SYNTHETIC AND BIOSYNTHETIC STUDIES ON THE MACRODIOLIDE COLLETODIOL

by

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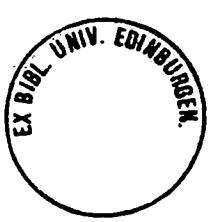

To Mum and Dad for all their help.

DECLARATION

I declare that this thesis is my own composition, that the work of which it is a record has been carried out by myself, and that it has not been submitted in any previous application for a higher degree.

The thesis describes the results of research carried out in the Department of Chemistry, University of Edinburgh, under the supervision of Dr. T.J. Simpson since 1st October 1984, the date of my admission as a research student.

Graeme I. Stevenson

POST GRADUATE LECTURE COURSES

The following is a statement of the courses attended during the period of research.

Organic research seminars (3 years attendance).

Current Topics in Organic Chemistry, various lecturers (10 lectures).

Industrial Chemistry, Drs. A. Nicoll, R. Sinclair and L. Mustoe, Paisley College of Technology, (5 lectures).

Departmental Technical German Lectures and Examination, (1984-1985).

Carbohydrate Chemistry, Prof. R. Ramage, (5 lectures).

Modern Synthetic Methods in Organic Chemistry, Prof. R. Ramage (5 lectures).

Multipulse Methods in nmr. Spectroscopy , Dr. I. Sadler, (5 lectures).

The Elements of Cell Biology, Dr. J. Phillips, (5 lectures).

Industrial Topics in Organic Chemistry, ICI Pharmaceutical and Organic Divisions and Beecham Pharmaceuticals, (5 lectures).

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The research described in the following pages required extensive use of n.m.r. spectroscopy, and many thanks are due to Dr. I. Sadler and Dr. D. Reed, for the 360 MHz studies. I would also like to thank Mr. J.R.A. Miller of the departmental n.m.r. service for his help and patience, and also for his excellent tuition on practical techniques. I also wish to thank all of the technical staff in the department of chemistry for their support and help during the past three years.

Finally I would like to extend my thanks to my family, for all their support over the years, especially my wife, Dian, for her advice and encouragement during our university careers.

ABSTRACT

The first chapter is a general introduction to natural product chemistry, in particular the biosynthesis of polyketides. The relationship between polyketides (secondary metabolites), and fatty acids (primary metabolites), is discussed in detail, as this relationship is further discussed in later chapters. The use of stable isotopes and n.m.r. spectroscopy in modern biosynthetic studies is also described with respect to direct and indirect methods.

Chapter two describes the results of stable isotope studies on the macrodiolide colletodiol. The origins of all the oxygen and hydrogen atoms in colletodiol have been determined by the incorporation of label from [1-^{13}C, ^{2}H$_3$] and [1-^{13}C,^{18}O$_2$]-acetate and ^{18}O$_2$ gas, into colletodiol by *Cytospora Sp.* ATCC 20502. From the resultant labelling pattern, the structures of the enzyme bound precursors can be deduced and these give some indication of the reactions occurring during the early stages of polyketide chain assembly. The macrodiolide colletotriene is proposed as a possible prime precursor to colletodiol.

Chapter three describes synthetic studies carried out towards a total synthesis of colletotriene. The C_6 unit has been successfully achieved by previous workers during the synthesis of other macrodiolides, and was repeated without difficulty. The C_6 unit has not been prepared as yet, and three possible routes were explored.

Chapter four describes the synthesis of *mono* esters of malonic acid and their possible use as biosynthetic probes to

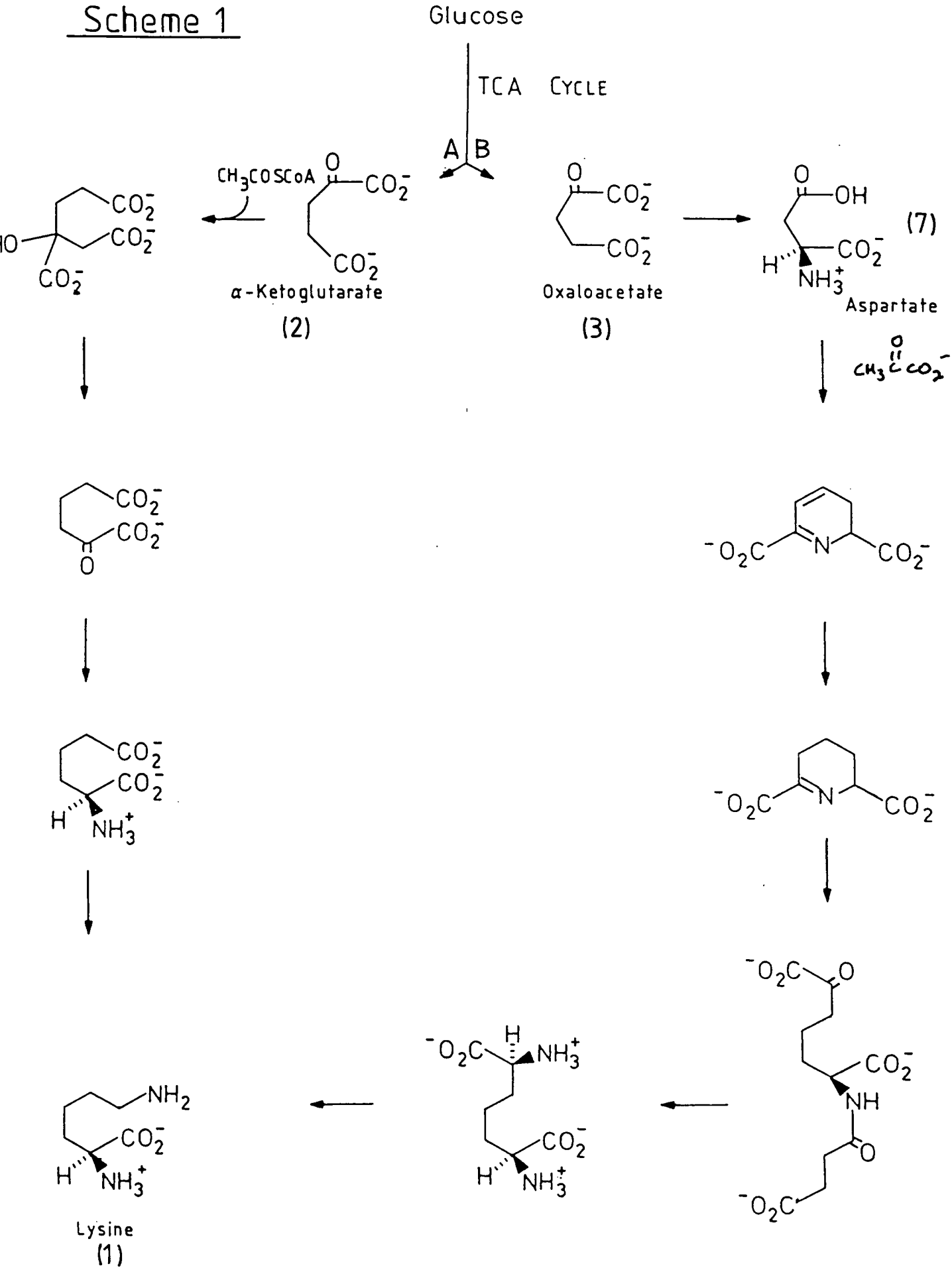
mimic malonyl Coenzyme A. The synthesis described is based on the reaction of CO_2 with the Lithium enolates of simple esters. Incorporation studies using labelled *mono* *n*-butyl and *S*-ethyl esters of malonic acid, prepared by this route have been carried out on the polyketide metabolites Alternariol (*Alternaria tenuis*) and 6-Methyl salicylic acid (*Penicillium urticae*).

CONTENTS

CHAPTER ONE

NATURAL PRODUCTS AND ORGANIC CHEMISTRY

Scheme 1


Glucose
TCA CYCLE
A
B
α-Ketoglutarate
(2)
Oxaloacetate
(3)
Aspartate
(7)
Lysine
(1)

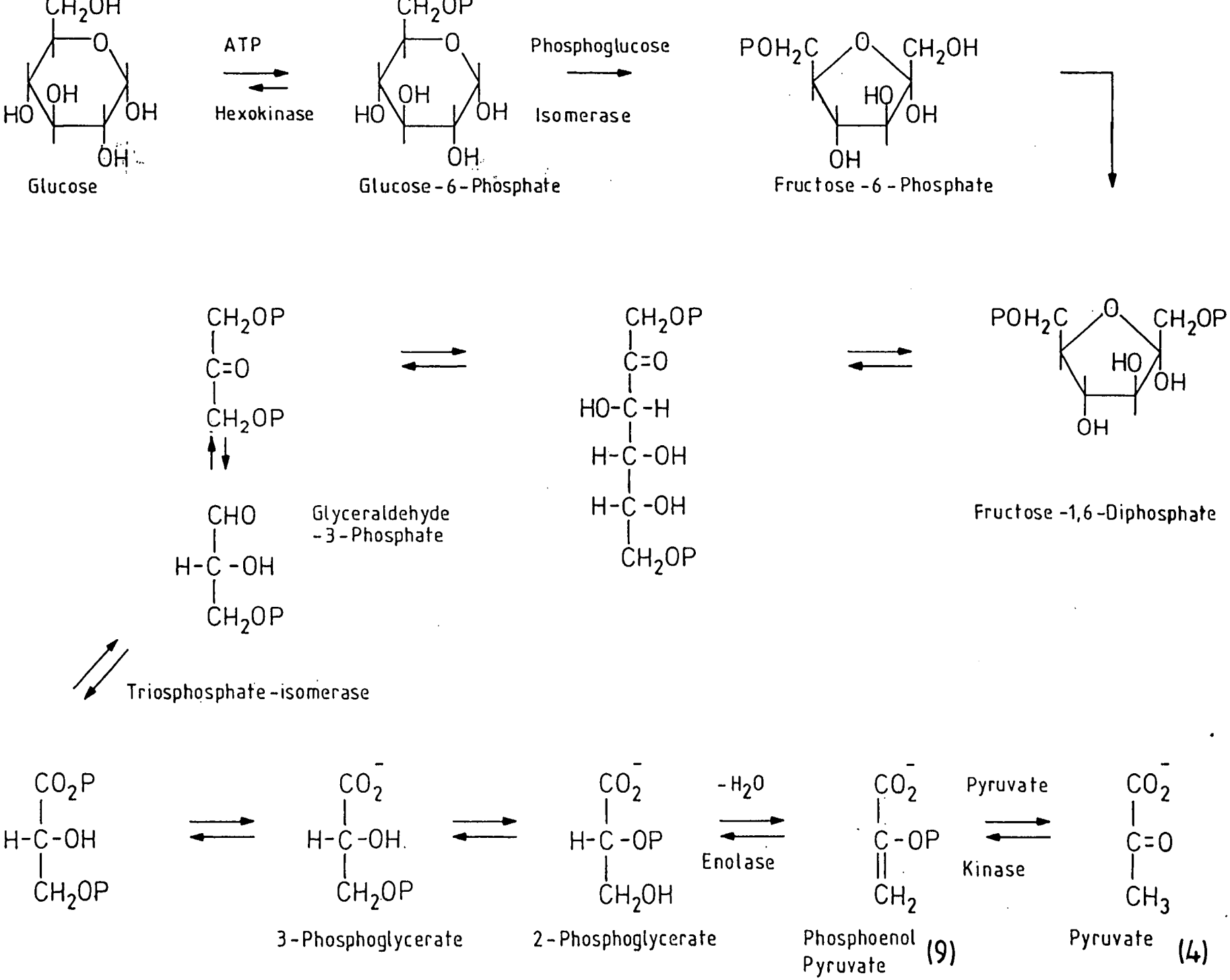
1. NATURAL PRODUCTS AND ORGANIC CHEMISTRY

INTRODUCTION

Organic chemistry as it stands today, stems from the study of "natural products" during the 19th century. A "natural product" can be considered as any compound produced by a living organism. However as living organisms produce a vast range of compounds some form of classification is needed.

1.1. PRIMARY AND SECONDARY METABOLISM[1]

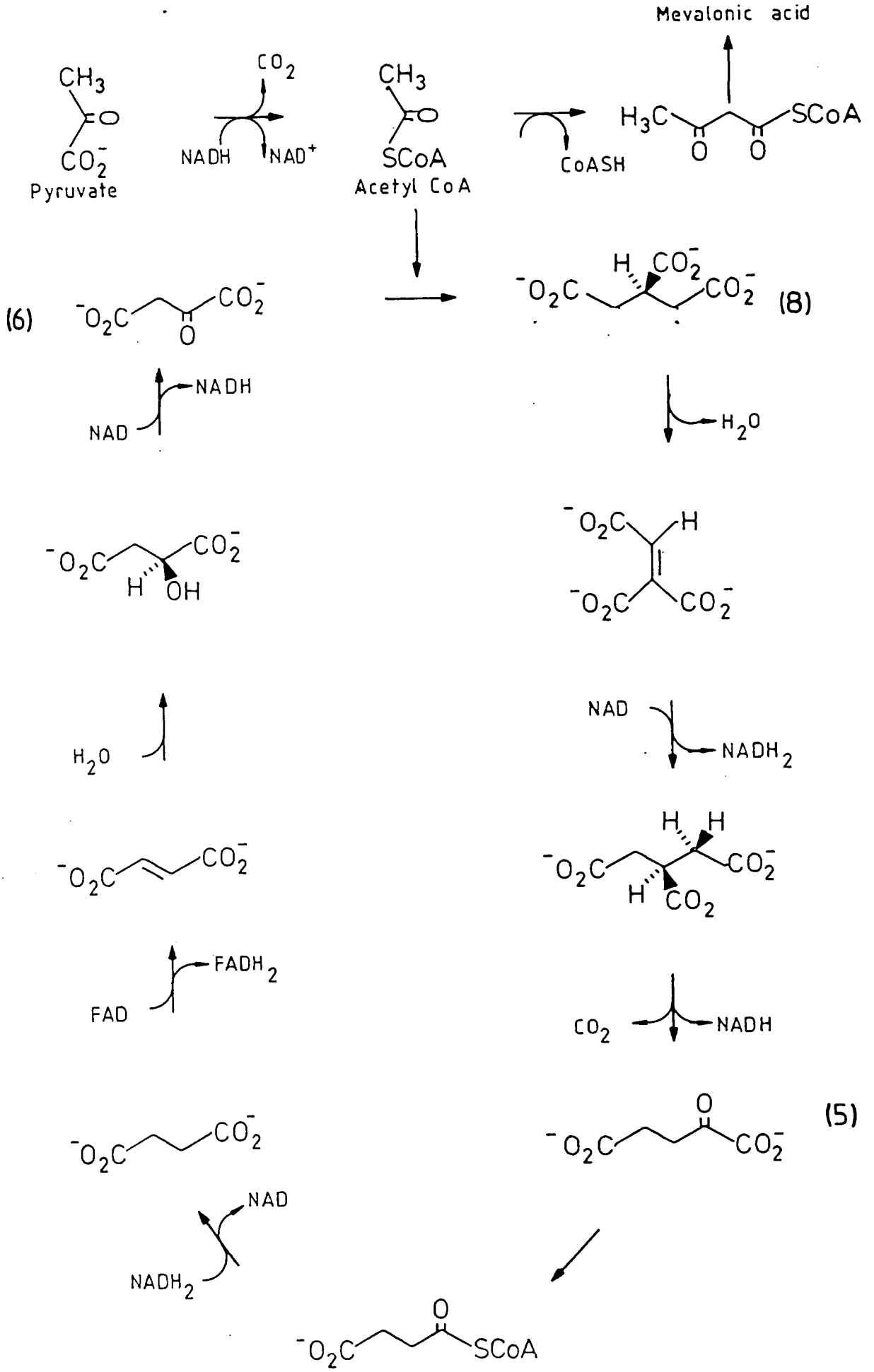
The processes of enzyme mediated reactions by which an organism synthesises and degrades compounds are collectively called metabolism. This encompasses anabolism, the synthesis of compounds, and catabolism, the degradation of compounds. The metabolic pathways that lead to the essential and ubiquitous molecules of life such as sugars, amino acids, fatty acids, and the heterocyclic bases, and the polysaccharides, proteins, nucleic acids, and lipids that can be assembled from them are, in general common to all organisms. Collectively they are known as primary metabolism, and in general across species there is very little difference in mechanism. However there are exceptions, such as in the biosynthesis of lysine (1)[2] which is shown in

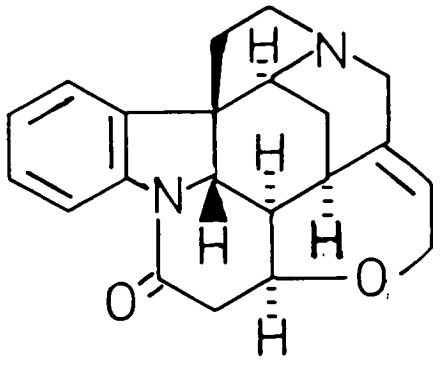

Glucose
ATP
Hexokinase
Glucose-6-Phosphate
Phosphoglucose
Isomerase
Fructose-6-Phosphate
Glyceraldehyde
-3-Phosphate
Fructose-1,6-Diphosphate
Triosphosphate-isomerase
3-Phosphoglycerate
2-Phosphoglycerate
-H2O
Enolase
Phosphoenol
Pyruvate
(9)
Pyruvate
Kinase
Pyruvate
(4)

scheme 1. Glucose via glycolysis and the tricarboxylic acid cycle provides the starting material in both yeasts and moulds (path A), where α-keto glutarate (2) is combined with acetate. On the other hand in bacteria and plants, oxaloacetate (3) is combined with pyruvate (4) (path B).

The basic starting materials and energy for primary metabolism are provided by the breakdown of glucose by glycolysis, scheme 2, and the tricarboxylic acid cycle, scheme 3. The end product of glycolysis is pyruvate (4) which is decarboxylated and esterified to acetyl CoA, the two carbon unit used to synthesise fatty acids and the essential steroid hormones[3]. Acetyl CoA also serves to propagate the tricarboxylic acid cycle scheme 3, which provides oxaloacetate (5) and α-keto glutarate (6), which are converted to aspartate (7) and glutamate (8), and subsequently the other aliphatic amino acids. Combination of phosphoenol pyruvate (9) an intermediate in glycolysis and erythrose-4-phosphate (10) from the pentose phosphate cycle gives shikimic acid (11), the precursor to the aromatic amino acids, which combine with the aliphatic amino acids to form proteins.

As well as primary metabolism, many organisms also utilize other metabolic pathways which have no apparent purpose. These pathways are classed as secondary metabolism, and their products secondary metabolites. Unlike primary metabolism secondary metabolism has a limited taxonomic distribution, and particular pathways are often species specific. Secondary metabolites are the "natural products" of organic chemistry. They


Mevalonic acid
Pyruvate
NADH
NAD+
Acetyl CoA
CoASH
SCoA
(6)
(8)
NADH
NAD
H2O
H2O
NAD
NADH2
FADH2
FAD
CO2
NADH
NAD
NADH2
(5)
SCoA



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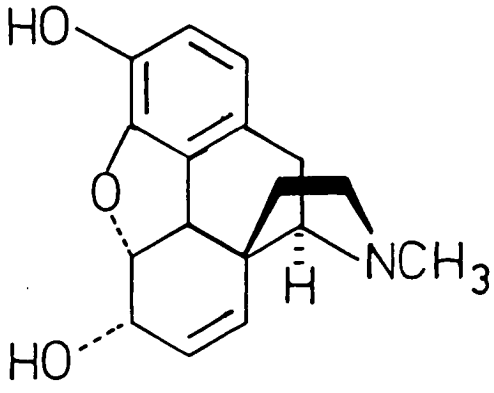

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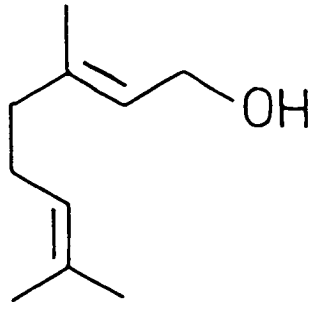


(14)

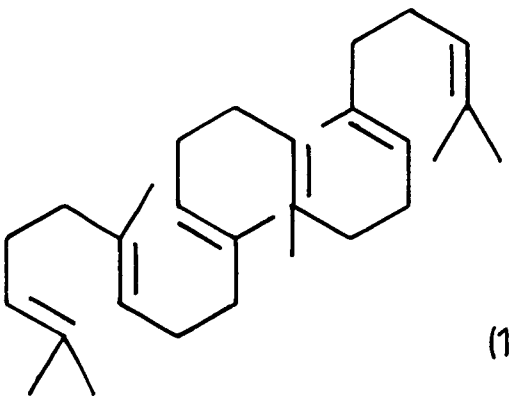
were first studied usually because of some specific property that crude extracts of living matter possessed, such as smell, flavour, colour, toxicity, or narcotic action. In this way compounds such as strychnine (12), morphine (13) and geraniol (14) were first isolated, although determination of the structure of such compounds did not begin until the early 20th century. The actual function of secondary metabolism and its products is not known. Some ideas that have been proposed are;[4]

i) Reserve foodstuffs; The two main objections to this are the diversity of the structures involved, and all organisms lay down fat as food anyway.

ii) Detoxification products; Higher organisms have livers for this, lower organisms may use secondary metabolism

iii) Chemical messengers; Many secondary metabolites are sex attractants or defensive / aggresive weapons.

The dividing line between primary and secondary metabolism is not clear. Compounds such squalene (15) were initially classed as secondary metabolites, only to be later shown to have a primary role. Squalene is an intermediate in the synthesis of the steroid hormones. However the two areas are closely linked in that primary metabolism provides the starting materials and energy for secondary metabolism. Acetyl CoA is the two carbon unit involved in both the polyketide and isoprenoid pathways. The amino acids are involved in alkaloid and penicillin synthesis. Shikimic acid is the precusor to the cinnamic acids and plant phenols. This input of primary metabolism into secondary metabolism is summarised in scheme 4



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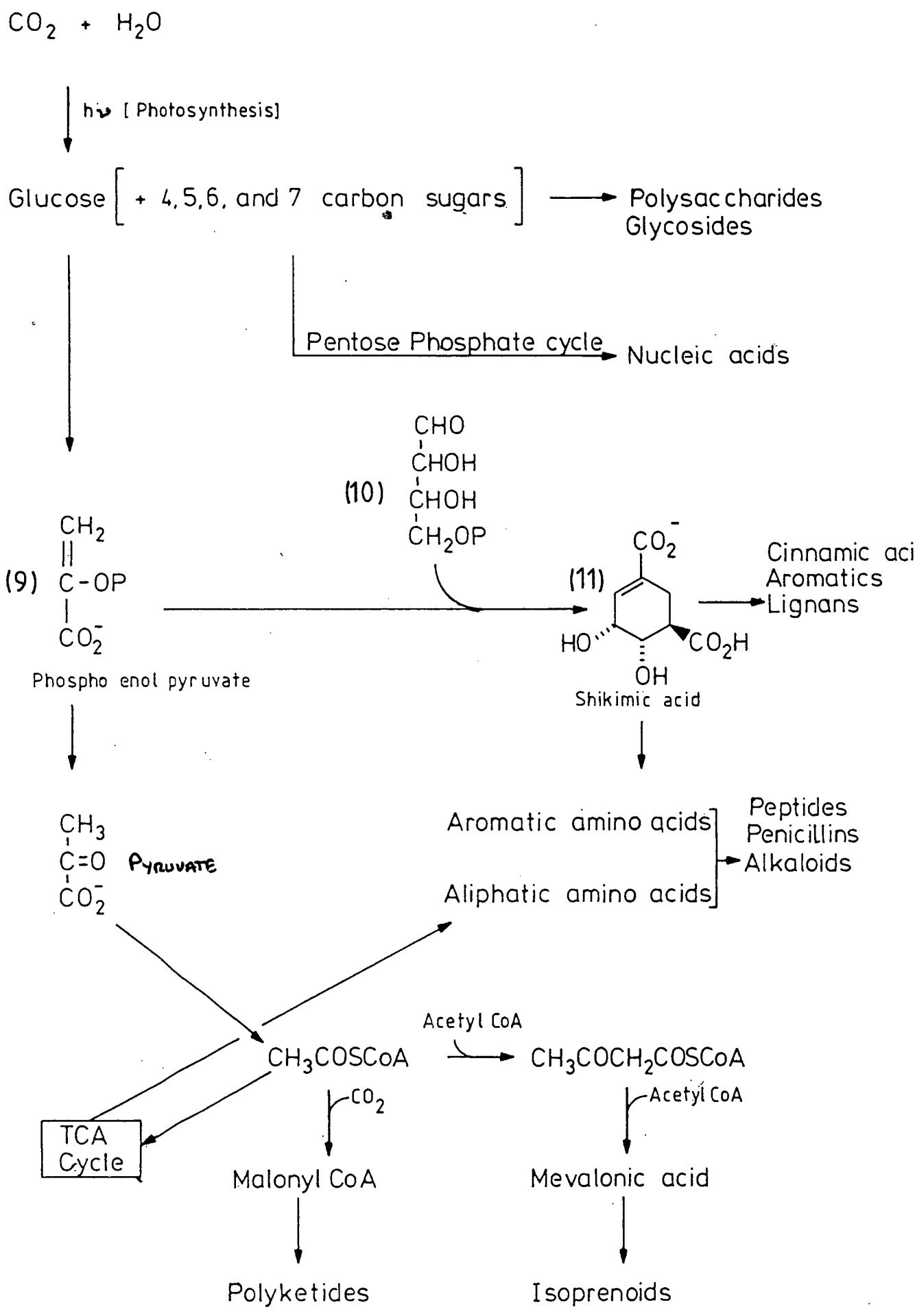
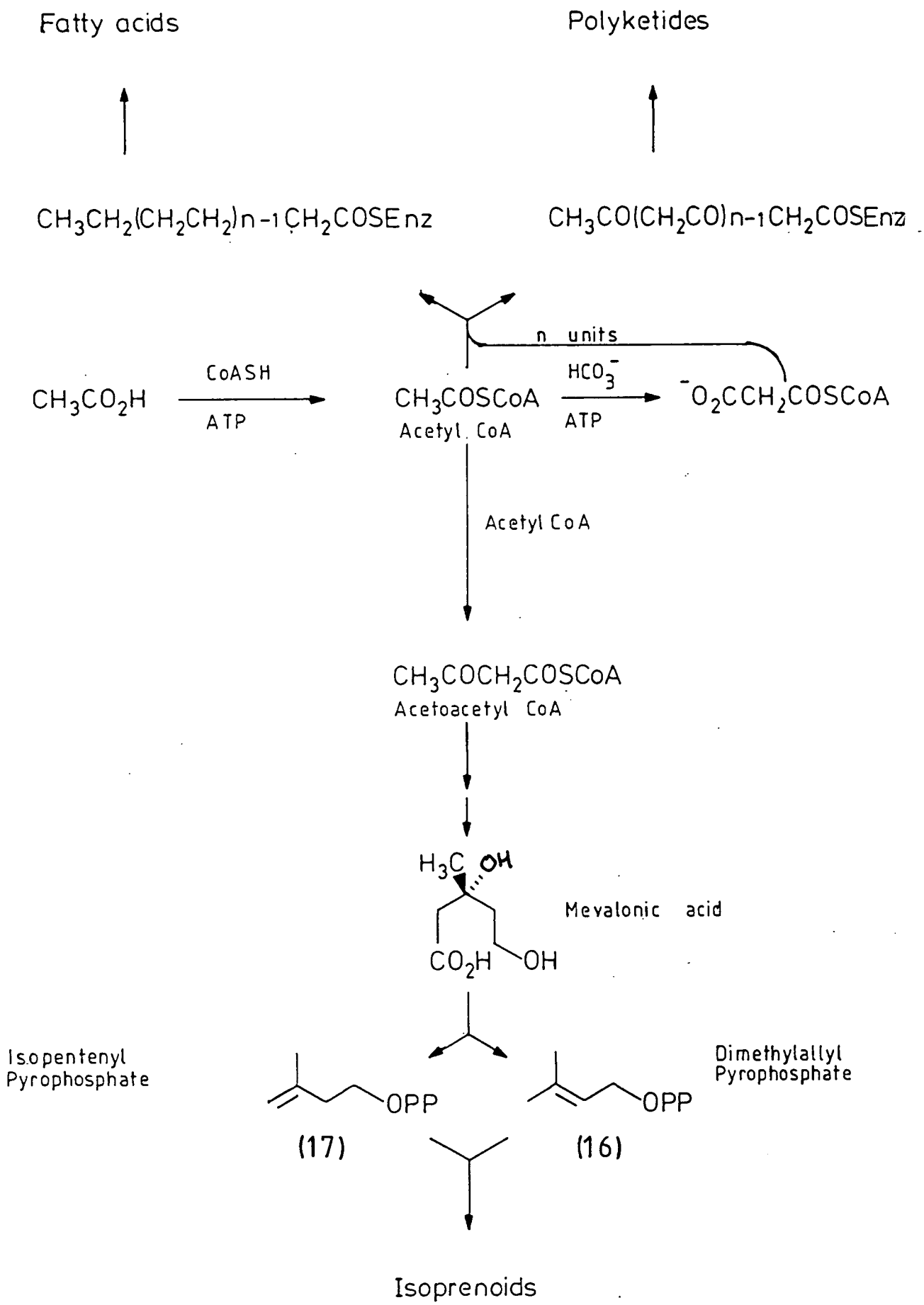

CO2 + H2O
hν [Photosynthesis]
Glucose [+ 4,5,6, and 7 carbon sugars]
Polysaccharides
Glycosides
Pentose Phosphate cycle
Nucleic acids
(10)
CHO
CHOH
CHOH
CH2OP
(9)
CH2
C-OP
CO2-
Phospho enol pyruvate
(11)
CO2-
HO
CO2H
OH
Shikimic acid
Cinnamic aci
Aromatics
Lignans
CH3
C=O
CO2-
Pyruvate
Aromatic amino acids
Aliphatic amino acids
Peptides
Penicillins
Alkaloids
Acetyl CoA
CH3COSCoA
CH3COCH2COSCoA
CO2
Acetyl CoA
TCA Cycle
Malonyl CoA
Mevalonic acid
Polyketides
Isoprenoids

As the study of secondary metabolism has progressed, the study of polyketide metabolites derived from acetyl CoA has attracted much interest, not least because of the similarities to fatty acid biosynthesis.

The polyketide pathway produces metabolites with a wide range of structural diversity. They are produced by microorganisms in general and by the filamentous fungi in particular. The fungi are ideal organisms for the study of secondary metabolites such as polyketides: they produce metabolites in relatively high yields over short periods of time; isolation of metabolites is generally easy ; and the uptake of precursor compounds is generally efficient. All the work described here concerns polyketides produced by filamentous fungi.


Fatty acids
Polyketides
CH3CH2(CH2CH2)n-1CH2COSEnz
CH3CO(CH2CO)n-1CH2COSEnz
n units
CoASH
ATP
CH3CO2H
CH3COSCoA
Acetyl CoA
HCO3-
ATP
-O2CCH2COSCoA
Acetyl CoA
CH3COCH2COSCoA
Acetoacetyl CoA
H3C
OH
CO2H
OH
Mevalonic acid
Isopentenyl Pyrophosphate
OPP
(17)
OPP
(16)
Dimethylallyl Pyrophosphate
Isoprenoids

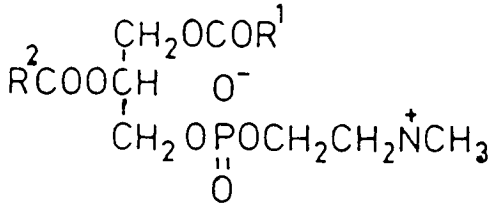
1.2 FATTY ACIDS AND POLYKETIDES: METABOLITES OF ACETYL CoA

Acetyl CoA is the basic building block for a large group of primary and secondary metabolites. This is outlined in scheme 5. Essentially there are two pathways, one involving the stepwise addition of C_2 units to form fatty acids and polyketides the other involving the condensation of three acetate units to form the C_5 unit of mevalonic acid, the basic unit for the formation of the isoprenoids via the intermediates dimethylallyl pyrophosphate (16) and isopentenyl pyrophosphate (17).

1.2.1 Fatty acids.

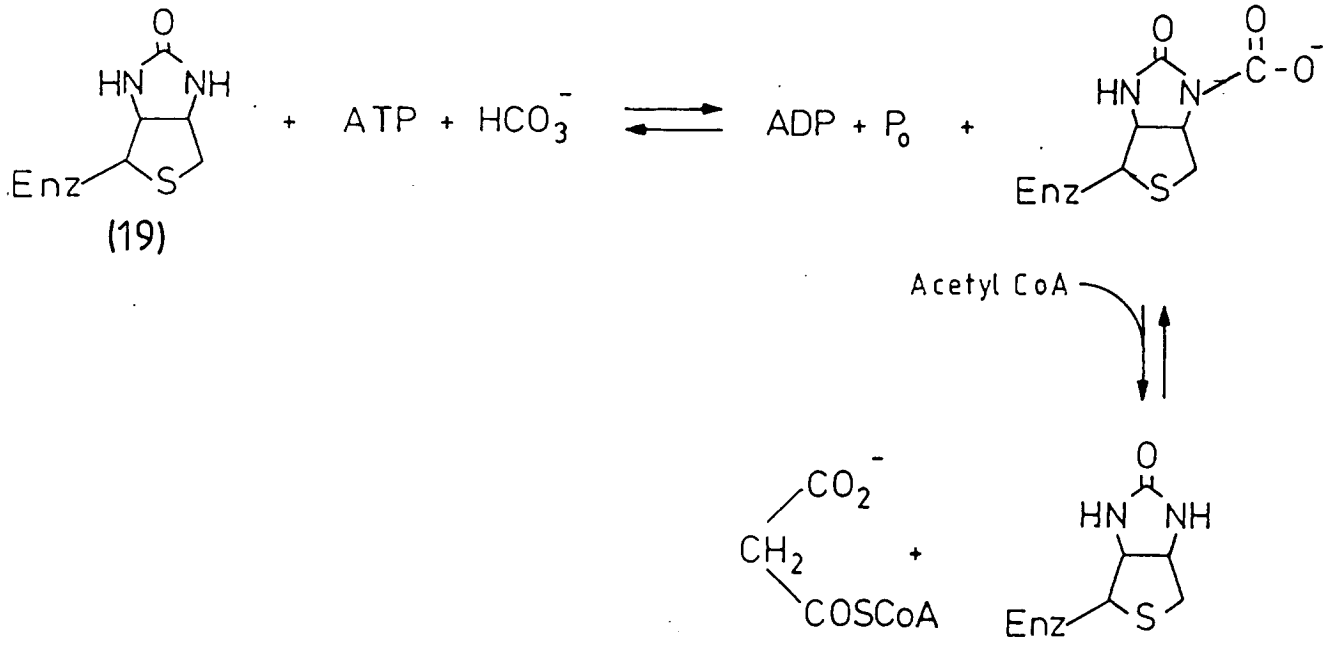
Saturated fatty acids have the general formula $CH_3CH_2(CH_2CH_2)_nCH_2CO_2H$, commonly with n = 2 to 7. They occur mainly as the major component of natural waxes and oils, and more importantly as phospholipids such as phosphatidyl choline (18). Polar lipids such as this are vital for the structure of living cells, and because of this most common fatty acids are classed as primary metabolites. Only the rarer longer chain fatty acids may be classed as secondary metabolites. However as polyketide biosynthesis is closely related to fatty acid biosynthesis, the details of fatty acid biosynthesis are fully discussed here.

The first serious suggestion for the source of fatty acids came from Fischer[5] in 1890. He proposed that sugar units condensed together to form fatty acids. This was followed by

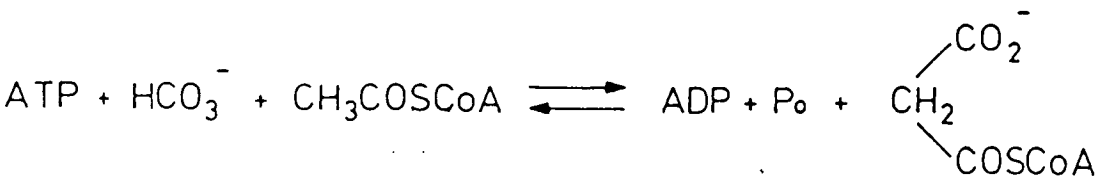


(18)

Scheme 6



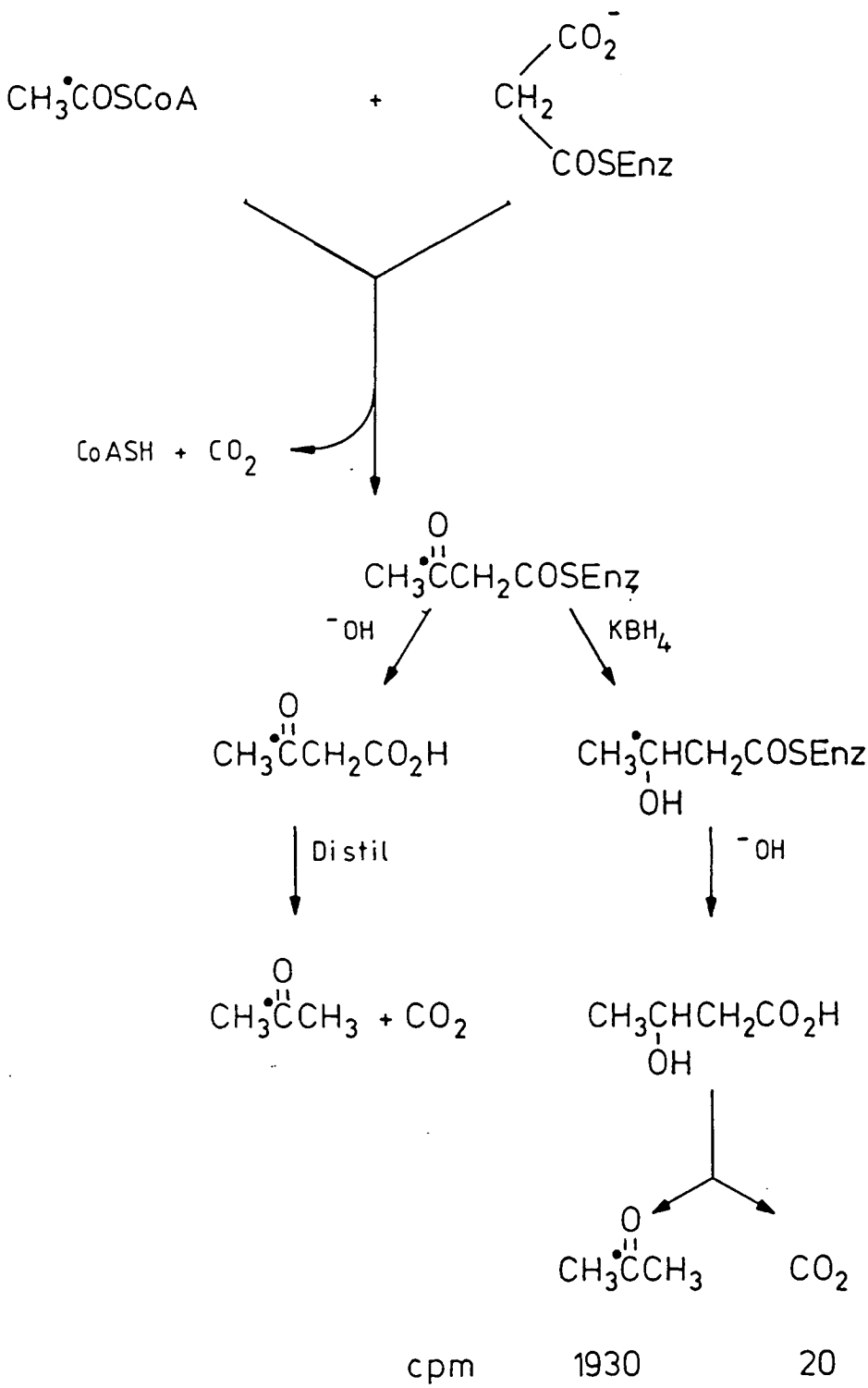
Overall:



Nencki[6] in 1898, and Raper[7] in 1907, who suggested that acetaldehyde was the basic unit for building fatty acids. It was not until 1945 that acetate was shown to be the true precursor by Rittenberg and Bloch[8]. They showed that both isotopes were retained from [1-^{13}C,^{2}H$_3$]acetic acid in the fatty acids of rats and mice. The fate of the isotope labels was monitored as follows. The fatty acids were burnt and the water isolated, the concentration of $^{2}H_2O$ in H_2O was then calculated by comparing the refractive index of the solution with that of pure H_2O. In order to follow the incorporation of the acid carbon, the fatty acids were decarboxylated and the amount of $^{13}CO_2$ measured using mass spectrometry. Subsequent studies showed that acetate, via acetyl CoA was the fatty acid precursor in microorganisms[8]. The next major discovery did not come until 1961 when malonyl CoA was identified as the chain extension unit[9], along with a requirement for ATP, NADPH, and CO2 in cell free systems. However administration of $^{14}CO_2$ did not lead to labelled products, and therefore CO2 was assigned to a catalytic role.

One of the enzymes involved in the pathway was shown to have a high biotin content[10]. The relationship between biotin (19) and fatty acid biosynthesis was established using the basic protein avidin[11]. Avidin is found in egg white and has a very high affinty for biotin, and so can successfully inhibit fatty acid biosynthesis. Lynen went on to show that the role of biotin was to carboxylate acetyl CoA to form malonyl CoA[12], as shown in scheme 6. As CO_2 is lost in the subsequent condensation of acetyl CoA with malonyl CoA, this explained why no labelling could be

Scheme 7


CH3COSCoA
+
CO2-
CH2
COSEnz
CoASH + CO2
O
CH3CCH2COSEnz
-OH
KBH4
O
CH3CCH2CO2H
CH3CHCH2COSEnz
OH
Distil
-OH
O
CH3CCH3 + CO2
CH3CHCH2CO2H
OH
O
CH3CCH3
CO2
cpm
1930
20

• = ^{14}C

introduced via labelled CO_2. Further studies using ^{14}C labelled acetyl CoA and malonyl CoA allowed the sequence of events in the pathway to be determined.

[1-^{14}C]Acetyl CoA was incubated with a fatty acid synthetase (FAS) derived from baker's yeast. The incubation was stopped by precipitation of the enzyme by addition of trichloro - acetic acid. Analysis of the precipitated enzyme showed the presence of bound acetoacetate which was released by mild base hydrolysis. Decarboxylation gave acetone labelled in the 2-position. If the precipitated enzyme was treated with KBH_4 and then hydrolysed, labelled β-hydroxybutyric acid was released. Oxidation by van Slykes procedure[13] showed that all the label was in the acetone fraction, the CO_2 being virtually unlabelled. This is summarised in scheme 7.

Incubation of the same FAS system with both enantiomers of (*S*)-β-hydroxybutyryl-*N*-acetylcysteamine, and monitoring the system by the appearance of absorbtion in the α,β-unsaturated region of the U.V. spectrum showed that only the 3-(R)-hydroxybutyrate was a substrate for dehydration. Hence the reduction of the β-ketoacyl system must occur stereospecifically to give the 3-(R)-hydroxythiol ester, as shown in scheme 8. The final step in the sequence was monitored in a similar fashion using S-crotonyl-N-acetyl-cysteamine as the substrate and observing the U.V. spectrum for the loss of the α,β-unsaturated system. Only the *trans* isomer was found to be a substrate

From these experiments Lynen summarised fatty acid biosynthesis as shown in scheme 8. It was also shown that no free

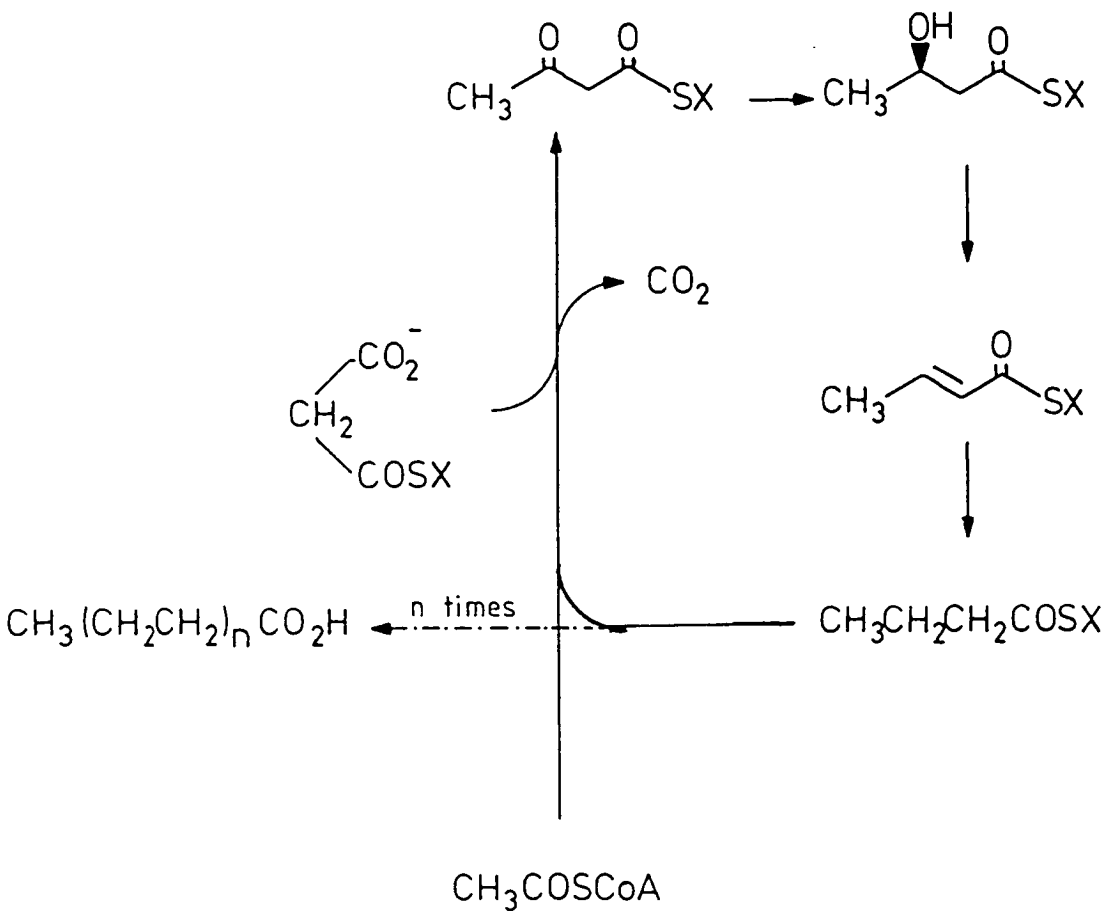
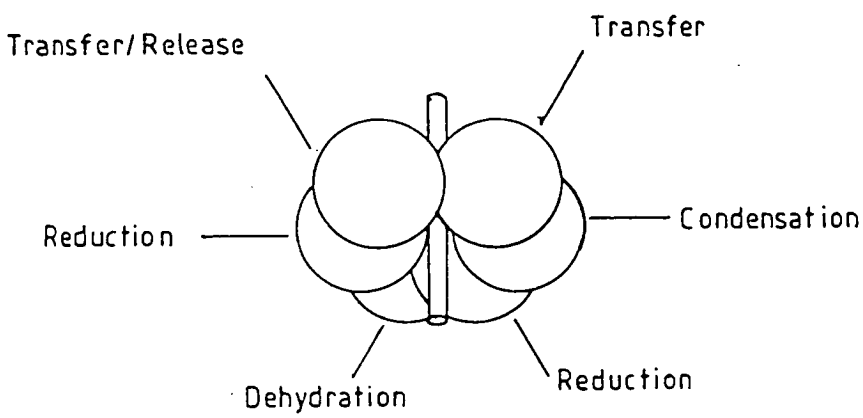

CH3COSCoA
CO2
n times
CH3(CH2CH2)nCO2H
CH3CH2CH2COSX

Figure 1


Transfer/Release
Transfer
Reduction
Condensation
Dehydration
Reduction

intermediates were present in the system, this coupled with the fact that the purified enzyme system appeared to have only one component, led Lynen to propose a multienzyme complex bound around a central core for the structure of yeast fatty acid synthetase, see figure 1. Lynen proposed that acetyl and malonyl CoA units were introduced via "peripheral" SH groups on to "central" SH groups where the condensation occurred. The growing chain was retained on the "central" SH group until ready for release.

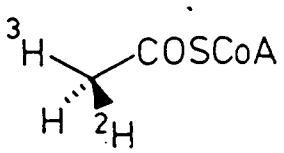
1.2.2. Stereochemistry of Fatty acid Biosynthesis.

Lynen[1,2] had shown that the β-ketoacylthiol ester was stereospecifically reduced to the 3-(R)-hydroxythiol ester, before being dehydrated to give the *trans*-2,3-enoylthiol ester. The stereochemical courses of the carboxylation, condensation, dehydration, and reduction steps are dicussed below

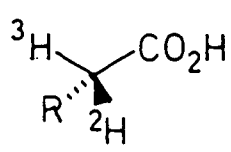
1.2.2.a. Carboxylation of Acetyl CoA

To elucidate the mechanism and stereochemistry of this step, Cornforth *et al*[1,4] prepared chiral forms of acetyl CoA. The chirality was created by the introduction of ^{3}H and ^{2}H labels into the methyl group using enzymatic methods. Incubation of both enantiomers with yeast FAS and subsequent isolation of the product fatty acid showed that more ^{3}H label was retained from the 2-(*S*) isomer than from the 2-(*R*). The discrimination was

Scheme 9



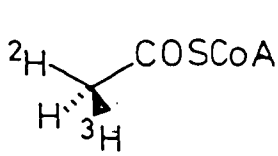
FAS →



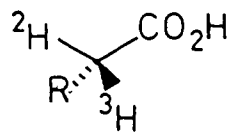
2(S)-[$^{2}H_1$,$^{3}H_1$] Acetyl CoA

2(S)-[2-$^{2}H_1$,$^{3}H_1$] Palmitic acid

71% ^{3}H Retained



FAS →

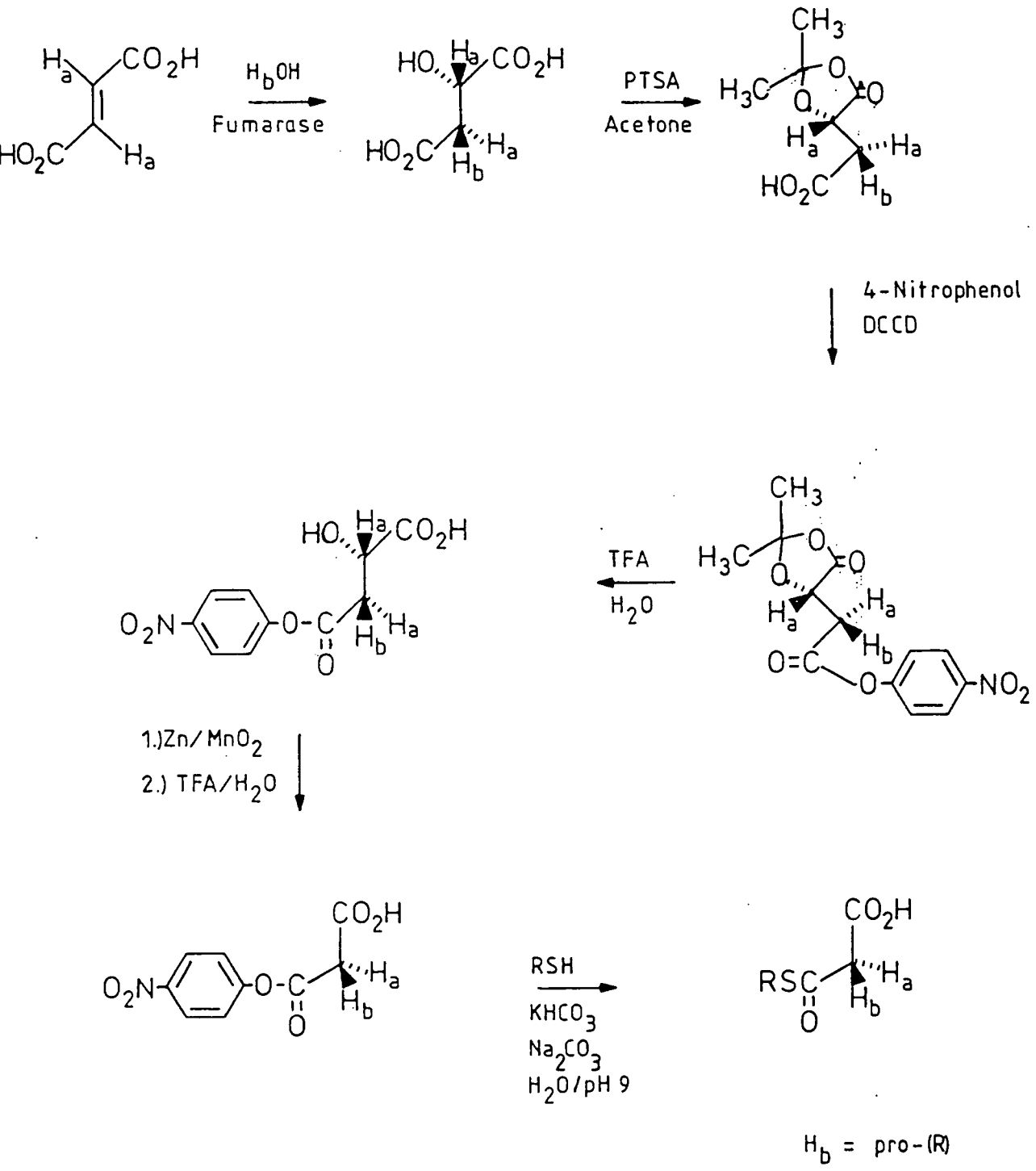


2(R)-[$^{2}H_1$,$^{3}H_1$] Acetyl CoA

2(R)-[2-$^{2}H_1$,$^{3}H_1$] Palmitic acid

64% ^{3}H Retained

Scheme 10



H_b = pro-(R)

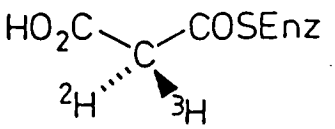
small as the hydrogen isotope effect, active in this system, led to nearly equal amounts of 2-(*R*)-, and 2-(*S*) malonyl CoA being formed and incorporated into the fatty acid. However the difference was large enough to indicate that there was an overall stereospecificity to the reaction. The experiment is summarised in scheme 9.

To examine the reaction further chiral forms of malonyl CoA were used. Both 2-(*R*)-, and 2-(*S*)-[2-$^{3}H_{1}$]-malonyl CoA were prepared[15] as shown in scheme 10. Incubation with yeast FAS and isolation of the fatty acid product showed 51% ^{3}H retention from 2-(*S*)-[2-$^{3}H_{1}$]-malonyl CoA and only 23% ^{3}H retention from 2-(*R*)-[2-$^{3}H_{1}$]-malonyl CoA. When these results are compared to those from the chiral acetyl CoA study it is clear that the carboxylation step occurs with overall retention of configuration. The course of the overall reaction is shown in scheme 11.

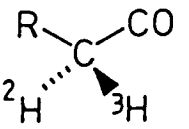
1.2.2.b. Condensation of Acetyl CoA with Malonyl CoA

Sedgwick et al prepared[16] specifically labelled β-hydroxybutyric acid by treating 2,3-(*R,R*)-, and 2,3-(*S,S*)-epoxybutyric acid with $LiAl^{3}H_{4}$, see scheme 12. The product was converted via a mixed anhydride into the S-N-acetylcysteamine ester. As only the 3-(*R*)-hydroxythiol ester acts as a substrate for the yeast FAS complex[12], the presence of the other did not affect the reaction.

Scheme 11



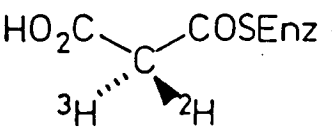
FAS →



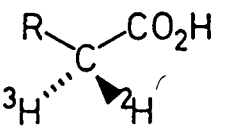
2-(S)-[2-$^{2}H_{1}$,$^{3}H_{1}$]Malonate

2-(S)-[2-$^{2}H_{1}$,$^{3}H_{1}$]Palmitic acid

51% ^{3}H Retained



FAS →

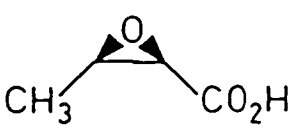


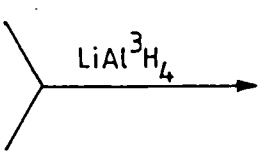
2-(R)-[2-$^{2}H_{1}$,$^{3}H_{1}$]Malonate

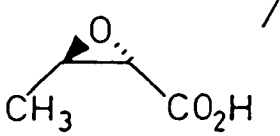
2-(R)-[2-$^{2}H_{1}$,$^{3}H_{1}$]Palmitic acid

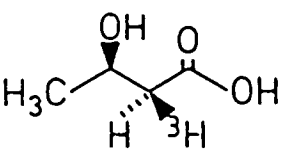
24% ^{3}H Retained

Scheme 12









+

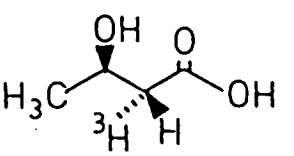


Schéma 15

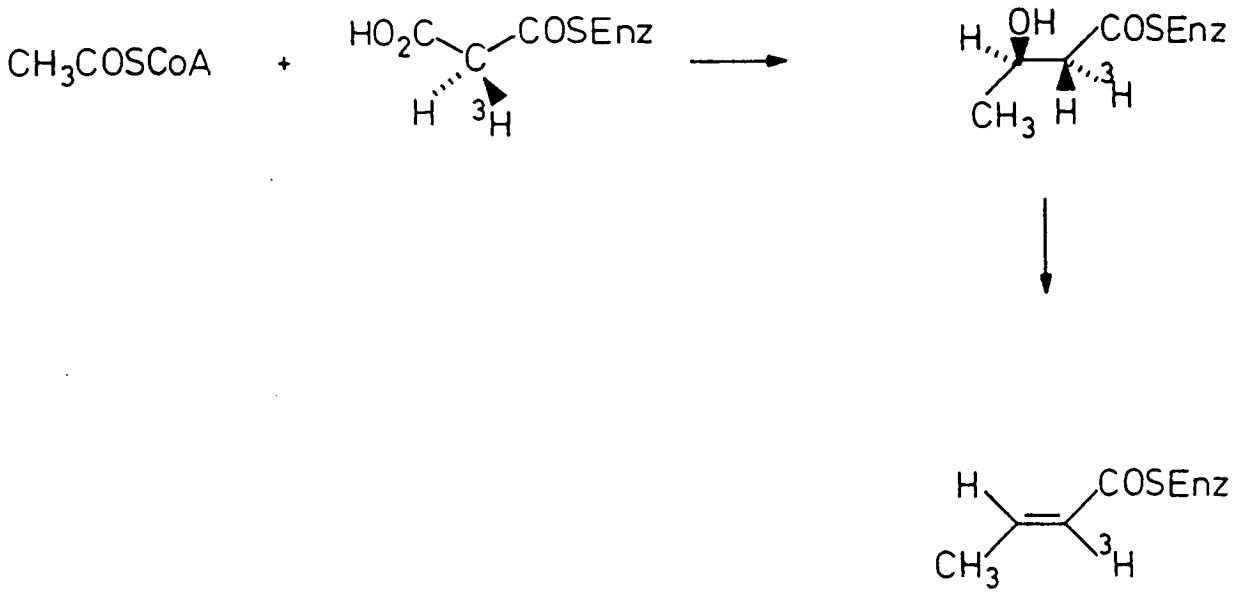
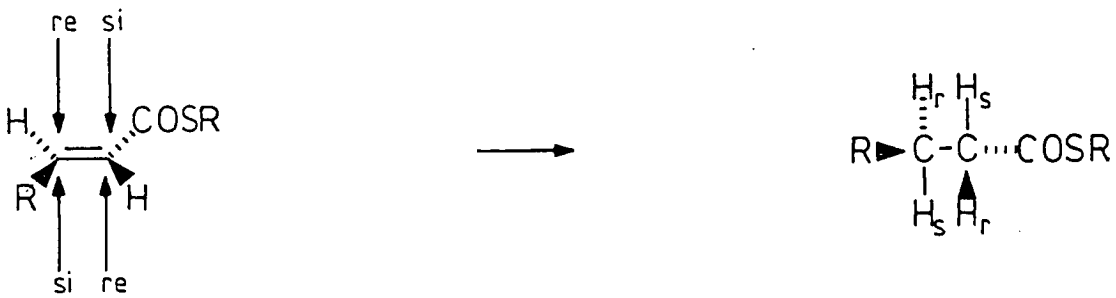


Figure 2

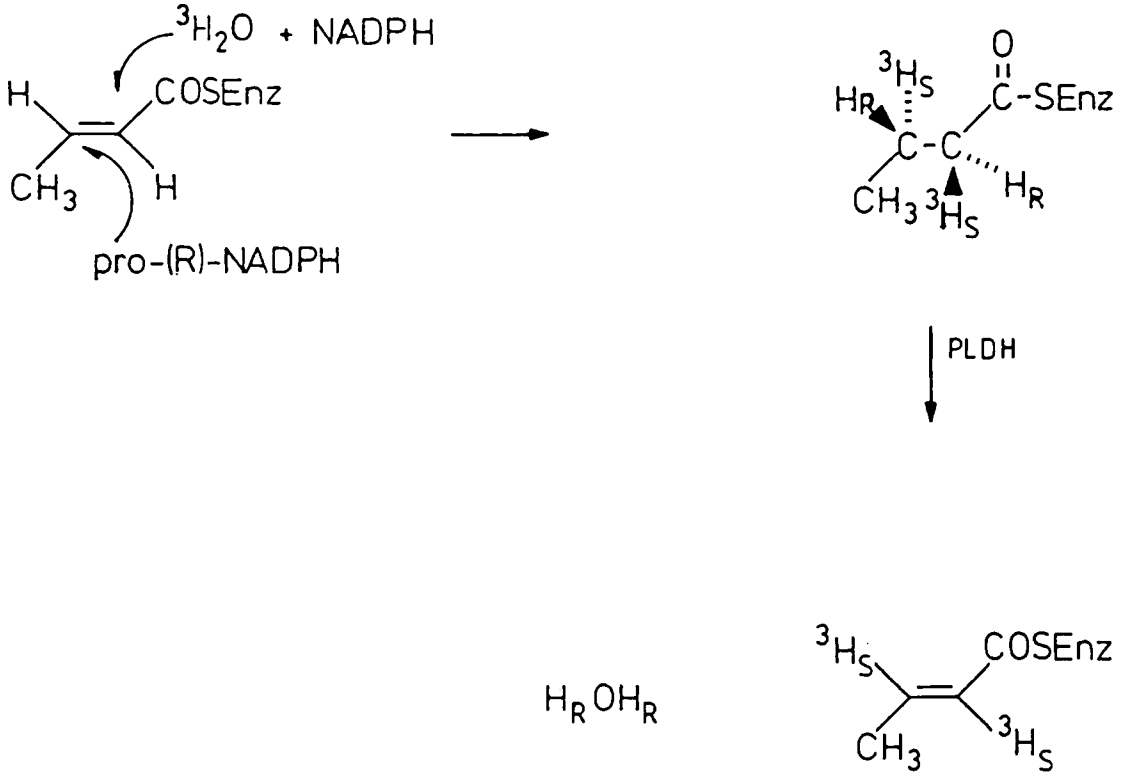


In the absence of NADPH the *trans*-2,3-enoylthiol ester could be isolated from the system. Analysis showed that ^{3}H label from the 2,3-(*R,R*)-substrate as well as ^{3}H label from the 2,3-(*R,S*)-substrate had been retained in the isolated material and not in the medium. This indicated a *syn* elimination of water from the 3-(*R*)-hydroxythiol ester. As one of the labels was retained in the *pro*-2-(*R*) position, and as the study using 2-(*S*)-[2-$^{3}H_1$]-malonyl CoA showed retention of the label from the *pro*-2-(S) position, it was concluded that the condensation of acetyl CoA and malonyl CoA must proceed with inversion of configuration. These results are summarised in scheme 13.

1.2.2.c. Reduction of the *trans*-2,3-Enoylthiol ester.

The stereochemistry of the final step of fatty acid biosynthesis was elucidated in an elegant experiment by Sedgwick and Morris[17]. The experiment was based on the reduction of the *trans*-2,3-enoylthiol ester via an NADPH dependant reaction, in which the C-3 position is reduced by hydrogen from the *pro*-4-(*R*) position of NADPH[18] The C-2 hydrogen is introduced from the medium. By using *pro*-4-(*R*)-NADPH, or incubation in $^{3}H_2O$, either C-3 or C-2 of the product butyric acid could be labelled. Assuming the reactions to stereospecific this gives four possible isomers, 2,3-(*R,R*), 2,3-(*S,S*), 2,3-(*R,S*), and 2,3-(*S,R*) configurations. This corresponds to *re-re, si-si, re-si, and si-re* addition of hydrogen respectively, see figure 2. Incubation of S-*trans*-2,3-crotonyl-N-acetylcysteamine with yeast FAS in the

Scheme 14


^3H_2O + NADPH
H
COSEnz
CH_3
H
pro-(R)-NADPH
^3H_S
H_R
O
C-SEnz
C-C
CH_3
^3H_S
H_R
PLDH
$H_R OH_R$
^3H_S
COSEnz
CH_3
^3H_S

presence of either, *pro*-4-(*R*)-$^{3}H_1$ NADPH or unlabelled NADPH and $^{3}H_2O$, butyric acid was prepared labelled either at C-2 or C-3.

To determine the stereochemistry of the addition, Pig Liver Dehydrogenase (PLDH) was used to re-oxidise the butyric acid. This enzyme has been shown to remove the *pro*-2-(*R*) and *pro*-3-(*R*) hydrogens of carboxylic acids[19,20] to give the *trans*-2,3 enoyl acid. Incubation of the labelled butyric acids with PLDH resulted in 69.8% ^{3}H retained at C-3 indicating that its original insertion was to a *pro*-3-(*S*) position. Similarly 78.3% ^{3}H was retained at C-2, indicating that it was originally inserted into a *pro*-2-(*S*) position. These results were confirmed by using chemically synthesised 3-(*R*)-[3-$^{3}H_1$]-butyric acid which lost all activity on treatment with PLDH, and chemically synthesised 2-(*RS*)-[2-$^{3}H_1$]-butyric acid which lost 50% of its activity on treatment with PLDH. These results indicate that yeast FAS carries out the final reduction in fatty acid biosynthesis via a 2,3 *si-si* addition of hydrogen, see scheme 14. However the mechanism of this particular step does appear to vary from species to species[23]. Combining the results of all this work allows us to draw up a detailed pathway for fatty acid biosynthesis, see scheme 15.

Later work by Lynen et al[21] confirmed that yeast FAS was indeed a multi-enzyme complex. Yeast FAS is homogeneous by electrophoresis and ultracentrifugation, and can be crystallised from ammonium sulphate solution. The condensation of acetyl CoA and malonyl CoA, and subsequent additions of malonyl CoA is achieved by intermediates covalently bound to two types of SH

Scheme 15

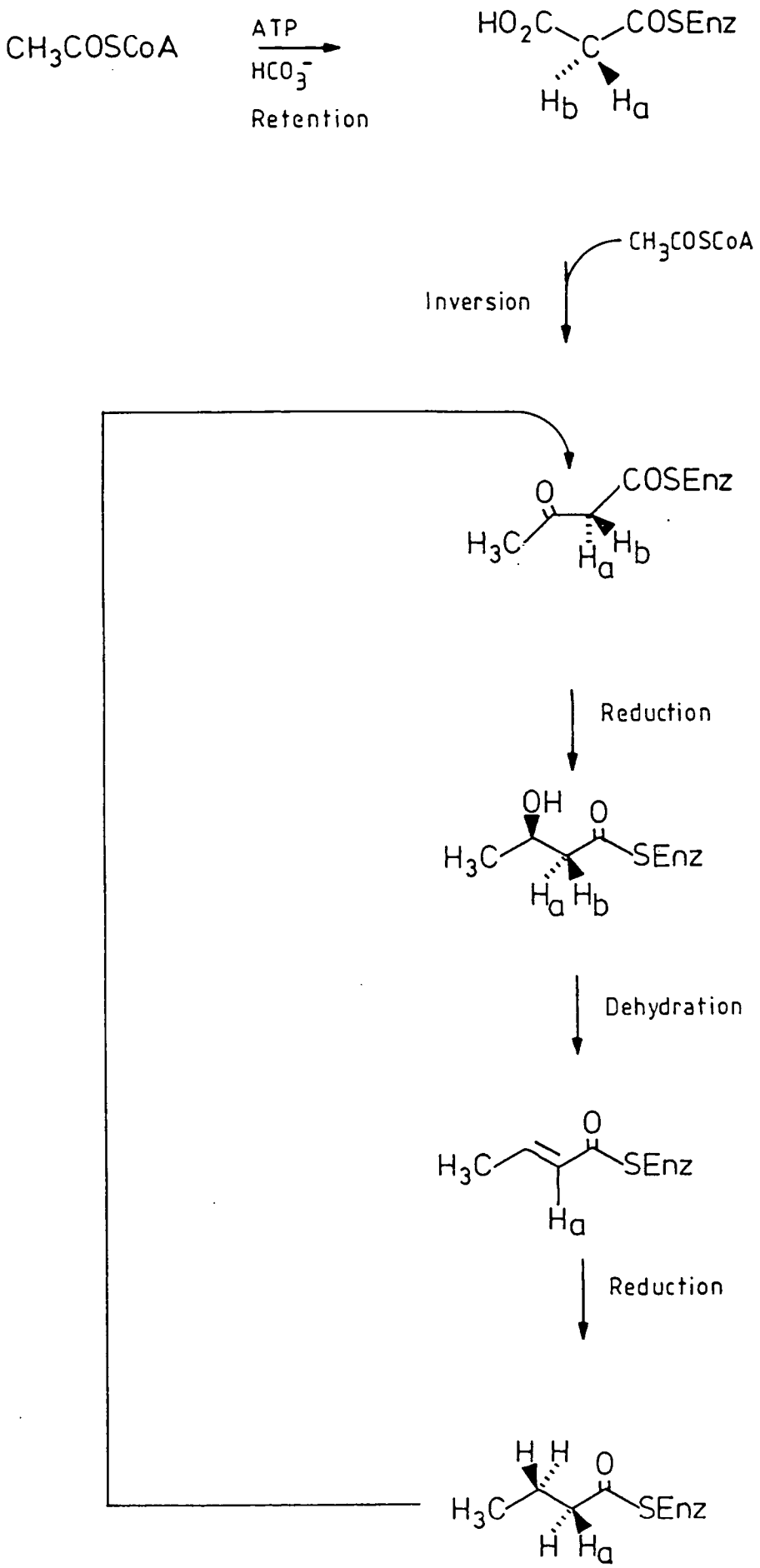

CH3COSCoA
ATP
HCO3-
Retention
HO2C
COSEnz
Hb
Ha
CH3COSCoA
Inversion
O
COSEnz
H3C
Ha
Hb
Reduction
OH
O
H3C
SEnz
Ha
Hb
Dehydration
O
H3C
SEnz
Ha
Reduction
H
H
O
H3C
SEnz
H
Ha

Figure 3

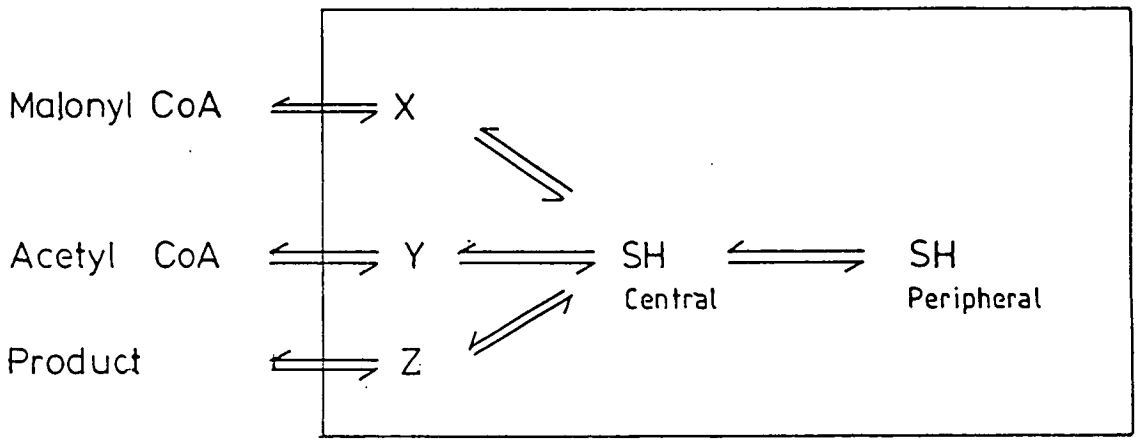
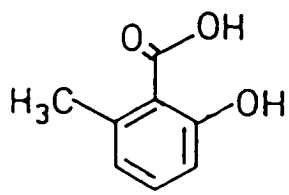

Malonyl CoA
X
Acetyl CoA
Y
SH
Central
SH
Peripheral
Product
Z

Table 1

Source	Products
Pigeon Liver	C16
Rat Liver	C16
Mammary Gland	C8-18
Yeast	C16, C18

Table 2

Source	Products
Avocado	C16, C18
E.coli	C16, C18
Lettuce	C16 C18
B.subtilis	C15 C17



(20)

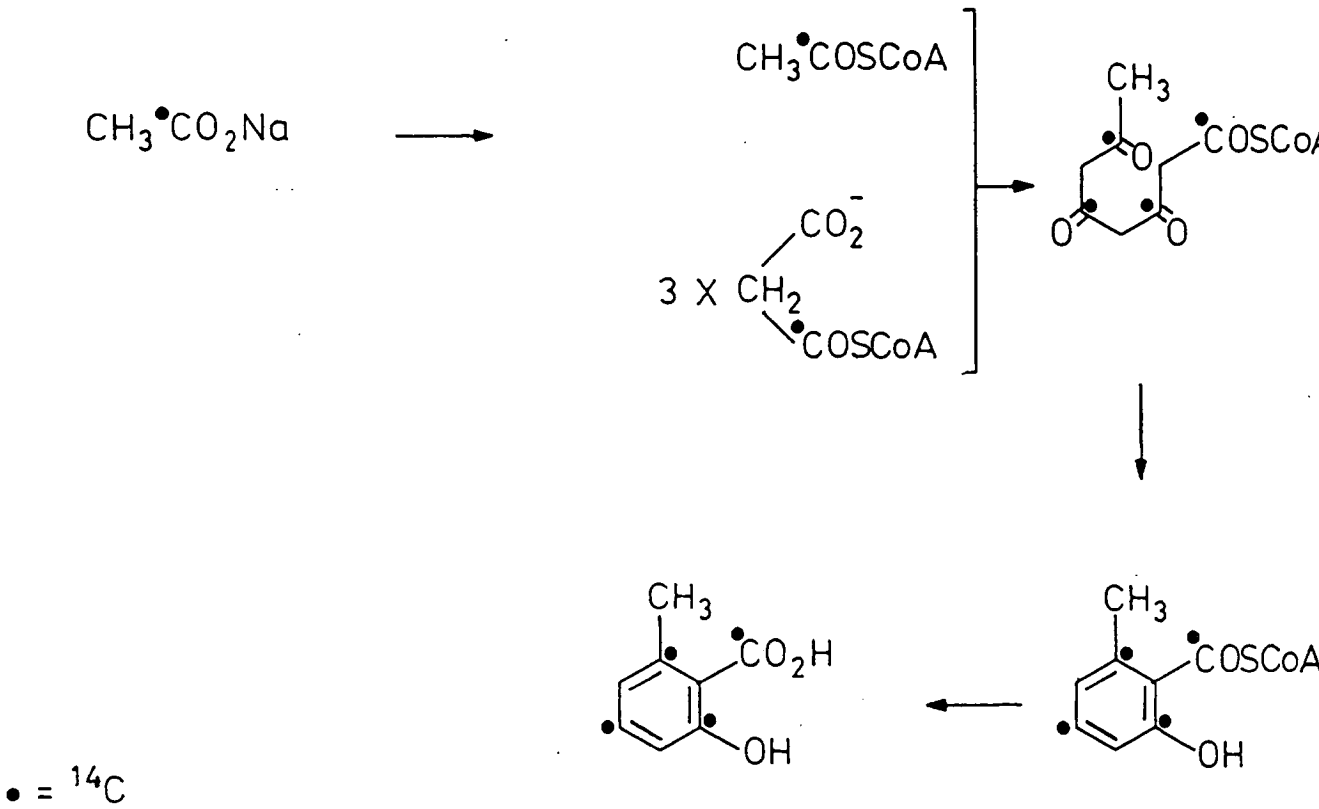
groups on the enzyme, one "peripheral" and one "central". Acetyl CoA is transferred from the "peripheral" group to the "central" group, here condensation occurs with malonyl CoA. All the subsequent reactions occur at this "central" SH group. Finally the product is transferred back to a "peripheral" group and released leaving the "central" group free for the next turnover, see figure 3.

