# INTERMEDIATES IN THE BIOSYNTHESIS

# OF BISLACTONE ANTIBIOTICS

A Thesis Presented By

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Summary

In this research a detailed investigation of the possible biosynthetic pathways to the bislactone antibiotics canadensolide (3) and dihydrocanadensolide (4) having a "type A" structure, *i.e.* an alkylitaconic acid skeleton, and the bislactone antibiotics ethisolide (1) and avenaciolide (2) having a "type B" structure, *i.e.* an isomeric skeleton has been undertaken. Previously, a number of possible pathways had been envisaged to the type B compounds, but this has now been virtually resolved by the synthesis of a number of suspected intermediates and a series of incorporation studies using these compounds.

The type B bislactones have been shown to be formed *via* the appropriate α-methylene-β-*n*-alkylglutaric acid (32) - the proposed product of a $B_{12}$-coenzyme catalysed rearrangement of the corresponding alkylitaconic acid (30), thus ruling out a number of other possible intermediates. More detailed study of these α-methylene-β-*n*-alkylglutaric acids (32) has suggested that the corresponding coenzyme A esters may be the genuine substrates utilised by the enzymes involved.

Further incorporation experiments using synthetically labelled compounds have ruled out one of two possible monolactonic precursors to these type B bislactones - strongly suggesting that the correct precursor is of the ethisic acid type (27). A number of synthetic approaches to these compounds have been examined and one route, which incorporates a degree of stereoselectivity, has been pursued to near completion.

A study of the non-specificity of the enzymes involved in the utilisation of the α-methylene-β-*n*-alkylglutaric acids (32) to form the appropriate bislactone has also been undertaken. The efficient incorporation of a shorter chain analogue by cultures of *A. avenaceus* clearly demonstrates this enzymatic non-specificity.

Similarly the synthesis of a number of metabolites of *P. canadense* has been undertaken in order to unravel the details of the biosynthesis of the type A bislactones (3) and (4). Isocanadensic acid (92), which had been previously isolated as its methyl ester has been synthesised and the isomeric compound, protoisocanadensic acid (96), which is the proposed key intermediate to (3) and (4), has also been obtained. Initial results of feeding its deuterated counterpart to cultures of *P. canadense* are in the process of being assessed.

The first synthesis of racemic canadensic acid (22) has been achieved. The conditions used in the four-step synthesis, however, appeared more likely to give a diastereoisomeric mixture. One possible route from *n*-butylitaconic acid (30a) to ethisolide (1) might involve hydroxylation and lactonisation to give an analogue (122) of canadensic acid (22) and this was also synthesised by the same route. Again the synthesis led almost exclusively to final isolation of the *trans* isomer. Since the α-methylene-β-*n*-propylglutaric acid (32a) is so efficiently incorporated into ethisolide (1) and since the lactone (31a) is not incorporated, it is most unlikely that this acid (122) would be incorporated even as a part of a minor pathway.

In an unrelated project, the natural substrate of a pigment producing enzyme of an *Aspergillus nidulans* mutant has been shown to be *N*-acetyl-6-hydroxytryptophan (136) by synthesis. A series of substrate studies on this and other related analogues has been carried out.

# CHAPTER 1

## Introduction

(i) Fungi and Secondary Metabolism

(ii) Secondary Metabolites resulting from Condensation of Fatty Acids with Tricarboxylic Acid Cycle Intermediates

(iii) Fatty Acid Biosynthesis

(iv) The Tricarboxylic Acid Cycle

(v) Biosynthesis of Fungal Metabolites

(i) Fungi and Secondary Metabolism

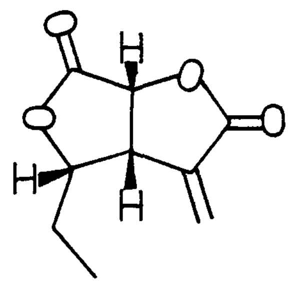
Fungi (Mycota)[1] have over thousands of years contributed both to the quality of life and to its calamities. Early biblical accounts mention not only plant diseases (*e.g.* Amos, 4, 9), but also that Noah consumed rather too much wine - a popular product of fermentation (Genesis, 9, 21).

The term fungi envelopes two distinct types of microorganism, namely yeasts which are predominantly unicellular organisms (often described as microfungi) and moulds or filamentous fungi. Often yeasts are erroneously omitted when fungi are referred to and the term is used only to describe filamentous fungi. Both yeasts and filamentous fungi are eukaryotic organisms (*i.e.* they have true nuclei, unlike simpler prokaryotic organisms such as bacteria) differing from other members of the plant kingdom by their lack of chlorophyll and therefore are unable to synthesise their own source of carbohydrates as green plants do. Most fungi are saprophytes, obtaining these essential carbohydrates from the remains of other plants or animals. Some are parasitic, deriving these benefits from a living host[2] and in a few cases both the fungi and the host derive some benefit from the association (*e.g.* lichens).

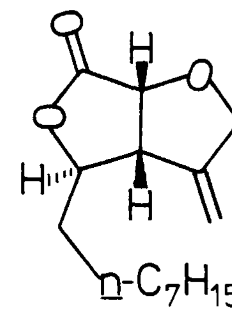
There are more than five hundred known species of yeast and at least one hundred times that number of filamentous fungi. Collectively, they are divided into four main classes based according to the mode of reproduction, which can be either sexual or asexual.[3] The first is the Ascomycetes which includes the yeast *Saccharomyces cerevisiae*, strains of which are used in brewing, wine making, baking and sake (rice wine) production. A number of harmful moulds also belong to this class *e.g.* *Ceratocystis ulmi* (Dutch elm disease), *Chaetomium* (digests cotton fabric) and *Claviceps purpurea* (ergotism). The second class, the Deuteromyces or Fungi Imperfecti, contains

*Aspergillus flavus* (aflatoxins), the mould *Coccidioidies immities* responsible for coccidioidomycosis or San Joaquin fever in man and some animals, and the yeast *Phaffia rhodozyma* which produces the carotenoid, astaxanthin - the reddish pigment found in salmon. The other two classes are the Basidomycetes and the Zygomycetes (moulds only).

