR spectrum of the alkene (130eii) was complicated as the acetalisation afforded a mixture of diastereomers which could not be separated easily by flash column chromatography on silica. The ^{13}C NMR spectra (Figure 8) were much simpler and show each of the alkene (130eii) carbons represented as a pair of signals suggesting the presence of two diastereomers in approximately equal proportions.

The aldehyde (131ii) was also a mixture of diastereomers which were separated by flash column chromatography on silica. Figure 9 shows the more polar aldehyde (upper spectrum) and a mixture of both aldehydes (lower spectrum), it can be seen from the intensity of their H_{1ax} protons that the diastereomers are present in approximately equal proportions. It is notable in the spectrum of the more polar aldehyde that the H_{1ax} and H_{4ax} protons are at a higher chemical shift than the respective protons of the other diastereomers shown in the lower spectrum of the mixture of aldehydes (131ii). It can be deduced that since the ethylidene methyl is likely to be equatorial (thus avoiding destabilising 1,3 diaxial interactions) that the more polar aldehyde (upper spectrum) must have an axial formyl group (increasing the axial proton chemical shifts) and the other diastereomer consequently must have an equatorial formyl group (having lower axial proton chemical shifts).

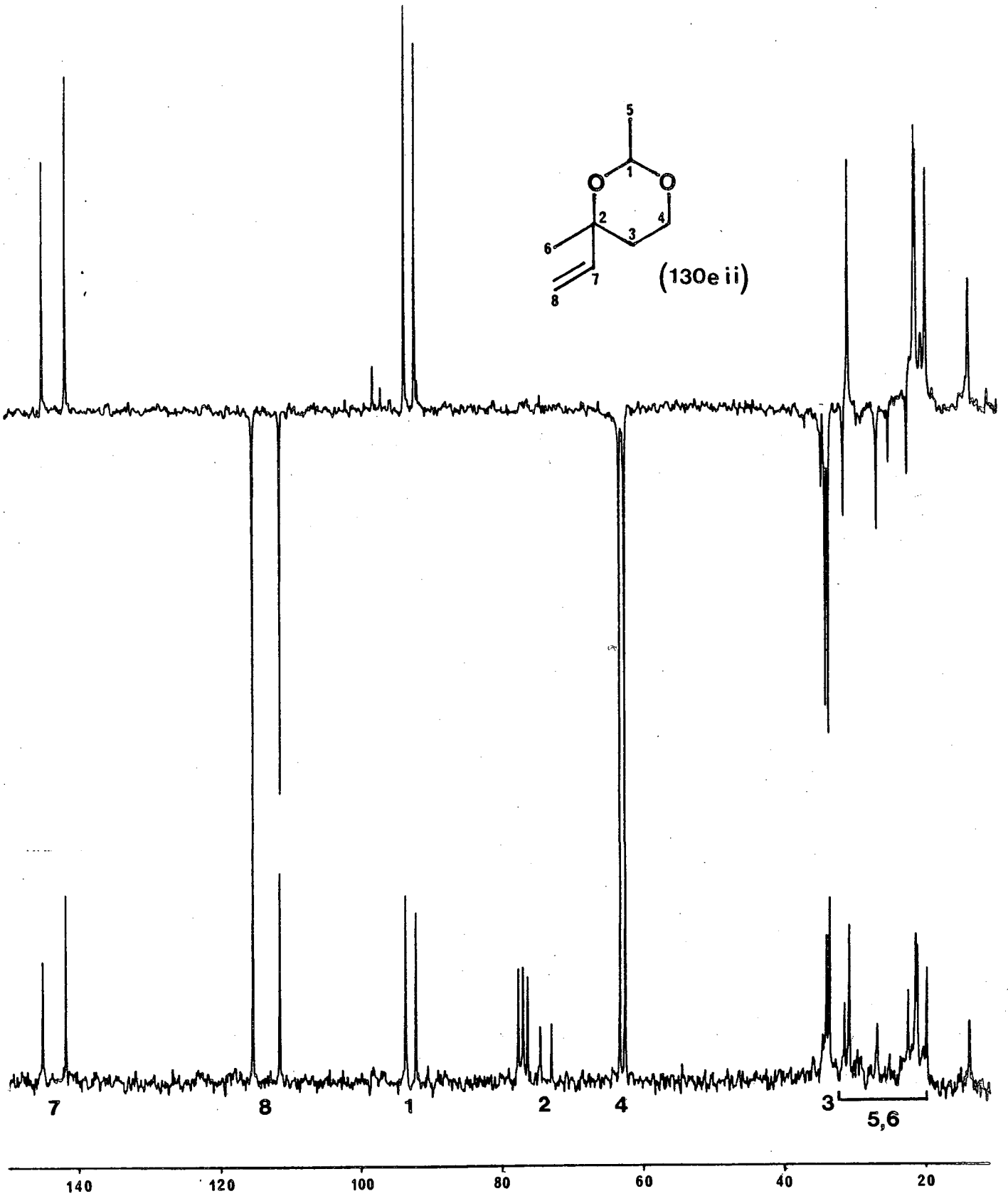


FIGURE 8

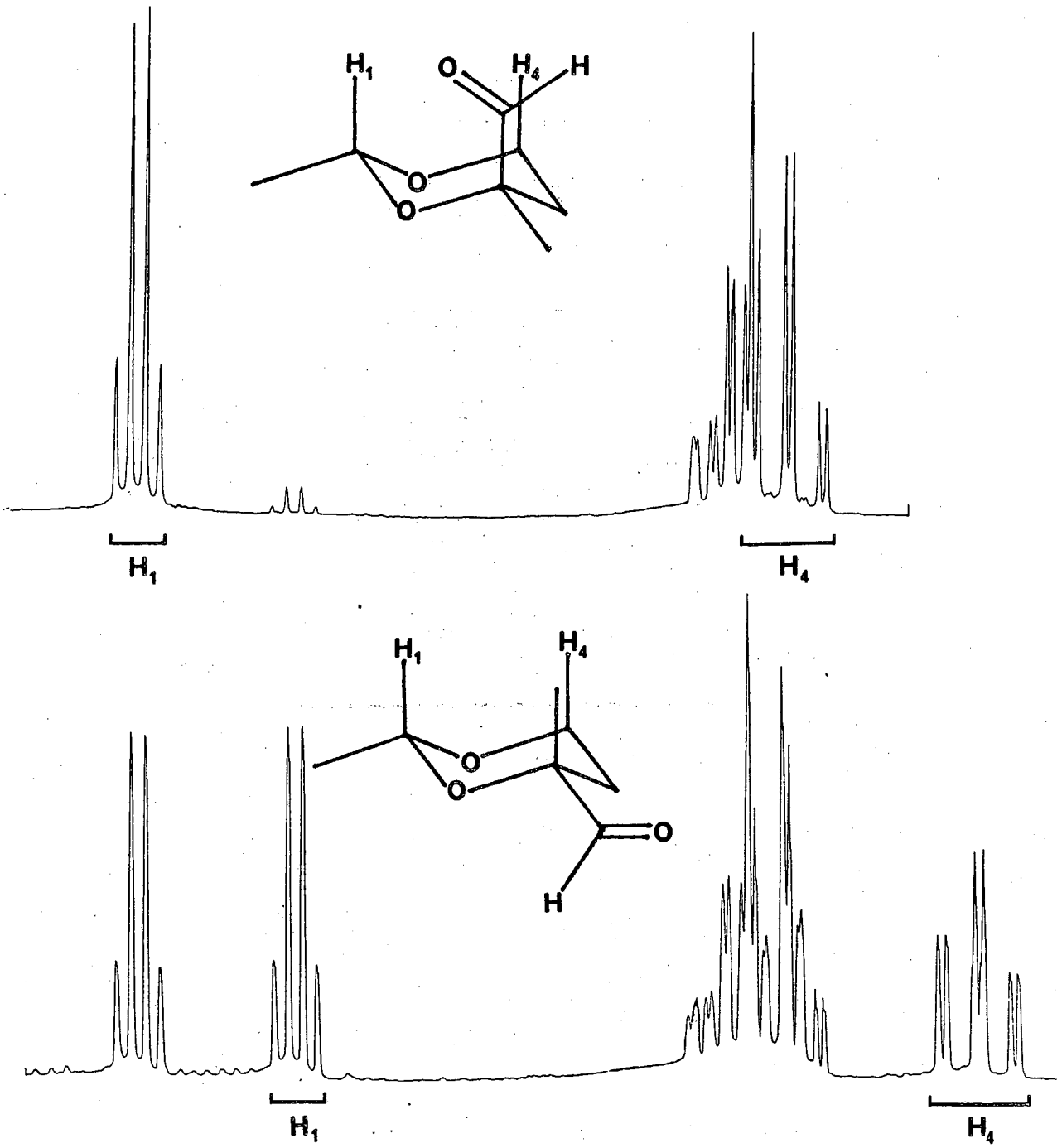
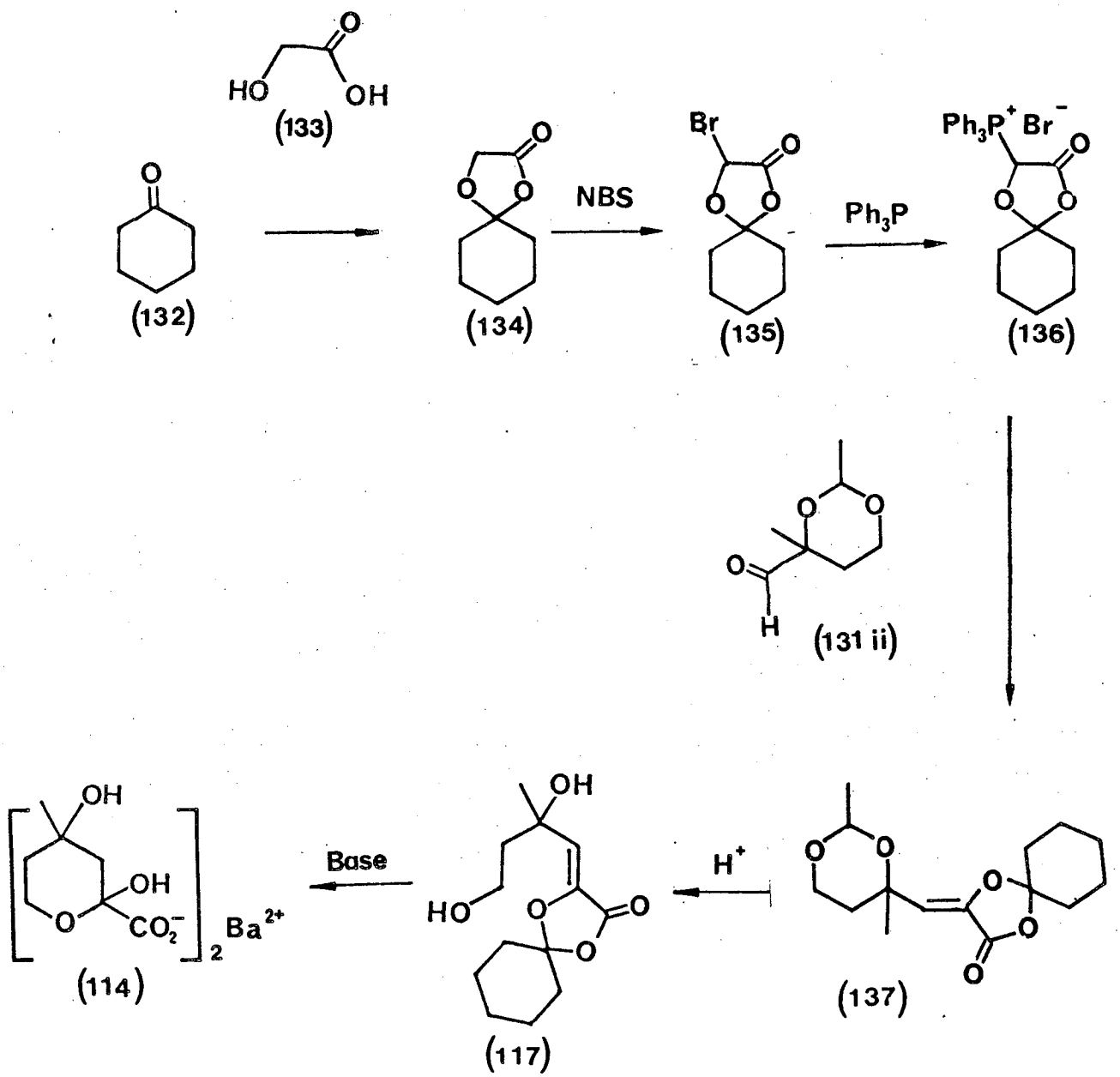


FIGURE 9

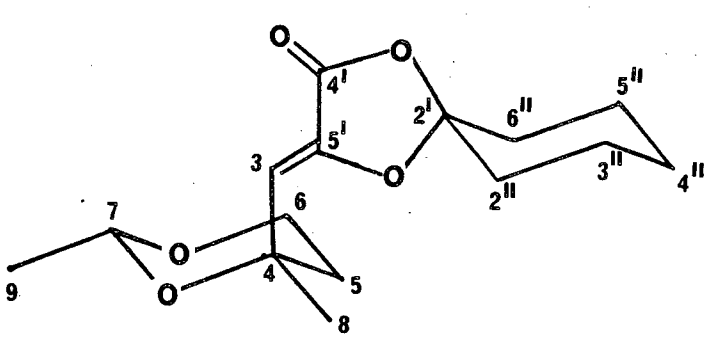
The synthesis of the Wittig reagent (136) is shown in Scheme (29). Reaction of cyclohexanone (132) with glycolic acid (133) in the presence of p-toluene sulfonic acid gives the 1,3-dioxolan-4-one (134)[83]. This was brominated α to the carbonyl with N-bromosuccinimide to give the 5-bromo-1,3-dioxolan-4-one (135). The Wittig salt (136) was formed on reaction with triphenyl phosphine.



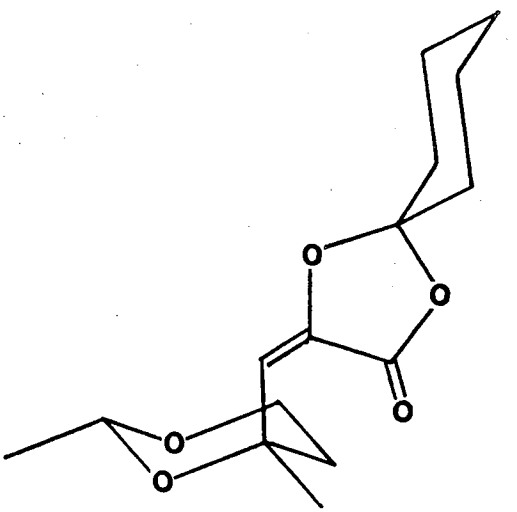
SCHEME 29

The Wittig reaction of the aldehyde (131ii) with the reagent (136) gave a mixture of isomers which could be partially separated into two groups by flash column chromatography on silica. Figure 10 shows the ^{13}C NMR spectrum (F), the ^{13}C DEPT NMR spectrum (E) and the ^{1}H NMR spectrum (B) of the more polar components.

There are eight possible isomers in total from the Wittig reaction as there are two chiral centres (at C-7 and C-4) and a double bond capable of geometric isomerism. It can be assumed that the C-9 methyl is equatorial in the product as it was equatorial in the starting material and is unlikely to take up the more sterically interactive axial position even though epimerisation is possible under the reaction conditions. It can also be conjectured that the more polar components have an equatorial methyl as the more polar starting material aldehyde (131ii) had an equatorial methyl. These assumptions would infer that the more polar group of isomers (spectra B, E and F) consisted of the pair of geometrical isomers (137i and 137ii) shown below. From the ratios of the H7 and H3 signals (Spectrum B) they are present in the ratio of 3:1.



(137i)



(137ii)

The observation that the C-7 proton (marked H7 in Spectrum B) appears at two different resonances is consistent with the assignment of an axial C-4 E and Z alkene substituent (i.e. equatorial C-4 methyl). The observation of two olefinic resonances (marked H3 in Spectrum B) in the same ratio as the C-7 proton intensities is also consistent with the assignment of an axial E and Z alkene substituent.

It is believed that the major component of the pair (spectrum B) is the isomer (137i) as calculation of the δ_H of the olefinic proton[84,85] suggests that a *cis* carbonyl *trans* oxygen configuration has a 0.1 ppm higher chemical shift than a *cis* oxygen *trans* carbonyl configuration.

Geometric isomerisation of this more polar pair by refluxing under U.V. in the presence of bromine afforded one major component. Figure 10 shows the ^{13}C NMR spectrum (D), the ^{13}C DEPT NMR spectrum (C) and the ^{1}H NMR spectrum (A) of this product. It can be seen from the acetal proton integrals (H7) in the ^{1}H NMR spectrum (A) that the isomers are present in the ratio of 1:10 in favour of previously minor component. The ^{13}C NMR spectra (C) and (D) show single carbon resonances where there had previously been two. The major isomer after the Wittig reaction (137i) was therefore isomerised to the more stable isomer (137ii) on irradiation in the presence of bromine. This is consistent with model builidng which suggests that (137i) is the more crowded of the two and

that (137ii) is the favoured form.