Recent genetic studies[22] have shown that the transfer enzyme responsible for the transfer of malonyl CoA is also responsible for the transfer of the product fatty acid. Thus as long as the completed fatty acid remains on the enzyme complex, further synthesis is inhibited. This may be the method of regulating fatty acid biosynthesis.

Two different types of fatty acid synthetase systems have been identified. Table 1 shows type 1, which are all multi-enzyme complexes, where the constituent enzymes are assenmbled in a compact unit that behaves as a single entity. Table 2 shows type 2, which exist as distinct enzymes with no physical interaction.

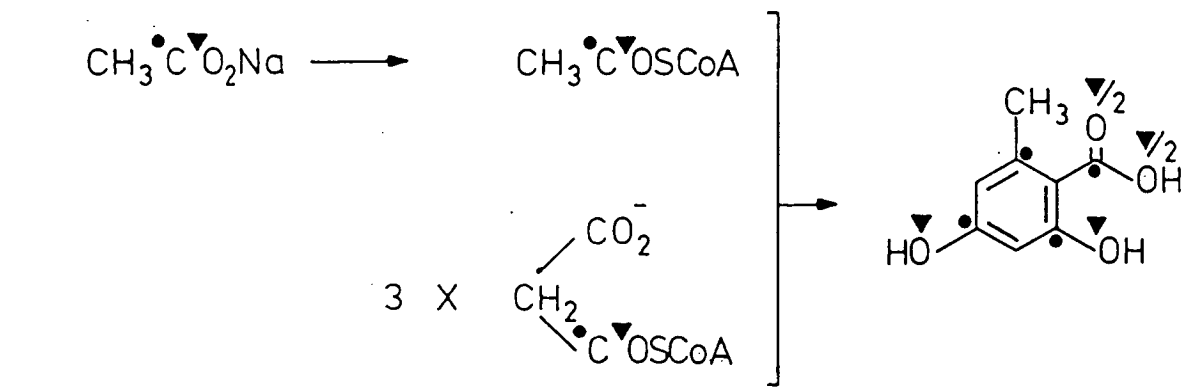
Lynen recognised in 1961[12] that the aromatic metabolite, 6-methylsalicylic acid (20) from *Penicillium patulum* was also of acetyl CoA/malonyl CoA origin, and that it too was assembled on a multi-enzyme complex. 6-Methylsalicylic acid is now a well documented metabolite and has been used to compare the biosynthesis of fatty acids and polyketides.

More fungal metabolites are produced by the polyketide pathway than by any other route[23]. the biosynthesis of



• = ^{14}C

Scheme 17



• = ^{14}C

▼ = ^{18}O

polyketides is intimately related to that of fatty acids, the main difference being in the oxidation level of the final product.

1.3. POLYKETIDES AND THE ACETATE HYPOTHESIS

The idea of an activated C_2 unit as the building block for many natural products as well as fatty acids was first put forward by Collie[24] in 1907. The suggestion went largely unnoticed until 1953 when Birch and Donovan[25] independently postulated the polyacetate origin of some natural phenols. Careful study of the structure of these phenols led them to conclude that they could be derived from the head to tail linkage of acetate units. In some cases the acetate oxygen appeared to be retained, sometimes lost as water during dehydration to form double bonds, or even reduced completely. Even at this early stage it was obvious that such a system must be related to fatty acid biosynthesis.

The first firm evidence came in 1955[26] with the incorporation of [1-^{14}C] acetic acid into 6-methylsalicylic acid from *Penicillium patulum* (scheme 16). This was quickly followed by the incorporation of [1-^{14}C,$^{18}O_2$] acetic acid into orsellenic acid[27] (scheme 17). Thus the acetate hypothesis was confirmed, These metabolites were of polyacetate origin and were called polyketides.

The basic theory of polyketide biosynthesis is that acetyl CoA provides a "starter" unit which is then condensed with

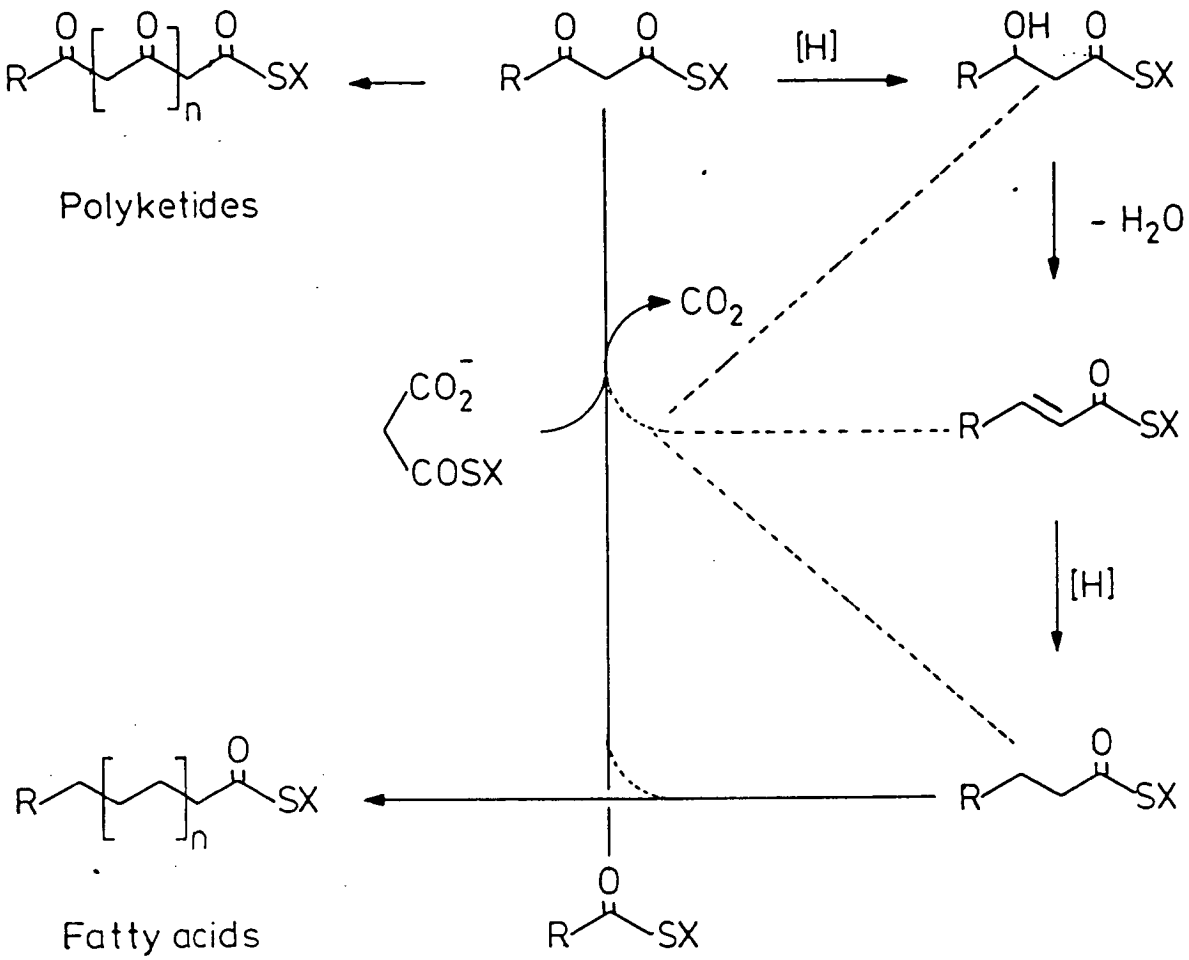
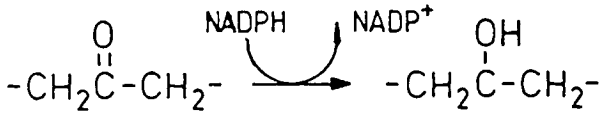
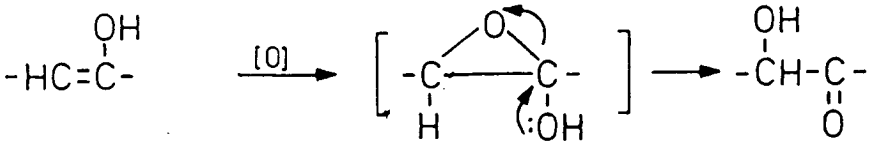


Figure 4

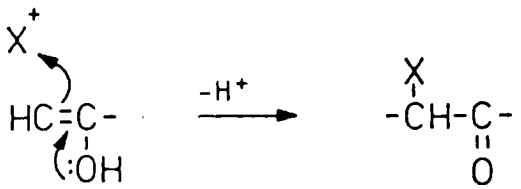
1.) Reduction



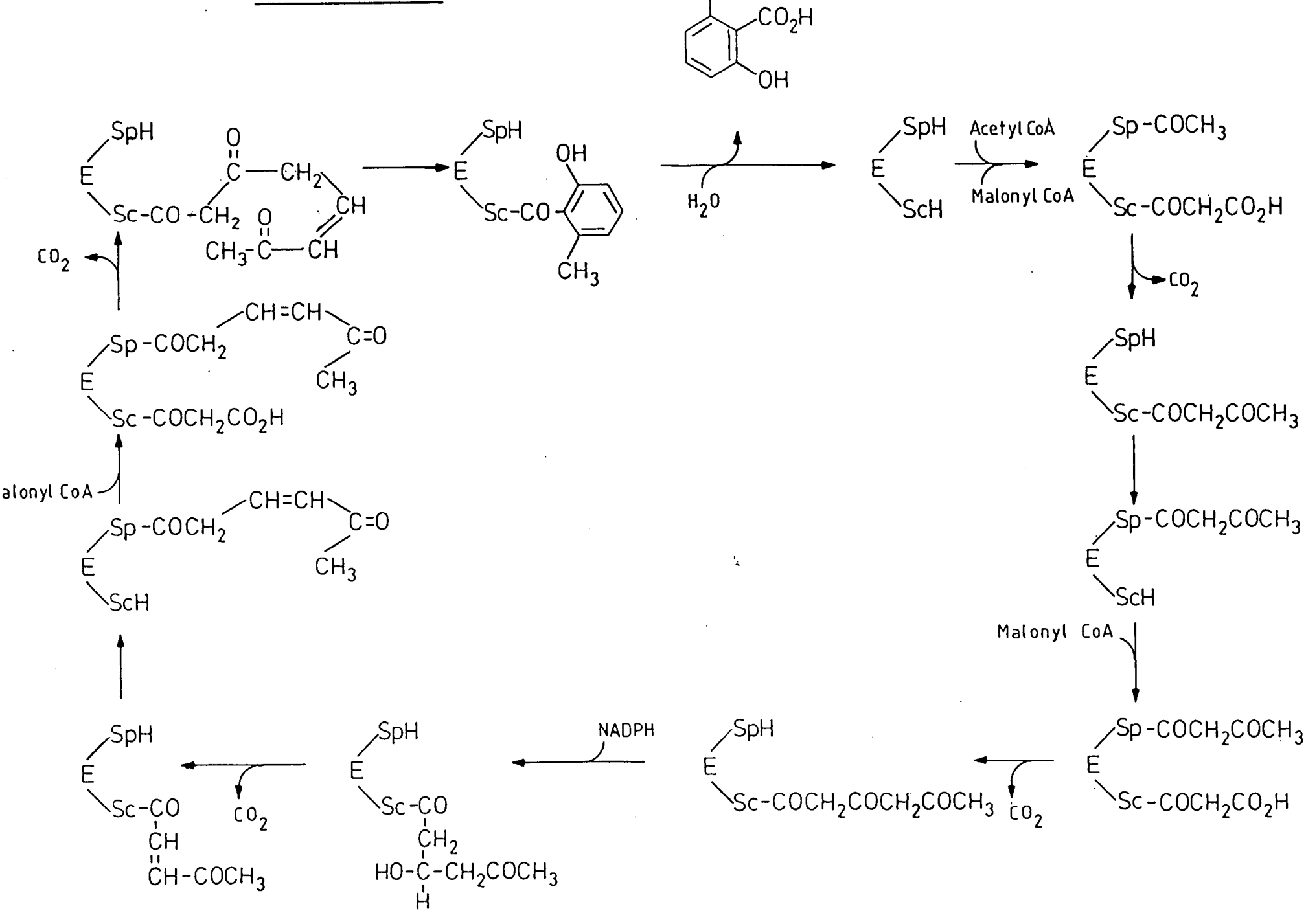
2.) Oxidation



3.) Alkylation



X = CH_3 or C_5, C_{10} or C_{15} Isoprenoid

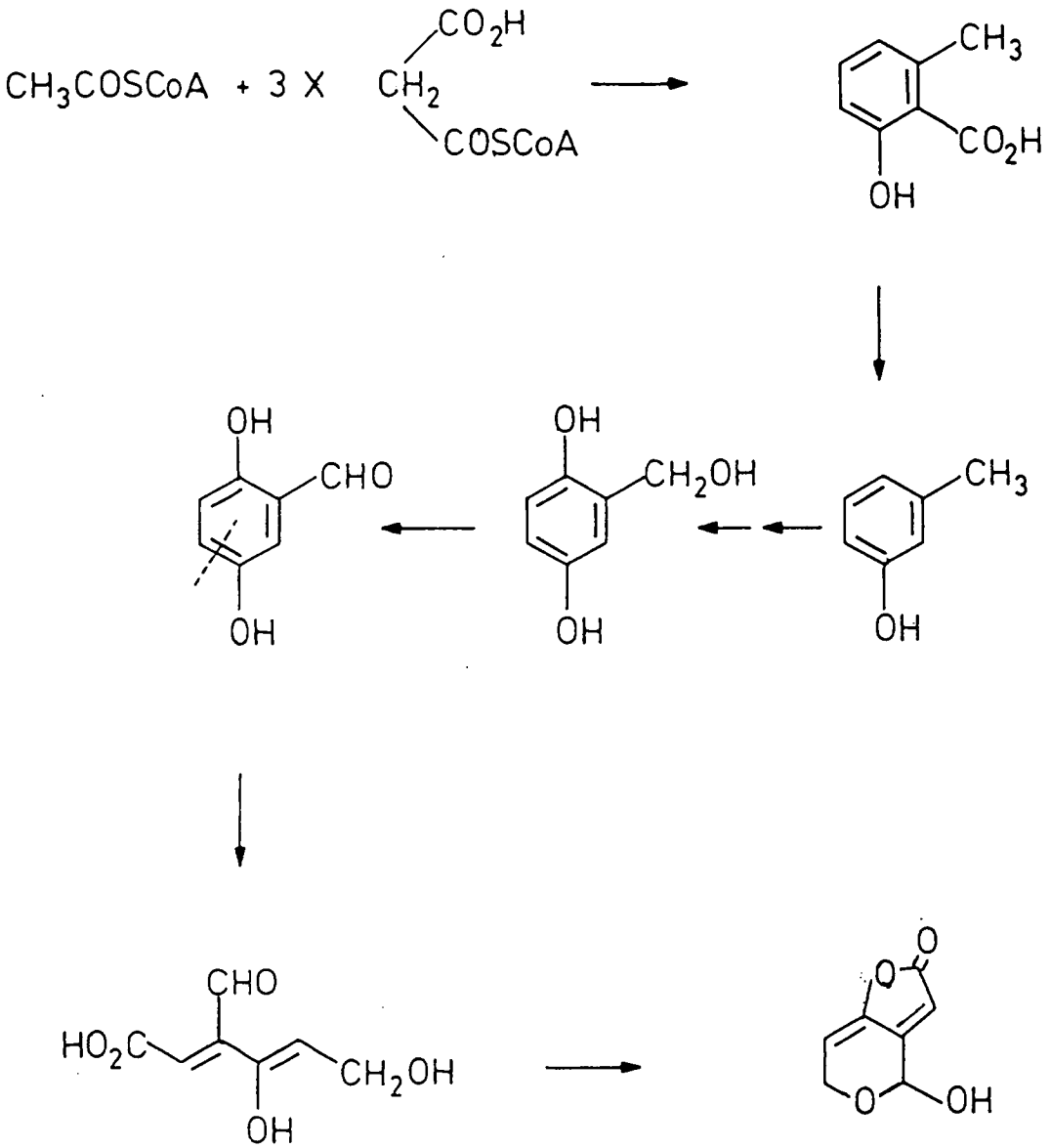

CO2H
OH
SpH
E
Sc-CO-CH2
O
C—CH2
CH
CH3-C—CH
O
OH
CH3
H2O
ScH
Acetyl CoA
Malonyl CoA
Sp-COCH3
Sc-COCH2CO2H
CO2
Sp-COCH2
CH=CH
C=O
CH3
Sc-COCH2CO2H
Malonyl CoA
Sp-COCH2COCH3
Sc-COCH2COCH2COCH3
NADPH
Sc-CO
CH
CH-COCH3
CH2
HO-C-CH2COCH3
H

n units of malonyl CoA to form a poly-β-keto-methylene chain of general structure, [(CHRCO)$_n$SEnz, n = 4 - 20)] which is bound in some fashion to an enzyme. This chain is then condensed and altered to give the polyketide metabolite, see scheme 18. This sequence is similar to that already discussed for fatty acids, the difference being that each keto-methylene group is not fully reduced after condensation. Aldol and Claisen type reactions of the polyketide chain provide the wide variety of structural forms known for polyketides.

Scheme 19[21] shows the proposed mode of assembly of a tetraketide chain and its subsequent conversion to 6-methyl salicylic acid. There is a carrier site and a condensing site on the enzyme complex. This is again similar to fatty acid biosynthesis. A crucial observation[28] was that the absence of NADPH in a cell free system results in the shunt product tri-acetic acid lactone. This suggests that the growing polyketide chain is modified during its assembly and not after. This again fits in with the evidence from fatty acid biosynthesis. Due to evidence such as this it is not unreasonable to suggest that the growing polyketide chain could utilize similar reduction/ elimination/reduction pathways to those found in fatty acid biosynthesis, this is outlined in scheme 18, which shows the possible relationship between the two pathways

In many polyketides that have been studied using labelled acetate precursors, the initial starter unit derived carbons are more intensely labelled than the subsequent sites. This is the so called "starter" effect[29], the other sites in the

Scheme 20

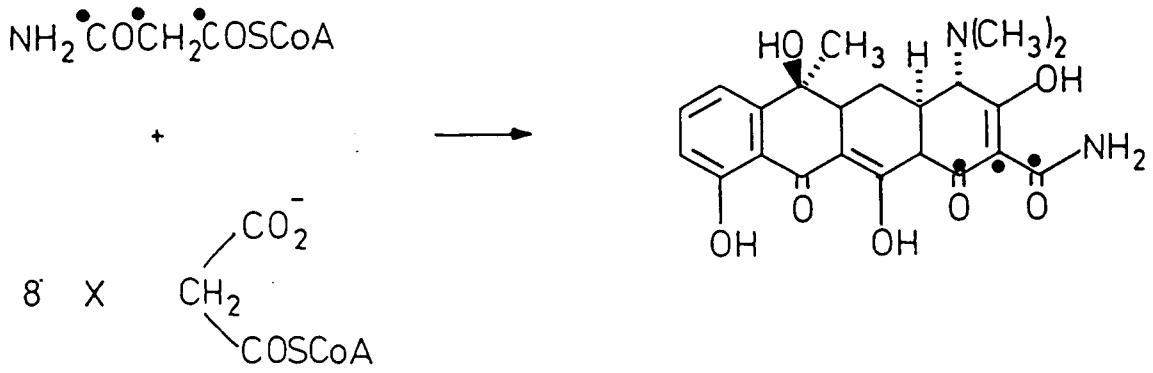

CH3COSCoA + 3 X
CO2H
CH2
COSCoA
CH3
CO2H
OH
CH3
OH
CH2OH
OH
OH
CHO
OH
CHO
HO2C
CH2OH
OH
O
O
O
OH

molecule can be labelled efficiently by using labelled malonate precursors. However the rapid the equilibration between acetate and malonate sites may lessen the effect.

At some point during their assembly polyketides are subjected to chemical modification. This is the main reason for the diversity of compounds produced. The three principle reactions that occur are reduction, oxidation, and alkylation. The groups used in alkylation reactions are CH_3 from methionine and/or C_5, C_{10}, or C_{15} groups derived from the isoprenoid pathway. Figure 4 summarises these reactions. Modifications can of course be combined in sequence to produce further alterations. For instance methylation may be followed by oxidation to produce a carboxylic acid. Similarly demethylation can occur by oxidation of the methyl group to a carboxylic acid, followed by loss of CO_2 to produce the demethylated compound.

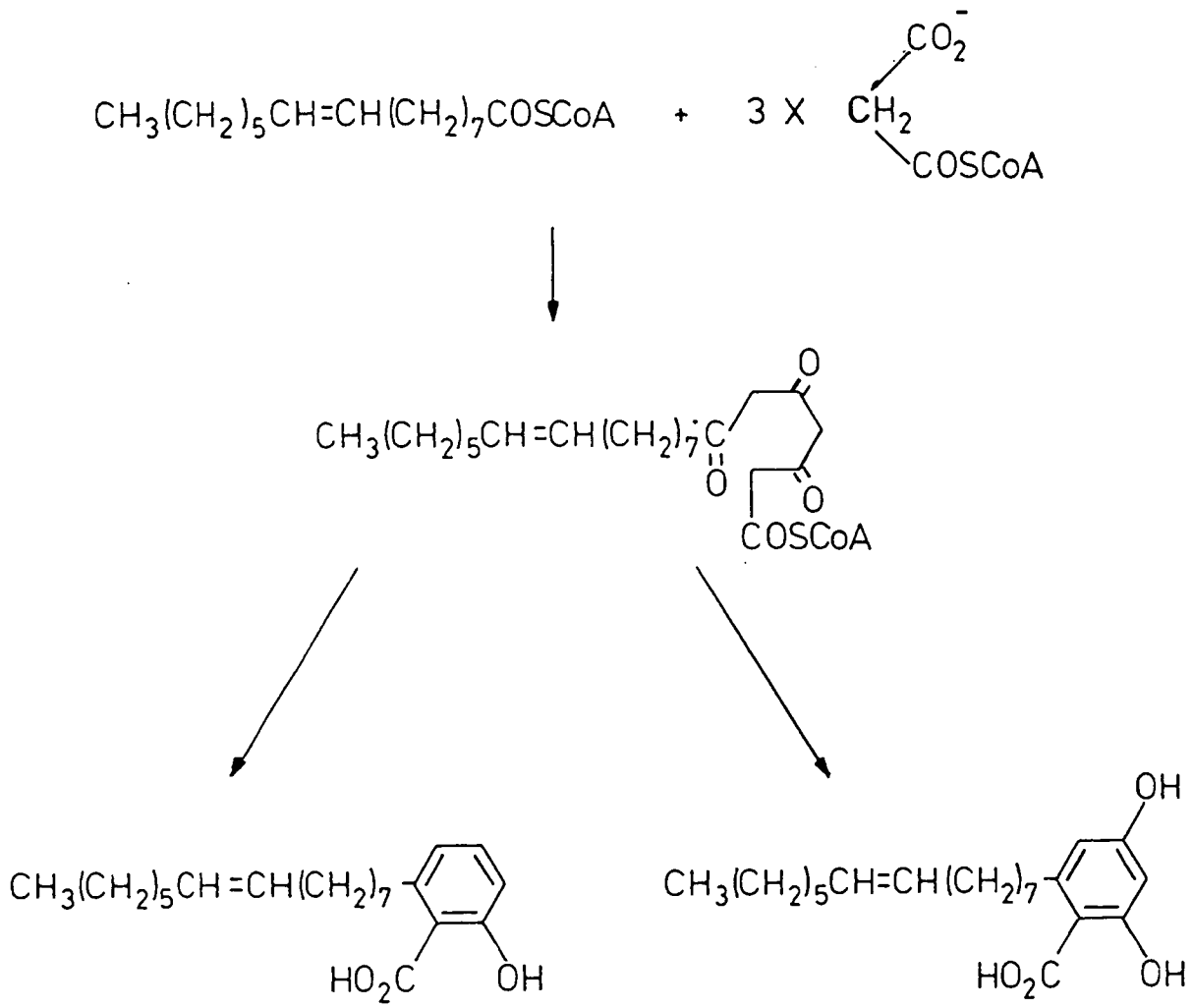
The formation of some polyketides involves the cleavage of aromatic rings, followed by recombination into a different configuration. This is well illustrated by the biosynthesis of patulin[30] (21) from *Penicillium patulum*. Decarboxylation gives *m*-cresol which is in turn oxidised to give the ring opened product, which then recyclises to patulin. scheme 20.

Although most polyketides are assembled from acetyl CoA and malonyl CoA they are by no means the only units that are used. The tetracyclines for instance use malonamido CoA as the starter unit[31] and malonyl CoA as the extension unit, see scheme 21. The *Anacardiacaes* family of plants use a series of unsaturated fatty acids[32] as starter units and three or four



• = ^{13}C

Scheme 22



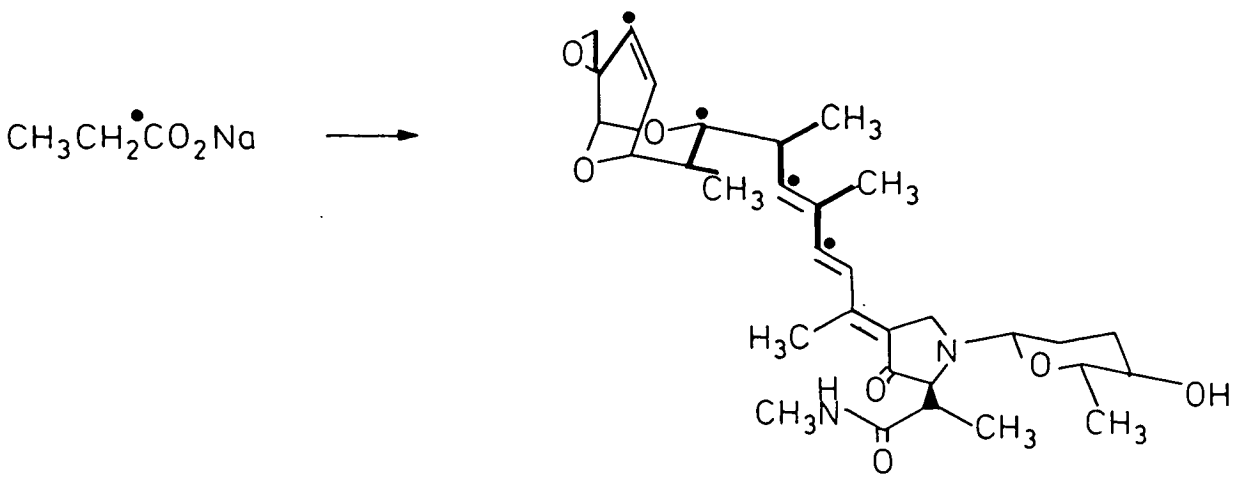
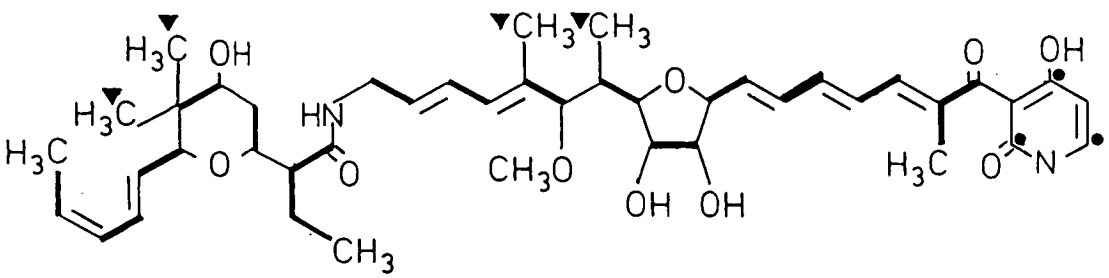


Figure 5

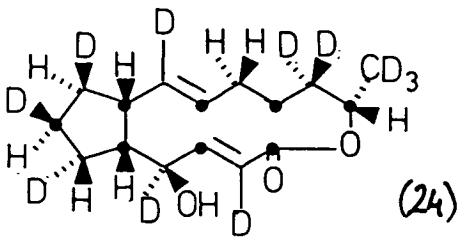


Methionine ▼

$CH_3\overset{\bullet}{C}O_2Na$ ▬

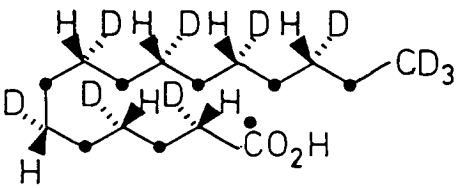
Propionate

Butyrate

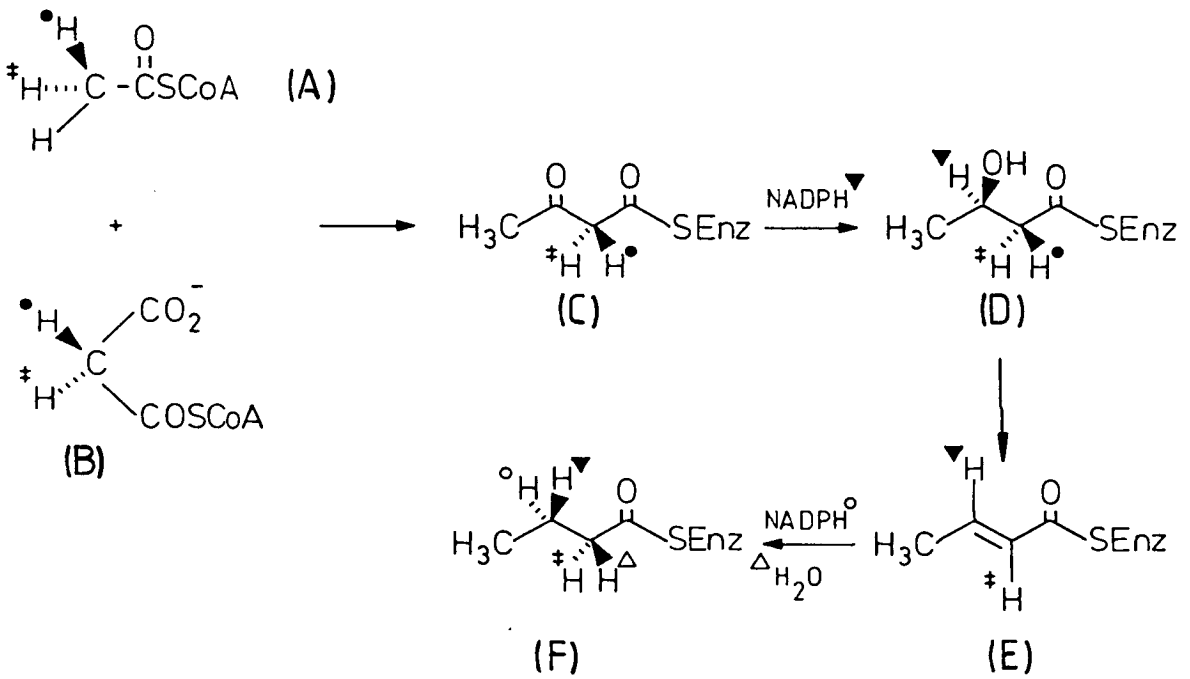


(24)

$CD_3\overset{\bullet}{C}O_2H$ ⟶



• = ^{13}C

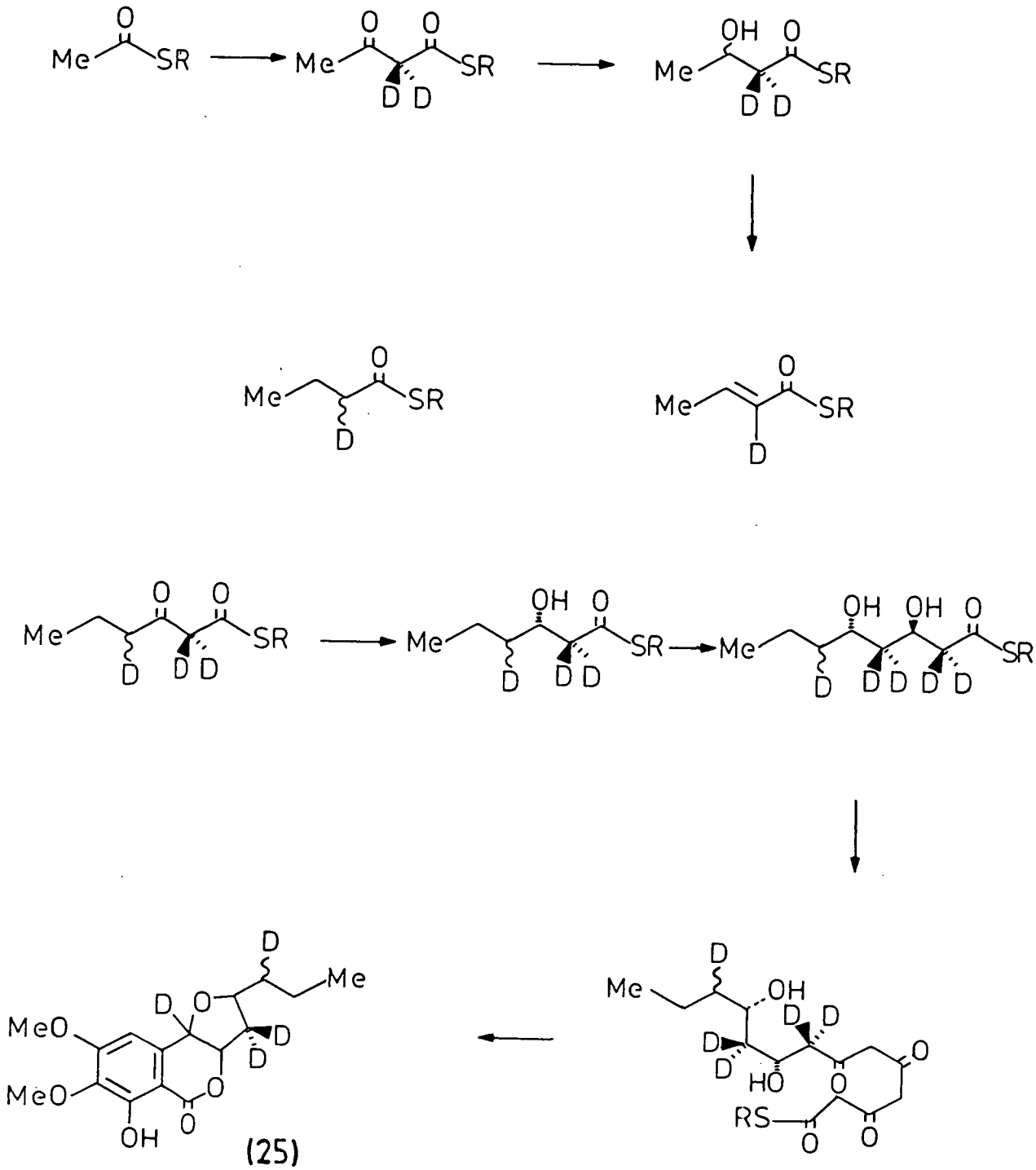


malonyl CoA units to produce the phenols shown in scheme 22. Propionate and butyrate are also used as chain extension units. Streptolydigin (22) a metabolite of *Streptomyces lydicus* has polketide chain methyl groups derived from propionate[33], see scheme 23. Aurodox (23) a metabolite of *Streptomyces goldiensis* has sections of its structure derived from both propionate and butyrate[34], see figure 5.

To date the elegant analysis of the stereochemistry of fatty acid biosynthesis has no parallel in the study of polyketides. Several studies have however contributed to the understanding of the relationship between the two pathways.

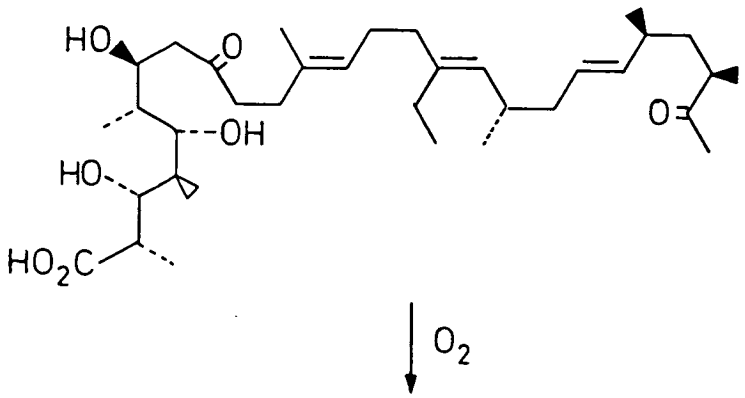
Brefeldin A (24)[35] from *Penicillium brefeldianum* has been extensively studied. The work has centered around the idea that any labelling produced in brefeldin A might be similar to that produced in palmitic acid from the same organism. Scheme 24 shows both compounds and a section of fatty acid biosynthesis. Positions 2,3 and 11 of brefeldin A, may be derived via (E). Positions 4,6,8 and 12 may be formed via the α carbon of (F) and positions 5,7,9 and 13 via the β carbon of (F). The results of this study in fact showed that positions 6 and 8 of brefeldin A were of the opposite stereochemistry to the corresponding positions in palmitic acid. Position 14 of brefeldin A retained two ^{2}H labels and hence cannot be derived via (C).The conclusion from this study was that the enzymes responsible for the assembly of brefeldin A were unique and did not use the route (A)-(F). However the retention of both deuterium labels at C_{-14} indicated that the polyketide chain was being altered as it was assembled.

Scheme 25

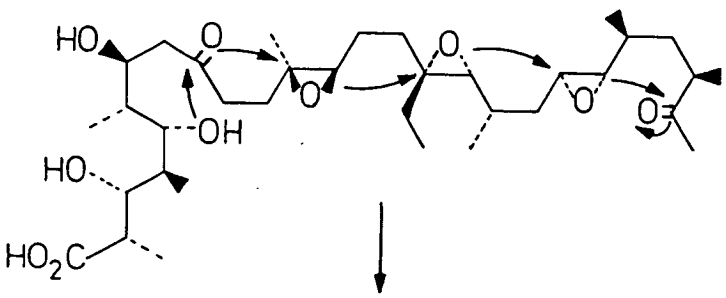


(25)

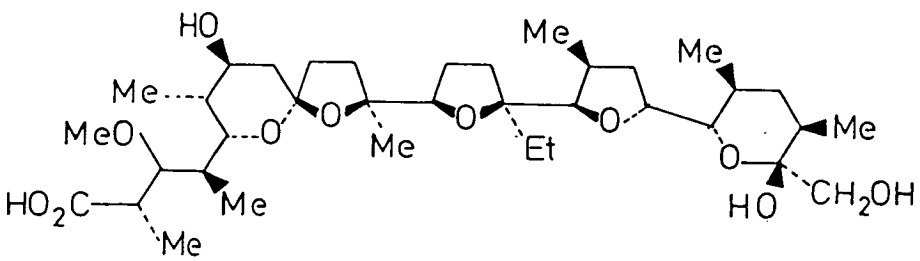
Scheme 26



(28)



(27)



(26)

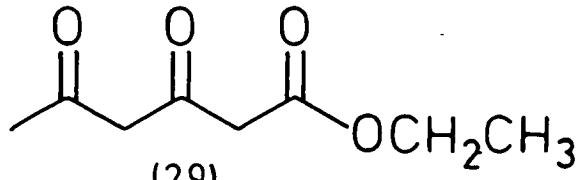
A similar result was obtained during he study of monocerin (25) from *Drechslera ravenelli*. The results of ^{2}H and ^{18}O labelling studies showed that monocerin was derived from a reduced polyketide and not a classical heptaketide[36]. Scheme 25 shows the proposed biosynthesis from these results.

$^{18}O_2$ Labelling studies have been used to examine the precursors to the polyether metabolite monensin (26). The results are summarised in scheme 26. Labelling from $^{18}O_2$ gas was found to be present in the positions bridging carbons 13,16,17,20,21, and 25. This led to the proposal of a triepoxide (27) derived from a triene (28) as the immediate precursor[37].

The work presented in this thesis was carried out with the intention of expanding what is known about polyketide biosynthesis and its relationship to fatty acid biosynthesis.

1.4. YEAST FATTY ACID SYNTHETASE AND 6-METHYLSALICYLIC ACID SYNTHETASE: SIMILARITIES IN STRUCTURE AND FUNCTION.

6-Methylsalicylic acid is one of the simplest of all polyketides. It is one of the few polyketides where the synthetase enzyme has been isolated. For this reason it has been used extensively to examine similarities between the biosynthesis of fatty acids and that of polyketides[21]. Lynen[12] was the first to point out that the two systems were very similar and has applied the knowledge gained from the study of yeast fatty acid synthetase to the study of 6-methylsalicylic acid synthetase[21].



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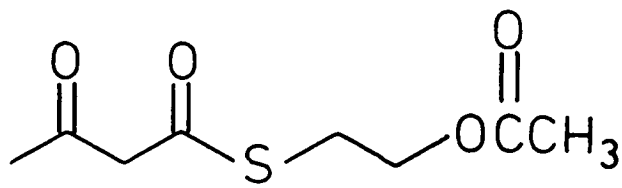


(30)

The overall reaction to produce 6-methylsalicylic acid is shown in Scheme 19. All the components shown here are also present in fatty acid biosynthesis. The enzyme system responsible for the assembly of 6-methylsalicylic acid was isolated from *Penicillium patulum*, and was found to display many of the characteristics of yeast FAS, such as the proposed "central" and "peripheral" SH groups and the assembly of 6-methylsalicylic acid is thought to proceed as shown in scheme 19. Acetyl CoA and malonyl CoA are transferred to SH groups and condensed to form acetoacetyl residues, addition of a second malonate unit gives triacetic acid. Reduction and elimination of water gives the γ,δ-unsaturated chain. This is extended by addition of a third malonate unit, and cyclisation occurs *via* an Aldol type reaction. 6-methylsalicylic acid is then released from the enzyme by hydrolysis.

In order to gain more information about the two systems Lynen[21] et al administered two substrate models to both complexes. Triacetic acid ethyl ester (TAE), (29) and S-acetoacetyl-N-acetylcysteamine (AAC), (30). Yeast FAS readily reduced AAC, but 6-MSA synthetase did not. Both complexes catalysed the reduction of TAE, but yeast FAS only did so at a greatly reduced rate to 6-MSA synthetase. This indicates that the specificity of the reducing enzyme determines the final product. In both systems AAC is a model for the product of the condensation of acetate and malonate. Hence yeast FAS reduces this compound but 6-MSA synthetase does not as it requires the β-carbonyl group to remain intact to participate in the ring closure reaction.

AAC is a model for the product of the condensation of acetate and malonate. Hence yeast FAS reduces this compound but 6-MSA synthetase does not as it requires the β-carbonyl group to remain intact to participate in the ring closure reaction.

Bioimmunoassay studies[21] indicate that in fact the two enzyme systems may have a common ancestor. Specific antibodies were prepared to 6-MSA synthetase and tested against FAS and 6-MSA synthetase from *Penicillium patulum* and also against yeast FAS. A cross reaction was observed between the antibodies and the FAS from *Penicillium patulum* but not with the yeast FAS. This indicates that there is a close relationship between the two enzyme complexes derived from *Penicillium patulum*, and that it is possible that they have evolved from a common ancestor.

1.5. THE ELUCIDATION OF BIOSYNTHETIC PATHWAYS

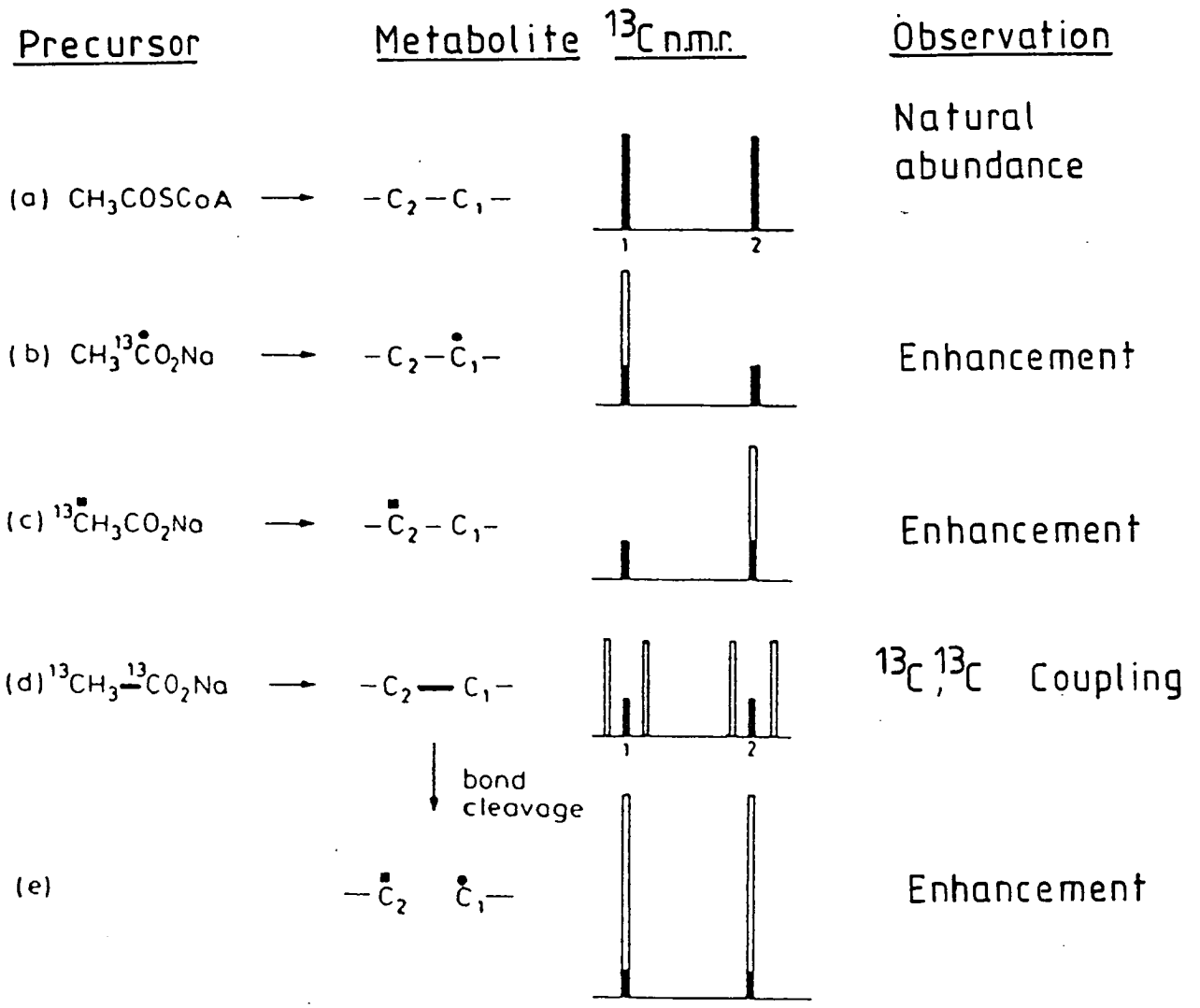
The earliest biosynthetic studies were carried out using precursors containing radiolabels, commonly ^{3}H and ^{14}C. The amount of precursor incorporated into a metabolite was assessed by measuring the specific activity of the metabolite. Using techniques such as this, the level of precursor incorporation can be determined very accurately. The technique is very sensitive and only small quantities of material are required. However biosynthetic labelling in this way does not give direct evidence about the specificity of the labelling. This can only be determined by chemical degradation, which is wasteful and labour intensive. In modern biosynthetic studies radiolabelling is

generally confined to measuring isotopic dilution, although in some situations it is still used for the actual biosynthetic study itself. Biosynthetic studies now rely heavily on the use of stable isotopes which can be analysed by high resolution nuclear magnetic resonance spectroscopy (n.m.r.). Good reviews of current n.m.r. technology and methodology are now available[40], and a reasonable working knowledge of the subject is assumed here.

The stable isotopes commonly used are ^{13}C, ^{2}H, ^{17}O, ^{18}O, and ^{15}N. These isotopes have several advantages ; they are all stable and therefore do not require specialist handling, with the exception of ^{13}C they all have very low natural abundance and so are easily detectable in small quantities, with the exception of ^{18}O all are directly observable by n.m.r., ^{18}O can be observed using indirect n.m.r. methods.

The use of stable isotopes in biosynthesis is not without its disadvantages, Despite recent advances in Fourier transform n.m.r. it is still not as sensitive as radiochemical methods. This means that larger quantities of material are required for analysis. However due to their low natural abundance the successful incorporation of a stable isotope allows smaller amounts of material and greater isotopic dilutions to be used. The exception to this is ^{13}C where the natural abundance is 1.1%, Hence isotopic dilutions of 100 or less are required to reduce the amount of material required for successful analysis by n.m.r.

Figure 6


Precursor
Metabolite
^{13}C n.m.r.
Observation
(a) $CH_3COSCoA$ → $-C_2-C_1-$
Natural abundance
1
2
(b) $CH_3{}^{13}\dot{C}O_2Na$ → $-C_2-\dot{C}_1-$
Enhancement
(c) $^{13}\dot{C}H_3CO_2Na$ → $-\dot{C}_2-C_1-$
Enhancement
(d) $^{13}CH_3-{}^{13}CO_2Na$ → $-C_2-C_1-$
^{13}C, ^{13}C Coupling
1
2
bond cleavage
(e)
$-\dot{C}_2$ $\dot{C}_1-$
Enhancement

1.5.1 Direct Observation of Stable Isotopes by n.m.r.

1.5.1.a. ^{13}C Enrichment studies

The successful incorporation of a ^{13}C labelled precursor into a metabolite results in the enhancement of the appropriate signals in the pnd. ^{13}C n.m.r. spectrum. This is represented in figure 6, which shows the effect of incorporation of a two carbon precursor, singly labelled with ^{13}C in either carbon, or doubly ^{13}C labelled. Incorporation of the singly labelled precursors results in signal enhancement in C-1, or C-2 as appropriate. Incorporation of the doubly labelled precursor results in ^{13}C-^{13}C coupling if the precursor carbon carbon bond is incorporated intact. If for some reason during the biosynthesis of the metabolite this bond is broken. the coupling is lost and only enhancement is observed, as in the case of the singly labelled precursors.

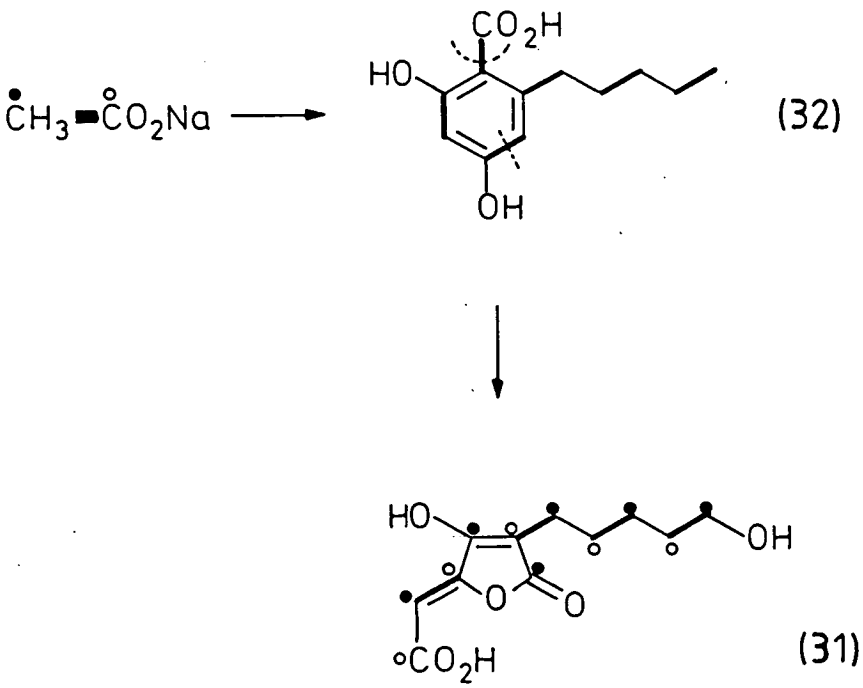
Observation of ^{13}C-^{13}C coupling from doubly labelled acetate was used to determine the biosynthesis of multicolic acid[41] (31). The absence of ^{13}C-^{13}C coupling in certain cases led to the conclusion that the tetronic acids were formed by oxidative cleavage of a benzenoid intermediate (32). (scheme 27).

In theory it should be possible to measure quantitatively levels of ^{13}c incorporation as follows[42] ;

% enrichment = 1.1 x (intensity at labelled site) - 1.1

(intensity at unlabelled site)

Scheme 27

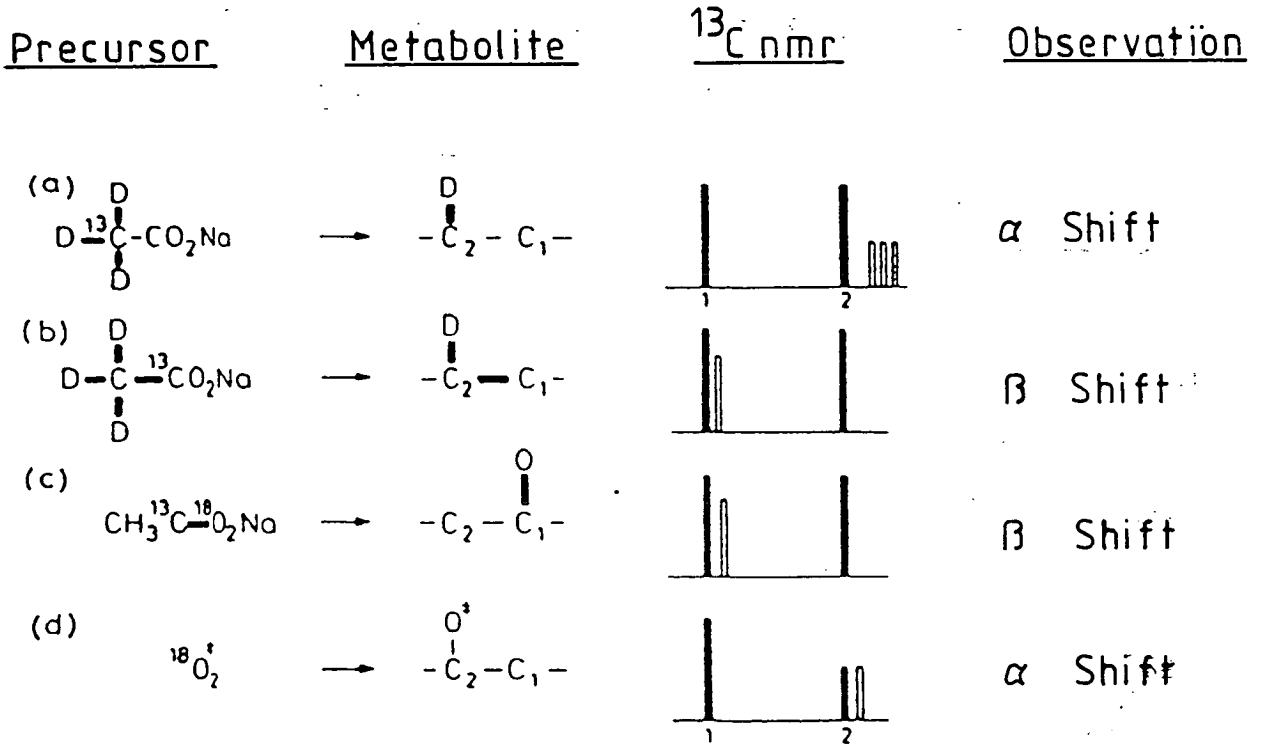


However due to a wide range of effects, the integration of carbon n.m.r. spectra is not a reliable technique. A wide range of spin lattice relaxation times, and variable n.O.e. effects due to proton decoupling, give rise to a wide variety of intensities and line shapes. The problem is further complicated by Fourier transform techniques, and digitisation of spectra, both of which lead to poor line shape. The physical side of the problem can be overcome by the use of paramagnetic suppresing agents such as chromium *tris*-aceto acetonate which reduces n.O.e.'s and spin lattice relaxation times. The acquisition side of the problem can only be overcome by better instrumentation and the use of more data points, this requires longer instrument time.

1.5.1.b ^{2}H Enrichment studies

The use of ^{2}H to determine the biosynthetic fate of hydrogen is now common[42]. Direct observation of ^{2}H by n.m.r. has several disadvantages. ^{2}H is a quadrupolar nucleus (spin 1), and is therefore very efficiently relaxed, this coupled with a low gyromagnetic ratio, and a narrow chemical shift window lead to poorly resolved spectra. However the use of ^{2}H n.m.r. does have several advantages. The natural abundance of ^{2}H is 0.012%, therefore far greater isotopic dilution can be tolerated. The efficient relaxation and the lack of n.O.e. allows accurate integration. The chemical shift range of ^{2}H is approximately the same as for ^{1}H and so a ^{1}H n.m.r. spectrum can be used to assign

Figure 7


Precursor
Metabolite
^{13}C nmr
Observation
(a)
D
D—^{13}C—CO$_2$Na
D
D
—C$_2$—C$_1$—
1
2
α Shift
(b)
D
D—C—^{13}CO$_2$Na
D
D
—C$_2$—C$_1$—
β Shift
(c)
CH$_3$^{13}C=^{18}O$_2$Na
O
—C$_2$—C$_1$—
β Shift
(d)
^{18}O$_2$
O*
—C$_2$—C$_1$—
1
2
α Shift

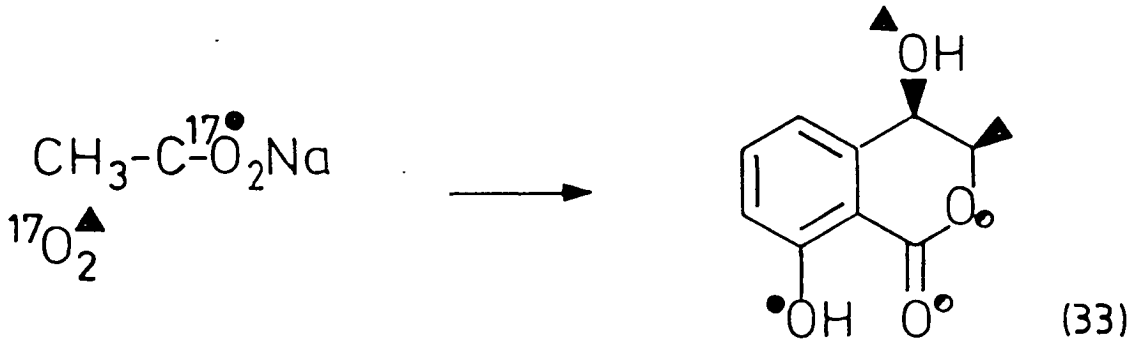
a ^{2}H n.m.r. spectrum. However the biosynthetic fate of ^{2}H is more commonly followed using indirect n.m.r. methods.

1.5.1.c Indirect observation of Stable isotopes by n.m.r.

The indirect observation of stable isotopes by n.m.r.involves the observation of a "reporter" nucleus such as ^{13}C. The presence of an isotope such as ^{2}H α or β to a ^{13}c nucleus, results in a change in the chemical shift for the ^{13}C resonance, usually to high frequency. These effects are known as α and β isotope shifts[42] and are illustrated in figure 7, Incorporation of [2-^{13}C, $^{2}H_{3}$]acetate into a metabolite results in an α isotope shift for C-2 in the pnd ^{13}C spectrum. The single ^{2}H retained causes a shift of 0.3-0,9 ppm. to low frequency. In the case of the α isotope shift the effect is complicated by ^{2}H-^{13}C coupling, which appears as a triplet as ^{2}H has spin 1. In a simple case such as this the coupling is not a problem. However where a variety of ^{2}H has been retained such as a methyl group which may be a mixture of $C^{2}H_{3}$, $C^{2}H_{2}H$, and $C^{2}H_{1}H_{2}$ the pattern can be difficult to interpret.

The effects of ^{2}H-^{13}C coupling can be avoided by placing the ^{2}H label β to the ^{13}C. This results in a β isotope shift being observed, which is smaller than the α isotope effect but still resolvable. However the two bond ^{2}H-^{13}C coupling is not resolvable and the shifted signal appears as a singlet. This is shown in figure 7.

Scheme 28



(33)

1.5.1.d ^{18}O Enrichment studies

The biosynthetic fate of oxygen can be followed by direct ^{17}O n.m.r. However ^{17}O is a quadrupolar nucleus with spin 5/2, and is very efficiently relaxed. There are also inherent difficulties involved in the acquisition of ^{17}O n.m.r. spectra. Recently direct ^{17}O n.m.r. has been used to sudy the biosynthesis of hydroxymellein (33), in particular the C-4 hydroxyl group[43]. Incorporation of [$^{17}O_2$]acetate and $^{17}O_2$ gas showed the origin of all the oxygen in (33), and indicated that the C-4 hydroxyl was derived from the atmosphere (scheme 28).

Despite this recent application, the biosynthetic fate of oxygen is more commonly followed by indirect observation of ^{18}O by ^{13}C n.m.r. This is an α shift technique, and as ^{18}O has no nuclear spin there is no coupling to carbon, and so the shifted signal appears as a singlet. This is illustrated in figure 7, for the incorporation of [1-^{13}C, $^{18}O_2$]acetate and $^{18}O_2$ gas. The ^{18}O isotope effect is small, generally not being more than 0.05 ppm. Fermentations using $^{18}O_2$ gas were used to determine the source of the oxygen atoms in aspyrone after no acetate derived oxygen was retained. The proposed biosynthetic route to aspyrone is shown in scheme 7, Ch 2.

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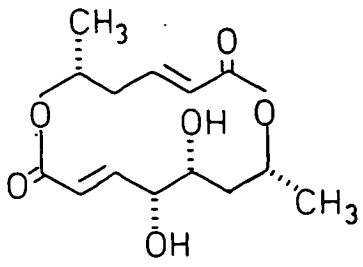
CHAPTER TWO

BIOSYNTHETIC STUDIES ON COLLETODIOL

A MACROCYCLIC DILACTONE METABOLITE

FROM *Cytospora Sp.* ATCC 20502.



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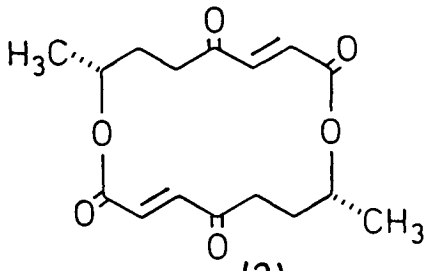

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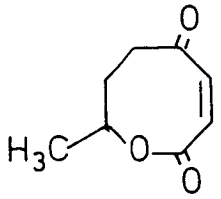

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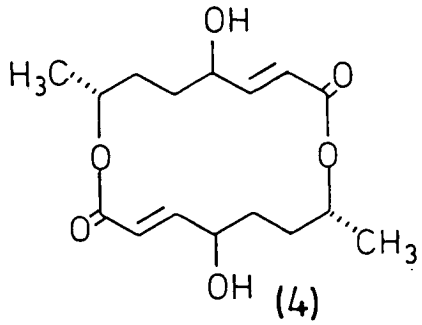

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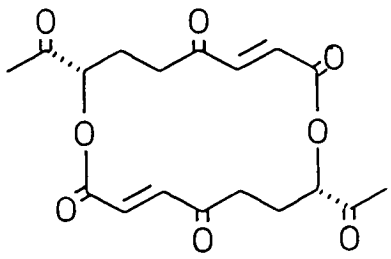

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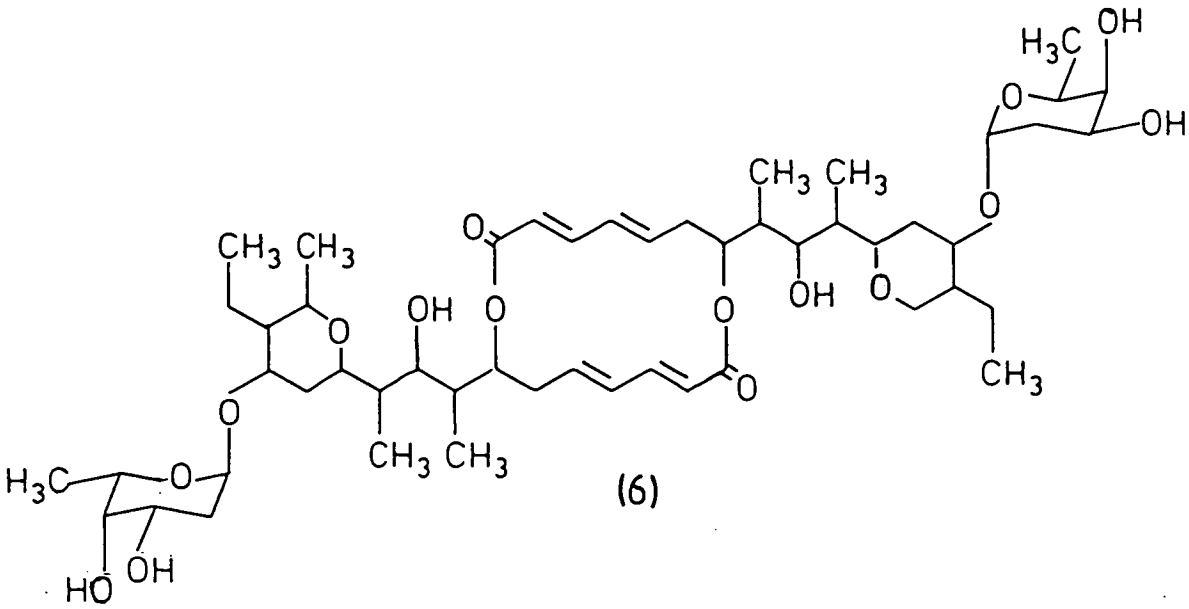
BIOSYNTHETIC STUDIES ON COLLETODIOL: A MACROCYCLIC DILACTONE METABOLITE FROM *CYTOSPORA Sp* ATCC 20502

2.1. Introduction

Colletodiol (1) is a macrocyclic dilactone first isolated by Grove *et al*[1] from the plant pathogen *Colletotrichum capsici*. More recently it has been isolated from *Cytospora Sp* ATCC 20502, a pathogenic fungus found in pine trees.

Colletodiol is a member of a family of macrocyclic dilactone compounds collectively known as the macrodiolides[2]. Many of these compounds show appreciable biological activity, although colletodiol itself is inactive[3]. The macrodiolides can be divided into two groups, sixteen member "symmetrical" dilactones and fourteen member "unsymmetrical" dilactones.

Of the sixteen member group pyrenophorin (2) from *Pyrenophora avenae* and *Stemphylium radicinum* can be considered the parent compound. It was first isolated in 1962[4], although the structure was incorrectly assigned as (3) until 1964[5]. The closely related pyrenophorol (4) is isolated from *Byssochlamys nivea*[6]. There are three other macrodiolides belonging to this group, vermiculin (5) from *Penicillium vermiculatum*[7], elaiophylin (6) from *Streptomyces violaceoniger*[8], and congloblatin (7) from *Streptomycees congloblatus* [9]. All of these compounds have a pyrenophorin type ring system.



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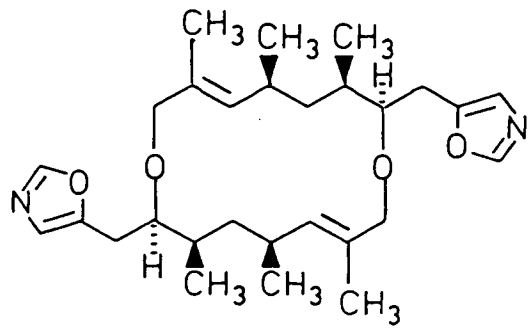

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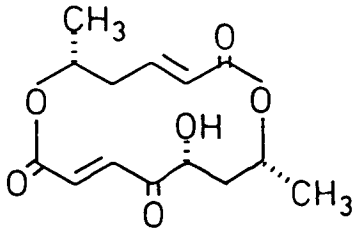

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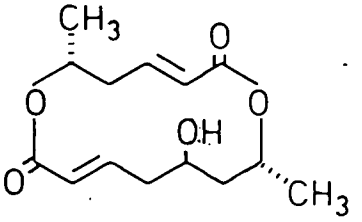

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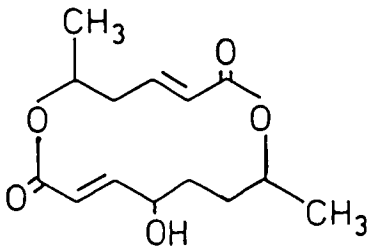

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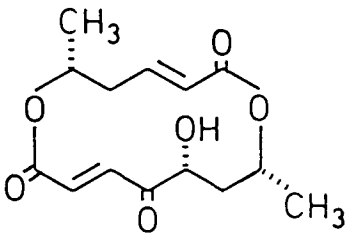

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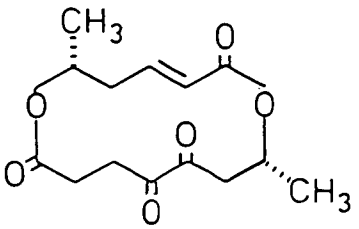

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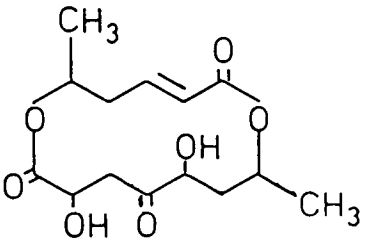

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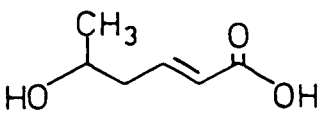
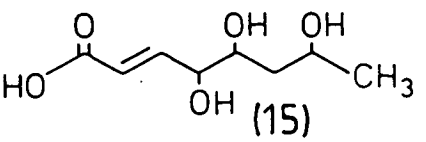
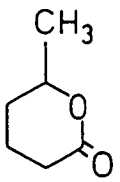


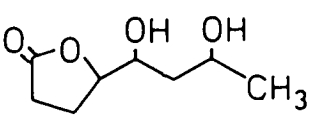
(14)



(15)



(16)



(17)

Colletodiol (1) can be considered to be the parent compound of the fourteen member unsymmetrical group. This is the larger of the two groups, and the structural relationships are much closer.

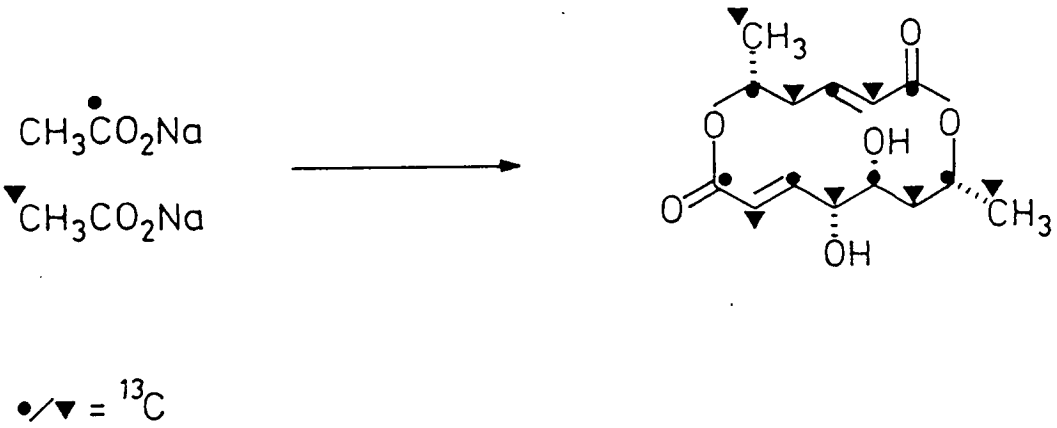
Colletodiol was first isolated from *Colletotrichum capsici*[1] and was also reported in *Chaetomium funicula*[10]. The structure of colletodiol was assigned mainly by use of n.m.r. studies[11]. Three co-metabolites of colletodiol, colletoketol (8), colletol (9), and colletalol (10) have also been isolated from *Colletotrichum capsici*[12].

The most recent additions to the fourteen member group are the grahamimycins[13]. Grahamimycin A (11), A_1 (12) and B (13) were all isolated from *Cytospora Sp ATCC 20502*. Grahamimycin A (11) was subsequently shown to be identical to colletoketol (8). However colletodiol was not reported from *Cytospora Sp ATCC 20502*

MacMillan and Simpson[12] examined the absolute stereochemistry of colletodiol. Mild base hydrolysis of colletodiol gave the two hydroxy acids (14) and (15). Hydrogenation and lactonisation gave (16) and (17). Optical rotation studies, and the application of the Hudson/Klyne lactone rules[14] showed colletodiol to have the configuration 7(*R*),5'(*R*),4(*R*),5(*S*) (18). However subsequent x-ray studies by Seebach[15] showed the configuration to be 7(*R*),5'(*R*),4(*R*),5(*R*) (1).

To date only macrodiolides from the fourteen member group have undergone biosynthetic examination. The first biosynthetic study was carried out in 1976[16], in one of the

Scheme 1



●/▼ = ^{13}C

earliest uses of singly labelled ^{13}C precursors. Sodium [1-^{13}C]- and [2-^{13}C]acetate was used to derive the origins of the carbon skeleton of colletodiol. This is summarised in scheme 1 and suggests that colletodiol is formed from the union of a triketide and a tetraketide fragment. The pnd ^{13}C n.m.r. spectrum was assigned by the methods of Birdsall *et al*[17]. This is an off resonance technique where selective irradiation of a ^{1}H signal causes collapse of the attached ^{13}C resonance to a singlet, but leaves all other carbons showing the off resonance ^{1}H coupling patterns. In practice the ^{1}H irradiation frequency is plotted against the ^{13}C chemical shift, where the two lines cross gives the corresponding ^{1}H and ^{13}C frequencies.

The work described here was initiated as the fourteen member macrodiolides were considered to be good model systems for the examination of polyketide chain assembly processes. Very little progress has been made in the study of the early intermediates of polyketide chain assembly by direct methods. By studying a system where the final product has a close resemblance to possible enzyme bound precursors, and using indirect methods of analysis, such as n.m.r., the following questions may be asked:-

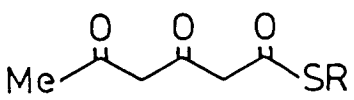
1.) Is the polyketide chain assembled in its entirety and then modified? OR

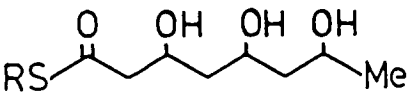
2.) Do the modifications occur during assembly? OR

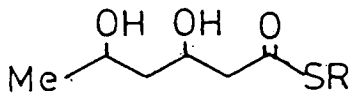
3.) Do they occur both during and after assembly?

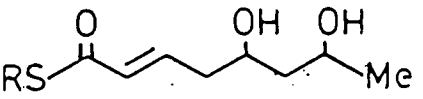
4.) What is the stereochemistry of the reduction and deoxygenation processes?