Many fungi produce commercially important products and are exploited on a very large scale. Apart from the well known (and much appreciated) uses in the food and drink industry, fungi produce enzymes (*e.g.* amylases and proteases), industrial chemicals (*e.g.* ethanol, citric acid) and occupy a key position in pharmaceutical production, ranging from steroid transformations (*Rhizopus nigricans*) to antibiotics. Commercially, antibiotics are the most important class of drug made by microbial techniques, with the penicillins (*Penicillium chrysogenum*) and cephalosporins (*Cephalosporium acremonium*) dominating.[4] *Penicillium decumbens*, the metabolites of which form part of this present work, also produces large quantities of citric acid (*ca.* 7.5g per litre of culture medium). *Penicillium canadense* produces fumaric and succinic acids. Apart from these accumulations of essentially primary metabolites, the fungi investigated produce a number of compounds related not only structurally, but, as will be discussed later, biogenetically to these metabolites and to their metabolic pathway. The compounds investigated include the antibiotics ethisolide (1) from *P. decumbens*,[5] avenaciolide (2) from *Aspergillus avenaceus*[6] and canadensolide (3) and the antiulcer compound dihydrocanadensolide (4) from cultures of *P. canadense*.[7]



(1)



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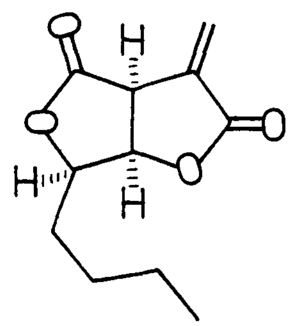
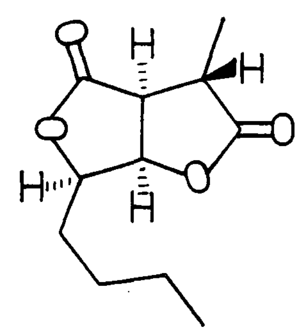


(3)



(4)

These compounds belong to the class of natural product known as secondary metabolites and like many secondary metabolites have no obvious role in the growth and maintenance of the cell, unlike primary metabolites which are essential for the well-being of the organism.[8] The occurrence and nature of secondary metabolites is not clearly understood and is subject to several differing viewpoints. Bu'lock[9,10] argues that secondary metabolism serves to maintain mechanisms essential to cell multiplication in operative order when multiplication is no longer possible. The process of secondary metabolism therefore, extends the activity of fundamental enzyme systems and prevents their loss so that they do not have to be regenerated whenever the restraint on growth is removed. Thus, it is the activity of secondary metabolism that is important, not the products themselves and this would account for their diversity of structural type.[11] An alternative hypothesis[12] argues that the diversion from primary to secondary metabolism is initiated when maximum growth has been achieved or is just declining and the considerable supply of carbon fragments available regulate, by feed back mechanisms, the enzymes in the primary biosynthetic processes to produce the more unusual secondary metabolites. The fact that most secondary metabolites can arise in fungi from the same basic primary metabolic processes is seen as support for this view.

The production of new enzymes as a consequence of metabolite accumulation has been observed in a *Neurospora* mutant[13] and the bacterium *Clostridium barkeri* develops two new enzymes for the fermentation of nicotinic acid when grown with this substance[14] (the role of one of these enzymes is of particular importance in this present work and its chemical and stereochemical properties will be discussed in more detail later). This tends to favour the original idea that secondary metabolism was a consequence of lack of effective enzymatic control under abnormal conditions.[15] Part of the reasoning behind Bu'lock's argument is also lost when you consider that enzyme synthesis usually recommences in favourable metabolic circumstances anyway, so these fundamental enzymes are normally produced regardless of whether or not they are lost due to lack of growth of the microorganism concerned. A further criticism of this hypothesis is that the fungi do, in some cases, benefit from the production of secondary metabolites such as antibiotics which may serve to inhibit the growth of competing microorganisms.[16] This conflicts with the view that it was the activity of producing secondary metabolites, thereby preventing the breakdown of enzymes, which was the important factor, not the properties of the secondary metabolites themselves.

Needless to say, the actual function of secondary metabolic activity is, almost certainly, a combination of all these possible hypotheses depending, to a large degree, on the fungi and the environment under investigation at the time.

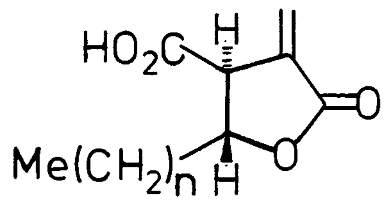
(ii) Secondary Metabolites resulting from condensation of Fatty Acids with Tricarboxylic Acid Cycle Intermediates

Currently, more than fifty fungal natural products have been isolated which probably result from condensation of acetate derived fatty acid chains with a tricarboxylic acid cycle (T.C.A.) intermediate, commonly oxaloacetate. Those resulting from condensation at the α-position of the fatty acid chain are referred to as type A metabolites and include the major metabolites of *P. canadense*, namely canadensolide (3) and dihydrocanadensolide (4). The α-linked type of compound is relatively common and includes a number of lichen lactones *e.g.* protolichesterinic acid (5a), nephrosterinic acid (5b) and murolic acid[17] (6) all of which contain a terminal methylene group. Others, such as lichesterinic acid (7) and two new lichen acids from *Neuropogon trachycarpus*,[18] isomuronic (8a) and neuropogolic acid (8b) have the double bond in the lactone ring. A number of α-linked, saturated lactones are also known *e.g.* nephrosteranic acid (9a) and roccellaric acid[17] (9b).

Type A metabolites also include anhydrides and the dimeric anhydrides known as nonadrides,[19,20,21] such as glauconic (10a) and glaucanic acid (10b).

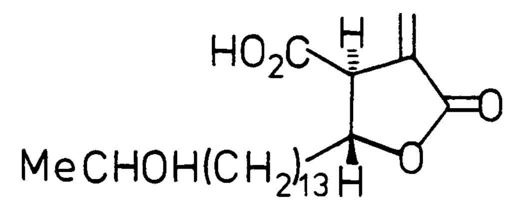
All of these type A metabolites can be formally derived *via* an alkylcitric acid from condensation of oxaloacetate with a fatty acid (Scheme 1) and, as will be discussed in more detail later, the existence of some naturally occurring alkylcitric acids helps support this pathway.