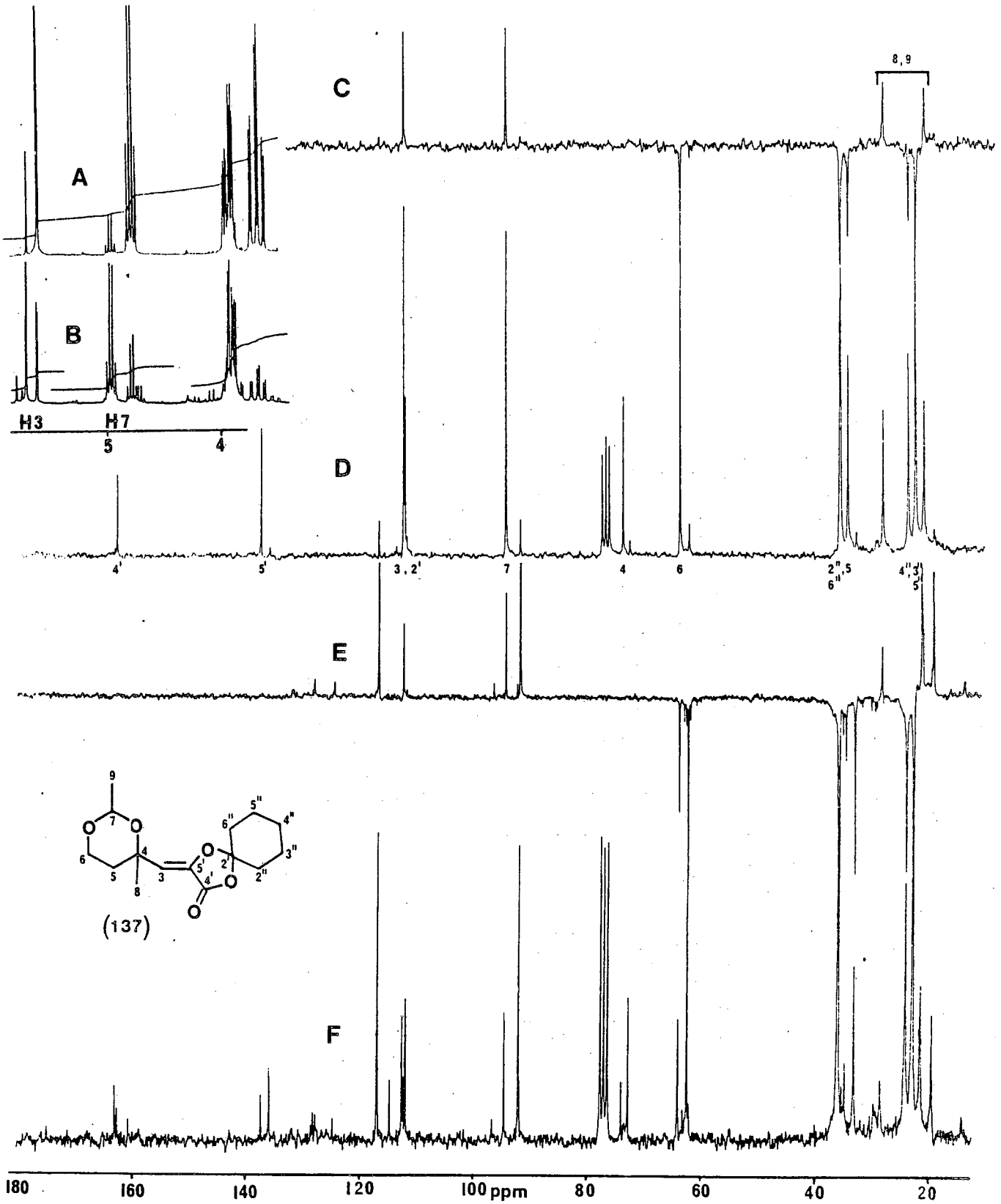


FIGURE 10

Attempted synthesis of the diol (117) by acid catalysed deprotection of the diol gave a complex mixture. ^{1}H NMR spectra of the crude mixture showed the absence of the acetal quartet, indicating that the protecting group had been removed. However, the spectrum showed no sign of the characteristic pair of triplets from the $-CH_2CH_2OH$ fragment. It is suggested that the deprotection of this acetal is unsuccessful because, unlike the KDO synthesis, a tertiary alcohol is involved in the deprotection stage and the ethylidene group is more difficult to remove than the isopropylidene protecting group[86]. These factors may encourage an elimination reaction to take place providing a conjugated diene capable of further reaction. In conclusion it would appear that the ylidene dioxolanone methodology is ineffective in the synthesis of the tetrahydropyran (114).

CHAPTER 3: FEEDING STUDIES

3.1 GROWTH CURVES

Before feeding the ^{13}C isotopically enriched tetrahydropyrans (63) and (99) to the bacterium *Rh. capsulata*, it was necessary to monitor both the cell and metabolite growth so that the tracers could be introduced at the optimum time for incorporation.

Figures 11.1 and 11.2 show culture growth curves for anaerobic growth of *Rh. capsulata* on medium S. Cell growth was determined by measuring the weight of *Rh. capsulata* residue after exhaustive acetone extraction (Figure 11.1) and U.V. adsorption of the culture at 600 nm (Figure 11.2). The ubiquinone-10 (51) concentrations (Figure 11.1) were measured by HPLC analysis of the acetone extract.

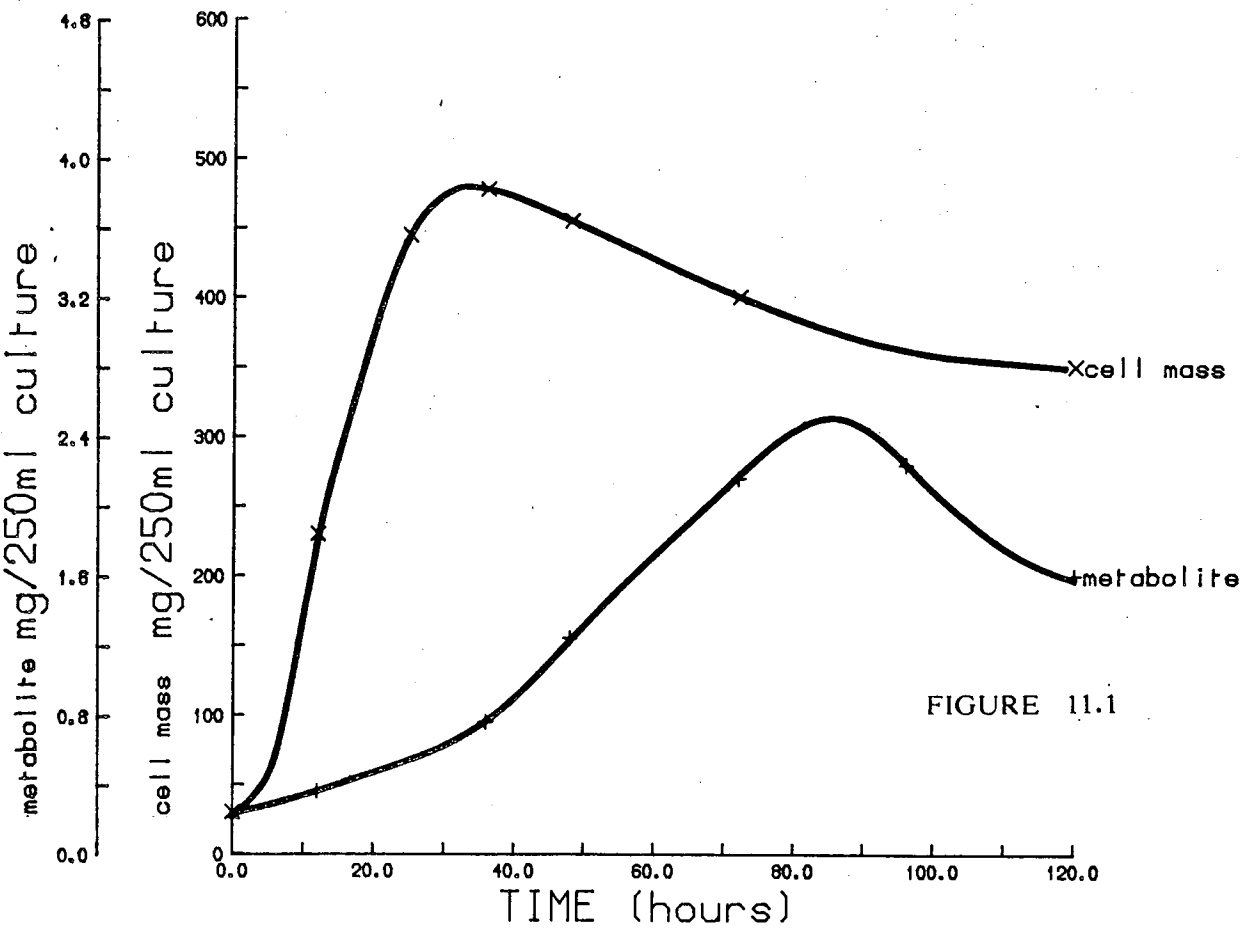


FIGURE 11.1

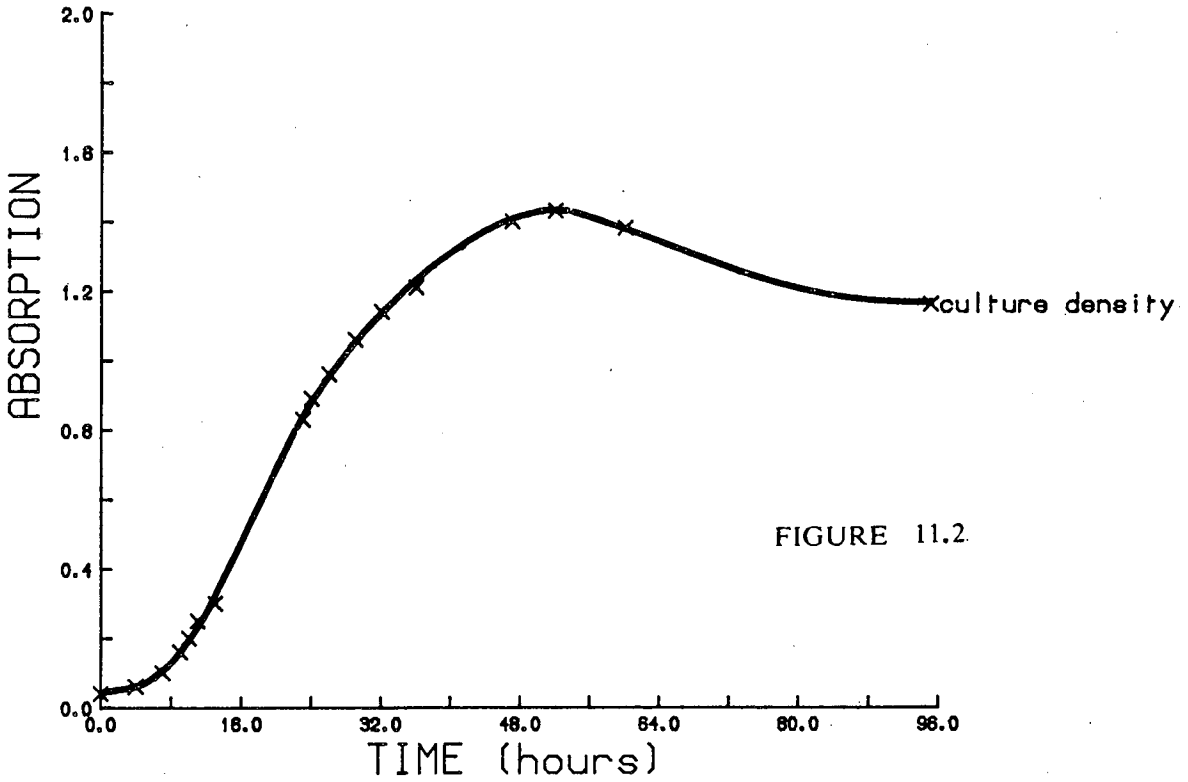


FIGURE 11.2

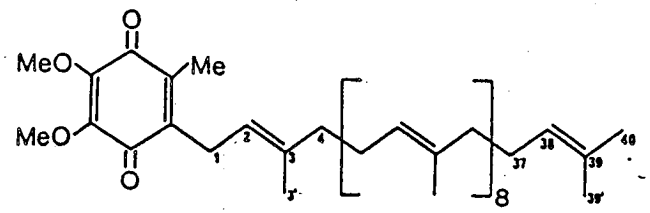
The cell mass growth curves show that there is a lag phase of about 8 hours followed by a log phase of 20 hours. Cell density then decreases from about fifty hours onwards as the cells die through lack of nutrients. The ubiquinone-10 (51) production has a longer lag phase of about 30 hours and peaks at around 80 hours before tailing off. These curves indicate that the optimum innoculation time for feeding isotopic precursors is after about 38

hours. This favours incorporation of the isotopically enriched precursors into the ubiquinone (51) rather than into the cell structures formed during the log phase of cell mass growth. The optimum time for harvesting is about 84 hours after inoculation when the ubiquinone (51) concentration is at its peak.

3.2 TYPICAL FEEDING STUDY : [3-^{13}C] SODIUM PYRUVATE

A typical feeding study procedure is outlined in the experimental section. Figures 12.1 and 12.2 show ^{13}C NMR spectra of ubiquinone-10 (51); Figure 12.2 shows natural abundance ^{13}C ubiquinone-10 (51) and Figure 12.1 shows isotopically enriched ^{13}C ubiquinone-10 (51) isolated from *Rh. capsulata* by preparative t.l.c. after feeding [3-^{13}C] sodium pyruvate.

The five largest peaks are due to superposition of eight isoprene units of the polyprenyl side chain, the smaller peaks are due to terminal isoprene units and the quinone ring. Assignment of the polyprenyl side chain is shown in Figure 12.2. The three largest saturated peaks at approximately δ 16, 27 and 40 ppm correspond to the methyl (δ 16 ppm) and two methylenes of the isoprene unit. The methylene at δ 40 ppm is closest to the methyl group. The two largest unsaturated peaks correspond to the methine (δ 125 ppm) and the quarternary (δ 135 ppm) carbons of the isoprene unit. ^{13}C assignments were based on carbon proton correlation spectroscopy on ubiquinone-10



(51)

FIGURE 12.1

FIGURE 12.2

(51) and comparison with the spectrum of geraniol which was assigned by INADEQUATE spectra[87].

Comparing the two Figures 12.1 and 12.2, it can clearly be seen that the relative intensities of the five main peaks are different. As a measure of incorporation both standard and enriched spectra were normalised so that an arbitrary peak (one assumed to correspond to an unenriched carbon) was assigned as having an intensity of 1.0. The normalised enriched intensities were then expressed as a multiple of their respective normalised standard intensities (see Table 4).

3.3 FEEDING STUDY RESULTS

Table 4 shows the observed incorporation patterns of the ubiquinone-10 (51) isoprene units after enrichment with the precursors indicated. Table 4 also shows the expected alternative incorporations based on the condensation of pyruvate (55) with acetoacetyl-CoA (14).

[^{13}C] sodium bicarbonate showed the same patterns as observed before[53] (see introduction) with most of the label going into the C-5 position. This study showed the reproducibility of the result and that the isolation procedure was effective.

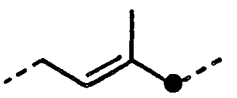
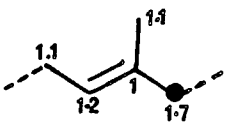
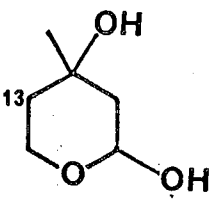
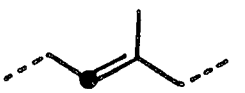
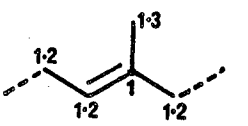
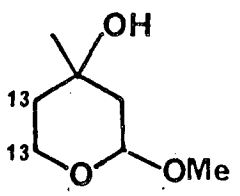
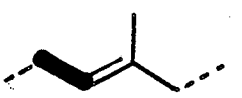
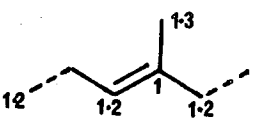
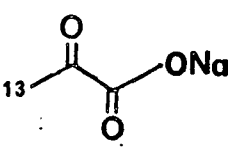
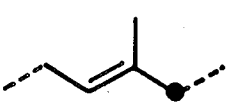
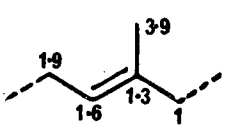
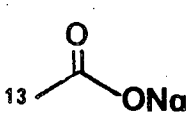
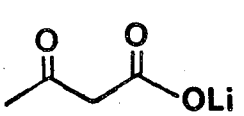
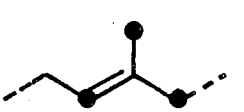
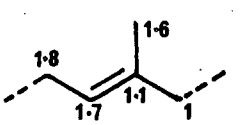
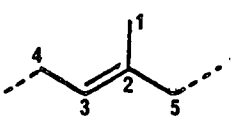
PRECURSOR	PREDICTED INCORPORATIONS	OBSERVED INCORPORATIONS
$NaH^{13}CO_3$		
		
		
		
 		
		

TABLE 4

The [4-^{13}C] Dihydroxytetrahydropyran (63) (Table 4) showed the same incorporation in each of the five isoprene carbons. This can be interpreted as the result of breakdown of the tetrahydropyran (63) and scrambling of the label before incorporation. While disappointing, the lack of incorporation of intact (63) does not preclude the intermediacy of this compound. It can for example, be argued that the lack of observed incorporation was due to lack of transport into the cell or catabolism of the bulk material (for example by oxidation to mevalonic acid and degradation to acetyl-CoA) prior to incorporation. It is worthy of note that if (63) is oxidised to mevalonate *in vivo* that no direct incorporation of the derived mevalonate occurs. This observation is consistent with there being no radioactivity in the ubiquinone-10 (51) after [2-^{14}C] mevalonate had been fed to *Rh. capsulata* as a control along with ^{13}C-sodium bicarbonate (see also reference 53).

To avoid the above effects, doubly enriched [4,5-$^{13}C_2$] tetrahydropyran acetal (99) was fed to *Rh. capsulata* reasoning that, passive diffusion of the non polar acetal into the cell would produce a pool from which the hemi-acetal (63) could be liberated by slow *in vivo* hydrolysis. This approach, of using a non-polar hydrolysable derivative of a biosynthetic intermediate has been used successfully in other studies[88]. Additionally, the use of a doubly labelled probe increases the sensitivity of the

experiment since it is easier to detect the presence of ^{13}C-^{13}C satellite peaks, than small increases in single resonance intensities. In the event, the spectrum of ubiquinone-10 (51) showed no ^{13}C-^{13}C satellites and approximately equal incorporation of stable ^{13}C isotope into all of the carbons. This was probably due to breakdown and scrambling of the label prior to incorporation.

In the light of the negative tetrahydropyran results [3-^{13}C] pyruvate was fed to see if it was induced in the isoprenoid biosynthesis in the way that had been hypothesised. It was found, like alanine (which deaminates to pyruvate) that it did not label the isoprene C-5 position as would have been expected in the event of it condensing with acetoacetyl-CoA. Instead, it labelled the methyl position quite strongly (3.9x) and had a pattern of intensities similar to that for [2-^{13}C] acetate (Table 1). It is logical to interpret this result as arising from pyruvate decarboxylation to acetyl-CoA before incorporation.

From these results it seemed clear that the postulated pathway, whereby pyruvate condensed with acetoacetyl-CoA rather than a third acetate, is not involved in isoprenoid biosynthesis. The results however, taken in conjunction with the preliminary studies (introduction), are still different to the normal acetoacetate pathway incorporation patterns and require explanation.

3.4 *RH. CAPSULATA* METABOLISM

Rh. capsulata is capable of growth in five distinctly different ways:- anaerobically as a photoautotroph on H_2 and CO_2 with light as an energy source, anaerobically as a photoheterotroph on various carbon sources with light as an energy source, as a fermentative anaerobe in darkness on sugars as sole carbon and energy sources, aerobically as an ordinary chemotroph in darkness and aerobically as a chemoautotroph in darkness with H_2 as a source of reducing power, this gives it a varied and versatile metabolism[89]. Closer investigation of the metabolism revealed many other anomalous features relating to all of the precursors that were fed. All of the following remarks are concerned with photoheterotrophic growth which was the standard condition for the feeding studies.