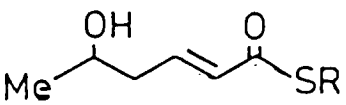
Scheme 2

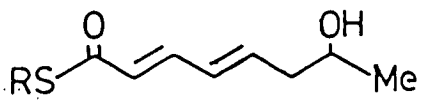

Me
O
O
O
SR

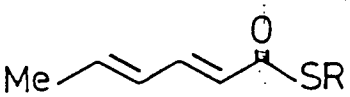

RS
O
OH
OH
OH
Me

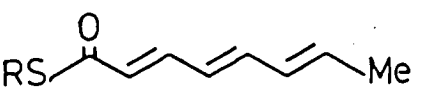

Me
OH
OH
O
SR

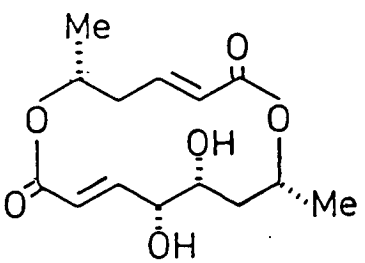

RS
O
OH
OH
Me


Me
OH
O
SR


RS
O
OH
Me


Me
O
SR


RS
O
Me

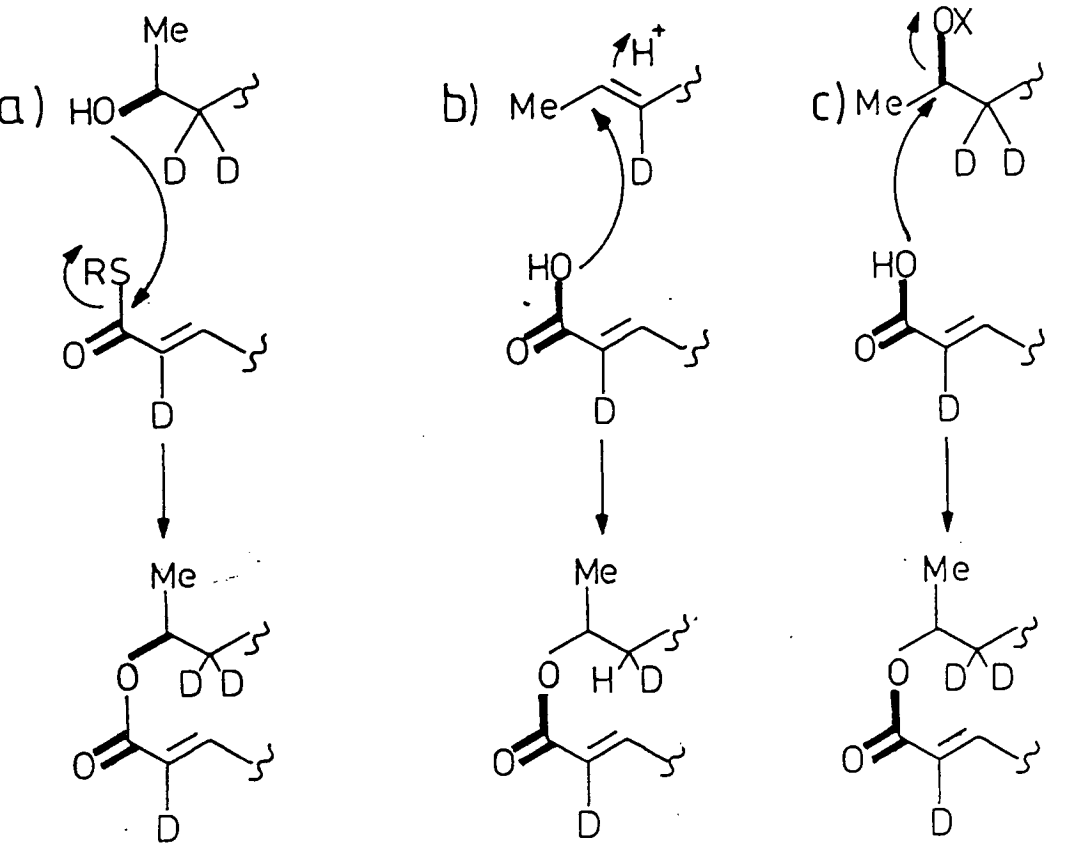

Me
O
O
O
OH
O
Me
OH

5.) What is the timing and mechanism of the introduction of extra oxygen.

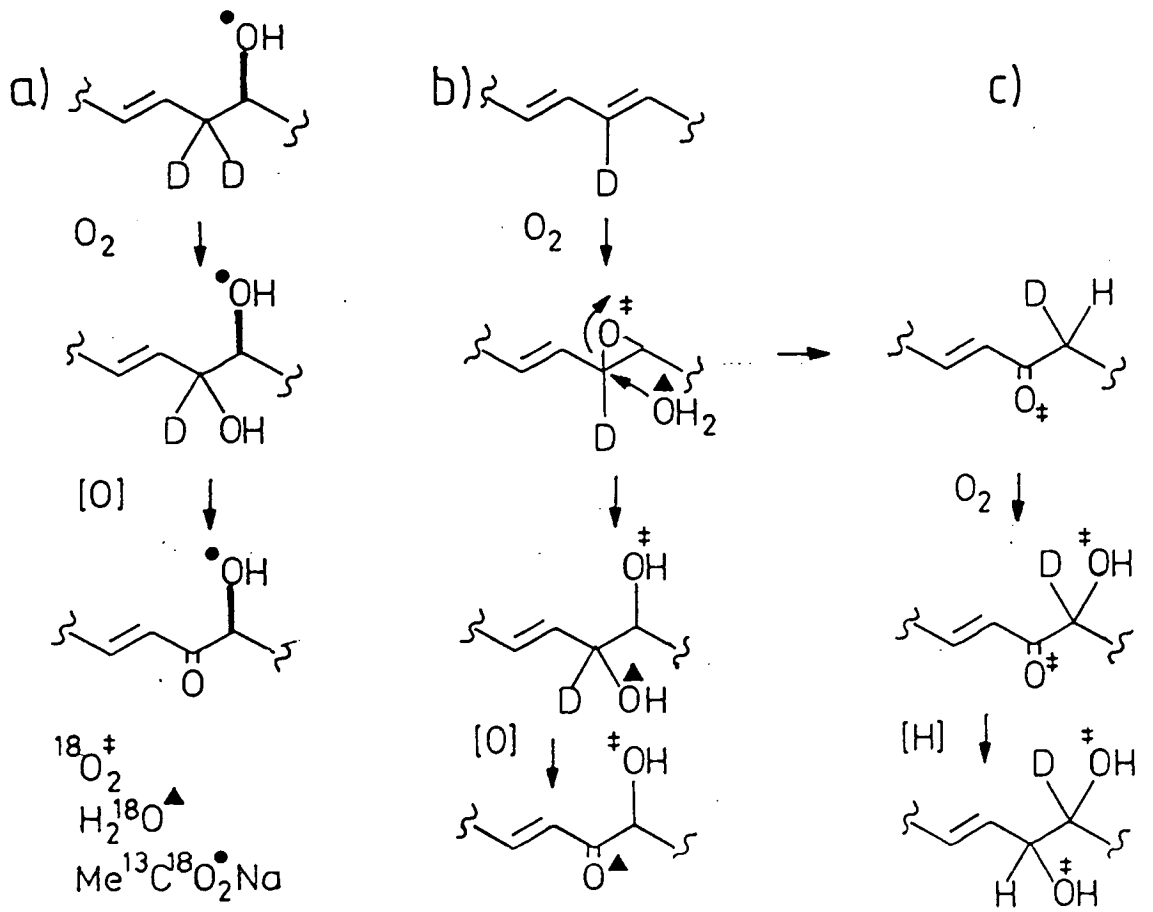
The system chosen for this study was the grahamimycins from *Cytospora Sp* ATCC 20502. A secondary aim of the study was to show that colletoketol (8) and grahamimycin A (11) were in fact identical. Colletoketol was initially isolated as a minor metabolite of *Colletotrichum capsici*. However later fermentations produced colletoketol as the major metabolite[16]. If in fact grahamimycin A and colletoketol were identical, then the opportunity existed to study fully the biosynthesis of colletoketol. The two metabolites were confirmed as being identical before the work commenced[19].

In our hands *Cytospora Sp* ATCC 20502 did not produce colletoketol in significant amounts. However it did produce colletodiol in high yield *ca.* 600 mg per litre. This was previously unreported .

As noted before colletodiol appears to be formed from a triketide and a tetraketide fragment[16]. Scheme 2 shows some of the possible C_6 and C_8 fragments that may be involved. In the case of the C_6 fragment the oxidation level could correspond to that of a classical poly-β-ketomethylene chain. Other possible intermediates represent various reduced forms, the reduction of carbonyl groups to alcohol functions poses the question of the stereochemical course of such modifications. Similarly elimination of water to form double bonds could occur with different possibilities for the stereochemistry of the


a)
Me
HO
D D
RS
O
D
Me
O
D D
O
D
b)
Me
H+
D
HO
O
D
Me
O
H D
O
D
c)
Me
OX
D D
HO
O
D
Me
O
D D
O
D

Scheme 4

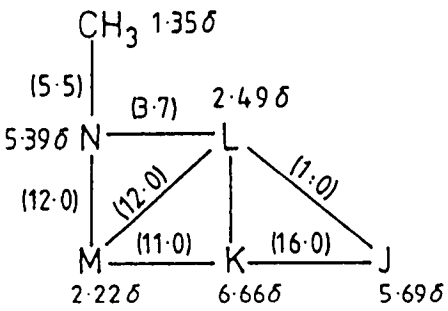

a)
OH
D D
O2
OH
D OH
[O]
OH
O
18O2‡
H2 18O▲
Me 13C 18O2• Na
b)
D
O2
O‡
OH2
D
OH
D OH
[O]
‡OH
O▲
c)
D H
O‡
O2
D ‡OH
O‡
[H]
D ‡OH
H ‡OH

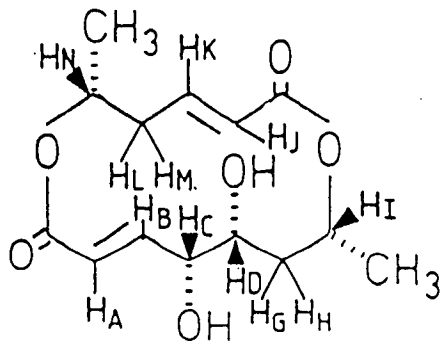
elimination. A similar set of intermediates is possible for the C_6 fragment.

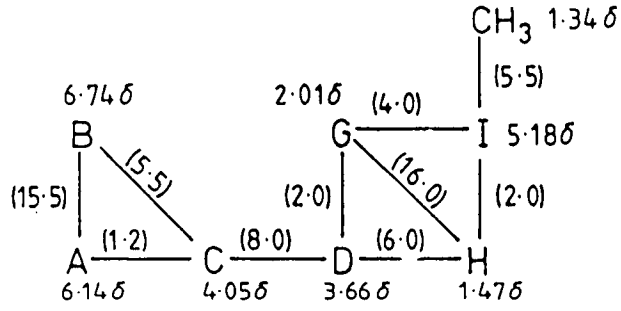
The exact structure of the intermediates involved will dictate the actual mechanism of the formatiom of the lactone functions. Scheme 3 shows some of the possibilities for this. Mechanism a) shows the attack of a hydroxyl group on a carbonyl function, activated as its thioester, with retention of configuration; b) involves a Michael-type reaction of a free acid on a carbon carbon double bond, with subsequent protonation from the medium, c) shows nucleophilic substitution by an acid on a carbon activated via the hydroxyl group, with net inversion of stereochemistry.

A similar set of proposals are shown in scheme 4 for the formation of the 4,5-diol system of colletodiol and the corresponding α ketol system of colletoketol. Mechanism a) shows the introduction of oxygen from the atmosphere *via* hydrxoylation to give colletodiol, with possible oxidation to give colletoketol; b) again involves the introduction of oxygen from the atmosphere *via* epoxidation of a double bond, hydrolysis gives colletodiol and oxidation gives colletoketol; c) shows a possible rearrangement of the epoxide formed in b), followed by a hydroxylation step to form colletoketol, subsequent reduction gives colletodiol. Note that in a) and b), colletodiol is the precursor to colletoketol, whereas in c) colletoketol is the precursor to colletodiol. In both schemes 3 and 4 each mechanism necessitates different origins and stereochemistry for the oxygen and hydrogen atoms involved. From this information it should be

Figure 1 300MHz n.m.r. of corticosteroid







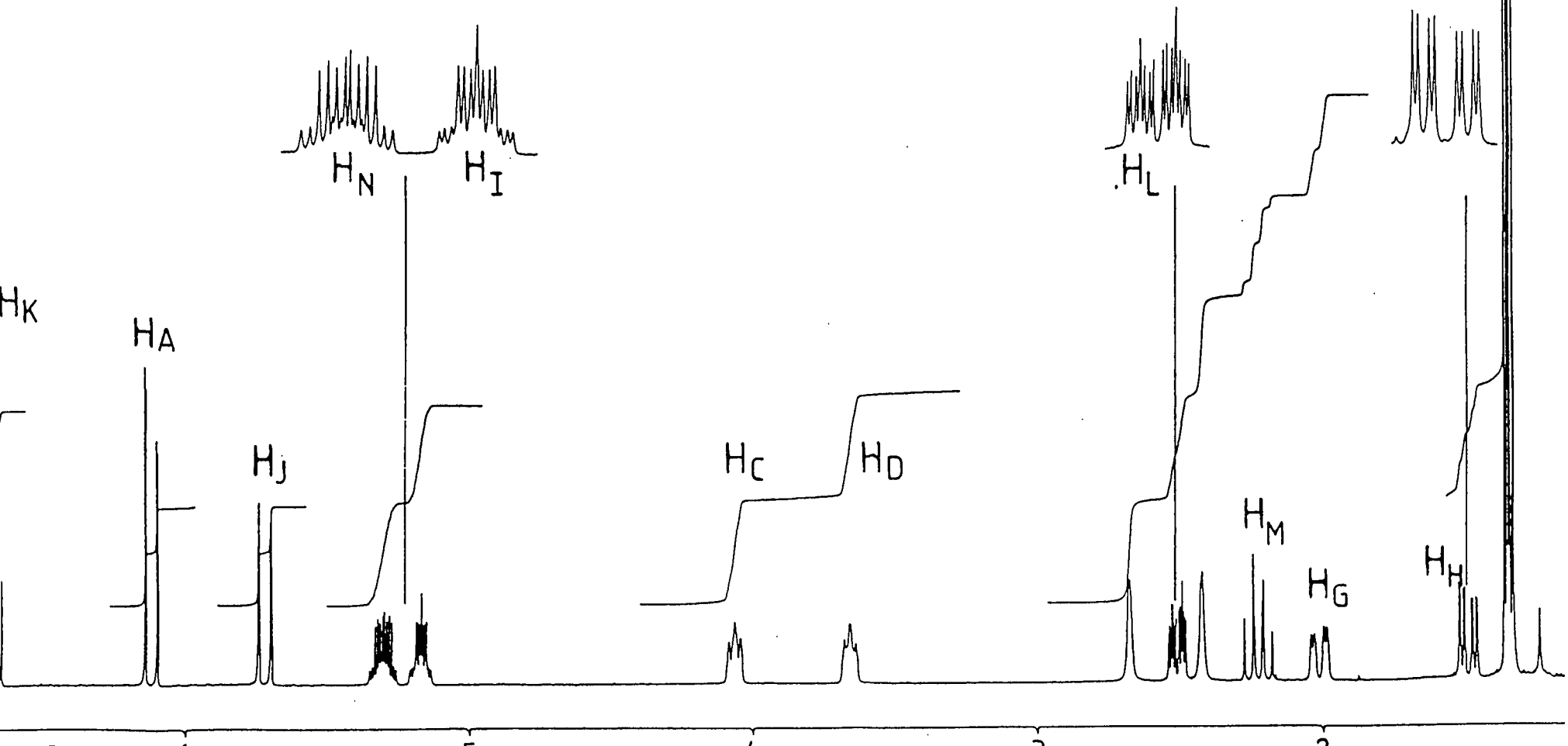
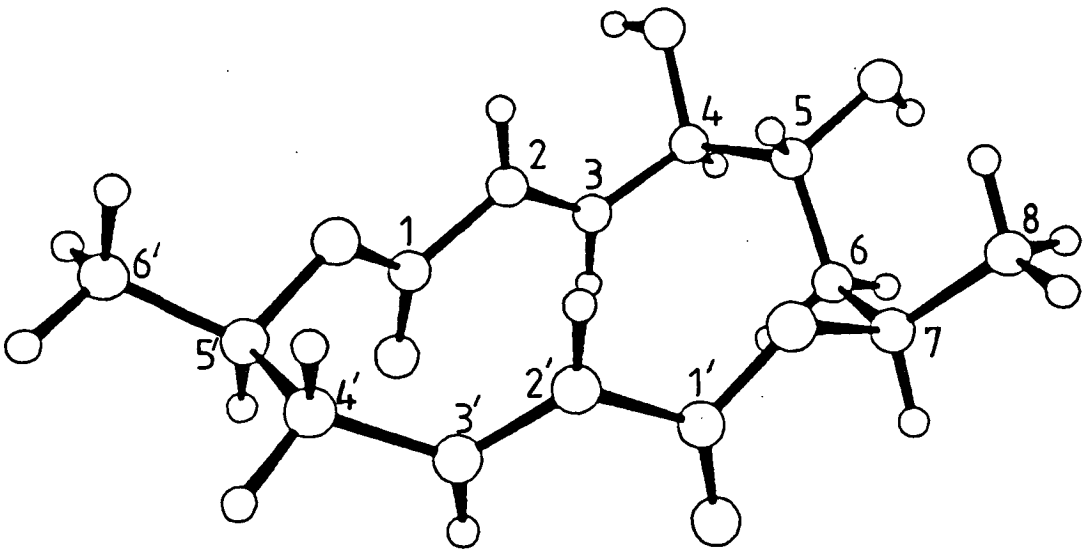


Figure 2



possible to establish the true nature of the enzyme bound intermediates, and the overall biosynthesis of colletodiol.

2.2 SPECTRAL ASSIGNMENT STUDIES.

In any biosynthetic study which concerns the observation of stable isotopes or their effects by n.m.r., it is essential that the assignment of the natural abundance spectrum is completely unambiguous. This is particularly true in the case of ^{13}C n.m.r. spectra or ^{1}H spectra if ^{2}H labelling is being used

2.2.1. ^{1}H n.m.r. Study.

A high field ^{1}H n.m.r. study was carried out on colletodiol to fully assign the ^{1}H spectrum which is shown in figure 1. The results of a series of selective homodecoupling experiments agreed with the published results[11], and gave the coupling pattern shown in figure 1.

The conformation of colletodiol in the solid state has been reported[15,20], and is shown in figure 2. This conformation was used as a model to interpret the observed coupling patterns, and the results of a series of difference n.O.e. experiments carried out to try to determine the conformation of colletodiol in solution.

The earlier n.m.r. studies[11] did not assign a specific solution conformation to colletodiol, and each of the diastereotopic hydrogens on carbons 4' and 6 remained to be

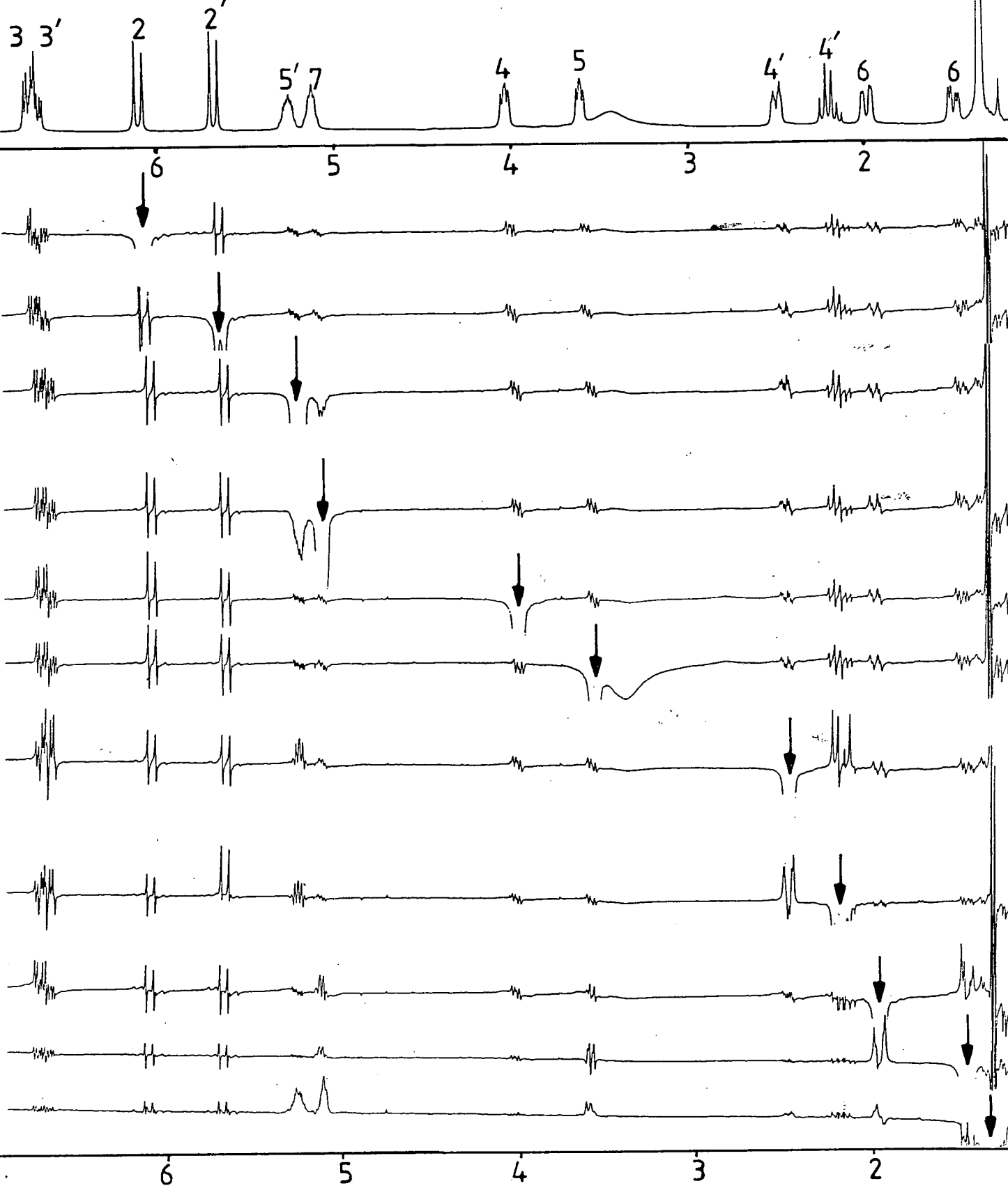


Figure 3 Difference n.O.e. studies on Colletodiol

rigorously assigned The complete coupling pattern of colletodiol is shown in figure 1. Careful examination of the H-4' multiplets at 2.22 and 2.49 ppm. reveals a 12.0 Hz coupling from H-5' to the high frequency multiplet. This is consistent with two protons being in a *trans* antiperiplanar relationship, whereas the lower frequency multiplet shows a 3.7 Hz coupling to H-5', characteristic of a *gauche* type relationship

The situation with the C-6 methylene group is more complex. Examination of the H-6 multiplet at 1.47 ppm. reveals a 6.0 Hz coupling to H-5, and a 2.0 Hz coupling to H-7, whereas the H-6 signal at 2.01 ppm. shows couplings of 4.0 Hz and 2.0 Hz to H-7 and H-5 respectively. With such small differences in coupling constants it is difficult to assign these signals on this evidence alone.

In order to support the coupling evidence summarised in figure 1, difference n.O.e. studies were carried out. These are shown in figure 3 and in the first instance the solid state conformation shown in figure 2 was used to interpret the results. Irradiation of the H-4' signal at 2.22 ppm. results in a 5% enhancement of the H-2' signal, whereas irradiation of the other H-4' signal at 2.49 ppm. results in a 5% enhancement of the H-3' signal. Examination of the solid state conformation shown in figure 4, confirms the proximity of *pro*-4'-(*R*) proton and H-2', and the *pro*-4'-(*S*) proton to H-3'. This conformation also confirms the *trans* antiperiplanar relationship of the *pro*-4'-(*R*) proton and H-5'. Therefore both the coupling and n.O.e. results are consistent with a solution conformation that is essentially

Figure 4

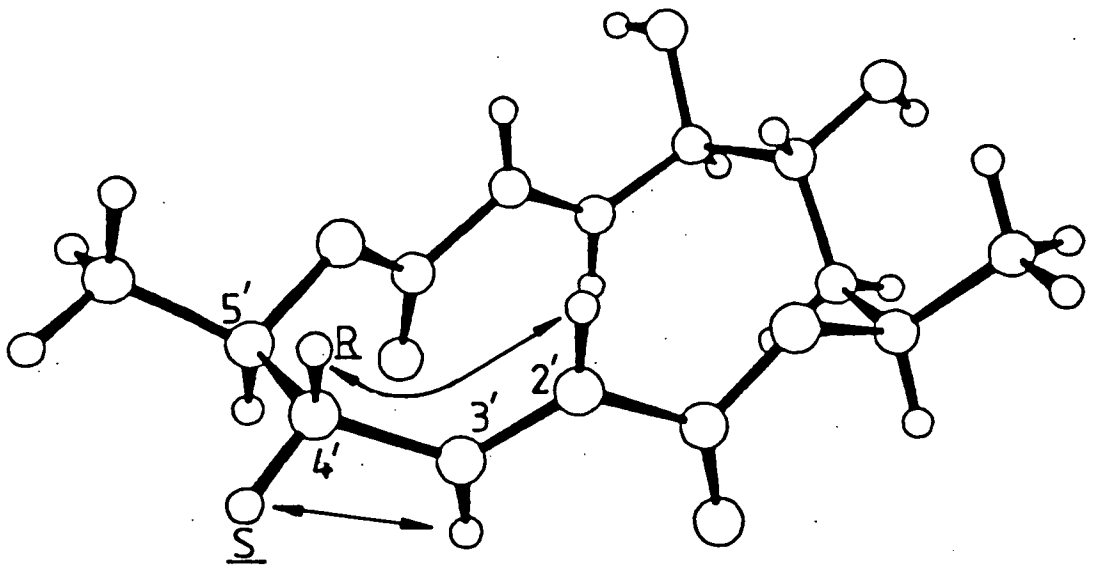
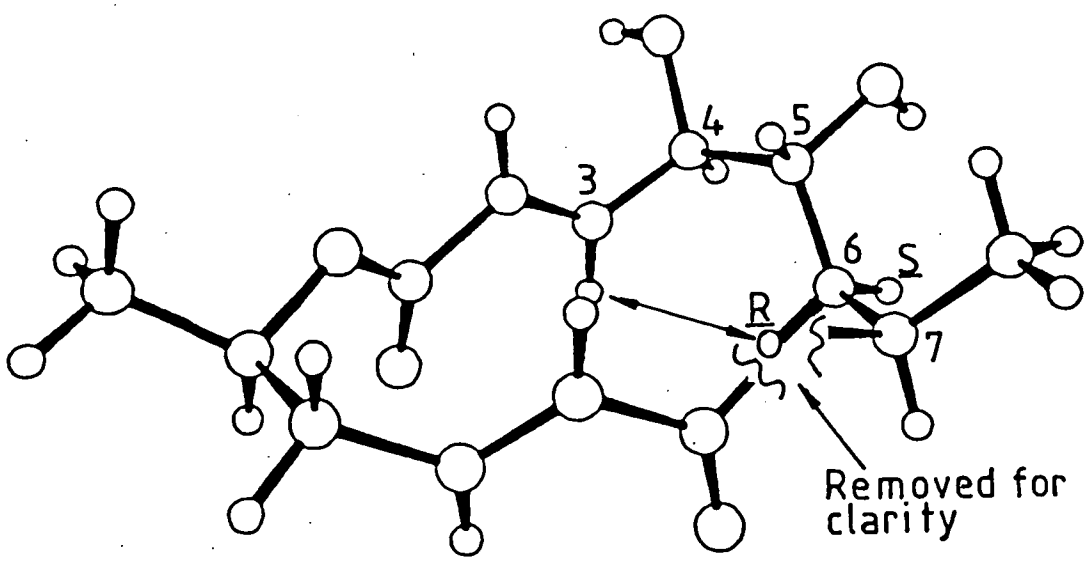


Figure 5



the same as the solid state conformation. The *pro*-4'-(*R*) hydrogen can therefore be assigned to 2.22 ppm., and the *pro*-4'-(*S*) hydrogen can be assigned to 2.49 ppm. Irradiation of the H-6 multiplet at 1.47 ppm. results in a 2% enhancement of the H-7 signal, and a 1% enhancement of the H-5 signal, indicating that these protons are close in space. Irradiation of the H-6 multiplet at 2.01 ppm. results in a 1% enhancement of the H-7 signal and a 2% enhancement of the H-3 signal. Again these results are consistent with the solution conformation being essentially identical to the crystal state. The interaction of H-3 and the *pro*-6-(*R*) proton can be seen in figure 5. Clearly the *pro*-6-(*S*) proton cannot interact with H-3, and so the multiplet at 2.01 ppm. is assigned to the *pro*-6-(*R*) proton, and the multiplet at 1.47 ppm. can therefore be assigned to the *pro*-6-(*S*) proton. The C-6 methylene group would therefore seem to adopt a *gauche* conformation with both H-7 and H-5. Again this is consistent with the solid state conformation shown in figure 2.

All of the observed n.O.e.'s are shown in table 1. In particular note the 2% enhancement in the C-3 proton signal when the *pro*-6-(*R*) signal at 2.01 ppm. is irradiated. Hence these two protons are close enough in space to interact. Similarly there is an interaction between the C-8 methyl protons and the C-5 methine proton of 3%. These two results are again consistent with the solution state conformation being the same as the solid state conformation shown in figures 2-5. Finally there is an interaction between the C-4 methine proton and the C-2 olefinic proton and this also supports the conformation described above.

Table 1 Observed n.O.e's

Proton Irradiated	Interactions	n.O.e.
2'(5·69δ)	4' (2·22δ)	1%
4 (4·05δ)	3 (6·74δ)	4%
	2 (6·14δ)	2%
7 (5·18δ)	8 (1·34δ)	6%
	6 (1·47δ)	2%
5'(5·3δ)	6' (1·36δ)	3%
6 (1·47δ)	7 (5·18δ)	2%
	5 (3·6δ)	1%
	6 (1·47δ)	9%
6 (2·01δ)	6 (1·47δ)	6%
	7 (5·18δ)	1%
	3 (6·74δ)	2%
4' (2·22δ)	4' (2·49δ)	7%
	5' (5·3δ)	1%
	2' (5·69δ)	5%
4' (2·49δ)	4' (2·22δ)	9%
	5' (5·3δ)	5%
	3' (5·66δ)	5%
6'+8 (1·35δ)	4' (2·22δ)	3%
	5 (3·66δ)	3%
	7 (5·18δ)	7%
	5' (5·13δ)	7%

Figure 6 90 MHz ^{13}C-NMR of corticolin

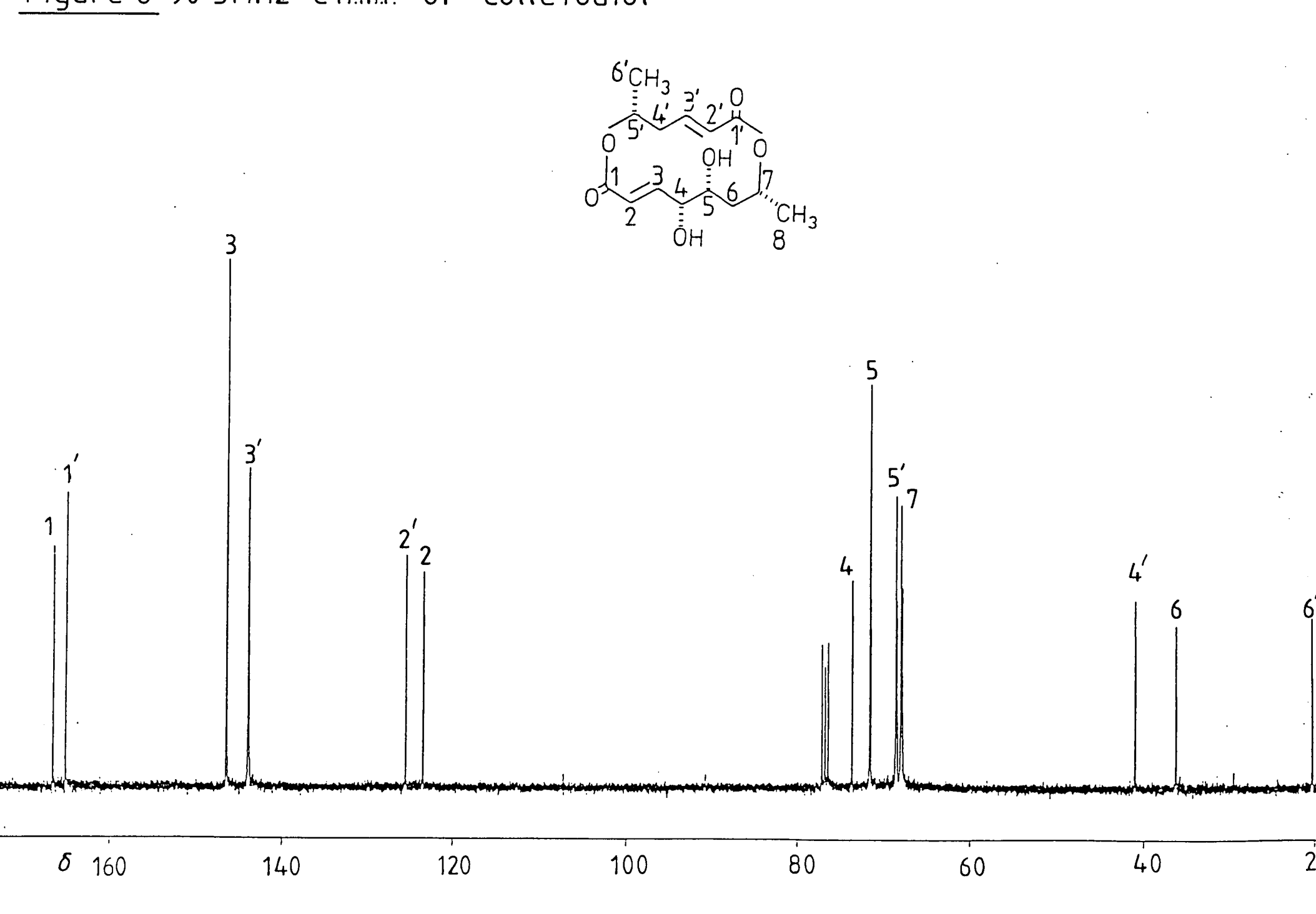
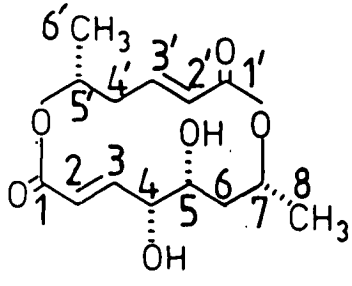
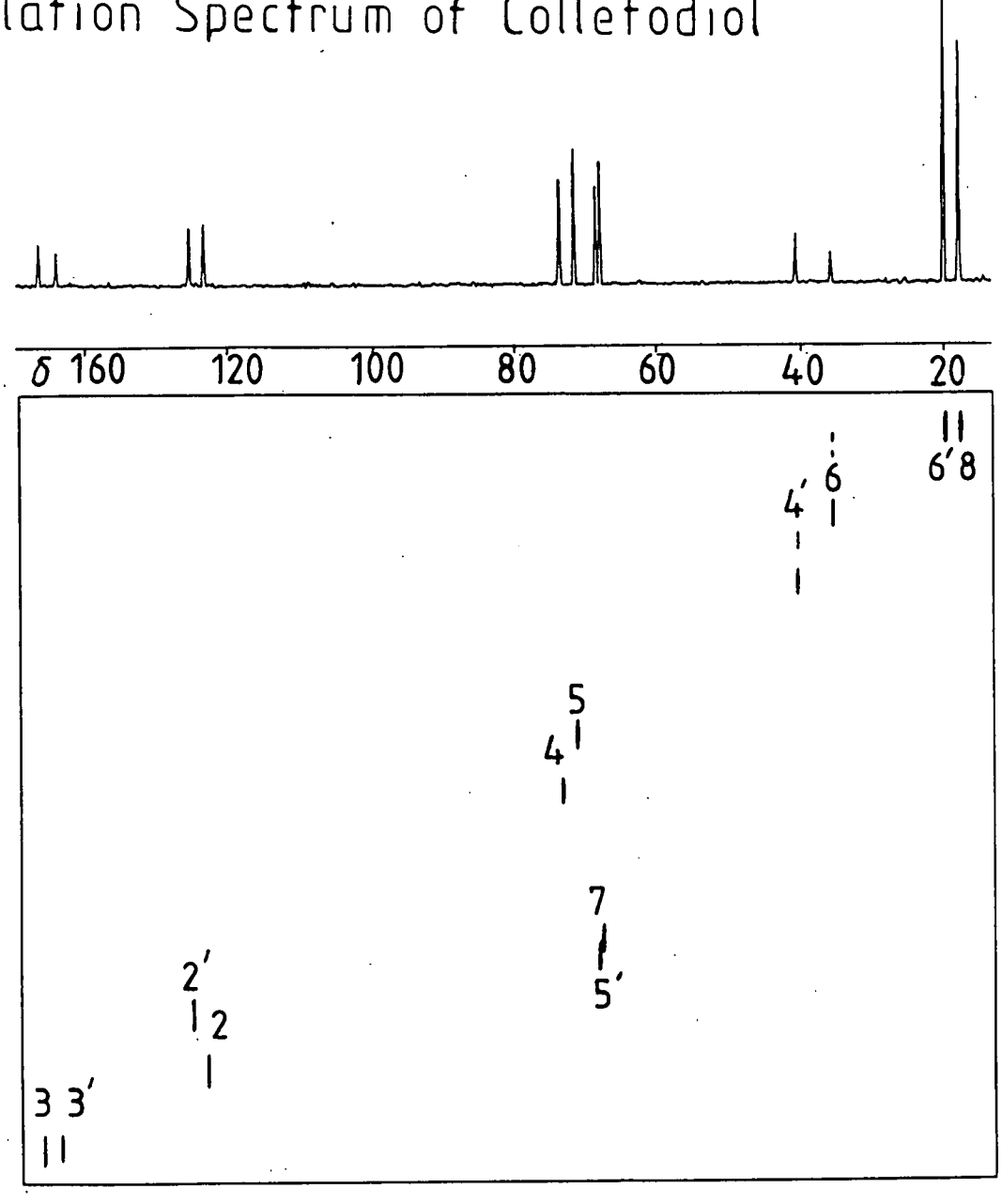


Figure 7 ^1H-^{13}C Correlation Spectrum of Colletodiol





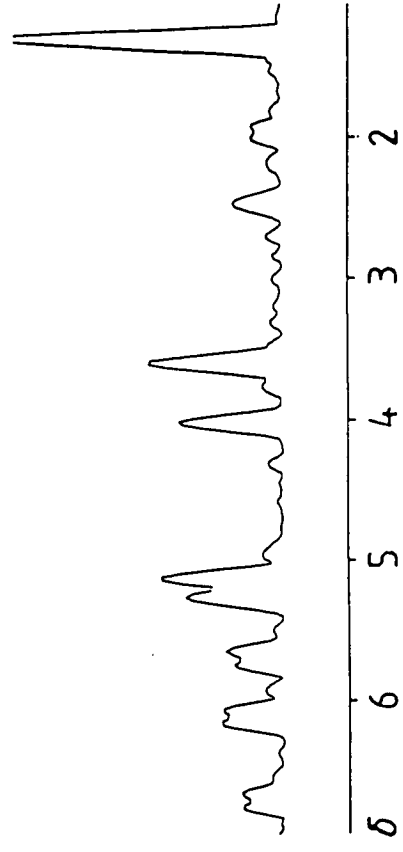


Table 2 Proton Carbon Correlations

^{1}H Signal/ppm	^{13}C Correlation	Assignment
2 (6·14)	123·17	C-2
2' (5·69)	125·63	C-2'
3 (6·74)	146·19/143·91	C-3/C-3'
3' (5·66)	143·91/146·19	C-3'/C-3
4 (4·05)	73·87	C-4
4' (2·22, 2·49)	40·99	C-4'
5 (3·66)	71·74	C-5
5' (5·13)	68·64	C-5'
6 (1·47, 2·01)	36·32	C-6
6' (1·3)	20·29/18·04	C-6'/C-8
7 (5·18)	67·92	C-7
8 (1·3)	18·04/20·29	C-8/C-6'

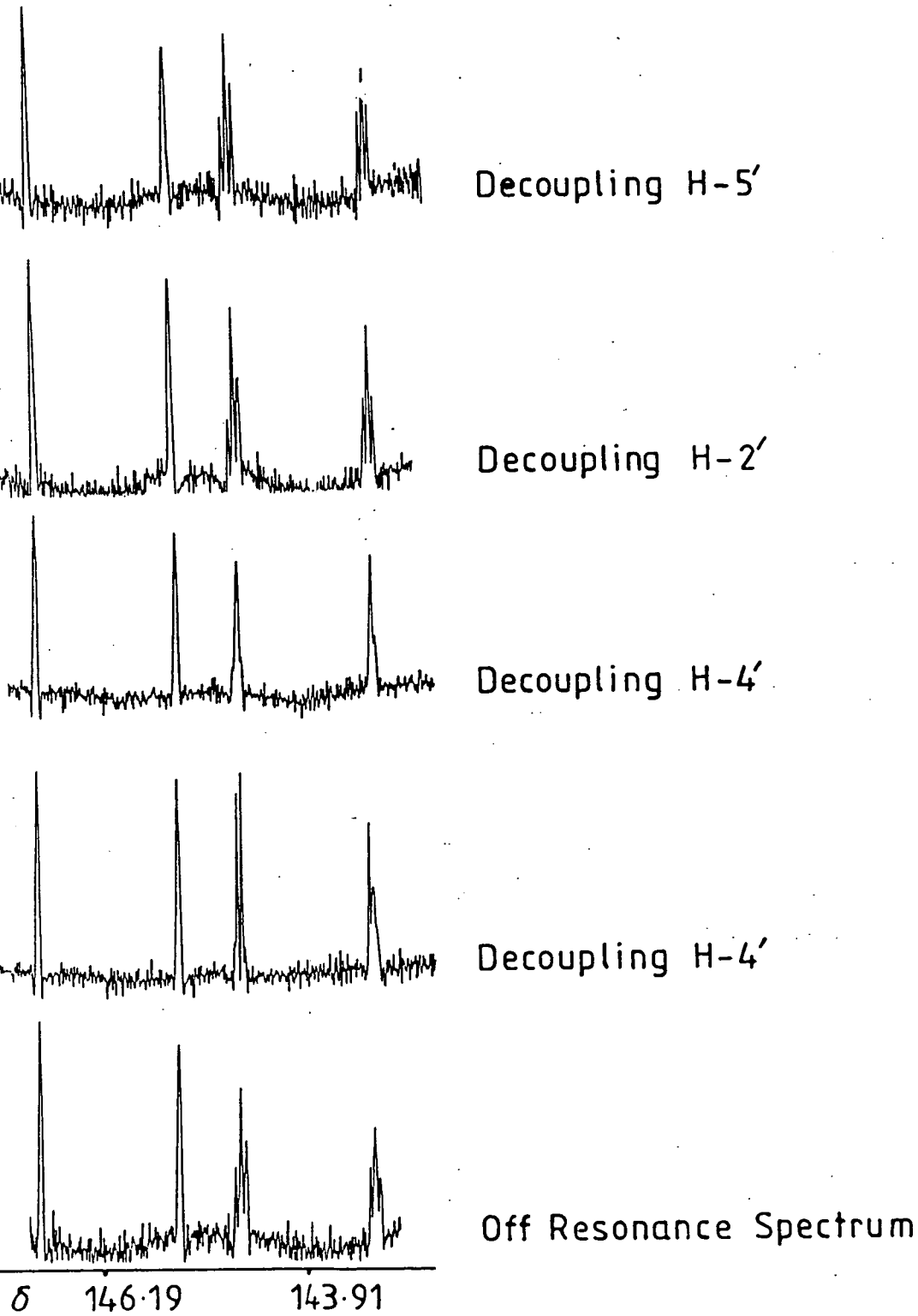
2.2.2 Assignment of the ^{13}C NMR Spectrum of Colletodiol

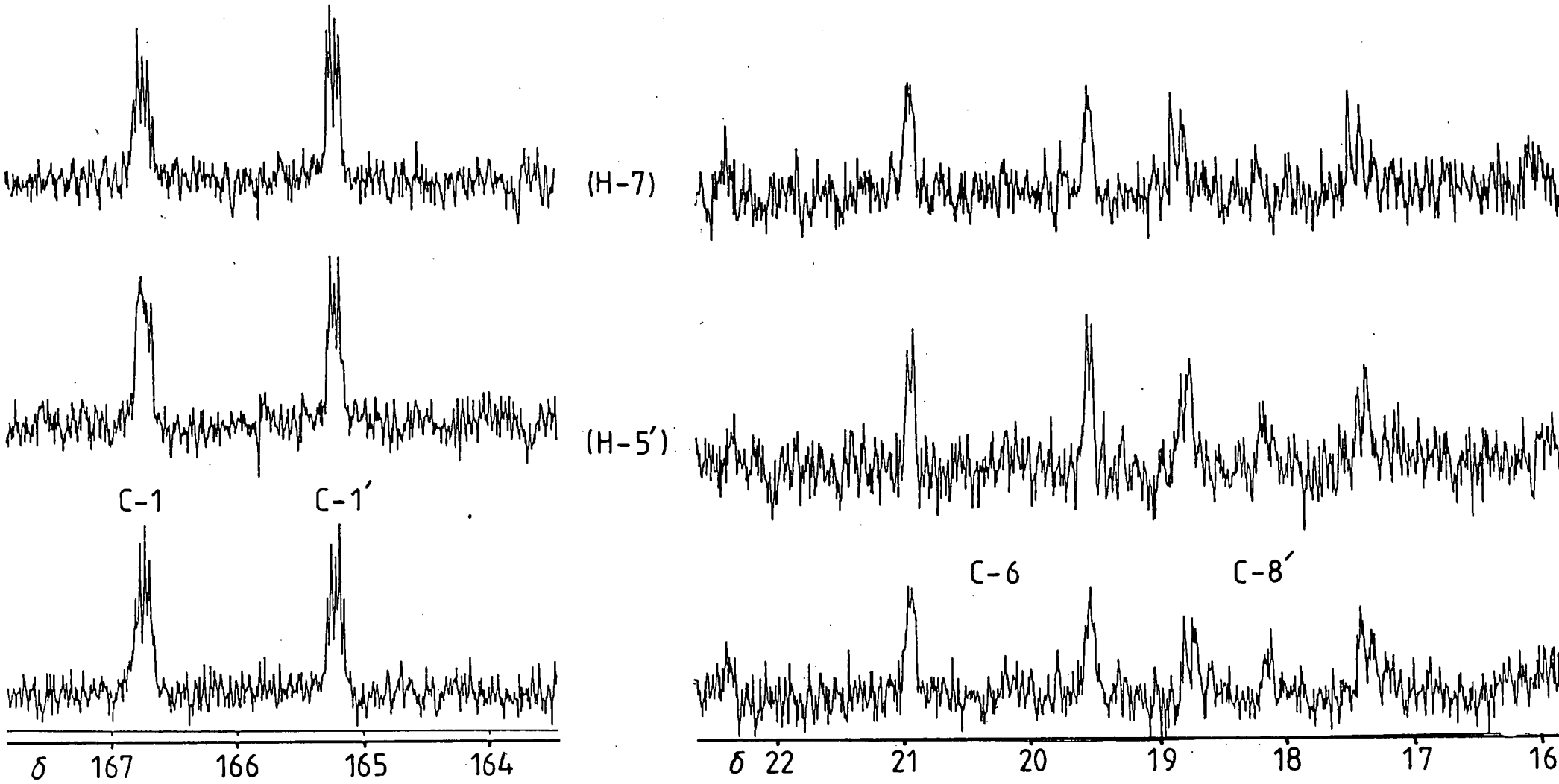
The information gained from the assignment of the ^{1}H n.m.r. spectrum could now be used in the assignment of the ^{13}C n.m.r. spectrum, shown in figure 6. This was achieved first by carrying out a ^{1}H-^{13}C correlation experiment to assign some of the corresponding proton bearing carbon signals. The spectrum is shown in figure 7 and table 2 details the correlations found.

Using this method the ^{1}H signals at 6.14, 4.05,3.66, 2.01 and 1.47, 5.18, 5.69, 2.22 and 2.49, and 5.30 ppm. correlated with the ^{13}C signals at 123.77, 73.87, 71.24, 36.32, 67.92, 125.63, 40.99, and 68.64 ppm. Thus allowing these signals to be assigned to C-2, C-4, C-5, C-6, C-7, C-2', C-4', and C-5' respectively. However due to the low digital resolution the assignment of the olefinic carbons C-3 and C-3' and the methyl carbons C-8 and C-6' could not be considered definitive. The carbonyl resonances also remained to be assigned.

In order to assign all of the remaining carbon resonances, fully ^{1}H coupled ^{13}C spectra were examined. By doing this, the long range proton carbon coupling constants could be seen and selective decoupling of ^{1}H signals allowed the remaining carbon signals to be identified. Figure 8 shows the fully ^{1}H coupled ^{13}C n.m.r. and the effect of selective decoupling of both H-4' resonances in turn. The signal at 143.91 ppm. collapses from a doublet of triplets to a doublet of doublets. Selective decoupling of H-5' or H-2' also causes a significant sharpening

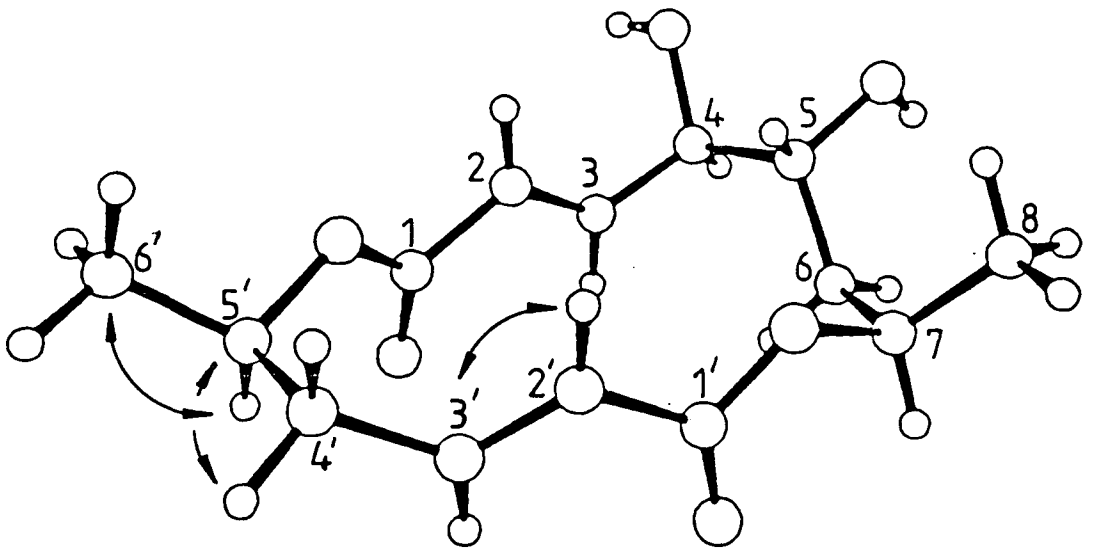
Figure 8 Assignment of the C signals for C-3 and 3'





(Figures in parenthesis indicates proton being irradiated)

Figure 10 Long range proton-carbon couplings observed in Colletodiol



Carbon	δC
C-1	166·3
C-1'	164·9
C-2	123·6
C-2'	125·6
C-3	146·4
C-3'	143·9
C-4	73·7
C-4'	40·9
C-5	71·7
C-5'	68·6
C-6	36·2
C-6'	20·2
C-7	67·9
C-8	17·9

of the triplet structure at 143.91 ppm. On the basis of these two experiments C-3' was assigned to 143.91 ppm and therefore C-3 was assigned to 146.19 ppm.

A similar analysis of the long range ^{1}H-^{13}C couplings was used to assign the carbonyl resonances at 165.00 ppm. and 166.42 ppm. The fully ^{1}H coupled ^{13}C n.m.r. spectrum is shown in figure 9. Selective decoupling of H-7 causes the collapse of the signal at 165.00 ppm. from a multiplet to a doublet of doublets. Similar decoupling of H-5' results in the collapse of the multiplet at 166.42 ppm. to a doublet of doublets. These two results represent the removal of a three bond coupling through oxygen to C-1' and C-1 respectively. The remaining couplings being the two bond and three bond couplings to the respective olefinic hydrogens. Therefore C-1 was assigned to 166.42 ppm. and C-1' to 165.00 ppm.

The selective decoupling of H-7 also had an effect on the methyl signal at 18.04 ppm. This is shown in figure 9, removal of the H-7 coupling causing the loss of the doublet structure of the signal at 18.04 ppm. Therefore the C-8 methyl group was assigned to 18.04 ppm. and the C-6' methyl group was assigned to 20.29 ppm.

Figure 10 shows the other long range couplings that were observed. Irradiation of H-5' removed a two bond coupling to C-6'. Coupling was observed between C-3' and H-2', both H-4' protons and H-5'. The full assignment of the ^{13}C n.m.r. spectrum derived from these results is also shown in figure 10.

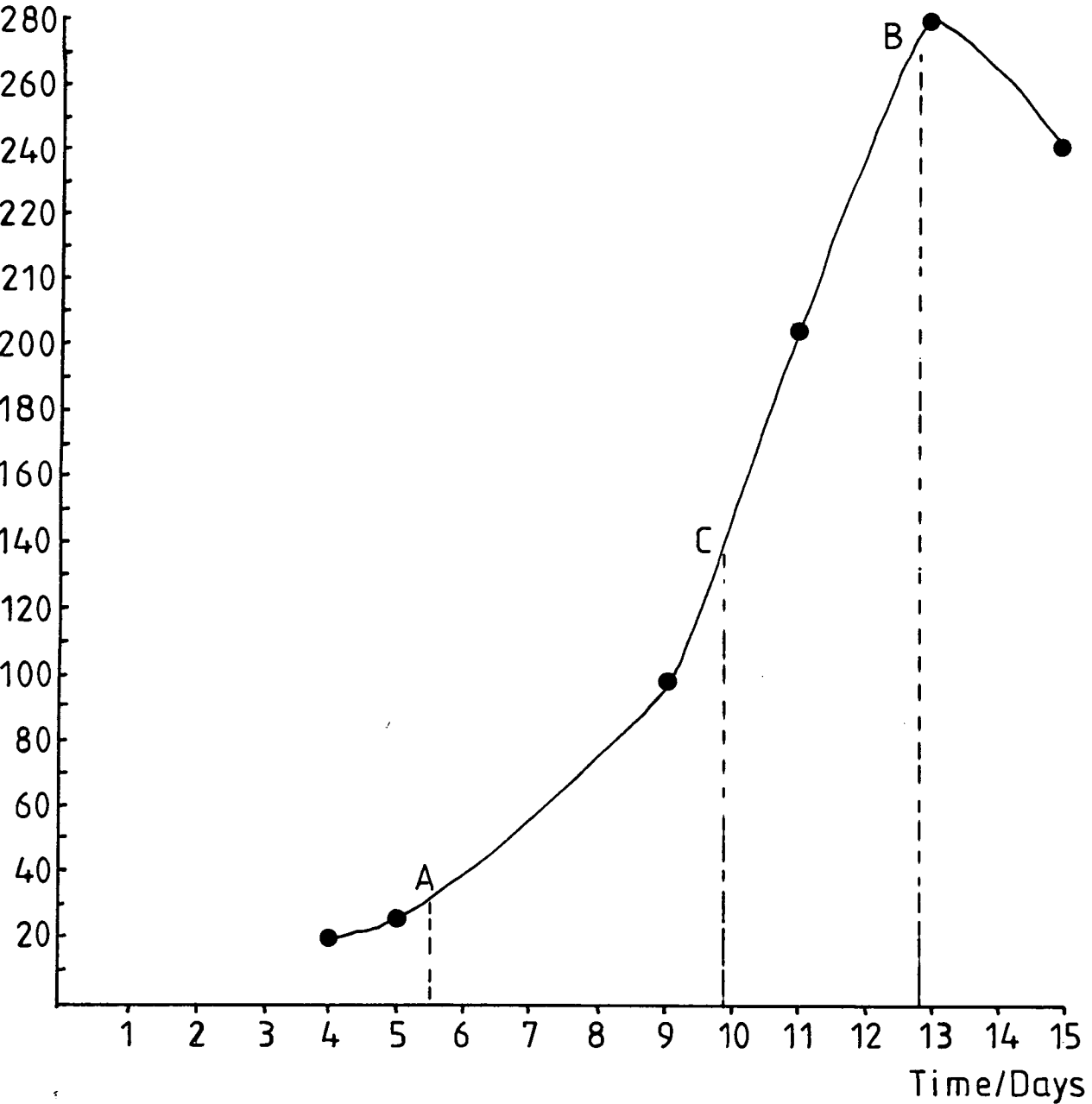


Figure 11 Growth/Production curve for Monocerin from D. ravenelii

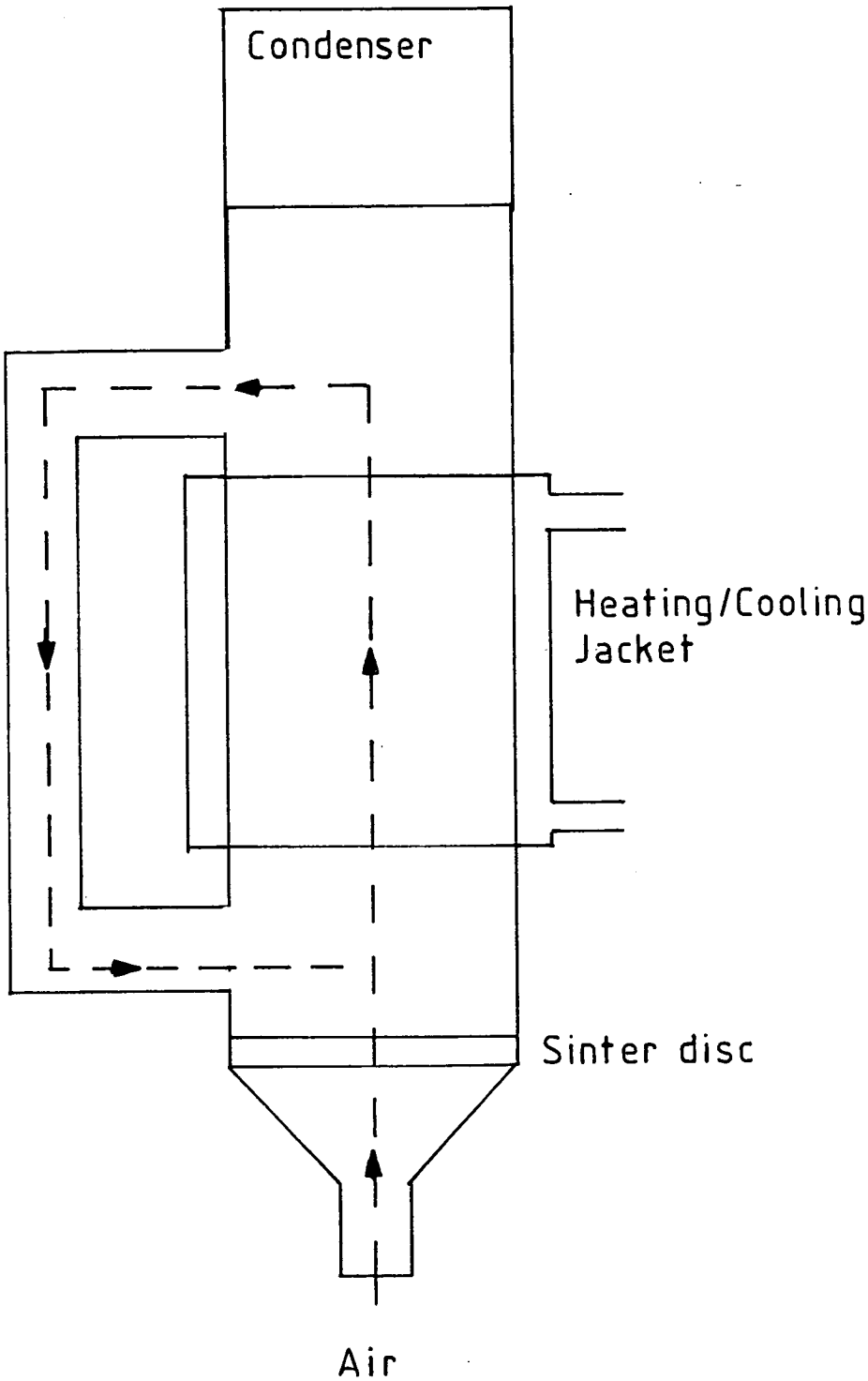
2.3 RESULTS AND DISCUSSION.

2.3.1 Preliminary Fermentation Studies.

In this study isotopically labelled precursors, notably sodium acetate precursors were to be administered to *Cytospora Sp.* The labels to be used were all stable isotopes observable directly or indirectly by n.m.r. namely ^{13}C, ^{2}H, and ^{18}O. To achieve any measure of success with these methods it was neccessary to carry out some preliminary fermentation studies on *Cytospora Sp.*

To achieve the successful incorporation of a precursor into a given metabolite, the pattern of production of that metabolite by the parent organism must be examined closely. Such growth production studies allow an efficient feeding protocol to be established. The result of such a study on the production of monocerin[21] (11) from *Drechslera ravenelli* is shown in figure 11. This type of curve is commonly found in the production of secondary metabolites, there is a lag phase, production phase and a limit to the maximum concentration of the metabolite, usually controlled by the growth conditions. If the requisite precursor is administered at time A, and the metabolite isolated at time B, the amount of metabolite produced may work against the experiment by causing unacceptable isotopic dilution. The ideal situation is to isolate the metabolite at time C. Thus a compromise between the yield of metabolite and the isotopic dilution may be neccessary. However if the labelling has been successful then the

Figure 12 Airlift Fermentor



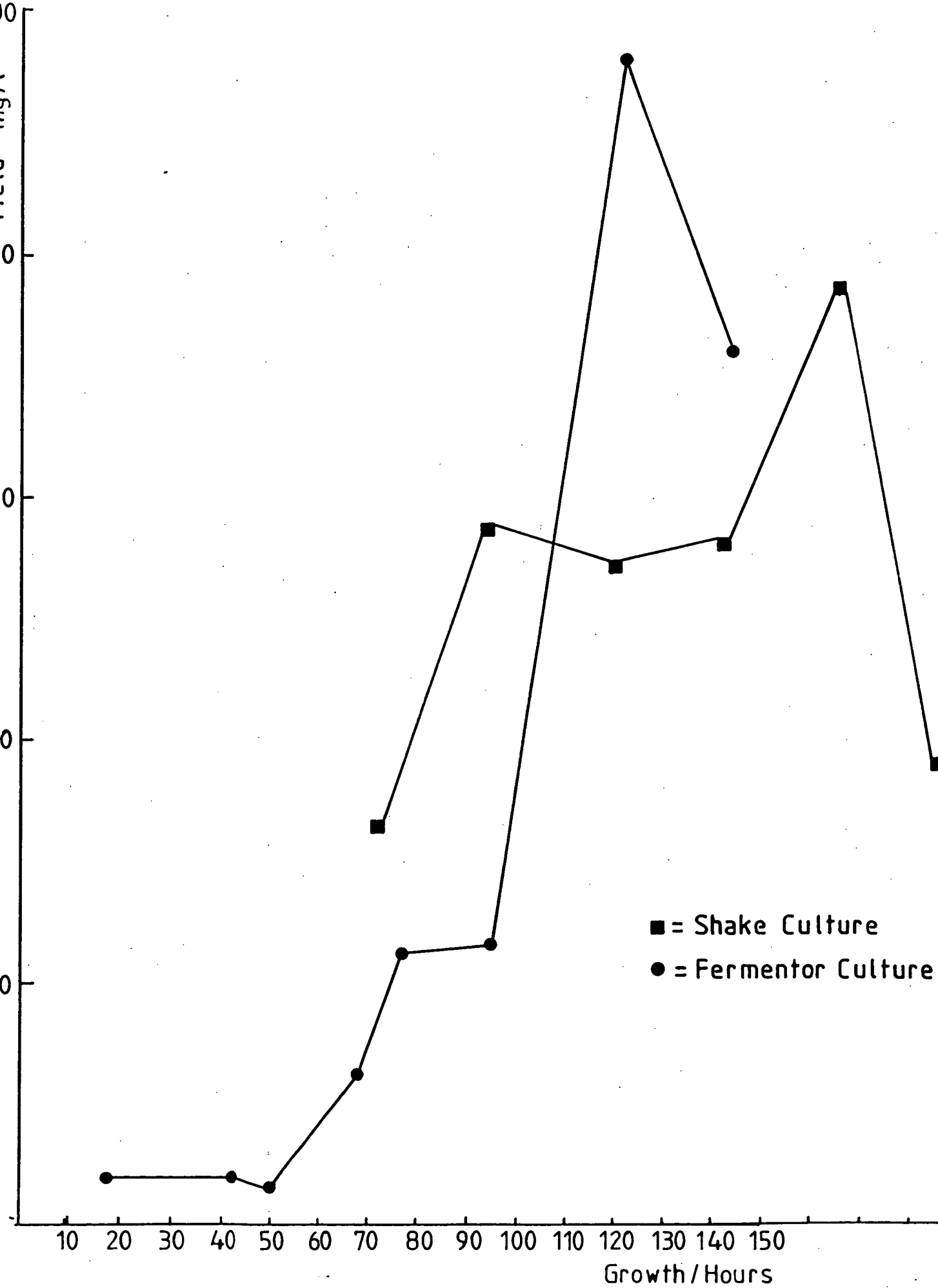
absolute amount of metabolite recovered becomes less important as the labels used have very low natural abundances and so any increase in this level by incorporation of labelled precursor is immediately observable. The exception to this is the case of ^{13}C labels, here the natural abundance figure is 1.1% and so isotopic dilutions of less than 100 are required.

In the present study *Cytospora Sp* was grown under two sets of conditions. The first method was to use shake flask culture in 500 ml conical flasks each containing 100 ml of medium. These were grown on an orbital shaker. The second method was to use an airlift fermentor of the type shown in figure 12, originally designed for fermentations using $^{18}O_2$ gas.

Under both these sets of conditions *Cytospora Sp* did not produce colletoketol (8) in significant amounts as had been reported[13], but rather it unexpectedly produced large amounts of colletodiol (1). Therefore the study centred around the biosynthesis of colletodiol.

The growth/production curves for colletodiol isolated from *Cytospora Sp* in both shake and airlift culture are shown in figure 13. These are clearly different from the typical situation shown in figure 11. In the case of colletodiol a peak in production is achieved after a lag phase, but then the colletodiol is rapidly metabolised. This phenomena is particularly striking in the case of the airlift fermentor culture where the period of production of colletodiol only lasts about thirty hours.

Figure 13 Growth/Production of Colletodiol



Based on the results of these studies it was decided to administer aqueous solutions of acetate precursors by injection into the medium. The exact timing of the injection would be determined by monitoring the concentration of colletodiol in the medium by glc., (2½% OVI, 225°C) when there was a significant rise in the concentration the acetate solution was injected. In this fashion it was hoped that the acetate would be available at the onset of maximum production *ie* the bottom of the curve in figure 13. The concentration of colletodiol continued to be monitored by glc until an acceptable level was reached, usually *ca.* 100mg/l, the system was then extracted and the colletodiol isolated. It was found that there was a lot of variation between fermentations and to achieve reproducible results it was necessary to follow each fermentation carefully. In particular it was easy to miss the production period and so lose all the colletodiol. In general precursors were administered sometime during the first seventy two hours of the fermentation, and colletodiol was isolated no later than 150 hours after the administration.

To gain some information about the exact isotopic dilution with respect to carbon a preliminary feeding study was carried out using [1-^{14}C]acetate. A solution of sodium acetate, 1g in 10mls of water, was spiked with 10μCi of [1-^{14}C]acetate. This solution was administered as 1ml aliquots to each of ten 500ml shake flasks each containing 100ml of *Cytospora Sp.* after twenty four hours of growth. The production of colletodiol was followed in the usual manner by glc., This indicated that very

Table 3

CH_3CO_2Na/ mgl^{-1}	Colletodiol/ mgl^{-1}
Control	290
200	190
300	140
400	140
500	250

Table 4

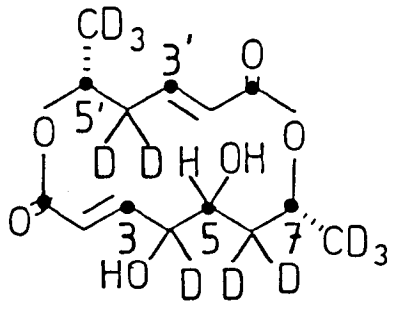
CH_3CO_2Na/mgl^{-1}	Colletodiol/mg	dpm/mmol	Dilution/Labelled Site
900 + 10μ ^{14}C	6·1	$2{\cdot}1\times10^6$	22·4
700 + 10μ ^{14}C	6·0	$1{\cdot}7\times10^6$	27·8
500 + 10μCi ^{14}C	6·3	$1{\cdot}2\times10^6$	38·1

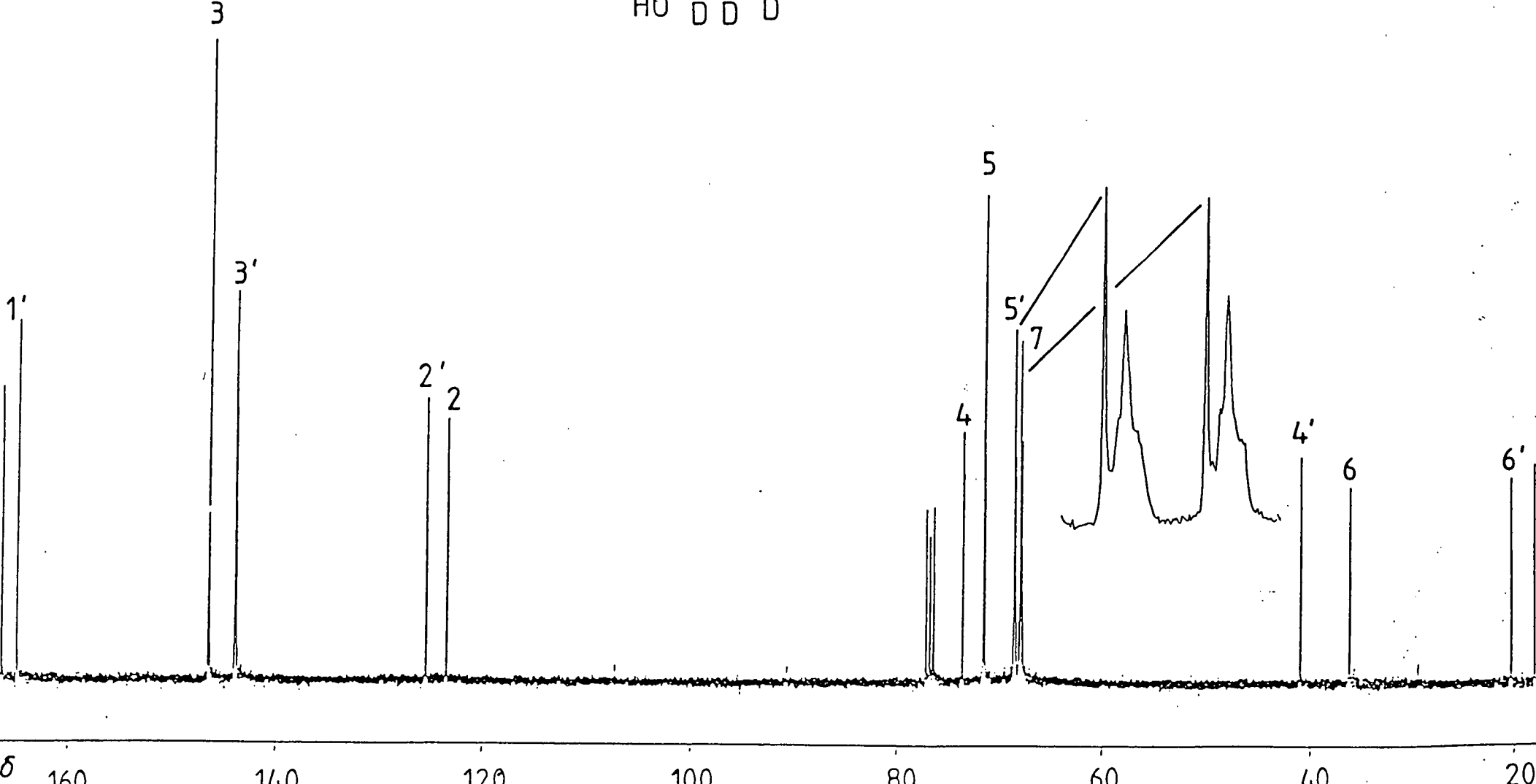
little colletodiol was being produced. Attempted extraction of the colletodiol after 143 hours of fermentation yielded nothing. From this experiment it was clear that high levels of sodium acetate in the medium impaired the growth of the culture and greatly reduced the levels of colletodiol produced. Before proceeding further with labelling studies it was necessary to find out the maximum sodium acetate concentration that was tolerable by *Cytospora Sp*, but which still allowed production of colletodiol.

Sodium acetate solutions were prepared representing concentrations of 200, 300, 400,and 500 mg/l. These solutions were pulse fed in three aliquots to a set of three flasks at 36, 48, and 60 hours. The results are summarised in table 3. The production of colletodiol was still impaired but remained at an acceptable level. The optimum concentration of sodium acetate seemed to be 700mg/l., fed in three aliquots at 36, 48, and 60 hours. All subsequent feeding experiments were based on this feeding protocol.

To determine the isotopic dilution involved using this feeding protocol [2-^{14}C]acetate was used. Solutions containing 360, 280, and 200 mg sodium acetate in 24 mls of water were prepared representing concentrations of 900, 700, and 500 mg/l sodium acetate. Each solution was spiked with 10 μCi of [2-^{14}C]-acetate. The solutions were administered as 2 ml aliquots at 36, 48, and 60 hours to a set of four shake flasks. The fermentation was monitored by glc. and the colletodiol recovered after 139

Figure 14 90·5 MHz ^{13}C n.m.r. Spectrum of Cotteroolot enriched with [1-^{13}C, 2-$^{2}H_3$] Acetate





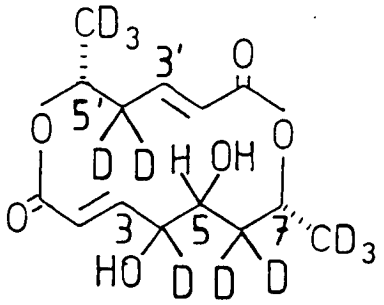
hours. The results are summarised in table 4. The weight of recovered colletodiol is also shown.

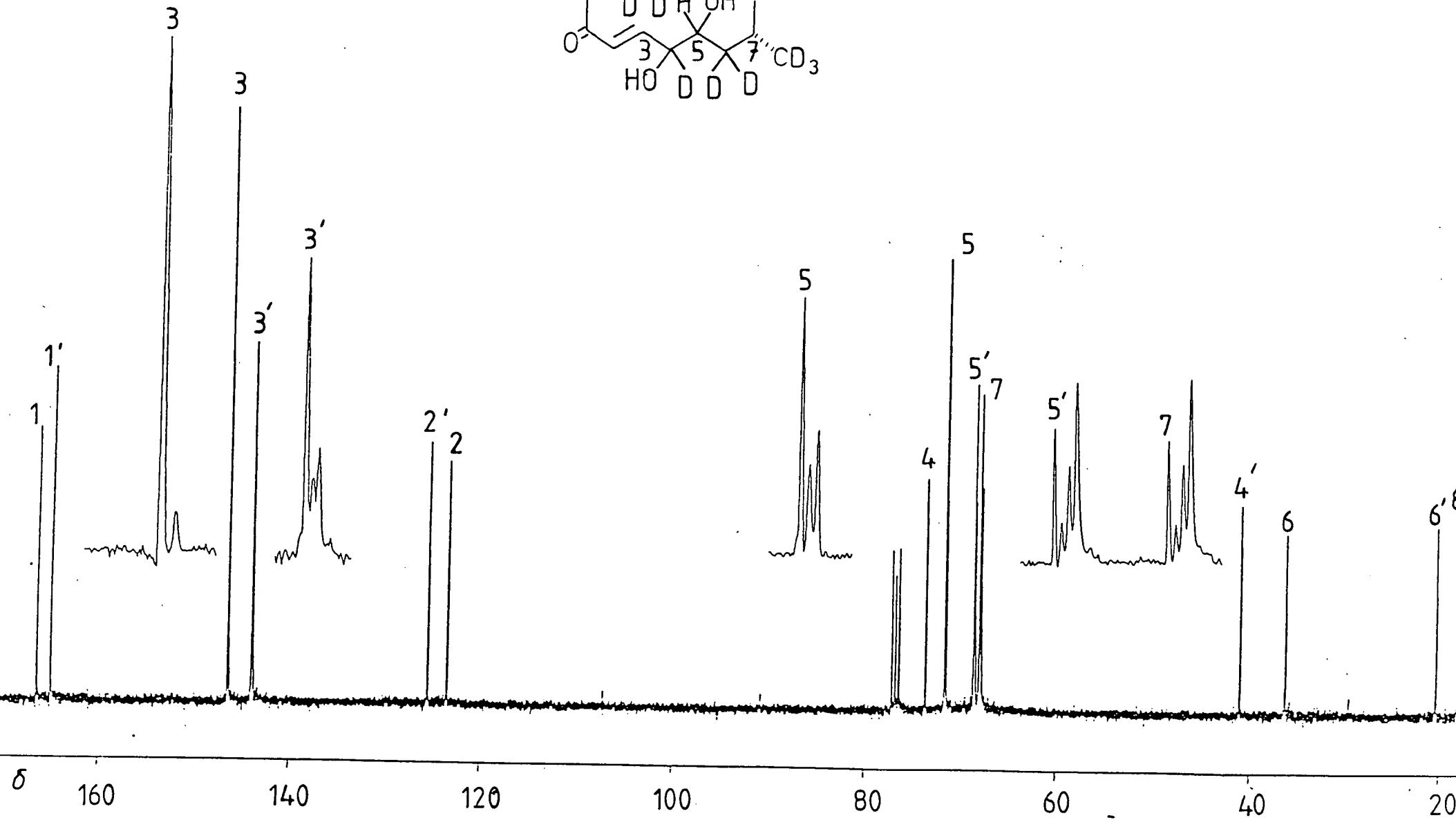
The colletodiol samples were purified initially by thin layer chromatography and then by repeated recrystallisation to constant activity from methylene chloride/n-hexane. The activity of each sample was determined by liquid scintillation counting, and the results are summarised in table 4. The best result was obtained from the 700 mg/l feed which gave a dilution per labelled site of 89.3 after initial purification and 27.8 after crystallisation to constant activity. Having now established the optimum conditions for feeding labelled acetate precursors, incorporation studies using precursors labelled with stable isotopes could be carried out.