The type B metabolites, in which a $C_3$ unit is linked to the β-position of a fatty acid chain, are far fewer in nature and ethisolide (1) and avenaciolide (2) which are included in this present study are two well known examples, the biosynthetic details of which will be discussed later.



(5a) n = 12

(5b) n = 10



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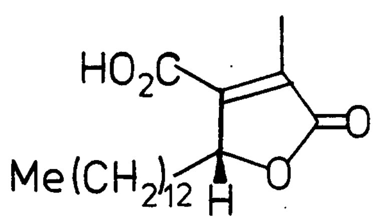

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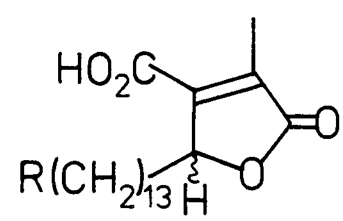
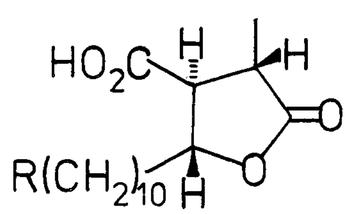


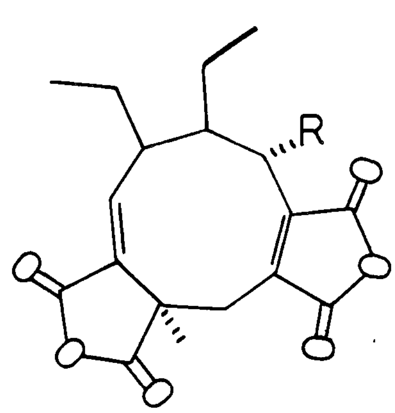
(8a) R = MeCO

(8b) R = MeCHOH



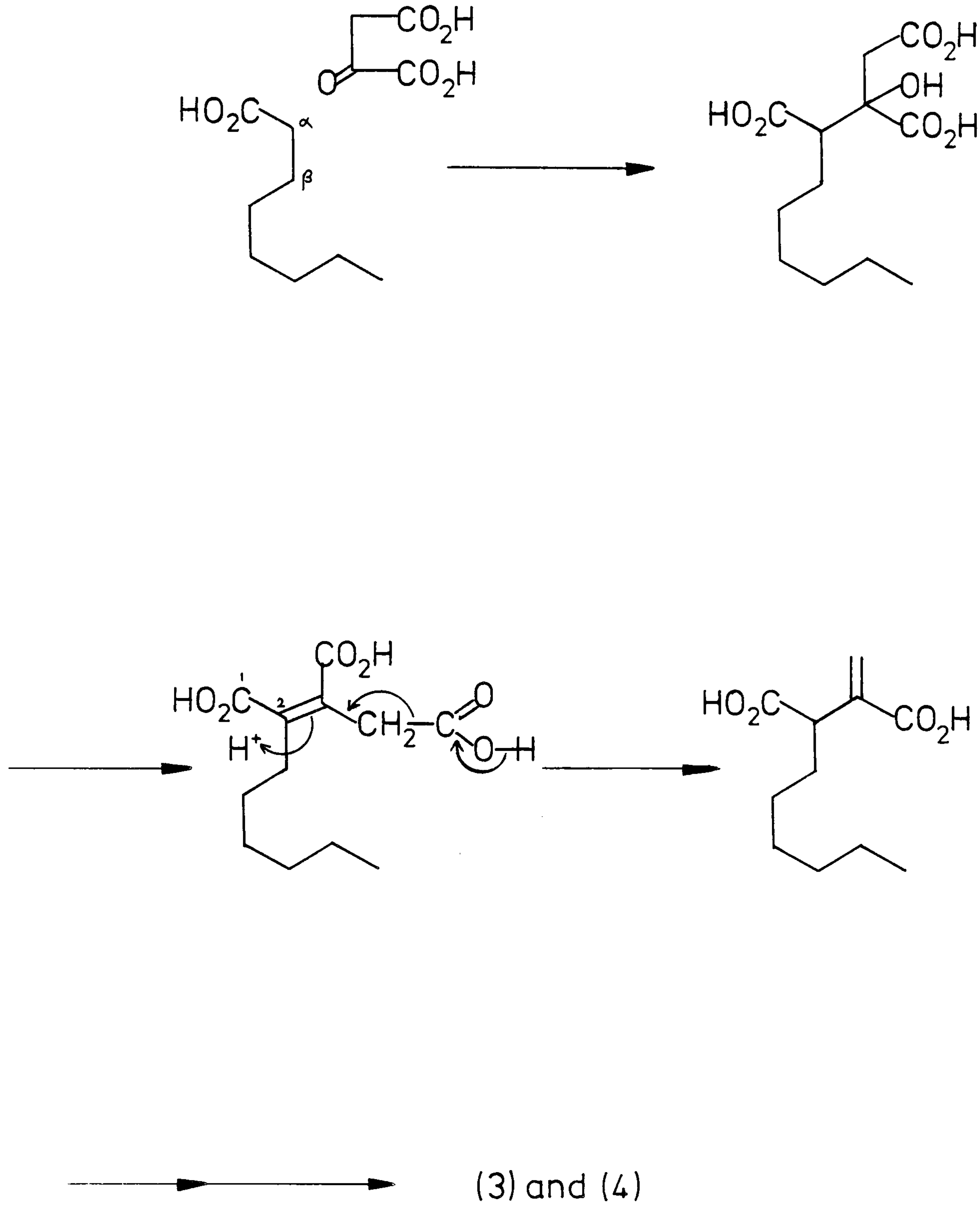
(9a) R = Me

(9b) R = $MeCH_2CH_2$



(10a) R = OH

(10b) R = H

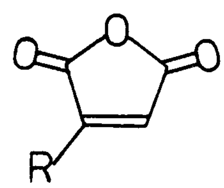


(3) and (4)