Although *Rh. capsulata* photoheterotrophically uses light as its main source of energy it is capable of growth on fructose, glucose and sucrose. While glucose is catabolised *via* the Entner Doudoroff pathway (EDP), fructose is catabolised *via* fructose-1-phosphate and the Embden Meyerhoff pathway (EMP)[90]. Thus there are two separate pathways of sugar catabolism present in *Rh. capsulata*. The enzymes of the EDP are induced irrespective of which sugar is present whereas the EMP enzymes are only induced in the presence of fructose which *Rh. capsulata* seems to prefer. Glucose catabolism is

regulated by glucose-6-phosphate dehydrogenase which is inhibited by phosphoenol pyruvate.

The citric acid cycle, which catabolises acetyl-CoA from sugar catabolism to provide reducing power for oxidative phosphorylation and important biosynthetic intermediates (oxaloacetate, α-ketoglutarate and succinyl-CoA) in aerobic metabolism appears only to be used as a source of intermediates in *Rh. capsulata*[91]. It has been shown that α-ketoglutarate dehydrogenase, the regulatory catalyst in the citric acid cycle is five times more active in chemotrophic than phototrophic growth and that even small amounts of oxygen produce large increases in respiratory enzymes.

Acetate metabolism seems to be variable and strain dependent which may be a result of different micro environments from which various strains were isolated. Studies on acetate utilisation by *Rh. capsulata* strain St. Louis[92] indicated that the wild type grew poorly on acetate. A spontaneous mutant *Acl.* however, was found to be capable of vigorous and immediate growth on acetate and exhibited high levels of the glyoxylate cycle (see Appendix) enzyme isocitrate lyase. A similar situation has been reported for the utilisation of glycerol in *Rh. capsulata* in which two enzymes not present in the parental type are synthesised by a spontaneous glycerol utilising mutant[93]. It has been suggested that some of the isolates that had not encountered acetate or fatty acids in their

environment for many generations may be in the process of losing the ability to synthesise what would be the useless enzymes of the glyoxylate cycle. Some of those organisms may in fact have lost completely the structural genes for the enzymes involved while others may have retained the structural genes while simply losing the ability to express them[92]. Thus a simple explanation of the differences in reported *Rh. capsulata* glyoxylate enzyme compliments is that under strong selective pressure mutants expressing long silent genes of the glyoxylate cycle possess a selective advantage and are enriched in culture.

The Calvin cycle (see Appendix), which incorporates carbon dioxide into 3-phosphoglycerate, has been shown to have two forms of the key enzyme (I and L) ribulose-1,5-diphosphate carboxylase/oxygenase (Rubisco). These enzymes are repressed by high amounts of carbon dioxide, the L form in particular is totally repressed by the amount of carbon dioxide produced on photoheterotrophic metabolism of malate. *Rh. capsulata* was the first bacterium in which two forms of Rubisco were demonstrated (1984)[94].

Pyruvate as well as being metabolised to D-lactate and acetyl-CoA has the possibility of forming acetaldehyde as pyruvate decarboxylase is present[95,96].

Alanine dehydrogenase (ADH) does not function as an NH_4^+ assimilating enzyme, instead its function is to

deaminate the substrate, L-alanine, to supply the growing cells with the proper amount of organic carbon (i.e. source of pyruvate). However, in culture media with L-alanine and D,L-malate *Rh. capsulata* AD2 does not excrete NH_4^+ into the culture medium although the cells contained ADH. Moreover, its deaminating capacity was inhibited by pyruvate (50% inhibition by 4 mM pyruvate). It seems that the "feedback" inhibition of the enzyme by C_3 and C_4 compounds turns off ADH activity under conditions where cellular nitrogen requirements can be satisfied by transamination of L-alanine[97].

3.5 REVISED INTERPRETATION OF FEEDING STUDIES

After close analysis of the feeding study results it was found that it was possible to interpret them in a way consistent with the acetoacetate pathway. The results that require explanation are the singly labelled acetate studies and the bicarbonate study. The [U-$^{13}C_6$] glucose and [1,2-$^{13}C_2$] acetate results are already consistent with the acetoacetate pathway.

One rationalisation of the anomalously low incorporation of [2-^{13}C] acetate into the C-5 isoprene position invokes the existence of a starter effect when the total amount of enrichment in each of the three constituent acetate units is calculated, (i.e. C-1 and C-2, C-3 and C-4, and C-5) a gradual reduction from the first to the third (C-1 and C-2 to C-5) is observed. This

effect is essentially a dilution of the label and occurs as a result of the amount of time it takes for the latter units to be incorporated. That is, the longer the isotopically enriched acetate remains in the cell the greater its chance of being randomly incorporated and the lower its chance of being incorporated into ubiquinone-10 (51). The observed C-5 isoprene ^{13}C NMR intensity might therefore constitute a low incorporation rather than none at all. This starter effect would also be enhanced by the poor utilisation of acetate by *Rh. capsulata* and would explain how the observed result could be consistent with the biosynthesis *via* the acetoacetate pathway.

In an attempt to overcome this starter effect and encourage incorporation of isotope into the isoprene C-5 position, [2-^{13}C] acetate was fed together with lithium acetoacetate. It was hoped that the lithium acetoacetate would form a pool of acetoacetyl-CoA with which the [2-^{13}C] acetate would condense rather than condensing with itself to form [2,4-$^{13}C_2$] acetoacetyl-CoA. In the event, the pattern of incorporation was very similar to that obtained with [2-^{13}C] acetate alone. This could have been due to random metabolism of acetoacetate or slow formation of acetoacetyl-CoA.

The high level of enrichment in the isoprene C-5 position after [^{13}C] sodium bicarbonate feeding can also be explained by a time lag in metabolism. [^{13}C] Sodium bicarbonate is incorporated into the C-3 position of

glucose-6-phosphate *via* the C-1 of 3-phosphoglycerate in the Calvin cycle. Glucose-6-phosphate is then metabolised *via* the Entner-Doudoroff pathway to [3-^{13}C] pyruvate and to [2-^{13}C] acetyl-CoA by oxidative decarboxylation of pyruvate. It is suggested that by the time the bacterium has metabolised the [^{13}C] sodium bicarbonate to [2-^{13}C] acetyl-CoA it has already built up a pool of natural abundance acetoacetyl-CoA with which the [2-^{13}C] acetyl-CoA condenses. This mechanism would explain how the [^{13}C] sodium bicarbonate and [1-^{13}C] acetate (which loses its ^{13}C label in the form of carbon dioxide *via* the Kreb's cycle) results could be consistent with biosynthesis *via* the acetoacetate pathway.

Proving that the acetoacetate pathway exists in *Rh. capsulata* using feeding studies is made difficult by the varied metabolism it exhibits. It may be possible to prove its existence by monitoring the label using ^{13}C NMR as it moves through the metabolite pools. However, a more direct way would be to assay for the acetoacetate pathway enzymes. From the existence of these enzymes one could invoke the use of the acetoacetate pathway in isoprenoid biosynthesis.

CHAPTER 4: ENZYMOLOGY

4.1 INTRODUCTION

The initial precursor feeding studies, as described previously (Introduction part 6) had suggested that the *Rh. capsulata* isoprenoid pathway might possibly involve pyruvate condensation with acetoacetyl-CoA rather than a normal acetyl-CoA, acetoacetyl-CoA condensation. Feeding studies with the [5-^{13}C] dihydroxytetrahydropyran (63) and the [5,6-$^{13}C_2$] hydroxymethoxytetrahydropyran (99) seemed to disfavour this hypothesis and a revised interpretation of the feeding studies as a whole with respect to the metabolism of *Rh. capsulata* seemed to suggest the use of the classical acetoacetate pathway.

To resolve these apparent contradictions it was decided to investigate whether specific enzymes of the acetoacetate pathway were present in *Rh. capsulata*. The enzyme 3-hydroxy- 3-methylglutaryl-CoA (HMGCoA) synthase was selected for investigation. Although it is the key enzyme involved in the condensation of acetyl-CoA and acetoacetyl-CoA, HMGCoA synthase activity has not previously been detected in bacteria. The enzyme was originally discovered in bakers yeast[98] and shown to catalyse the reaction of acetyl CoA (13) with acetoacetyl-CoA (14) shown in Scheme 1.

While it is recognised that the bacterial enzyme, should it exist, might possess different properties (with regards to solubility, K_m etc.) to the eukaryotic HMGCoA synthase, it was decided to repeat the work of Middleton and Tubbs[25] for the assay of this enzyme in yeast and having successfully repeated this work to apply the methodology directly to *Rh. capsulata*.

Figure 13 shows the previously described[25] assay system. The principle of this spectrophotometric assay is based on the absorption of the enol form of acetoacetyl-CoA at 303 nm. The synthase activity is measured by observing the acetyl-CoA stimulated decrease in absorption. The protein determination was carried out by Bradford microassay procedure which is described in detail in the experimental section along with the full description of the assay procedure.

The observed rate was converted to specific activity and compared with the specific activity obtained by Middleton and Tubbs. The respective specific activities after ethanolic-fractionation of the bakers yeast lysate supernatant were 0.099 units mg^{-1} as compared with 0.04 units mg^{-1}.

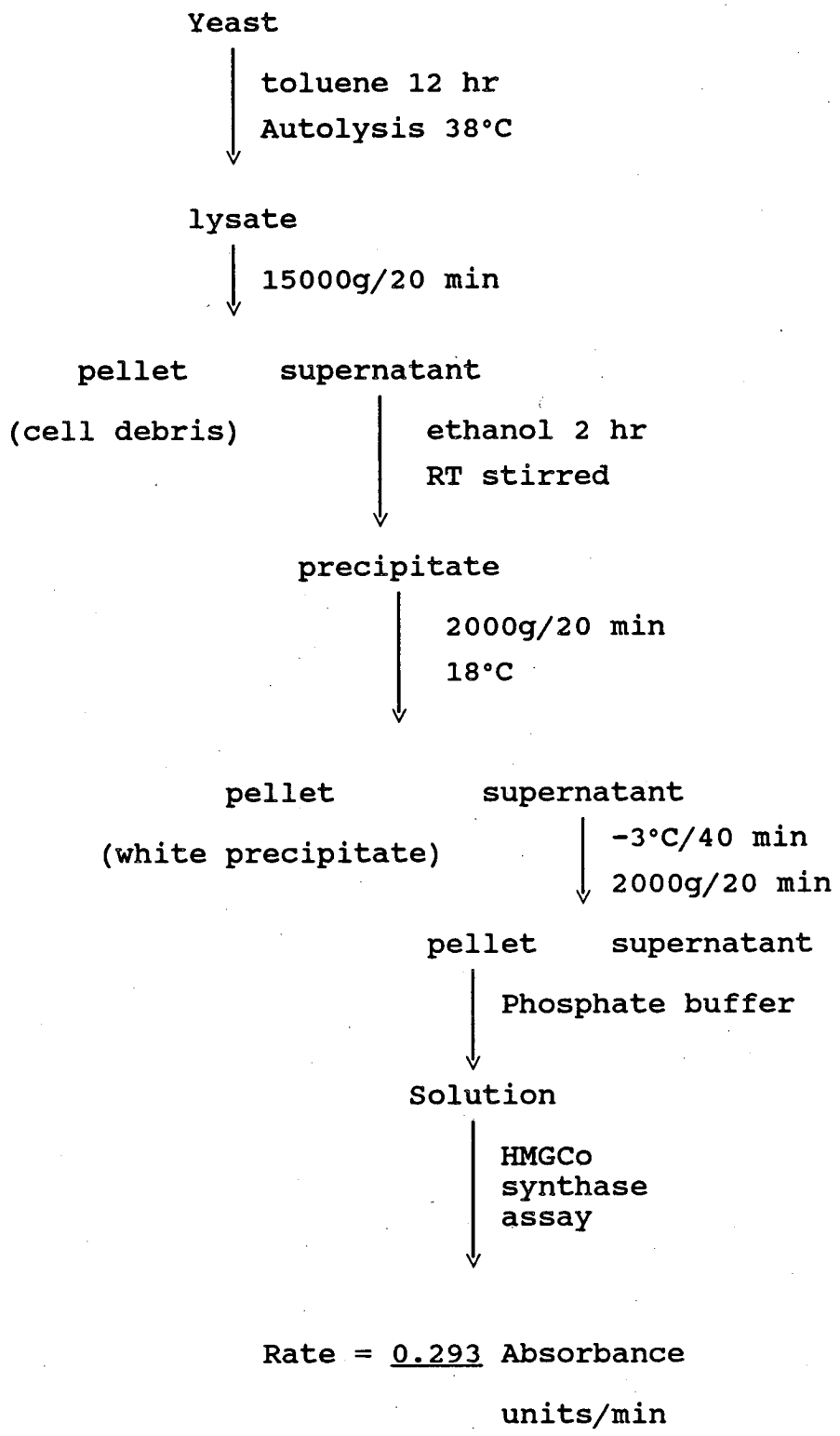

Yeast
toluene 12 hr
Autolysis 38°C
lysate
15000g/20 min
pellet
(cell debris)
supernatant
ethanol 2 hr
RT stirred
precipitate
2000g/20 min
18°C
pellet
(white precipitate)
supernatant
-3°C/40 min
2000g/20 min
pellet
supernatant
Phosphate buffer
Solution
HMGCo
synthase
assay
Rate = 0.293 Absorbance
units/min

Figure 13

It should be noted that Ferguson and Rudney[98] have observed that erroneously high activities can be observed as a result of the presence of non specific thiolase activity. To obtain the correct HMGCoA synthase rate, the thiolase rate was first determined by observing the decrease in absorption when only acetoacetyl-CoA was present in the cuvette with the enzyme solution. This thiolase rate was then subtracted from the rate of acetoacetyl-CoA disappearance after acetyl-CoA addition. Typical thiolase rates were of the order of 0.08 absorbance unit min^{-1} and all rates quoted here have been corrected for thiolase activity. It should be noted that this adjustment does not take into account any loss of acetyl CoA from thiolysis.

To confirm the presence of HMGCoA synthase in yeast the cells were lysed by another more gentle method used routinely in the preparation of yeast mitochondria[99] outlined in Figure 14. This method involves hand homogenisation of sphaeroplasts obtained from the action of xymolase on the cells. Table 5 shows the result of the assay on the supernatant (1) after centrifugation of the cell homogenate and supernatant (2) after homogenisation of the pellet and centrifugation. The specific activities were calculated as before and are corrected for thiolase activity.

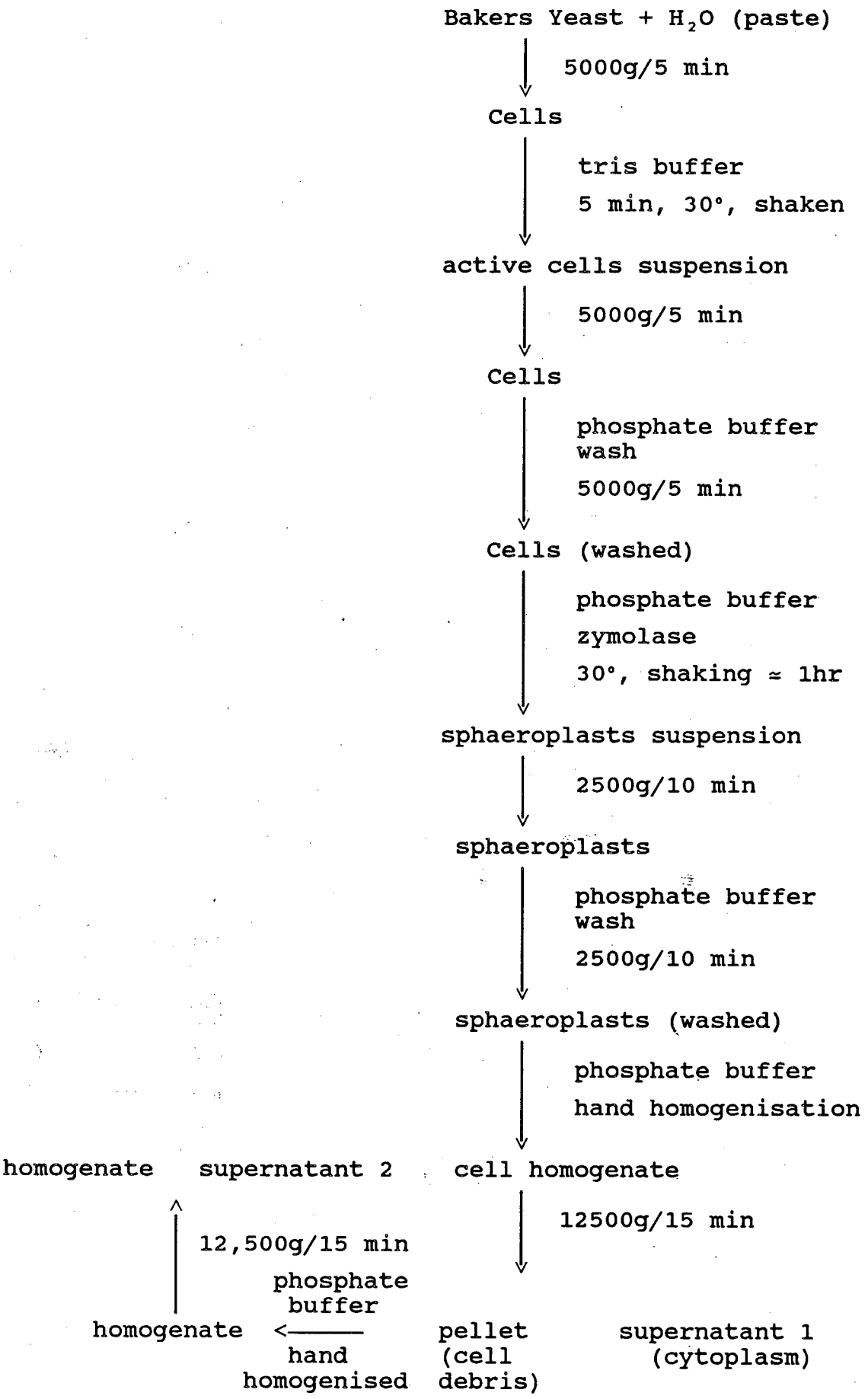

Bakers Yeast + H_2O (paste)
5000g/5 min
Cells
tris buffer
5 min, 30°, shaken
active cells suspension
5000g/5 min
Cells
phosphate buffer wash
5000g/5 min
Cells (washed)
phosphate buffer
zymolase
30°, shaking ≈ 1hr
sphaeroplasts suspension
2500g/10 min
sphaeroplasts
phosphate buffer wash
2500g/10 min
sphaeroplasts (washed)
phosphate buffer
hand homogenisation
cell homogenate
12500g/15 min
pellet (cell debris)
supernatant 1 (cytoplasm)
hand homogenised
phosphate buffer
12,500g/15 min
homogenate
supernatant 2
homogenate

Figure 14

	Rate (min^{-1})	Protein ($mgml^{-1}$)	Specific Activity (units mg^{-1})
Supernatant 1	0.035	4.45	0.045
Supernatant 2	0.028	2.15	0.065

Table 5

These results confirm the presence of HMGCoA synthase in yeast at a specific activity of the order of that observed by Middleton and Tubbs after ethanolic fractionation of bakers yeast. The thiolysis rates were much lower in this preparation accounting for approximately one fifth of the observed rate as opposed to approximately one quarter from the other preparation.