2.3.2 ^{2}H Labelling Studies.

Using the feeding protocol described above [1-^{13}C,$^{2}H_3$]-acetate was administered to *Cytospora Sp* and the colletodiol isolated. The pnd ^{13}C n.m.r. spectrum of the resultant enriched metabolite is shown in figure 14. β Isotope shifts were observed on carbons C-3', C-5', C-3, C-5, and C-7. This corresponds to the retention of ^{2}H at C-4', C-6', C-4, C-6, and C-8. However in addition to the anticipated *intra* acetate β shifts extra isotopically shifted signals were observed on C-5' and C-7. These were due to *inter* acetate β shifts caused by incorporation of two labelled precursor molecules adjacent to each other. This

Figure 15 90·5 MHz ^{13}C nmr of Colletodiol enriched with [1-^{13}C, 2-$^{2}H_3$]Acetate





has been brought about by efficient incorporation of the precursor and the net effect is poor resolution in the ^{13}C n.m.r.

To reduce the possibility of this happening the experiment was repeated using an acetate concentration of 750mg/l consisting of unlabelled acetate:[1-^{13}C,$^{2}H_3$]acetate in a 1:2 ratio. This allows comparison with other feeds by keeping the concetration the same, but minimising the chances of observing *inter* acetate β shifts. The pnd ^{13}C n.m.r. spectrum of the enriched colletodiol from this experiment is shown in figure 15. β isotope shifts were observed on carbons C-3', C-5', C-3, C-5, and C-7. These correspond to the retention of acetate derived hydrogen on C-4', C-6', C-4, C-6, and C-8 repectively. These results are summarised in table 6. The size of the β shifts observed for colletodiol are typical (generally 0.05 ppm. maximum[22]) and compare well with published data on other metabolites. The origin of the some of the hydrogens in *O*-methylasparvenone (12) from *Aspergillus parvulus* is shown in scheme 5, and figure 16 shows some of the observed β isotope shifts[23], which compare well with those found in colletodiol.

No ^{2}H β isotope shift could be detected from the C-1 or C-1' carbonyl resonances that would have indicated the presence of acetate derived hydrogen at C-2 or C-2'. However carbonyl groups are known to be poor reporter nuclei for this effect. In the case of *O*-methylasparvenone (12) an *in vitro* experiment involving exchanging the C-2 methylene hydrogens with $MeO^{2}H$, showed that the β-^{2}H shift in a carbonyl resonance was to high

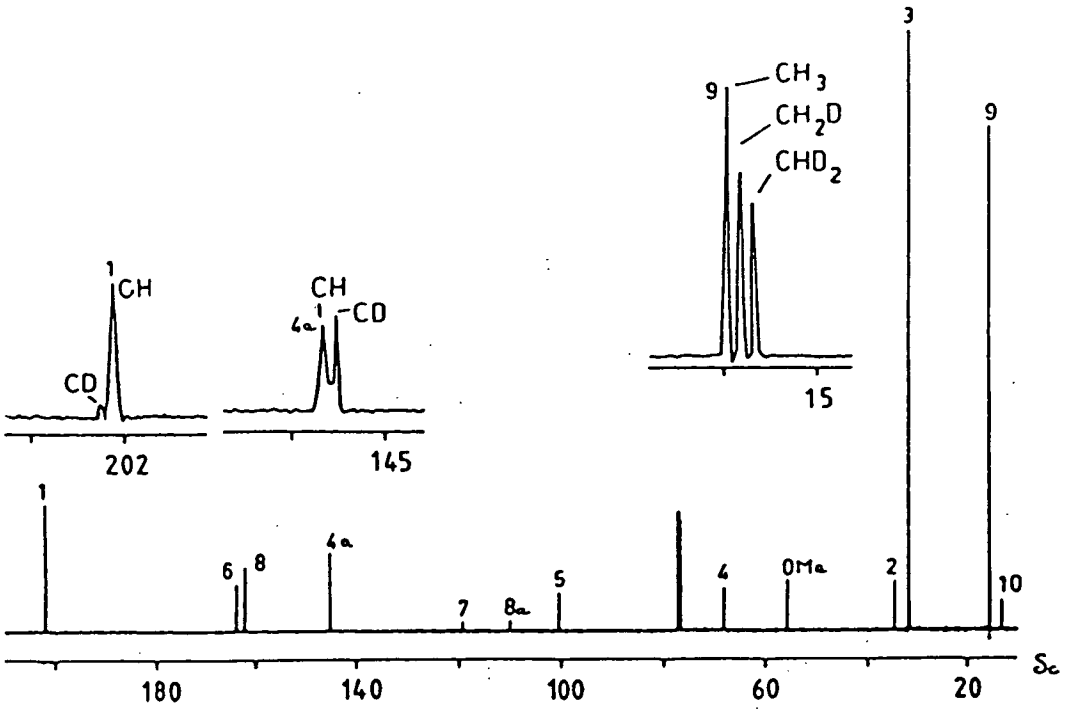
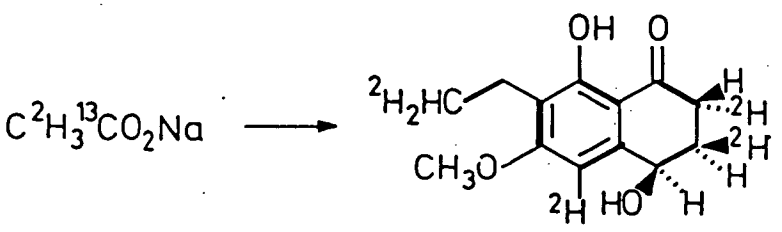


Figure 16 90·5 MHz ^{13}C n.m.r. of (12) enriched with [1-^{13}C,$^{2}H_3$]Acetate

Scheme 5



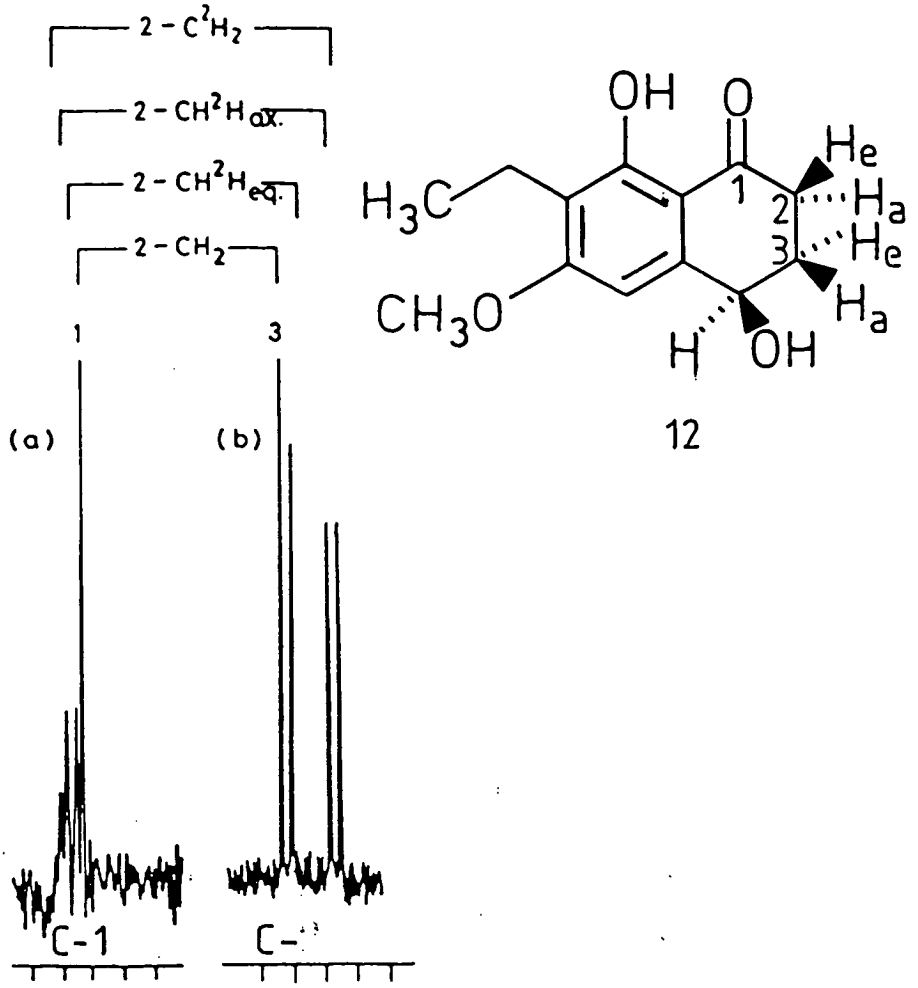


Figure 17 90·5MHz ^{13}C n.m.r. of (12) partially deuterated at C-2

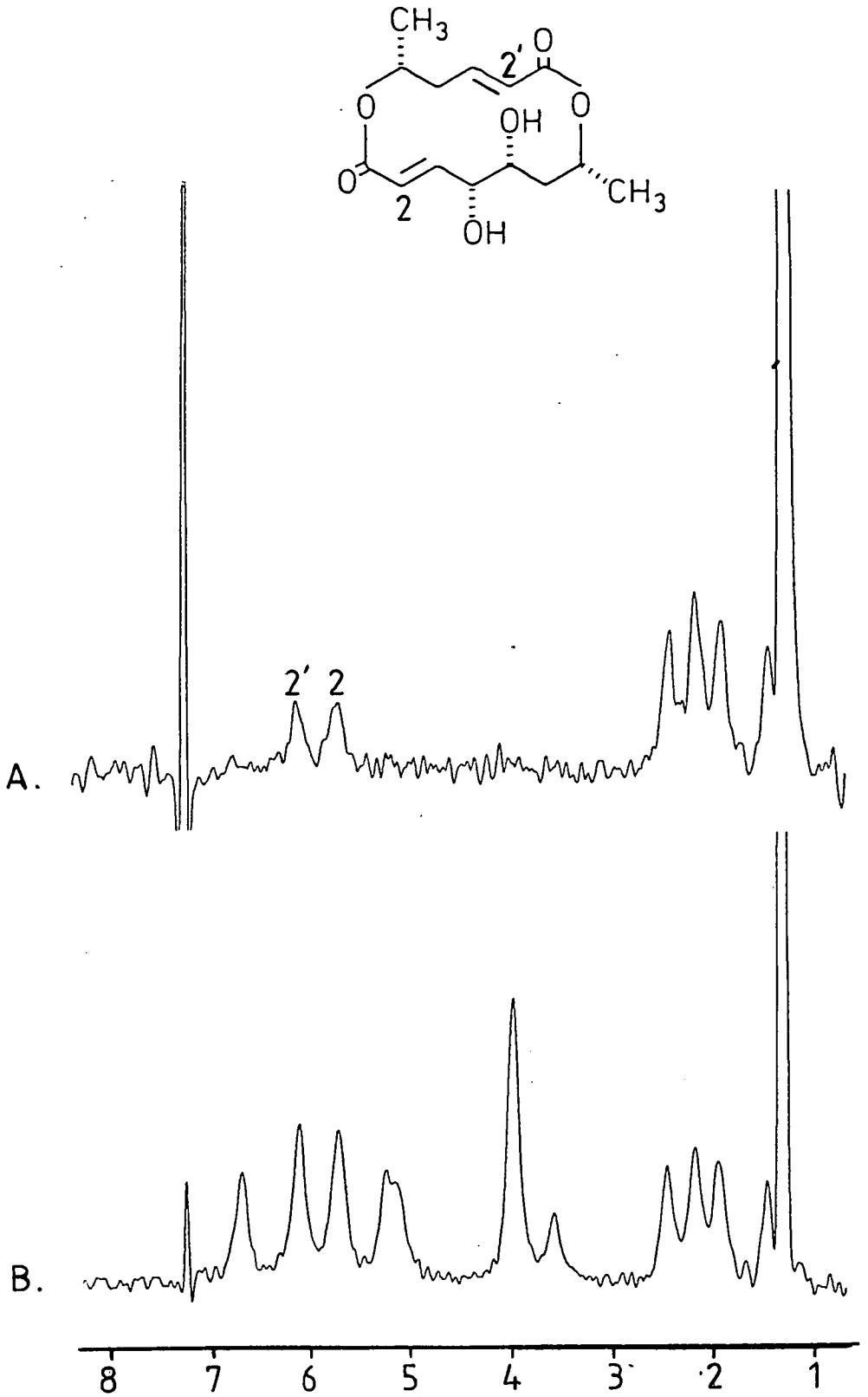


Figure 18 H n.m.r. Spectra of Colletodiol enriched with A) [1-^{13}C, $^{2}H_3$]Acetate
B) 5% $^{2}H_2O$

frequency in contrast to the usual case of being to low frequency[24,25]. This is illustrated in figure 17.

To look for possible ^{2}H retention at C-2 and C-2' the alternative use of direct ^{2}H n,m,r. was therefore employed. To achieve this it was necessary to have a sample of colletodiol containing a high percentage of deuterium at each possible carbon in order to assign the ^{2}H n.m.r. from the ^{1}H n.m.r. spectrum. *Cytospora Sp.* was grown for the usual period of time, but on a medium containing 5% $^{2}H_{2}O$. The ^{2}H n.m.r. of the isolated colletodiol is shown in figure 18. All the signals are well resolved and the assignment was made on the basis of the ^{1}H n.m.r. spectrum. The ^{2}H n.m.r. spectrum of the colletodiol isolated from the experiment in which the precursor solution of [1-^{13}C,$^{2}H_{3}$] acetate was diluted with unlabelled acetate is shown in figure 18a. This clearly shows the presence of ^{2}H at C-2 and C-2' which was not discernable from the β shift experiments. The retained ^{2}H on carbons C-4', C-6', C-6, and C-8 can also be seen in this spectrum. It is interesting to note that from the β shift experiment the level of ^{2}H labelling at C-4 was lower than in any other part of the molecule. However the sample of colletodiol that was obtained from the fermentation in a 5% $^{2}H_{2}O$ medium shows a very high level of incorporation of ^{2}H label at C-4. This has not been rationalised. The complete pattern of ^{2}H labelling is shown in figure 19.

Figure 19

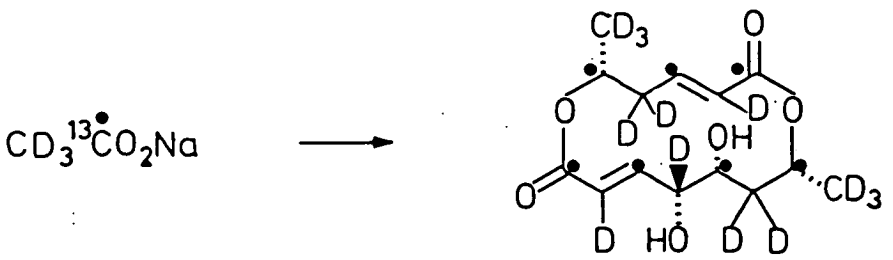
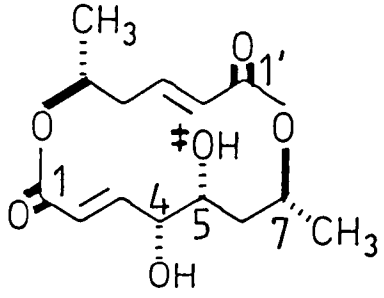


Figure 20 90·5 MHz ^{13}C n.m.r. Spectrum of Colletodiol enriched with $[^{13}C, ^{18}O_2]$Acetate, and $^{18}O_2$



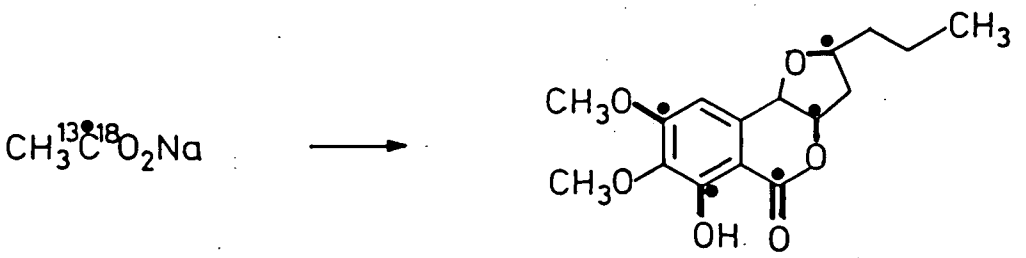
2.3.4. ^{18}O Labelling Studies: Origin of the Oxygen atoms in Colletodiol.

[1-^{13}C,$^{18}O_2$]Acetate was administered to *Cytospora Sp* in the manner described above and the colletodiol produced isolated. The pnd ^{13}C n.m.r. spectrum of the enriched metabolite is shown in figure 20. α Isotope shifts can be seen for C-1 and C-1' the ester carbonyl functions and also for C-5' and C-7 the ether derived carbons. The shifts are of 0.034, 0.032, 0.039, and 0.037 ppm respectively.

The shifts observed for C-1 and C-1' are consistent[22] with those observed for the incorporation of ^{18}O into the carbonyl oxygen portion of an ester. If at any time the biosynthesis involved a free carboxylate function then due to resonance we would expect an equilibration of the label. This would result in the observation of two α shifts in the carbonyl signal due to the mixture of C^{18}OOR and CO^{18}OR in the molecule.

The fact that C-1, C-1', C-5' and C-7 all show only one α isotope shift means that the carbonyl and ether oxygens are derived from different acetate units. This means that during the biosynthesis each of the ester oxygens has remained bound to its original carbon and has not passed through a free acid intermediate. This is similar to the case in monocerin (11) the labelling pattern of which from an experiment using [1-13C,$^{18}O_2$] acetate is shown in figure 21, the labelling pattern of the lactone is identical to that found in colletodiol.

Figure 21



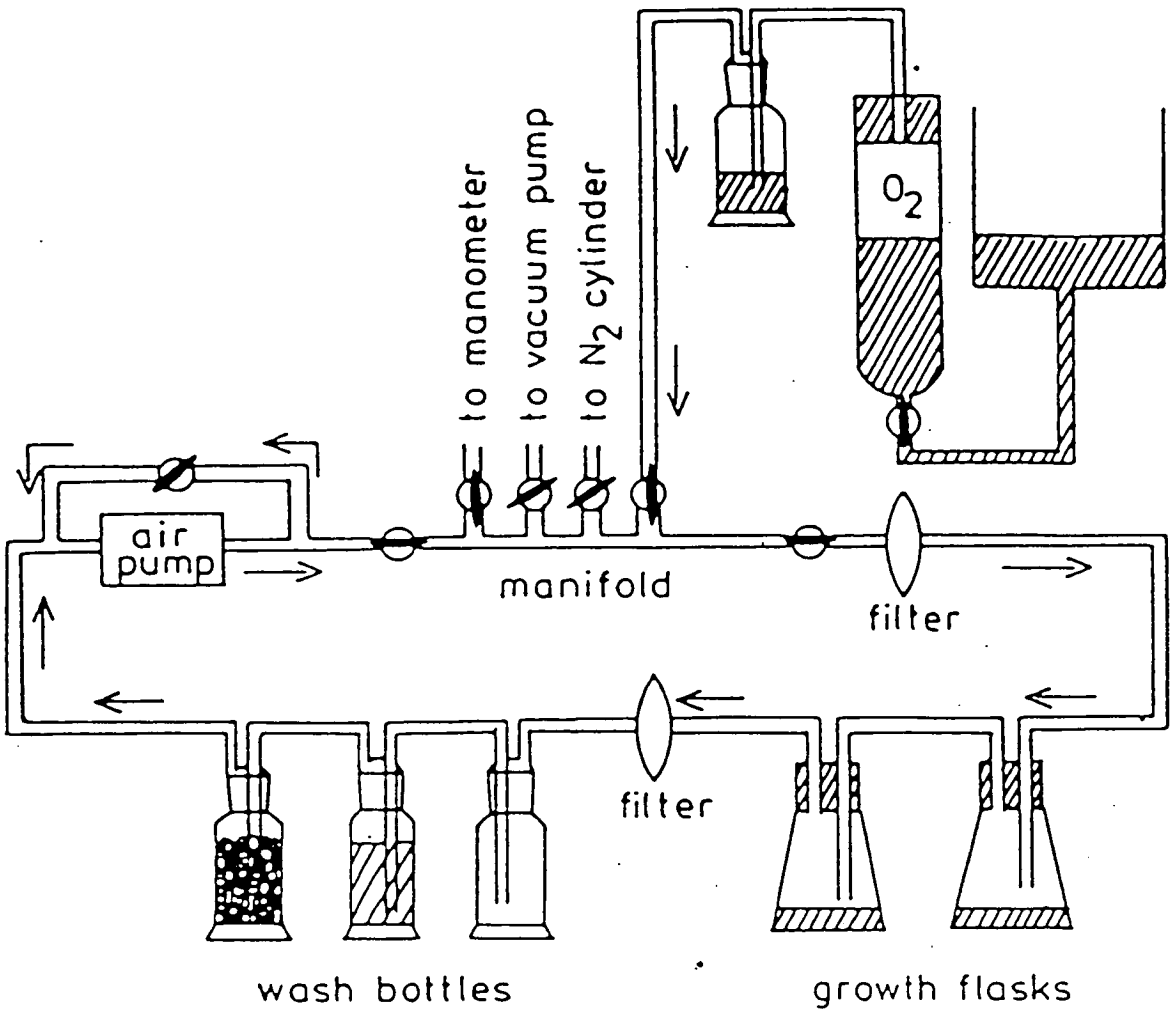
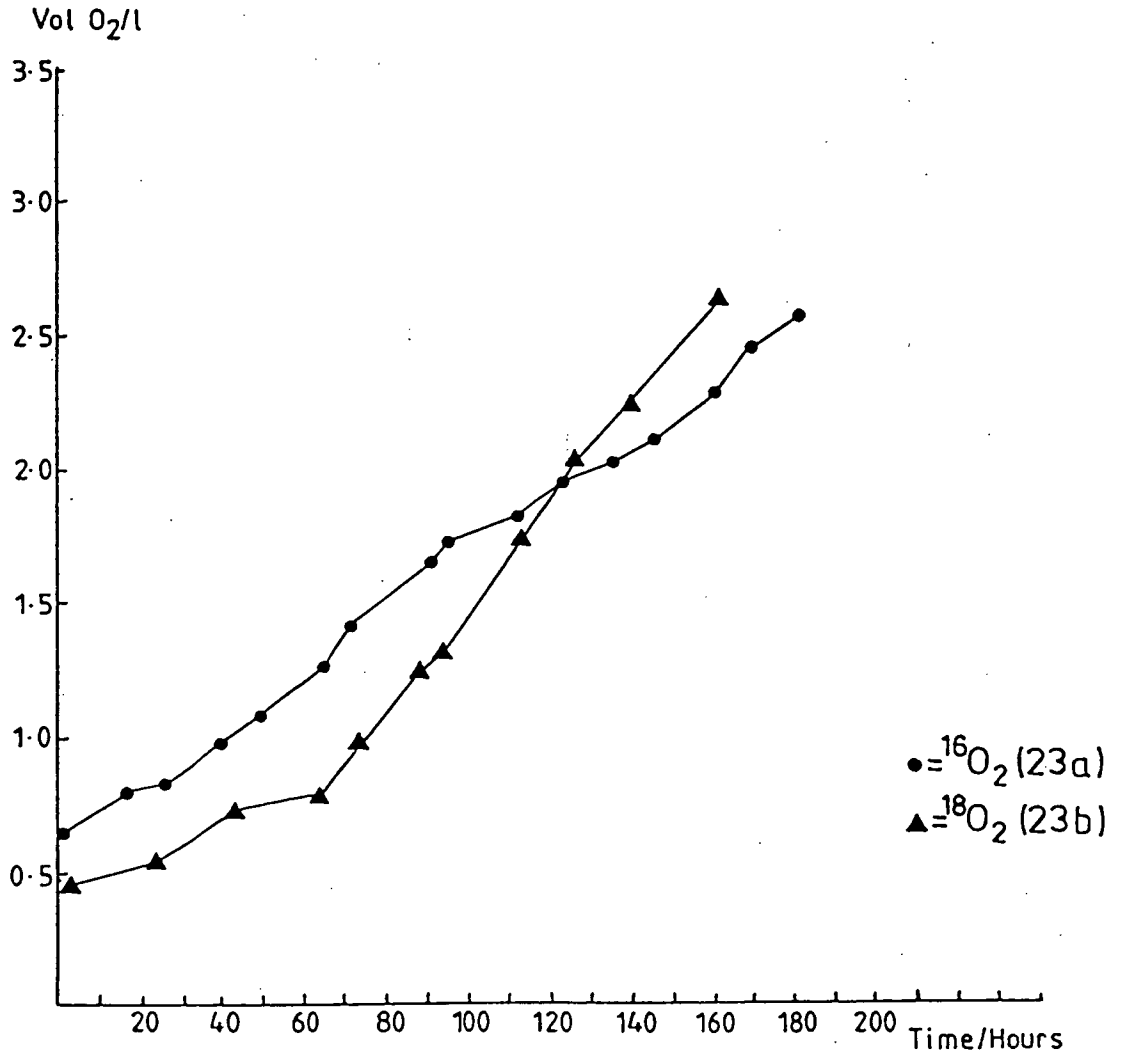


Figure 22 Constant Pressure $^{18}O_2$ Apparatus

Figure 23 Oxygen uptake of Cytospora Sp



No α isotope shifts were observed for C-4 or C-5 and therefore neither of the diol oxygens can be derived from acetate. This would seem to indicate that these oxygens come either from aerially derived oxygen or from medium derived oxygen *via* water. To obtain more information on this point a fermentation of *Cytospora Sp* in an atmosphere containing $^{18}O_2$ gas was carried out.

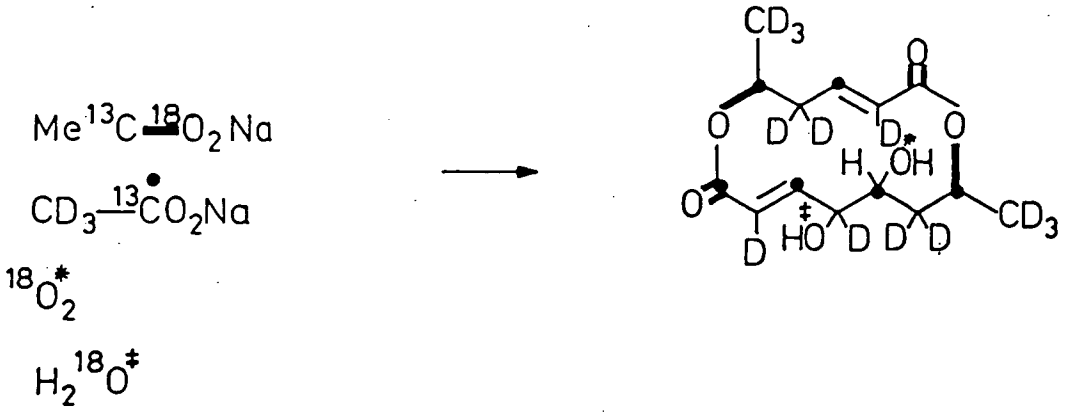
2.3.5. Incorporation of ^{18}O from $^{18}O_2$ gas.

To successfully use $^{18}O_2$ gas as a precursor, fermentations have to be carried out under strictly controlled conditions. The apparatus used is shown in figure 22. This is a constant pressure system in which CO_2 is scrubbed out, and the volume of oxygen used can be measured. The system is usually operated using an atmosphere of 20% oxygen and 80% nitrogen.

To assess the volume of oxygen required a preliminary experiment was carried out using $^{18}O_2$. The uptake of oxygen by *Cytospora Sp.* is shown in figure 23a. The experiment was repeated using $^{18}O_2$ and the uptake for this is shown in figure 23b. It can be seen that these curves are essentially the same.

The enriched colletodiol from the labelled experiment was isolated and the pnd ^{13}C n.m.r. spectrum is shown in figure 20. Only one α isotope shift was observed, on the signal corresponding to C-5. The magnitude of the shift was 0.022 ppm. with 46% incorporation. No labelling was found at C-4 and so by

Figure 24


Me13C18O2Na
CD313CO2Na
18O2*
H2 18O‡
CD3
O
D
D
D*
O
H
OH
O
D
HO‡
D
D
D
CD3

default the hydroxyl function at C-4 must be derived from the medium *via* water.

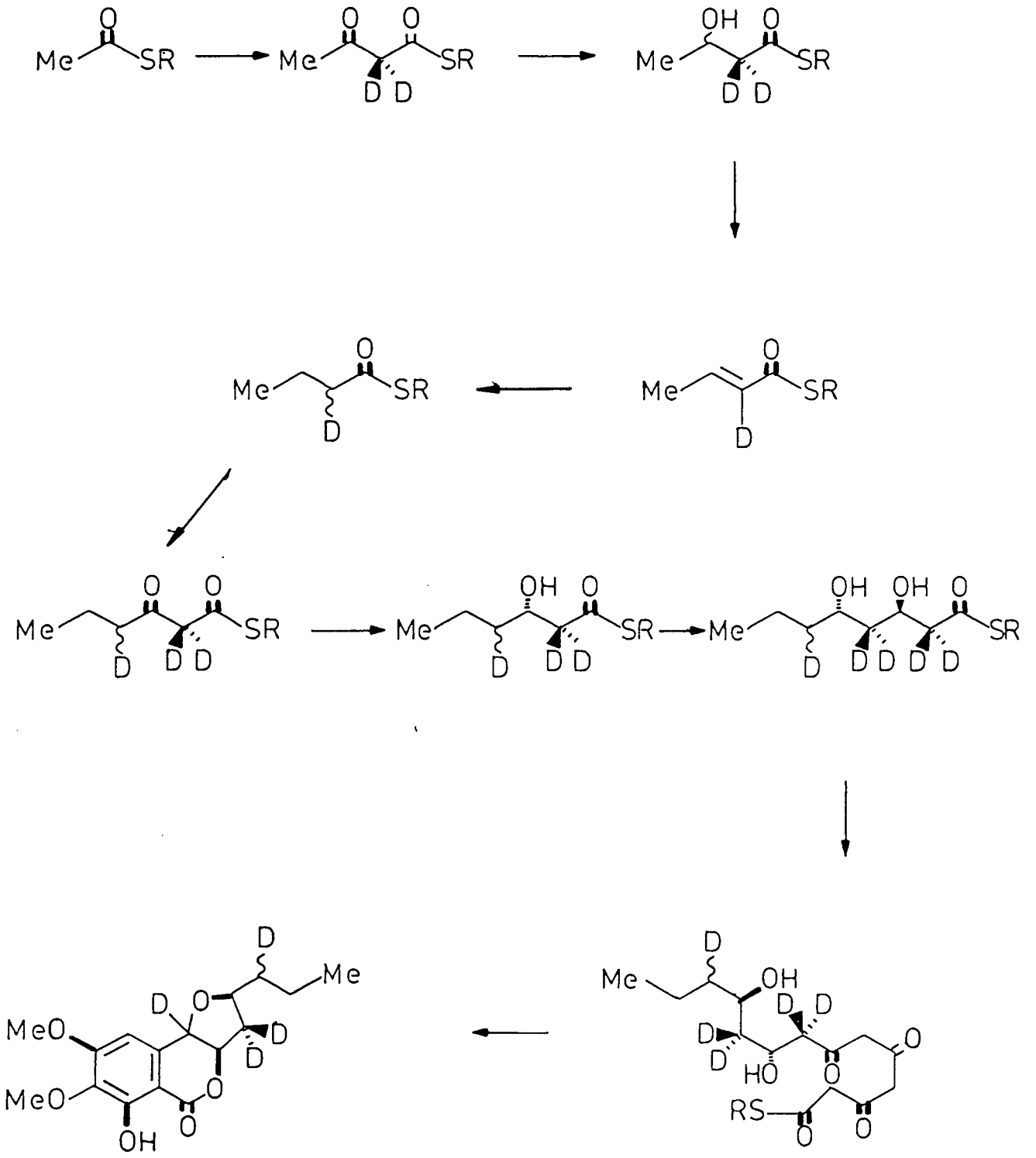
2.4. CONCLUSIONS FROM THE LABELLING STUDIES.

A summary of the labelling pattern from the studies described above is shown in figure 24. The conclusions in terms of the mechanism of the lactone formation and the 4,5-diol formation, and in turn for the nature of the polyketide precursors assembled by the polyketide synthetase are discussed below.

2.4.1 Mechanism of the Lactone formation.

The results of the incorporation of [1-^{13}C,$^{18}O_2$]-acetate into colletodiol support the acyl substitution mechanism a) of scheme 3. Attack of a hydroxyl function on a carbonyl group, probably activated as its thioester. This is supported by the evidence from the [1-^{13}C,$^{2}H_3$]acetate incorporation which showed retention of acetate derived hydrogen on both C-4' and C-6 methylene groups. This means that the oxygen function of the acetate "starter" unit is retained in the form of a secondary alcohol with the (*R*) configuration, this is similar to the situation in fatty acid biosynthesis where the acetoacetyl residue is reduced to the β-(*R*) configuration in each case. However in the case of colletodiol the reduction stops at this point.

Scheme 6



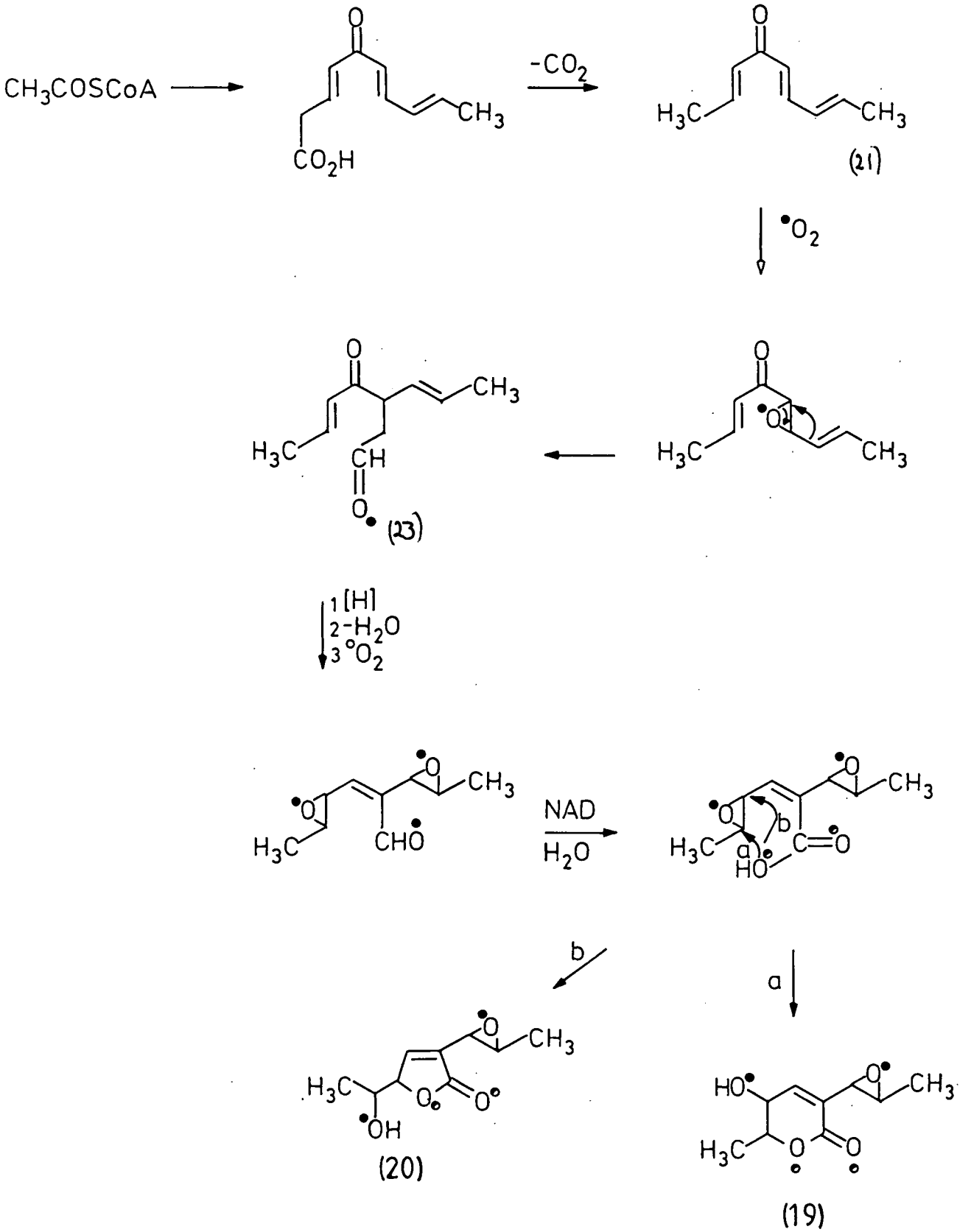
The labelling pattern observed in colletodiol from the studies using [1-^{13}C,$^{2}H_3$]- and [1-^{13}C,$^{18}O_2$]acetate is similar to that produced in monocerin[21] (11) biosynthesis. The proposed reduced polyketide chain, with the appropriate labelling from the administration of [1-^{13}C,$^{2}H_3$]- and [1-^{13}C,$^{18}O_2$]acetate is shown in scheme 6. It can be seen that this labelling pattern also suggests an acyl substitution mechanism for the formation of the lactone.

We can conclude by saying that the lactone linkages of colletodiol are formed *via* an acyl substitution mechanism, involving two reduced polyketide chains, the reaction occuring with retention of configuration at C-5' and C-7.

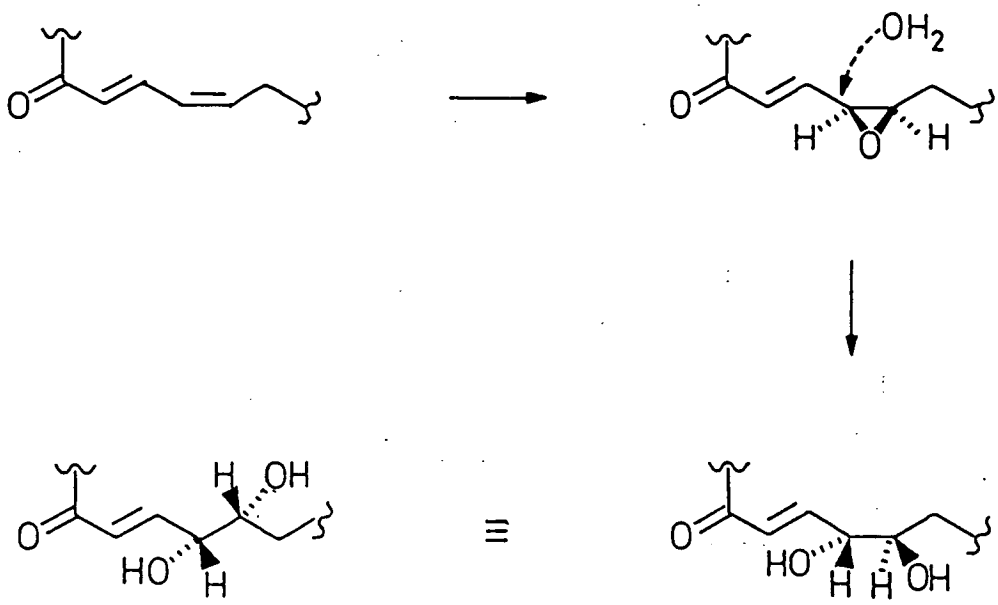
2.4.2 Formation of the 4,5 Diol system.

No acetate derived oxygen was found in the 4,5 diol system. This immediately rules out the hydroxylation pathway shown in mechanism a), of scheme 4. The C-5 hydroxyl group however showed the presence of oxygen derived from the atmosphere, and this suggests that it was introduced *via* epoxidation of a double bond. The epoxidation of a double bond is not without precedent, aspyrone (19) and asperlactone (20) two closely related metabolites from *Aspergillus ustus* have been studied using $^{18}O_2$ fermentation[26]. The proposed mechanism of their biosynthesis is shown in scheme 7. Reduction of the chain to the trienone (21), followed by decarboxylation, epoxidation and rearrangement gives the aldehyde (22). This is then converted

Scheme 7


CH3COSCoA
-CO2
CO2H
CH3
H3C
(21)
•O2
(23)
1 [H]
2 -H2O
3 °O2
CHO
NAD
H2O
HO
a
b
OH
(20)
(19)

Scheme 8



to the epoxy carboxylic acid (23) by epoxidation and oxidation of the aldehyde. Ring closure gives aspyrone (19) and asperlactone (20).

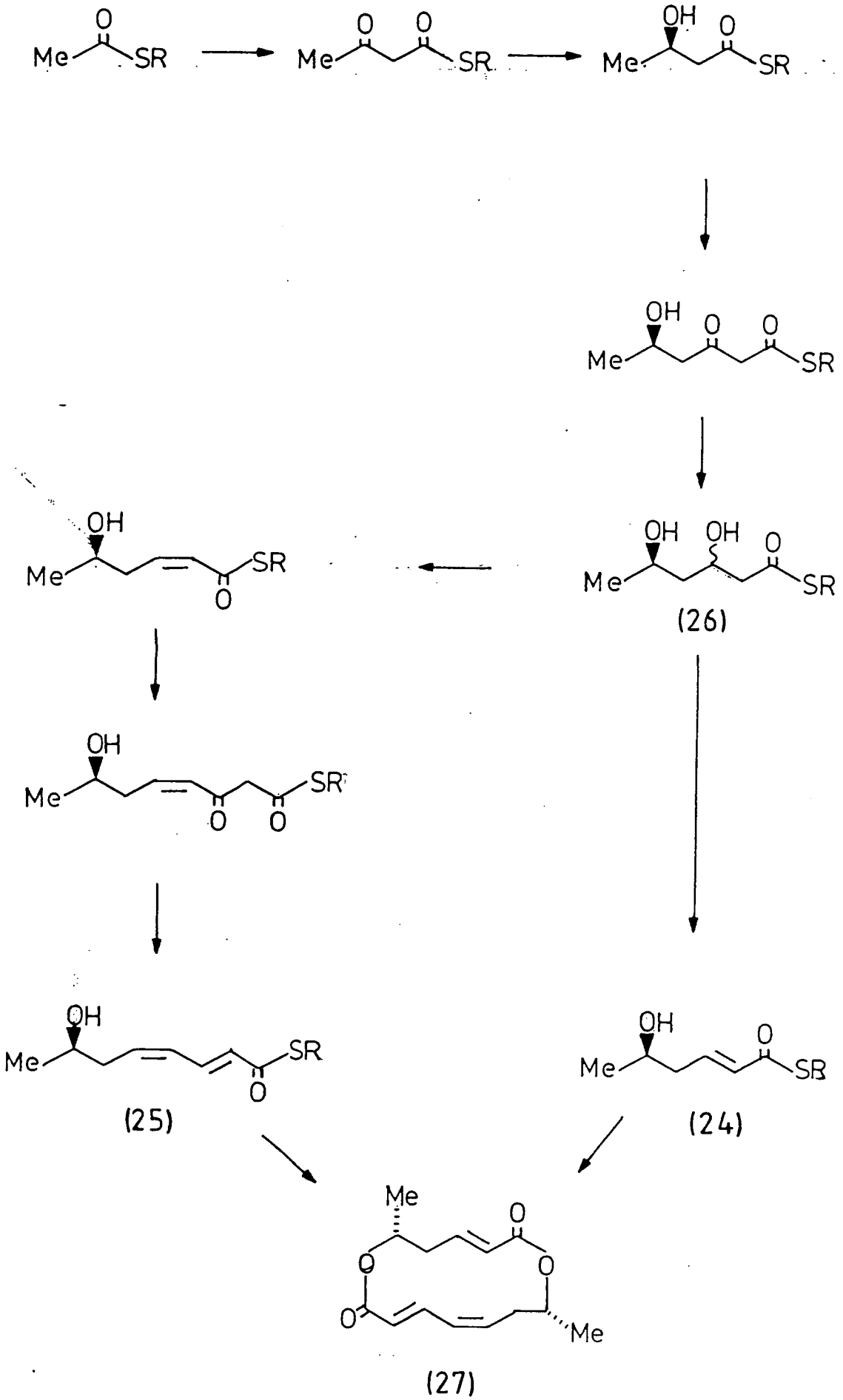
In the case of colletodiol, the two remaining possibilities shown in scheme 4 both involve epoxidation. The epoxidation, rearrangement and hydroxylation pathway, shown in c) involves a 1,2 hydride shift. This would result in ^{2}H label at C-5, which could not be observed by isotope shift methods but would have been observed by direct ^{2}H n.m.r. No ^{2}H label was observed in this position by direct ^{2}H n.m.r. However a small but significant amount of acetate derived hydrogen was retained in the C-4 position indicating that hydrolysis of the epoxide proceeds as shown in path b) and not path c) of scheme 4. This must mean that COLLETODIOL is not formed by reduction of colletoketol.

The stereochemistry of colletodiol is 4(*R*),5(*R*),7(*R*),5'(*R*). To achieve this stereochemistry in the diol system, the epoxide must be formed on the β face of a *Z* alkene, followed by hydrolysis of the epoxide at C-4 from the α face. This is represented in scheme 8. Although hydrolysis could occur at C-5 the observed mechanism allows formation of the more stable carbocation.

2.4.3 Colletodiol: Overall biosynthesis.

The main conclusion from this study is that the triketide and tetraketide fragments of colletodiol are modified

Scheme 9



during assembly. The results described above imply that the thioesters (24) and (25) are the immediate enzyme bound precursors. Scheme 9 shows the proposed assembly of (24) and (25). Acetyl CoA and malonyl CoA combine to form an acetoacetyl thioester, which is then stereospecifically reduced at C-3 to give the 3-(*R*) hydroxybutyryl thioester. Addition of a further unit of malonyl CoA and reduction would give a common intermediate (26), in which the stereochemistry of the C-3 hydroxyl group is uncertain. In the following step *trans* elimination of water from (26) would give the C_{ϵ} fragment (24) directly, whereas *cis* elimination of water from (26) followed by a further addition of malonate , reduction and *trans* elimination of water would give the C_{ε} fragment (25)

The actual timing of the formation of the diol system is uncertain, but it may occur from an intermediate such as the triene lactone (27). Careful examination of molecular models of (27) have led to the conclusion that an *E,Z* diene system in the C_{ε} fragment can be accomodated in the fourteen member ring with the β face of the *Z* double bond unhindered. On the other hand an *E,E* diene system produces considerable strain in the ring, and the α face of the γ,δ double bond is now more exposed, and could also be subject to epoxidation.

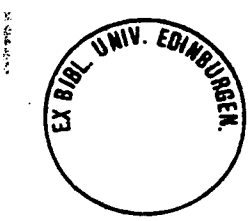
2.4.5 Future work.

From a biosynthetic point of view there are two areas to be explored from the present state of the study. The synthesis

of specifically labelled precursors, such as the intermediates shown in scheme 9, and subsequent feeding studies with them would allow some of the remaining details to be determined. For example the stereochemistry of C-3 in (26) could be examined by the synthesis of both enantiomers, and feeding suitably labelled forms of them to *Cytospora Sp.* Research towards the synthesis of (24) and (25) is described in the following chapter.

The C-6 and C-4' methylene groups of colletodiol are derived from the C-2 of malonyl CoA. With such a good model system where the *pro-(R)* and *pro-(S)* hydrogens are well resolved in the ^{1}H n.m.r. and ^{2}H n.m.r., studies using chiral malonate models would be feasible.

Finally the biosynthesis of colletodiol has now been well studied, and significant evidence has been found for the structure of the enzyme bound intermediates. Work is currently underway to try to isolate some of the enzymes responsible for colletodiol. This would allow cell free studies to be carried out and hence gain direct evidence about polyketide chain assembly processes.



2.5 EXPERIMENTAL.

2.5.1 General procedures and instrumentation

Melting points were determined on a Kofler hot stage apparatus and were uncorrected. ^{13}C, and ^{1}H Nuclear magnetic resonance spectra were recorded on Bruker WH 360, WP 200, and WP 80 instruments, referenced on the deuterated solvent, or on chloroform in the case of ^{2}H n.m.r. spectra. Routine ^{1}H spectra were recorded on a Perkin Elmer EM 360 spectrometer.

Mass spectrometry was carried out on a Kratos MS 50, or A.E.I. MS 902 spectrometer. Unless otherwise stated ionisation was achieved using electron impact.

Scintillation counting was carried out on a Beckmann LS7000 liquid scintillation counter. Programme four was used without automatic quench correction, butyl PBD was used as the scintillant. The counting efficiency was determined by using both standard channels ratio and H-number quench curves. Optical rotations were recorded on a Perkin Elmer 141 polarimeter. Infra red spectra were recorded on a Perkin Elmer 781 spectrometer. Gas chromatography was carried out on a Pye 204 chromatograph.

Preparative thin layer chromatography was carried out on glass plates (20 x 20 cm.), coated with a 0.5 mm. layer of silica gel (Fluka 60765 Kieselgel Gf 254). Bands were visualised using ultra violet light (254 nm.). Flash chromatography was carried out on silica gel 400-200 mesh (Kieselgel 60 Merck).

All materials and equipment involved with culture experiments were sterilised before use by autoclaving for fifteen minutes at 15 p.s.i. Precursor solutions were sterilised by autoclaving, or by passage through a sterile fertile. Unless otherwise stated cultures were incubated in a constant temperature room at 25-27°C for the required period of time. All solvents were distilled before use, and dried using standard procedures when required. Nitrogen was dried by passage through a series of traps, sulphuric acid, glass wool, sodium hydroxide, and self indicating silica gel.

2.5.2 Culture Details

Cytospora Sp ATCC 20502 was stored on agar slopes (Oxoid CM 139) at 4°C. Large scale fermentations were carried out using potato extract broth, prepared by homogenising 60-70 g. of potatoes in one litre of water and adding 20 g. of glucose.

Seed cultures were prepared in 500 ml. conical flasks each containing 100 ml. of medium. These were in oculated with a spore suspension in distilled water prepared directly from an agar storage slope. The seed culture was allowed to incubate for forty eight hours at 26°C, and was then used to in oculate larger volumes of medium, either 100 ml aliquots in shake culture or in an airlift fermenter.

2.5.3 Isolation of Colletodiol.

Cultures of *Cytospora Sp* ATCC 20502 were normally incubated for six to nine days at 26°C. The exact length of the fermentation depending on the concentration of colletodiol present as determined by glc (2½% OVI 225°C). The mycelial mass developed as distinct white spherical structures, which developed a green pigmentation after 72 hours.

The mycelial mass was separated from the liquor by filtration under reduced pressure, through a double layer of muslin cloth in a Buchner funnel. The aqueous liquor was extracted with chloroform (3 x equivalent volume), dried over $MgSO_4$, and the solvent removed *in vacuo*. The mycelial mass was homogenised in acetone (50 ml per flask), and filtered through a Whatman No 1 filter paper. The acetone was then removed *in vacuo*, and the residual liquor extracted with chloroform (3 x equivalent volume), dried over $MgSO_4$, combined with the original liquor extract and the solvent removed *in vacuo* to give a green solid.

The green solid was redissolved in the minimum volume of chloroform and added dropwise to petroleum ether (80-100). This produced a brown precipitate which was filtered off and extracted exhaustively with ether. Any solid material remaining in the ethereal solution was filtered off, and the ether extract dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow/white solid, typically 2 g. per litre of culture.

The extracts from small scale fermentations were purified by preparative thin layer chromatography on silica gel.

The plates were eluted with ether/methanol/methylene chloride, (40:4:56). Bands were scraped off the plate and the silica exhaustively extracted with ethyl acetate. Colletoketol appeared at Rf 0.75, and crystallised as white needles from methylene chloride/n-hexane (mp. 136-137°C, lit[12] 138-139°C). All ^{1}H and ^{13}C n.m.r data agree with the published data. Colletodiol appeared at Rf 0.2, and crystallised as white needles from methylene chloride/n-hexane (mp. 161-163°C, lit[12] 163-164°C). All ^{1}H and ^{13}C n.m.r, data agree with the published data. Extracts from large scale fermentations were purified by flash chromatography using a gradient elution of 1% to 20% ether in methylene chloride.

Colletodiol : $[\alpha]_D$ = +31°, c = 0.99 in chloroform

lit[12] $[\alpha]_D$ = +33°, c = 1.0 in chloroform

2.5.4 Growth production study of Colletodiol

2.5.4.a. Shake culture

Cytospora SP ATCC 20502 was incubated as described above in twenty seven flasks. From day two after inoculation three flasks were removed and the colletodiol isolated as described above.

Results

Time/days	Colletodiol(liquor) mg/l	Colletodiol(mycelium) mg/ l
2	-	-
3	120	47
4	283	56
5	235	37
6	239	42
7	-	- *
8	289	97
9	116	75

* No colletodiol produced in these flasks

2.5.4.b. Airlift fermentor culture

Seed cultures of *Cytospora Sp* ATCC 20502 were prepared as described above and were used to inoculate an airlift fermentor of the type shown in figure12, containing 1300 ml. of medium. Samples containing both mycelia and liquor (10 ml.) were removed from the fermentation at regular intervals. These samples were not chromatographed but were homogenised in chloroform (50 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo*. The residue was analysed by glc. (2½% OVI, 225°C), against a standard sample of colletodiol.

Results

Time/hours	colletodiol mg/l
18	79
26	110
48	126
91	156
113	142
121	158
135	332
143	578
155	162

2.5.5 Study to determine the tolerance of *Cytospora Sp* ATCC 20502 to sodium acetate

Cytospora Sp was grown in shake culture as described above in ten flasks. Two of the flasks were allowed to incubate as usual without any interference to act as controls. Sterile solutions of sodium acetate were prepared in the following way :

Sodium acetate (6·0 mg, ; 9·0 mg, ; 12·0 mg, ; and 15·0 mg,) was dissolved in water (30 ml.). These solutions represent sodium acetate concentrations of 200, 300, 400, and 500 mg l respectively.

To a set of two flasks an aliquot (5 ml.) of one of the solutions was injected into the medium, 36, 48, and 60 hours after in oculation. The fermentation was continued for seven days and the colletodi ol isolated in the usual fashion.

Results

sodium acetate mg/ l	colletodiol mg/l
200	190
300	140
400	140
500	250

2.5.6 Incorporation of [2-^{14}C]acetate into Colletodiol

Three sterile solutions spiked with [2-^{14}C]acetate were prepared as follows :

1.) Sodium acetate (200 mg, 2.43 mmol.) was dissolved in water (23 ml.). To this solution was added [2-^{14}C]acetate in water (1 ml, 10 μCi, 9.10 x 10^6 dpm/mmol). The solution was divided into four aliquots (6 ml.)

2.) Solution prepared as above but using sodium acetate (280 mg 3.4 mmol.)

3.) Solution prepared as above but using sodium acetate (360 mg 4.4 mmol.)

These solutions represent sodium acetate concentrations of 500, 700, and 900 mg l respectively. *Cytospora Sp.* was grown as described above in shake culture in twelve flasks. At 36, 48, and 60 hours after innoculation an aliquot (2 ml.) of each solution was injected into each of a set of four flasks. The fermentation was allowed to continue for six days and the colletodiol isolated as usual. Each colletodiol sample was crystallised to constant activity as determined by liquid scintillation counting. The isotopic dilution values for each precursor solution are shown in parentheses : 900 mg/l, (22.4), 700 mg/l, (27.8), 500 mg/l, (38.1). These results are summarised in table 5.

2.5.7 Fermentation of *Cytospora Sp* ATCC 20502 using a constant pressure $^{18}O_2$ apparatus

Cytospora Sp. was grown in five shake flasks as described above. Immediately after innoculation the flasks were connected to the "constant pressure" apparatus shown in figure 22. Fermentation was continued for seven days in an atmosphere of $^{18}O_2/N_2$ 1:4, and the colletodiol isolated as usual. (21 mg, 42 mg l), The total volume of oxygen consumed was 2595 ml. This is represented in figure 23a.

2.5.7.a. Incorporation of $^{18}O_2$ gas into Colletodiol

Cytospora Sp. was grown in five shake flasks as described above. Immediately after inoculation the flasks were connected to the "constant pressure" apparatus. The fermentation was continued for seven days in an atmosphere of $^{18}O_2/N_2$ 1:4, and the colletodiol isolated as usual (41 mg. 82 mg/l). The total volume of oxygen consumed was 2658 ml. This is shown in figure 23b. The pnd ^{13}C n.m.r. of this sample is shown in figure 20.

2.5.8 Incorporation of [1-^{13}C,$^{2}H_3$]acetate into Colletodiol

Method 1. *Cytospora Sp.* was grown as described above in ten shake flasks. A sterile solution of [1-^{13}C,$^{2}H_3$]acetate (750 mg, 9.1 mmol.) in water (30 ml.) was prepared. After 36, 48, and 60 hours of fermentation an aliquot (1 ml.) of this solution was injected into each flask. Fermentation was continued for six days, and the colletodiol isolated as usual (87 mg. 87 mg l). The pnd ^{13}C n.m.r. of this sample is shown in figure 14.

Method 2. *Cytospora Sp.* was grown as described above in five shake flasks. A sterile solution consisting of acetate (175 mg, 2.1 mmol.), and [1-^{13}C, $^{2}H_3$] acetate (250 mg, 3.05 mmol.), in water (15 ml.), was prepared. After 36, 48, and 60 hours of fermentation an aliquot (1 ml.) of this solution was injected into each flask. The fermentation was continued for six days and the colletodiol isolated as usual (41 mg. 820 mg/l.) The pnd ^{13}C n.m.r. of this sample is shown in figure 15.

2.5.9 Incorporation of [1-^{13}C,$^{18}O_2$]acetate into colletodiol

Cytospora Sp. was grown as described above in five shake flasks. A sterile solution consisting of [1-^{13}C,$^{18}O_2$]-acetate (375 mg, 4.6 mmol.) in water (15 ml.) was prepared. After 36, 48, and 60 hours of fermentation an aliquot (1 ml.) of this solution was injected into each flask. The fermentation ws allowed to continue for six days and the colletodiol isolated as usual (22 mg. 44 mg l./) The pnd ^{13}C n.m.r. of this sample is shown in figure 20.

2.5.10 Preparation of universally Deuterated Colletodiol

Cytospora Sp. was grown as described above in five shake flasks. The water used to prepare the potato extract broth consisted of 5% $^{2}H_2O$, 95% H_2O. The fermentation was continued for six days and the colletodiol isolated as usual (140 mg. 280 mg/l.). The ^{2}H n.m.r. of this sample is shown in figure 18.

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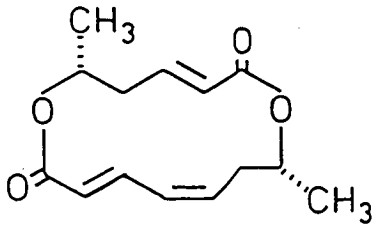
CHAPTER THREE

SYNTHETIC ROUTES TOWARDS COLLETOTRIENE

A NOVEL FOURTEEN MEMBER MACRODIOLIDE.



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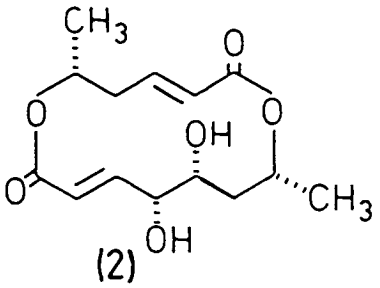

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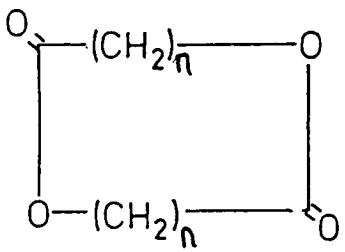

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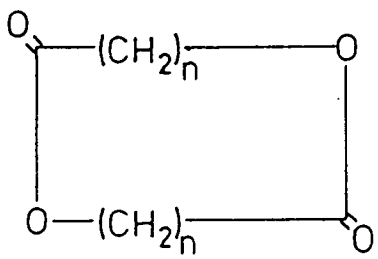
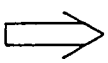
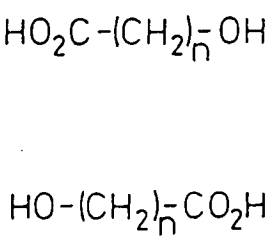
Figure 1







SYNTHETIC ROUTES TOWARDS COLLETOTRIENE A NOVEL FOURTEEN MEMBER MACRODIOLIDE

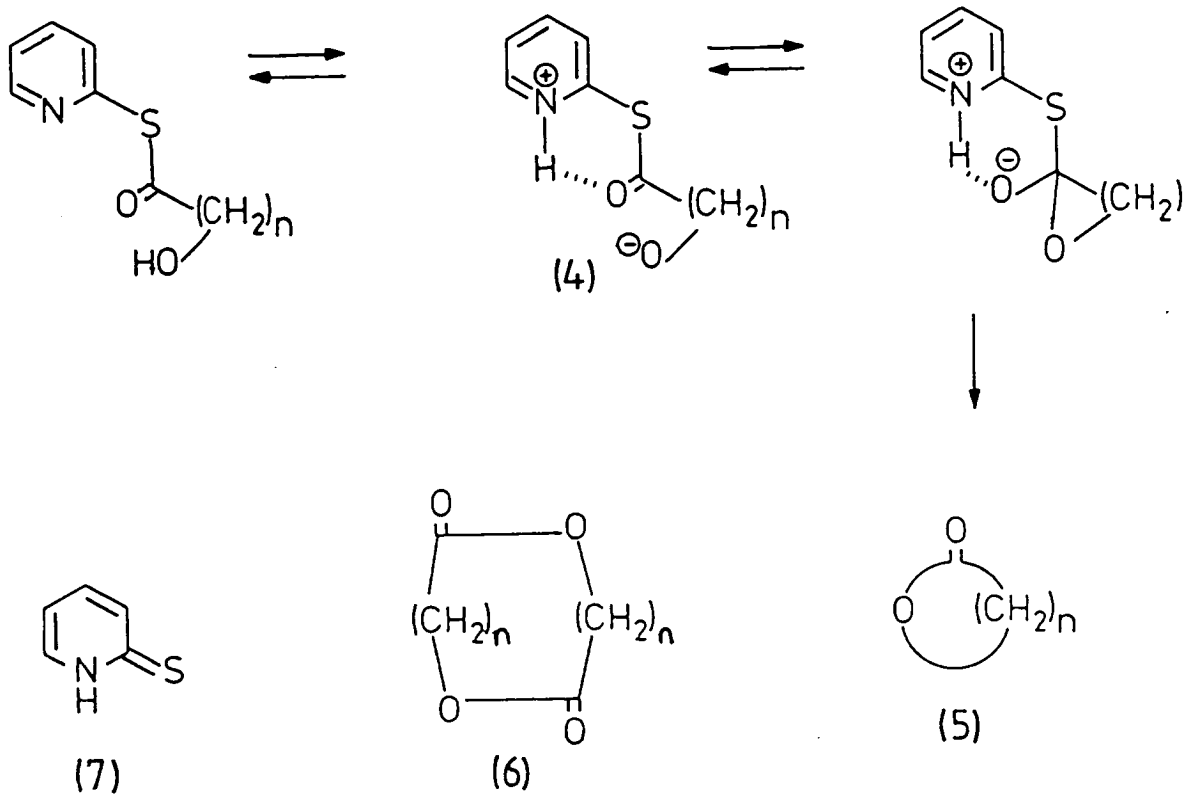
3.1 INTRODUCTION.

In the previous chapter the evidence for the involvement of the macrodiolide (1) in the biosynthesis of colletodiol (2) from *Cytospora Sp* ATCC 20502 was presented. In this chapter synthetic routes to this macrodiolide, for which we propose the trivial name colletotriene (1) are described. It is hoped that by synthesising colletotriene more evidence can be gained for the formation of the 4,5 diol system in colletodiol, *via* the epoxidation hydrolysis mechanism discussed in chapter 2. In order to discuss these synthetic routes fully the previous synthetic work on macrodiolides is discussed below.

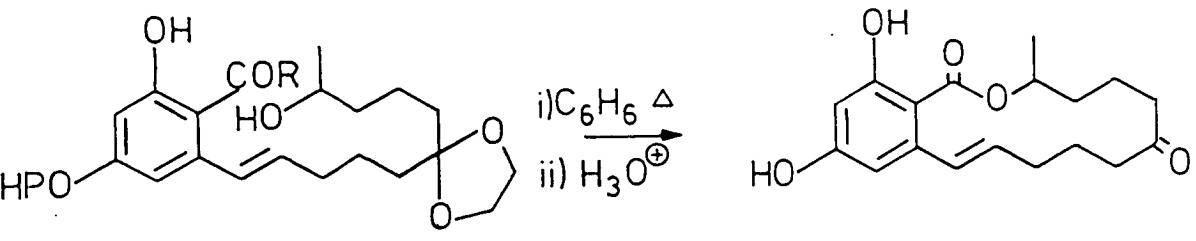
3.1.1. Macrolides and Macrodiolides

In recent years much attention has been focussed on the group of compounds known as the macrolides[1]. In general a macrolide is a molecule containing a large ring lactone. Systems with two ester linkages are called macrodiolides. Macrodiolides have the general formula shown in (3) with varying degrees of substitution, reduction and oxidation in the the straight chain moiety. Mainly due to their biological activity, many approaches have been made towards their synthesis. Generally this involves

Scheme 1



Scheme 2



the cyclisation of an open chain precursor, usually *via* a lactone forming step, although there are exceptions as discussed below.

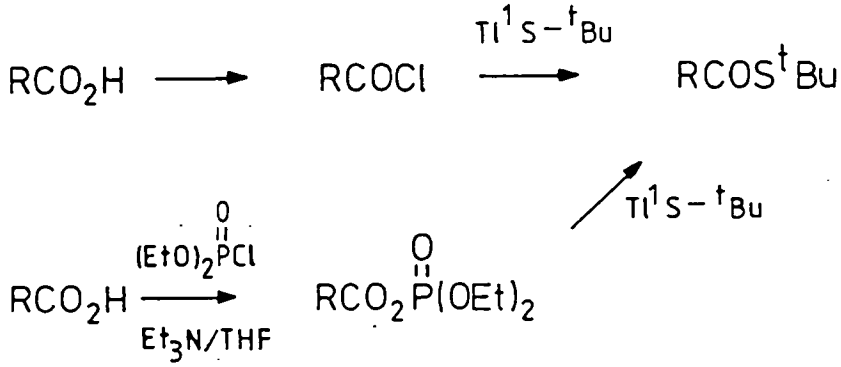
3.1.1.a. Macrocyclic Lactonisation

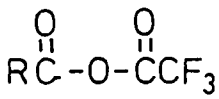
The most common approach to the synthesis of a macrodiolide is to disconnect at both ester linkages to give two hydroxy acid fragments. This is illustrated in figure 1. In the case of a symmetrical system it is common to synthesise the monomer and then form the ring in a dimerisation step, for which a number of methods have been developed.

Corey and Nicolaou[2] developed a method which is based on simultaneous activation of the acid and alcohol functions of a hydroxy acid, *via* a 2-pyridinethiol ester, as shown in scheme 1. The dipolar intermediate (4), generated by internal proton transfer cyclises to give the lactone (5), diolide (6) and also 2-pyridinethione (7). This method was used to cyclise a number of ω hydroxy acids to their corresponding lactones in excellent yield[3]. In each case the lactone was the major product.

This method has become known as the "double activation" method due to the simultaneous activation of the carboxyl and hydroxyl functions. Its use has largely been confined to lactones rather than diolides. Zearalenone (8) was hydrolyzed and protected to give the hydroxy acid (9). This was then activated as its 2-pyridinethiol ester (10) by reaction with 2,2'-dipyridylsulphide and triphenylphosphine[4]. Refluxing in benzene

Scheme 3





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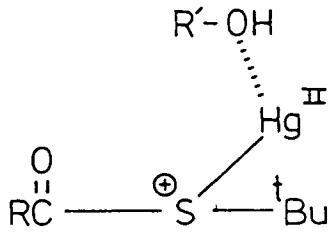

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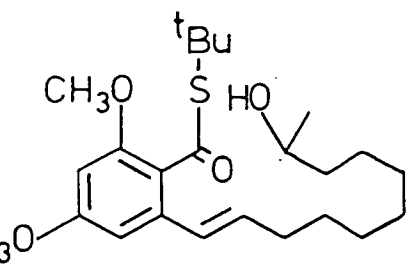
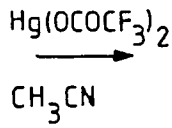
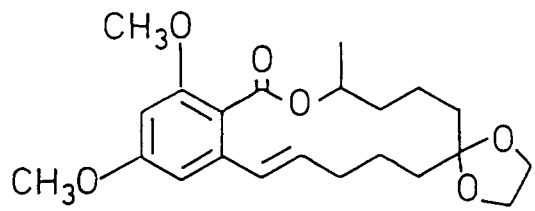
Scheme 4



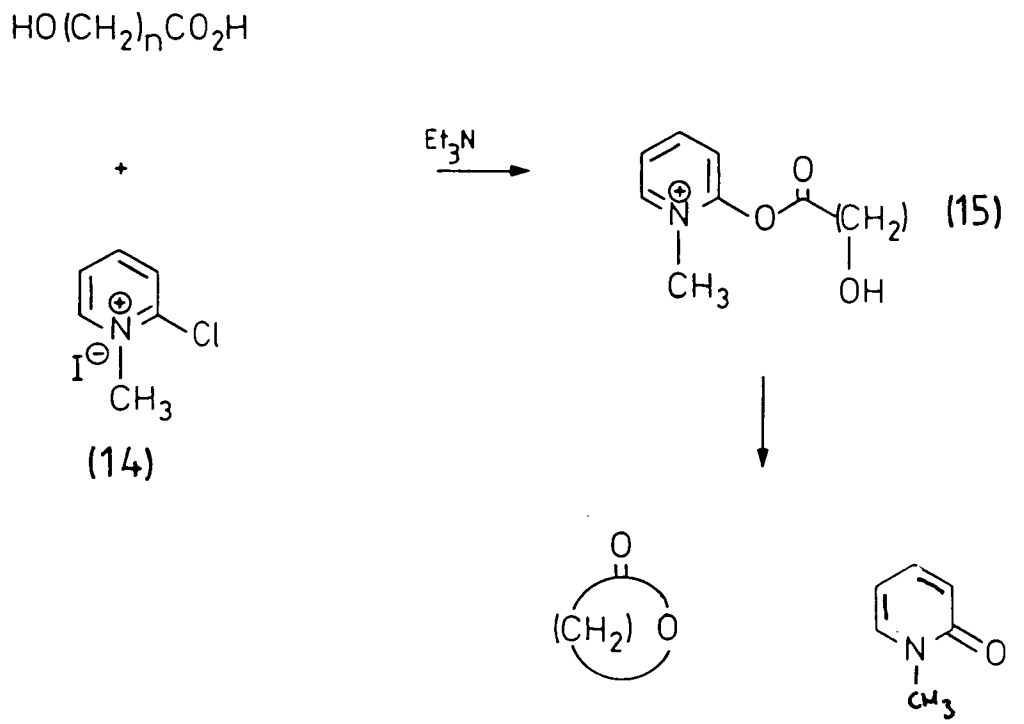
(12)



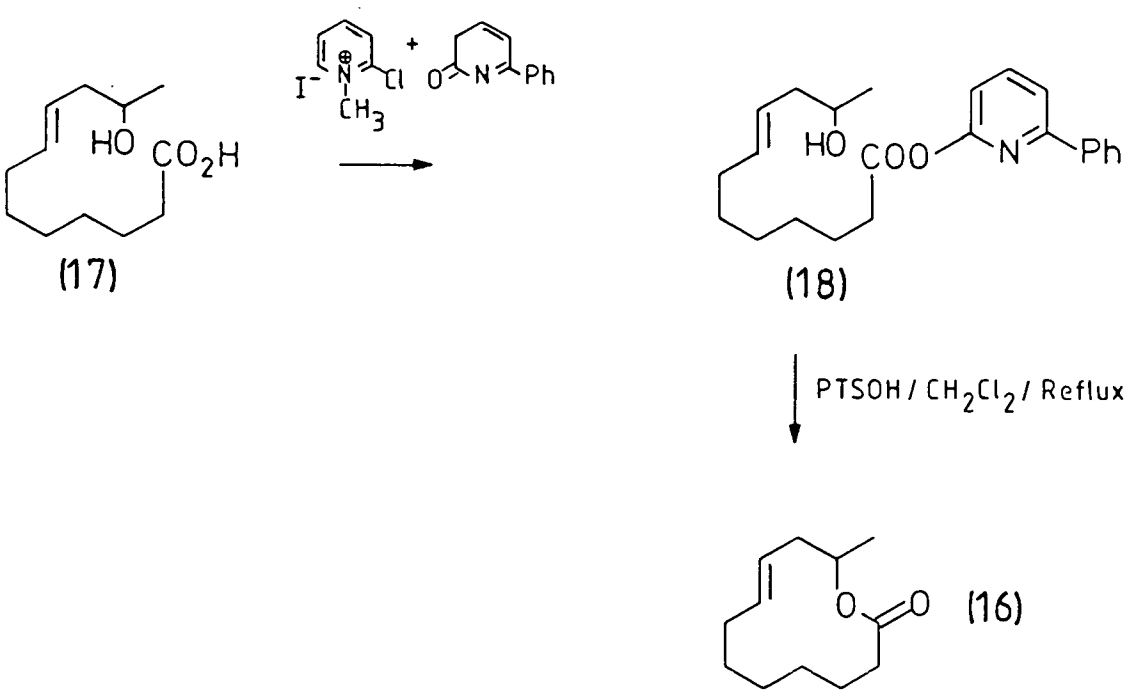


(13)

Scheme 5



Scheme 6



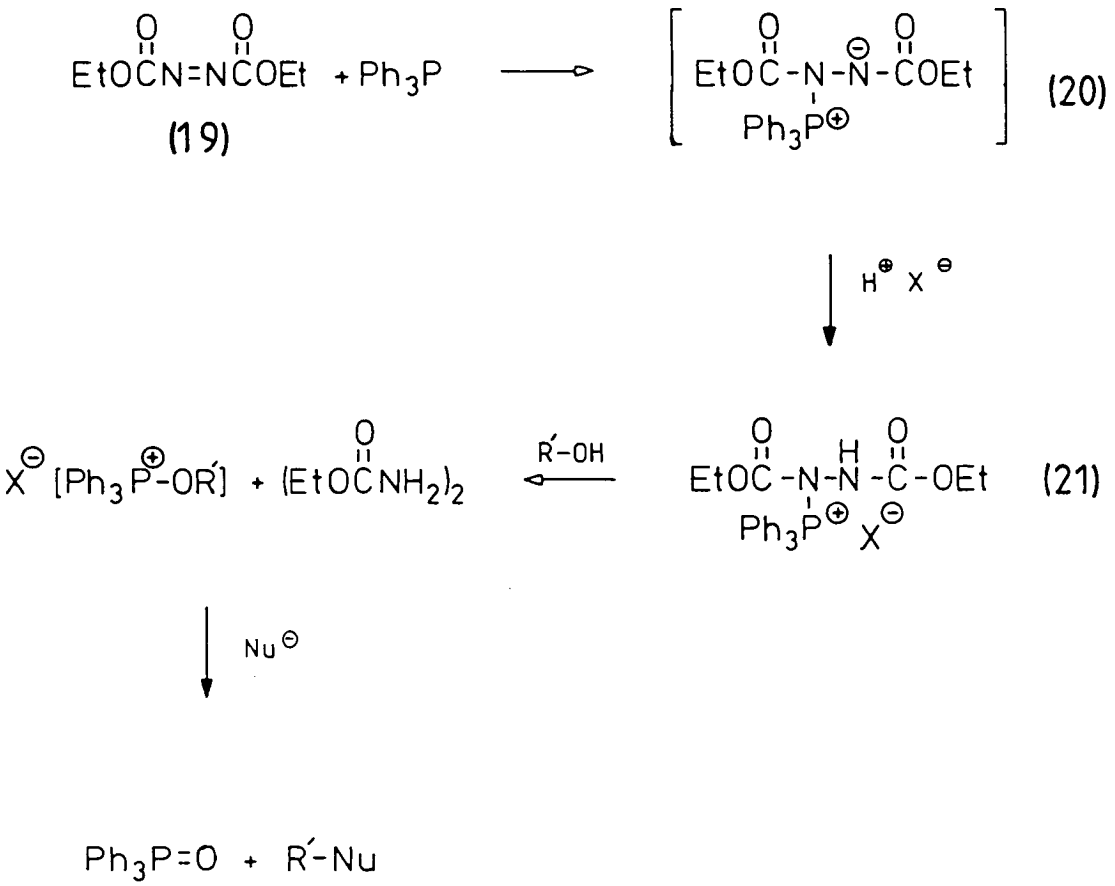
and removal of the protecting groups gave zearalenone[5] (8) in 75% yield (scheme 2).