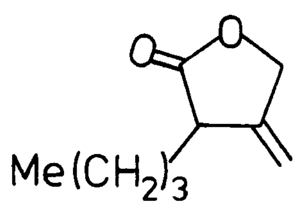
Scheme 1

Turner[22] lists thirty-nine examples of metabolites derived from the condensation of fatty acids with T.C.A. intermediates. Some more recently isolated metabolites, which may belong to the same class, are a number of citraconic anhydride derivatives (11a - 11d) from *Aspergillus wentii*,[23] two $\gamma$-butyrolactones (12) and (13) from *Hypoxylon serpens*[24] and a new disubstituted anhydride (14) from cultures of *Paecilomyces variotii*.[25] The structure of a pair of lichen acids from *Acarospora chlorophana*, namely acaranoic (15) and acarenoic (16) acids have recently been revised to $\delta$-lactones.[26]

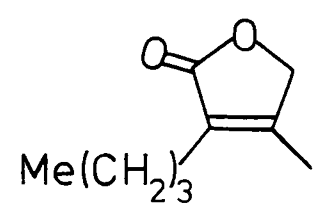
The biosynthetic details of fatty acid synthesis and the T.C.A. cycle have been well documented, but it is perhaps worthwhile to mention the more essential aspects here.



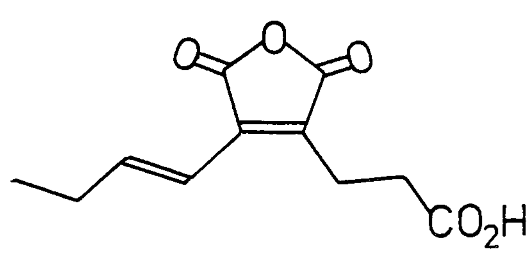
(11) a. R = $(CH_2)_{16}CH_2OAc$
b. R = $(CH_2)_{14}CHOAc.Me$
c. R = $(CH_2)_{14}CHOH.Me$
d. R = $(CH_2)_{14}CO.Me$



(12)



(13)



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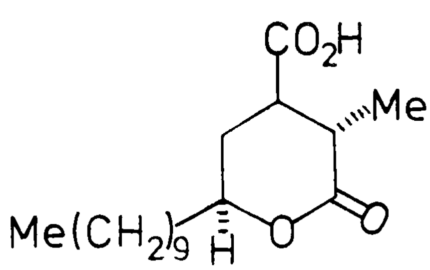

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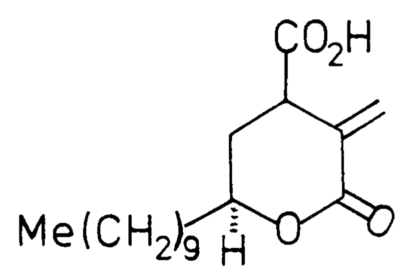
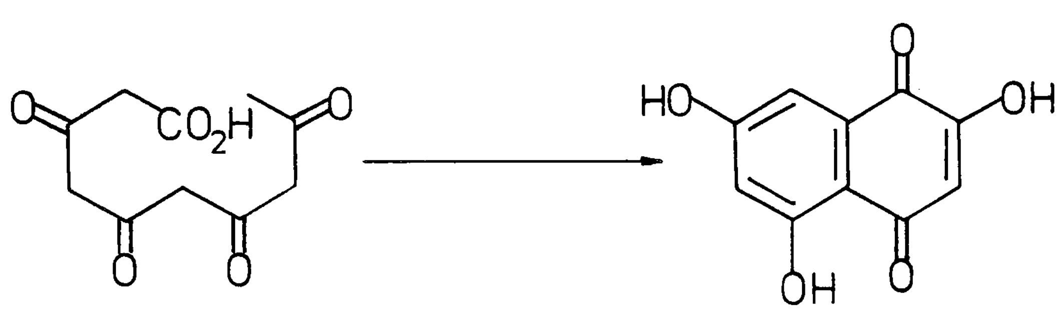


(16)

## (iii) Fatty Acid Biosynthesis: Its Chemistry and Stereochemistry

The mechanism of fatty acid synthesis[27] is important because of its implications for the biosynthesis of a large number of fungal natural products. An even greater number of natural products, the polyketides, arise from the closely related process of polyketide biosynthesis which was the basis of a number of early ideas on fatty acid biosynthesis itself. It was in 1953 that Birch suggested that the oxygenation pattern of a number of aromatic natural products indicated that they were produced by head to tail linkage of acetate units to form a polyketo methylene acid $CH_3(COCH_2)_nCO_2H$ which could be cyclised and modified to produce the desired structure.[28] For example, flaviolin (17) was formed by cyclisation of (18) which was itself derived from intact incorporation of five acetate units.[29] (Fig. 1).



(18) (17)

Fig. 1

It was wrongly thought at the time that fatty acids resulted from reduction of the polyketo acids formed and although the process of fatty acid biosynthesis closely parallels that of polyketides in that the formation of linear chains proceeds by the addition of $C_2$ units, there are a number of differences. The most important being that chain extension only occurs after a three-stage reduction step in fatty acid biosynthesis, the chemistry of which is shown in Scheme 2.

| | | | |
|---|---|---|---|
| (a) | $CH_3COSCoA + CO_2$ or $HCO_3^-$ | $\rightarrow$ | $HO_2CCH_2COSCoA$ |
| (b) | $CH_3COSCoA + HSACP$ | $\rightarrow$ | $CH_3COSACP + HSCoA$ |
| (c) | $CH_3COSACP + HSE$ | $\rightarrow$ | $CH_3COSE + HSCoA$ |
| (d) | $HO_2CCH_2COSCoA + HSACP$ | $\rightarrow$ | $HO_2CCH_2COSACP + HSCoA$ |
| (e) | $CH_3COSE + CH_2(CO_2H)COSACP$ | $\rightarrow$ | $CH_3COCH_2COSACP + CO_2 + HSE$ |
| (f) | $CH_3COCH_2COSACP$ | $\rightarrow$ | $CH_3CH(OH)CH_2COSACP$ |
| (g) | $CH_3CH(OH)CH_2COSACP$ | $\rightarrow$ | $CH_3CH{=}CHCOSACP$ |
| (h) | $CH_3CH{=}CHCOSACP$ | $\rightarrow$ | $CH_3CH_2CH_2COSACP$ |
| (i) | $CH_3CH_2CH_2COSE + CH_2(CO_2H)COSACP$ | $\rightarrow$ | $CH_3CH_2CH_2COCH_2COSACP + CO_2 + HSE$ |