4.2 *RH. CAPSULATA* HMGCoA SYNTHASE ASSAY

To disrupt the cells of *Rh. capsulata* the subcellular preparation developed by Cox, Beatty and Flavinger[91] was used, this preparation is outlined in Figure 15.

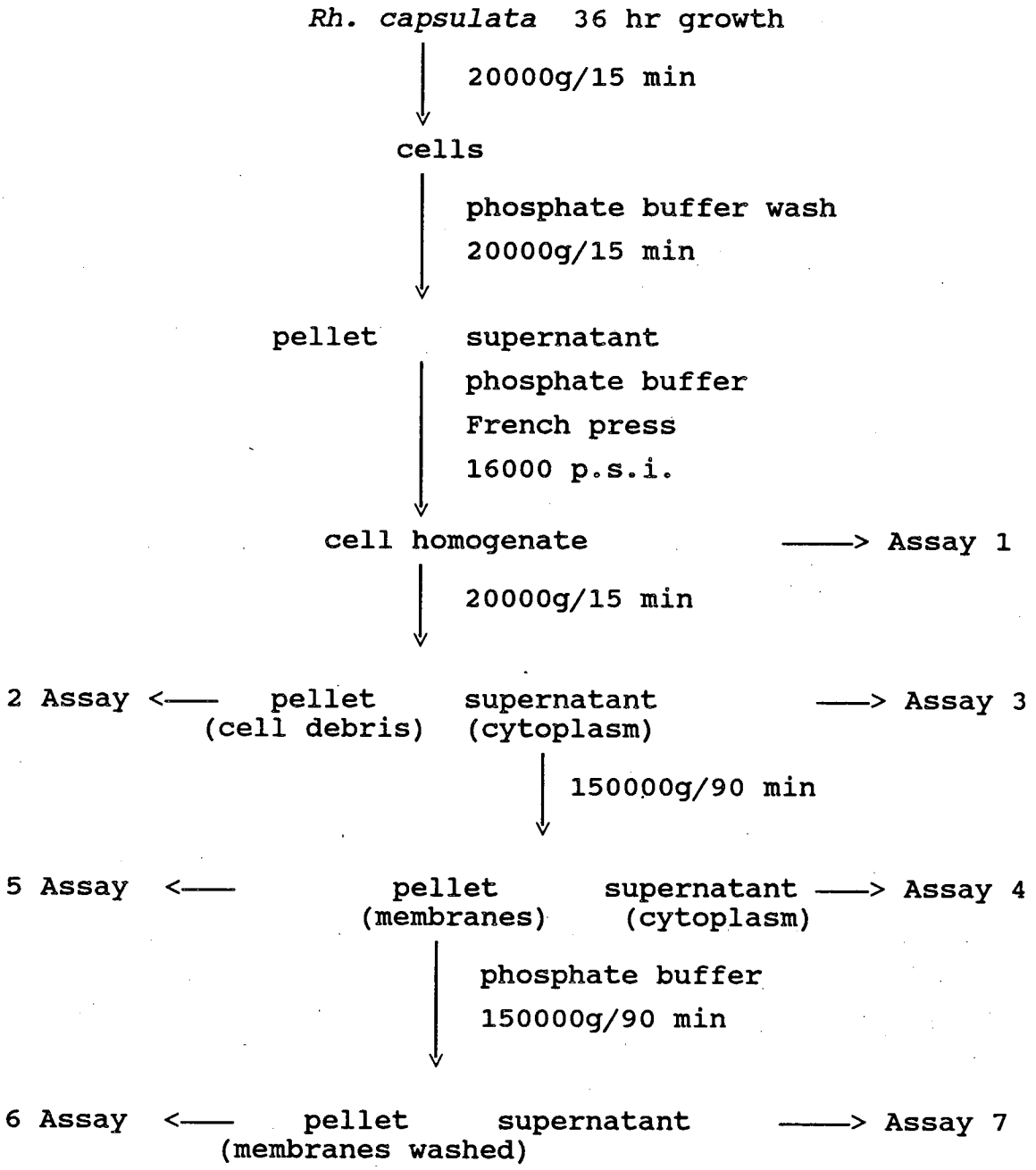

Rh. capsulata 36 hr growth
20000g/15 min
cells
phosphate buffer wash
20000g/15 min
pellet
supernatant
phosphate buffer
French press
16000 p.s.i.
cell homogenate
Assay 1
20000g/15 min
2 Assay
pellet (cell debris)
supernatant (cytoplasm)
Assay 3
150000g/90 min
5 Assay
pellet (membranes)
supernatant (cytoplasm)
Assay 4
phosphate buffer
150000g/90 min
6 Assay
pellet (membranes washed)
supernatant
Assay 7

Figure 15

It was decided to assay for succinate dehydrogenase as well as HMGCoA synthase from this preparation as it would ensure that the preparation was carried out effectively (Cox, Beatty and Flavinger determine succinate dehydrogenase activity using this preparation) and it would give some information on the location of HMGCoA synthase in the cell. Succinate dehydrogenase was shown to be membrane bound and not in the cytosol of *Rh. capsulata*[91].

It should be noted that in chicken liver (eukaryotes) HMGCoA synthases are found both in the cytosol[100] and in the mitochondria[24] where their distinctly different properties are found to be consistent with the independent regulation of cholesterogenesis and ketogenesis respectively. It is therefore not possible to predict solubility etc. of the bacterial protein which may be soluble (cytosolic) or bound to some membrane fraction.

The assay method used for succinate dehydrogenase was based on a method of King[101] as developed by Owen and Freer[102]. Succinate was used as a substrate along with potassium cyanide as an inhibitor of the terminal oxidase, phenazine methosulphate (PMS) was used as the intermediate electron acceptor and dichlorophenolindophenol (DCPIP) as terminal electron acceptor. The activity of succinate dehydrogenase was measured by the decrease in absorption at 600 nm of DCPIP. Figure 16 shows the cascade of electron transfers with PMS as the intermediate electron

acceptor, which results in the oxidative decolouration of the DCPIP dye. The precise mechanisms of succinate oxidation by ferricyanide and PMS are not known although it can be presumed that oxidation occurs *via* a series of one electron transfer steps.

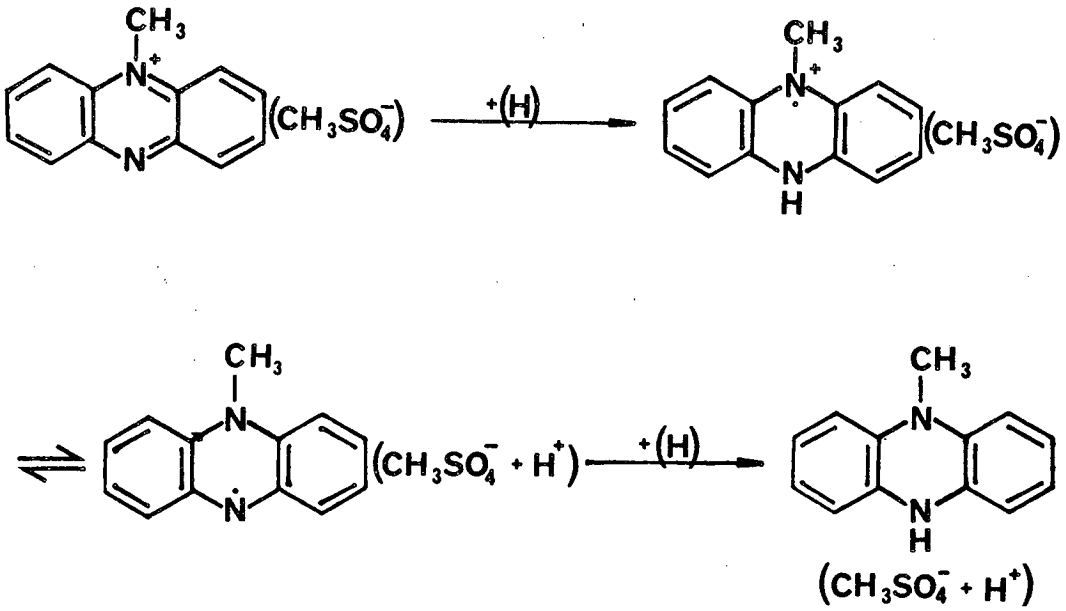


FIGURE 16

Table 6 shows the thiolase corrected rates, protein concentrations and specific activities of the various stages of the preparation (Figure 15).

	HMGCoA Synthase Rate (min^{-1})	Succinate Dehydrogenase Rate (min^{-1})	Protein conc. ($mgml^{-1}$)	HMGCoA Synthase Specific Activity (units mg^{-1})
1 cell homogenate	0.0064		2.65	0.0120
2 cell debris	0.0037		18.25	0.0010
3 cell supernatant	0.0040		2.7	0.0074
4 cytosol	0.0023	0	6.0	0.0019
5 membranes	0.0010	0.00473	6.6	0.0007
6 membranes (washed)	0		2.2	0
7 washings	0.0020		6.9	0.0014
1 cell homogenate (after 3 hrs)	0.0047		2.65	0.0090

Table 6

These results show several important features:

1. HMGCoA synthase exists in *Rh. capsulata* and is present mainly in the cytosol.
2. Succinate dehydrogenase is present in *Rh. capsulata* and is completely membrane bound.
3. *Rh. capsulata* HMGCoA synthase is probably not present in the membranes although this negative result may be

due to cleavage of the enzyme by peptidases.

4. *Rh. capsulata* HMGCoA synthase is unstable. Activity decreases by ≈ 25% in three hours at RT.
5. *Rh. capsulata* HMGCoA synthase has no affinity for pyruvate as its addition made no change to the rate.

In conclusion, it appears likely that isoprenoids are biosynthesised by the classical acetoacetate pathway in *Rh. capsulata*. To further confirm this theory the assay of other enzymes in this pathway such as HMGCoA reductase is necessary.

EXPERIMENTAL

GENERAL METHODS

All starting materials were purchased from commercial sources mainly Aldrich, Fluka and Sigma. Reagents were routinely redistilled or recrystallised before use. The following solvents were dried using the reagents given in parenthesis before distillation:- chloroform, dichloromethane, ethyl acetate (calcium chloride); ethanol, methanol (magnesium sulphate); diethyl ether, tetrahydrofuran, 1,2-dimethoxyethane (lithium aluminium hydride); toluene, benzene (sodium); ethyl formate (phosphorous pentoxide).

Merck Kieselgel 100 (70-230 mesh) silica was used for flash column chromatography. Thin layer chromatography (t.l.c.) was carried out on 20x20cm glass sheets precoated with Merck Kieselgel 60 silica. Solvent systems were either ethyl acetate/hexane (1:1) or chloroform/methanol 5% unless otherwise stated. Visualisation of the components was achieved by either: iodine vapour, UV absorption at 254 nm or phosphomolybdic acid spray. High pressure liquid chromatography (HPLC) was carried out using a Gilson 303 pump and 802 manometer, a LDG 1203 UV III monitor and a BCG Goerz servoger 210 printer.

Infra red spectra were recorded on a Perkin Elmer 781 spectrophotometer using the polystyrene 1603 cm^{-1} peak as a standard. Solid samples were tested as nujol mulls and

oils were tested as thin films.

Electron Impact (EI) mass spectra were recorded on an AEI MS902 spectrometer. Fast atom bombardment (FAB) and chemical ionisation (CI) spectra were recorded on a Kratos MS50TC spectrometer.

Proton nuclear magnetic resonance (NMR) spectra were recorded on either a Bruker WP80 (80 MHz), WP200 (200 MHz) or WH360 (360 MHz) spectrometer in the solvent indicated using tetramethylsilane (TMS) (δ 0.0) as an external standard. Carbon 13 NMR spectra were recorded on either a Bruker WP200 operating at 50.3 MHz or a Bruker WP360 operating at 90 MHz using $CDCl_3$ as an internal standard (δ 77.0 ppm).

Melting points were recorded on an electrically heated Reichert 7905 melting point apparatus and are uncorrected.

2-HYDROXYTETRAHYDROPYRAN (82):-

2,3-Dihydropyran (81) (168 ml : 155g : 1.84 mol) was added slowly to a stirred, ice cooled, solution of water (300 ml) and conc hydrochloric acid (25 ml). The temperature was maintained below 37°C and the reaction ran for ½ hour. The mixture was neutralised using 5N sodium hydroxide (phenolphthalein indicator) until a faint pink colour remained, and then continuously extracted with ether for 20 hours. The extract was dried with magnesium sulphate, filtered, evaporated and distilled through a vigreux column to give a clear colourless oil (125.5 g,

67%); b.p. 66-69°C (1-3 mm Hg) (lit. 50-52°C, 0.7 mm Hg); δ_H($CDCl_3$, 80 MHz), 1.37(6H, m, 3xCH_2), 340(1H, m, CH), 3.80(1H, m, CH), 4.75(1H, bs, OH), 4.75(1H, m, CH); E.I.M.S. m/z 101$(M-1)^+$, 85, 86, 43.

1-(TETRAHYDROPYRANYL-2-OXY)-BUTAN-3-ONE (74):-

Methyl vinyl ketone (83) (70.0 g, 83 ml, 1 mol) was added over 3/4 hour to a stirred solution of 2-hydroxy-tetrahydropyran (82) (102 g, 1 mol) and methanolic potassium hydroxide (3N) (4.00 ml) at -5°C. The mixture was stirred at below 5°C for 2 hours and room temperature for 1½ hours. The mixture was then neutralised using hydrochloric acid (2N) (phenolphthalein indicator), diluted with ether (400 ml) and dried over sodium sulphate before being evaporated and distilled to give a clear colourless oil (115.8 g, 67%); b.p. 77-83°C, 2 mm Hg (lit[59]. 110°C, 13 mm Hg); δ_H($CDCl_3$, 80 MHz), 1.37(6H, m, 3xCH_2), 1.95(3H, s, Me), 2.47(2H, t, J=5H, CH_2), 3.2-3.9(4H, m, 2xCH_2), 4.35(1H, m, CH); ν_{max}(thin film), 1720 cm^{-1} (C=O); EIMS m/z 171$(M-1)^+$, 127, 99, 85, 71, 55, 43.

1-(TETRAHYDROPYRANYL-2-OXY)-3-HYDROXY-3-METHYLHEX-5-ENE (84):-

Allyl bromide (3.62 g, 30 mmol) and 4-(tetrahydro-pyranyl-2-oxo)-butan-3-one (74) (3.44 g, 20 mmol) in dry tetrahydrofuran (40 ml) were added to magnesium turnings

(0.96 g, 40 mmol) under nitrogen at such a rate that the mixture refluxed gently. After a further 3 hours refluxing, the mixture was cooled, diluted with water (40 ml), relieved of excess magnesium *via* a sieve and acidified with conc. sulphuric acid (2 ml). The solution was then extracted with ether (3 x 50 ml) and the extract washed with saturated sodium bicarbonate (50 ml), dried over magnesium sulphate, filtered and evaporated to give a clear colourless oil. Purification by flash column chromatography on silica gave a clear colourless oil (2.18 g, 50%); b.p. 102-106°C, 1 mm Hg; $\delta_H(CDCl_3$, 80 MHz), 1.10(3H, s, Me), 1.50(8H, bm, 4xCH_2), 2.35(2H, d, J=7Hz, CH_2), 3.1(1H, bs, OH), 3.3-4.1(4H, m, 2xCH_2), 4.5(1H, bs, CH), 4.9(1H, m, CH), 5.05(1H, m, CH), 5.5-6(1H, m, CH); FABMS m/z 215.16473 (MH^+ $CH_{12}H_{23}O_3$ requires 215.16471), EIMS 173, 101, 85, 43; Found: C, 67.3; H, 10.6 requires C, 67.3; H, 10.3% along with 1,3-dihydroxy-3-methylhex-5-ene (71) (0.58 g, 22%); b.p. 85-87 1 mm Hg (lit. 175-180°C, 20 mm Hg); $\delta_H(CDCl_3$, 80 MHz), 1.20(3H, s, Me), 1.70(2H, td, J=6Hz, 2Hz, CH_2), 2.30(2H, d, J=7Hz, CH_2), 3.40(2H, bs, 2xOH), 3.85(2H, t, J=6Hz, CH_2), 5.0(1H, m, CH), 5.2(1H, m, CH), 5.6-6.1(1H, m, CH); FABMS m/z 131.10721 (MH^+ $C_7H_{15}O_2$ requires 131.10720); FABMS m/z 131(MH^+), 102, 94.

1,3-DIHYDROXY-3-METHYLHEX-5-ENE (71):-

1-(tetrahydropyranyl-2-oxo)-3-hydroxy-3-methylhex-5-ene (84) (626 mg, 3 mmol) was stirred in a mixture of

ethanol (15 ml) and hydrochloric acid (2N) (1 ml) at 50°C for 20 minutes. The mixture was then neutralised with sodium hydroxide (5N) (phenolphthalein indicator), dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (280 mg, 73%). The product was tlc, NMR and b.p. homogeneous with a standard sample.