An alternative method developed by Masamune *et al*[6] uses the S-t-butyl thiol esters of hydroxy acids, and mercuric trifluoroacetate as an activating agent. The S-t-butyl thiol esters are prepared from the corresponding acid chloride[7] and thallous-t-butylthioate. Alternatively a phosphorous mixed anhydride can be used instead of the acid chloride[8], as illustrated in scheme 3. Lactonisation of such systems occurs rapidly at room temperature. The exact role of the mercuric trifluoroacetate is not clear, but a mixed trifluoroacetic anhydride (10) or the mercury complex (11) have been suggested.

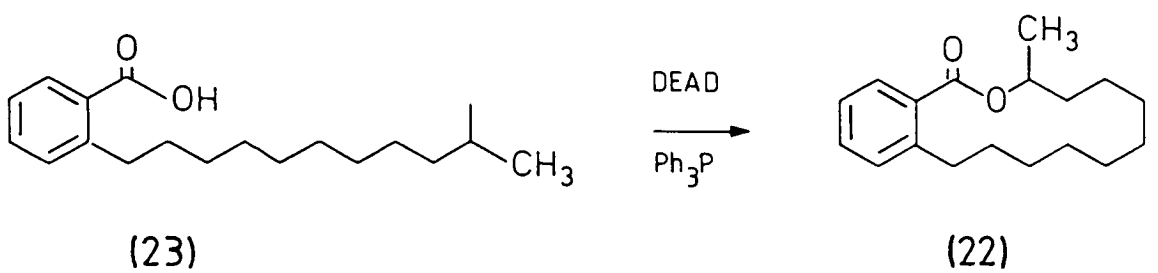
Using this method the *seco* ester (12) was cyclised to the ethylene ketal of methyl zearalenone (13)[8] in dilute acetonitrile at 25°C using two equivalents of mercuric trifluoroacetate (scheme 4).

A further method, closely related to the "double activation" method described above involves the use of 1-methyl-2-chloropyridinium iodide (14)[9]. The mechanism of the reaction with ω hydroxy acids is shown in scheme 5. Slow addition of the hydroxy acid to (14) in the presence of triethylamine in refluxing methylene chloride/acetonitrile results in the formation of the reactive species (15). The 1-methyl-2-pyridone acts as a better leaving group, and the reaction is driven to completion by the increase in entropy. Using a modification of this method, the lactone recifeiolide[10] (16) was cyclised from

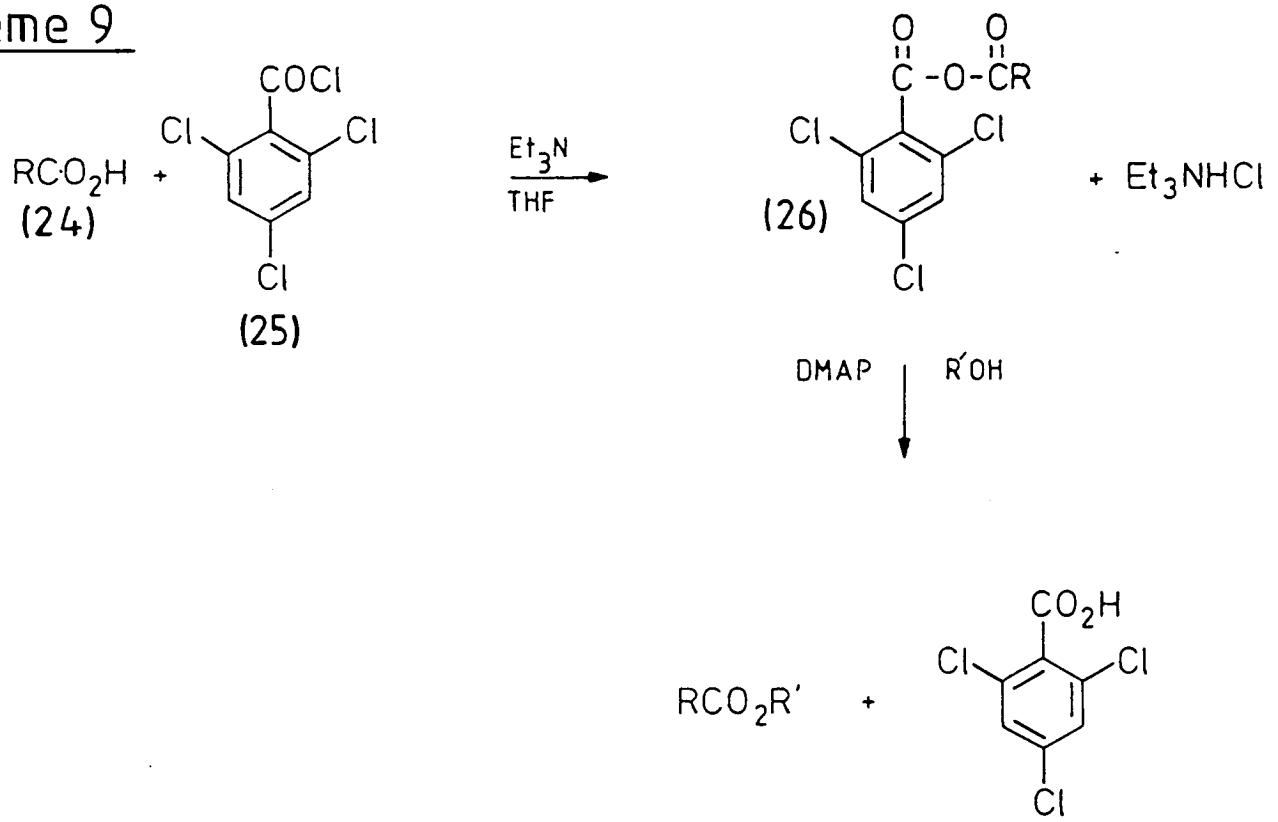
Scheme 7



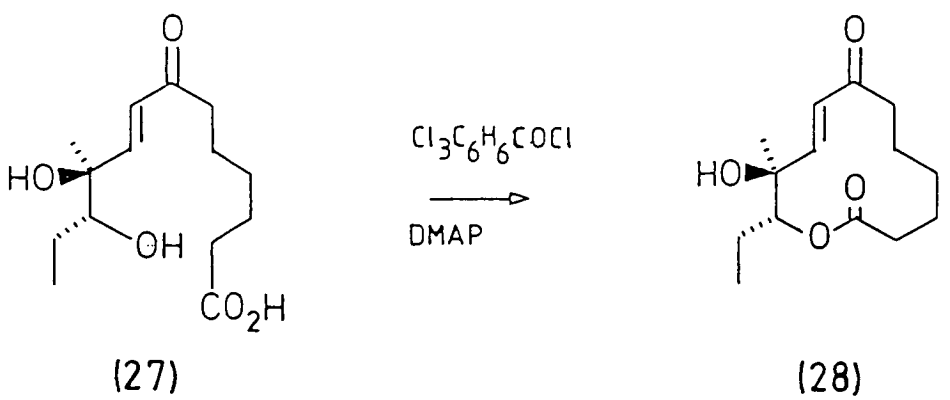
Scheme 8



Scheme 9



Scheme 10



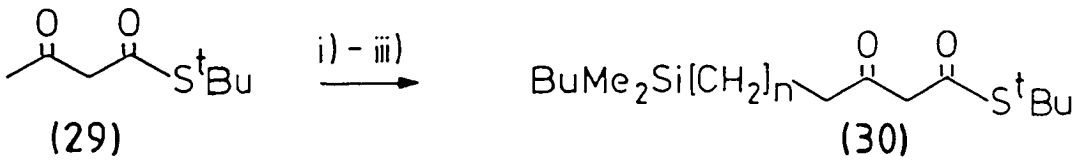
the hydroxy acid (17) *via* the intermediate (18) in 87% yield as shown in scheme 6.

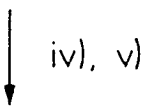
The Mitsonobu reaction of diethylazidodicarboxylate (DEAD) (19) and triphenylphosphine has found many uses in organic synthesis[11], and is used frequently in macrolide synthesis. The reaction is shown in scheme 7 and unlike the other lactonisation techniques described in this section, it proceeds with inversion of configuration at the hydroxyl carbon. The triphenylphosphine attacks the DEAD to form the dipolar intermediate (20). Protonation followed by treatment with an alcohol leads to the formation of an alkoxyphosphonium salt (21). This is rapidly followed by Sn2 displacement by a nucleophile Nu, to give the product. Using this method didehydroxyzearalenone (22) has been cyclised from the hydroxy acid (23)[12] as shown in scheme 8

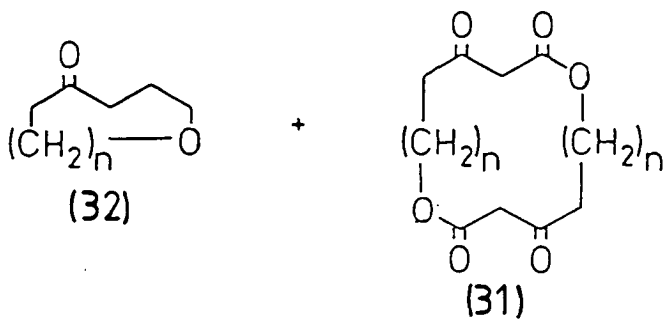
Another method developed by Yamaguchi[13] utilizes bulky mixed anhydrides as the leaving group and the catalytic activity of 4-dimethylaminopyridine (DMAP) in acyl transfer reactions[14]. The general outline of the method is shown in scheme 9. Reaction of an acid (24), with 2,4,6-trichlorobenzoylchloride (25) results in the mixed anhydride (26), which is then reacted with the appropriate alcohol in the presence of DMAP to give the ester. Many other substituted benzoyl chlorides were investigated[13], but none showed the activity of (25). Using this method the hydroxy acid (27) was successfully cyclised to (DL)-2,4,6-tridemethyl-3-deoxymethynolide (28) in 40% yield (scheme 10)[13].

Ley has used a thiol ester method (scheme 11)[15], similar to the Masamune[6] method. Treatment of t-butylacetoacetate

Scheme 11

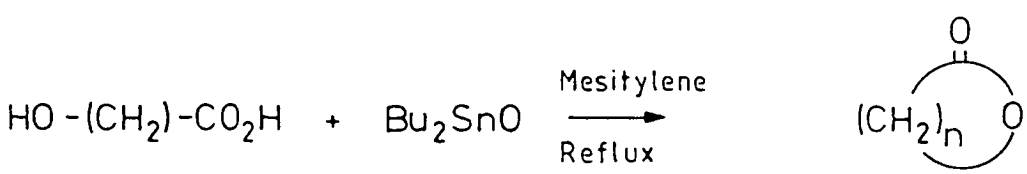






i) NaH / DME
ii) $^{n}BuLi$
iii) $^{t}BuMe_2SiO[CH_2]_nI$
iv) HF
v) $CuOCOCF_3$

Scheme 12

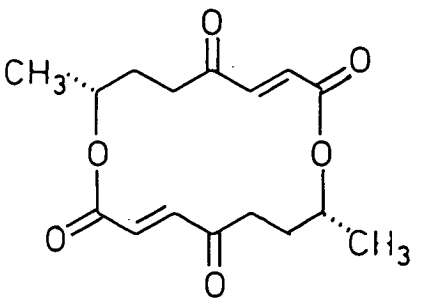


(29) with sodium hydride and then with n-butyllithium gives the dianion which is then treated with a t-butyldimethylsilyl protected iodo- alcohol to give the γ alkylated species (30). Treatment with cuprous trifluoroacetate in methylene chloride solution containing a sodium hydrogen phosphate buffer to give a mixture of the macrodiolide (31) and the lactone (32). The use of the cuprous salt is reported to be superior to the corresponding mercurous salt.

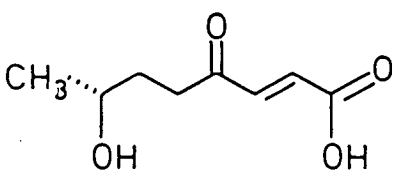
The methods described above are the most common ones in use. None are universal and each finds its own particular application. There are however other methods which do not possess even this generality. Imidazole active esters have been used[16] and an example of this is described below, and a tin mediated esterification[17], which has been used on ω hydroxy acids is shown in scheme 12. The use of some of the methods described above is discussed below in relation to the synthesis of some of the known macrodiolides.

3.2. Macrodiolide synthesis

The synthetic work that will be described later concerning the synthesis of colletotriene (1) was based to a large extent on the methods used to synthesise some of the other known macrodiolides. For this reason some of the more relevant synthetic work in this field is described below.

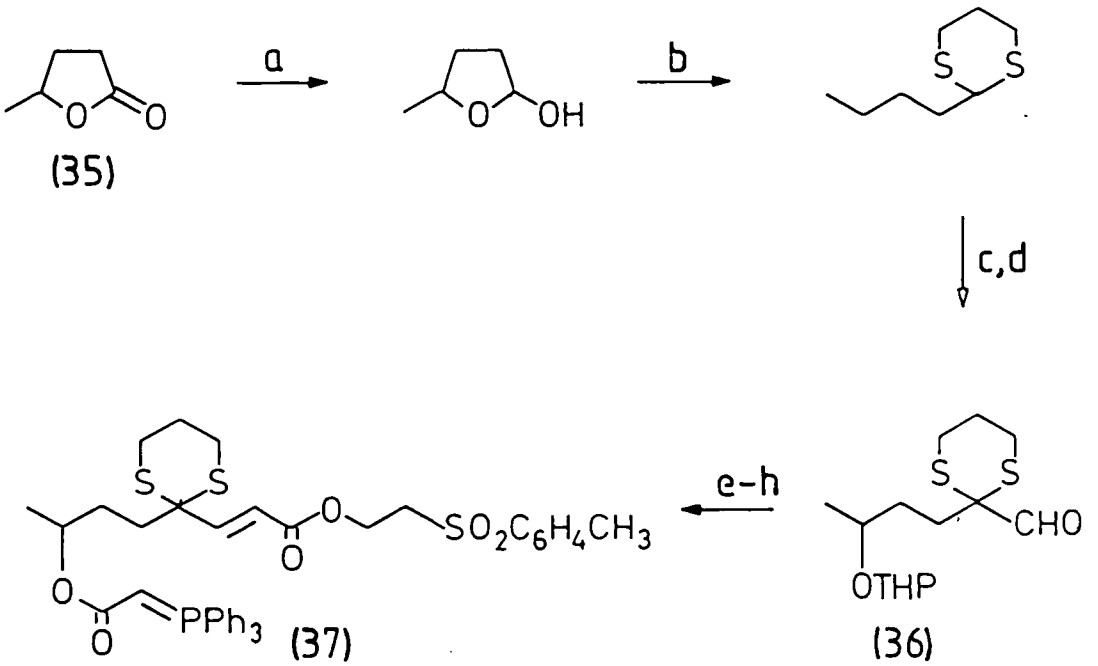


(33)



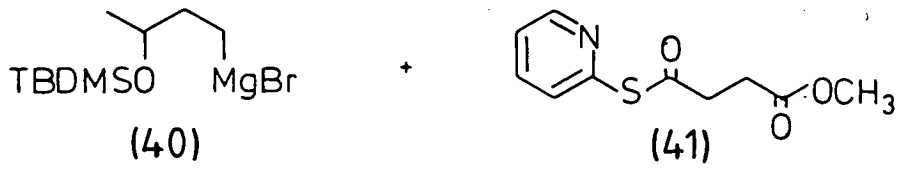
(34)

Scheme 13

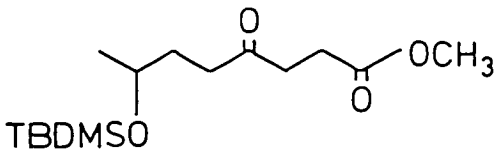


a) $NaAlH_4$
b) $HS(CH_2)_3SH / BF_3$
c) DHP/PTSA
d) nBuLi / HCOOEt
e) $(C_6H_5)_3P{=}CHCO_2(CH_2)_2SO_2C_6H_4CH_3$
f) $H_3O^{\oplus}$
g) $ClCOCH_2Br$
h) $(C_6H_5)_3P$
i) (36)
j) $H_3O^{\oplus}$
k) DBU
l) Carbonyldiimidazole (34)
m) DBN
n) $NCS/AgNO_3$

Scheme 14

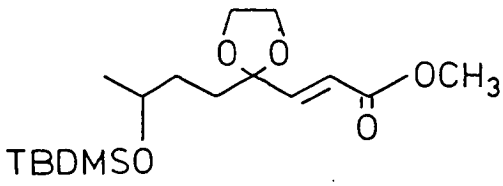


(40) (41)

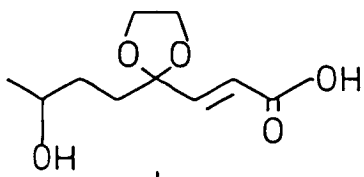


(42)

a - d

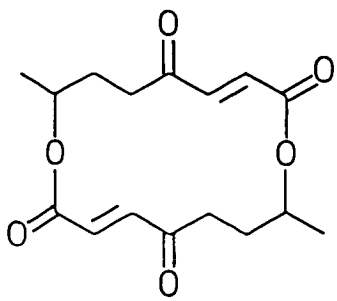


e, f



(43)

g, h



(33)

a) $(CH_2OH)_2$
b) LDA
c) C_6H_5SeBr
d) H_2O_2
e) Bu_4NF
f) KOH
g) DEAD/$(C_6H_5)_3P$

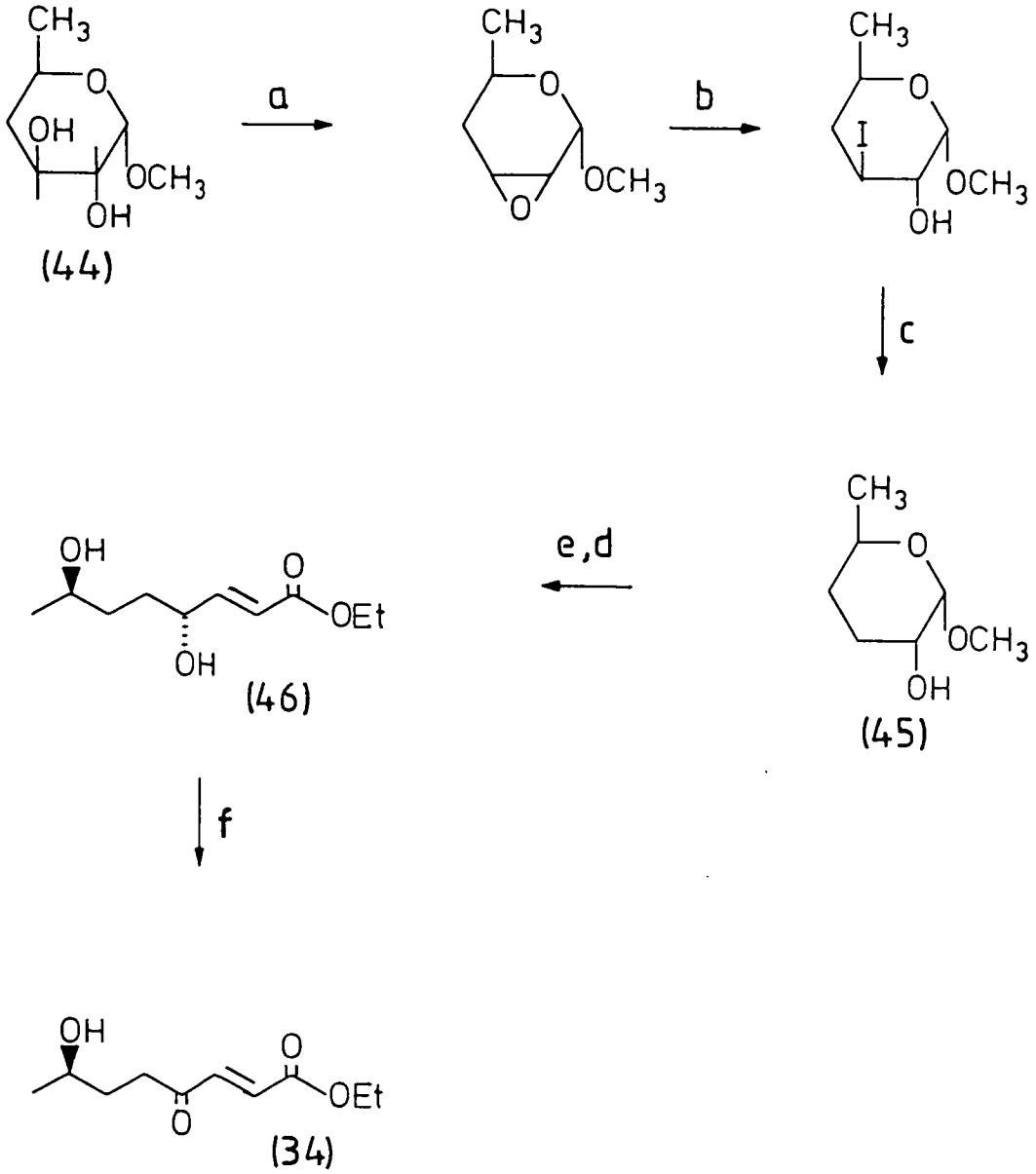
3.2.1.a. Pyrenophorin.

Pyrenophorin (33) is the parent structure of the sixteen member macrodiolides. To date twenty three syntheses have been reported[18]. Most of the routes involve the synthesis of some suitably protected form of the monomer (34), which is then cyclised and deprotected to give (33). The most common method of lactonisation is the Mitsonobu method[11] described above. Some aspects of these studies are described below.

The first synthesis of Raphael *et al*[16], which is outlined in scheme 13 started from γ valerolactone (35) which was converted to the key aldehyde (36). The chain was then extended in a Wittig reaction and then modified to give the stabilised ylid (37). Reaction of (36) with (37) gave the acyclic precursor (38). The hydroxyl group was then deprotected and the system activated as its imidazole ester [19] by the method of Staab[19], treatment of which with DBN. in benzene, followed by deprotection gave pyrenophorin (33).

The route of Gerlach *et al*[20] is shown in scheme 14 and is a good example of the dimerising cyclisation approach to pyrenophorin using the Mitsonobu method[11]. Reaction of the Grignard reagent (40) with the ester (41) gives the key intermediate (42). Protection of the C-4 carbonyl group allows the introduction of the α,β unsaturated bond *via* a selenation elimination reaction to give the protected monomer (43), which was then cyclised under Mitsonobu conditions[11], subsequent deprotection giving pyrenophorin (33).

Scheme 15



DEAD/$(C_6H_5)_3P$

MgI_2

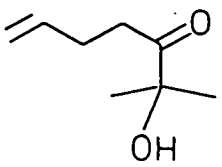
$LiAlH_4$

$H_3O^{\oplus}$

$(C_6H_5)_3P{=}CHCO_2Et$

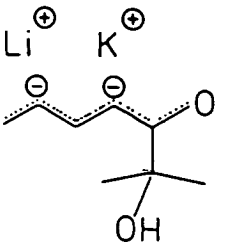
MnO_2

Scheme 16

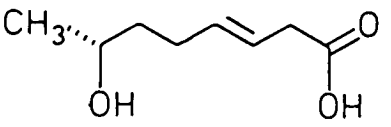


(48)

a, b

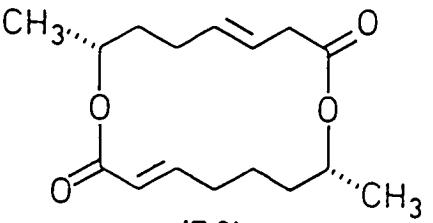


c - e



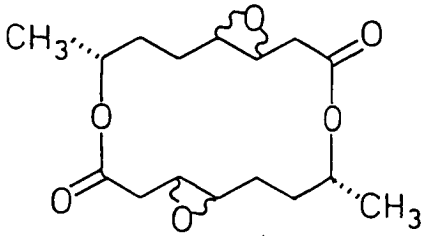
(49)

d

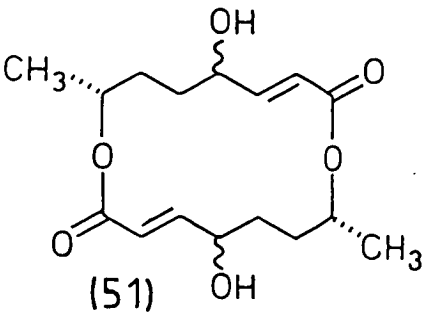


(50)

e

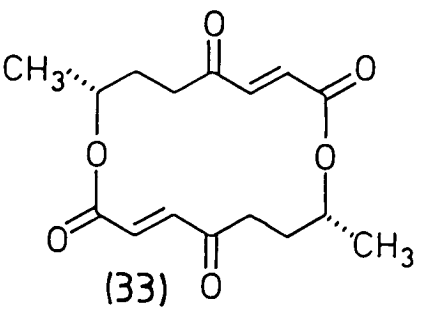


f



(51)

g



(33)

a) KH
b) nBuLi
c) (S)-Propylene Oxide (47)
d) NH_4OHCl
e) HIO_4

d) DEAD / $(C_6H_5)_3P$
e) MCPBA
f) LDA
g) PCC

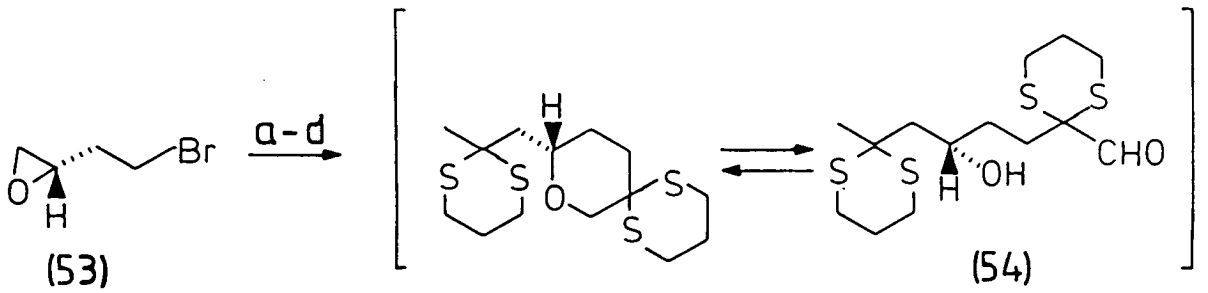
Both of the methods described above gave pyrenophorin as a (+/-)/*meso* mixture. In a recent synthesis Mitsonobu *et al*[21] used a modified sugar to produce the monomer (34). The hexopyranoside (44) was converted as shown in scheme 15 to (45) which was then extended in a Wittig reaction to give the diol (46) which was subsequently oxidised to the pyrenophorin monomer (34), which could easily be converted to pyrenophorin (33).

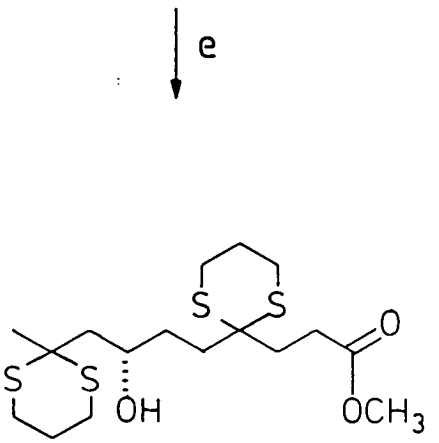
In a study aimed at developing a short but enantiomerically pure synthesis of pyrenophorin, Seebach *et al*[18] examined the possibility of introducing the C-4 carbonyl functions after the sixteen member ring had been completed. This is outlined in scheme 16. The starting material (*S*)-propylene oxide (47) was combined with the dianion of (48) and converted to the hydroxy acid (49) *via* the oxime and a Beckmann rearrangement, using periodate cleavage, followed by a dimerising cyclisation[11] to give the macrodiolide (50). Treatment *m*-chloroperbenzoic acid gave a mixture of diastereomeric diepoxides, base catalysed rearrangement gave the pyrenophorols (51) which were subsequently oxidised to pyrenophorin (33).

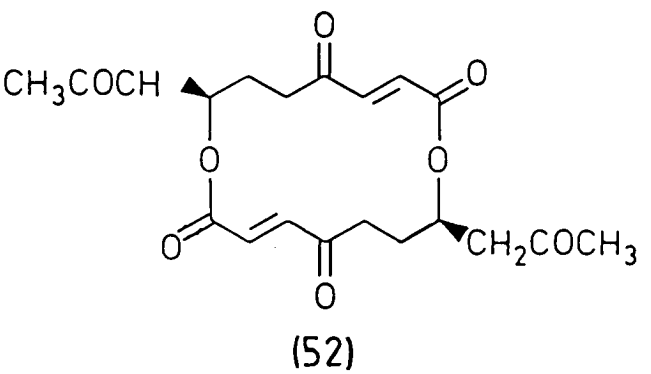
3.2.1.b. Vermiculine

Vermiculine (52) is closely related to pyrenophorin (33). It has been the target of several synthetic routes[18], again these routes have been based on a dimerising cyclisation approach[11]. The route used by Seebach *et al*[22] is outlined in scheme 17. The chiral starting material (53) was synthesised from

Scheme 17

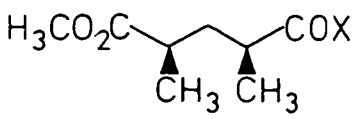






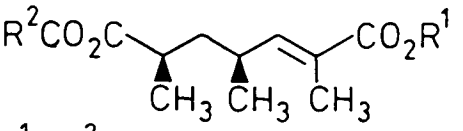
a HS $(CH_2)_3SH$ / n BuLi
b 2-Methyl 1,3 Dithiane / n BuLi
c nBuLi / TMEDA
d DMF
e $(C_6H_5)_3P{=}CHCO_2CH_3$
f LiOH / CH_3OH / H_2O
g $^{\ominus}OH$
h DEAD / $(C_6H_5)_3P$
i HgO / $BF_3 \cdot Et_2O$

Scheme 18



X = OCH_3 (58)
X = OH
X = Cl
X = H

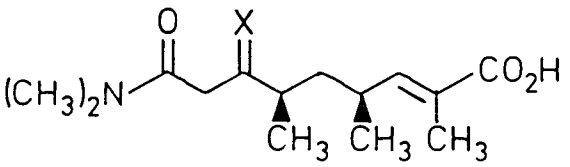
a - e



$R^1 = R^2 = CH_3$ (59)
$R^1 = R^2 = H$
$R^1 = H$ $R^2 = CH_3$

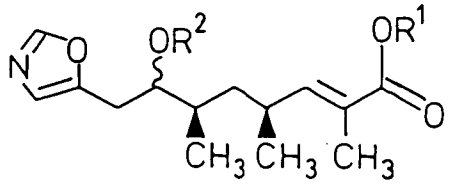
CH_3OH/ TosOH
Chymotrypsin Ester Hydrolysis
$(COCl)_2$
Pd/C / H_2
$(C_6H_5)P{=}CHCO_2CH_3$

f - h



X = O (60)
X = H/OH (61)

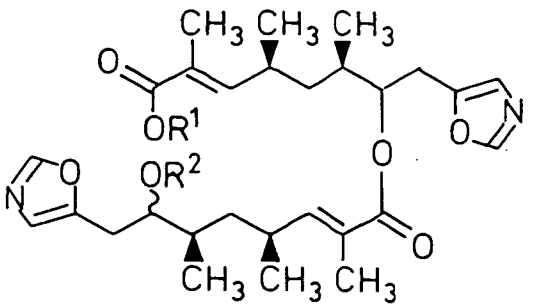
i,j



(62) $R^1 = R^2 = H$
(63) $R^1 = CH_2CCl_3$ $R^2 = H$
(64) $R^1 = H$ $R^2 = COCH_3$

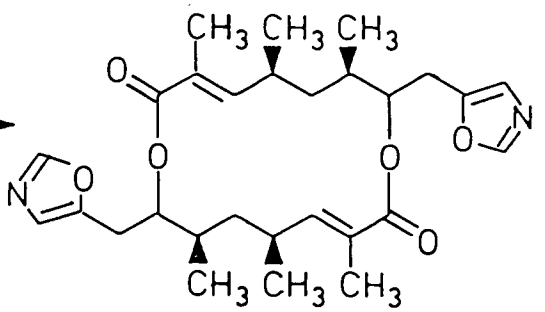
f) CH_3OH/NaOH
g) $(CH_3O)_2C(CH_3)_2$ / CH_3O/HCl
h) LDA/ $CH_3CON(CH_3)_2$
i) $NaBH_4$/EtOH
j) n BuLi/CH_3NC

k - m



(65) $R^1 = CH_2CCl_3$ $R^2 = COCH_3$

o - p



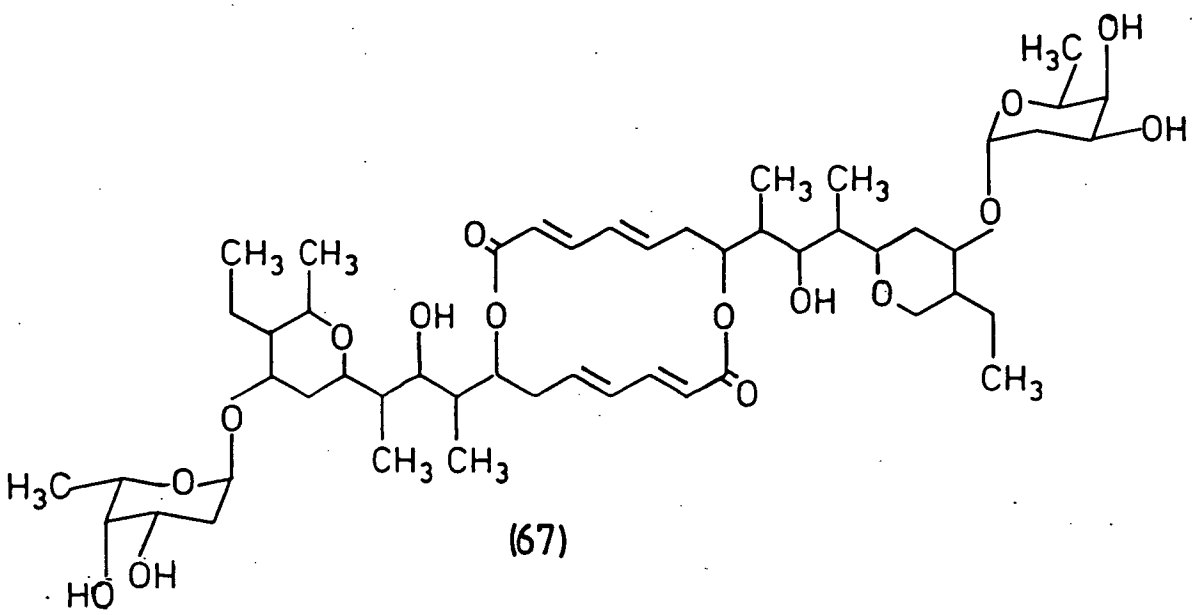
(57)

k) DCC/DMAP/Cl_3CCH_2OH
l) DMAP/Ac_2O
m) $Cl_3C_6H_2CO_2H$/DMAP
n) AcOH/Zn
o) CH_3OH /K_2CO_3

malic acid[23]. Using the dithiane method, three successive C-C bond forming reactions were carried out in a one pot method. At low temperature the anion of 1,3-dithiane reacts selectively with (53) to displace bromine, which is in turn opened at higher temperature with the anion of 2-methyl-1,3-dithiane, ubsequent formylation gives (54) which is combined in a Wittig reaction with methoxycarbonylmethylene-triphenylphosphorane (55) to give the protected hydroxy ester (56). The ester was then hydrolysed and cyclised under Mitsonobu conditions[11], ubsequent deprotection gave (*S,S*) vermiculine (52) in 60% yield.

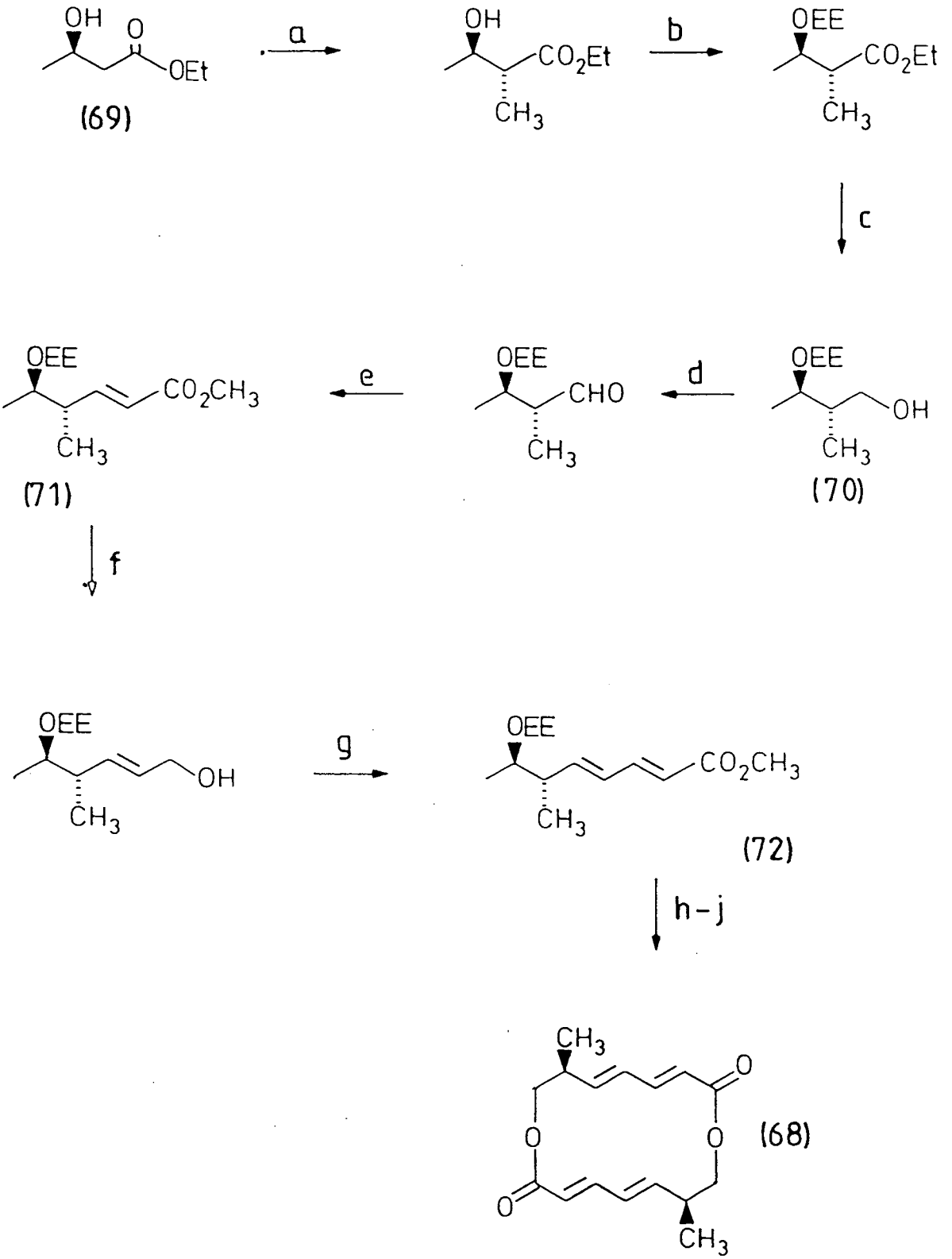
3.2.1.c. Congloblatin

Congloblatin (57) is the latest of the pyrenophorin group to be reported[24], and to date only one synthesis has been achieved and this is shown in scheme 18.[25] The chiral starting material (58)[26] is extended first by a Wittig reaction (59) and then by reaction with the enolate of dimethylacetamide to give (60), subsequent reduction gave the hydroxy acid (61). Treatment of (61) with excess lithiated methyl isocyanate[27] gave (62) as an inseparable mixture of diastereomers. Dimerising cyclisation of (62) could not be brought about and so the two complementary fragments (63) and (64) were prepared and coupled in a stepwise fashion using the Yamaguchi method[13] with pyrrolodinopyridine as the acyl transfer agent. This gave the ester (65) and subsequently the macrodiolide (66). Deprotection gave congloblatin as a mixture of diastereomers, which were separated



(67)

Scheme 19



a) LDA/CH_3I
b) EE
c) $LiAlH_4$
d) PCC
e) $(C_6H_5)_3P{=}CHCO_2CH_3$
f) DIBAL
g) PCC
h) $(C_6H_5)_3P{=}CO_2CH_3$
i) $H_3O^{\oplus}$
j) $Cl_3C_6H_2COCl$/DMAP

by chromatography, the natural isomer being isolated in 10% yield.

3.2.1.d. Elaiophylin

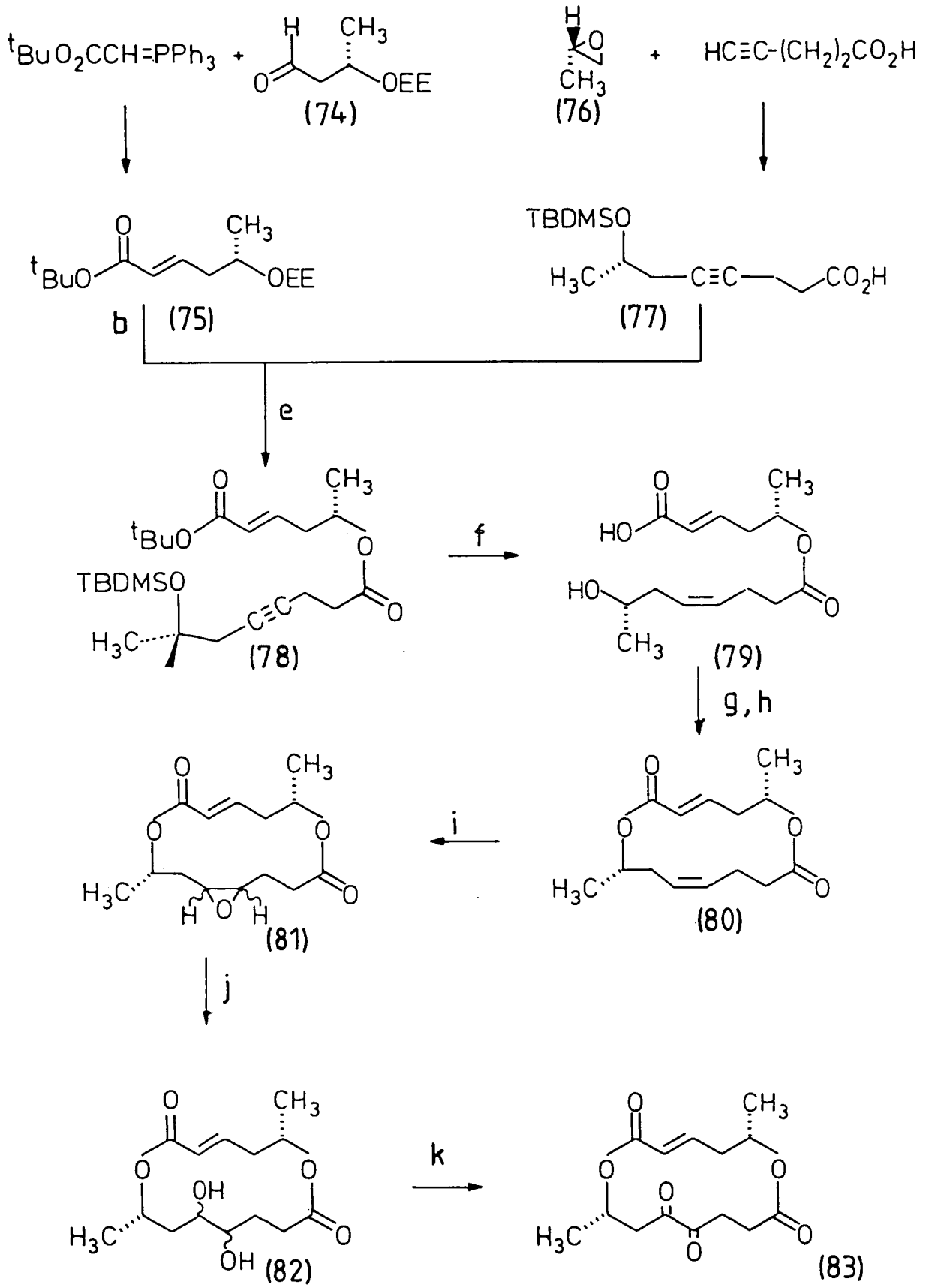
Elaiophylin (67)[28] also known as azlomycin B has not as yet been completely synthesised. However the synthesis of the central pyrenophorin type ring (68) has been achieved and is outlined in scheme 19. (*R*)-Ethyl-3-hydroxybutyrate (69) is methylated, protected and reduced to give the alcohol (70). This is then oxidised to the aldehyde and extended in a Wittig reaction to give (71). This sequence is then repeated in a milder fashion to give the protected ester (72). Deprotection and hydrolysis of (72) gave the free hydroxy acid (75), which was subjected to dimerising cyclisation using Yamaguchi conditions[13] to give (68).

3.2.1.e. Grahamimycin A_1

Grahamimycin A_1 (73) is a fourteen member unsymmetrical macrodiolide. The natural isomer has the (*R,R*) stereochemistry (99), there are two routes to the unnatural (*S,S*) isomer (83) and one to the (*R,R*) isomer.

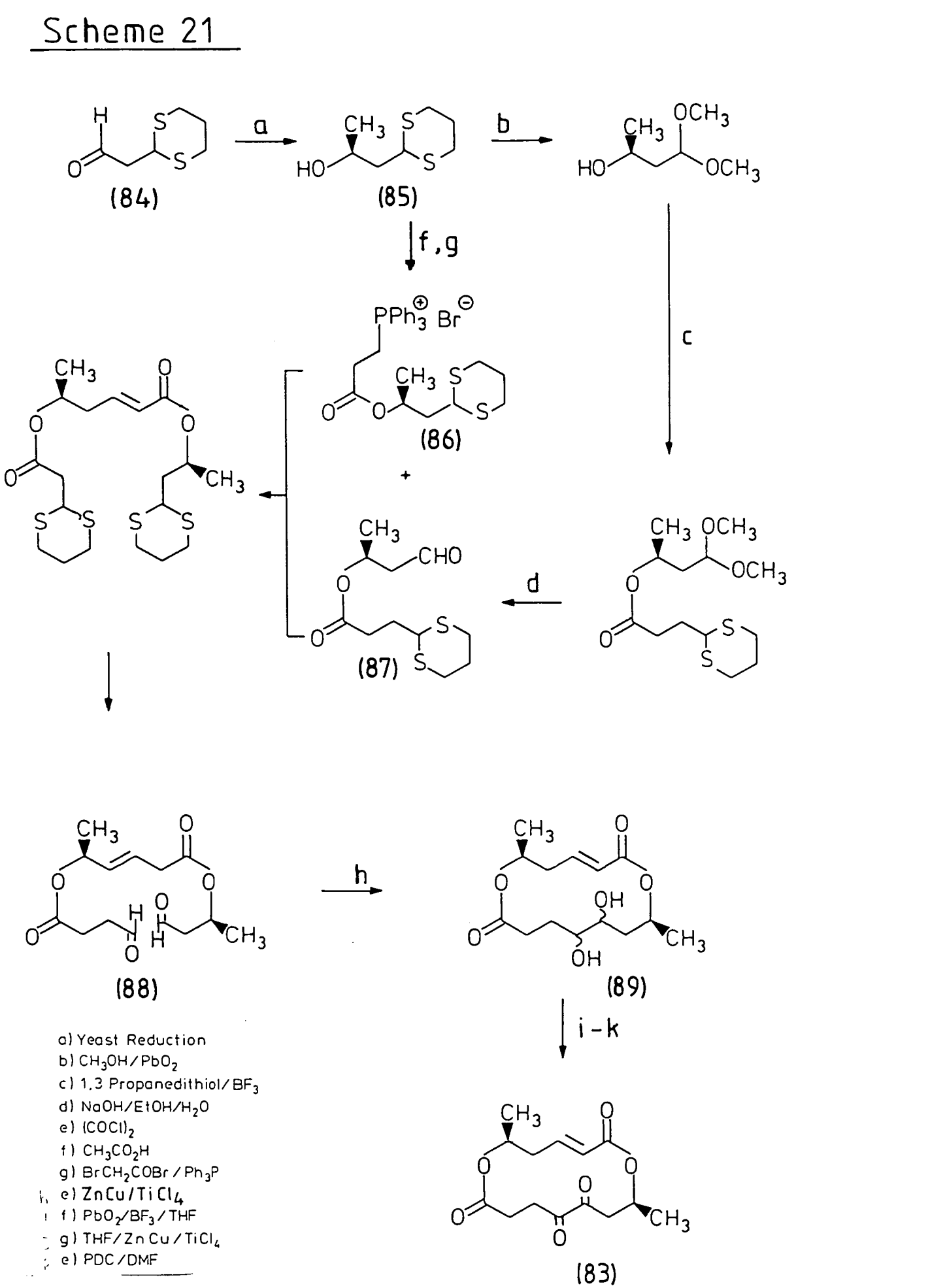
Scheme 20 outlines the approach of Seebach *et al*[29] *to the* (*S,S*) isomer. The aldehyde (74) was extended *via* a Wittig reaction to give the protected C_6 fragment (75) directly. The C_6 fragment was formed from reaction of (*S*)-propylene oxide (76) and

Scheme 20



a) DMF/Reflux
b) $THF/HClO_4$
c) nBuLi/HMPT
d) TBDMSCl/DMF/Imidazole
e) DCC/DMAP
f) H_2/Lindlar
g) CF_3CO_2H
h) Et_3N/2-Chloro-1-Methyl-Pyridinium Tosylate/CH_3CN
i) MCPBA/$CHCl_3$
j) $THF/HClO_4$
k) PCC/CH_2Cl_2

Scheme 21



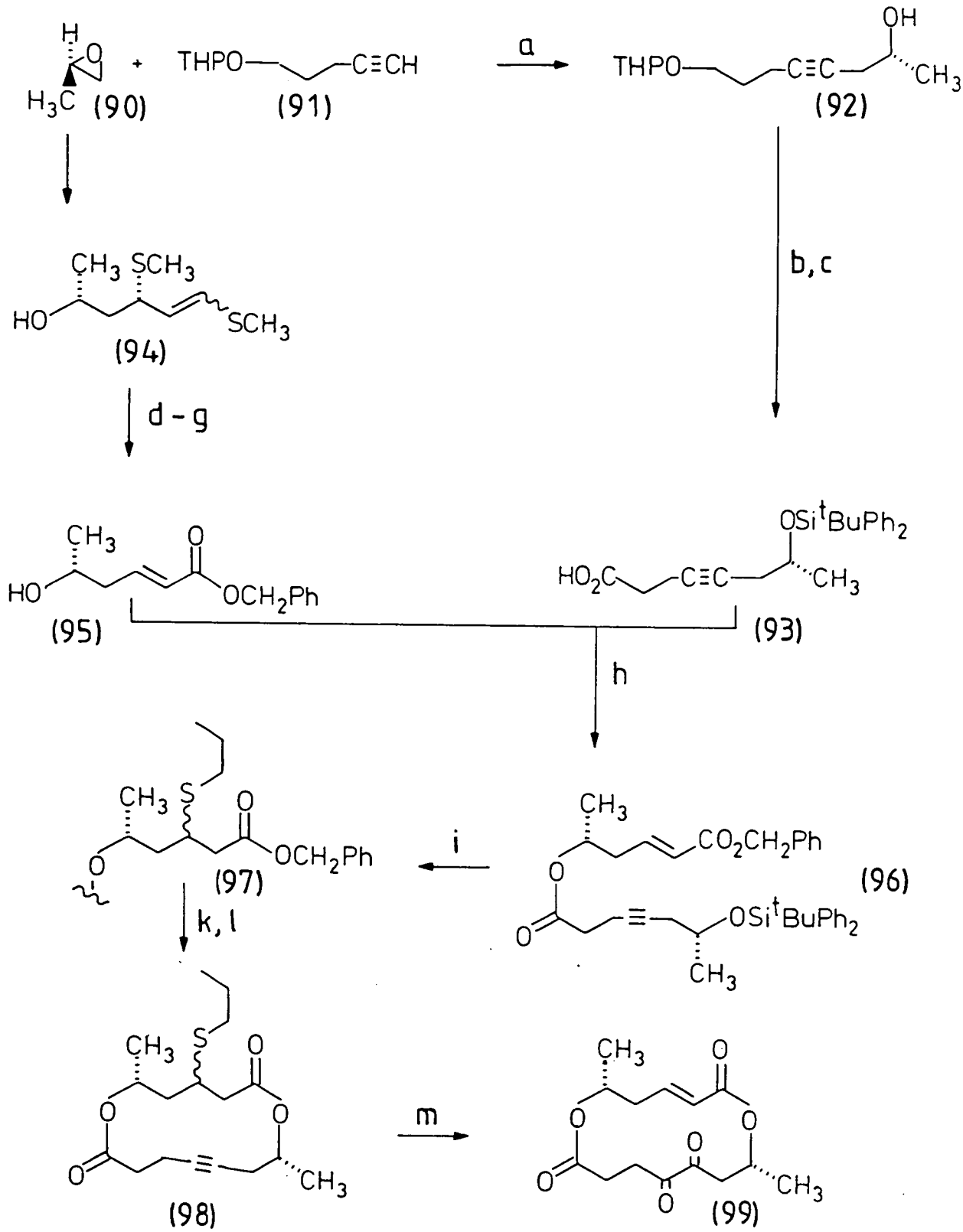
a) Yeast Reduction
b) CH_3OH/PbO_2
c) 1,3 Propanedithiol/BF_3
d) $NaOH/EtOH/H_2O$
e) $(COCl)_2$
f) CH_3CO_2H
g) $BrCH_2COBr / Ph_3P$
e) $ZnCu/TiCl_4$
f) $PbO_2/BF_3/THF$
g) $THF/ZnCu/TiCl_4$
e) PDC/DMF

the dianion of 4-pentynoic acid, subsequent protection giving the C_6 fragment as its free acid (77). Coupling of (75) and (77) gave the *seco* ester (78). Catalytic hydrogenation of (78) over Lindlars catalyst, and deprotection gave the *seco* acid (79), which was cyclised under Mukaiyama conditions[8] to give the macrodiolide (80). Epoxidation of this gave a 1:8:8:1 mixture of diastereomers (81) which were hydrolysed to give the diols (82), and then oxidised to (83) in 16% yield.

In a somewhat different approach Ghirringhelli[8c] synthesised (83) by stepwise formation of the ester linkages, followed by ring closure to form a 4,5 diol system, which was then oxidised to the product. The chiral centre was introduced *via* reduction of 1,3-dithian-2-yl acetone (84) to (*S*)-1-(1,3-dithian-2-yl)-2-hydroxypropane (85) by fermenting bakers yeast. This was then converted to the ylid (86) and also to the aldehyde (87). Combination of (86) and (87) in a Wittig reaction gives the protected *seco* ester, deprotection gives the dialdehyde (88) and a pinacolic coupling gave a mixture of diastereomeric diols (89). Oxidation gave (83) in 20% yield. (scheme 21).

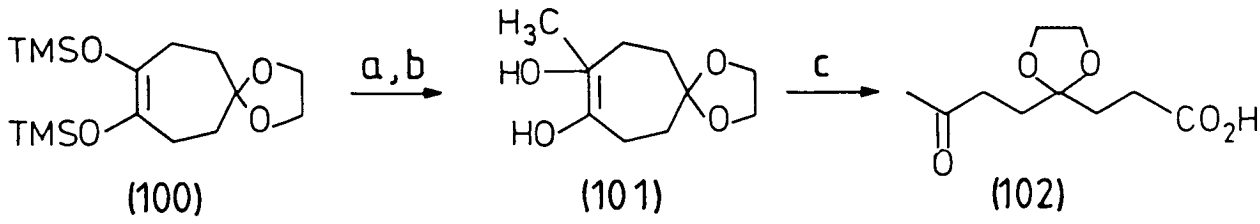
The natural isomer of grahamimycin A_1 (99) has been synthesised by Hillis *et al*[8d] and is shown in scheme 22. The chiral centre of both fragments was introduced from (*R*)-propylene oxide (90). Reaction of (90) with the anion of 1-(2-tetrahydropyranyl)-4-pentyne (91) gave the alcohol (92), protection of the secondary alcohol and oxidation of the primary alcohol gave the C_6 acid (93).

Scheme 22

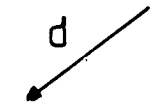


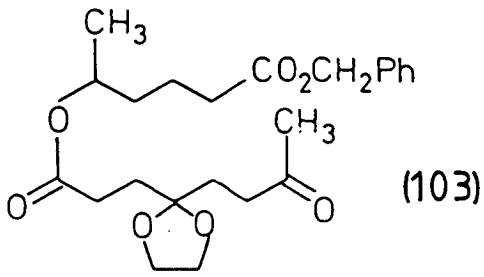
a) n BuLi / THF
b) DMF / $^{t}BuPh_2SiCl$ / Imidazole
c) Jone's Reagent
d) 1,3 bis(Methylthio)allyllithium / THF
e) $^{t}BuOH$ / 2-Methyl-2-butene / $NaClO_2$
f) $NaOH / H_2O$
g) Ph_2CN_2 / EtOAc
h) DCC / DMAP
i) $CH_3CH_2CH_2SH$ / THF
j) $HF / CH_3NO_2 / CH_3CO_2H$
k) 2-Pyridylthiochloroformate / Et_3N
l) $CH_3I / NaBF_4 / CH_2Cl_2$
m) OsO_4 / Pyr

Scheme 23

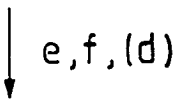


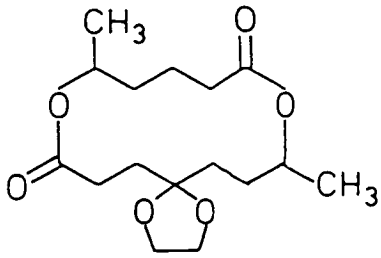
(100) (101) (102)



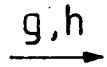


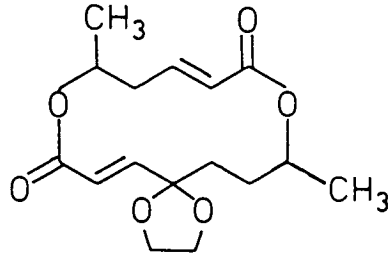
(103)



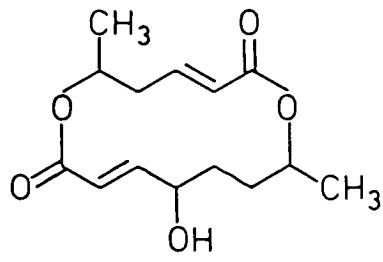


(104)





(105)



(106)

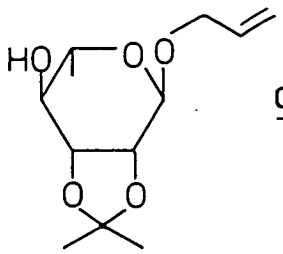
a) THF/Reflux
b) CH_3Li/THF
c) $Pb(OAc)_4$
d) $CH_3CHOH(CH_2)_3CO_2CH_2Ph$/Diethyl Phosphochloridate/Et_3N
e) $NaBH_4/C_6H_6$
f) H_2/PdC
g) PhSeBr/LDA / THF
h) H_2O_2/AcOH
i) CF_3CO_2H/CH_2Cl_2
j) $NaBH_4$ / CH_3OH

Similarly addition of (90) to a 1,3-bismethylthioallyllithium solution yielded (94) as an isomeric mixture. This was subsequently converted to the the C_6 fragment (95). The two fragments (93) and (95) were then esterified to give the *seco* ester (96), and the α,β-unsaturated bond protected by 1,4 addition of 1-propanethiol (97), the ring was then formed using the Corey Nicolaou method[83] (98). Direct oxidation of the triple bond and reformation of the double bond gave (*R,R*)-grahamimycin A_1 (99) in 10% yield.

3.2.1.f. Colletalol.

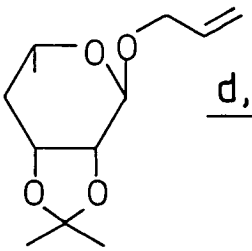
Colletalol (106) is closely related to colletodiol (2) and only one synthesis is reported[82] as shown in scheme 23. The TMS ether (100) was prepared by a modified acyloin condensation, hydrolysis and reduction giving the vicinal diol (101). Oxidative cleavage and subsequent reduction gave the C_6 hydroxy acid (102). Esterification with the appropriate hydroxy ester , gave the *seco* ester (103), which was subsequently converted to the macrodiolide (104). The *trans* double bonds were then introduced *via* a selenation elimination sequence to give (105) which was deprotected and reduced to give (+/-) Colletalol (106) in low yield.

Scheme 24



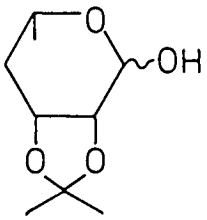
(107)

a-c

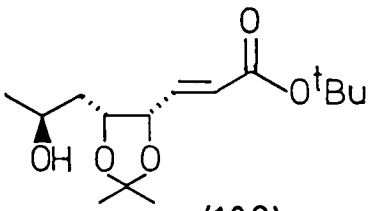


(108)

d,e

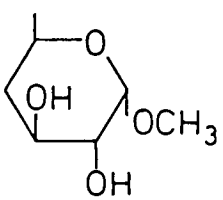


a) NaH/CS_2/ Imidazole/ CH_3I
b) Bu_3SnH / Tol/Reflux
c) $RhCl(Ph_3P)_3$ / DABCO
d) $HgCl_2$/Acetone
e) $Ph_3P{=}CHCO_2{}^tBu$



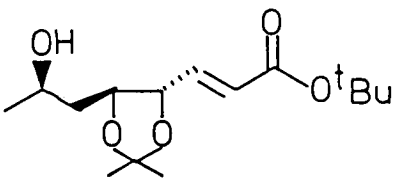
(109)

Scheme 24A



(110)

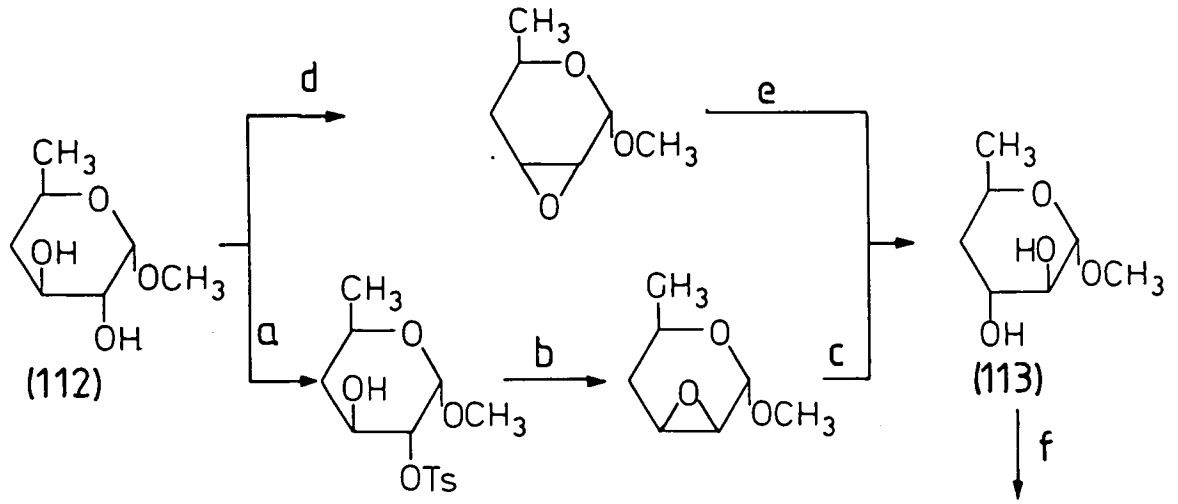
a,c

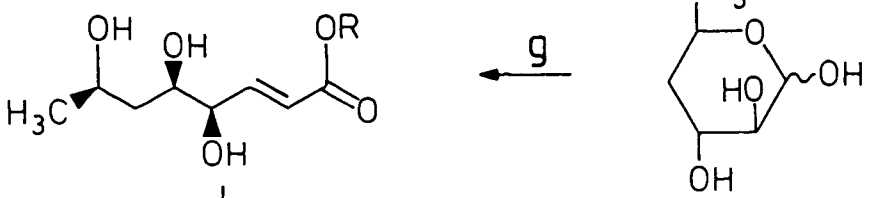


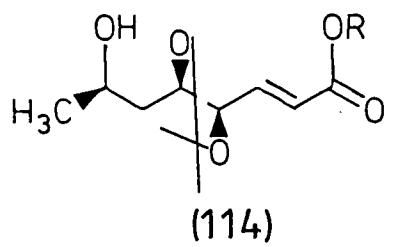
(111)

a) H_2O/Dowex 50 / $H^{\oplus}$
b) $Ph_3P{=}CHCO_2{}^tBu$
c) Acetone/$Me_2C(OMe)_2$/p-TsOH

Scheme 25





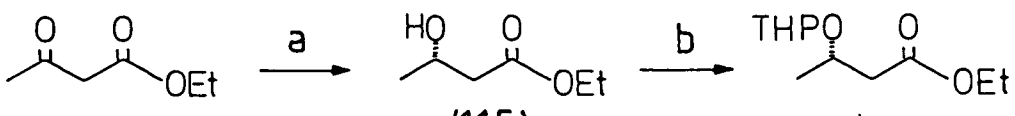


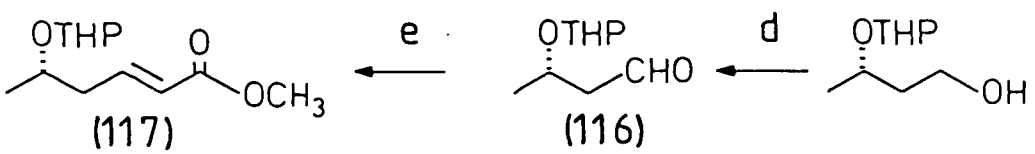
(114)

= $CH_2CH_2SO_2C_6H_4CH_3$

a) Dowex/$H^{\oplus}$
b) KOH/H_2O
c) p-TsOH
d) $NaOCH_3/CH_3OH$
e) KOH/H_2O
f) Dowex 50/$H^{\oplus}$
g) $Ph_3P{=}CHCO_2CH_2CH_2SO_2C_6H_4CH_3$
h) Acetone/$(CH_3O)_2C(CH_3)_2$/p-TsOH

Scheme 26





a) Yeast Reduction
b) DHP/PPTS
c) $LiAlH_4$/THF
d) PCC
e) $Ph_3P{=}CHCO_2CH_3$

a) $Cl_3C_6H_2COCl$/DMAP
b) DEAD/Ph_3P
c) $CF_3CO_2H/CH_3CN/H_2O$

3.2.1.g. Colletodiol.

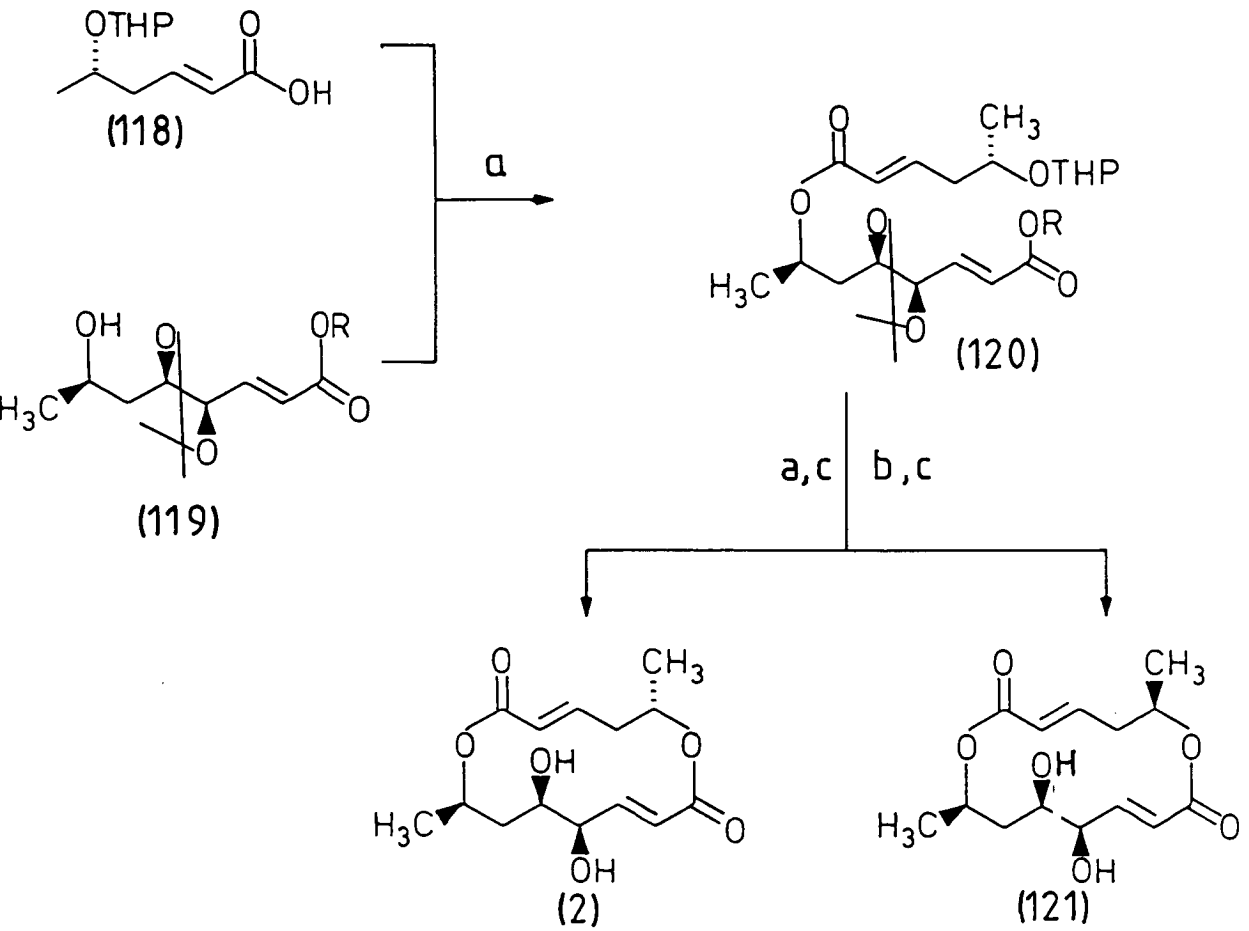
Colletodiol (2) can be considered the parent structure of the fourteen member macrodiolides. It has proved to be a difficult synthetic problem, mainly due to the 4,5 diol system which is prone to elimination during synthesis.

Scheme 24 shows the approach of Mitsonobu *et al*[33] to the synthesis of the C_8 fragment and its stereoisomers. In each case the starting material was a modified sugar. Thus (107) was converted to (108), which was extended by a Wittig reaction to to give the C_8 fragment in the 4-(*S*),5-(*R*),7-(*S*) configuration (109). In a similar fashion (110) was converted to (111) which had the 4-(*S*),5-(*S*),7-(*R*) configuration.

In a subsequent paper Mitsonobu *et al*[34] prepared the C_8 fragment in an analogous fashion but with the 4-(*R*),5-(*R*),7-(*R*) configuration, as shown in scheme 25. The hexopyranoside (112) was converted to (113) by two independant routes, and then extended *via* a Wittig reaction to the triol, which was protected as the acetonide (114).

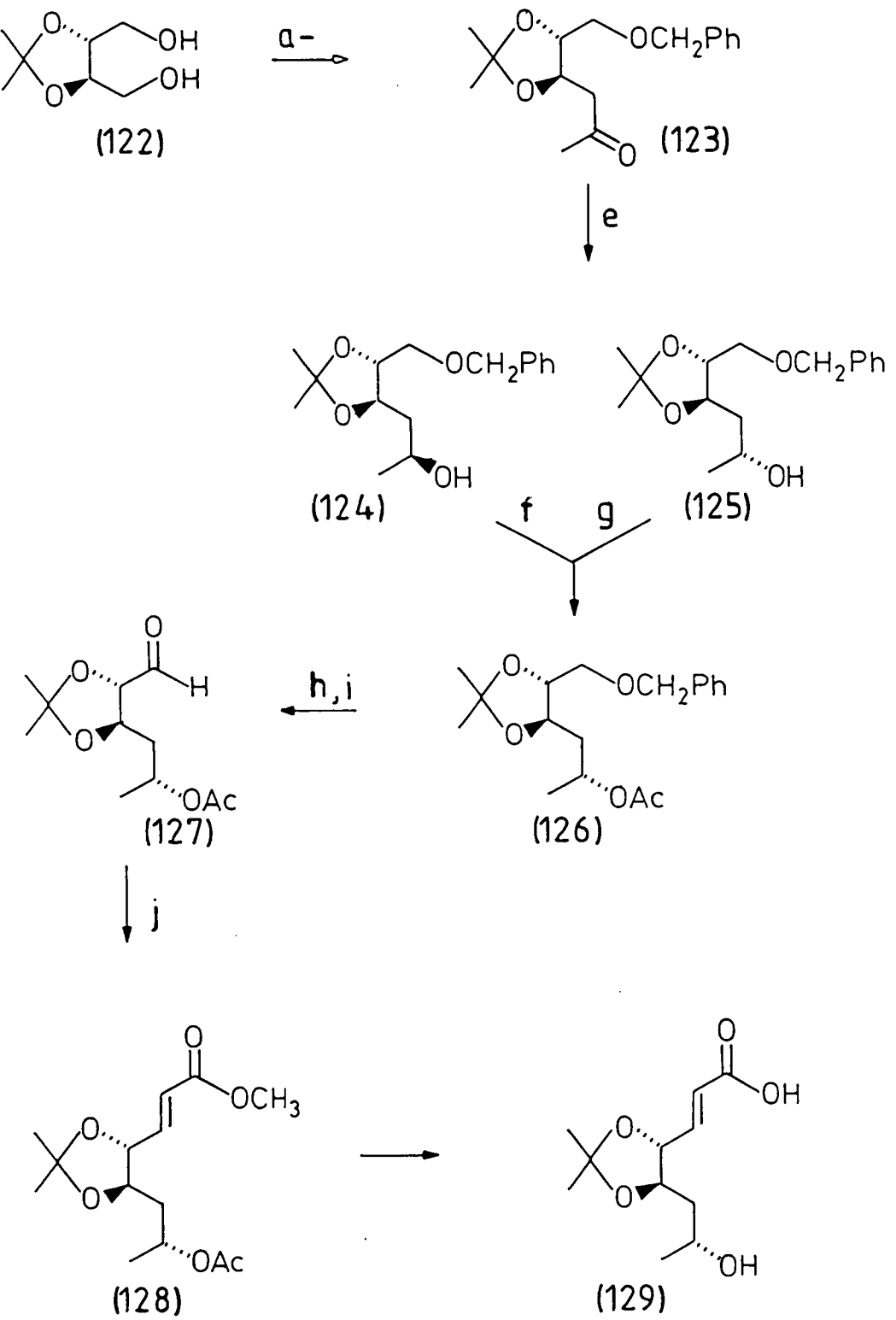
Scheme 26 shows the preparation of the C_6 fragment *via* a yeast reduction of ethyl acetoacetate to (*S*)-ethyl-3-hydroxy butyrate (115), conversion to the aldehyde (116), and extension to the C_8 ester (117). The first coupling was carried out between the C_6 acid (118) and the C8 alcohol (119) using Yamaguchi conditions[13] as shown in scheme 27. The *seco* ester (120) was then deprotected and successfully lactonised[35] using the Mitsonobu method[11], and then deprotected to give colletodiol (2). The ring

Scheme 27



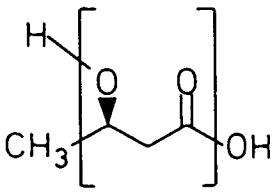
a) $Cl_3C_6H_2COCl$ / DMAP
b) DEAD / Ph_3P
c) CF_3CO_2H / CH_3CN / H_2O

Scheme 28

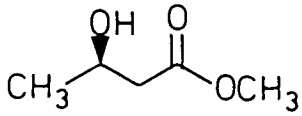


a) C_6H_5Br/DMF
b) $CBr_4/Ph_3P/CH_2Cl_2$
c) 2-Methyl 1,3-Dithian
d) $HgCl_2/CH_3CN$
e) $NaBH_4/CH_3OH$
f) Ph_3P/DEAD/CH_3CO_2H
g) CH_3CO_2Et/ $Ti(OC_2H_5)_4$
h) $H_2/Pd(OH)_2/C$
i) $(COCl)_2$/ Swern
k) CH_3OH/H_2SO_4
l) EE
m) $LiAlH_4$
n) PCC
o) $Ph_3P{=}CHCO_2CH_3$
p) DBN
q) LiOH / THF
r) NaOH / THF
s) $Cl_3C_6H_2COCl$/DMAP

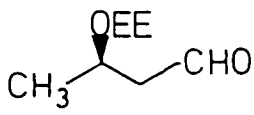
Scheme 29

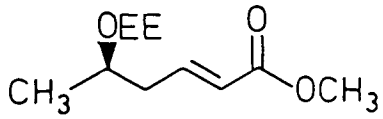


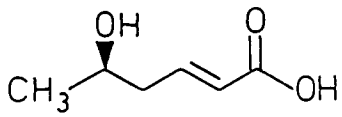
(130)



(131)



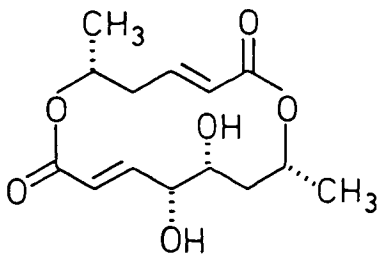




(132)

a) H_2SO_4/CH_3OH
b) EE
c) $LiAlH_4$
d) PCC
e) $Ph_3P{=}CHCO_2CH_3$
f) $NaOH/THF/H_2O$

(132)
+
(129)



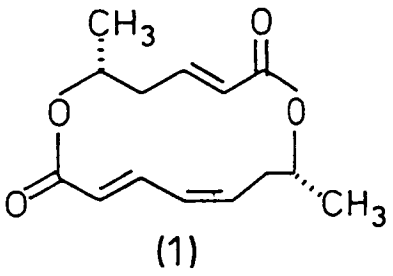
closure was also carried out using the Yamaguchi method[13] to give 5'-epi colletodiol (121).