ACP = acyl carrier protein, E = enzyme

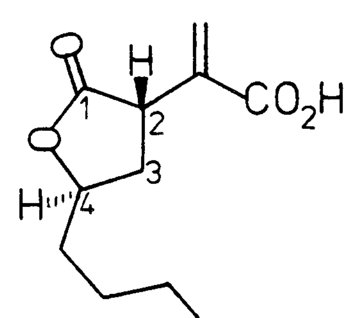
Scheme 2

Acetylcoenzyme A is carboxylated (a) to give malonyl CoA in the presence of $CO_2$ or $HCO_3^-$. The acetyl moeity is then transferred to an enzyme *via* acyl carrier protein (ACP) (b and c). Malonyl CoA is also transferred to ACP and condensation occurs to give acetoacetyl ACP and $CO_2$ (d and e). This is reduced, dehydrated and reduced again to give butyryl ACP (f, g and h). After transferring to the enzyme, the butyryl residue can be further extended by the addition of malonyl CoA (i). This series of reactions continues until, usually, a $C_{16}$ or $C_{18}$ fatty acid is transferred to CoA. All of the reactions take place on an enzyme complex called fatty acid synthetase and no free intermediates are released.

The stereochemical aspects of fatty acid biosynthesis are important when considering the fate of, say, labelled acetate. Upon feeding deuterio-acetate ($CD_3CO_2Na$), it follows from the sequence of reactions in Scheme 2, that each even numbered carbon atom (apart from the methyl group) in the resulting fatty acid retains only one acetate derived deuterium atom, and the configuration of the deuterium atom will depend on the stereochemistry of reduction of the unsaturated acid moeity (19) (Scheme 3). The formation of crotonate (19) has been found to be *via* a (3R) hydroxyfatty acyl derivative (20),[30,31] followed by a *syn* dehydration process eliminating the pro-2S hydrogen atom to yield (19).[32]

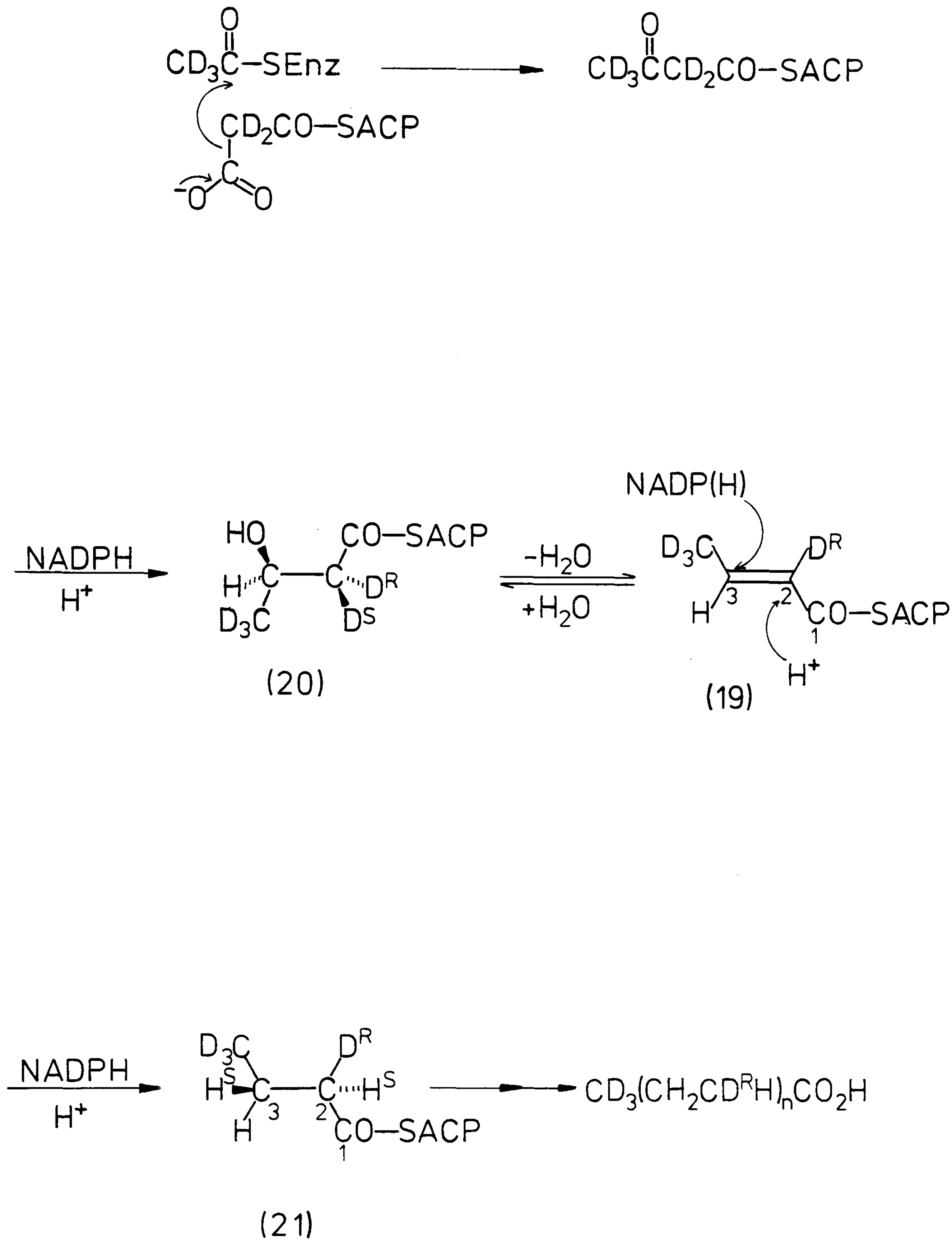
Recent work by Sedgwick and Morris[33] using the enoyl reductase enzyme of yeast fatty acid synthetase has established that the reduction of crotonate (19) to the saturated alkyl thioester (21) occurs by means of an *anti*-addition of hydrogen to the pro-2S and pro-3S positions as shown in Scheme 3. This gave the same result as some earlier work by McCorkindale *et al.* using $^{13}CD_3CO_2Na$ which helped to resolve the stereochemistry of the reduction process in fungi, as well as providing information on the absolute stereochemistry of the bislactone antibiotics (1 - 4).[34]