2,4-DIHYDROXY-4-METHYLTETRAHYDROPYRAN (63):-

A solution of 1,3-dihydroxy-3-methyl-5-ene (71) (280 mg, 2.15 mmol) in methylene chloride (25 ml) was subjected to ozonolysis for ½ hour at -78°C. Triphenylphosphine (676 mg, 1.2 eq, 2.58 mmol) was added and the mixture allowed to warm up to room temperature overnight. The solvent was removed and the residue purified by flash column chromatography on silica gel to give a mixture of diastereomers as a clear colourless oil (230 mg, 90%), b.p. ≈ 80°C 1 mm Hg dec., $\delta_H(CDCl_3$, 360 MHz), 1.18-1.25(6H, m, 2xCH_3), 1.28-1.82(8H, m, 4xCH_2), 2.0-2.25(2H, bs, 2xCH), 3.48-4.25(6H, m, 2xCH_2 + 2xOH), 4.9-5.25(2H, m, 2xCH); $\delta_C(CDCl_3$, 50 MHz), 30.2(Me), 37.9(C4), 41.0(C2), 55.6(C5), 68.0(C3), 92.1(C1); and 30.9(Me), 37.8(C4), 45.2(C2), 61.2(C5), 69.2(C3), 93.0(C1); CIMS m/z 133(MH^+), 131, 115, 98; FABMS m/z 132.078643 (M $C_6H_{12}O_3$ requires 132.07864). One of the diastereomers was obtained in sufficient purity to assign its proton coupling constants ($CDCl_3$, 360 MHz), 1.20(3H,

s, Me), 1.54(1H, ddddd, $^{2}J_{4eq4ax.}$ = 13.7Hz, $^{4}J_{4eq,5ax}$ = 2.8Hz, $^{4}J_{4eq2eq}$ = 2.6Hz, $^{3}J_{4eq5eq}$ = 2.0Hz, $^{5}J_{4eq1eq}$ = 0.7Hz, H4EQ), 1.65(1H, dd, $^{2}J_{2ax2eq}$ = 14.0Hz, $^{3}J_{2ax1eq}$ = 3.5Hz, H2AX), 1.70(1H, ddd, $^{2}J_{4ax4eq}$ = 13.7Hz, $^{3}J_{4ax5Ax}$ = 12.4Hz, $^{3}J_{4ax5eq}$ = 5.3Hz, H4AX), 1.79(1H, ddd, $^{2}J_{2eq2Ax}$ = 14.0Hz, $^{4}J_{2eq4eq}$ = 2.6Hz, $^{5}J_{2eq5ax}$ = 0.6Hz, H2EQ), 2.25(1H, bs, OH), 3.61(1H, dddd, $^{2}J_{5eq5ax}$ = 11.9Hz, $^{3}J_{5eq4ax}$ = 5.3Hz, $^{3}J_{5eq4eq}$ = 2.0Hz, $^{4}J_{5eq1eq}$ = 0.4Hz, H5EQ), 3.68(2H, bs, 2xOH), 4.17(1H, dddd, $^{3}J_{5ax4ax}$ = 12.4 Hz, $^{2}J_{5ax5eq}$ = 11.9Hz, $^{3}J_{5ax4eq}$ = 2.8Hz, $^{5}J_{5ax2eq}$ = 0.6Hz, H5AX), 5.22(1H, ddd, $^{3}J_{1eq2ax}$ = 3.5Hz, $^{4}J_{1eq5eq}$ = 0.4Hz, H1EQ). The average ratio of the intensities of the isomerically equivalent carbon atoms in the ^{13}C spectrum was 3:10 with the isomer described in detail above being the major component.

METHYL-3-(TETRAHYDROPYRANYL-2-OXY)-PROPANOATE (86):-

Methyl acrylate (85) (50 g, 5.0 ml, 60 mmol) was added to a stirred solution of 2-hydroxytetrahydropyran (82) (5.1 g, 50 mmol) and methanolic potassium hydroxide (3N) (0.2 ml) at 50°C over 1 hour. A further portion of methyl acrylate (2.5 ml, 30 mmol) and methanolic potassium hydroxide (3N) (0.2 ml) were added and the reaction left to stir at 50°C for another hour. Finally, methanolic potassium hydroxide (3N) (0.2 ml) was added and after ½ hour when the solution was neutral the excess methyl acrylate was evaporated off and the residue purified by

flash column chromatography on silica gel to give a clear colourless oil (6.8 g, 73%); b.p. 69-71°C 1 mm Hg; δ_H($CDCl_3$, 200 MHz), 1.50(6H, m, 3xCH_2), 2.53(2H, t, ^3J=6.4Hz, CH_2), 3.63(3H, s, Me), 3.3-4.2(4H, m, 2xCH_2), 4.56(1H, m, CH); ν_{max}(thin film) 1745 cm^{-1} (C=O); FABMS m/z 189.11268 (MH^+ $C_9H_{17}O_4$ requires 189.11267); FABMS m/z 189(MH^+), 85.

3-(TETRAHYDROPYRANYL-2-OXY)-PROPANOIC ACID (87):-

The esters (86) (1.14 g, 6 mmol) was stirred at 60°C in 10% sodium hydroxide (10 ml) for 2 hours then diluted with water (10 ml) and stirred for a further 2 hours at 60°C. The solution was then extracted with ethyl acetate, acidified with hydrochloric acid (2N) to pH 4-5 and extracted again with ethyl acetate. The extract was dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (894 mg, 86%); b.p. 96-99°C, 1 mm Hg; δ_H($CDCl_3$, 60MHz), 1.67(6H, m, 3xCH_2), 2.33(2H, t, ^3J=6Hz, CH_2), 3.0-4.1(4H, m, 2xCH_2), 4.31(1H, bs, CH), 10.1(H, bs, OH); ν_{max}(thin film) 1650 (C=O) 3400 cm^{-1} (OH); FABMS m/z 175.09702 (MH^+ $C_8H_{15}O_4$ requires 175.09703); FABMS m/z 175(MH^+), 157, 101, 85.

ATTEMPTED PREPARATION OF 1-(TETRAHYDROPYRANYL-2-OXY)-BUTAN-3-ONE (74):-

The acid (87) (474 mg, 2.75 mmol) in dry

dimethoxyethane (2 ml) was added dropwise over 10 minutes to a stirred solution of lithium hydride (26 mg, 3.2 mmol) in dimethoxyethane (2 ml). The mixture was then refluxed for 2 hours, before the then bright pink suspension was cooled (ice bath) and an ethereal solution of methyl lithium (2.28 ml of 1.4M soln., 3.2 mmol) added. The mixture was stirred at room temperature overnight before being diluted with water (8 ml), acidified to neutrality with hydrochloric acid (2N, 3ml) and extracted with ether. The extract was dried over magnesium sulphate, filtered and evaporated to give a complex oil (229 mg) the bulk of which was t.l.c. homogeneous with 2-hydroxytetrahydropyran (82).

1-(TETRAHYDROPYRANYL-2-OXY)-PROPAN-3-OL (89):-

Dihydropyran (81) (54.74 ml, 50.5 g, 0.6 mol) was added to a solution of propane-1,3-diol (88) (36 ml, 38 g, 0.5 mol) and dry methylene chloride (400 ml) along with p-toluenesulfonic acid (1.9 g, 0.01 mol) at 0°C under nitrogen and stirred for 1.5 hours. Solid sodium bicarbonate (35 g) was added, and the solution filtered through a plug of Celite before being evaporated to give a yellow oil (90 g). Purification of this crude mixture (5 g) by silica flash column chromatography gave a clear colourless oil (1.65 g, 37%); b.p. 62-64°C, 1 mm Hg; $\delta_H(CDCl_3$, 60 MHz), 1.60(6H, bm, $3xCH_2$), 1.80(2H, m, CH_2), 2.64(1H, bs, OH), 3.3-4.1(6H, bm, $3xCH_2$), 4.53(1H, bs,

CH); FABMS m/z 161.11777(MH^+ $C_8H_{17}O_3$ requires 161.11776); EIMS m/z 159($M-1^+$) 101, 85.

1-(TETRAHYDROPYRANYL-2-OXY)-PROPAN-3-OL (89):-

The ester (86) (1.7 g, 9 mmol) in dry tetrahydrofuran (10 ml) was added to a suspension of lithium aluminium hydride (684 mg, 18 mmol) in tetrahydrofuran (40 ml). The mixture was stirred under nitrogen at room temperature and refluxed for 1 hour each before water (0.76 ml), 10% sodium hydroxide (1.14 ml) and water (2.28 ml) were added in succession. After stirring for a further 15 minutes the mixture was diluted with ether (100 ml) and filtered. The filtrate was then dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (960 mg, 67%). The product was t.l.c., NMR and b.p. homogeneous with a standard sample.

1-(TETRAHYDROPYRANYL-2-OXY)-PROPAN-3-AL (90):-

The alcohol (89) (1.46 g, 9 mmol) was stirred with pyridinium chlorochromate (3.25 g, 15 mmol), sodium acetate (120 mg, 1.5 mmol) and dry methylene chloride (35 ml) at room temperature for 1 hour. The reaction mixture was diluted with ether and filtered through a silica plug before flash column chromatography on silica gave a clear colourless oil (600 mg, 42%); b.p. 48-51°C 1 mm Hg; δ_H($CDCl_3$, 60 MHz), 1.67(6H, m, 3xCH_2), 2.71(2H, td,

^3J=6.2Hz, CH_2), 3.2-4.3(4H, m, 2xCH_2), 4.63(1H, bs, CH), 9.82(1H, t, ^3J=2Hz, CHO); ν_{max}(thin film) 1725 cm^{-1} (C=O); FABMS m/z 159.10210(MH^+ $C_8H_{15}O_3$ requires 159.10211); FABMS m/z 159(MH^+), 102, 87, 47.

1-(TETRAHYDROPYRANYL-2-OXY)-BUTAN-3-OL (91):-

The aldehyde (90) (600 mg, 3.8 mmol) in dry ether (5 ml) was added to a stirred solution of magnesium (91 mg, 3.8 mmol), iodomethane (0.24 ml, 540 mg) and ether (5 ml) under nitrogen. The mixture was refluxed for 1 hour before being allowed to cool and then acidified with ammonium chloride (satd., 50 ml). The mixture was then extracted with ether and the extract dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (216 mg, 33%); b.p. 61-63°C 0.5 mm Hg; δ_H($CDCl_3$, 60 MHz), 1.2(3H, d, ^3J=7Hz, CH_3), 1.67(6H, m, 3xCH_2), 1.73(2H, m, CH_2), 3.2(1H, bs, OH), 3.2-4.2(5H, m, 2xCH_2 + CH), 4.55(1H, bs, CH); FABMS m/z 175.133411 (MH^+ $C_9H_{19}O_3$ requires 175.133410), EIMS m/z 173($M-1^+$), 129, 101, 85, 73, 45.

1-(TETRAHYDROPYRANYL-2-OXY)-BUTAN-3-ONE (74):-

The alcohol (91) (210 mg, 1.2 mmol) was stirred with pyridinium chlorochromate (388 mg, 1.8 mmol), sodium acetate (15 mg, 0.18 mmol) and dry methylene chloride (5 ml) at room temperature for 3 hours. The reaction

mixture was diluted with ether (20 ml), filtered through a silica plug, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (144 mg, 70%). The product was t.l.c., NMR and b.p. homogeneous with a standard sample.

1,1-DIMETHOXYBUTAN-3-ONE (97):-

Anhydrous ethyl formate (96) (0.62 g, 10.3 mmol) and acetone (0.5 g, 8.6 mmol) in anhydrous ether (2 ml) were added dropwise to a stirred suspension of sodium methoxide (0.47 g, 8.6 mmol) in anhydrous ether (5 ml) under nitrogen. The reaction mixture was then refluxed for 1 hour, cooled and the ether carefully removed at aspirator pressure. Methanol (2.06 ml, 1.65 g, 51.5 mmol) was added to the residue and the suspension stirred for a few minutes before the addition of methanolic hydrogen chloride (17.4 mmol, 1.2 ml of a 15M soln.). After 2 hours at room temperature the pH was adjusted to 8 by addition of saturated methanolic potassium hydroxide. Ether (5 ml) was added and the mixture filtered, the filtrate concentrated on a rotary evaporator and the residue redissolved in ether. The mixture was once again filtered and evaporated, (care was taken to avoid loss of product by prolonged evaporation) and the residue was distilled via a short path condenser using liquid nitrogen to cool both flasks (receiver and reaction) before applying a high vacuum. After 1 hour the distillate was

dissolved in ether and left over Linde sieve 4Å and sodium sulphate to remove methanol and water. The ether was finally evaporated off to give a clear colourless oil (160 mg, 14%); b.p. 62-64°C 760 mm Hg; (lit[72]. 64°C); δ_H($CDCl_3$, 60 MHz), 2.1(3H, s, CH_3), 2.68(2H, d, ^3J=7Hz, CH_2), 3.3(6H, s, 2xCH_3), 4.72(1H, t, ^3J=7Hz, CH); ν_{max}(thin film), 1720 cm^{-1} (C=O); EIMS 101, 85, 75, 43, 32, 31.

ETHYL 3-HYDROXY-3-METHYL-5,5-DIMETHOXY PENTANOATE (98):-

To diisopropylamine (7 ml, 50 g, 50 mmol) in dry tetrahydrofuran (150 ml) at -78°C under nitrogen was added butyllithium (32 ml of 1.6M soln., 50 mmol) over 10 minutes and the mixture allowed to warm up to 0°C. The mixture was recooled to -78°C, ethyl acetate (4.9 ml, 4.4 g, 50 mmol) added slowly and the mixture stirred at -78°C for ½ hour. Acetoacetaldehyde dimethyl acetal (6.7 ml, 6.6 g, 50 mmol) was added and the mixture left at -78°C and room temperature for ½ hour each before saturated ammonium chloride (75 ml) was added and the tetrahydrofuran evaporated. The residue was extracted with ether, washed with brine, dried over magnesium sulphate filtered and evaporated to give a clear colourless oil (7.6 g, 67%), b.p. 98-100°C 0.5 mm Hg (lit. 70°C, 0.01 mm Hg), δ_H($CDCl_3$, 60 MHz), 1.2(3H, s, CH_3), (3H, t, ^3J=7Hz, CH_3), 1.8(2H, d, ^3J=7Hz, CH_2), 2.43(2H, s, CH_2), 3.25(6H, s, 2xOCH_3), 3.85(1H, bs, OH), 4.1(2H, q, ^3J=7Hz), 4.5(1H, t, ^3J=7Hz, CH); ν_{max}(thin film) 1735

(C=O), 3400 cm^{-1} (OH); FABMS m/z 189($M-31^+$), 171, 131.

1,1-DIMETHOXY-3,5-DIHYDROXY-3-METHYLPENTANE (75):-

A solution of the ester (98) (7.13 g, 32 mmol) in dry tetrahydrofuran (50 ml) was added to a suspension of lithium aluminium hydride (2.43 g, 64 mmol) in tetrahydrofuran (100 ml) and the mixture refluxed for 1 hour. Water (2.43 ml), 10% sodium hydroxide (2.43 ml) and water (7.3 ml) were added in succession and the mixture was filtered, dried with magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (5.3 g, 92%); b.p. 103-105°C, 1 mm Hg; $\delta_H(CDCl_3$, 60 MHz), 1.2(3H, s, CH_3), 1.8(2H, d, ^3J=7Hz, CH_2), (1.8, t, ^3J=7Hz, CH_2), 3.25(6H, s, 2xOMe), 3.7(2H, t, ^3J=7Hz, CH_2), 4.5(1H, t, ^3J=7Hz, CH); FABMS m/z 179.12831 (MH^+ $C_8H_{19}O_4$ requires 179.12832), EIMS m/z 133, 115, 101, 75.

2-METHOXY-4-HYDROXY-4-METHYLTETRAHYDROPYRAN (99):-

The diol (75) (534 mg, 3 mmol) was stirred at room temperature in dry methanol (10 ml) along with AG50 WX2 acidic ion exchange resin (1 g) for 1 hour. The resin was filtered off and washed with methanol. The washings and filtrate combined were evaporated and purified by flash column chromatography on silica gel to give two clear colourless oils (75 mg, 17%); b.p. 60-62°C 8 mm Hg; R_f(hexane/ethylacetate 1:1)=0.5; δ_H ($CDCl_3$, 360 MHz),

1.17(1H, s, CH_3), 1.54(1H, ddddd, $^2J_{4eq4ax}$ = 13.6Hz, $^3J_{4ex5ax}$ = 2.7Hz, $^4J_{4eq2eq}$ = 2.5Hz, $^3J_{4eq5eq}$ = 1.7Hz, $^5J_{4eq1eq}$ = 0.8Hz, H4EQ), 1.7(1H, dd, $^2J_{2ax2eq}$ = 14.1Hz, $^3J_{2ax1eq}$ = 3.6Hz, H2AX), 1.7(1H, ddd, $^2J_{4ax4eq}$ = 13.6Hz, $^3J_{4ax5eq}$ = 5.3Hz, $^3J_{4ax5ax}$ = 12.7Hz, H4AX), 1.78(1H, dddd, $^2J_{2eq2ax}$ = 14.1Hz, $^4J_{2eq4eq}$ = 2.5Hz, $^3J_{2eq1eq}$ = 1.6Hz, $^5J_{2eq5ax}$ = 0.6Hz, H2EQ), 3.37(3H, s, OMe), 3.6(1H, dddd, $^2J_{5eq5ax}$ = 11.8Hz, $^3J_{5eq4ax}$ = 5.3Hz, $^3J_{5eq4eq}$ = 1.7Hz, $^4J_{5eq1eq}$ = 0.6Hz, H5Eq), 3.94(1H, dddd, $^2J_{5ax5eq}$ = 1.8Hz, $^3J_{5ax4ax}$ = 12.7Hz, $^3J_{5ax4eq}$ = 2.7Hz, $^5J_{5ax2eq}$ = 0.6Hz, H5AX), 4.04(111, bs, OH), 4.79(1H, dddd, $^3J_{1eq2ax}$ = 3.6Hz, $^3J_{1eq2eq}$ = 1.6Hz, $^5J_{1eq4eq}$ = 0.8Hz, $^4J_{1eq5eq}$ = 0.6Hz, H1EQ) δ_C($CDCl_3$, 50 MHz), 29.8(Me), 37.9(C4), 40.9(C2), 54.9(C5), 55.7(OMe), 66.5(C3), 98.8(C1) and (245 mg, 56%); b.p. = 46-49°C, 0.75 mm Hg; R_f(hexane/ethyl acetate 1:1)= 0.3; δ_H($CDCl_3$, 360 MHz), 1.27(3H, s, Me), 1.50(1H, dddd, $^2J_{4eq4ax}$ = 13.7Hz, $^3J_{4eq5eq}$ = 4.0Hz, $^3J_{4eq5ax}$ = 3.2Hz, $^4J_{4eq2eq}$ = 1.9Hz, H4EQ), 1.51(1H, ddd, $^2J_{2ax2eq}$ = 13.4Hz, $^3J_{2ax1ax}$ = 7.8Hz, $^4J_{2ax4ax}$ = 0.8Hz, H2AX), 1.63(1H, ddd, $^2J_{4ax4eq}$ = 13.7Hz, $^3J_{4ax5ax}$ = 9.9Hz, $^3J_{4ax5eq}$ = 4.9Hz, $^4J_{5ax2ax}$ = 0.8Hz, H4AX), 1.77(1H, dddd, $^2J_{2eq2ax}$ = 13.4Hz, $^3J_{2eq1ax}$ = 2.7Hz, $^4J_{2eq4eq}$ = 1.9Hz, $^5J_{2eq5eq}$ = 0.3Hz, H2EQ), 3.41(3H, s, OMe), 3.76(1H, ddd, $^2J_{5ax5eq}$ = 11.7Hz, $^3J_{5ax4ax}$ = 9.9Hz, $^3J_{5ax4eq}$ = 3.2Hz, H5AX), 3.84(1H, ddddd, $^2J_{5eq5ax}$ = 11.7Hz, $^3J_{5eq4ax}$ = 4.9Hz, $^3J_{5eq4eq}$ = 4.0Hz, $^4J_{5eq1ax}$ = 0.4Hz, $^5J_{5eq2eq}$ = 0.3Hz, H5EQ), 4.61(1H, ddd, $^3J_{1ax2ax}$ = 7.8Hz, $^3J_{1ax2eq}$ = 2.7Hz, $^4J_{1ax5eq}$ = 0.4Hz,

H1Ax), δ_C($CDCl_3$, 50 MHz), 30.6(Me), 38.4(C4), 43.8(C2), 55.9(C5), 60.6(OMe), 69.0(C3), 99.8(C1); FABMS m/z 147.10212 (MH^+ $C_7H_{15}O_3$ requires 147.10211); FABMS m/z 146(M^+) 130, 116, 99, 72, 46.