The route of Seebach *et al*[35,36] to colletodiol (2) is shown in scheme 28. The diol (122) was converted to the monobromide and then *via* the anion of 2-methyl-1,3-dithian-2-yl to the ketone (123). This was reduced non stereospecifically and the epimers separated by chromatography. Acetylation of (124) with retention of configuration and acetylation of (125) with inversion of configuration gave (126). Debenzylation and oxidation gave the aldehyde (127) which was converted to the ester (128) by a Wittig-Horner reaction, hydrolysis giving the free hydroxy acid (129). The C_6 fragment was prepared from the polymer, poly-β-hydroxy butyrate (130), by depolymerisation and conversion of the monomer to the aldehyde (131), as shown in scheme 29. A Wittig reaction followed by deprotection giving the C_6 hydroxy acid (132). A 1:1 mixture of (129) and (132) was then subjected to macrocyclic lactonisation under Yamaguchi conditions[13]. Colletodiol was isolated in low yield.

3.3. Proposed Synthetic route to Colletotriene

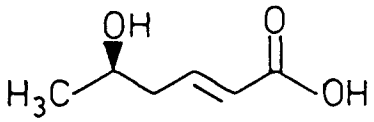
The proposed synthetic route to colletotriene (1) is shown in scheme 30. As with the majority of the macrodiolide synthesis described here, it involves the disconnection of the ring into the two component hydroxy acids (133) and (134) Both of these fragments have the (*R*) configuration at the secondary

Scheme 30

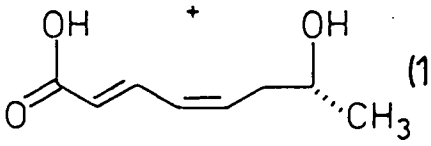


(1)

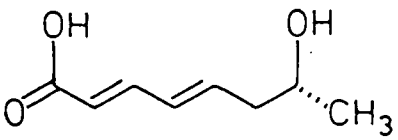
⇨



(133)

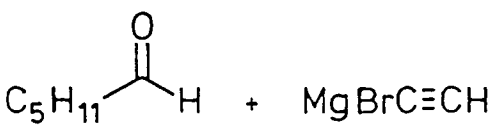


(134)

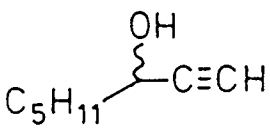


(135)

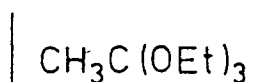
Scheme 31

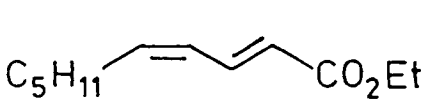


(138) (139)

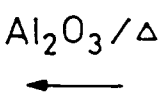


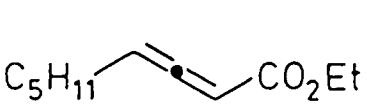
(140)



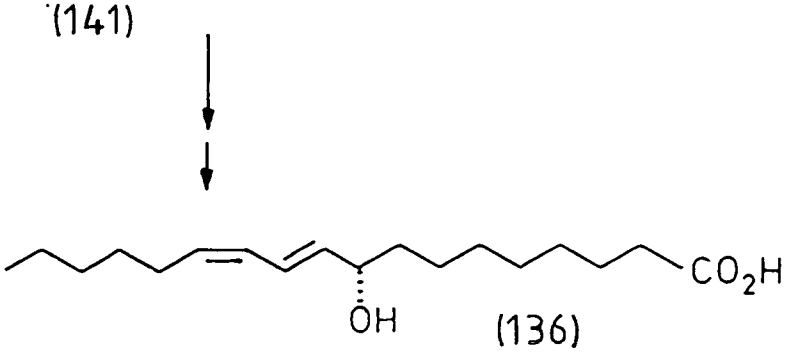


(141)





(137)



(136)

alcohol and it is intended to form each of the ester linkages with retention of configuration.

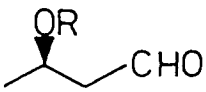
In developing a synthesis of colletotriene it was hoped that the methods used could later be adapted to allow the specific incorporation of isotopic label. This would allow feeding studies to be carried out on *Cytospora Sp* using advanced precursors.

The C_6 fragment, (*R*)-5-hydroxy-2-(*E*)-hexenoic acid (133) will be synthesised using the route of Seebach *et al*[36]. (scheme 29). This synthesis is not only short but also efficient. There is also the possibility to introduce isotopic label at two stages, at the reduction step using $LiAl^2H_4$, or using a stabilised ylid in the Wittig reaction that had been synthesised from labelled sodium acetate.

In designing a route to the C_8 fragment, (*R*)-7-hydroxy-2-*E*,4-*Z*-octenoic acid (134), The major problem encountered is the *E,Z* diene system. The *E,E* diene (*R*)-7-hydroxy-2-*E*,4-*E*-octanoic acid (135) should be relatively easy to produce by reduction of the C_8 ester to the alcohol, oxidation and a further Wittig reaction. However such reactions to produce the *Z* double bond are not very efficient[37].

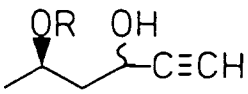
A system analagous to (134) has been achieved by Tsuboi *et al* in the synthesis of dimorphicolic acid (136)[38]. This synthesis is outlined in scheme 31. It is based on the rearrangement of an allene on an alumina catalyst. The allene (137) was prepared by reaction of an aldehyde (138) with acetylene Grignard (139) to give the carbinol (140). This was

Scheme 32



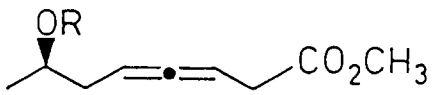
(131)

$MgBrC{\equiv}CH$



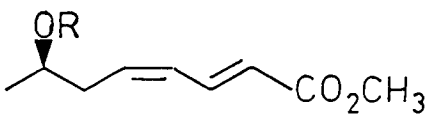
(143)

$CH_3C(OCH_3)_3$



(144)

Al_2O_3/Δ



(134)

then converted to the allenic ester (137) and subsequently rearranged on an alumina catalyst to the *E,Z* diene (141), which was then successfully converted to (136) in a series of simple steps.

The application of this route to (134) is outlined in scheme 32. It makes use of the aldehyde (131) formed during the synthesis of the C_6 fragment, reaction with acetylene grignard producing the required carbinol (143). Reaction with trimethyl ortho acetate should give the allene (144) which can be rearranged on an alumina catalyst to the diene (134). Such a route will not lend itself to the introduction of isotopic label, but should provide a good synthesis of the *E,Z* diene system.

Once the synthesis of the two hydroxy acids is complete it is proposed to couple them using the Yamaguchi method[139] or the Mukiayama method[99]. With colletotriene in hand it will be interesting to study its reaction with epoxidation reagents. The synthesis of the *E,E* isomer will also be prepared and its behaviour to epoxidation studied. This should give some chemical evidence to support the biosynthetic evidence described in chapter 2.

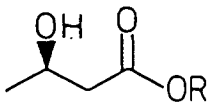
3.4. RESULTS AND DISCUSSION.

3.4.1. Synthesis of the C_6 unit of Colletotriene

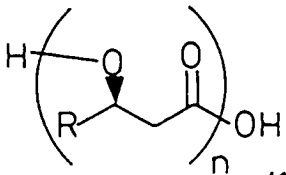
During the early stages of the design of the synthetic route to colletotriene (1), no particular method of esterification/lactonisation was under consideration. Therefore to allow for all possibilities, such as the use of the Mitsonobu method[11], which proceeds with inversion of configuration, both optical isomers of the starting material ethyl-3-hydroxybutyrate were prepared.

The synthesis of ethyl-(*S*)-3-hydroxybutyrate (145) shown in scheme 27, involves the reduction of an achiral substrate by bakers yeast. In this case the subtrate is ethyl acetoacetate. This type of reaction is well documented[39], and various bulk methods are available. In our hands the best results were obtained when the reduction was carried out using fresh yeast rather than dried yeast, which did not work at all. The concentration of the substrate in the broth had a severe effect on the yield of product. Using a system incubated in 500ml shake flasks, an ethyl acetoacetate concentration of 1g/l gave the optimum results. This is consistent with the results published by Wipf *et al*[39], who found that continuous feeding of the substrate to the yeast culture, maintaining the concentration below 1g/l gave the best results.

The progress of each fermentation was followed by glc [2½% OVI, 100°C]. When the reaction seemed to be complete, the



(146)

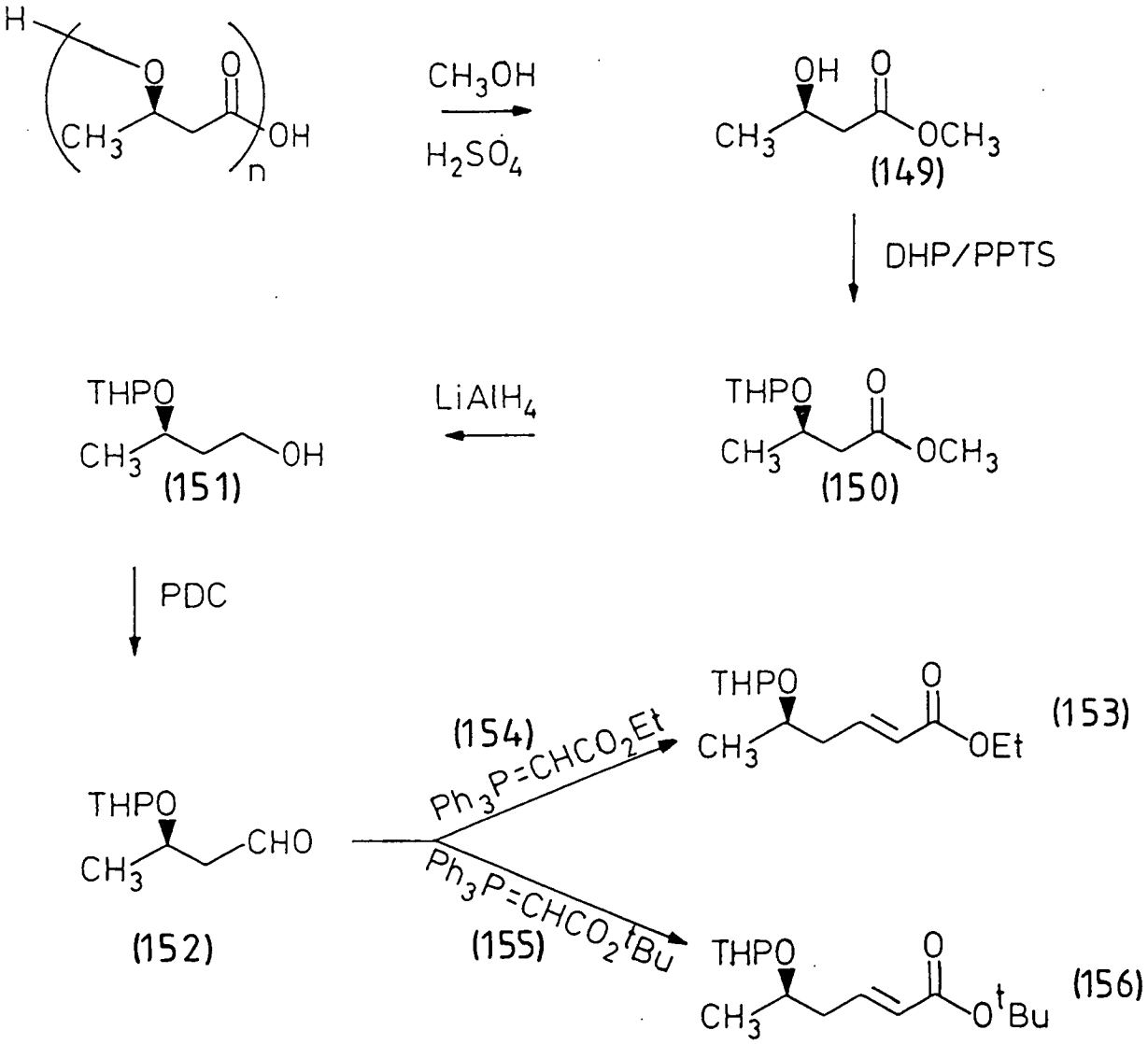


(147)

R = CH_3

R = CH_3CH_2 (148)

Scheme 33



broth was extracted and the product was isolated. In most cases the reduction reached approximately 60% conversion and then stopped. This caused minor problems when purifying the product as ethyl acetoacetate boils at 181°C and ethyl-(*S*)-3-hydroxybutyrate boils at 170°C. At this stage the synthesis of the ethyl-(*R*)-3-hydroxybutyrate became more accessible, and so the work on the (*S*) isomer was suspended.

Ethyl-(*R*)-3-hydroxybutyrate (146) can also be produced *via* a microbial reduction of ethyl acetoacetate, *Geotrichum candidum*[39] being used rather than bakers yeast. However the reaction is not very efficient, 25-30% chemical yield, 89% ee. The ready availability of the polymer poly-β-hydroxy butyrate[40] (147) makes this method somewhat redundant. Poly-β-hydroxy butyrate (PHB) is a stereochemically pure polymer of (*R*)-hydroxybutyric acid produced by *Alcaligenes eutrophus H16*, which under the correct conditions will produce up to 80% of its dry weight as PHB[41]. Another strain *Alcaligenes eutrophus NC1B* produces a mixed (*R*)-3-hydroxybutyrate, (*R*)-3hydroxyvalerate copolymer (148)[41]. These polymers are of high optical purity and are recognised as being excellent sources of chiral building blocks[42]. Using an alcoholic depolymerisation PHB. can easily be converted to methyl-(*R*)-3-hydroxybutyrate (149) as shown in scheme 33 in 86% yield[43].

The hydroxyl group of methyl-(*R*)-3-hydroxybutyrate was now protected to allow reduction of the ester. This was carried out using using dihydropyran with pyridinium *para* toluene sulphonic acid (PPTS) as the catalyst to produce the THP ether

(150)[44]. (scheme 33). The reduction of (150) to the alcohol (151) was carried out using $LiAlH_4$, the product being recovered in 87% yield.

To complete the synthesis of the C_6 fragment using a Wittig reaction, it was neccessary to oxidise (151) to the aldehyde (152). This compound was not only required for this synthesis but also for the synthesis of the C_8 fragment. To carry out this conversion it was decided to use either pyridinium dichromate (PDC)[45], or pyridinium chlorochromate (PCC)[46]. Both of these reagents are available commercially, However as the best results are reported using freshly prepared reagent[45] each was prepared as required.

In contrast to the 1.5 molar excess quoted as being sufficient in these reactions[45,46], it was found that a 5-8 molar excess of the reagent was required to complete the reaction. In this system PDC gave better results than PCC, which despite the vast excess would not complete the reaction. Isolation of (152) was difficult as it was volatile and air sensititve. Purification was carried out by subjecting the entire reaction mixture to flash chromatography[47]. (scheme 33).

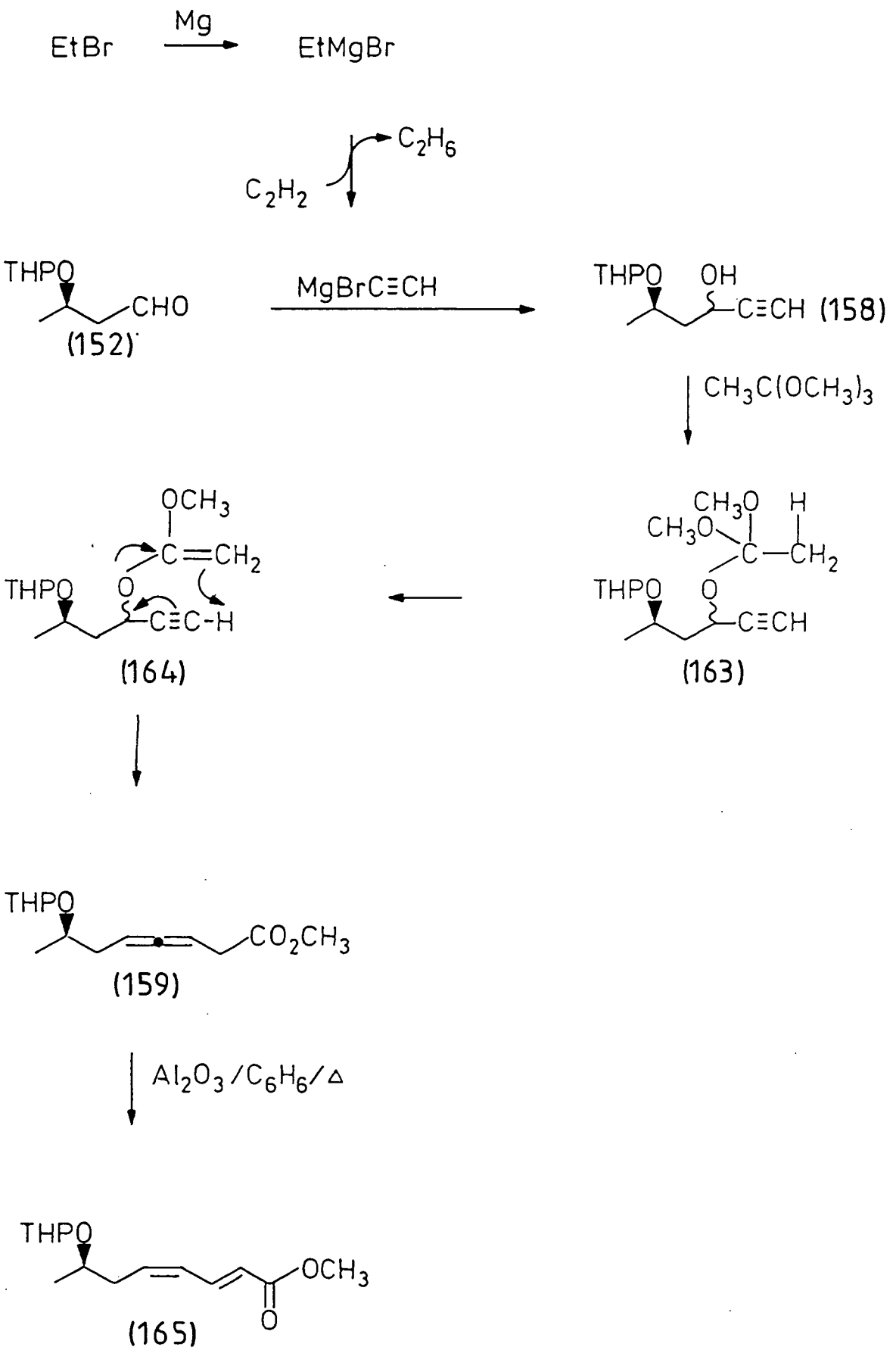
To try to overcome the problems of this method a modified form of the reaction was attempted[48]. The oxidation was carried out as usual but with a five-fold excess of powdered 3A molecular sieve in the flask. Under these conditions it was possible to reduce the PDC to a threefold excess, but there was no improvement in the reaction time as reported[48].

The final step in this synthesis involved extending the aldehyde (152) to the C_6 ester (153) using a Wittig reaction. As at some stage during the synthesis of colletotriene the free acid of (153) will be required, the choice of ylid for the Wittig reaction is important. Initially as a test system ethoxy-carbonylmethylene-triphenylphosphorane (154) was used. This was prepared as its bromide salt from ethyl bromoacetate and triphenylphosphine. Neutralisation with mild base giving the stabilised form of the ylid, which could be recrystallised from benzene and stored indefinitely at 0°C. Combination of (152) and (154) in refluxing methylene chloride produced the protected ethyl ester (153) in 80% yield, (scheme 33), 46% overall from (147).

As ethyl esters are quite stable and therefore difficult to hydrolyse under mild conditions, the t-butyl ester was also prepared using t-butyloxycarbonylmethylene-triphenyl-phosphorane (155), prepared in an analogous fashion to (154). Reaction with (152) under the same conditions as described above gave the t-butyl ester (156) in 74% yield, (scheme 33), 35% overall from (147).

The overall yields for these reactions compare favourably with those reported for similar systems. Seebach *et al* using a similar synthesis (scheme 19) produced the ester (153) in overall 40% yield.

Scheme 34

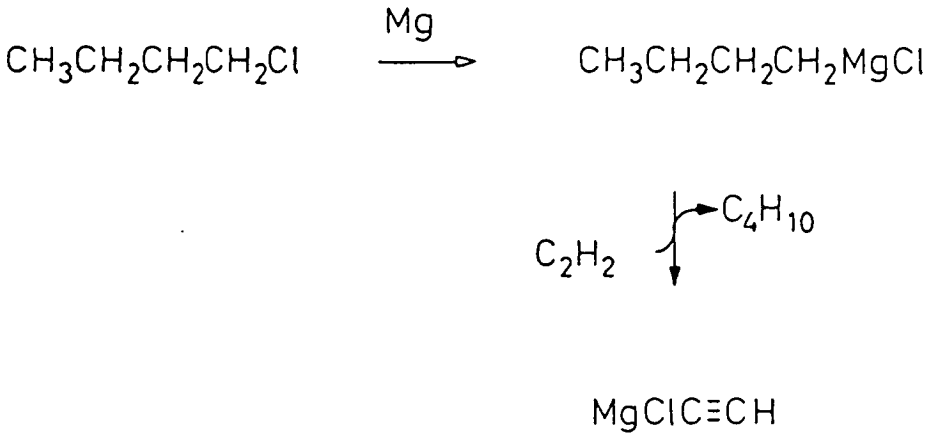

EtBr
Mg
EtMgBr
C_2H_2
C_2H_6
THPO
CHO
(152)
MgBrC≡CH
THPO
OH
C≡CH (158)
$CH_3C(OCH_3)_3$
CH_3O
H
CH_3O
C
CH_2
THPO
O
C≡CH
(163)
OCH_3
C
CH_2
THPO
O
C≡C-H
(164)
THPO
CO_2CH_3
(159)
$Al_2O_3/C_6H_6/\Delta$
THPO
OCH_3
O
(165)

3.4.2. Synthesis of the C_6 fragment of Colletotriene

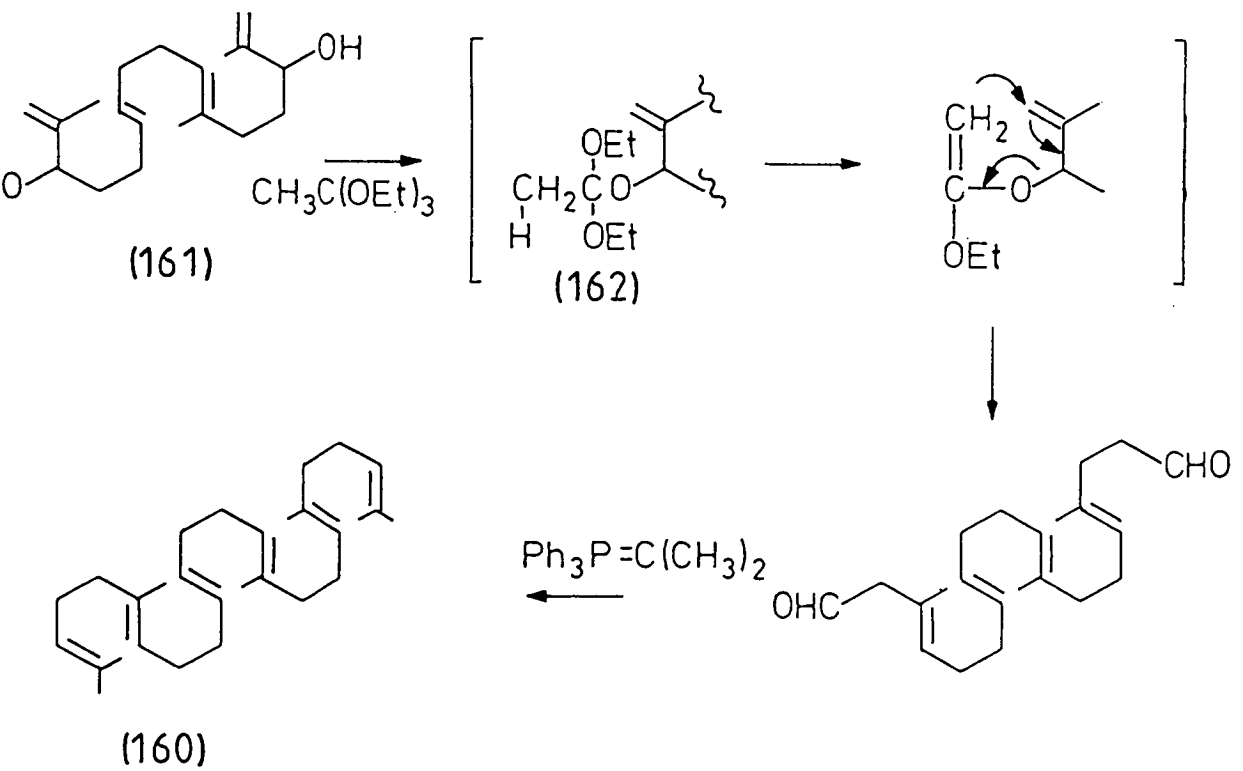
This synthesis was based on a method described by Tsuboi for the preparation of E,Z diene systems as described above[37,38]. The first step was to convert the aldehyde (152) to the carbinol (158), by reaction with acetylene magnesium bromide as shown in scheme 34. Acetylene magnesium bromide is formed by the reaction of acetylene gas with a "primer" Grignard reagent, usually ethyl magnesium bromide in this case[49]. The standard method of passing acetylene gas through a solution of ethyl magnesium bromide in ether[49] results in the formation of not only the mono Grignard reagent but also the bis Grignard reagent. The bis reagent is extremely insoluble in ether, and although it initially precipitates out as a fine white solid, it rapidly forms a black tar, even at low temperature[50]. This black solid is reported to react as the mono Grignard[49], but in this study it would not react with (152).

To overcome this the method of Jones *et al* was used and is shown in scheme 34[50]. In this method the ethyl magnesium bromide is formed in tetrahydrofuran and slowly added to a solution of tetrahydrofuran saturated with acetylene at -10°C. Under these conditions, the by product ethane escapes as a fine froth, quite distinct from the acetylene bubbling into the solution. As the reaction proceeds the solution turns pink and a fine pink/white precipitate forms, which can be redissolved on warming to 0°C. The yield of the reaction with (152) can be

Scheme 35



Scheme 36



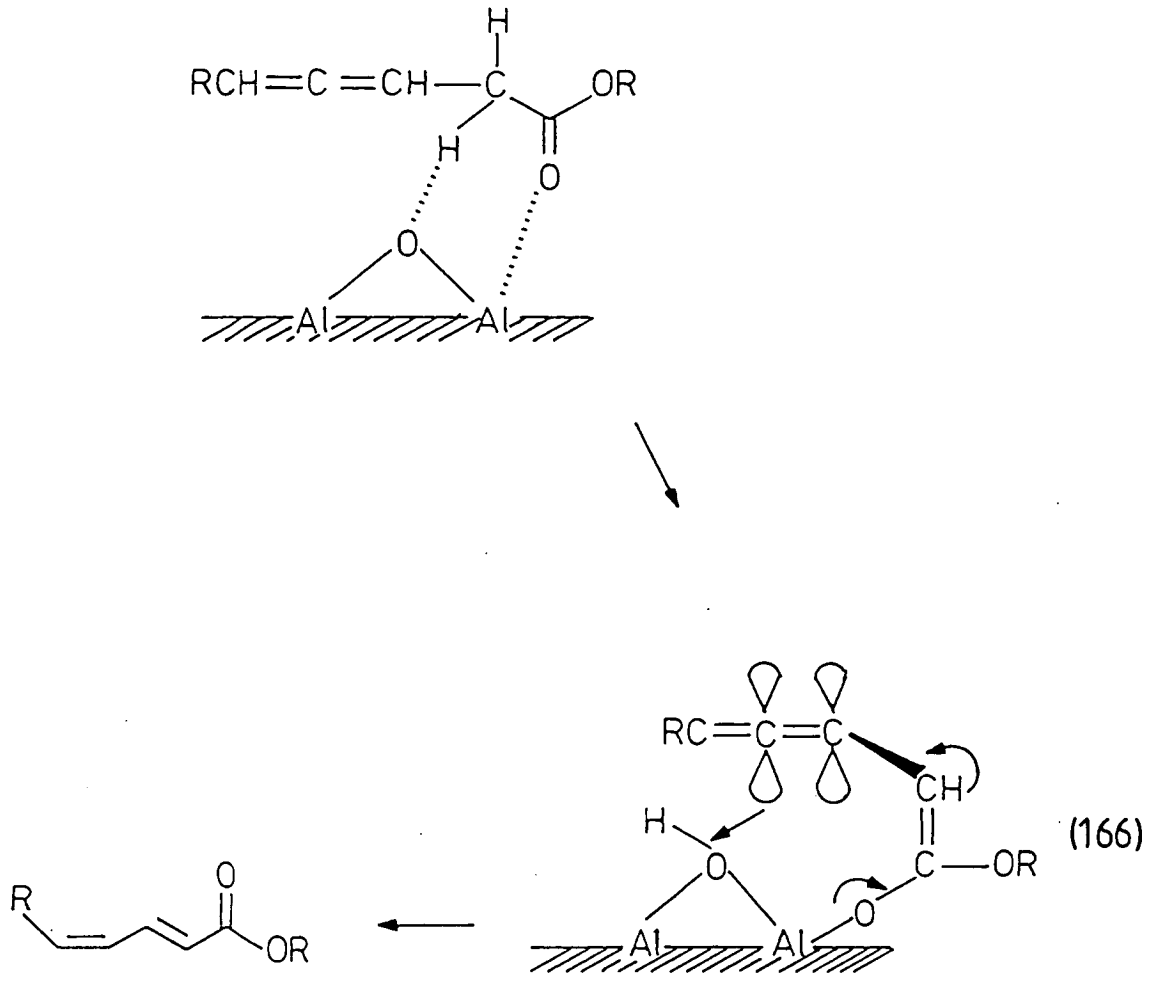
significantly improved if the ethyl magnesium bromide solution is filtered before addition to the acetylene solution.

Holmes *et al* have recently suggested a further improvement on this method[51]. In place of ethyl magnesium bromide they have used butyl magnesium chloride as the "primer" Grignard reagent. This is more soluble in tetrahydrofuran and the by product butane is less volatile. This modification is reported to give fewer by-products. (scheme 35).

Using the original method of Jones *et al*[50] acetylene magnesium bromide was allowed to react with (152)(scheme 34). The carbinol (158) was unstable, and various attempts to purify it proved wasteful as most of the product polymerised in the distillation flask. However a small quantity of pure material was obtained and its identity confirmed. The problem of purification was alleviated by the observation that the crude product could be used in the formation of the allene without any difficulty.

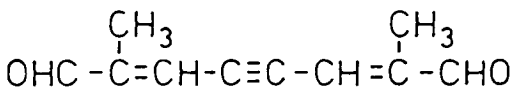
The conversion of the carbinol (158) to the allene (159) is shown in (scheme 34). The crude carbinol was combined with a sixfold excess of trimethyl-orthoacetate and heated to reflux. The reaction proceeds *via* a modified form of the acid catalysed oxy-Claisen rearrangement[52]. This type of rearrangement has been observed before and has been applied synthetically. Scheme 36 shows its use in the synthesis of squalene (160)[53]. Reaction of the triethylorthoacetate with the allylic alcohol (161) produces the intermediate (162), loss of ethanol then sets the molecule up for the oxy-Claisen rearrangement which occurs spontaneously. Replacement of the allylic alcohol system by a

Figure 2

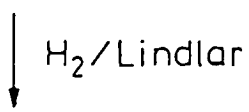


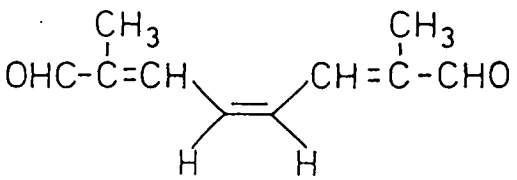
(166)

Scheme 37



(167)





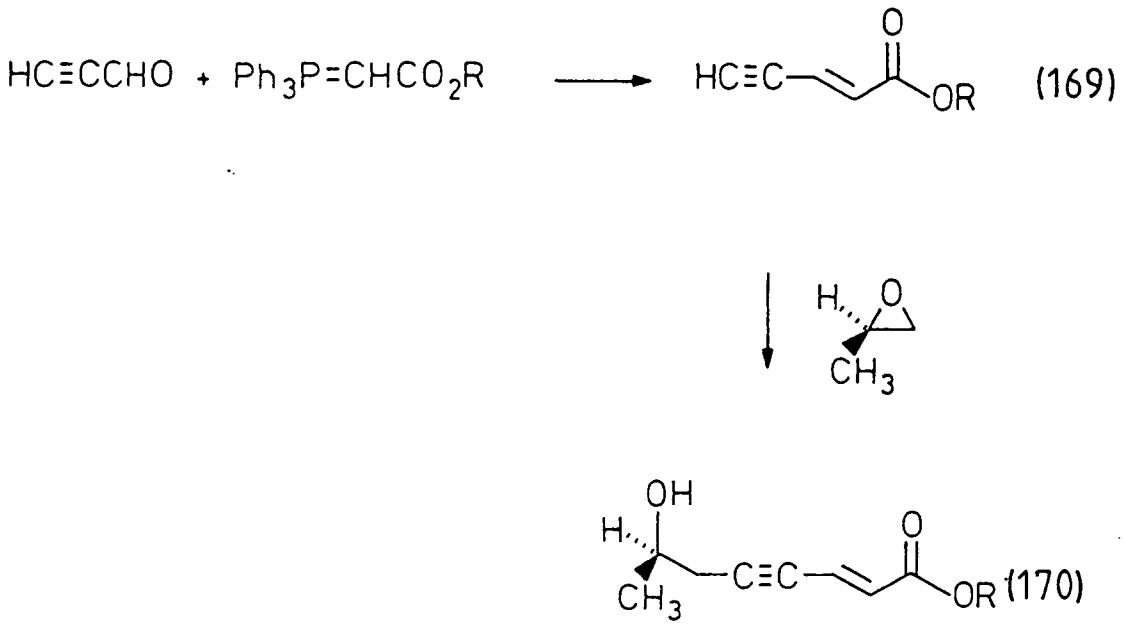
(168)

carbinol was found to produce the same reaction but with the allene as the product. The mechanism of the reaction is shown in scheme 34. reaction of the carbinol (158) with the trimethylorthoacetate produces the intermediate (163), which then loses methanol to give (164), the oxy-Claisen rearrangement then occurs to give the allene (159), in 46% yield. The reaction is reported to work only with the methyl or ethyl ortho esters.[37].

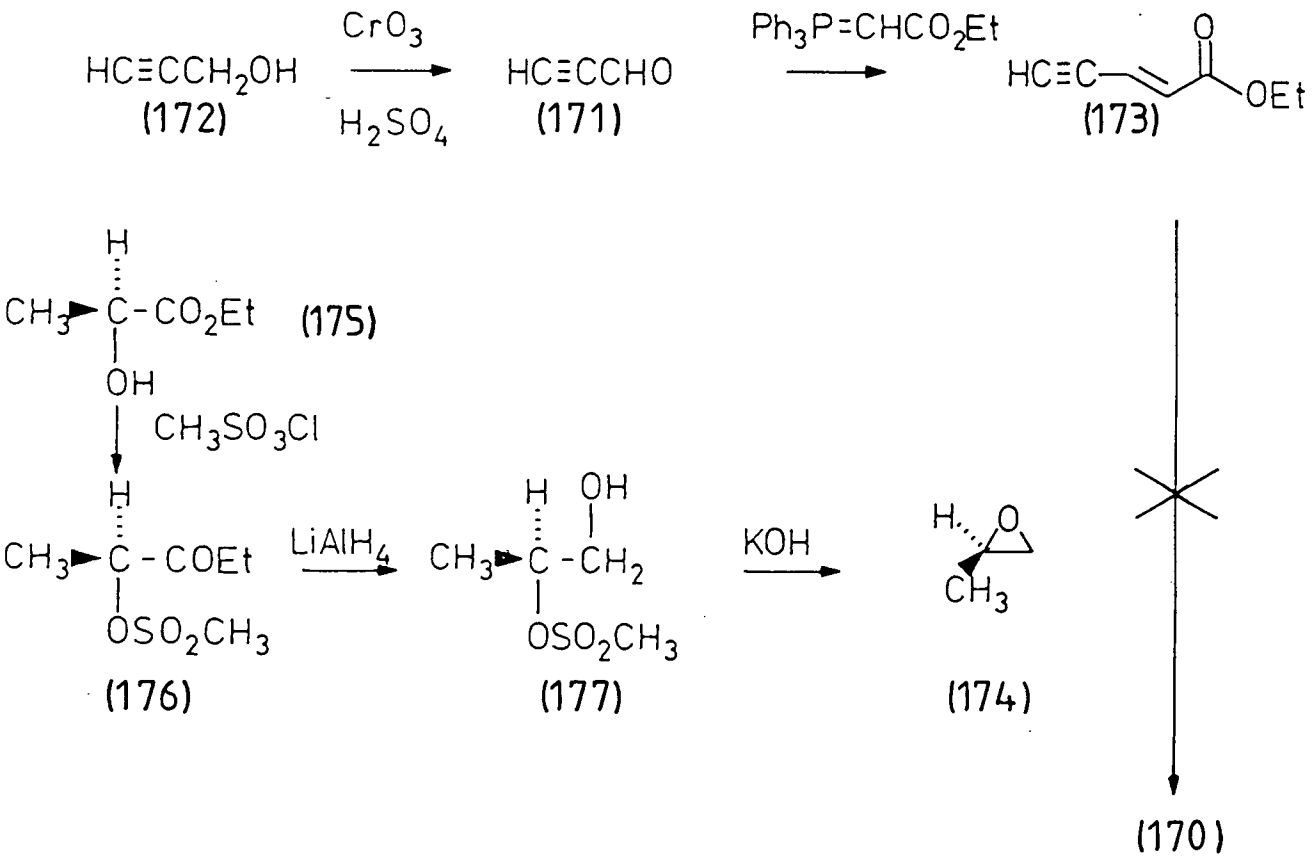
The conversion of the allene (159) to the *E,Z* diene (165) was achieved by a thermal rearrangement on an alumina catalyst as shown in scheme 34. The suggested mechanism for this reaction[37] is shown in figure 2. Coordination of the carbonyl oxygen to the aluminium and loss of a proton to the catalyst oxygen results in the enolate (166). The reapproach of the proton to the central carbon must occur from the least hindered side to produce the *cis* geometry in the γ,δ bond of the product.

Using this technique (165) was isolated in overall 10% yield. However the final step presented a problem which severly limited the synthesis. A five fold excess of the alumina catalyst was required and despite stirring and/or reaction in large vessels it was difficult to prevent severe bumping. This limited the scale of this step to around 50 mg, too little for practical purposes considering the subsequent steps still to be achieved. The route was further hampered by the fact that only the methyl or ethyl ester could be produced *via* the oxy-Claisen rearrangement. In the later stages when esterification and lactonisation were intended, more labile esters would be more

Scheme 38



Scheme 39



helpful. Due to these difficulties it was decided to redesign the synthesis.

A *cis* olefinic bond can be introduced *via* the partial catalytic hydrogenation of a triple bond using Lindlars catalyst[54]. In the synthesis of (*S,S*) grahamimycin A_1, Seebach[23] used this method to introduce a *cis* double bond at the *seco* ester stage (scheme 20), which was later converted to the 4,5 dione system of (*S,S*) grahamimycin A_1. In the grahamimycin A_1 system the triple bond was not conjugated to an α,β unsaturated ester. In a potential synthesis of colletotriene by this route, the triple bond would be conjugated to just such a unit. Catalytic semi hydrogenation of triple bonds in the presence of such sensitive groups has been achieved, scheme 37 shows the partial reduction of the dialdehyde (167) to the triendial (168) using Lindlars catalyst[55].

The revised synthesis using this method to produce the *cis* double bond is shown in scheme 38. A Wittig reaction provides the ester (169), the anion of which should react (*R*)-propylene oxide to form the hydroxy ester (170) (*cf* scheme 20). The next step would be to couple (170) to the C_6 fragment *via* the free acid and then introduce the *cis* double bond.

Propargyl aldehyde (171) was prepared using the method of Sayer *et al*[56]. This involves the slow addition of chromic acid to a solution of propargyl alcohol (172) in sulphuric acid boiling gently under reduced pressure. The initial product of the oxidation, propargyl aldehyde distils out immediately into a trap at -78°C. The yield of the reaction is low, 20-30%, but the

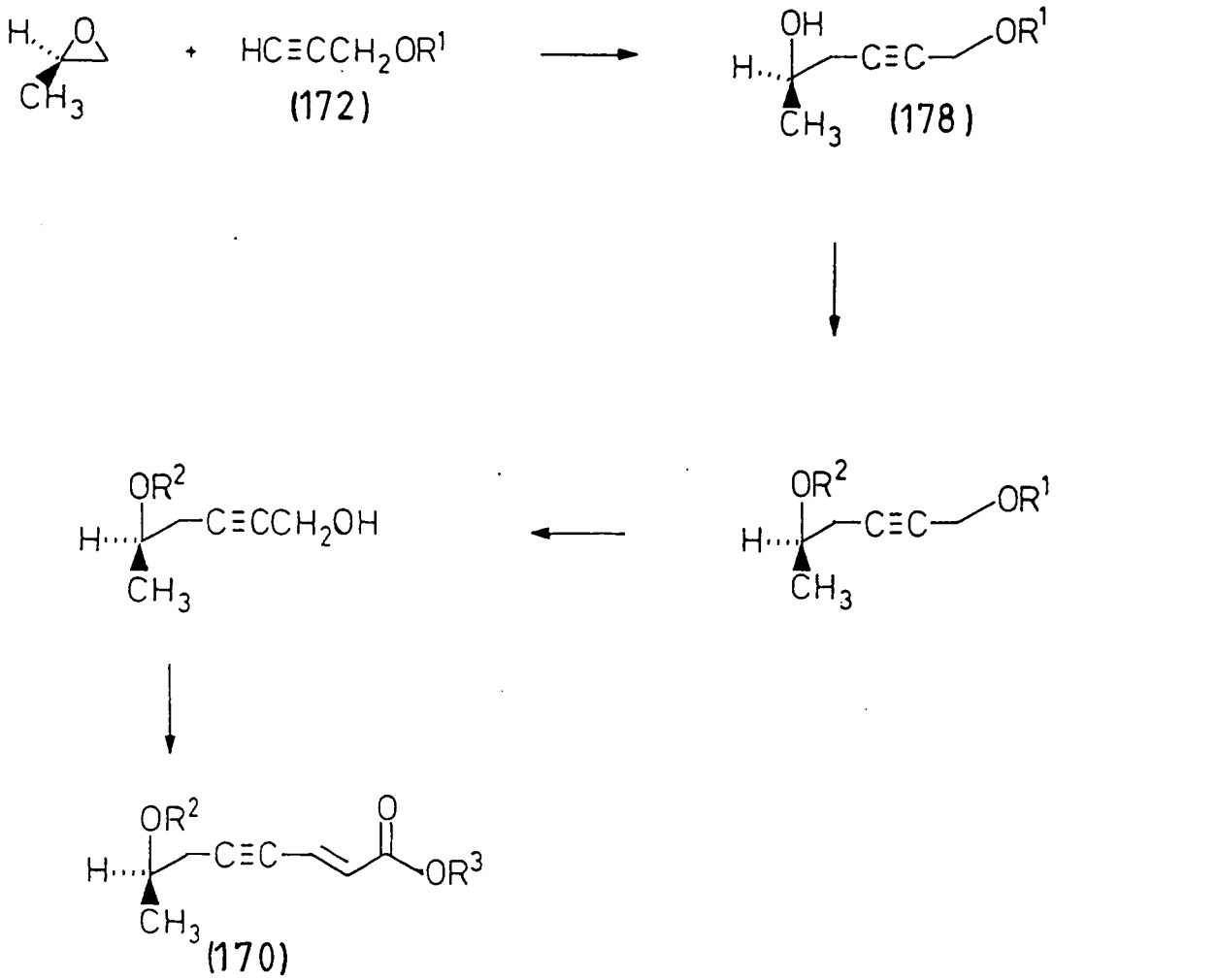
aldehyde is very pure. To test the feasibility of the route shown in scheme 36 propargyl aldehyde (171) was allowed to react with ethoxycarbonylmethylene-triphenyphosphorane to produce the ester (173) in 65% yield, scheme 39.

To complete the synthesis of the essential carbon chain (*R*)-propylene oxide (174) was required (scheme 38). Both (*R*) and (*S*)-propylene oxide are commercially available, but at high cost. Fortunately good syntheses are available for both from inexpensive starting material[57,42]

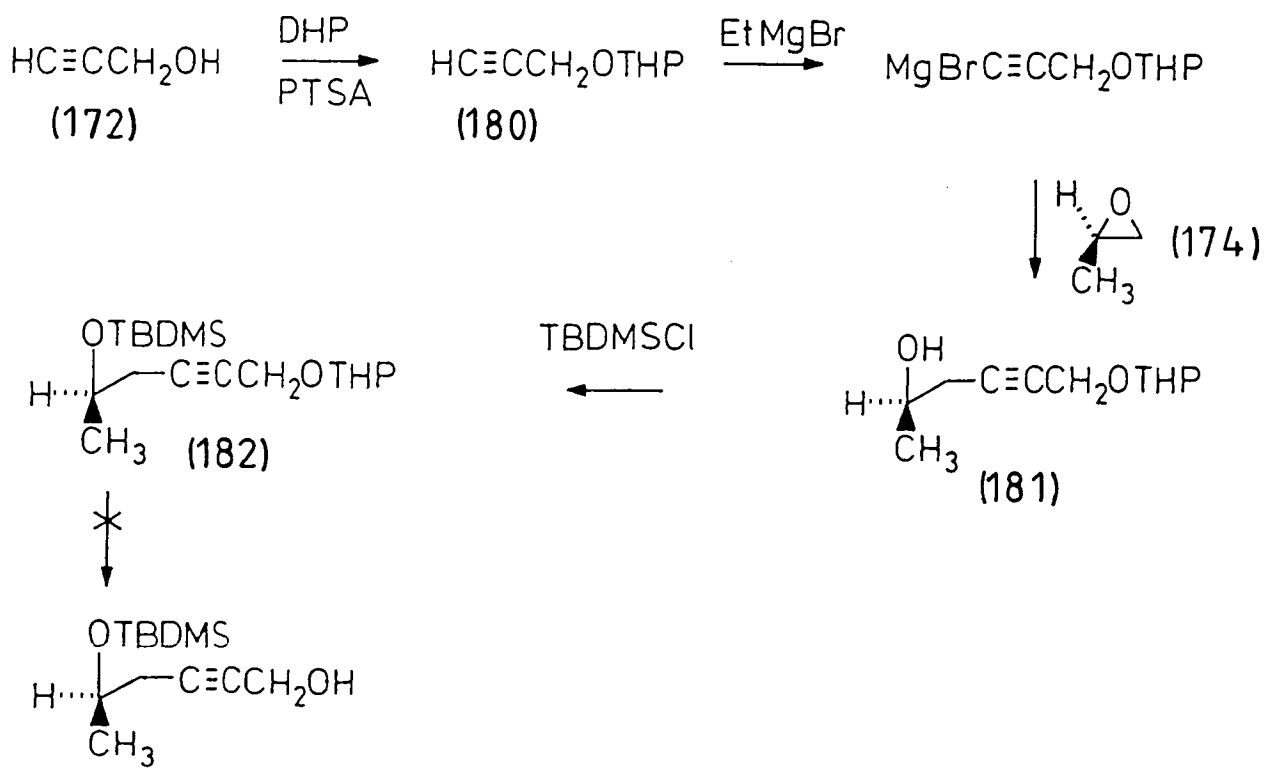
The synthesis of (*R*)-propylene oxide (174) is shown in scheme 39[57]. Ethyl-(*S*)-lactate (175) is converted to the mesylate (176) and purified. The ethyl ester is then reduced using AlH_3. This was prepared by the method of Brown *et al*[58], $LiAlH_4$ was dissloved in tetrahydrofuran and filtered, the filtrate was then added to concentrated sulphuric acid at 0°C. The mesylate (176) was added to this, after 30 minutes the product was isolated but not purified as it is prone to explosive decomposition (177) [57]. The epoxide was then formed by reaction of (177) with boiling KOH to produce (174) in 41% yield.

Having now produced the ester (173) and the epoxide (174), attempts were now made to react the anion of (173) with (174) as shown in scheme 39. All of these attempts failed, the only product being a black tar and reisolated epoxide. One of the main problems was the solubility of the anion, which could not be overcome by the use of solvents such as HMPA. It is also possible that the anion reacted with itself in an inter-molecular Michael

Scheme 40



Scheme 41



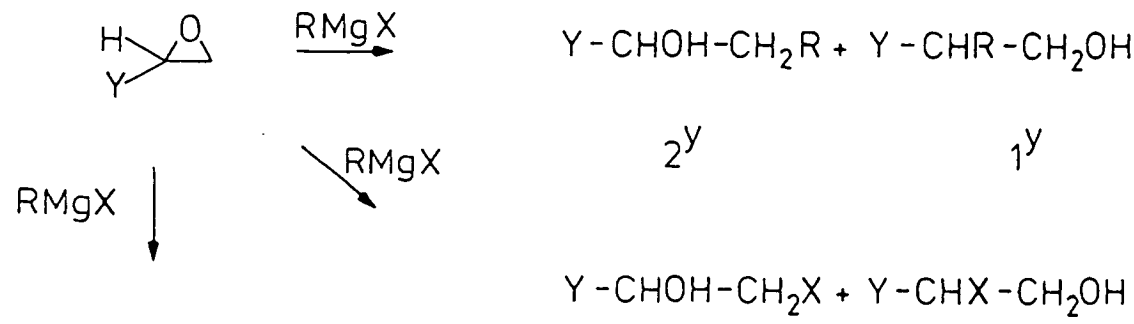
reaction, at the α,β unsaturated ester. As no progress could be made in this direction the synthesis was again reviewed.

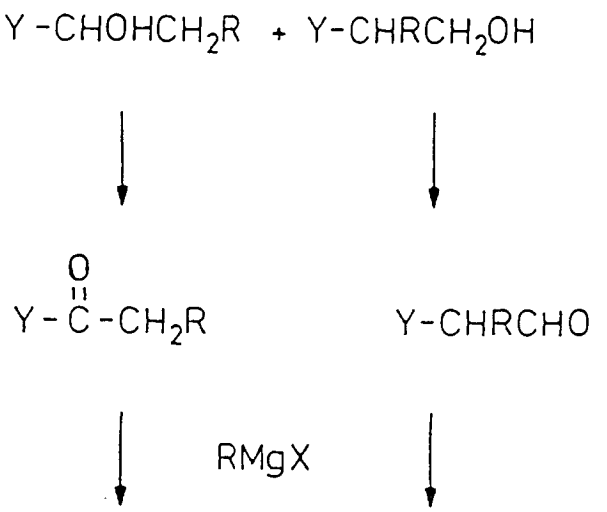
It was decided to retain the idea of introducing the *cis* double bond *via* a triple bond and so the target product was still the ester (170). Similarly the chirality is still derived from (*R*)-propylene oxide (scheme 40). However in this route the triple bond is introduced from propargyl alcohol (172) in a protected form. The product (178) will then be protected at the secondary alcohol, deprotected at the primary alcohol, and oxidised to the aldehyde, a Wittig reaction completing the synthesis.

Propargyl alcohol was protected by reaction with dihydropyran in the usual fashion, scheme 41. Having experienced difficulty with lithium acetylides in the previous synthesis, the Grignard reagent was used in this case. The protected alcohol (180) was allowed to react with ethyl magnesium bromide in a manner analogous to that described above. Reaction with (*R*)-propylene oxide (174) provided the semi protected diol (181) in 35% yield. (scheme 41).

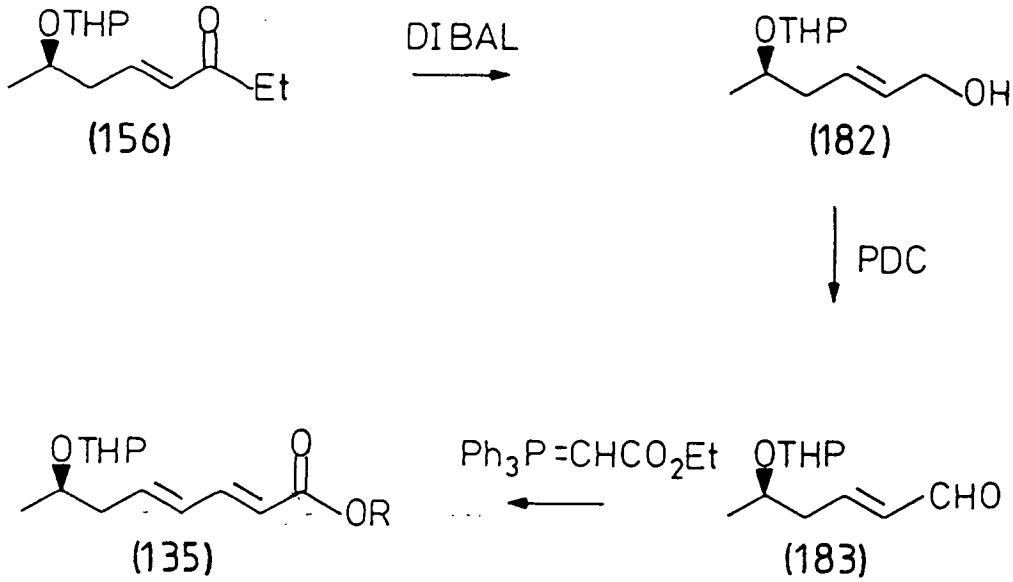
The reaction of Grignard reagents with terminal epoxides is well documented[59]. In general an epoxide will react with a Grignard reagent to give a secondary alcohol as the major product, a primary alcohol and sometimes halogenoalcohols due to the presence of halide in solution as shown in figure 3. There is also the possibility that the initial product will rearrange to a carbonyl compound which can react further with the Grignard to give isomeric tertiary and secondary alcohols; figure 3. Thus in

Figure 3

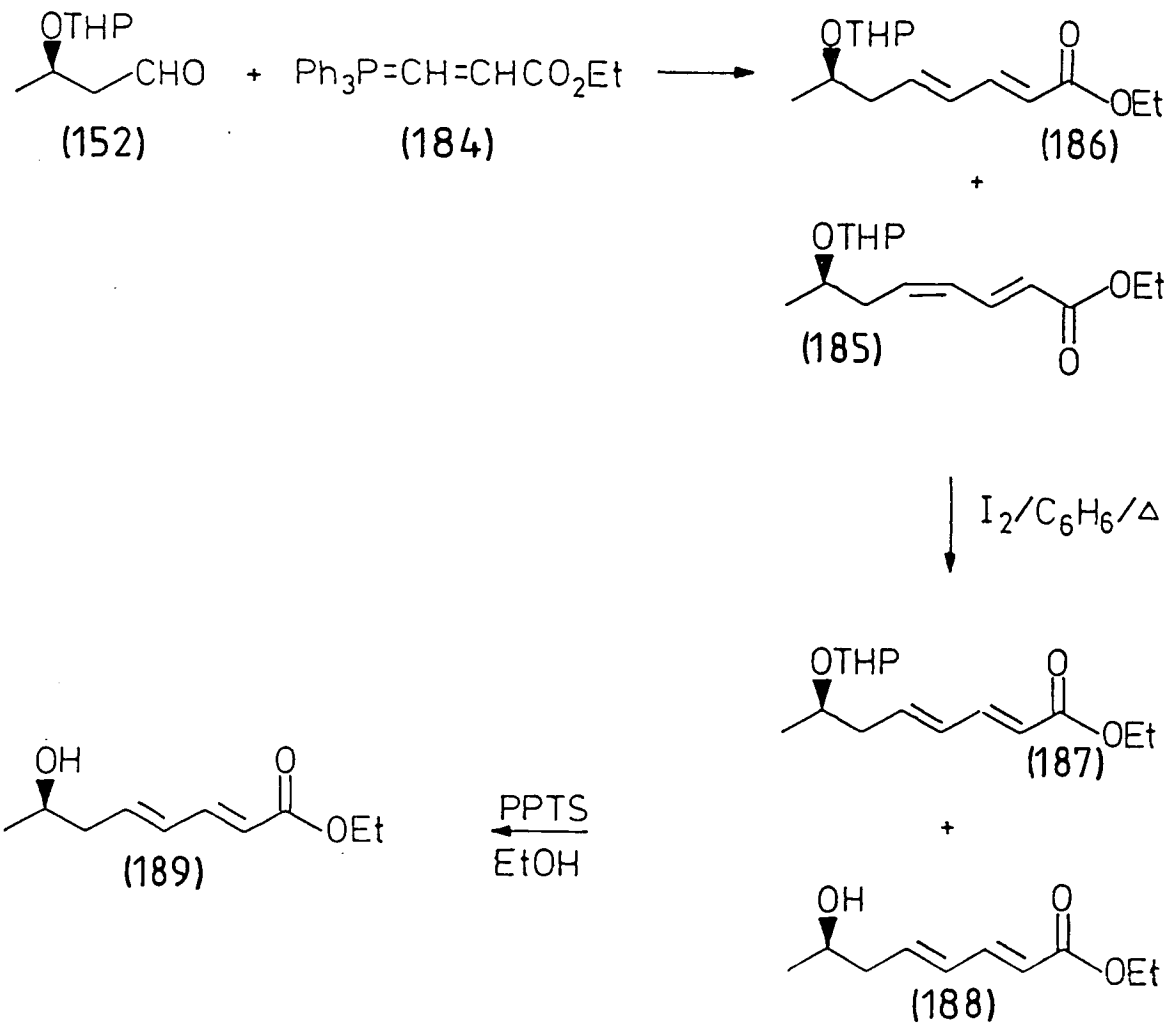

RMgX
Y-CHOH-CH2R + Y-CHR-CH2OH
2y
1y
RMgX
RMgX
Y-CHOH-CH2X + Y-CHX-CH2OH


Y-CHOHCH2R + Y-CHRCH2OH
Y-C(=O)-CH2R
Y-CHRCHO
RMgX
Y-C(OH)(R)-CH2R
Y-CHR-C(OH)(R)-H

Scheme 42



Scheme 43



all there are six possible products from this type of reaction. In this study no significant amounts of the by products were found.

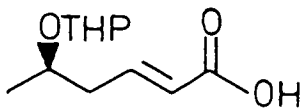
The semi protected diol (181) was fully protected using a t-butyl-dimethylsilyl (TBDMS), group[60] (182) as shown in scheme 41. All attempts to selectively deprotect the primary alcohol, so as to allow oxidation to the aldehyde failed, in each case the (TBDMS) group was removed as well. A recent report[61] suggested that this type of deprotection could be achieved with $MgBr_2$, but this too has been unsuccessful. Possible solutions to the problem are discussed in the section on future work at the end of this chapter.

3.4.3. Synthesis of the *E,E* isomer of the C_8 fragment.

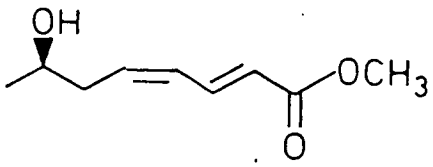
The synthesis of the C_8 fragment in the *E,E* configuration (135) could have been achieved by two routes. Reduction of the C_8 ester (156) to the alcohol (182), oxidation to the aldehyde (183) and reaction with an appropriate ylid in a Wittig reaction giving the desired *E,E* configuration (135) as shown in scheme 42. This route is analogous to that used in the synthesis of the sixteen member ring unit of elaiophylin (68) by Seebach *et al* (scheme 19)[28].

The alternative route is shown in scheme 43. The starting material (152) is readily available from PHB. as described above, and the ylid (184) is derived from ethyl-4-bromo-crotonate. This route was chosen as it was the shorter of

Scheme 44

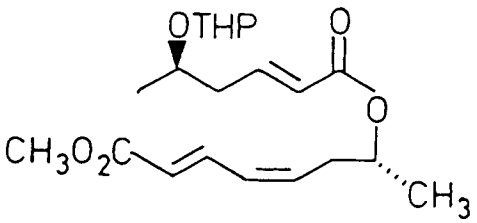


(190)



(191)

DCC
DMAP



(192)

the two. The reaction went smoothly to produce two major products in approximately 1:1 ratio as determined by glc. (2½% OVI 130°C). From the ^{1}H n.m.r. it appeared that both the *cis* (185) and the *trans* (186) isomers had been produced in equal amounts. To convert the mixture to a single product, it was treated with molecular iodine in benzene, with irradiation from a 100w light bulb. This procedure was successful in producing the *E,E* isomer, (187) but it also resulted in some of the product being deprotected to the free alcohol (188). This mixture was therefore treated with PPTS in ethanol to complete the deprotection to give the pure $C_{\ominus}$ hydroxy ester (189) in a few percent yield. This material was unstable at room temperature, decomposing to a black tar. (scheme 43).

3.4.4. Attempted Esterification reactions

With the two fragments of colletotriene in hand, an esterification was attempted between the $C_{\ominus}$ acid (190) and the $C_{\ominus}$ alcohol (191) as shown in scheme 44. The acid (190) was prepared from (156) by mild base hydroysis, and the hydroxy ester (191) was prepared by treatment of (165) with HCl in methanol.

The esterification was attempted using DMAP as the acyl transfer reagent and dicyclohexyldicarbodiimide DCC as the activation reagent. (scheme 44). These conditions were used by Mitsonobu *et al* in the synthesis of colletodiol (scheme 27). From this reaction a product was isolated which appeared to be the *seco* ester (192). The isolated material appeared to consist of

one major component, by tlc. and the 1H n.m.r. showed both sets of olefinic resonances, the methyl ester, and the THP. protecting group.

3.4.5. Future Work

As can be seen from the above studies, the main problem in this synthesis is in the preparation of the C_8 fragment. To sucessfully conclude the study, the acetylenic ester (170) must be prepared. The route shown in scheme 41 probably offers the best hope for this. The only problem to be overcome is that of the complementary protecting groups. A more labile protecting group such as ethoxy-ethyl could be used[28,42].

The esterification and lactonisation steps to form colletotriene should be easily achieved using Yamaguchi conditions now that 2,4,6-trichlorobenzoyl chloride is more readily accessible[16]. Failing this the use of DCC/DMAP has been shown to work for at least the esterification step, and one of the other lactonisation steps such as the Mukaiyama method should prove applicable. Alternatively it should be possible to prepare colletotriene from colletodiol itself, by elimination of the 4,5 diol system.

When the synthesis of colletotriene is complete, the synthesis can then be adapted to introduce isotopic label. This will not only be of use in *in vivo* studies but also in any *in vitro* studies that result out of research currently underway on the *Cytospora Sp*/colletodiol system. Studies on the behaviour of

colletotriene towards epoxidation of the 4,5 double bond should also be carried out.

3.4.6. EXPERIMENTAL.

For general procedures and instrumentation see section 2.4.1 on page 52.

Ethyl-(*S*)-3-hydroxybutyrate

The method that is outlined below was achieved after many abortive attempts. The best results were achieved using fresh yeast, dried yeast gave very poor results. Baker's yeast, *Saccharomyces cerivisae* was incubated in 10 x 500ml shake flasks each containing 100ml of water. After forty eight hours a solution of ethyl acetoacetate (1.0 g, 8.47 mmol) in 1ml of ethanol was added to each flask, and the incubation continued for a further forty eight hours. The contents of each flask were then slurried with 10-15g of celite and filtered through a sintered glass disc. The filtrates were combined and slurried with a further 100g of celite and refiltered. The resulting clear solution was then extracted with ethyl acetate (4 x 100ml). The organic layer was separated and dried over $MgSO_4$, and the solvent removed *in vacuo* to give a pale yellow oil. This was distilled under reduced pressure, the fraction boiling at 23-24°C at 0.15 mmHg being collected to give ethyl-(*S*)-3-hydroxybutyrate (5.05 g, 38.2 mmol, 49%). (lit[42] 80-84°C at 18 mmHg).

I.r. : neat film, ν_{max} 1710s cm^{-1}

^1H n.m.r. : (80 MHz, $CDCl_3$), δ1,07 (3H, t 6.0 Hz), 2.14 (2H, d 5.0 Hz), 3.91 (1H bs), 4.06 (2H, q 6.0 Hz), and 4.01 ppm (1H m).

$[\alpha]_D$ = +29.07 c=.901 $CHCl_3$ (lit[42] + 38.6, c=1 $CHCl_3$)

Pyridinium *para*-toluene sulphonic acid (PPTS).

para-Toluene sulphonic acid (5.70g, 30 mmol) was added to pyridine (12.1 ml, 150 mmol). The mixture was stirred vigorously at room temperature for twenty minutes. The excess pyridine was distilled off *in vacuo* to give a colourless solid, which was recrystallised from acetone to give colourless needles m.p. 118-119°C. (lit[44] m.p. 120°C).

Methyl-(*R*)-3-hydroxybutyrate

Poly-β-hydroxybutyrate (50 g) was disolved in 1,2-dichloroethane (250 ml). To this solution was added a solution of concentrated H_2SO_4 (2.5 ml) in methanol (250 ml). The resulting mixture was refluxed for forty eight hours and then allowed to cool. The light brown solution was then washed with brine solution (500 ml, 50% w/w), sodium hydrogen carbonate solution (500 ml, saturated), and brine (500 ml, saturated). The organic and aqueous layers were separated, and the aqueous layer extracted with methylene chloride (1 l.). The organic extracts were combined dried over $MgSO_4$, and the solvent removed *in vacuo* to give a dark red oil. This was distilled under reduced pressure, the fraction boiling at 65-67°C at 1 mmHg being collected to give (*R*)-methyl-3-hydroxybutyrate (35.3 g, 0.3 mol.) (lit[43] bp 62-73°C at 1.5 mmHg)

I.r. : neat film, ν_{max} 1710s cm^{-1}

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ0.99 (3H d 6.0 Hz), 2.23 (2H d 5.0 Hz), 3.37 (1H bs), 3.46 (3H s), and 4.01 ppm (1H m).

$[\alpha]_D$ = -43.1 c=1.14 $CHCl_3$ (lit[4] -48.6 c= 1.1 $CHCl_3$).

Methyl-(*R*)-3-(tetrahydropyranyloxy)-butyrate

Methyl-(*R*)-3-hydroxybutyrate (25 g, 0.21 mol.) was dissolved in methylene chloride (100 ml.). To this solution was added dihydropyran (26.69 g, .32 mol.) and the catalyst PPTS (5.35 g, 0.32 mol.), and the mixture stirred at room temperature for twenty four hours. It was then diluted with ether (100 ml.) and washed with brine (100 ml, 50% w/v). The organic layer was separated and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a clear oil, which was shown to be a single compound by glc (2½% OVI, 130°C). The product was not purified but used directly in the next step. (31.74 g, 0.16 mol. 76%)

I.r. : neat film, ν_{max} 1750s cm^{-1}

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ1.02 (3H d 6.0 Hz), 1.4 (6H m), 2.3 (2H m), 3.51 (3H s), 3.6 (2H m), 4.41 (1H q 6.0 Hz), and 4.51 ppm. (1H bs).

(*R*)-3-(Tetrahydropyranyloxy)-butan-1-ol

Methyl-(*R*)-3-(tetrahydropyranyloxy)-butyrate (31.74 g, 0.16 mol.) was dissolved in dry ether (100 ml.) and added slowly to a stirred suspension of $LiAlH_4$ (6.08 g, 0.16 mol.) in dry ether (200 ml.) at 0°C. The mixture was stirred at this

temperature for thirty minutes and for a further 2½ hours at room temperature. The reaction was then stopped by addition of water (6.08 ml.), NaOH (6.08 ml. of a 10% w/v solution) and water (6.08 ml.) in rapid succession, and allowing the mixture to stir for a further one hour. This resulted in the formation of a grey/white granular precipitate, which was filtered off, and washed with terahydrofuran (100 ml.). The combined organic layers were dried over $MgSO_4$, and the solvent removed *in vacuo* to give a clear oil. This was distilled under reduced pressure, the fraction boiling at 86-89°C at 0.65 mmHg being collected. This was shown to be a single compound by glc (2½% OVI 130°C) (22.14 g, 0.13 mol. 80%).

I.r. : neat film, ν_{max} 3400 cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ1.25 (3H d 6.5 Hz), 1.35 (6H bm), 2.5 (2H m), 3.7 (1H m), 4.25 (2H bm) 4.6 (1H bm) and 9.75 ppm. (1H t)

Pyridinium dichromate

Pyridine (80.6 ml.) was added slowly to an ice cold solution of chromium trioxide (100 g, 1 mol.) in water. The solution was allowed to stir for five minutes and was then diluted with acetone (400 ml.) and cooled to -20°C. After three hours the resulting orange crystalline solid was filtered off washed with acetone and dried under high vacuum. The product was stable for long periods of time if stored in a vacuum desiccator.

(*R*)-3-(Tetrahydropyranyloxy)-butanal

(*R*)-3-(Tetrahydropyranyloxy)-butan-1-ol (2g, 11.4 mmol.) was dissolved in methylene chloride (50 ml.), along with pyridinium dichromate (32 g, 80 mmol.) and stirred at room temperature for twenty four hours. The solvent was then removed *in vacuo*, and the residual brown slurry poured on to the top of a short flash column (2.5 cm, 50 g silica). The column was eluted once with ether (100 ml.) and the solvent rmoved *in vacuo* to give the product which was air sensitive and had to be used immediately. (1.4 g, 8.1 mmol. 72%).

^{1}H n.m.r. (80MHz, $CDCl_3$) : δ1.25 (3H d 6.0 Hz), 1.35 (6H bm), 2.5 (2H bm), 4.25 (2H bm), 4.5 (1H m), 4.6 (1H bm), and 9.75 ppm. (1H t 3 Hz).

Ethoxycarbonylmethylene-triphenylphosphorane

Ethyl bromoacetate (20 g, 0.12 mol.) was added to a solution of triphenylphosphine (31.44 g, 0.12 mol.) in toluene (100 ml.), and the mixture allowed to stir at room temperature overnight. The resulting white precipitate was filtered off and dried, (50.6 g, 0.117 mol. 97%). (mp. 156-159°C, lit[82] 158°C).

^{1}H n.m.r. (80MHz, $CDCl_3$) : δ0.33 (3H t 8.0 Hz), 3.31 (2H q 8.0 Hz), 4.78 (1H bd 14 Hz), and 6.75-7.5 ppm. (15H bm).

The salt (1 g, 2.51 mmol.) was dissolved in water (50 ml.) and titrated against a sodium hydroxide (0.05 mol.) solution with vigorous stirring using phenolphthalein as an

indicator. The solution was then extracted with methylene chloride (50 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow gum which crystallised from benzene as white needles (mp. 115-119°C, lit[62] 117°C). (0647 g, 2.04 mmol. 95%).

^1H n.m.r. (80 MHz, $CDCl_3$) : δ0.84 (3H t 8.0 Hz), 2.99 (1H bs), 3.74 (2H q 8.0 Hz), and 6.75-7.5 ppm. (15H bm).

Ethyl 5-(*R*)-(tetrahydropyranyloxy)-2-(*E*)-hexenoate

(*R*)-3-(tetrahydropyranyloxy)-butanal (2 g, 11.6 mmol.) was added to a solution of ethoxycarbonylmethylene-triphenylphosphorane (7.35 g, 23.2 mmol.) in methylene chloride (50 ml.), and refluxed for six hours. The resulting pink solution was poured into water (50 ml.) extracted with ether (2 x100 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a red oil. This was absorbed onto flash silica (10 g.) and purified using a flash column with the following gradient : n-hexane/ethyl acetate ; 99/1 to 50/50 in 5% steps. The product was isolated as a clear oil (840 mg, 3.47 mmol. 30%).

I.r. : neat film, ν_{max} 1725s cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ1.15 (3H t, 8.0 Hz *CH_3*-CH_2), 1.25 (3H d, *6.0 Hz* *CH_3*-CH), 1.3-2 (6H bm), 2.3 (2H m, *CH_2*-CH), 3.9 (2H bm), 4.09 (1H q, 6.0 Hz *CH*-OH), 4.12 (2H q 8.0 Hz CH_3-*CH_2*), 4.55 ppm. (1H bm), 5.25 (1H dt, 12.0 Hz *CH*-CHCO), and 6.9 ppm (1H dt, 12 Hz *CH*-CO)

t-Butyoxycarbonylmethylene-triphenylphosphorane

t-Butyl-bromocetate (10 g, 51 mmol.), and triphenyl-phosphine (13.36 g, 51mmol.) were stirred together in dry benzene (50 ml.) under a dry nitrogen atmosphere for 18 hours. The resulting white precipitate was filtered off and washed with dry benzene (22.89 g, 50.3 mmol. 98%). (mp.174-177°C, lit[63] 177°C). ^{1}H n.m.r. (80 MHZ, $CDCl_3$) : δ0.9 (9H s), 4.9 (2H d, 14 Hz), and 7.5 ppm (15H bm).

The salt (5 g, 10.9 mmol.) was dissolved in water (100 ml.), stirred vigorously and titrated against a NaOH solution (2 molar), using phenolphthalein as an indicator. When the end point had been reached the solution was extracted with benzene (100 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give pale yellow crystals (3.52 g, 9.4 mmol. 86%). ^{1}H n.m.r. (80MHz, $CDCl_3$) : δ1.19 (9H s), 2.6 (1H bs), and 7.2 ppm (15H bm).

t-Butyl-(R)-5-(tetrahydropyranyloxy)-hex-2(E)-enoate

(*R*)-3-(Tetrahydropyranyloxy)-butanal (1 g, 5.8 mmol.) was combined with t-butyloxycarbonylmethylene-triphenyl-phosphorane (3 g, 8 mmol.), in dry benzene (50 ml.) and refluxed under a dry nitrogen atmosphere for 3 hours. The solution was cooled and poured into water (50 ml.), and extracted with ether (2 x 100 ml.). The organic layers were separated, dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow oil.