The $^{13}C$ n.m.r. spectra of the derived ethisolide (1), avenaciolide (2), canadensolide (3) and canadensic acid (22) showed no signs of deuterium either at C-2 or, except in the case of



(22)

ethisolide (1), at C-4. The loss of deuterium at C-2 can be explained by considering the formation of alkylitaconic acids from alkylcitric acids when the acetate derived H atom is lost from C-2 (Scheme 1). The fact that none of the antibiotics retained deuterium



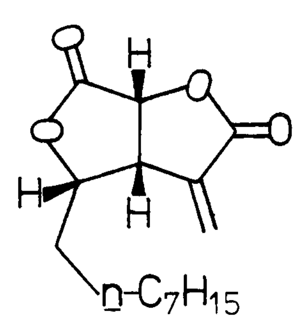
CD3C-SEnz
CD2CO-SACP
CD3CCD2CO-SACP
NADPH
H+
HO
CO-SACP
DR
DS
D3C
-H2O
+H2O
NADP(H)
(20)
(19)
CD3(CH2CDRH)nCO2H
(21)


Scheme 3

at C-2 supports a common biosynthetic pathway for these two types of fungal metabolite (this pathway will be discussed in detail later). The observation that ethisolide (1) retained deuterium at C-4 whereas deuterio-acetate derived deuterium is lost at C-4 in all the other compounds indicates that, assuming hydroxylation at C-4 prior to lactonisation takes place with retention of configuration, the absolute stereochemistry at C-4 is the same in all the lactones examined, apart from ethisolide (1).

At the time of this work, the absolute stereochemistry of the lactones was not known, but a series of elegant syntheses by Anderson and Fraser-Reid from D-glucose of avenaciolide (2),[35] canadensolide (3)[36] and isoavenaciolide (23)[37] showed the absolute configuration at C-4 to be R, apart from ethisolide (1) which had the S configuration at C-4.

It was now possible to determine the absolute configuration of the acetate derived hydrogens in the lactones, as canadensolide (3), *etc.* retains the pro-4S hydrogen then hydroxylation must have removed the pro-4R hydrogen and as deuterium is known to be lost at C-4 then the acetate derived deuterium must be in the R configuration. This is confirmed by the retention of deuterium in ethisolide (1) which has the S configuration at C-4.




(23)

These findings about the pro-R configuration of the acetate derived hydrogen were extended to the stereochemistry of fatty acid biosynthesis in *P. canadense*.[34]

Desaturation of stearic acid to give oleic acid had been shown to proceed in several systems with loss of the pro-9R and pro-10R hydrogens,[38] hence if the acetate derived hydrogen at C-10 in stearic acid is again pro-R it should be lost in the desaturation to oleic acid, The $^{13}C$ n.m.r. spectrum of methyl oleate isolated from cultures of *P. canadense* fed with $^{13}CD_3CO_2Na$ indicated the loss of deuterium from C-10 and therefore was probably in the R configuration (Fig. 2).