2,4-DIHYDROXY-4-METHYLTETRAHYDROPYRAN (63):-

The acetal (146 mg, 1 mmol) was dissolved in dry THF (2 ml) along with AG50 WX2 ion exchange resin (1 g) and water (180 μl, 1 mmol). The mixture was then stirred at 50°C for 1 hour. After cooling, the mixture was evaporated onto silica and purified by flash column chromatography to give a mixture of diastereomers as a clear colourless oil (119 mg, 91%). The product was t.l.c. and NMR identical to a standard sample.

n-BUTYL ACETATE (101):-

Sodium acetate (100) (1.5 g, 18 mmol) was mixed with tri-n-butylphosphate (6 ml) and refluxed for 6 hours until the solution was homogeneous. The mixture was then cooled to room temperature and distilled through the condenser and an ice cooled trap into a liquid nitrogen cooled trap at oil pump pressure. This gave a clear liquid which smelt strongly of pear drops (1.85 g, 89%), b.p. 124-126°C (lit. 124-126°C); δ_H 0.9(3H, m, CH_3), 1.5(4H, m, 2xCH_2), 2.05(3H, s, Me), 4.05(2H, t, ^3J=7Hz, CH_2); ν_{max}(thin film) 1725 cm^{-1} (C=O).

2-^{13}C-n-BUTYL-(3-HYDROXY-3-METHYL-5,5-DIMETHOXY)-PENTANOATE (102):-

Butyl lithium (7.5 ml of a 1.6M soln., 12 mmol) was added to a stirred solution of diisopropylamine (1.67 ml, 1.21 g, 12 mmol) and THF (40 ml) at -78°C under nitrogen over 10 minutes. The mixture was then warmed to 0°C before being recooled to -78°C for the addition of n-butylacetate (101) (1.38 g, 12 mmol). The mixture was left for a further ½ hour at -78°C before the addition of the ketone (97) (1.77 ml, 1.76 g, 12 mmol) and finally left at -78°C and room temperature for ½ hour each. The mixture was then diluted with saturated ammonium chloride (25 ml) and the tetrahydrofuran evaporated. The residue was extracted with ether and the extract was washed with saturated sodium chloride and dried over magnesium chloride before being filtered and evaporated to give an essentially pure (tlc) clear colourless oil (3.0 g, 100%); δ_H($CDCl_3$, 60 MHz), 0.9(3H, m, CH_3), 1.1(3H, d, $^3J_{CH}$=6Hz, CH_3), 1.5(4H, m, 2xCH_2), 1.7(2H, dd, $^3J_{CH}$=6Hz, $^3J_{HH}$=9Hz, CH_2), 2.4(2H, dm, $^1J_{CH}$=128Hz, CH_2), 3.25(6H, s, 2xOCH_3), 3.6(1H, bs, CH), 4.1(2H, t, $^3J_{HH}$=9Hz, CH_2), 4.6(1H, t, $^3J_{HH}$=9Hz, CH).

4-^{13}C-1,1-DIMETHOXY-3,5-DIHYDROXY-3-METHYLBUTANE (75):-

The ester (102) (3.0 g, 12 mmol) was dissolved in THF (10 ml) and added to a stirred suspension of lithium aluminium hydride (912 mg, 24 mmol) and THF (80 ml) under

nitrogen. The mixture was refluxed for 1 hour, cooled, and diluted with water (1.8 ml), 10% sodium hydroxide (2.7 ml) and water (1.8 ml) in succession and stirred for 15 minutes. The mixture was diluted with ether (200 ml), filtered, dried over magnesium sulphate, filtered and evaporated to give an essentially pure oil which was tlc homogeneous with a standard sample (1.84 g, 86%); $\delta_H(CDCl_3$, 60 MHz), 1.1(3H, d, $^3J_{CH}$ =6Hz, CH_3), 1.6(2H, dt, $^1J_{CH}$=128Hz, $^3J_{HH}$=9Hz, CH_2), 1.7(2H, dd, $^3J_{CH}$=6Hz, $^3J_{HH}$= 9Hz, CH_2), 3.25(6H, s, 2xOCH_3), 3.6(2H, s, 2xOH), 3.7(2H, dt, $^3J_{HH}$=9Hz, $^3J_{CH}$=6Hz, CH_2), 4.6(1H, t, $^3J_{HH}$=9Hz, CH).

2-METHOXY-4-HYDROXY-4-METHYLTETRAHYDROPYRAN (99):-

Mevalonic lactone (61) (520 mg, 4 mmol) in dry methylene chloride (40 ml) at -78°C was treated with diisobutylaluminium hydride (4.4 ml of a 1M soln., 4.4 mmol) and allowed to warm up to 5°C. The mixture was recooled to -78°C and treated with excess methanol before being warmed up to room temperature and evaporated to a gum. The gum was covered with methanol and dissolved on the addition of 2N hydrochloric acid to give a clear solution. The mixture was finally neutralised with 5N sodium hydroxide and extracted with chloroform to give after silica flash column chromatography two clear colourless diastereomeric oils (92 mg, 16%) and (192 mg, 33%) which were tlc, NMR and b.p. homogeneous with compounds prepared by another route.

3-TRIMETHYLSILYLPROP-2-YN-1-OL (111):-

Prop-2-yn-1-ol (110) (7 g, 7.27 ml, 0.125 mol) in THF (40 ml) was added to ethylmagnesiumbromide (freshly prepared from magnesium (6.1 g, 0.25 mol) and ethylbromide (28 g, 19.18 ml, 0.25 mol)) and stirred at 0°C for 1.5 hours. Trimethylsilylchloride (28 g, 32.7 ml, 0.25 mol) was added over 1 hour and the mixture was kept at 50°C for 1 hour and left at RT overnight. Saturated ammonium chloride was added (200 ml) and the mixture was extracted with ether (3 x 100 ml). The extract was washed with brine (100 ml) and concentrated before being stirred with ice (7.5 g), ethanol (25 ml) and conc. HCl (2-3 drops) for 1 hour. The mixture was then extracted with ether, the extract was dried with magnesium sulphate, filtered, concentrated and distilled to give a clear colourless oil (5.47 g, 34%); b.p. 79-82°C, 10 mm Hg (lit[78]. 76°C 11 mm Hg); δ_H($CDCl_3$, 60 MHz), 0.0(9H, s, 3xCH_3), 2.2(H, bs, OH), 4.2(2H, s, CH_2); EIMS m/z 113(M-15), 85, 75, 73, 61, 45.

3-BROMO-1-TRIMETHYLSILYLPROP-1-YNE (112):-

The alcohol (111) (12.8 g, 0.1 mmol) in DMF (80 ml) was added to a solution of DMF (400 ml), triphenyl phosphine (100 g, 0.37 mol) and bromine (14 ml, 0.27 mol) and stirred under nitrogen at 0°C for 3 hours and room temperature overnight. The mixture was extracted with petrol (40-60) (3 x 500 ml) and the extract was washed with saturated sodium bicarbonate (330 ml), dried over

magnesium sulphate, filtered, evaporated and distilled after passage through a short silica column to give a clear colourless oil (10.6 g, 55%), b.p. 39-42°C 1 mm Hg (lit[78]. 44-45°C, 2mm Hg); δ_H($CDCl_3$, 60 MHz), 0.0(9H, s, 3xMe), 3.7(2H, s, CH_2); ν_{max}(thin film) 2180 cm^{-1} (C≡C); EIMS m/z $(M-15)^+$ 177, 175, 149, 147, 139, 137, 111, 110, 85.

4-HYDROXY-4-METHYL-6-(TETRAHYDROPYRANYL-2-OXY)-1-TRIMETHYLSILYLHEX-1-YNE (113):-

The bromide (112) (1.9 g, 10 mmol) was stirred overnight with activated zinc (1.95 g, 30 mmol) in dry THF (20 ml) at RT. The ketone (74) (1.72 g, 10 mmol) was then added and the mixture refluxed for four hours. The zinc was filtered off and the yellow gum remaining after evaporation of the THF was purified by flash column chromatography on silica gel to give a clear colourless oil (1.80 g, 63%), b.p. 93-95°C 1mm Hg; δ_H($CDCl_3$, 80 MHz), 0.1(9H, s, Me_3Si), 1.3(3H, s, Me), 1.6(6H, bm, 3xCH_2), 1.9(2H, dd, J=5.7Hz, 1.4Hz, CH_2) 2.4(2H, d, J=1.7Hz, CH_2), 3.2(1H, bs, OH), 3.5-4.0(4H, m, 2xCH_2), 4.6(1H, bs, CH); EIMS m/z 284(M^+), 266, 200, 182, 173; ν_{max}(thin film) 2180 cm^{-1}(m) (-C≡C-), 3500 cm^{-1} (bs)(OH); FABMS m/z 285.18860 (MH^+ $C_{15}H_{29}O_3Si$ requires 285.18858).

1-TRIMETHYLSILYL-4,6-DIHYDROXY-4-METHYLHEX-1-YNE (109):-

The alkyne (113) (1.1 g, 3.7 mmol) was stirred at 40°C

for 1 hour in ethanol (920 ml) along with 2N hydrochloric acid (1.2 ml). The mixture was neutralised with sodium hydroxide (5N) (phenolphthalein indicator) and dried over magnesium sulphate before being filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (709 mg, 96%), b.p. 55-57°C, 0.5 mm Hg; $\delta_H(CDCl_3$, 80 MHz), 0.1(9H, s, Me_3Si), 1.3(3H, s, Me), 1.8(2H, t, ^3J=5Hz, CH_2), 2.4(2H, s, CH_2), 2.9(2H, bs, 2xOH), 3.9(2H, t, ^3J=5Hz, CH_2); ν_{max}(thin film) 2180 cm^{-1} (C≡C) 3400 cm^{-1} (OH); FABMS m/z 201.131071 (MH^+ $C_{10}H_2O_2Si$ requires 201.13107); FABMS m/z 201, 183.

REACTION OF OSMIUM TETROXIDE WITH 1-TRIMETHYLSILYL-4,6-DIHYDROXY-4-METHYL HEX-1-YNE (109):-

Tertiary butyl hydroperoxide (2 ml of 3M soln. in toluene, 6 mmol) was added at 0°C under nitrogen to a solution of the alkyne (109) (240 mg, 1.2 mmol) in methanol (10 ml) followed by osmium tetroxide (17 mg, 0.06 mmol) and stirred for 2 hours. After stirring at room temperature overnight, the solution was diluted with saturated sodium metabisulphite (2 ml) and the bulk solvents were removed *in vacuo*. Saturated ammonium chloride was added and the mixture extracted with methylene chloride (3 x 5ml). The extract was dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (50 mg, 21%). The product was t.l.c., NMR

and b.p. homogeneous with a standard sample of the starting material.

OZONOLYSIS OF 1-TRIMETHYLSILYL-4,6-DIHYDROXY-4-METHYL HEX-1-YNE (109):-

The alkyne (109) (900 mg, 4.5 mmol) was dissolved in methanol (20 ml) and ozonised at -78°C for forty minutes. The mixture was then allowed to warm up to room temperature over 30 minutes before being evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (290 mg); R_F = 0.2-0.3 (50/50 ethyl acetate/hexane); FABMS m/z 173, 155, 131, 113; IR(thin film) 1770-1700 cm^{-1} (C=O), 3400 cm^{-1} OH; δ_C($CDCl_3$, 50 MHz) 26.5, 41.7, 45.0, 51.4, 58.7, 71.5, 95.0, 172.5 (C=O); δ_H($CDCl_3$, 200 MHz), 1.26(s, CH_3), 1.32(s, CH_3), 1.75(m, CH_2), 2.5(q, J≈15.3Hz, CH_2), 3.66(s, OCH_3), 3.7(m, CH_2), 4.3, 4.6(m, CH_2).

METHYL TRIPHENYLPHOSPHONIUM IODIDE:-

Triphenylphosphine (27.5 g, 0.11 mol) was dissolved in dry benzene (22.5 ml) and placed in a pressure bottle which was cooled in an ice salt bath before addition of iodomethane (9.35 ml, 21.3 g, 0.15 mol). The bottle was then left to stand sealed at RT for 1 day. The white precipitate was filtered off and washed with hot benzene before being dried at 100°C overnight in a vacuum pistol (41 g, 100%), m.p. 182-184°C (lit[81]. 183-184°C).

1-(TETRAHYDROPYRANYL-2-OXY)-3-METHYLBUT-3-ENE (123):-

Dry ether (250 ml) and triphenylmethylphosphonium iodide (17.85 g, 44 mmol) were stirred at room temperature under argon while butyl lithium (32 ml of 1.6M, 51 mmol) was added over 10 minutes. The solution was left for 4 hours at room temperature before the ketone (74) (8.6 g, 50 mmol) was added and the solution stirred overnight. The mixture was then refluxed for 5 hours, allowed to cool and filtered. The filtrate and ether washings of the filter cake were washed with water until neutral then dried over calcium chloride, filtered, evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (1.3 g, 15%); b.p. 58-60°C 8 mm Hg; δ_H($CDCl_3$, 60 MHz), 1.6(6H, bm, 3xCH_2), 1.8(3H, s, CH_3), 2.3(2H, t, ^3J=7Hz, CH_2), 3.3-4.1(4H, bm, 2xCH_2), 4.6(H, bs, CH), 4.8(2H, bs, CH_2); FABMS m/z 171.13849 (MH^+ $C_{10}H_{19}O_2$ requires 171.13850), EIMS m/z 169, 129, 101, 85, 69, 41.

1-(TETRAHYDROPYRANYL-2-OXY)-3-METHYLBUT-3-ENE (123):-

The alcohol (122) (10.1 ml, 0.1 mol), dihydropyran (81) (13.7 ml, 0.15 mol) and p-toluenesulfonic acid (50 mg, 0.2 mmol) were stirred at room temperature for 20 minutes in dry methylene chloride (200 ml). The mixture was then diluted with ether (200 ml), washed with brine (100 ml), dried over sodium sulphate, and evaporated to

give an essentially pure oil (17 g, 100%) which was t.l.c., NMR and b.p. identical to a standard sample.

1-(TETRAHYDROPYRANYL-2-OXY-)3-METHYLBUT-3-ENE EPOXIDE (124):-

The alkene (123) (3.4 g, 20 mmol) in methylene chloride (50 ml) was refluxed with m-chloroperbenzoic acid (4.3 g, 20 mmol) for 3 hours and left stirring overnight at room temperature. The precipitate was filtered off and the filtrate was washed with 20% sodium sulphite (5 ml), 10% sodium bicarbonate (3 x 5 ml) and brine (5 ml) before being dried over magnesium sulphate filtered and evaporated to give an essentially pure clear colourless oil (3.7 g, 100%), b.p. 65-67°C 1 mm Hg; δ_H($CDCl_3$, 200 MHz), 1.34(3H, s, CH_3), 1.5-2.0(8H, m, 4xCH_2), 2.6(2H, dddd, $^2J_{HH}$=14.2Hz, J=2.5, H, 0.6), 3.5(2H, m, CH_2), 3.85(2H, m, CH_2), 4.57(1H, s, CH); FABMS m/z 187.13340 ($C_{10}H_{19}O_3$ requires 187.13341); 187$(MH)^+$ 185$(M-H)^+$.

1,2-DIHYDROXY-2-METHYL-4-(TETRAHYDROPYRANYL-2-OXY)-BUTANE (125):-

The epoxide (124) (380 mg, 2 mmol) was refluxed for 3 days in water/THF (50:50) (10 ml) along with sodium hydroxide (5N, 2 ml). The mixture was neutralised and evaporated onto silica to give, after flash column chromatography, a clear colourless oil (95 mg, 23%), b.p. 79-81°C; 1 mm Hg; δ_H($CDCl_3$, 60 MHz); 1.2(3H, s, CH_3),

1.7(8H, bm, 4xCH_2), 3.4(2H, s, CH_2), 3.3-4.1(6H, bm, 2xCH_2, 2OH), 4.6(H, bm, CH); FAB MS m/z 205$(MH)^+$ 101, 85.

OXIDATION OF 1,2-DIHYDROXY-2-METHYL-4-(TETRAHYDROPYRANYL-2-OXY)-BUTANE (125):-

The diol (125) (300 mg, 1.5 mmol) in dry methylene chloride (10 ml) was stirred along with sodium acetate (20 mg) and pyridinium chlorochromate (485 mg, 2.25 mmol) at room temperature for 4 hours. The solution was diluted with ether and filtered through a silica plug to give, after flash column chromatography on silica gel, a clear colourless oil (200 mg, 77%). The product was t.l.c., NMR and b.p. identical to 1-(tetrahydropyranyl-2-oxy)-butan-3-one (74).

ETHYL-3-HYDROXY-3-METHYLPENT-1-ENOATE (128e):-

Butyl lithium (12.5 ml of a 1.6M soln., 20 mmol) was added to a stirred solution of THF (75 ml) and diisopropylamine (2.8 ml, 20 mmol) at -78°C under nitrogen and the mixture was allowed to warm up to 0°C over ½ hour. The mixture was recooled to -78°C and ethyl acetate (1.95 ml, 20 mmol) was added. After ½ hour at -78°C the ketone (83) (1.06 ml, 20 mmol) was added and stirred for ½ hour at -78°C and ½ hour at room temperature. The mixture was diluted with ammonium chloride (50 ml) and the THF evaporated *in vacuo*. The residue was then extracted with ether and the extract washed with brine, dried over

magnesium sulphate, filtered, evaporated and purified by flash column chromatography to give a clear colourless oil (3.2 g, 100%); b.p. 64-66°C, 11 mm Hg; δ_H($CDCl_3$, 60 MHz), 1.4(3H, s, CH_3), 1.35(3H, t, $^3J_{HH}$=7Hz, CH_3), 2.7(2H, s, CH_2), 3.9(1H, bs, OH), 4.2(2H, q, $^3J_{HH}$=7Hz, CH_2), 5.0-6.3(3H, m, CH_2CH); ν_{max}(thin film) 1740 cm^{-1} (C=O), 3500 cm^{-1} (OH); FAB MS m/z 159.10210 (MH^+ $C_8H_{15}O_3$ requires 159.10211); 159, 141.