This was absorbed onto flash silica (10 g.) and purified using flash chromatography using the following gradient : n-hexane 100% to ethyl acetate/n-hexane 1:1, in 5% increments. The product was isolated as a clear oil (590 mg, 2.18 mmol. 37%).

I.r. : neat film, ν_{max} 1730s cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ1.1 (3H d, 6.0 Hz *CH_3*-CHOR), 1.35 (9H s, *t-Bu*), 1.25 (6H bm), 2.25 (2H m, *CH_2*-CH), 3.3 (2H bm), 3.9 (1H q, 6.0 Hz CH_3-*CH*), 4.5 (1H bm), 5.6 (1H dt, 16.0 Hz CH=*CHCO$_2$R*), and 6.75 ppm. (1H dt *16.0 Hz CH*=CHCO$_2$R).

3-Hydroxy-5-(*R*)-(tetrahdropyranyloxy)-1-hexyne

Method 1. Ethyl bromide (0.5 g, 4.6 mmol.), was allowed to react with magnesium turnings (0.11 g, 4.6 mmol.), for thirty minutes and then refluxed gently for ten minutes. Acetylene gas was dried by passage through H_2SO_4/glass wool/KOH/$CaCL_2$, and then introduced in a fine stream into the ethereal solution of ethyl magnesium bromide. A fine white precipitate formed, which turned into a viscous black ether insoluble oil, which was totally unreactive towards 3-(tetrahydropyranyloxy)-butanal.

Method 2. Ethyl bromide (18.1 g, 0.17 mol.), was allowed to react with magnesium turnings (4.1 g, 0.17 mol.), in tetrahydrofuran (100 ml.), under a dry nitrogen atmosphere. Once the reaction had subsided, the solution was refluxed for fifteen minutes and then cooled to 0°C. The ethereal solution was

decanted off carefully into a dropping funnel attached to a three neck flask (250 ml.), containing tetrahydrofuran (100 ml.) that had been saturated with dry acetylene gas for thirty minutes at a temperature of -10°C. The ethereal solution of ethyl magnesium bromide was added slowly from the dropping funnel over a period of one hour with vigorous stirring. Each addition was accompanied by violent frothing due to the escape of the ethane. After the addition was complete a pink/white precipitate was present in the flask, this dissolved when the solution was allowed to come to room temperature. The solution was recooled to -10°C and (*R*)-3-(tetrahydropyranyloxy)-butanal (1 g, 5.81mmol.) was added in tetrahydrofuran (10 ml.). After one hour the solution was allowed to come to room temperature, and the acetylene flow turned off. The reaction was allowed to stir overnight at room temperature under a dry nitrogen atmosphere. The solution was then cooled in an ice bath and quenched with NH_4Cl (100ml saturated), and allowed to stir for twenty minutes. The mixture was then extracted with ether (3 x 100 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a brown oil (1.08 g, 94% crude yield), which could be used in the next step without purification. (bp. 84-88°C at 0.6-0.7 mmHg.)

I.r. : neat film, ν_{max} 3500s cm, 3300m cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ1.01 (3H d, *6.0 Hz* CH_3-CHOR), 1.15 (6H bm), 1.6 (2H m, CH_2-CHOH), 2.3 (1H d, 2.0 Hz C≡*CH*), 3.3 (2H bm), 3.5 (1H m, *CH*-OH), and 4.3 ppm. (1H bm).

Mass spectrum : m/z , (% base peak), M , 198 (1), 197 (4), 181 (2), 173 (10), 157 (4), 143 (3), 139 (2), 125 (20)

116 (3), 111 (1), 101 (100), 99 (5)

Exact mass $C_{11}H_{18}O_3$ found 198.1250 Δ 3.05 ppm. Requires 196.2614

Methyl 7-(*R*)-(tetrahydropyranyloxy)-3,4-octadieneoate

The crude product (1.08 g.) from the previous experiment, was combined with excess trimethyl-orthoacetate (5 g, 42 mmol.), and a catalytic amount of propionic acid in a round bottomed flask (50 ml.), and heated to reflux under a dry nitrogen atmosphere for twenty four hours. The mixture was cooled and the methanol produced removed *in vacuo*, to give a sweet smelling brown oil. This was absorbed onto flash silica (10 g.) and subjected to flash chromatograpy using the following gradient: 1% ether/methylene chloride to 10% ether/methylene chloride in 1% increments. The product was recovered as a sweet smelling clear oil (680 mg, 2.7mmol. 46% from aldehyde)

I.r. : neat film, ν_{max} 1720s cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ0.99 (3H d, 7.0 Hz CH_3-CHOR), 1.5 (6H bm), 2.1 (2H m, CH_2-CHC), 2.9 (2H dd, 7.0 Hz C=CH-CH_2CO_2R), 3.54 (3H s OCH_3), 3.65 (2H m), 3.8 (1H q, 7.0 Hz CH_3-CH-OR), 4.51 (1H bm), and 5.01 ppm (2H m, CH=C=CH).

Mass spectrum : m/z, (% base peak), M , 254 (30), 236 (12), 223 (10), 210 (23), 170 (8), 153 (17), 125 (27), 111 (20), 93 (21), 85 (100), 77 (18), 67 (29), 55 (28).

Exact mass $C_{14}H_{22}O_4$ found 254.1521 Δ 1.21 ppm. Requires 254.3254

Methyl (2*E*,4*Z*)-7-(*R*)-(tetrahydropyranyloxy)-octadienoate

Methyl-7-(*R*)-tetrahydropyranyloxy)-3,4-octadienoate (450 mg. 1.77 mmol.), was dissolved in dry benzene (5 ml.), and heated to reflux in the presence of basic alumina (pH 9, 3 g.) for six hours. The alumina was filtered off and the solvent removed *in vacuo* to give a clear oil. This was absorbed onto flash silica (5 g.) and purified using flash chromatography using ethyl acetate/n-hexane 1:8. (101 mg. 0.397mmol. 24%)

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ1.1 (3H d, 7.0 Hz CH_3-CHOR), 1.7 (6H bm), 2.5 (2H m, CH_2-CH=CH), 3.5 (2H bm), 3.7 (3H s, OCH_3) 3.95 (1H q, 7.0 Hz CH_3-*CH*OR), 4.6 (1H bm), 6.01 (2H m, CH=*CH*-CH=*CH*CO_2R), and 7.5 ppm. (2H m, *CH*=CH-*CH*=CHCO_2R).

Mass spectrum : m/z, (% base peak), M , 254 (12), 236 (5), 210 (83), 170 (57), 139 (65), 115 (95), 86 (100).

(*Trans*-3-Ethoxycarbonylallyl)-triphenylphosphonium bromide

Ethyl 4-bromocrotonate (15 mmol. 2.67 ml. of a 75% solution), was added to a stirred solution of triphenylphosphine (5.24 g, 20 mmol) in dry benzene (50 ml.). The solution was stirred at room temperature for forty eight hours by which time a white crystalline precipitate had formed. This was filtered and dried under high vacuum. (5.396 g. 14.4 mmol. 96%) (mp. 187-190°C lit[64] 189-191°C).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.01 (3H t 8.0 Hz), 3.9 (2H q 8.0 Hz),4.95 (1H dd 15 Hz), 6.2 (2H m), and 7.3 ppm. (15H m).

Ethyl (2*E*,4*E*)-7-(*R*)-(tetrahydropyranyloxy)-octadienoate

Sodium (0.04 g, 1.74 mmol), was allowed to react with super dry ethanol (5 ml.) under a dry nitrogen atmosphere. The solution was cooled to 25°C and tetrahydrofuran (25 ml.) added. To this solution was added (*trans*-3-ethoxycarbonylallyl)-triphenylphosphonium bromide (0.924 g, 2.47 mmol.), this produced a bright yellow solution. To the stirring solution was added (*R*)-3-(tetrahydropyranyloxy)-butanal (0.310 g. 1.8 mmol.), which caused the solution to turn bright red. The mixture was left stirring overnight at room temperature, and then refluxed for one hour, allowed to cool and the solvent removed *in vacuo*, to give a red oil which was analysed by glc. (2½% OVI 150°C). This showed that the oil was a mixture of two closely related compounds suspected to be the two possible geometric isomers of the product. The mixture was dissolved in dry benzene (25 ml.) along with iodine (0.5% w/v), and irradiated with light from a 100 w light bulb for nine hours. The solution was cooled and washed with sodium thiosulphate (2x50 ml. 10% w/v), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a clear oil. Analysis by glc. (2½% OVI 150°C) showed that the oil was now a mixture of two unrelated products. This mixture was dissolved in ethanol (5 ml.) along with PPTS (0.1 g, 0,4 mmol.) and stirred at room temperature for twenty four hours. The solution was passed down a short flash silica column, and the solvent removed *in vacuo* to

give a clear oil. This was purified by plc. using n-hexane/ethyl acetate 8:1 (0.83 g, 0.40 mmol. 22%) and identified as the free alcohol.

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ 1.1 (3H t, 8.0 Hz *CH_3*-CH_2), 1.25 (3H d, *6.0 Hz* *CH_3*-CHOH), 2.27 (2H dd, 11.0 Hz*CH_2*-CH=CH), 3.9 (1H m, CH_3-*CH*OH), 4.1 (2H q, 8.0 Hz *CH_2*-CH_3), 5.75 (1H dt, 12.0 Hz *CH*CO_2R), 6.1 (1H dd, 11.0 Hz CH=*CH*-CH=CHCO_2R), 6.25 (1H dd, 12.0 HzCH=CH-*CH*=CHCO_2R), and 7.25 ppm. (1H m, *CH*=CH-CH=CHCO_2R).

Prop-2-ynal (propargyl aldehyde)

A one litre three neck flask was fitted with a dropping funnel, N_2 inlet, and a distillation head connected to a vacuum line with three traps. In the flask was placed propargyl alcohol (50 g, 0.89 mol.), and a pre-cooled solution of H_2SO_4 (60 ml.) in water (100 ml.) The flask was cooled in an ice/salt bath to -5°C. The first trap in the vacuum line was cooled to -5°C and the remaining traps cooled to -78°C (CO_2/acetone). The pressure of the system was reduced to 40-60 mmHg, and the nitrogen flow started. A solution of CrO_3 (89 g, 0.89 mol.), in H_2SO_4 (60 ml.), and water (150 ml.), was then added dropwise over a three hour period. The reaction vessel was maintained at 2-12°C. After the addition was complete the pressure was reduced further to 10 mmHg. for fifteen minutes to complete the distillation. The contents of the three traps were combined and washed with brine (50 ml. saturated), the organic layer was dried

over $MgSO_4$ and the solvent removed *in vacuo*. The product was purified by distillation (bp 56-58°C, lit[56] 56°C). (10.04 g, 0.19 mol. 21%)

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ 3.59 (1H s, *HC*≡C),

and 9.07 ppm (1H s, *CHO*).

Ethyl pent-2-en-4-ynoate

Prop-2-ynal (0.5 g, 9.25 mmol.) was dissolved in tetrahydrofuran (5 ml.) and added to a solution of ethoxycarbonylmethylene-triphenylphosphorane (3.5 g, 10 mmol.) in tetrahydrofuran at -15°C. The solution was stirred at this temperature for thirty minutes, and then for one hour at room temperature. The solvent was then removed *in vacuo*, and the product distilled from the semi solid residue under high vacuum into a trap at -78°C (bp. 26-28°C at 0.9 mmHg)

(0.754 g, 6.0 mmol. 65%)

I.r. : neat film, ν_{max} 1720s and 2100w cm

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ 1.1 (3H t, 7.0 Hz CH_2-*CH_3*),

3.26 (1H d, 2.0 HZ *HC*≡C), 4.15 (2H q *7.0 Hz* *CH_2*-CH_3), 6.19 (1H d,

16.0 Hz *CH*CO_2R), and 6.67 ppm. (1H dd, *16.0 Hz* *CH*-C≡CH).

Mass spectrum : m/z, (% base peak), M , 124 (16), 96 (30),

79 (75), 69 (100), 55 (5), 51 (32).

Exact mass : $C_7H_8O_2$ found 124.0527 Δ 1.87 ppm. Requires 124.139

Ethyl (*S*)-2-(Mesyloxy)-propanoate

Ethyl-(*S*) lactate (82.0 g, 0.695 mol.) was combined with triethylamine (111.0 ml, 0.798 mol.), and dissolved in toluene (1l.) The mixture was placed in a two litre round bottomed flask equipped with a mechanical stirrer and cooled to 0°C under a dry nitrogen atmosphere. Methane sulphonyl chloride (56.6 ml, 0.73 mol.), was then added carefully, and the light orange solution stirred at 0°C for one hour. The solution was then allowed to stand at -20°C for eighteen hours, the solid was then filtered off, the filtrate dried over $MgSO_4$, and the solvent removed *in vacuo*. The residual oil was distilled under reduced pressure (bp.88-90°C at 0.6 mmHg. lit[57] 76°C at 0.3 mmHg.) to give a pale yellow oil. (107.4 g, 0.55 mol. 80%)

I.r. : neat film, ν_{max} 1750s, 1360m, and 1180w cm^{-1}

^1H n.m.r. (80 MHz, $CDCl_3$) : δ 1.1 (3H t 7.5 Hz), 1.5 (3H d7.0 Hz), 3.02 (3H s), 4.1 (2H q7.5 Hz), and 5.01 ppm. (1H q 7.0 Hz).

$[\alpha]_D$ = -52.47° c = 4.3 $CHCl_3$ lit[57] -52.8° c = 4.32 $CHCl_3$

(*S*)-2-(Mesyloxy)-1-propanol

A one litre three neck flask was equipped with a nitrogen inlet, reflux condenser, and a magnetic stirrer. The flask was charged with tetrahydrofuran (200 ml.), and with a solution consisting of $LiAlH_4$ in tetrahydrofuran (0.139 mol, 111 ml.). The flask was placed in an ice bath and cooled to 0°C. To the cooled solution was added H_2SO_4 (3.7 ml, 70 mmol.), and

the solution stirred at 0°C for one hour. The flask was then cooled to -5°C in an ice/salt bath, and ethyl (*S*)-2-(mesyloxy)-propanoate (23.5 g, 0.12 mol.) was added slowly in tetrahydrofuran (50 ml.), over a period of fifteen minutes. THe solution was allowed to stir for a further thirty minutes and was then quenched with a solution of tetrahydrofuran/water (20 ml, 1:1), and stirred for twenty minutes. The white granular precipitate was filtered off and the filter cake washed with tetrahydrofuran (3 x 100 ml.). The filtrate was dried over $MgSO_4$ and the solvent removed *in vacuo* to give the product as a clear oil (33.2 g). The product was not purified as it is prone to explosive decomposition, and was therefore used immediately in the next step.

(*R*)-Propylene oxide

A three neck round bottomed flask (250 ml.) was equipped with a rubber septum, magnetic stirrer, and a distillation head. Potassium hydroxide pellets (100g, 1.58 mol.) and water (50 ml.) were placed in the flask and the temperature raised to 70°C. The (*S*)-2-(mesyloxy)-1-propanol (33.2 g.) prepared above was introduced to the hot KOH solution by syringe, the product distilled out immmediately and was purified by redistillation (bp. 31-36°C lit[57] 33-34°C) from KOH pellets (3.1 g, 53 mmol. 45%,)

^1H n.m.r. (80 MHz, $CDCl_3$) : δ 1.13 (3H d 5.0 Hz), 2,2 (2H dd 6.0, 2.0 Hz), and 2.8 ppm (1H m). $[\alpha]_D$ = + 11° neat lit[57] + 13° neat.

Attempted preparation of Ethyl (*S*)-7-hydroxy-2-(*E*)-oct-2-en-4-ynoate

A solution of ethyl pent-2-en-4-ynoate (0.5 g, 4.03 mmol.) in tetrahydrofuran (50 ml.) was prepared and cooled to 0°C, under a dry nitrogen atmosphere. To this solution was added n-butyl lithium (2,7 ml of a 1.6 M solution in hexane, 4.43 mmol). the solution immediately turned black and a rubbery black solid precipitated out.

The reaction was repeated at -78°C with the addition of HMPA (10 ml.) in an attempt to solubilise the anion, however the black solid still formed. To test its reactivity (*R*) propylene oxide was introduced to the flask, but could be recovered unchanged after eighteen hours.

2-[(Propan-1-yl)oxyl-Tetrahydropyran

A mixture of propargyl alcohol (5 g, 89mmol.) and dihydropyran (8.8 g, 104 mmol.) was dissolved in tetrahydrofuran (75 ml.), along with a catalytic amount of *para*-toluene sulphonic acid and stirred at 0°C for six hours. The mixture was then diluted with ether (100 ml.), washed with sodium hydrogen carbonate solution (100 ml, 5% w/v), water (100 ml.) and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a clear oil which was distilled under reduced pressure (bp. 80-84°C at 19 mmHg, lit[55] 83°C at 20 mmHg.). (9.58 g, 68 mmol 77%).

I.r. : neat film, ν_{max} 3300s and 2120w cm^{-1}

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.4 (6H bm), 2.1 (2H d 2.0 Hz) 2.28 (1H t 2.0 Hz), 3.4 (2H bm), 4.03 (2H m), and 4.6 ppm. (1H bm). Mass spectrum : m/z, (% base peak), M , 140 (60), 112 (9), 101 (40), 85 (100), 57 (60), 56 (45), 53 (25), 42 (63), 39 (80).

1-(Tetrahydropyranyloxy)-5-(*R*)-hydroxyhex-2-yne

Ethyl bromide (2 g, 18 mmol.) was added to magnesium turnings (0.44 g, 18 mmol.) in tetrahydrofuran and allowed to react at gentle reflux under a dry nitrogen atmosphere. When the reaction had subsided the solution was heated to reflux for fifteen minutes, and then cooled to room temperature. 2-[(Prop-1-yl)oxy]-tetrahydropyran (2.5 g, 17.9 mmol) was then added slowly over a period of fifteen minutes. As the addition progressed there was violent frothing of the solution due to the escape of ethane. Once the addition was complete the solution was refluxed for thirty minutes, and recooled to 0°C. (+/-)-Propylene oxide (2.1 g, 18 mmol.) was then added, and once the addition was complete the reaction was brought back to reflux for four hours. The reaction was quenched with NH_4Cl solution (100 ml, saturated), extracted with ether (2 x 100 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a brown oil. This was absorbed onto flash silica (20 g.) and purified by flash chromatography using ethyl acetate/n-hexane 1:8 as eluant. The product was recovered as a clear oil (1.152 g, 5.8 mmol. 32%)
^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.18 (3H d, 7.0 Hz *CH_3*-CHOH), 1.7 (6h bm), 2.25 (2H dt, 6.0 Hz CHOH-*CH*-C≡C), 3.5 (2H bm), 3.9 (1H

h,7.0 Hz *CH*OH), 4.2 (2H dt, 6.0 HZ C≡C-*CH*$_2$-CHOTHP), and 4.75 ppm. (1H bm)

Mass spectrum : m/z, (% base peak), M , 198 (15), 197 (19), 181 (18), 169 (30), 129 (19), 101 (60), 55 (100), 79 (33) 67 (32), 56 (55), 52 (63), 43 (75), 41 (60).

Exact mass : $C_{11}H_{18}O_3$ found 198.123 Δ 9.3 ppm. Requires 198.261

1-(Tetrahydropyranyloxy)-5-(*R*)-(t-butyldimethylsilyloxy)-hex-2-yne

1-(Tetrahydropyranyloxy)-5-(*R*)-hydroxyhex-2-yne (1 g, 5.05 mmol.) was dissolved in dry dimethylformamide (30 ml.), and stirred under a dry nitrogen atmosphere. To this solution was added t-butyldimethylsilyl chloride, (0.765 g, 5.1 mmol.), and imidazole (0.69 g, 10.2 mmol.). The reaction was stirred overnight at room temperature, and then diluted with water (100 ml.), extracted with ether (3 x 50 ml.), washed with dilute HCl (100 ml, 5% solution), water (100 ml.), brine (100 ml, saturated), and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a pale yellow oil. This was absorbed onto flash silica (10 g.) and purified by flash chromatography using ethyl acetate/n-hexane 1:5. The product was recovered as a clear oil (1.5 g, 4.8 mmol. 95%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ -0.025 (6H s), 0.78 (9H s) 1.15 (3H d, 7.0 Hz *CH*$_3$-CHOSi), 1.5 (6H bm), 2.2 (2H m, *CH*$_2$-C≡CCH$_2$OTHP), 3.5 (2H bm), 3.9 (1H m, *CH*-OSi), 4.2 (2H dt, 8.0 Hz *CH*$_2$-OTHP), and 4.75 ppm. (1H bm).

Mass spectrum : m/z, (%base peak), M ,313 (30), 284 (10), 256 (50), 230 (21), 212 (24), 196 (5), 172 (40), 160 (95), 86 (100) 76 (93), 58 (79), 43 (87).

Exact mass $C_{17}H_{32}O_3Si$ found 312.21162 Δ5 ppm. Requires 312.5073

Attempted preparation of 1-Hydroxy-5-(*R*)-(t-butyldimethyl-silyloxy)-hex-2-yne

Repeated attempts to prepare this compound using various methods to remove the tetrahydropyranyl group failed. The deprotection was attempted using dilute mineral acid in methanol which decomposed the starting material, PPTS in methanol, which did not react at all, the starting material being recovered, and anhydrous magnesium bromide in tetrahydrofuran, which again did not react. No other methods were attempted.

2-(*E*)-5-(*R*)-(Tetrahydropyranyloxy)-hexenoic acid

Lithium hydroxide (0.42 g, 17.5 mmol.) was dissolved in a mixture of water and tetrahydrofuran (100 ml, 1:1). Ethyl-2-(*E*)-5-(*R*)-(tetrahydropyranyloxy)-hexenoate (0.181 g, 0.75 mmol.), was added to the lithium hydroxide solution (10 ml.), and stirred for twenty four hours at room temperature. The solution was then diluted with ether (50 ml.), and the layers separated. The aqueous layer was extracted with ether (2 x 50 ml.), and the organic layers combined and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a clear oil. (110 mg. 0.52 mmol. 69%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.1 (3H d 6.0 Hz), 1.5 (6H bm), 2.4 (2H m), 3.3 (2H m), 3.9 (1H q 6.0 Hz), 5.8 (1H dd 12.0 Hz), 6.9 (1H dd 12.0 Hz), and 8.1 ppm. (1H bs).

Methyl 7-(*R*)-hydroxy-2-(*E*),4-(*Z*)-octadienoic acid

Methyl 7-(*R*)-(tetrahydropyranyloxy)-2-(*E*),4-(*Z*)-octadienoate (0.221 g, 0.87 mmol.) was dissolved in methanol (1 ml.) and a catalytic amount of HCl (0.1 ml. 5% solution) added. The solution was stirred at room temperature for three hours, and was then diluted with chloroform (10 ml.), washed with water (20 ml.), sodium hydrogen carbonate solution (5% w/v),dried over $MgSO_4$, and the solvent removed *in vacuo* to give a clear oil (0.138 g, 0.81 mmol, 93%)

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.25 (3H d6.0 Hz), 2.3 (2H dd 10.0 Hz),3.6 (3H s), 3.9 (1H q6.0 Hz), 6.01 (3H m), and 7.6 ppm. (1H dd 12.0 Hz).

Attempted preparation of the *seco* ester (192)

The acid and the alcohol prepared above were dissolved in dry methylene chloride along with DMAP (20 mg, 0.1 mmol), and the solution cooled to 0°C. DCC (0.113 g, 1.0 mmol.) was then added and the solution allowed to warm to room temperature and stirred for twenty hours. The white precipitate was then filtered off and the solution dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow gum. This was purified by plc. using

ether/methylene chloride 1:10 as eluant. A product suspected to be the *seco* ester (192) was isolated (21 mg.)

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.1 (3H d 6.0 Hz), 1.16 (3H d 6.0 Hz),1.6 (6H bm), 2.3 (2H dd), 2.5 (2H m), 3.6 (3H s), 3.9 (1H q 6.0 Hz), 4.05 (1H q 6.0 Hz), 5.01 (1H m), 6.03 (3H m), 6.03 (3H m), 6.9 (1H m), and 7.5 ppm. (1H dd 12 Hz).

The product was a shown to be a single compound by glc (2½% OVI, 180°C), but decomposed during an attempt to remove the tetrahydropyranyl group with mercuric chloride.

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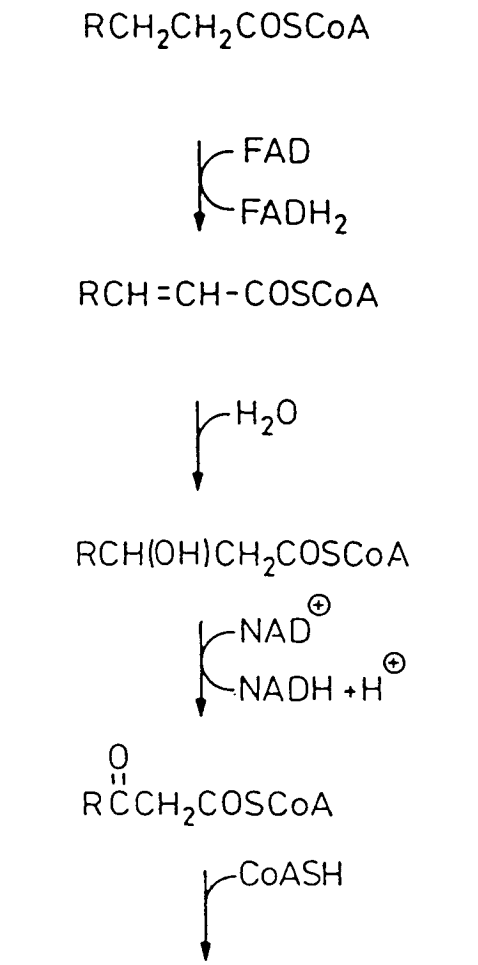
CHAPTER FOUR

NEW BIOSYNTHETIC PROBES BASED ON MALONYL CoA.

Scheme 1


RCH2CH2COSCoA
FAD
FADH2
RCH=CH-COSCoA
H2O
RCH(OH)CH2COSCoA
NAD⊕
NADH + H⊕
O
RCCH2COSCoA
CoASH
RCOSCoA + CH3COSCoA

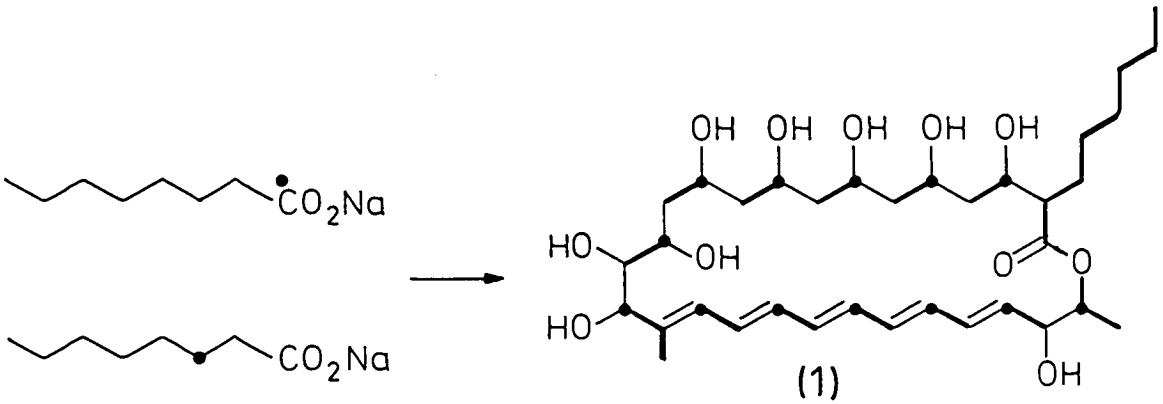
NEW BIOSYNTHETIC PROBES BASED ON MALONYL CoA

4.1. INTRODUCTION.

In the first chapter the role of acetyl CoA and malonyl CoA in both fatty acid and polyketide biosynthesis was described. The elucidation of these details in the polyketide pathway was determined largely by *in vivo* fermentation methods, using precursors such as sodium acetate and malonic acid derivatives. In this way the overall structural biosynthesis of many polyketides has been determined. However very little is known about the absolute stereochemistry of these processes, compared with what is known about fatty acid biosynthesis as determined by Cornforth *et al* [1]. This is due to two factors, the structural diversity of polyketides, and the absence of good cell free enzyme systems for *in vitro* studies, such as those used in the study of fatty acids[1].

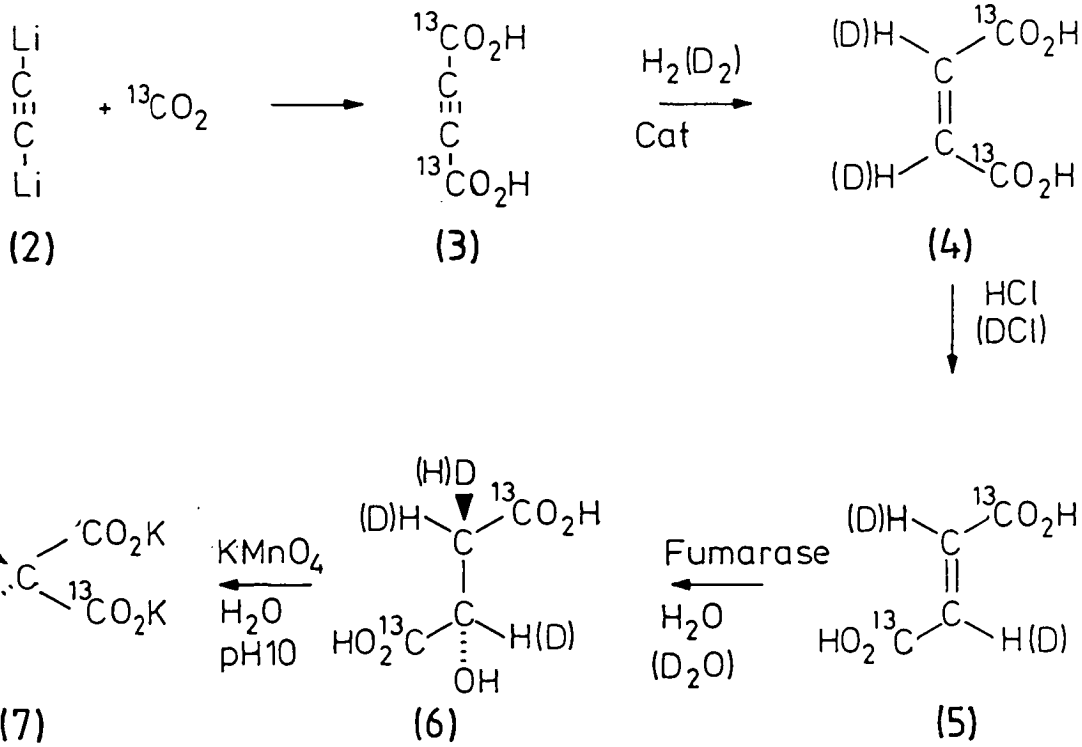
With studies limited to *in vivo* fermentation, problems arise with precursors larger than acetate or malonic acid derivatives. Incorporation of carboxylic acids or their derivatives larger than four carbons are rare. This is mainly due to the activity if the β oxidation pathway, by which organisms catabolise fatty acids[2] as shown in scheme 1. The pattern of intact and catabolised incorporation of octanoic acid into fungichromin[3] (1), a macrolide from *Streptomyces celluosae* is shown in scheme 2. [1-^{13}C]- and [3-^{13}C]octanoates were administered in separate experiments to *Streptomyces cellulosae*,

Scheme 2



(1)

Scheme 3



(2) (3) (4)

(7) (6) (5)

the resulting fungichromin showed intact incorporation of octanoate, but also labelling in positions derived from C-1 of acetate, as shown by [1-^{13}C]acetate experiments. This indicated that some of the octanoate had been metabolised to acetate and hexanoate. No further degradation of the chain occurred, as the [3-^{13}C]octanoate experiment did not show any labelling in the acetate derived positions. All attempts to incorporate labelled hexanoate failed.

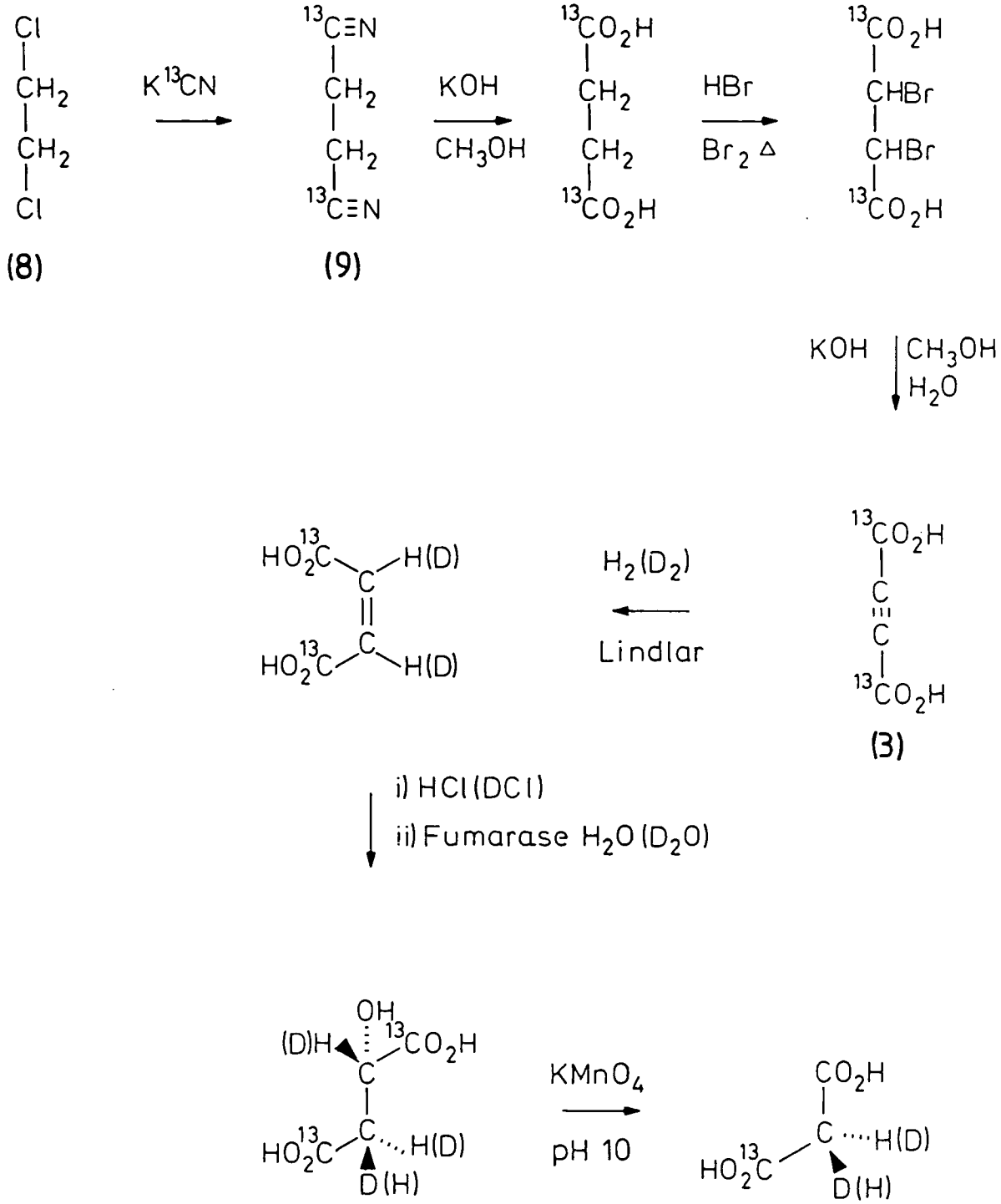
In these situations it is difficult to gain detailed stereochemical information about the advaced stages of polyketide biosynthesis, in particular the stereochemistry of reduction and elimination processes. Recently[4,5] intrest has focussed on the use of chiral malonate precursors in an attempt to gain more information about these steps.

As with fatty acid biosynthesis, the chain extension unit in polyketide biosynthesis is malonyl CoA (scheme 9, Ch 1). In certain organisms the equilibrium between acetyl CoA and malonyl CoA is in favour of the latter, and this leads to the so called "starter" effect[5]. This is observed as more intense labelling in the "starter" acetate unit than in subsequent malonate units, due to isotopic dilution by natural malonyl CoA. This effect can be overcome by using malonic acid derivatives as precursors. It is these very sites derived from the C-2 of malonyl CoA that are often involved in reduction/elimination steps. Therefore a malonic acid precursor that is specifically labelled at C-2 could potentially provide some of the desired stereochemical information.

Chiral malonic acid derivatves are the most important metabolic precursors still to be utilized in the study of both primary and secondary metabolism. Malonic acid or any of its symmmetrical derivatves has C_{2v} symmetry, and therefore the methylene group can be considered a *pro*-prochiral site of the Ca_2b_2 type[7]. Chirality can be conferred on a site such as this by isotopic labelling. Since the difference in size is very small, even in the extreme case of the hydrogen isotopes, the isotopically chiral substrate will bind to the enzyme in the same fashion as the prochiral substrate. The only observable difference will be in the rate of the reaction occuring at the active site, which will be affected by an isotope effect. A prochiral site of this kind can be converted into a chiral centre in two ways. In the studies on fatty acid biosynthesis Cornforth *et al* [1], abolished the structural symmetry of the molecule by preparing the half ester, therefore ^3H label at the C-2 position created a chiral centre. (scheme 10, Ch 1).

If the structural symmetry of the malonic acid derivative is retained, then isotopic labelling of one of the methylene protons is not sufficient to create chirality, one of the carboxyl carbons must also be labelled. Scheme 3 shows the route of Floss *et al* [4] to a malonic acid derivative of this kind. Lithium acetylide (2) was carboxylated with $^{13}CO_2$ to give acetylene dicarboxylate (3), which was partially reduced with the appropriate form of hydrogen to maleic acid (4) and then isomerised to fumaric acid (5). This was then specifically hydrated using the appropriate form of water and fumarase[1] to

Scheme 4


Cl
CH2
CH2
Cl
K13CN
13C≡N
CH2
CH2
13C≡N
KOH
CH3OH
13CO2H
CH2
CH2
13CO2H
HBr
Br2 Δ
13CO2H
CHBr
CHBr
13CO2H
(8)
(9)
KOH
CH3OH
H2O
HO2C13
C
H(D)
C
HO2C13
H(D)
H2(D2)
Lindlar
13CO2H
C
C
13CO2H
(3)
i) HCl (DCl)
ii) Fumarase H2O (D2O)
OH
(D)H
13CO2H
C
C
HO2C13
H(D)
D(H)
KMnO4
pH 10
CO2H
C
H(D)
HO2C13
D(H)

give chiral malic acid (6), permanganate oxidation giving malonic acid (7). A similar route has been used by Jordan *et al* [5] as shown in scheme 4. The starting material here is 1,2-dichloroethane (8) which is converted to acetylene dicarboxylate (3) *via* succinonitrile (9), and then to malonic acid in an identical fashion to that shown in scheme 3. Note that in both these cases the malonic acid derivative is only chiral by virtue of the labelled carboxyl function.

The major problem both in the synthesis and use of precursors such as (7) is their tendency to racemise. Malonic acid itself has a half life of ninety minutes in $^{2}H_{2}O$ at 35°C. Under mild conditions (pH 9, 30°C), the half life of (7) is 216 minutes[4]. Thus (7) must be generated and used above pH 8, purification steps such as chromatography are ruled out, and experiments must be carried out in minutes rather than hours. It is mainly because of the latter reason that no experiments using precursors such as (7) have been carried out on polyketide systems, which can take several days or even weeks to produce metabolites.

A further problem with such symmetrical derivatives was highlighted by Jordan[5]. The chiral malonate (7) was used in a study of mammalian fatty acid synthetase. With such a symmetrical system, the transferase enzyme is unable to distinguish between the two carboxyl groups, and as one of the methylene hydrogens is removed during the synthesis, there is an equal chance of incorporation of one but not both labels into the product fatty

Scheme 5

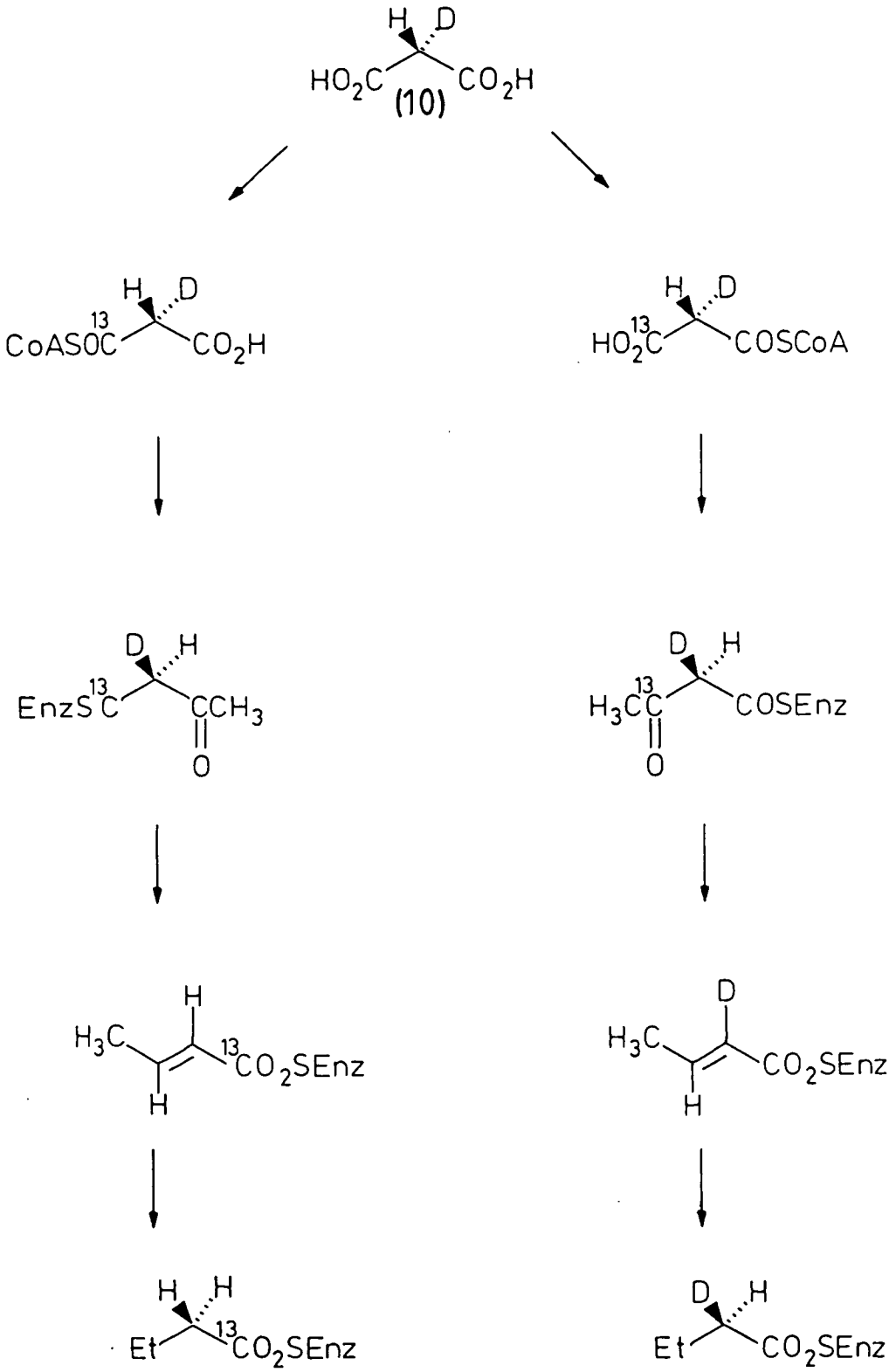
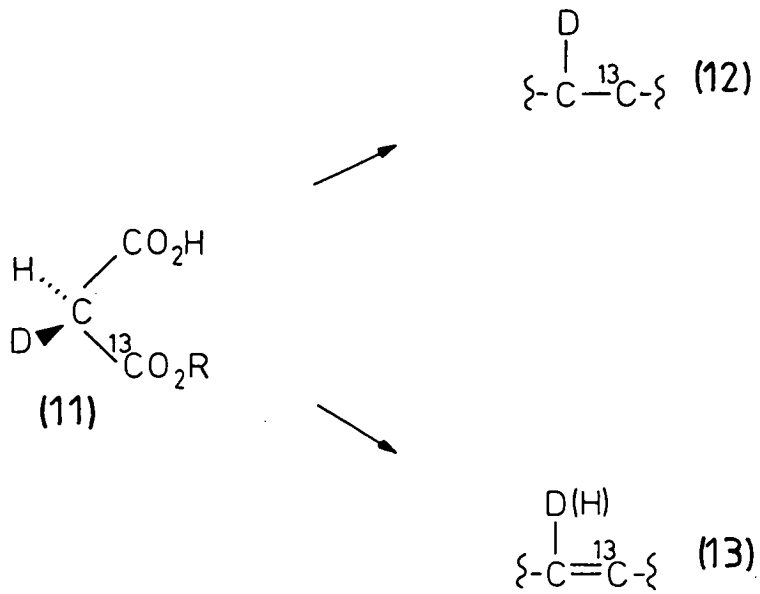

H D
HO2C CO2H
(10)
H D
CoASOC 13 CO2H
H D
HO2C 13 COSCoA
D H
EnzS 13 C CCH3
O
D H
H3C 13 C COSEnz
O
H
H3C 13 CO2SEnz
H
D
H3C CO2SEnz
H
H H
Et 13 CO2SEnz
D H
Et CO2SEnz

Figure 1

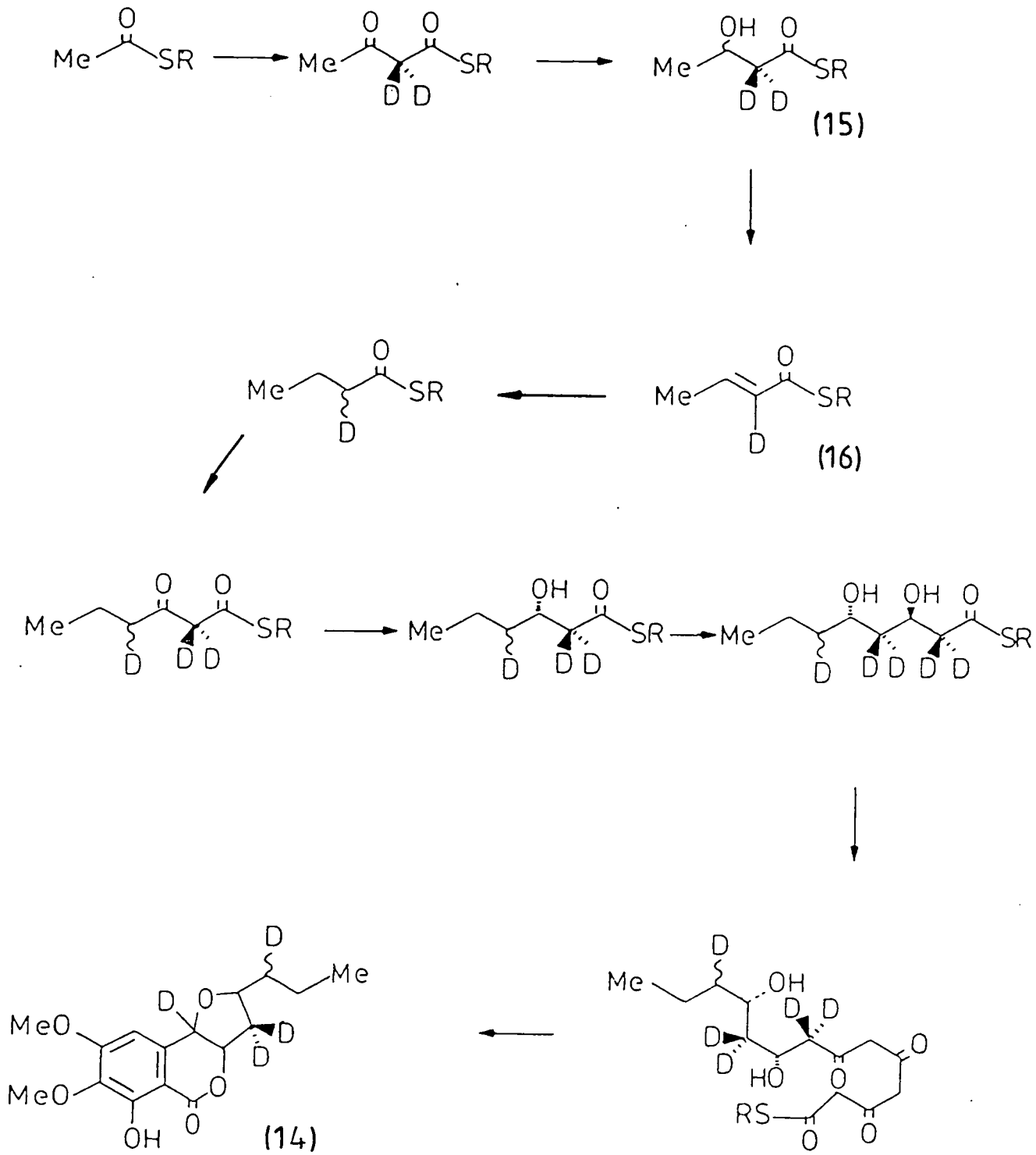


acid. This situation is illustrated in scheme 5 for the 2-(*R*)-malonic acid (10).

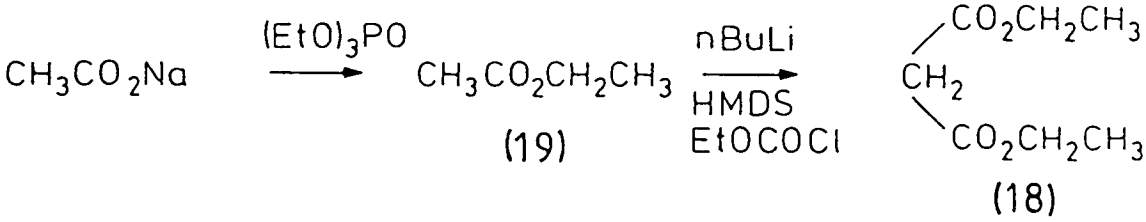
In a potential polyketide study it would be desirable to introduce not only a chiral methylene group, but also the the specific two carbon unit containing it. This situation is shown in figure 1. The malonic acid derivative (11) is chiral both by virtue of the label in the carboxyl group and the asymmetry of the structure. If the C-1, C-2 unit is selectively recognised by the enzyme system and incorporated into the hypothetical polyketide (12), then the ^{2}H label can be detected by observation of the carboxyl ^{13}C label in a β-isotope shift experiment. Similarly if (11) is converted to (13), *via* elimination then the loss of either the *pro*-(*R*) or *pro*-(*S*) hydrogen could be detected by use of the appropriate malonate and a β-isotope shift experiment.

This type of precusor , containing an "activated" two carbon unit would lend itself to an analysis of the biosynthesis of the side chain of monocerin (14)[6], which retains a solitary acetate derived hydrogen at C-12 of unknown stereochemistry.. The proposed overall biosynthesis of monocerin is shown in scheme 6. The key intermediate in this case is the crotonyl thiol ester (16) produced by elimination of water from the butyryl thiol ester (15). As the C-2 position of (16) is derived from malonyl CoA, experiments using 2-(*R*), and 2-(*S*)-[2-$^{2}H_{1}$] malonic acid derivatives should show which of the hydrogens is lost to give (16). The final stereochemistry of the product could only be predicted if the mechanism of the reduction of (16) is known. In

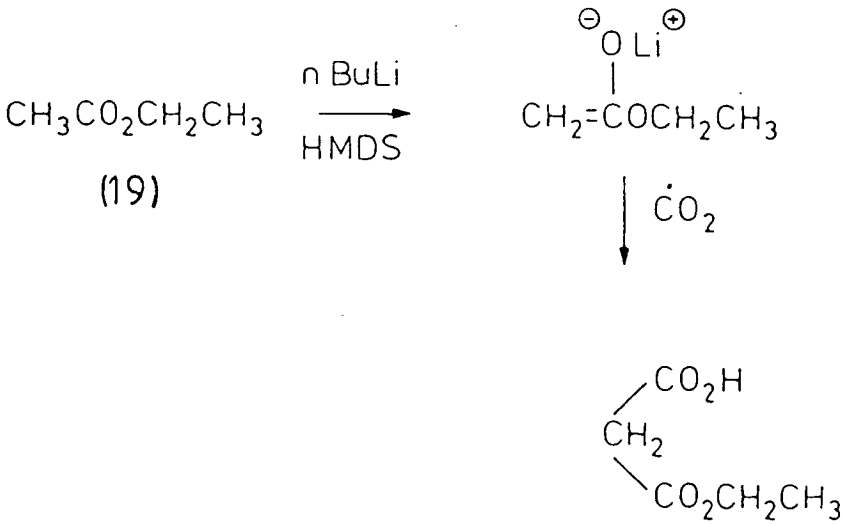
Scheme 6

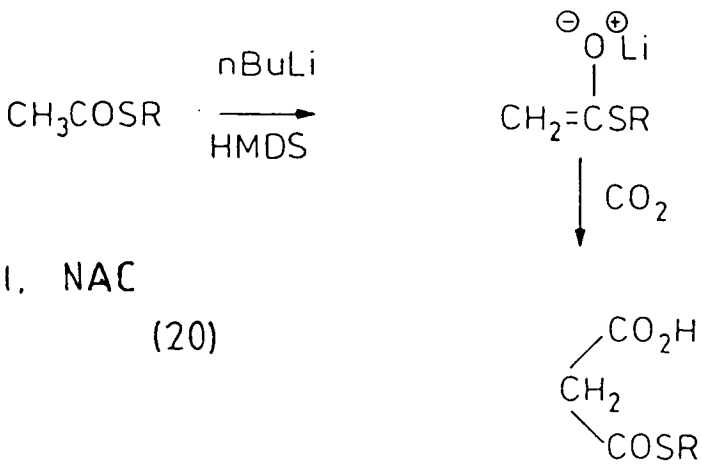


Scheme 7

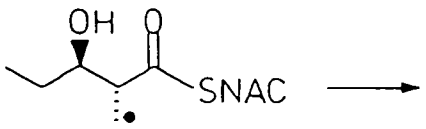


Scheme 8

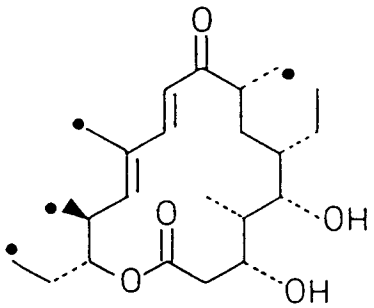




Scheme 9



(21)



(22)

NAC = SCH_2CH_2NAc

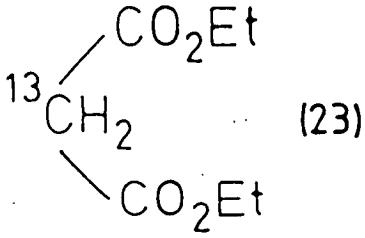
fatty acid biosynthesis this step has been reported to be species dependant[8]. The work described in the following chapter goes someway to achieving a precursor such as (11).

The proposal was to modify the synthesis of diethyl malonate (18) from sodium acetate as shown in scheme 7[10]. The key step that needs modification is the electrophile used to quench the enolate of ethyl acetate (19)[11]. As the synthesis is based on sodium acetate any degree of double labelling can be introduced to the product malonic acid derivative, to allow β-shift experiments to be carried out.

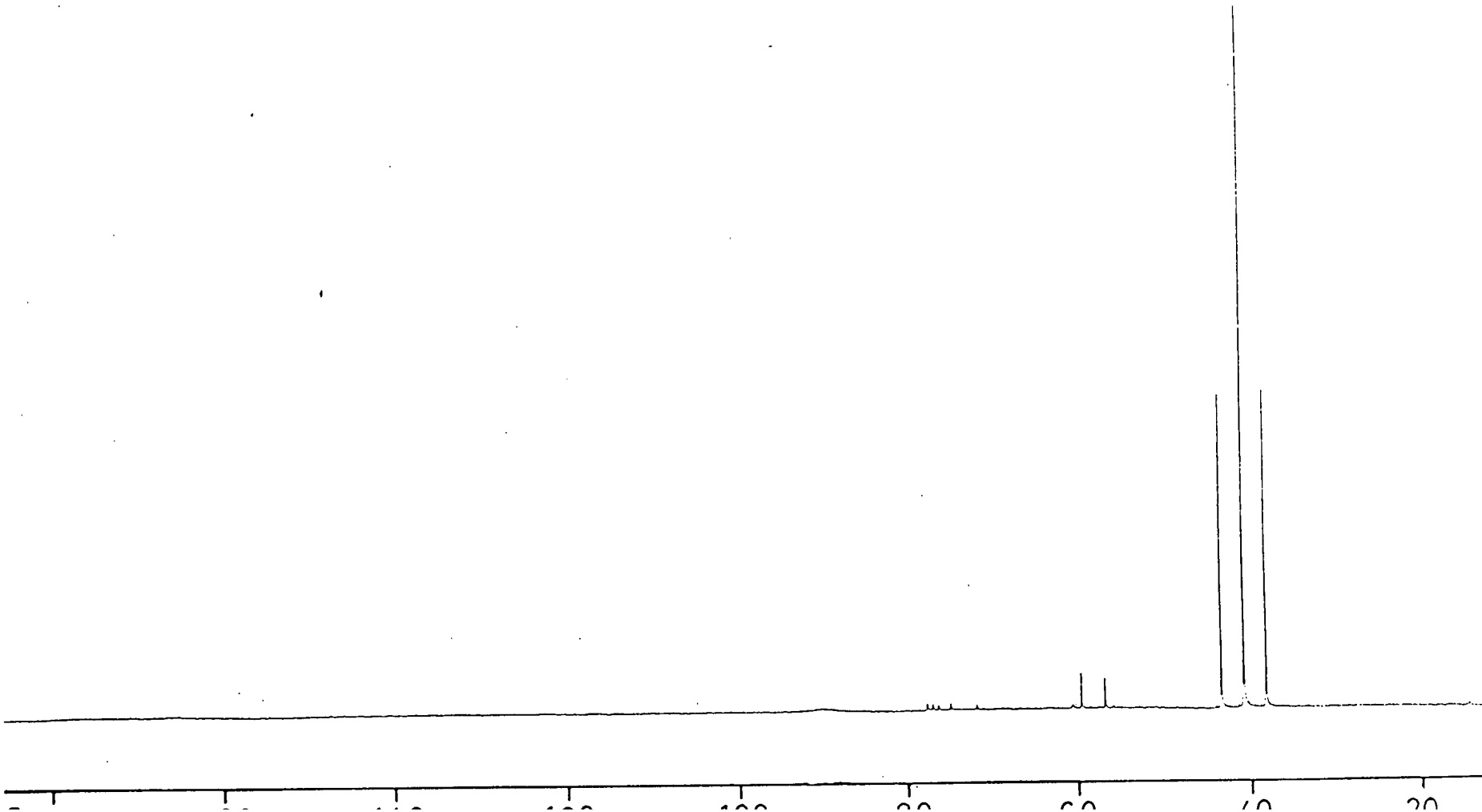
It was proposed to adapt this synthesis in two ways. Firstly the ethyl acetate (19) would be converted to the half ester of malonic acid by reaction of the enolate with CO_2 as shown in scheme 8. Secondly the ethyl ester would be replaced with longer chain alkyl groups to increase lipophilicity, and also with S-esters to mimic CoASH derivatives (scheme 8),in particular the S-N-acetylcysteamine ester (NAC) (20). This group has been successfully used incorporate large precursors into polyketides[12,13]. Scheme 9 shows the incorporation of the NAC. ester of 2-methyl-3-hydroxypentanoate (21) into tylactone (22) with a 1:1.1 ratio of intact to catabolic incorporation[12].

Having produced the various malonic acid derivatives shown in scheme 8, it is proposed to test them on a variety of organisms to look for specific incorporation of the labelled two carbon unit derived from acetate.

Figure 2 30.51MHz ^{13}C n.m.r. of (23)



(23)

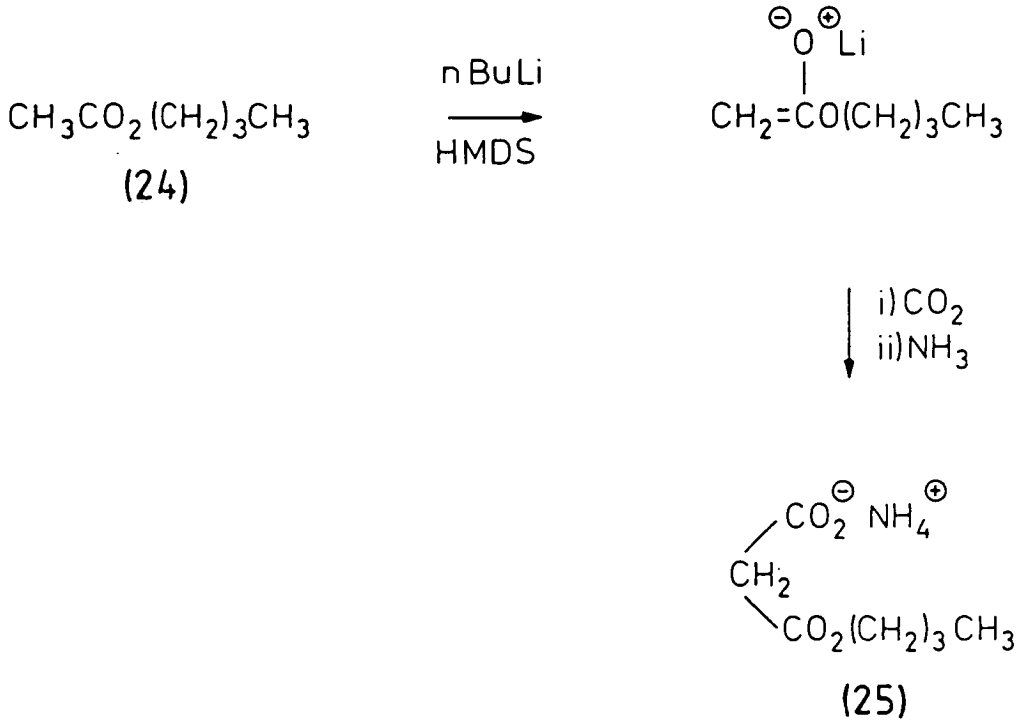


4.2. RESULTS AND DISCUSSION.

In order to succesfully alter the synthesis of diethyl malonate as shown in scheme 7 [10,11], the procedure was repeated, in order to become familiar with all the steps involved. In a synthesis such as this where isotopic label is being used it is important to avoid contamination of the product with by products, as they too are labelled and can lead to ambiguous results. The first stage of the reaction is the most sensitive, the sodium acetate must be completely anhydrous, and only freshly distilled triethyl phosphate gives reasonable yields of ethyl acetate (19). During the reaction of the ethyl acetate with lithium hexamethyldisilazide to form the enolate, the temperature must be maintained at less than -55°C to prevent self condensation. Finally the use of freshly distilled ethyl chloroformate prevents contamination of the diethyl malonate (18) with unwanted impurities.

Using the procedure described above diethyl-[2-^{13}C]-malonate (23) was prepared from [2-^{13}C]-acetate. The fully ^{1}H-^{13}C coupled n.m.r. spectrum of (23) is shown in figure 2. The C-2 methylene group appears as a triplet at 40.75 ppm. (J=129Hz). It was intended to use (23) to investigate metabolites from different organisms for "starter" effects in feeding studies. A pronounced "starter" effect would indicate that malonate incorporation is more efficient than acetate incorporation and so administration of one of the precursors postulated in scheme 8

Scheme 10


CH3CO2(CH2)3CH3
(24)
n BuLi
HMDS
O Li
CH2=CO(CH2)3CH3
i) CO2
ii) NH3
CO2 NH4
CH2
CO2(CH2)3 CH3
(25)


13CH3CO2(CH2)3CH3
(26)
CO2 NH4
13CH2
CO2(CH2)3CH3
(27)

may result in specific incorporation of an "activated" two carbon unit.

The first modification to be made to the route shown in scheme 7 was to attempt to prepare the *mono* ethyl ester of malonic acid (24). (scheme 8). Sodium acetate was converted to ethyl acetate (19) as described above, and the enolate prepared using lithium hexamethyldisilazide. Dry carbon dioxide gas was then introduced into the flask in a fine stream. Reaction occured almost immediately as indicated by the rapid rise in temperature from -78°C to -20°C in a few seconds, falling back to -78°C after two minutes. Isolation of the product from this system proved to be virtually impossible due to spontaneous decarboxylation during the work up.

To prove the feasibility of this type of reaction the synthesis was repeated using butyl acetate (24), as shown in scheme 10, rather than ethyl acetate (19). Initially commercial n-butyl acetate was used, but labelled n-butyl acetate can be prepared from sodium acetate by reaction with tri-n-butyl phosphate in a manner analogous to that described above. The enolate of n-butyl acetate was prepared using lithium hexamethyldisilazide, and allowed to react with carbon dioxide. This time the reaction was much slower with no rapid rise in temperature being observed. To prevent decarboxylation, the product (25) was isolated by passing anhydrous ammonia gas through the crude ethereal extract to give a white crystalline precipitate of the ammonium salt (25). This proved to be a convenient method of isolation and purification. The salt was

Figure 3 — 90.5 MHz ^{13}C n.m.r. of (26)

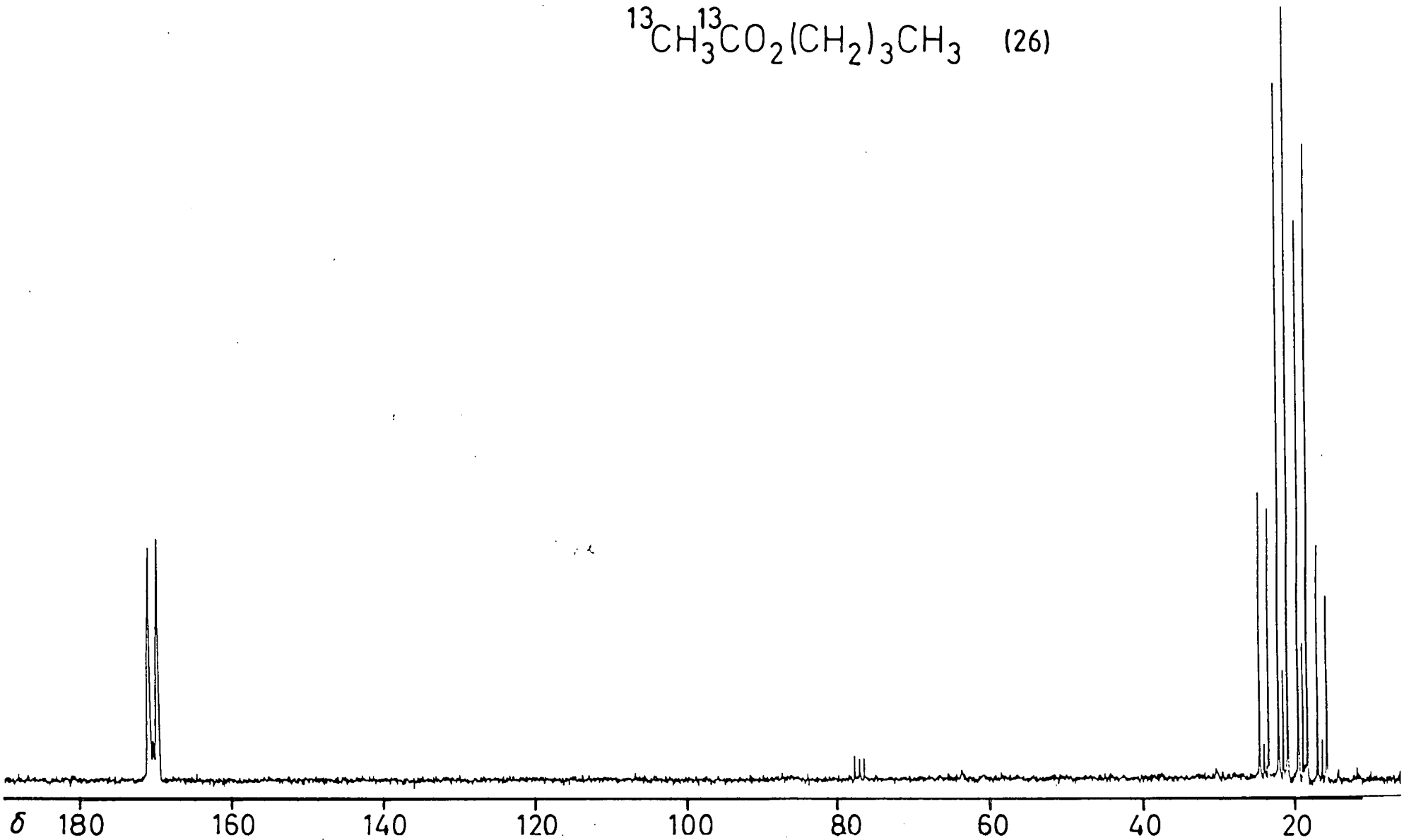
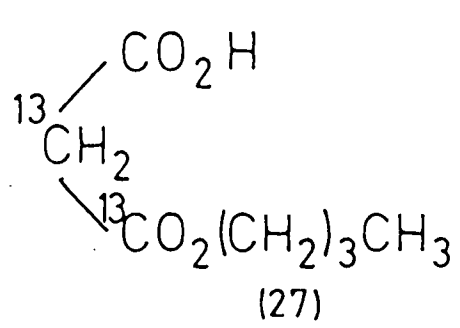
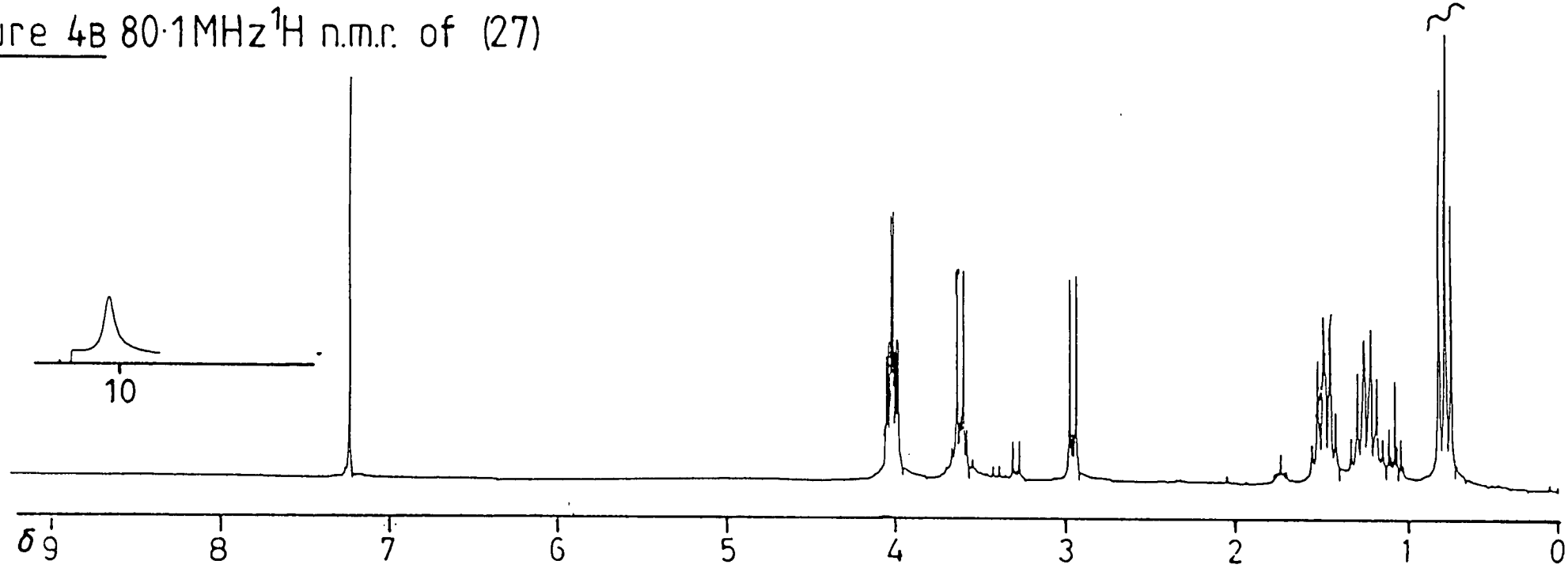


Figure 4A 20·1MHz ^{13}C n.m.r. of (27)



(27)

Figure 4B 80·1MHz ^{1}H n.m.r. of (27)



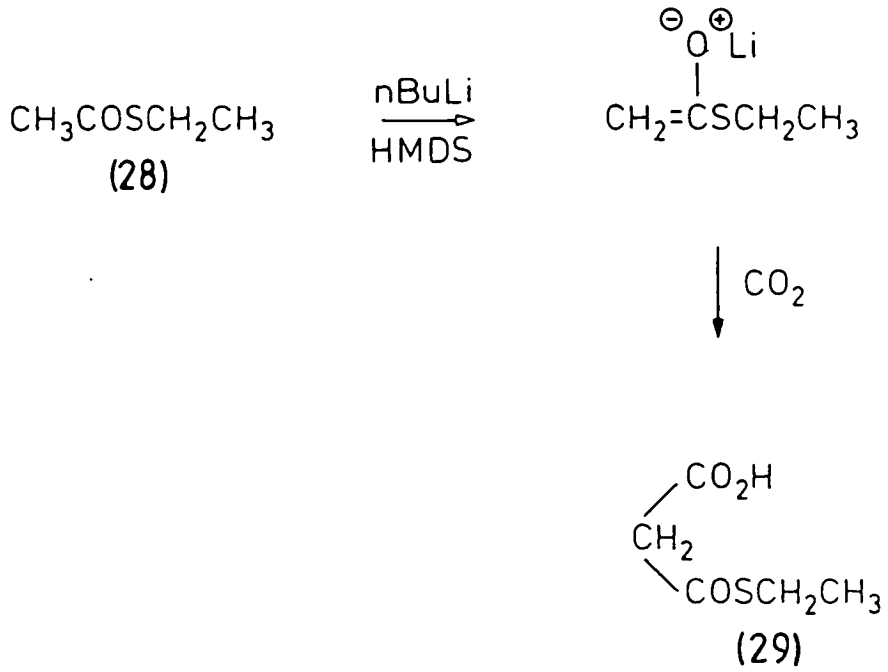
shown to be pure by high resolution FAB mass spectrometry. It also provided a conveniently water soluble precursor to use in feeding studies.

In an identical fashion [1,2-^{13}C]-acetate was converted *via* n-butyl-[1,2-^{13}C]acetate (26) to *mono*-n-butyl-[1,2-^{13}C]malonic acid (27). The fully ^{1}H coupled ^{13}C n.m.r. spectrum of (26) is shown in figure 3. Only the enriched carbons C-1 and C-2 are shown. The C-1 methyl group appears as a quartet of doublets at 20.17 ppm, the carbonyl group as a doublet at 170.9 ppm. The carbonyl resonance also shows signs of fine proton coupling through oxygen from the butyl protons.

Figure 4a shows the fully ^{1}H-^{13}C coupled n.m.r. spectrum of (27), again only the enriched carbons are observed. The C-2 methylene group now appears as a triplet of doublets at 40.14 ppm. and the carbonyl at 166.7 ppm as a doublet with signs of fine coupling through oxygen to the butyl fragment. Both of these ^{13}C spectra show signals due to residual singly labelled impurity *ca* 5%, present from the commercial material. Figure 4b shows the ^{1}H n.m.r. of (27). The labelled methylene group appears as a doublet of doublets at 3.26 ppm. Note the residual doublet due to singly labelled impurity. The coupling through oxygen from the carbonyl group to the butyl protons is also evident.