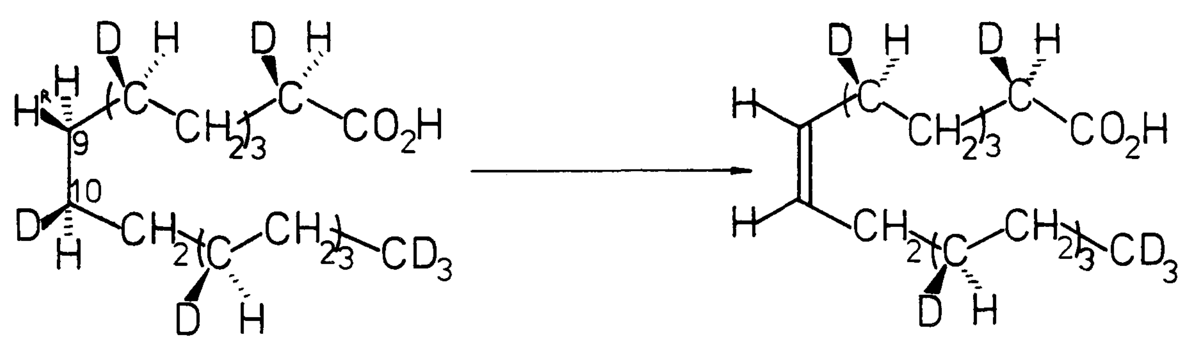


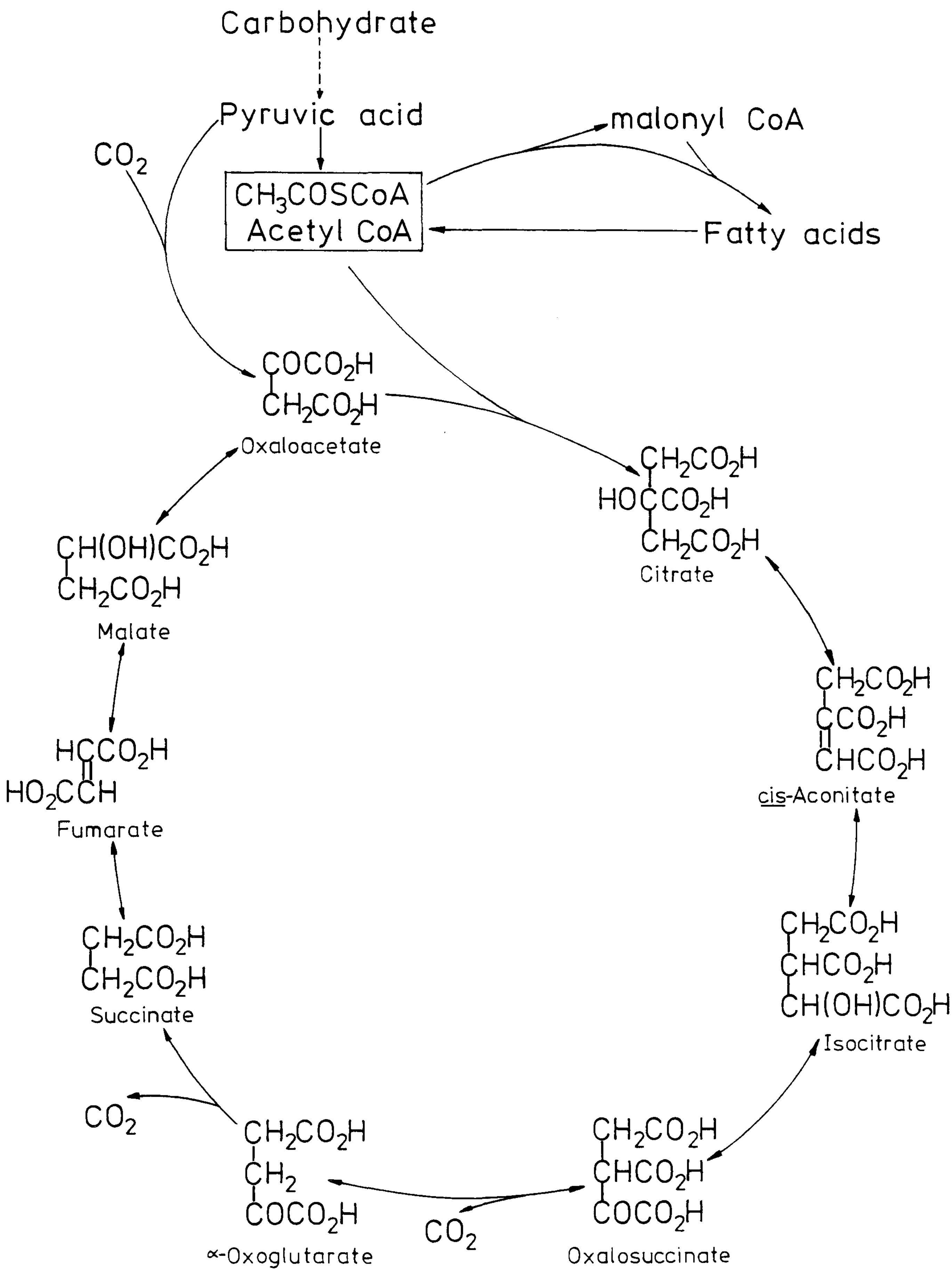
Fig. 2

The implication of this configuration in fatty acid biosynthesis was that the reduction at C-2 of crotonate (19) occurs from the *Si* face (Scheme 3), as was also found by later work on the reductase enzyme of the yeast fatty acid synthetase.[33]

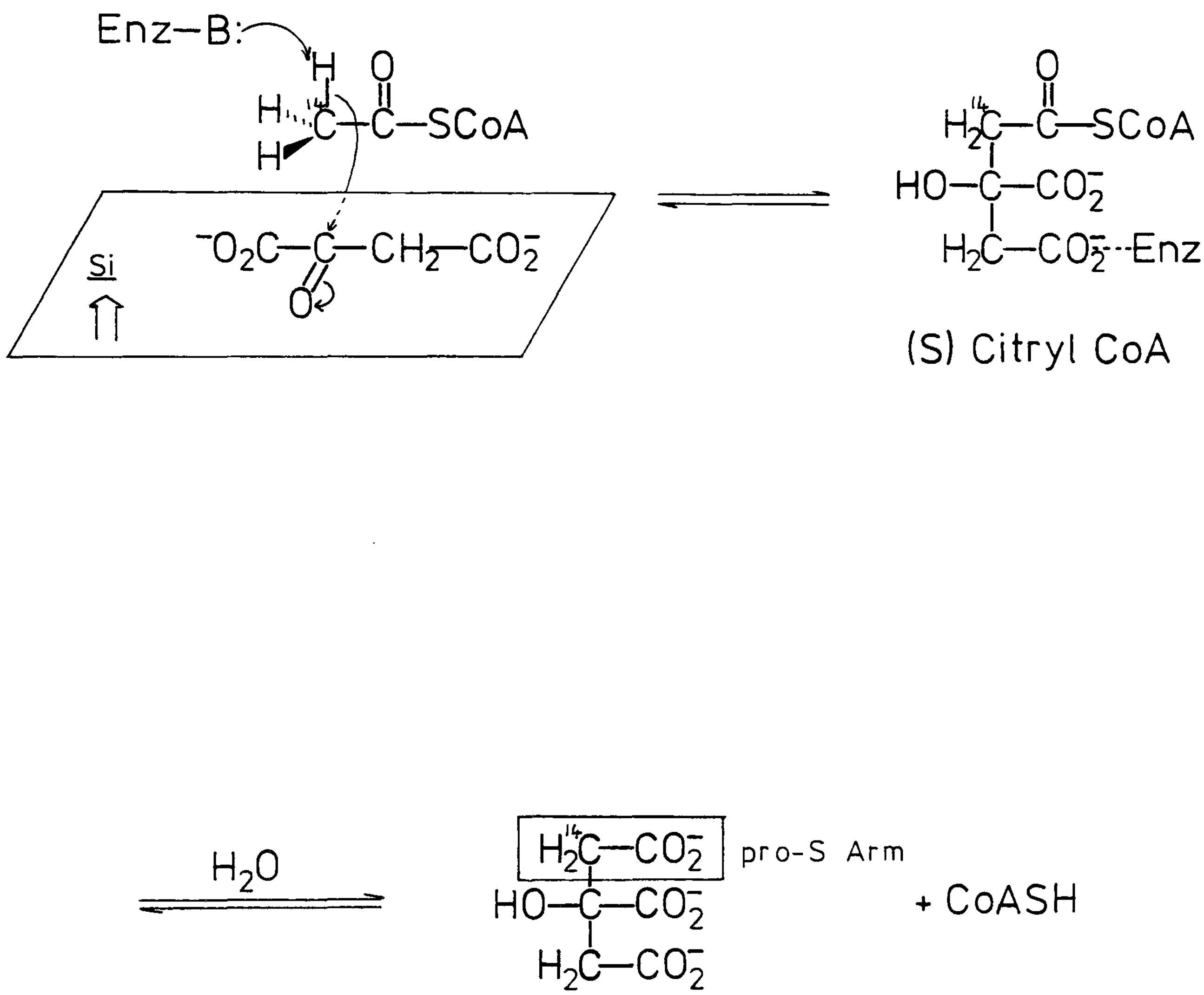
(iv) The Tricarboxylic Acid (T.C.A.) Cycle