3,5-DIHYDROXY-3-METHYLPENT-1-ENE (129e):-

The ester (128e) (3.2 g, 20 mmol) in THF (10 ml) was added to a solution of THF (100 ml) and lithium aluminium hydride (1.52 g, 40 mmol) and refluxed for 1 hour. Water (2.6 mol), 10% sodium hydroxide (4 ml) and water (7.8 ml) were added successively and the mixture stirred for a further 15 minutes. The mixture was diluted with ether, filtered, dried over magnesium sulphate, filtered and evaporated and purified by flash column chromatography on silica gel to give a clear colourless oil (2.1 g, 90%); b.p. 41-43°C 0.5 mm Hg; δ_H($CDCl_3$, 60 MHz), 1.4(3H, s, CH_3), 1.8(2H, m, CH_2), 3.4(2H, bs, 2xOH), 3.8(2H, t, $^3J_{HH}$=6Hz, CH_2), 5.0-6.3(3H, m, CH_2CH); FABMS m/z 117.09154 (MH^+ $C_6H_{13}O_2$ requires 117.09155); FABMS m/z 117, 99, 81.

2,4-DIMETHYL-4-ETHENYL-1,3-DIOXAN (130eii):-

The alcohol (129e) (9.1 g, 78 mmol) was stirred in dry methylene chloride (40 ml) with acetaldehyde (40 ml),

anhydrous copper sulphate (5 g, 2 eq) and p-toluene sulfonic acid (200 mg) for 2 hours at room temperature. The mixture was diluted with ether (100 ml), washed with saturated sodium bicarbonate, dried with magnesium sulphate, filtered and evaporated to give a clear colourless oil after flash column chromatography on silica. The product was characterised as a mixture of two diastereomers (10 g, 91%); boiling range 95-115°C; $\delta_H(CDCl_3$, 200 MHz), 1.20-1.37(12H, m, 4xCH_3), 1.5-2.0(4H, m, 2xCH_2), 3.6-3.9(4H, m, 2xCH_2), 4.8-5.9(6H, m, 2xCH_2CH); $\delta_C(CDCl_3$, 50 MHz), 19.8, 21.1, 21.3, 30.7(4xCH_3), 33.5, 33.9(2xC3), 62.5, 63.2(2xC4), 72.9, 74.4(2xC2), 92.1, 93.6(2xC1), 111.4, 115.3(2x$C8_2$), 141.7; 145.0(2x$C7_2$). FABMS m/z 143.10719 (MH^+ $C_8H_{15}O_2$ requires 143.10720); FABMS m/z 143, 141.

2,4-DIMETHYL-4-ALDEHYDO-1,3-DIOXAN (131ii):-

The alkene (994 mg, 7 mmol) in dry methylene chloride (30 ml) was subjected to ozonolysis for 15 minutes at -78°C until the solution became blue. Triphenylphosphine was then added and the mixture allowed to warm up to room temperature over 1 hour. The mixture was concentrated *in vacuo* and the residue purified by flash column chromatography on silica gel to give a clear colourless oil (910 mg, 90%). The product was characterised as a mixture of diastereomers. $\delta_H(CDCl_3$, 200 MHz), 1.1-1.4(12H, m, 4xCH_3), 1.5-2.0(4H, m, 2xCH_2), 3.54(1H,

td, $^{2}J_{6ax6eq}$= $^{3}J_{6ax5ax}$=11.8Hz, $^{3}J_{6ax5eq}$=2.8Hz, H6AX), 3.85(1H, td, $^{2}J_{6ax6eq}$=$^{2}J_{6ax5ax}$=11.7Hz, $^{3}J_{6ax5eq}$=2.7Hz, H6AX), 4.08(1H, ddd, $^{2}J_{6eq6ax}$=11.7Hz, $^{3}J_{6eq5ax}$=6.2Hz, $^{3}J_{6eq5eq}$=2.0Hz, H6Eq), 3.9(1H, m, H6EQ), 9.5(CHO, s) 4.68(1H, q, ^{3}J=5Hz, CH), 4.95(1H, q, ^{3}J=5Hz, CH); δ_{C}($CDCl_3$, 50 MHz) 16.2, 20.7, 21.1, 23.0(4xCH_3), 28.1, 30.1(C3x2), 61.9, 63.9(C4x2), 79.3(C2), 92.5, 96.7(C1x2), 202.1, 204.3(2xC=O); ν_{max}(thin film) 1740 cm^{-1} (C=O); FABMS m/z 145.08647 (MH^{+} $C_7H_{13}O_3$ requires 145.08646); FABMS m/z 145, 143, 115.

CYCLOHEXANESPIRO-2'-(1',3'-DIOXOLAN)-4'-ONE (134):-

To a solution of cyclohexanone (132) (65.2 g, 0.67 mol) and p-toluenesulfonic acid hydrate (0.15 g, 0.8 mmol) in toluene (300 ml) at reflux was added, dropwise over 4 hours, a solution of glycolic acid (133) (40.8 g, 0.54 mol) in water (20 ml). The mixture was heated for a further 5 hours (the water produced in the reaction was removed via a Dean and Stark apparatus), cooled to room temperature, treated with anhydrous sodium acetate (0.25 g, 3 mmol), and stirred for 1 hour. The mixture was filtered, and the filtrate was evaporated under reduced pressure to afford a yellow oil which was distilled to yield the dioxolan one as a colourless liquid (50.4 g, 60%), b.p. 76°C 1 mm Hg (lit[80]. 70°C, 0.5 mm Hg), δ_{H}($CDCl_3$, 60 MHz), 1.4-1.9(10H, m, (CH_2)x5), 4.3(2H, s, CH_2), ν_{max}(thin film) 1795 cm^{-1} (C=O).

5'-OXACYCLOHEXANESPIRO-2'-(1',3'-DIOXOLAN)-4'-YLTRIPHENYL PHOSPHONIUM BROMIDE (136):-

A mixture of N-bromosuccinimide (62.3 g, 0.35 mol), dioxolanone (134) (50.4 g, 0.32 mol) and azobisisobutyronitrile (200 mg, 1.2 mmol) in carbon tetrachloride (500 ml) was heated to reflux and irradiated by a 500W tungsten lamp. After vigorous reaction, the solution was left to reflux for one hour then the pale yellow suspension was cooled to 5°C and the white precipitate of N-succinimide filtered off and washed with carbon tetrachloride (2 x 50 ml). The combined filtrate and washings were concentrated *in vacuo* to afford 5'-bromocyclohexanespiro-2'-(1'3'-dixolan)-4'-one as a yellow lachrymatory oil (ν_{max}(thin film)=1810 cm^{-1}). The oil was stirred at 20°C in toluene (500 ml) whilst a solution of triphenylphosphine (49 g, 0.19 mol) in toluene (250 ml) was added dropwise over 1 hour. The solution was stirred overnight at room temperature then the white precipitate was filtered off and washed with toluene and ether. The crude product was purified by dissolution in ethanol then reprecipitation with ether to obtain the required phosphonium salt as a white solid (66.7 g, 42%), m.p. 187-189°C (lit[80]. 189°C); δ_H($CDCl_3$, 60 MHz), 1.1-1.7(8H, broad, cyclohexyl), 1.8-1.9(2H, bm, CH_2), 7.5-8.0(16H, b, $-PPh_3$), ν_{max}(nujol mull) 1795 cm^{-1} (C=O); FABMS m/z 497.08815 (M^+ $C_{26}H_{26}O_3PBr$ requires 497.08817).

4,6-(ETHYLIDENE)-4-METHYLBUTYLIDENECYCLOHEXANESPIRO-2'-(1',3'-DIOXOLAN)-4'-ONE (137):-

To the phosphorane generated from the phosphonium salt (136) (9.92 g, 20 mmol) and DABCO (2.7 g, 1.2 eq) in dry benzene (100 ml) under nitrogen at 80°C was added the aldehyde (131ii) (2.88 g, 20 mmol) in benzene (20 ml) in 5 ml portions. The mixture was stirred at 100°C for 1½ hours after which time it was cooled and a white precipitate filtered off. The filtrate was concentrated *in vacuo* and the residue applied to a silica dry flash column to give a mixture of diastereomeric pairs of geometrical isomers (3.9 g, 69%). The alkene (137) (1.9 g, 6.7 mmol) in hexane (125 ml) was then irradiated at reflux under a 400W tungsten lamp with iodine (139 mg, 1.09 mmol) for 3 hours. The solution was cooled and washed with sodium thiosulphate, and brine before being dried over magnesium sulphate, filtered, evaporated and purified by flash column chromatography to yield two almost pure white solid diastereomers (120 mg, 6.3%); δ_H($CDCl_3$, 200 MHz), 1.25-1.32(6H, m, 2xMe), 1.42-2.00(12H, m, 6xCH_2), 3.6(H, td, $^3J_{axeq}$=3.0Hz $^3J_{axax}$=$^2J_{axeq}$=8.6Hz, 6ax), 3.9(H, m, 6eq), 4.75(H, q, ^3J=5.1Hz -O(CH_3)C<u>H</u>-O-), 5.6(H, s, -C<u>H</u>=); δ_C($CDCl_3$, 50.3 MHz), 21.1(C-8 or C-9), 22.6(C-3", C-5"), 23.9(C-4"), 28.3(C-8 or C-9), 34.5(C-5), 35.9(C-2", C-6"), 63.9(C-6), 73.8(C-4), 94.5(C-7), 112.1 (C-2'), 112.4(C-3), 137.3(C-5'), 162.6(C-4'); ν_{max}(nujol)

1790 cm^{-1} C=O; FABMS m/z 283.15444 (MH^+ $C_{15}H_{23}O_5$ requires 283.15454) and (150 mg, 7.9%); δ_H($CDCl_3$, 200 MHz), 1.1-1.3(6H, m, 2xMe), 1.3-1.9(12H, m, 6xCH_2), 3.65(H, m, 6ax), 3.9(H, m, 6eq), 4.88(H, q, ^3J=5.1Hz), 5.76(H, s, -C<u>H</u>=); δ_C($CDCl_3$, 50.3 MHz), 21.3(C-8 or C-9), 22.8(C-3", C-5"), 24.2(C-4"), 28.8(C-8 or C-9), 35.2(C-5), 35.9(C-2", C-6"), 63.1(C-6), 74.2(C-4), 94.1(C-7), 113.3(C-2'), 119.7(C-3), 137.9(C-5'), 160.7(C-4'); ν_{max}(nujol) 1790 cm^{-1} (C=O); FABMS m/z 283.15455 (MH^+ $C_{15}H_{23}O_5$ requires 283.15454).

<u>CULTURE OR MICROORGANISMS</u>

Rhodopseudomonas capsulata was grown anaerobically with stirring between 25-30°C under light on the following medium S:- Glutamate, 3.8 g; DL-malate, 2.7 g; sodium hydroxide, 1.6 g; potassium dihydrogen phosphate, 0.5 g; dipotassium hydrogen phosphate, 0.5 g; diammoniumhydrogen-phosphate, 0.85 g; glass distilled water to 1 litre; then magnesium sulphate heptahydrate, 1 ml from stock of 200 g/l; nicotonic acid, 0.1 ml from stock of 10 mg/ml; thiamine hydrochloride, 0.1 ml from stock of 10 mg/ml; biotin, 0.1 ml from stock of 100 μg/ml; manganese chloride tetrahydrate, 0.1 ml from stock of 10 mg/ml; ferric citrate, 0.5 ml from stock of 5 mg/ml; then adjusted to pH 6.9; 0.2% yeast extract.

The medium was autoclaved at 121°C for 25 minutes before use, 2% agar was added for slopes. The

microorganism was cultured as follows: a single slope of *Rh. capsulata* was used to seed a start culture (100 ml, Medium S) which was grown as above for 2 days. This was used as a 10% inoculum for production cultures.

MEASUREMENT OF CULTURE GROWTH AND METABOLITE PRODUCTION

1. HPLC Method

Eight shake flasks (250 ml) of medium S were each inoculated with a 10% inoculum of freshly grown stationary phase *Rhodopseudomonas capsulata* culture. At initial intervals of 12 hours and latterly 24 hours, one flask was removed and the cells harvested, lysed, and extracted (see isolation procedure). The weight of residue after extraction was taken as a measure of cell mass and the extract was analysed for ubiquinone-10 (51) using reverse phase high pressure liquid chromatography. 100 x 1 mm column, Hypersil-ODS 5 μm; solvent, 10% ethyl acetate/1% acetic acid/methanol; detection at 280 nm.

2. Turbidity Method

1 ml Samples of mediums (300 ml) inoculated (10%) with *Rh. capsulata* were monitored for adsorption at 625 nm on an ultra violet spectrometer (see General Methods). The increase in absorption due to the increase in turbidity of the medium was taken as a measure of cell growth.

ISOLATION OF UBIQUINONE-10 (51)

The bacterial cells were harvested by centrifugation (15,000 g for 20 min) and washed free of medium with water. The material was suspended in ethanol (300 ml/litre of medium) and dried *in vacuo*. The dry powder was then extracted four times with hot acetone (100 ml/litre of medium) or until the extract was colourless. The combined acetone extract was evaporated to dryness and purified by preparative thin layer chromatography on silica. A red band (R_f=0.6) isolated after elution with hexane/ether 70/30 was repurified on silica using chloroform/hexane 50/50 as eluent to give ubiquinone-10 (51) (2-3 mg/litre of medium) which was t.l.c. homogeneous with a standard sample. FABMS m/z 864(MH^+), 863(M^+), 680, 683, 684, 666, 667, 408, 196, 182, 180. δ_C(360 MHz, $CDCl_3$) 11.8(ArMe), 15.9(C-3′,7′,11′,15′,19′,23′,27′,31′,35′), 17.6(C-39′), 25.2(C-1), 25.5(C-40), 26.6(C-5,9,13,17,21,25,29,33,37), 39.6(C-4,8,12,16,20,24,28,32,36), 61.0(OMe), 118.8(C-2), 124.2(C-6,10,14,18,22,26,30,34,38), 134.8(C-3, 7,11,15,19,23,27,31,35,39).

FEEDING STUDY PROCEDURE

Typically 3 shake flasks (250 ml) of medium S were inoculated with a 10% inoculum of freshly grown stationary phase *Rhodopseudomonas capsulata* and inoculated anaerobically under light at 25-30°C with stirring for 38

hours. The flasks were then inoculated with a stable isotope tracer (1 mmol per flask) and incubated for another 46 hours (84 hours in total). The ubiquinone-10 (51) was then isolated and analysed using ^{13}C NMR spectroscopy.

ASSAY METHODS

HMGCoA SYNTHASE ASSAY

In the standard assay a 1 cm silica cuvette contained (at 30°C), 40 mM tris-HCl, pH 8.2, 20 mM magnesium chloride, 16 μM acetoacetyl-CoA and enzyme (10-100 μl) in a total volume of 0.995 ml. The enzyme was added last and the rate of acetoacetyl hydrolysis measured (if any). Then acetyl CoA was added (5 μl of 8.6 mM soln.) and the increase in the rate of acetoacetyl-CoA disappearance at 303 nm measured to give the HMGCoA synthase activity. The apparent extinction coefficient (ϵ) of acetoacetyl CoA under these conditions is 20 x 10^3 litre mol^{-1} at 303 nm^{-1}.

The observed rate was converted to the specific activity and compared with the specific activity obtained by Middleton and Tubbs[25]. A unit of enzymatic activity is defined[23] as the amount of enzyme necessary to convert 1 μmol of acetoacetyl CoA into product per minute under the conditions described by Middleton and Tubbs. The change in absorption that would be observed if 1 μmol of

acetoacetyl CoA was converted into product in a 2 ml cuvette where cell path length is 1 cm is given by the Beer Lambert Law:-

$$dA = 20 \times 10^{3} \times \frac{1 \times 10^{-6}}{2 \times 10^{-3}} \times 1 = 10$$

$$\epsilon \quad . \quad c \quad . \quad l$$

Thus the rate in units for the yeast HMGCoA synthetic assay (pg. 82) is:-

$$\text{Rate} = 0.293/10 = 0.0293 \text{ units}$$

However, the specific activity is measured in units/mg of protein. Thus the amount of protein in 20 μl of enzyme solution causing this rate is given by

$$20 \times 10^{-6} \times 14.75 \times 10^{-3} = 0.2950 \text{ mg}$$

where the second term is the protein concentration obtained from the Bradford assay. The specific activity determined was:-

$$0.0293/0.2950 = 0.0994 \text{ units mg}^{-1}$$

Succinate Dehydrogenase Assay[102]

The test cuvette (1 cm silica) contained 10 μl of 5 mM DCPIP, 100 μl of 10 mM potassium cyanide, 50 μl of PMS (3 mg/ml freshly prepared), 4 μl of 1M succinate and typically 20 μl of membrane suspension. All the reagents were made up in phosphate buffer (50 mM, pH 7.7) which was added to give a final volume of 1 ml. All reagents except PMS were present in the blank cuvette. Succinate initiated the reaction. The absorption was measured at 600 nm and 15°C.

Preparation of Cell Free Extracts

1. Fresh bakers yeast (100 g) was autolysed at 38°C for 12 hours with toluene (11 ml). The mixture was then diluted with cold deionised water (90 ml) and centrifuged at 15000 g for 20 minutes.

   The supernatant (150 ml) was diluted with commercial ethanol (50 ml) and stirred at RT for 2 hours. The mixture was then centrifuged at 2000 g for 20 minutes at 18°C and the consequent supernatant left at -3°C for forty minutes. The precipitate was collected by centrifugation at 2000 g for 20 minutes and dissolved in 10 mM potassium phosphate buffer pH 7.8 containing 1 mM DTT to give a final volume of 10 ml. An assay was performed on this final solution.
2. *Fresh bakers yeast* was stirred to a paste with water and centrifuged to collect the cells (5,000 g for 15

min.). The cells were resuspended to 0.5 g/ml wet weight with tris-sulphate buffer (0.1 M pH 9.4/10 mM DTT) and incubated with shaking for 5 min at 30°C. The cells were collected by centrifugation (5,000 g for 5 mins), washed with phosphate buffer (20 mM pH 7.4/1.2M sorbitol), collected by centrifugation as above and resuspended in the phosphate buffer to which 0.15 g/ml Zymolase was added (5 mg/g wet weight). The cells were incubated with shaking at 30°C until sphaeroplast formation was 80% complete (~ 1 hr). The sphaeroplasts were collected by centrifugation (2,500 g for 10 min) and washed with phosphate buffer before being hand homogenised in phosphate buffer (20 mM pH 7.4/0.6M mannitol). The cell debris was spun out (12,500 g for 15 min) and the supernatant assayed for HMGCoA synthase activity.