The *mono* n-butyl ester (27) was much more stable than the equivalent ethyl compound , and could be stored as the free acid at 0°c for long periods of time. However for feeding studies the ammonium salt was prepared as above to allow aqueous

Scheme 11



Scheme 12

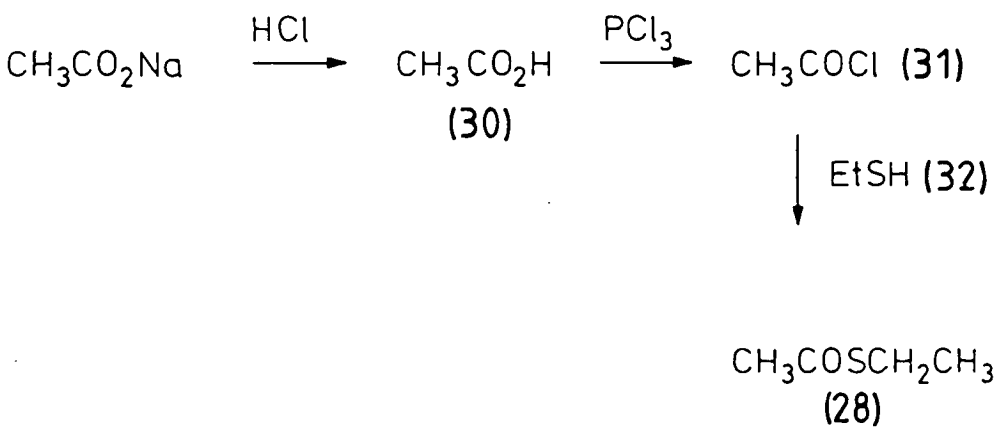
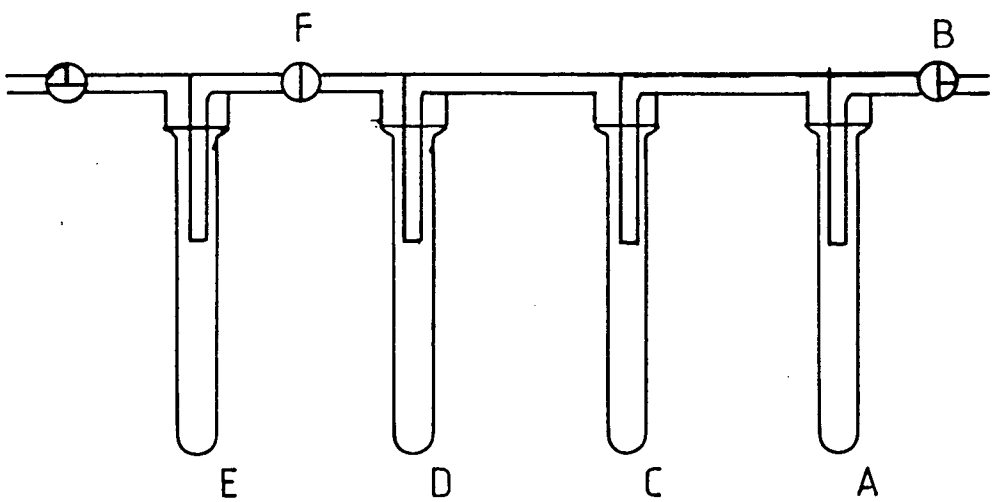


Figure 5



solutions to be used. This was not stable and had to be used within a few hours of preparation.

The next modification to the synthesis was to change the O-alkyl group to an S-alkyl group as shown in scheme 11. To test the feasibility of the carboxylation reaction on this type of substrate, commercial ethyl thiolacetate (28) was used initially. The enolate was prepared as usual using lithium hexamethyldisilazide, and allowed to react with carbon dioxide. The product (29) could be isolated from the reaction mixture by conventional extraction and was stable as the free acid at room temperature. The ammonium salt was easily prepared as described above and was found to be water soluble.

In order to prepare (29) in a labelled form, it was neccessary to prepare labelled ethyl thiolacetate (28) from sodium acetate. This was carried out using the route shown in scheme 12. Sodium acetate was converted to acetic acid (30) using the method of Cox *et al* [13]. This involves the reaction of sodium acetate with anhydrous HCl gas. The apparatus used is shown in figure 5. The sodium acetate is placed in A and the system flushed through B with dry nitrogen. Anhydrous HCl is then introduced through B and allowed to react with the acetate while the temperature is increased over thiry minutes to 100°C. The acetic acid formed distils out through C into trap D at -78°C. After thirty minutes the HCl flow was stopped and the system connected to a high vacuum line at tap F. Trap D is cooled down to -196°C and the remaining acetic acid is distilled into it. Tap F is then closed and trap D allowed to warm to room temperature.

Scheme 13

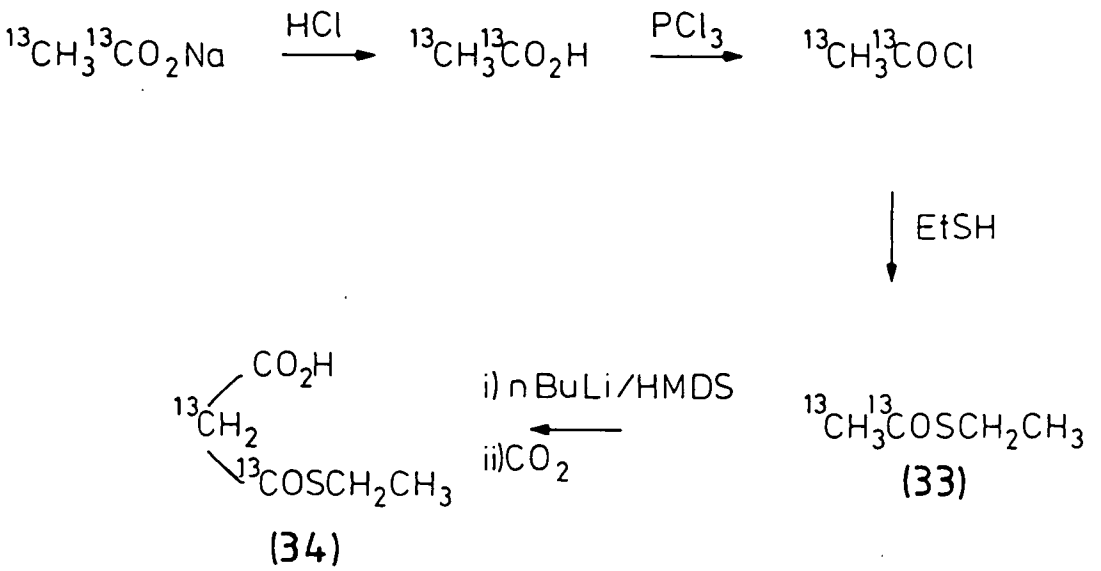
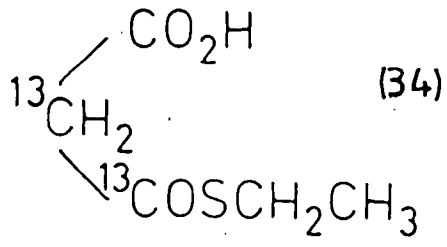

$^{13}CH_3{}^{13}CO_2Na$
HCl
$^{13}CH_3{}^{13}CO_2H$
PCl_3
$^{13}CH_3{}^{13}COCl$
EtSH
$^{13}CH_3{}^{13}COSCH_2CH_3$
(33)
i) n BuLi/HMDS
ii) CO_2
CO_2H
$^{13}CH_2$
$^{13}COSCH_2CH_3$
(34)

Figure 6 — 90 MHz ^{1}H n.m.r. of (34)



(34)

Dissolved HCl is then removed by briefly exposing the system to the high vacuum through tap F. The acetic acid is then distilled into trap E at -20°C.

To convert the acetic acid to acetyl chloride (31) it was treated with phthaloyl dichloride as described by Cox *et al*[13]. However this method proved to be inefficient, the product only being recovered in 60% yield. To overcome this the method of Roberts *et al* [14] for the preparation of acetyl bromide was adapted as shown in scheme 12. Treatment of acetic acid (30) with excess phosphorous trichloride at 40-50°c results in a two layer system of phosphorous acid and acetyl chloride (31) which can be distilled off easily in 80-85% yield. Combination of acetyl chloride (31) with ethane thiol (32) and allowing the reaction to proceed at room temperature for forty eight hours produced ethyl thiol acetate (28) in 60% yield[15].

Following the method described above [1,2-^{13}C]-acetate was converted to ethylthiol-[1,2-^{13}C]acetate (33) in 41% overall yield. The enolate was prepared using lithium hexamethyldisilazide, and allowed to react with carbon dioxide in the usual way to give *mono*-ethylthiol-[1,2-^{13}C]malonic acid (34) in overall 33% yield. (scheme 13). The ^{1}H n.m.r. of (34) is shown in figure 6. Extensive ^{13}C-^{1}H coupling through sulphur to the ester CH_2 can be seen in the multiplet at 2.9 ppm. This signal obscures one half of the C-2 methylene doublet of doublets, the other half of which can be seen at 4.25 ppm. The fully ^{1}H coupled ^{13}C coupled n.m.r. spectrum of (34) can be seen in figure 7. The C-2 methylene group appears as a triplet of doublets at 4.25 ppm.

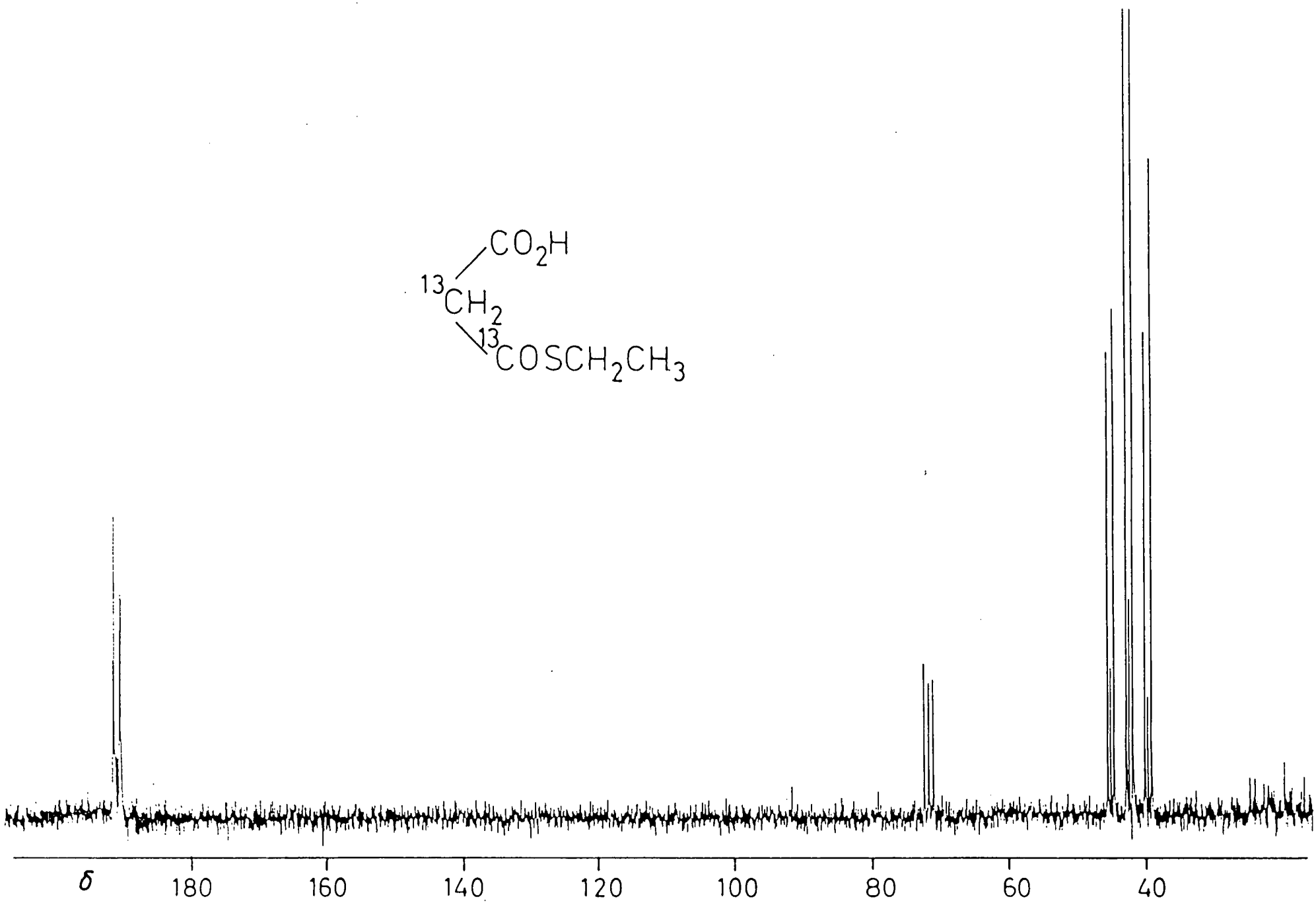
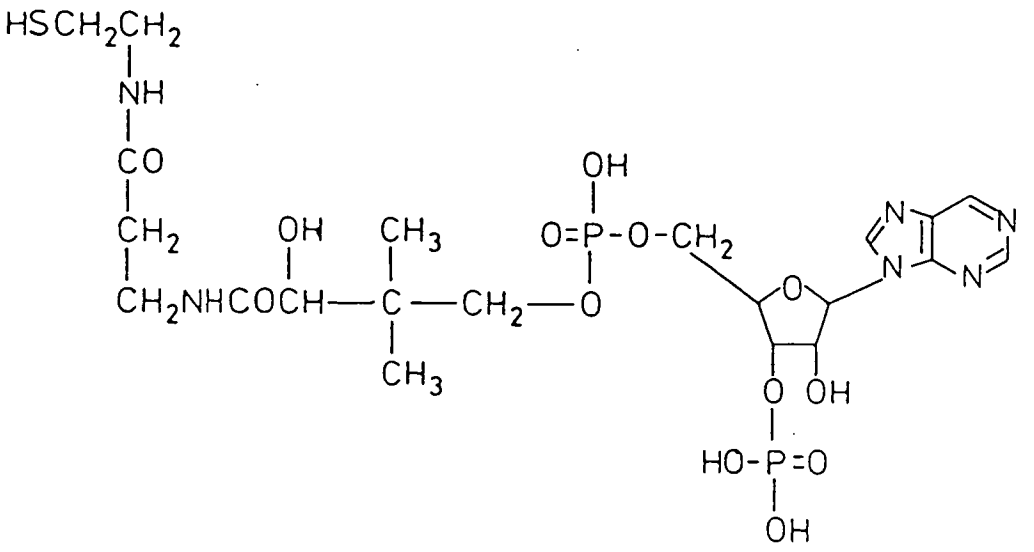
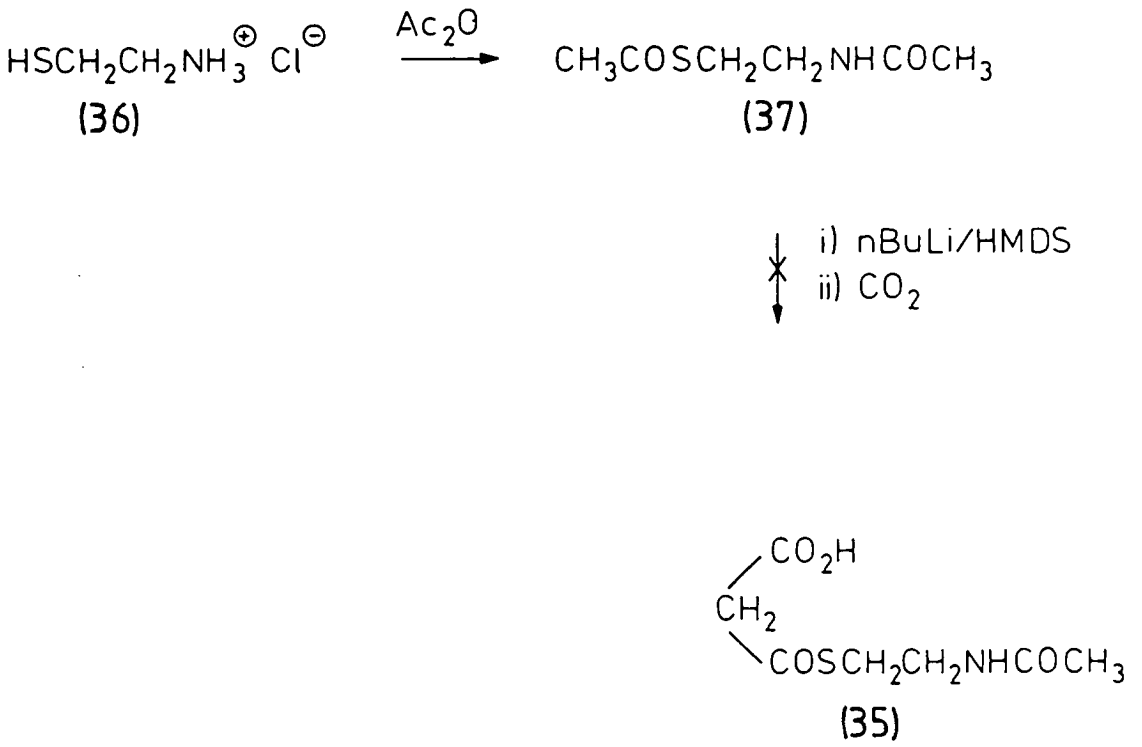

CO2H
13CH2
13COSCH2CH3
δ
180
160
140
120
100
80
60
40

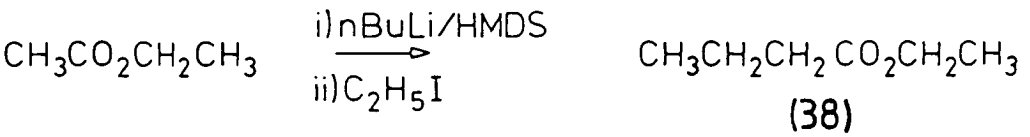
Figure 8


HSCH2CH2
NH
CO
CH2
CH2NHCOCH
OH
CH3
C
CH2
O
CH3
OH
O=P-O-CH2
O
N
N
N
N
O
O
OH
HO-P=O
OH

Scheme 14



Scheme 15



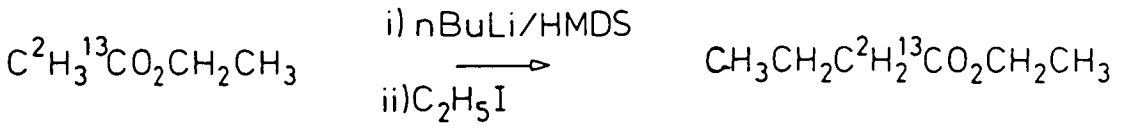
the singly labelled impurity appearing as a triplet. The C-1 carbonyl appears at 191,5 ppm. and as in the case of the O-n-butyl compound (29), there are signs of coupling through sulphur to the protons in the butyl fragment.

The final modification attempted was to try and prepare the S-N-acetylcysteamine ester (35) as shown in scheme 14. This group has proved useful in studies using large advanced precursors[12,13] with *in vivo* systems. The reason for this may be in part due to its similarity to the thiol side chain of coenzyme A (figure 8).

The proposed route to (35) is shown in scheme 14. 2-Mercaptoethylamine-hydrochloride (36) is acetylated to give N,S-diacetylcysteamine (37). It was then proposed to selectively enolise the S-acetyl group by reacting (37) with two moles of base. The first equivalent of base to remove the amino proton, the second to form the S-enolate. Reaction with carbon dioxide would then give the *mono*-NAC-malonic acid derivative (35) directly. However this reaction failed and resulted in polymerisation, probably due to the tendency of thiol esters to react with themselves, rather than other substrates. However no such reaction was observed with ethyl thiolacetate.

The reactions described above all use sodium acetate as the starting material, hence any desired form of labelling in the "activated" two carbon unit can be achieved from the appropriate acetate. To test the versitility of the reaction the enolate of ethyl acetate was prepared and allowed to react with ethyl iodide as shown in scheme 15. The product of this reaction was ethyl

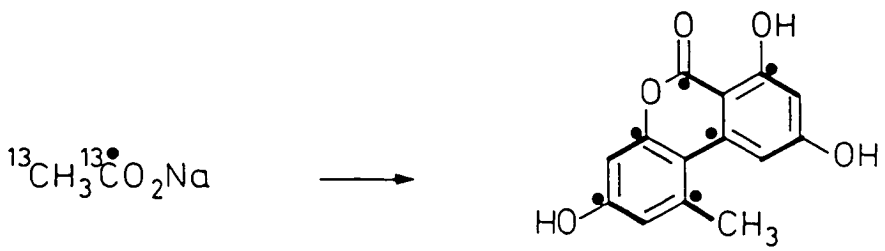
Scheme 16



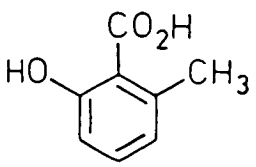
$CH_3CH_2^{14}CH_2CO_2CH_2CH$

(39)

Scheme 17



(40)



(41)

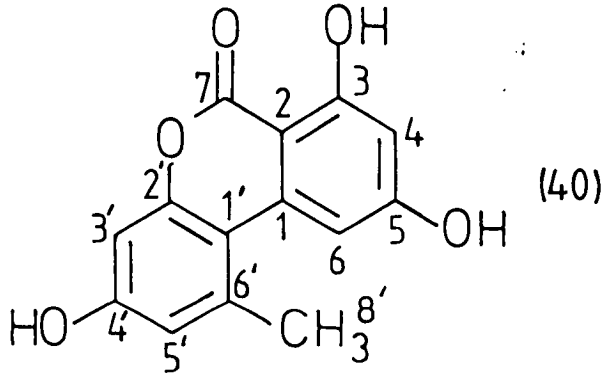
butyrate (38) was isolated in 56% yield. Ethyl butyrate (38) or butyric acid salts are available commercially with ^{13}C labelling at C-1,2,3, and 4, ^{14}C labelling at C-1, and with up to $^{2}H_7$ substitution[17]. However no doubly labelled material is available. This reaction therefore represents a convenient synthesis of doubly labelled ethyl butyrate from doubly labelled acetate. For instance ethyl-[1-^{13}C,2-$^{2}H_2$]butyrate could easily be prepared from [1-^{13}C,$^{2}H^3$]-acetate as shown in scheme 16. Using the method described above ethyl-[2-^{14}C]butyrate (39) was prepared from [2-^{14}C]acetate. This will be useful for studying the incorporation of intact butyryl fragments.

4.2.1 Incorporation studies

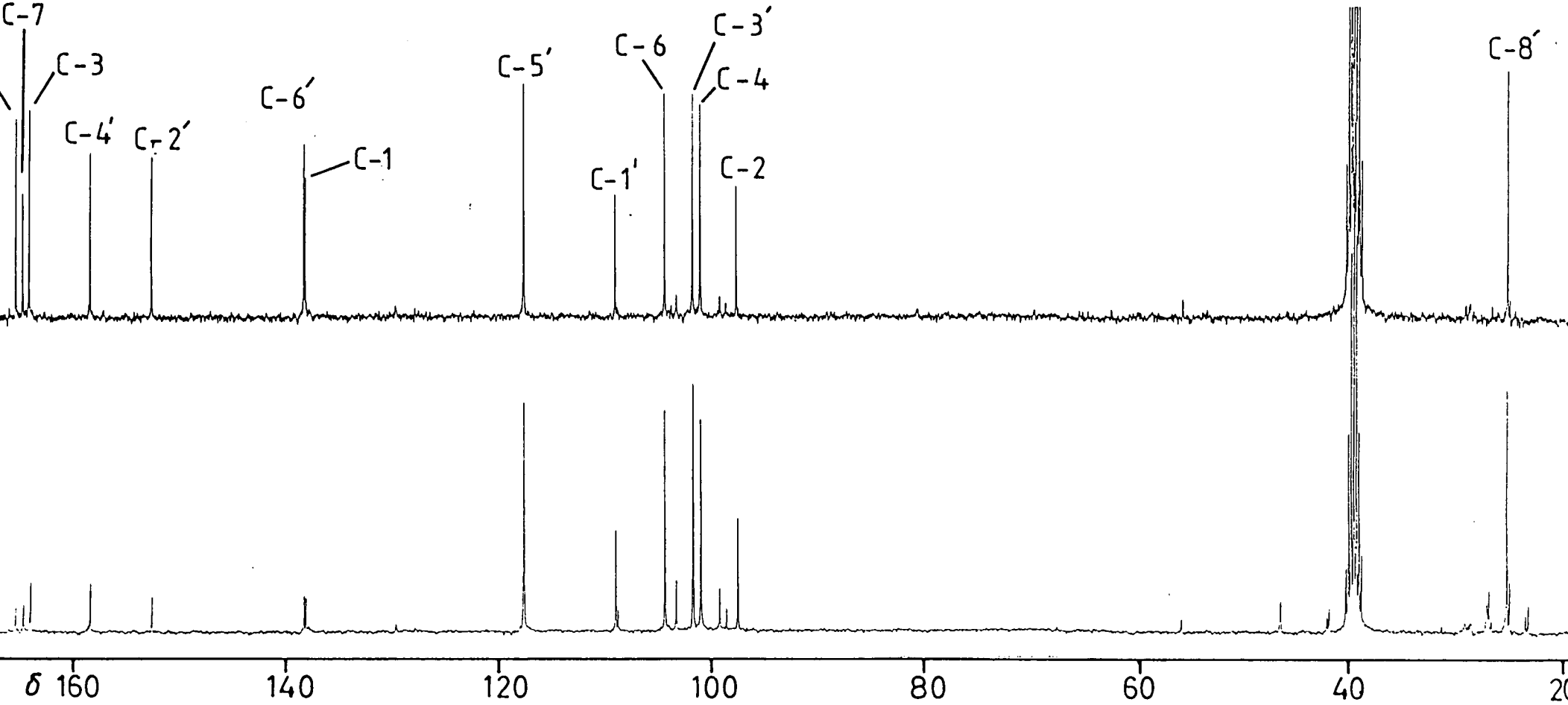
The *Alternaria* family of fungi produce a wide range of metabolites. The most common is the mycotoxin alternariol (AOH) (40) from *Alternaria tenuis* [18]. Alternariol is produced in high yield from the strain *Alternaria tenuis NRRL 6434*. The results of a previous biosynthetic study using [1,2-^{13}C]acetate are shown in scheme 17, and clearly show AOH to be of polyketide origin[18]. Growth production studies showed that precursor incorporation was most efficient between day twelve and day sixteen of the fermentation[18,19].

Using the literature conditions diethyl-[2-^{13}C]malonate (300 mg, 1.87mmol) prepared as described above was introduced by injection through the mycelial mat in three portions on days 12, 14, and 16. The mycelial mat was removed on day 18 and the

Figure 9 90 MHz ^{13}C n.m.r. of (40)



(40)



alternariol isolated. Earlier studies had shown that the isolated material was pure enough for n.m.r. purposes[19].

The result of this experiment can be seen in the ^{13}C n.m.r. spectrum shown in figure 9. The spectrum in figure 9a is the natural abundance spectrum, and that in figure 9b is of the AOH isolated from the diethyl-[2-^{13}C]malonate feed. The C-8' methyl signal shows the same intensity of labelling as the rest of the labelled signals. This must mean that in this system there is no significant starter effect, and in fact the diethyl-[2-^{13}C]malonate has been catabolised to [2-^{13}C]acetate before incorporation.

In an attempt to investigate the alternariol system further *mono*-ethylthiol-[1,2-^{13}C]malonic acid (300 mg, 1.7 mmol.) was injected into the medium as its ammoniun salt in an identical fashion to that described above. On day 18 the mycelial mat was removed and the AOH isolated. No evidence of any ^{13}C labelling of any kind could be detected. The liquor was extracted with chloroform to try and recover the precursor but none could be detected by ^{1}H n.m.r. of the crude extract.

The simple polyketide 6-methylsalicylic acid (44) is being extensively studied in these laboratories the present time. The studies being carried out will hopefully lead to the isolation of the 6-methylsalicylic acid synthetase enzyme complex from *Penicillium urticae*. As the work described in this chapter will eventually be used on such cell free systems, it was decided to examine the incorporation of the *mono*-n-butyl-[1,2-^{13}C]-

Figure 10 Growth/Production curve of 6-MSA

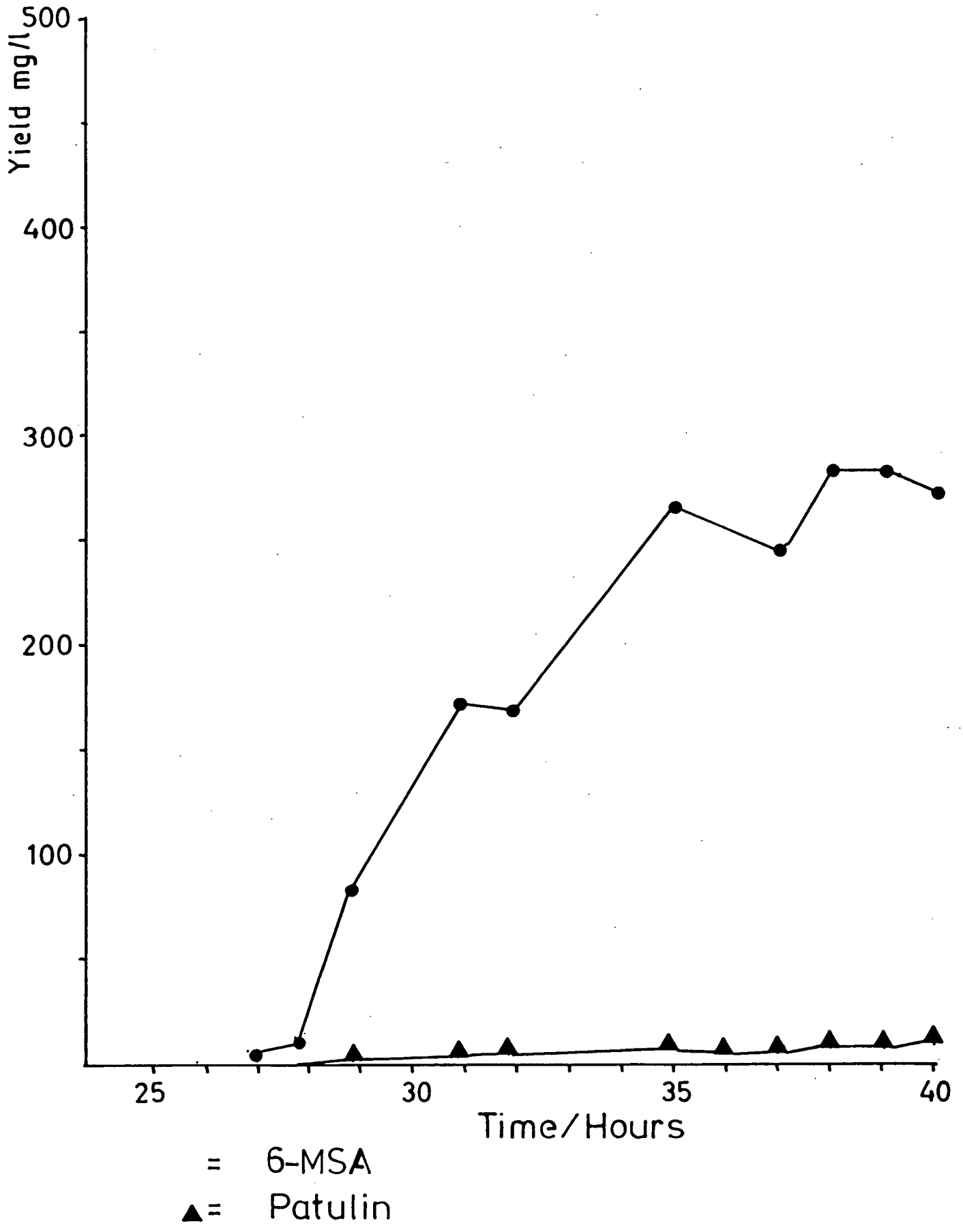
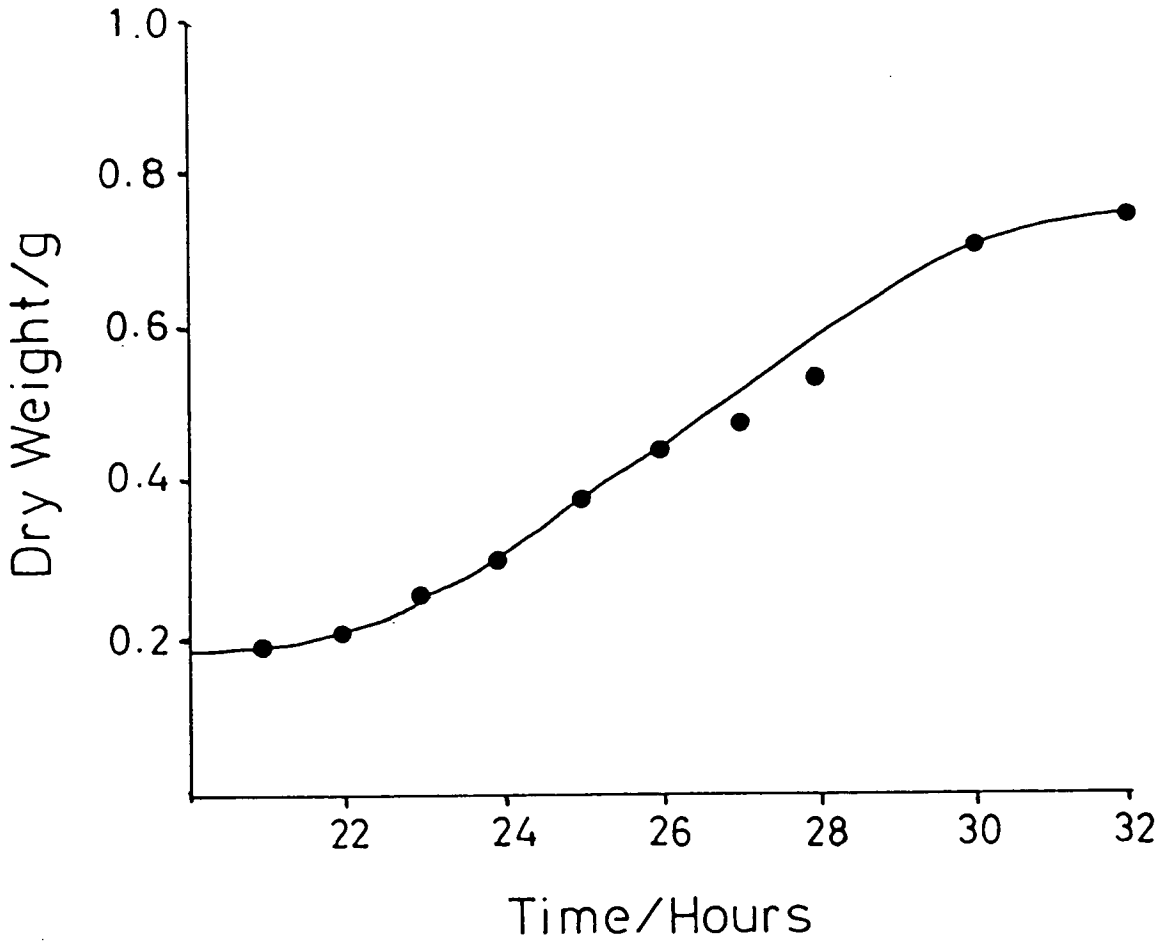


Figure 11

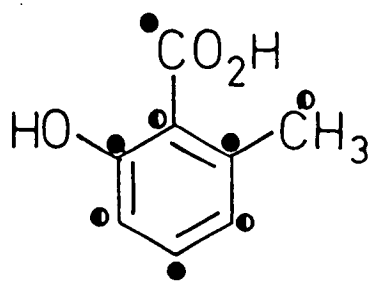


malonic acid into 6-methylsalicylic acid in an *in vivo* experiment.

The growth production curve for 6-methylsalicylic acid from *Penicillium urticae* is shown in figure 10[20]. As described in chapter one, 6-methylsalicylic acid is subsequently converted to patulin (scheme 21, Ch 1), and the levels of patulin produced are also shown. Figure 11 shows the dry weight of the cell mass over the same time period. This levels out at around thirty two hours, 6-MSA production commences at around twenty seven hours. This indicates that production of the secondary metabolite only starts when the processes of primary metabolism are slowing down.

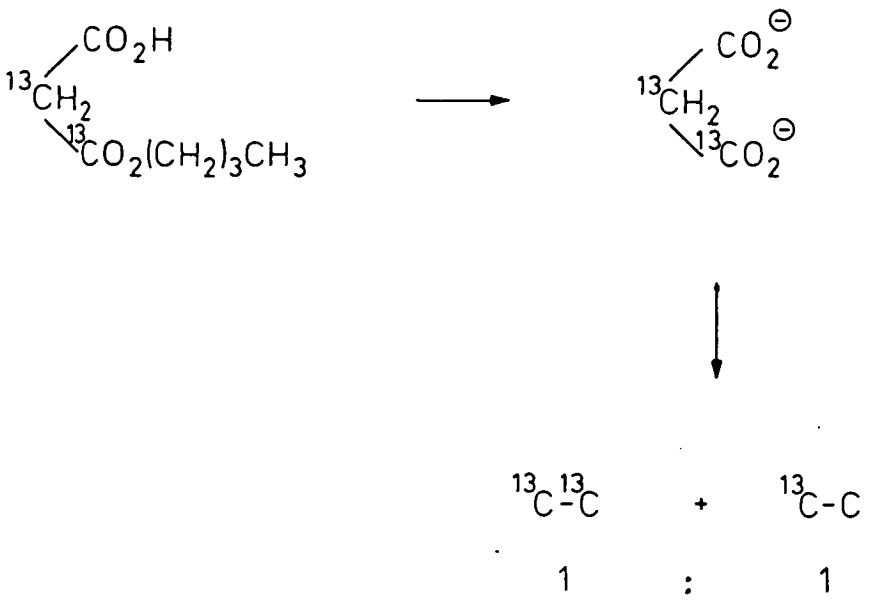
Using this information *mono*-n-butyl-[1,2-^{13}C]malonic acid (100 mg, 0.63 mmol.) was administered as the ammonium salt to *Penicillium urticae* after twenty three hours of fermentation. The fermentation was stopped at twenty nine hours and the 6-MSA isolated. The pnd. ^{13}C n.m.r spectrum is shown in figure 12. The appearance of the observed coupling pattern indicates that specific incorporation of the "activated" two carbon unit has not occurred. In signals corresponding to carbons derived from C-2 of malonyl CoA, the ^{13}C-^{13}C coupled signal shows only 50% of the natural abudance signal. However in sites corresponding to carbons derived from C-1 of malonyl CoA, the coupled signal is of equal intensity to the natural abundance signal. This effect has been caused by the precursor being catabolised to a symmetrical malonic acid derivative (scheme 18). In this state the enzyme complex cannot distinguish between the two carboxyl groups and so

Figure 12 90·6 MHz ^{13}C n.m.r. of (41) enriched with (27)



(41)

Scheme 18



there is only a 50% chance that C-1 will be incorporated intact with C-2.

4.3. Future work.

So far in this work successful incorporation of an "activated" two carbon unit from a malonic acid derivative has not been achieved. The next logical step is to prepare the *mono*-NAC ester of malonic acid and examine the pattern of its incorporation. The preparation described here failed but it should be possible to prepare it by adapting the route of Cornforth *et al* used for the synthesis of chiral malonyl CoA (scheme, 10 Ch 1). The CoASH being replaced by NAC in the final step.

This synthesis would also seem to offer the best route to a chiral derivative, as it only requires the introduction of one isotopic label. Combination of this with a suitable "activation" group would give a very useful precursor for biosynthetic studies. However the problem of racemisation still remins to be overcome, and for the present it seems likely that the use of such precursors will be confined to cell free systems.

4.4 EXPERIMENTAL

For general procedures and instrumentation see section 2.4.1. on p 52.

Ethyl acetate

Freshly distilled tri-ethyl phosphate (7.5 ml) was added to sodium acetate (2.0 g, 24 mmol.) in a round bottom flask (25 ml.). A small piece of glass wool was placed on top of the mixture, and then the system was heated to reflux for ninety minutes. The mixture was then allowed to cool to room temperature and the top of the condensor attached to a high vacuum line. The pressure was reduced carefully to avoid bumping and the ethyl acetate distilled out into a trap at -196°C. (1.99 g, 23.4 mmol. 95%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.2 (3H t 8.0 Hz), 1.9 (3H s), and 4.01 ppm. (2H q 8.0 Hz).

Diethyl malonate

n-Butyl lithium (20.25 ml. of a 1.6 molar solution in hexanes, 2.07 g, 32.4 mmol.) was added slowly to a stirred solution of hexamethylenedisilazane (4.04 g, 25.1 mmol.) in dry tetrahydrofuran under a dry nitrogen atmosphere at -78°C. The solution was allowed to warm to 20°C during thirty minutes, and was then recooled to -78°C. A solution of dry ethyl acetate (1.35 g, 15.34 mmol.) in dry tetrahydrofuran (4 ml.) was then

added slowly from a syringe, and the solution allowed to stir for thirty minutes at -78°C. Freshly distilled ethyl chloroformate (1.65 g, 17.84 mmol was then added slowly from a syringe, during the additon the temperature was maintained below -55°C. The mixture was then allowed to stir at -78°Cfor 2½ hours. Dilute HCl (4ml, 6 molar) was then added to the solution followed by water (20 ml.) and ether (100 ml.). The organic layer was separated and the aqueous layer extracted with ether (2 x 50 ml.). The ether extracts were combined and washed with HCl (50 ml, 3 molar), water (50 ml.), and sodium hydrogen carbonate (50 ml, 5% w/v). The aqueous washings were combined and re-extracted with ether (2 x 50 ml.). All ether extracts were combined and dried over $MgSO_4$. Removal of solvent *in vacuo* gave a clear oil (1.73 g, 10.8 mmol, 70%).

^1H n.m.r. (80 MHz, $CDCl_3$) : δ 1.1 (6H t 6.0 Hz), 3.1 (2H s), and 3.98 ppm. (4H q 6.0 Hz).

Ethyl-[2-^{13}C]acetate

[2-^{13}C]-Ethyl acetate was prepared using the procedure described above from [2-^{13}C] acetate (1.03 g, 12.6 mmol.), and freshly distilled tri-ethyl phosphate (4 ml.). The product was identified by ^1H n.m.r. (1.06 g, 12.4 mmol, 98%).

^1H n.m.r (80 MHz, $CDCl_3$) : δ 1.08 (3H t, 8.0 Hz CH_2-CH_3), 2.01 (3H d, 129.0 Hz $^{13}CH_3$-CO), and 4.01 ppm. (2H q, *8.0 Hz* CH_2-CH_3).

^{13}C n.m.r. (50 MHz, $CDCl_3$) : δ 26.68 ppm ($^{13}CH_3$ q, 129.0 Hz.).

Diethyl-[2-^{13}C]malonate

[2-^{13}C]-Diethyl malonate was prepared as described above from [2-^{13}C]-ethyl acetate (1.06 g, 12.4 mmol.), and ethyl chloroformate (1.52 g, 14 mmol.). The product was recovered as a clear oil (1.41 g, 8.81 mmol, 71%).

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ 1.08 (3H t, 6.0 Hz CH_2-*CH_3*), 2.01 (3H d, 132.0 Hz ^{13}*CH_3*-CO), and 4.01 ppm. (2H q, 6.0 Hz CH_3-*CH2*).

^{13}C n.m.r (50 MHz, $CDCl_3$) : δ 40.75 ppm. ($^{13}CH_2$ t 132.0 Hz)

Acetic acid

Sodium acetate (1.3 g, 15.8 mmol.) was placed in a three neck round bottom flask (100 ml.), attached to a gas inlet and a vacuum line. The system was flushed with dry nitrogen for twenty minutes, and then the nitrogen flow was replaced with dry HCl gas. The temperature of the reaction flask was raised slowly to 100°C. The acetic acid was allowed to distil out into two traps at -78°C. The HCl flow was switched off after thirty minutes and the system allowed to cool down to room temperature. The system was then exposed to high vacuum and the volatile contents of the flask and first trap distilled into the second trap at -196°C. Once the distillation was complete the trap was isolated from the vacuum and the contents allowed to warm to room temperature. The trap was then cooled to -78°C and the contents exposed briefly to the high vacuum, and then reisolated. The trap

was then warmed to -20°C and again the contents exposed briefly to the high vacuum. The identity of the product was confirmed by ^{1}H n.m.r. (0.94 g, 15.6 mmol, 97.5%)

^{1}H n.m.r. (80 MHz $CDCl_3$) : δ 1.9 (3H s), and 11.3 (1H s).

Acetyl chloride

1.) Acetic acid (2 g, 33 mmol.) was placed in a three neck round bottomed flask (50 ml.) along with phthaloyl dichloride (20 ml 400% excess), and the mixture heated to 60°C under a dry nitrogen atmosphere, for thirty minutes. At the end of this period the dry nitrogen source inlet was lowered beneath the surface of the liquid and the temperature raised to 120°C, the product distilled out into a trap cooled in ice. (1.69 g, 21.6 mmol, 65%).

2.) Acetic acid (1.88 g, 30 mmol.) was added to a stirred excess of phosphorous trichloride (1.6 g, 11.6 mmol.) at 0°C. The mixture was brought to room temperature and the to 45°C for one hour. At the end of this period the product was distilled (bp. 47-51°C) out at atmospheric pressure. (1.802 g, 23.1 mmol, 77%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 2.2 ppm (3H s)

Ethylthiol acetate

Acetyl chloride (0.9 g, 11.5 mmol.) was added slowly down a condenser to ice cold ethane thiol (1.01 g, 16.2 mmol.).

Once the addition was complete the top of the condensor was connected to the stem of a glass funnel which was submerged in water. The mixture was allowed to stand at room temperature for forty eight hours, and was then refluxed for four hours. The mixture was then diluted with ether (50 ml.) and poured into water (50 ml.). The organic layer was separated and washed with sodium hydrogen carbonate solution (50 ml, 5% w/v), water (50 ml.), and then dried over $MgSO_4$. The solvent was removed *in vacuo*, to give a pale yellow oil. This was distilled (bp. 115-120°C. lit[15] 116-117°C) at atmospheric pressure to give a clear oil (0.725 g, 7.0 mmol. 61%).

^1H n.m.r. (80 MHz, $CDCl_3$) : δ 1.1 (3H t 8.0 Hz), 2.25 (3H s), and 2.8 ppm. (2H q 8.0 Hz).

mono-(Ethylthiol)-malonic acid

n-Butyl lithium (10.5 ml. of a 2.5 molar solution in hexanes, 1.68 g, 26.25 mmol.) was added slowly to a solution of hexamethylenedisilazane (3.3 g, 20.5 mmol.) in tetrahydrofuran (30 ml.) stirring under a dry nitrogen atmosphere at -78°C. The solution was allowed to warm to 20°C over thirty minutes, and was then immediately recooled to -78°C. Ethylthiol acetate (1.3 g, 12.5 mmol.) was added dropwise from a syringe, the temperature was maintained below -55°C during the addition. The solution was then stirred at -78°C for one hour. A fine stream of dry carbon dioxide gas was then introduced under the surface of the liquid and maintained for three hours. The solution was then allowed to

warm to room temperature and allowed to stir overnight. The reaction was quenched with HCl (4 ml. 6 molar), diluted with water (50 ml.), and extracted with ether (100 ml.). The organic layer was separated and the aqueous layer extracted with ether (2 x 50 ml.). The combined ether layers were washed with HCl (20 ml, 3 molar), water (50 ml.), and dried over $MgSO_4$. Removal of solvent *in vacuo* gave a pale yellow oil. (1.77 g, 11.9 mmol. 95%)
^1H n.m.r. (80 MHz, $CDCl_3$) : δ 1.2 (3H t, 8.0 Hz CH_3-CH_2), 2.9 (2H q, *8.0 Hz* CH_2-CH_3), 3.52 (2H s, OC-CH_2-CO), and 8.6 (1H s, CO_2H) Mass spectrum : m/z (% base peak) : M , 148 (18), 130 (8), 104 (3), 89 (10), 87 (100), 71 (28), 62 (40), 51 (20).
Exact mass $C_5H_8O_3S$ found 148.0192 Δ 1.4 ppm. Requires 148.2794

[1,2-^{13}C]-Acetic acid

[1,2-^{13}C]-Acetic acid was prepared in an identical fashion to that described above from [1,2^{13}C]-acetate (0.95 g, 11.6 mmol.). The product was identified by ^1H n.m.r. (0.514 g, 8.6 mmol. 74%).
^1H n.m.r. (80 MHz, $CDCl_3$) : δ 2.1 (3H dd, *128, 6.0 Hz* CH_3-CO_2H), and 11.1 ppm. (1H bs)

[1,2-^{13}C]-Acetyl chloride

[1,2-^{13}C]-Acetyl chloride was prepared in an identical fashion to that described above from [1,2-^{13}C]-acetic acid

(0.514 g, 8.6 mmol.), and phosphorous trichloride (0.42 g, 3.1 mmol.). (bp. 47-51°C, 0.32 g, 4.1 mmol, 48%).

Ethylthiol-[1,2-^{13}C]acetate

Ethylthiol-[1,2-^{13}C]acetate was prepared in an identical fashion to that described above from [1,2-^{13}C]-acetyl chloride (0.42 g, 3.1 mmol.), and ethane thiol (0.3 g, 4.8 mmol.) (0.220 g, 2.11 mmol. 67%). The product was used immediately.

mono-(Ethylthiol)-[1,2-^{13}C]malonic acid

mono-(Ethylthiol)-[1,2-^{13}C]malonic acid was prepared in an identical fashion to that described above from ethylthiol-[1,2-^{13}C]acetate (0.22 g, 2.12 mmol.) The product was recovered as a clear oil (0.248 g,1.7 mmol, 80%), and was identified by ^{1}H and ^{13}C n.m.r.

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.3 (3H t, *8.0 Hz* CH_3-CH_2), 2.9 (2H dq, *16.0, 8.0 Hz* CH_2-CH_3), and 3.5 ppm. (2H dd, 126.0, 50.0 Hz OC-CH_2-CO).

^{13}C n.m.r. (50 MHz, $CDCl_3$) : δ 48.5 (C-2, td 126.0, 50.0 Hz), and 191.98 ppm (C-1, dm 50.0 Hz).

Mono-(n-Butyl)-malonic acid

n-Butyl lithium (22.5 ml. of a 1.6 molar solution in hexanes, 2.304 g, 36 mmol.), was added to a stirred solution of

hexamthylenedisilazane (4.53 g, 28.08 mmol.) in dry tetrahydrofuran (100 ml.) under a dry nitrogen atmosphere at -78°C. The solution was allowed to warm to 20°C over thirty minutes, and then immediately recooled to -78°C. A solution of n-Butyl acetate (2 g, 17.2 mmol.) in tetrahydrofuran (5 ml.) was then added, the temperature was maintained below -55°C during the addition. The solution was allowed to stir at -78°C for thirty minutes. A fine stream of dry carbon dioxide gas was then introduced beneath the surface of the liquid and maintained for two hours. After this period the solution was allowed to come to room temperature, and stirred for a further two hours. The reaction was quenched with HCl (4 ml, 6 molar), water (20 ml.), and extracted with ether (150 ml.). The organic layer was separated and the aqueous layer extracted with ether (3 x 50 ml.). The organic extracts were combined and washed with HCl (20 ml, 3 molar), water (3 x 50 ml.), and dried over $MgSO_4$. The solvent was removed *in vacuo* to give a pale yellow oil. (2.7 g.). This was shown to be contaminated with starting material by glc. (2½% OVI 180°C). The crude product was dissolved in ether (100 ml.), and the solution cooled in an ice bath. Anhydrous ammonia gas was passed through the solution and a white crystalline precipitate formed. (2.26 g, 12.72 mmol. 74%).

^{1}H n.m.r. (80 MHz, ^2H_2O) : δ 0.9 (3H t, 7.0 Hz CH_2CH_2-*CH_3*), 1.4 (4H m, *CH_2*-*CH_2*-CH_3), 3.4 (2H s, OC-*CH_2*-CO), 4.1 (2H t, 6.0 Hz CO_2-*CH_2*-CH_2), and 9.52 ppm. (1H s, CO_2-*H*).

Mass spectrum, FAB, glycerol matrix : m/z (% base peak), M -1, 159 (68), 115 (100), 102 (12), 91 (23), 59 (20), 41 (77).

Exact mass $C_7H_{11}O_4$ found 159.06572 Δ 1.0 ppm. Requires 159.1615

n-Butyl-[1,2-^{13}C]acetate

[1,2-^{13}C]Acetate (2 g, 24.3 mmol) was combined with tri-n-butyl phosphate (9.9 g, 42 mmol.), and refluxed for three hours. At the end of this period the top of the reflux condensor was connected to a high vacuum line and the flask heated to 100°C. The product was distilled out into a trap at -196°C. (2.6 g, 22.4 mmol, 92%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 0.81 (3H t, *7.0 Hz* CH_3-CH_2), 1.25 (2H m, CH_2-CH_2), 1.75 (3H dd, 130, 8.0 Hz OC-CH_2-CO), and 3.8 ppm (2H dt, CO_2-CH_2-CH_2).

^{13}C n,m.r. (50 MHz, $CDCl_3$) : δ 20.17 (C-2, qd 130.0, 75.0 Hz), and 170.35 (C-1, dm 75.0 Hz).

mono-(n-Butyl)-[1,2-^{13}C]malonic acid

mono-(n-Butyl)-[1,2-^{13}C]malonic acid was prepared using the procedure described above from [1,2-^{13}C]-n-butyl acetate (2.0 g, 17.2 mmol.). The product was isolated and stored as the free acid (1.87 g, 11.7 mmol, 68%).

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 0.78 (3H t, 7.0 Hz CH_2-CH_3), 1.3 (4H dm, *6.0 Hz* CH_2-CH_2), 3.30 (2H dd, 150, 8.0 HzOC-CH_2-CO), 4.04 ppm. (2H dt, CO_2-CH_2-CH_2), and 9.5 ppm. (1H bs, CO_2-H).

^{13}C n.m.r. (50 MHz, $CDCl_3$) : δ 40.15 (C-2, td 150.0, 51.0 Hz), and 166.9 ppm. (C-1, dm 51.0 Hz.)

N,S-Diacetylcysteamine

A three neck round bottom flask (250 ml.) was equipped with two addition funnels (250 ml.), and a pH meter electrode. The flask was charged with 2-mercaptoethylamine hydrochloride (5.77 g, 58 mmol.) in water (40 ml.). One of the funnels was charged with acetic anhydride (17.7 ml, 174 mmol.), and the other with KOH (45 ml, 8 molar.). The contents of the flask were cooled in an ice bath and the pH adjusted to 8.0 by the addition of some of the KOH. Acetic anhydride was then added dropwise along with sufficient KOH to maintain the pH at 8.0. After the addition was complete the mixture was acidified (HCL 2 molar) to pH 7.0, and the mixture allowed to stir for one hour. Sodium chloride was then added to the point of saturation, and the mixture extracted with methylene chloride (5 x 50 ml.), dried over $MgSO_4$, and the solvent removed *in vacuo* to give a clear oil. (8.52 g, 52.8 mmol, 91%).

I.r. : neat film, ν_{max} 3280m, 1655s, and 1545s cm

^{1}H n.m.r. (80 MHz, $CDCl_3$) : δ 1.83 (3H s), 2.19 (3H s), 2.88 (2H t 6.0 Hz), and 3.22 ppm. (2H t 6.0 Hz).

Attempted preparation of *Mono*-S-(N-Acetylcysteamine)-malonic acid

n-Butyl lithium (7.81 ml. of a 1.6 molar solution in hexanes, 0.8 g. 12.52 mmol.) was added to stirred solution of hexamethylenedisilazine (2.04 g, 12.5 mmol.) in tetrahydrofuran (50 ml.) under a dry nitrogen atmosphere at -78°C. The solution

was allowed to warm to 20°C over thirty minutes, and then recooled to -78°C. S,N-Diacetylcysteamine (1 g, 6.26 mmol) was added. The solution immediately solidified, and remained so on warming to room temperature. No product could be isolated.

Ethyl butyrate

n-Butyl lithium (20.25 ml. of a 1.6 molar solution in hexanes, 2.07 g, 32.4 mmol) was added to a solution of hexamethylenedislazine (4.04 g, 25.1 mmol.) in tetrahydrofuran (50 ml.) under a dry nitrogen atmosphere at -78°C. The solution was allowed to warm to 20°C over thirty minutes and was then recooled to -78°C. Dry ethyl acetate (1.35 g, 15.34 mmol.) was then added in tetrahydrofuran (5 ml.), and the solution stirred at -78°C for thirty minutes. Ethyl iodide (4.84 g, 31 mmol.) was then added, and the solution allowed to warm to room temperature over three hours, and then stirred overnight at room temperature. The reaction was quenched with HCl (4 ml, 6 molar), water (20 ml.), and extracted with ether (100 ml.). The organic layer was separated and the aqueous layer extracted with ether (3 x 50 ml.). The combined organic extracts were washed with HCl (50 ml. 3 molar), water (50 ml.), sodium hydrogen carbonate (50 ml. 5% w/v), and dried over $MgSO_4$. Removal of solvent *in vacuo* gave a clear oil. (1.73 g, 14.72 mmol, 96%).

I.r. : neat film, ν_{max} 1740s cm

^1H n.m.r (80 MHz, $CDCl_3$) : δ 0.9 (3H t 7.0 Hz), 1.1 (3H t 6.0 Hz), 1.9 (4H m), 4.1 (2H q 7.0 Hz), and 4.25 (2H q 6.0 Hz).

Ethyl-[2-^{14}C]acetate

[2-^{14}C]-Ethyl acetate was prepared using the procedure described above from [2-^{14}C]acetate (0.93 g, 116.25 μCi 11.3 mmol.) and triethyl phosphate (4 ml.). (0.86 g, 9.9 mmol. 88%).

Ethyl-[2-^{14}C]butyrate

[2-^{14}C]-Ethyl butyrate was prepared in an identical fashion to that described above from [2-^{14}C]-ethyl acetate (0.86 g. 9.9 mmol.), and ethyl iodide (3.82 g, 24.5 mmol.). The product was isolated by distillation (bp 120°C, lit 121°C), to give a clear oil (0.804 g, 6.8 mmol. 70.58 μCi, 69%).

Feeding studies

Alternariol

Alternaria tenuis NRRL 6434 was stored on agar slopes at 4°C in the dark. A spore suspension in distilled water was used to innoculate 250 ml. conical flasks each containing 60 ml. of the following medium ;

Modified Czapek-Dox medium : Glucose (40.0 g.), yeast extract (1.0 g.), $NaNO_3$ (1.0 g.), KH_2PO_4 (1.0 g.), $MgSO_4.7H_2O$ (0.5 g.), NH_4Cl (0.25 g.), KCl (0.25 g.), NaCl (0.25 g.), $FeSO_4.7H_2O$ (0.01 g.), and $ZnSO_4.7H_2O$ (0.01 g.), made up to one litre in

water. The culture was incubated stationary in the dark at 25°C. A dark coloured mycelial mat formed after three days.

Isolation of Alternariol

After eighteen days of fermentation the mycelial mat was removed, and extracted with methanol (100 ml./flask). The crude extract was diluted with water (1 equivalent), and extracted with chloroform (5 x equivalent volume). The organic extracts were dried over $MgSO_4$, and the solvent removed *in vacuo* to give a brown solid. This was the alternariol and no further purification was neccessary.

Incorporation of Diethyl-[2-^{13}C]malonate into Alternariol

Diethyl-[2-^{13}C]malonate (300 mg. 1.9 mmol.) was dissolved in ethanol (0.6 ml.). On days 11, 13, and 15 after innoculation 0.2 ml. of this solution was injected through the mycelial mat of a single 250 ml. flask. The mycelial mat was removed on day eighteen and the alternariol isolated as described above. (0.117 g, 1.95 g/l).

Incorporation of *mono*-(Ethylthio)-1[1,2-^{13}C]malonic acid into alternariol

The ammonium salt of *mono*-(ethylthiol)-[1,2-^{13}C]malonic acid (300 mg. 2.02 mmol.) was prepared as described above, and

dissolved in water (0.9 ml.). On days 11, 13, and 15 after innoculation 0.3 ml. of this solution was injected through the mycelial mat of a single flask. On day eighteen the mycelial mat was removed and the alternariol isolated as described above. (87 mg. 1.45 g/l). No incorporation could be detected, and repeated extraction of the residual liquor with ethyl acetate did not recover the precursor.

6-Methylsalicylic acid

Penicillium urticae IMI 92,273 was stored on Czapek-Dox slopes at 4°C. A spore suspension in distilled water was used to innoculate 500 ml conical flasks each containing 100 ml. of modified Czapek-Dox medium (as described above). The culture was incubated on a rotary shaker for forty eight hours.

Isolation of 6-Methyl salicylic acid

The contents of the culture flask were combined and filtered through a Whatman No 1 filter paper. The mycelial mass was discarded, and the liquor acidified to pH 2 with HCl (6 molar), and extracted with ether (2 equivalents). The combined ether extracts were extracted with an equal volume of sodium hydrogen carbonate solution (5% w/v). The aqueous layer was then acidified to pH 2, and re-extracted with ether (2 equivalents). This extract was dried over $MgSO_4$, and the solvent removed *in vacuo* to give a yellow crystalline solid. This was purified by

plc. using chloroform/acetic acid 1:9. A standard sample of 6-MSA was used to identify the correct band which was removed and the semi pure 6-MSA isolated. The semi pure material was then subjected to further plc. using ethyl acetate/n-hexane 1:9. The pure 6-MSA being identified using a standard sample.

Incorporation of *mono*-(n-Butyl)-[1,2-^{13}C]malonic acid into 6-Methyl salicylic acid

The ammonium salt of *mono*-(n-Butyl)-[1,2-^{13}C]malonic acid was prepared as described above (100 mg, 0.6 mmol), and dissolved in water (0.5 ml.). Twenty three hours after innoculation this solution was injected into the medium of a single culture flask. Twenty nine hours after innoculation the 6-methyl salicylic acid was isolated and purified as usual. (24 mg. 240 mg/l).

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Studies of Polyketide Chain-assembly Processes: Origins of the Hydrogen and Oxygen Atoms in Colletodiol

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The origins of all the oxygen and hydrogen atoms in colletodiol (**2**) have been elucidated by incorporation of label from [1-^{13}C,$^{18}O_2$]- and [1-^{13}C,$^{2}H_3$]-acetate and $^{18}O_2$ gas into (**2**) in cultures of *Cytospora sp.* (ATCC 20502); from the resultant labelling pattern the structures of the enzyme-bound precursors can be deduced and information obtained on the processes occurring during the early stages of polyketide chain-assembly.

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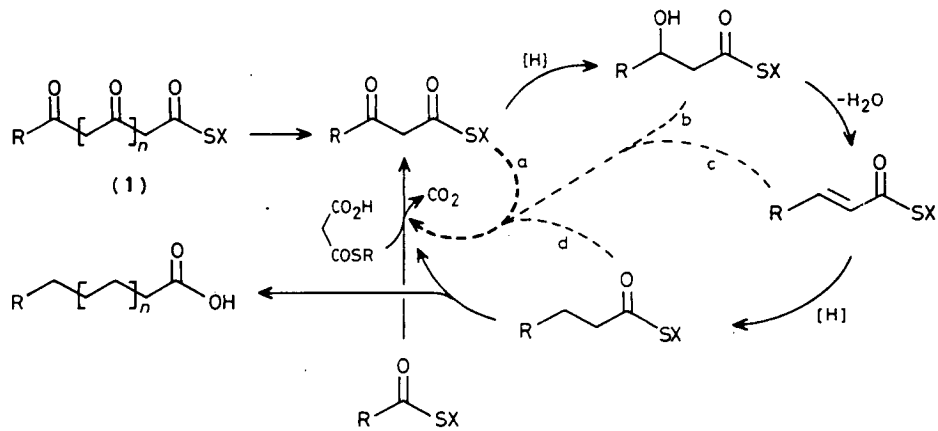
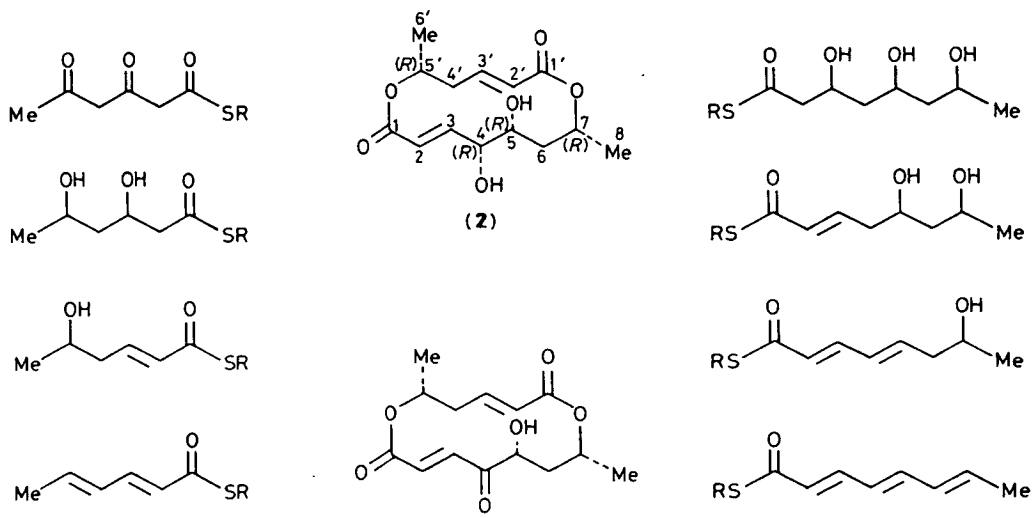
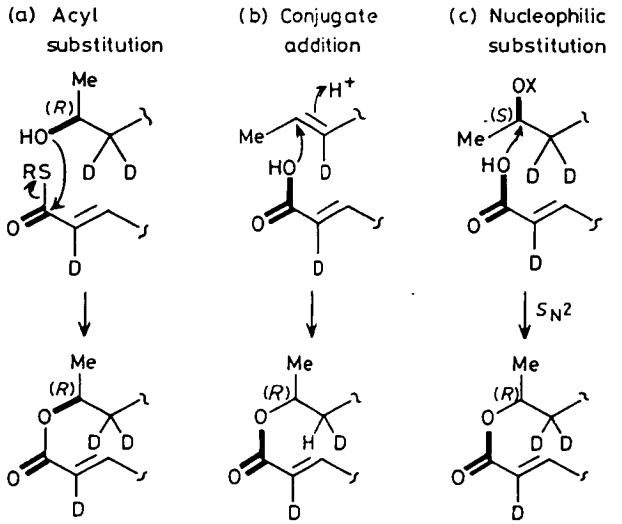
The polyketide pathway is one of the major pathways of secondary metabolism, but despite much effort over the 30 years since the recognition of the pathway,[1] little is known of the exact nature of the intermediates involved in the early stages of polyketide chain-assembly. At its simplest, it is thought that poly-β-ketide intermediates (**1**) are built up by a cyclic process (Scheme 1) analogous to fatty acid biosynthesis[2] but omitting the reduction–elimination–reduction sequence responsible for the loss of acetate oxygen. While some aromatic metabolites do retain the full oxygen content of intermediate (**1**) most metabolites show varying degrees of reduction and/or deoxygenation and an increasing body of evidence suggests that this occurs by processes analogous to fatty acid biosynthesis before the initial release of metabolites or intermediates from the chain-assembly enzymes. Thus, path a in Scheme 1 would simply produce poly-β-ketides but by invoking paths b, c, and d intermediates with varying degrees of reduction may be formed. There has been little progress in enzymatic or other direct methods of observing these early intermediates but recent developments in n.m.r.-based methods[3] (*viz.* ^{2}H and ^{18}O isotope-induced shifts in ^{13}C n.m.r., and ^{2}H n.m.r. spectroscopy) which facilitate determination of the biosynthetic origins of hydrogen and oxygen enable significant indirect evidence for the nature of the



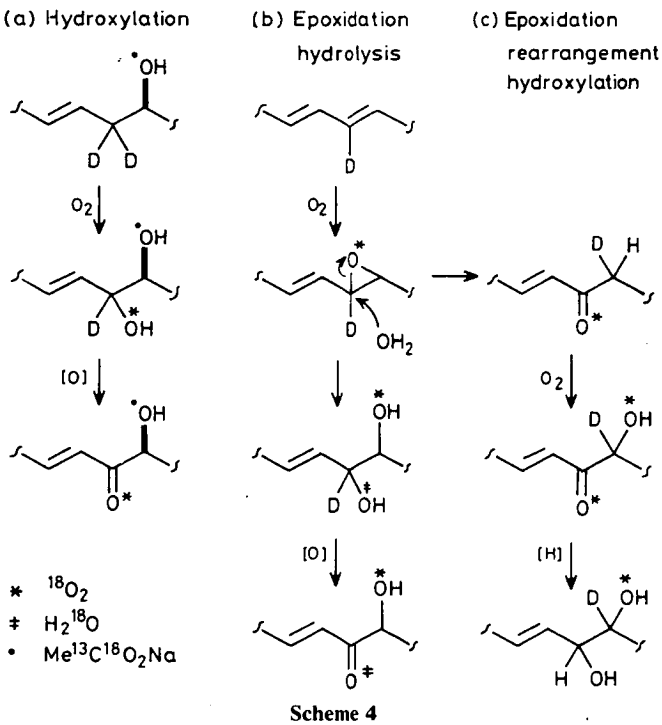
Scheme 1



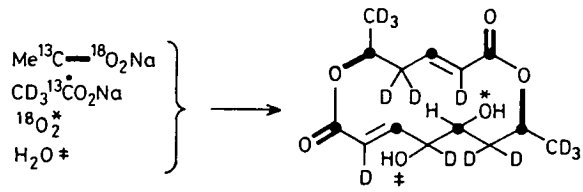
Scheme 2



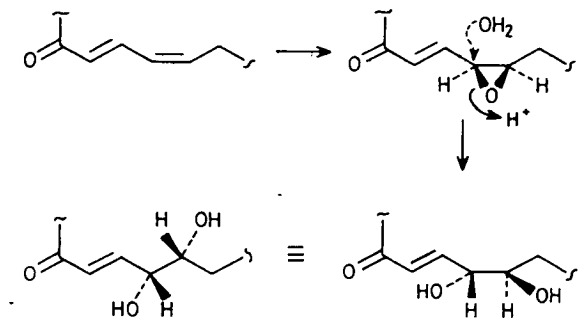
Scheme 3



Scheme 4



Scheme 5



Scheme 6

Table 1. ^2H and ^{18}O isotope-induced shifts observed in the 90.56 MHz ^{13}C n.m.r. spectrum of colletodiol (**2**).

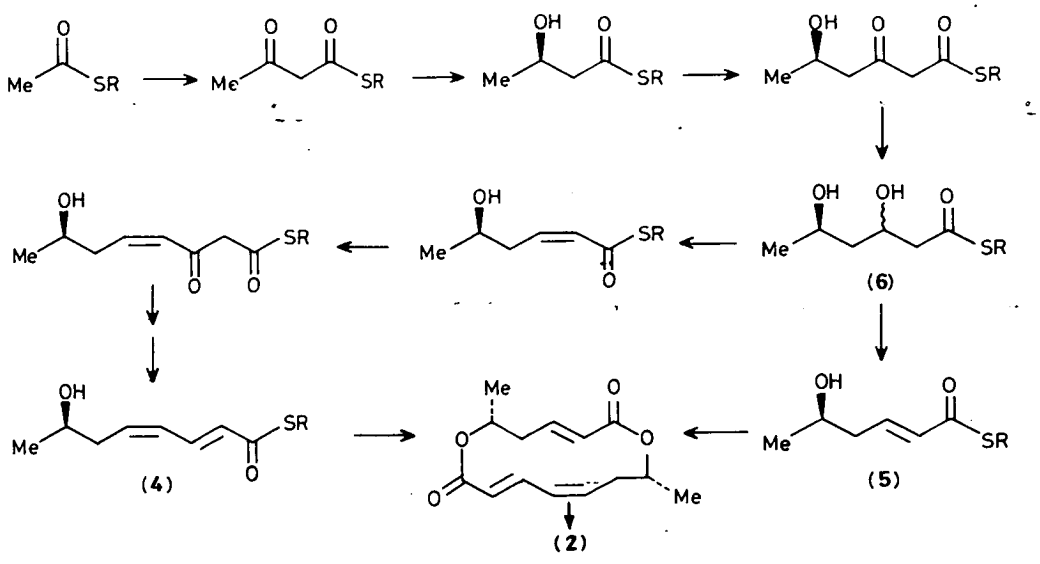
	δ_C	$\Delta\delta \times 100$	$^{16}O:{}^{18}O$	$^1H:{}^2H$
C-1	166.3	3.4[a]	69:31	
C-1′	164.9	3.2[a]	70:30	
C-3	146.4	9.0[c]		95:5
C-3′	143.9	4.4,4.2[c]		53:19:28
C-5	71.7	2.2[b]	54:46	
		4.7,4.6[c]		53:20:27
C-5′	68.6	3.9[a]	79:21	
		4.1,4.3,4.3[c]		30:10:22:38
C-7	67.9	3.7[a]	72:28	
		4.2,4.2,4.3[c]		27:9:23:41

[a] [1-^{13}C,$^{18}O_2$]acetate-enriched. [b] $^{18}O_2$-enriched. [c] [1-^{13}C,$^2H_3$]acetate-enriched.

intermediates to be obtained. We now report ^2H and ^{18}O labelling studies on colletodiol (**2**) designed to obtain information on the processes occurring during the early stages of polyketide chain-assembly.

Colletodiol (**2**) and colletoketol (**3**) are macrocyclic dilactonic metabolites originally isolated from the plant pathogen, *Colletotrichum capsici*.[4] More recently grahamimycin A was isolated as a broad spectrum antibiotic from a species of *Cytospora* and was subsequently shown to be identical to colletoketol.[5] All four chiral centres in colletodiol have the (*R*) configuration.[4,6] Incorporation studies with singly ^{13}C-labelled acetates have confirmed the acetate-origin of colletodiol in *C. capsici*.[7] These metabolites can be seen to be derived by combination of C_6 and C_8, moieties and *a priori* one can postulate a number of triketide- and tetraketide-derived moieties as the actual enzyme-bound precursors. Some of these are shown in Scheme 2. Depending on the nature of the actual intermediates a number of mechanisms can be proposed for the formation of the lactone functions. These are summarised in Scheme 3 along with the possible stereochemical outcome and the predicted origins of the associated oxygen and hydrogen atoms. Similarly a number of different mechanisms can be proposed for the formation of the 1,2-diol and α-ketol systems found in colletodiol and colletoketol respectively. These are shown in Scheme 4 and again they may be differentiated, as indicated, by appropriate ^2H and ^{18}O labelling experiments.

In our hands *Cytospora sp.* (ATCC 20502) has produced colletodiol as the major metabolite and only minor amounts of grahamimycin A. Fermentations were carried out in the presence of [1-^{13}C,$^2H_3$]- and [1-^{13}C,$^{18}O_2$]-acetate and under an atmosphere of $^{18}O_2$. The ^2H and ^{18}O isotope shifts observed in the proton noise decoupled ^{13}C n.m.r. spectra of colletodiol isolated in each case are summarised in Table 1. No ^2H isotope-induced shifts could be observed for C-1 or C-1′ in the ^{13}C n.m.r. spectrum of the [1-^{13}C,$^2H_3$]acetate-enriched colletodiol. However carbonyl groups are known to be poor 'reporter' groups for ^2H shifts[8] and the presence of ^2H label at both C-2 and C-2′ was shown by ^2H n.m.r. analysis of the



Scheme 7

enriched metabolite. The labelling pattern resulting from these experiments is summarised in Scheme 5.

The retention of acetate-derived oxygen on both the carbonyl and ether oxygens of the lactone functions indicates that ring closure must proceed by mechanism (a) in Scheme 3 and so the enzyme-bound intermediates must retain the oxygen of the acetate 'starter' units as hydroxy functions with the (*R*) configuration.

Considering the formation of the 1,2-diol system, a low but significant level of acetate-derived hydrogen is retained at C-4. This means that colletoketol cannot be the precursor of colletodiol, and route (c) in Scheme 4 is ruled out. The oxygen labelling results indicate that the 5-hydroxy group is derived from the atmosphere *i.e. via* an oxidative process, whereas the 4-hydroxy group must be derived from the medium *cf.* route (b), Scheme 4. A mechanism consistent with the observed labelling and the (*R*) configuration at both centres is shown in Scheme 6; epoxidation of a (*Z*)-alkene from the β-face is followed by hydrolytic ring opening by attack of water from the α-face at C-4.

On the basis of these results, the thioesters (**4**) and (**5**) can be proposed as the actual enzyme-bound precursors for colletodiol. These may be built up by the sequence shown in Scheme 7 where the diol (**6**) in which the C-3 stereochemistry is uncertain, is proposed as a common intermediate, *trans*-elimination of water giving rise to the C_6 precursor directly, whereas *cis*-elimination followed by addition of a further C_2 unit produces the C_8 precursor. The relative timing of the diol formation step is not yet known but it may occur after lactonisation and release from the enzyme surface as indicated.

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