3. *Rhodopseudomonas capsulata* was grown anaerobically from a 10% inoculum on medium S (1 litre). The cells were grown for 36 hours at 25-30°C under illumination from 2 banks of 6 x 60W Osram lamps.

The cells were harvested by centrifugation (20,000 g for 15 minutes), washed with phosphate buffer (50 mM pH 7.0), and disrupted in a French pressure cell after resuspension in phosphate buffer at 16,000 p.s.i. Residual intact cells and the larger debris were removed from the extracts by low speed centrifugation at 4°C (20,000 g for 15 minutes) and

pigmented particles were then collected by ultra centrifugation (150,000 g for 90 minutes) of the supernatant. The particles were resuspended in phosphate buffer (50 mM pH 7.0) and recentrifuged under the same conditions. Assays were performed on the cell extract after French press, the cytoplasmic fraction and the cell debris after French press, the ultracentrifuged pellet and supernatant and the washed ultracentrifuged pellet and supernatant.

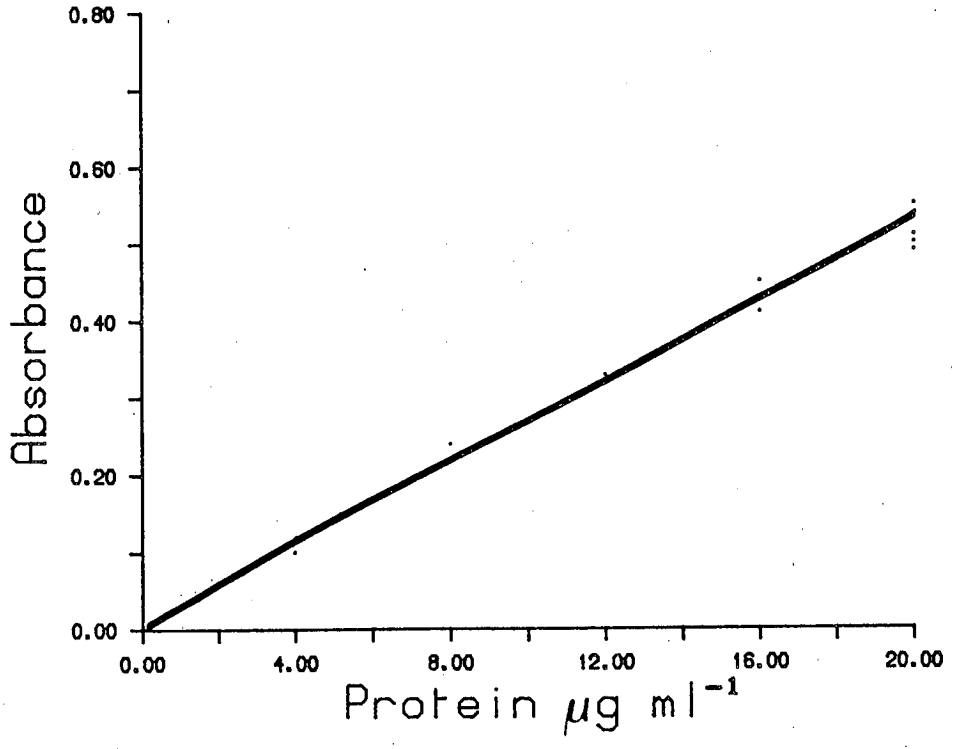
Protein Determination

The Bradford microassay procedure was used for all the steps of the purification.

Several dilutions of protein standard BSA were prepared 0 → 20 μl/ml in 4 μl steps and 0 → 100 ml in 10 μl steps.

0.8 ml of standards and appropriately diluted samples were placed in clean, dry test tubes and 0.8 ml of buffer was placed in a blank tube. Biorad reagent concentrate (0.2 ml) was then placed into the test tubes and the solutions were mixed by inversion taking care to avoid excess foaming.

After a period of ½ hour the absorbances were measured at 595 nm against reagent blank. A plot of absorbance 595 nm versus standards then meant that unknowns could be read from the standard curve. Figure 17 shows two graphs of the standard absorptions obtained.


0.80
0.60
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0.20
0.00
Absorbance
0.00
4.00
8.00
12.00
16.00
20.00
Protein μg ml^{-1}

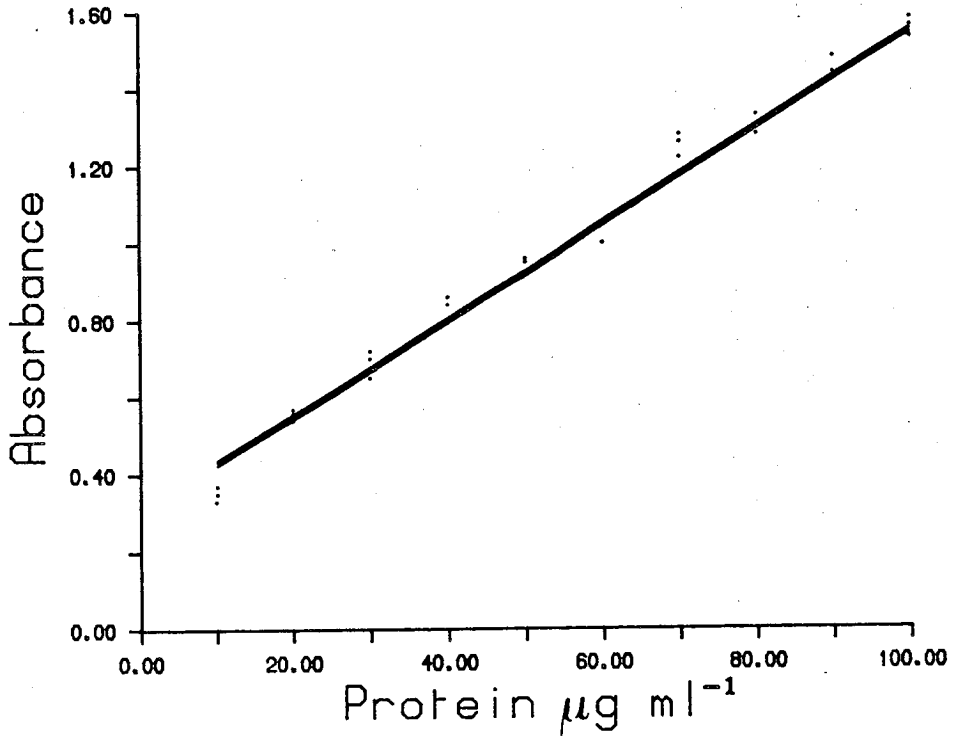
1.60
1.20
0.80
0.40
0.00
Absorbance
0.00
20.00
40.00
60.00
80.00
100.00
Protein μg ml^{-1}

FIGURE 17

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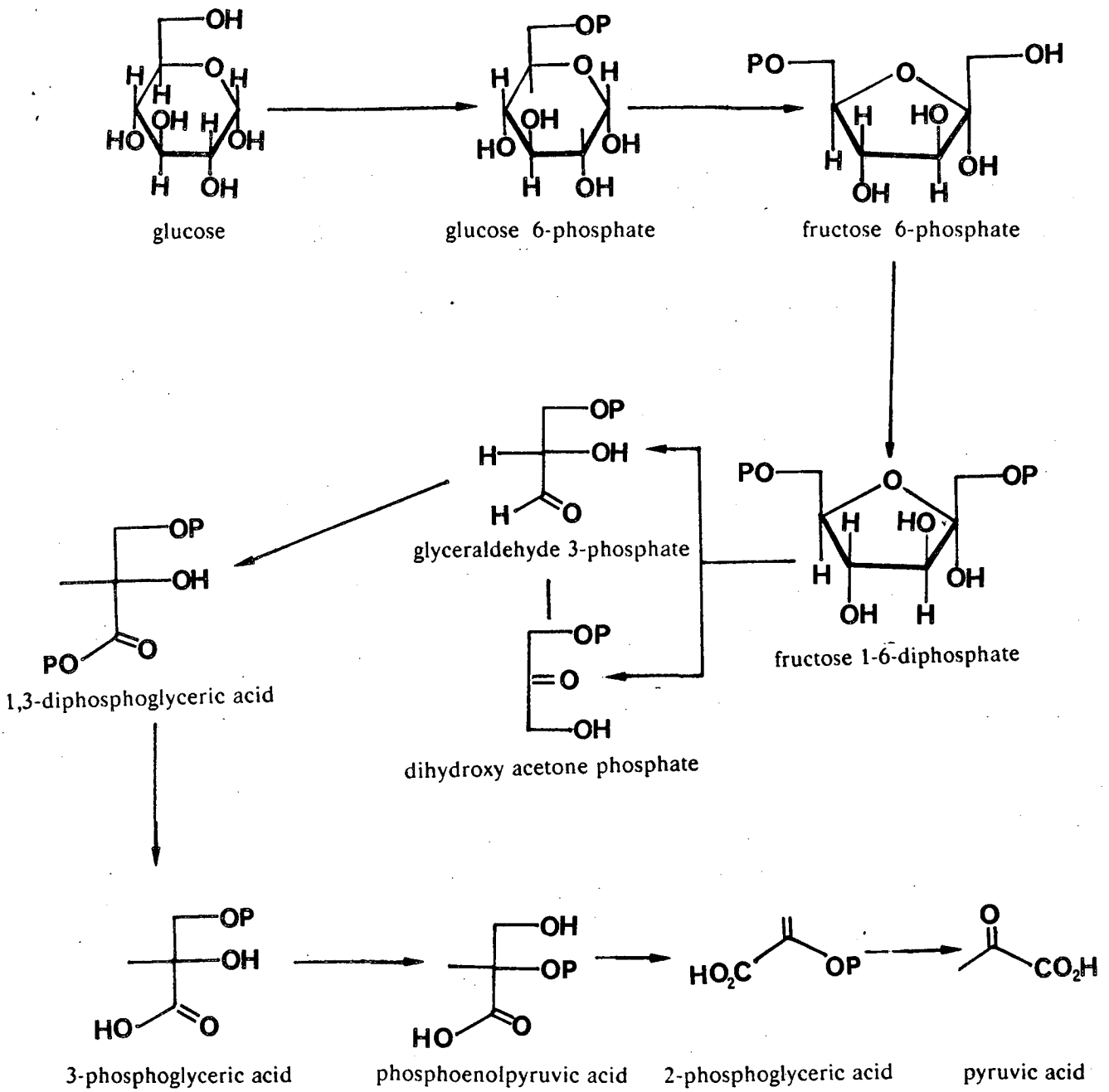
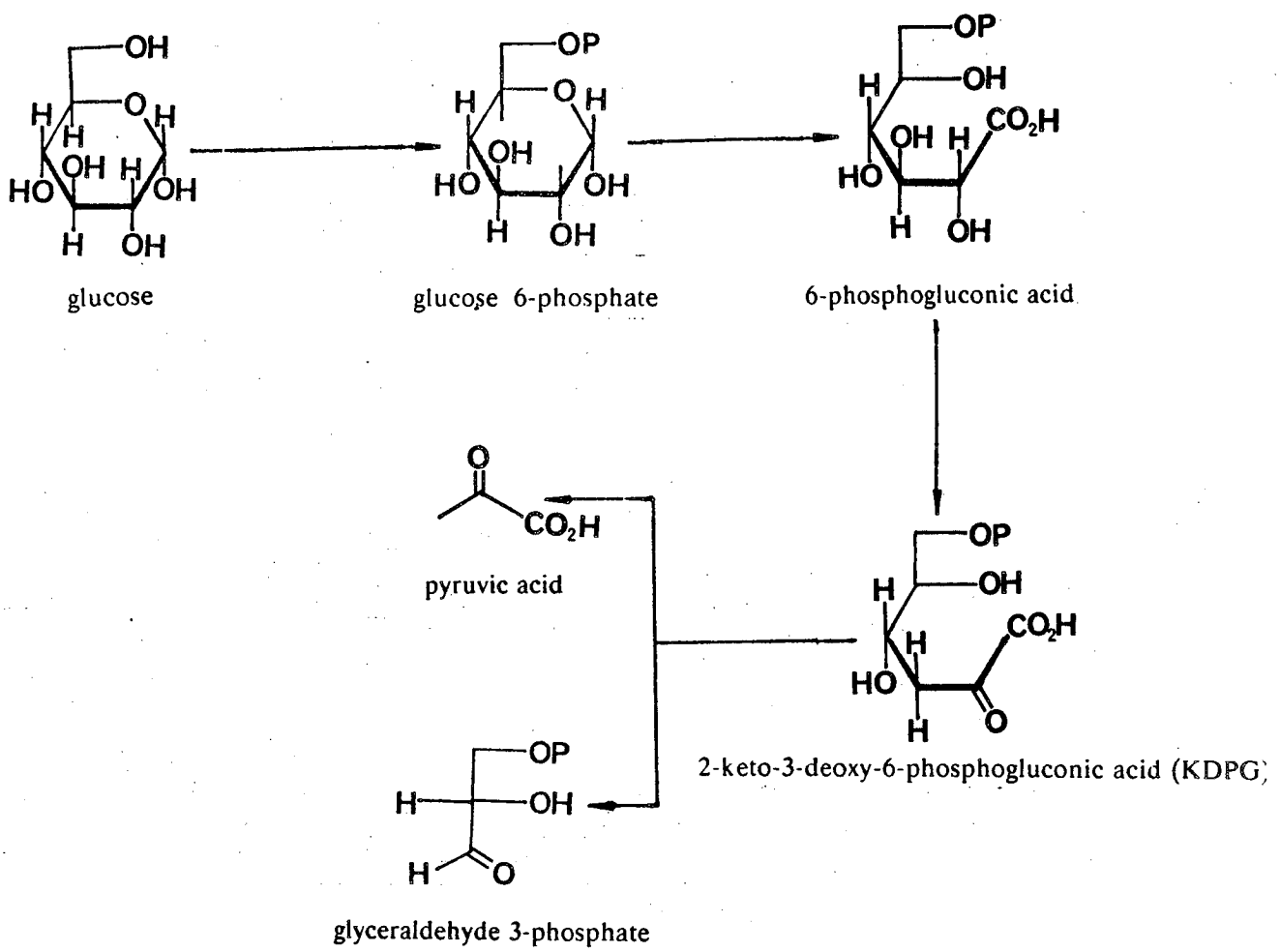
APPENDIX

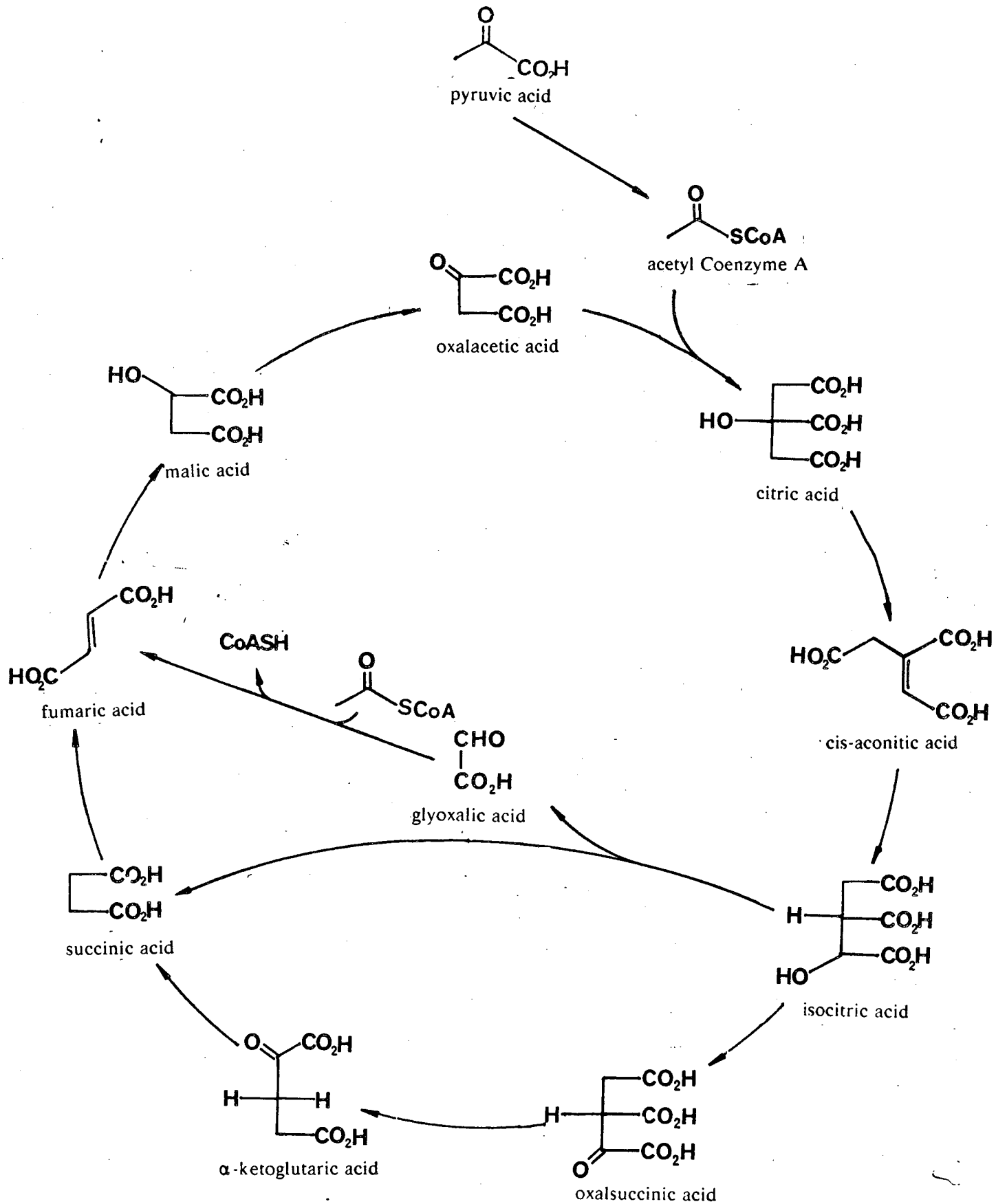
THE EMBDEN-MEYERHOF (GLYCOLYTIC) PATHWAY



THE ENTNER-DOUDOROFF PATHWAY



THE TRICARBOXYLIC ACID (KREB'S) CYCLE AND THE GLYOXYLATE BYPASS



THE CALVIN CYCLE