The tricarboxylic acid cycle (Scheme 4) provides a mechanism for carbohydrate (*e.g.* glucose) oxidation in cells and also establishes a link between fatty acid biosynthesis *via* a key intermediate acetyl CoA. The condensation of acetyl CoA with the T.C.A. cycle intermediate oxaloacetic acid to produce citric acid is one of the vital primary metabolic processes of a vast range of living organisms. In some cases, massive over production of this essentially primary metabolite results in it being also classed as a secondary metabolite (*e.g. Aspergillus niger, P. decumbens*). The enzyme responsible for this condensation, citrate synthase, has been extensively studied[39] and the two important features seem to be the thermodynamically favourable formation of citrate by hydrolysis of the acylthioester[40] and furthermore, labelled acetyl CoA produces citrate with only the pro-S arm labelled,[41] *i.e.* addition of the acetyl CoA carbanion is always to the *Si* face (with the exception of a few bacteria) of the oxaloacetate carbonyl at the active site[41] (Scheme 5).

At least two other citrate synthases responsible for the formation of alkylcitric acid secondary metabolites have been isolated and investigated. The first, methylcitrate synthase, has been shown to condense propionyl CoA with oxaloacetate to produce (-) methylcitric acid along with other alkylated T.C.A. cycle intermediates formed by other alkylcitrate specific enzymes.[42] Another catalyses the formation of (-) decylcitric acid isolated from cultures of *Penicillium spiculisporum*[43] and although the original claim that (+) decylcitric acid was also produced has since been corrected,[44] the enzyme catalysing the condensation between lauryl CoA and oxaloacetate was isolated and purified.[45] This study, along with more recent work on decylcitrate synthase,[46] showed that the enzyme, while very specific for the $C_4$ diacid species, was relatively non-specific with respect


Carbohydrate
Pyruvic acid
malonyl CoA
CO2
CH3COSCoA
Acetyl CoA
Fatty acids
COCO2H
CH2CO2H
Oxaloacetate
CH2CO2H
HOCCO2H
CH2CO2H
Citrate
CH(OH)CO2H
CH2CO2H
Malate
CH2CO2H
CCO2H
CHCO2H
cis-Aconitate
HCCO2H
HO2CCH
Fumarate
CH2CO2H
CHCO2H
CH(OH)CO2H
Isocitrate
CH2CO2H
CH2CO2H
Succinate
CO2
CH2CO2H
CH2
COCO2H
α-Oxoglutarate
CO2
CH2CO2H
CHCO2H
COCO2H
Oxalosuccinate

Scheme 4



Enz—B:
H
O
H
C
C—SCoA
H
Si
$^-O_2C$—C—$CH_2$—$CO_2^-$
O
O
$H_2^{14}C$—C—SCoA
HO—C—$CO_2^-$
$H_2C$—$CO_2^-$···Enz
(S) Citryl CoA
$H_2O$
$H_2^{14}C$—$CO_2^-$
pro-S Arm
HO—C—$CO_2^-$
$H_2C$—$CO_2^-$
+ CoASH


Scheme 5

to the fatty acid moeity used and that a range of shorter chain substrates could be utilised. A study of this type is included in the present work. Another alkylcitric acid, namely *n*-butylcitric acid, has been isolated from cultures of *P. decumbens*[47] and this further supported the intermediacy of an alkylcitric acid intermediate in the biosynthesis of the type B lactones ethisolide (1) and avenaciolide (2). This aspect will be discussed in more detail later.

Although there has been little investigation of the stereochemistry of methylcitrate synthase and decylcitrate synthase they probably operate in a similar manner to citrate synthase itself with addition of the fattyacyl CoA carbanion to the *Si* face of oxaloacetate (Fig. 3).

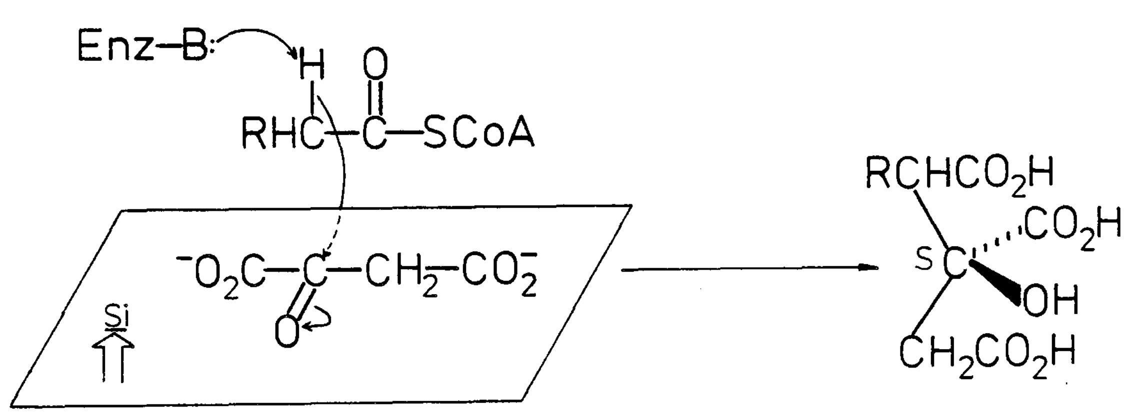



Fig. 3

In the context of this present work, however, the absolute stereochemistry of the alkylcitric acid formed is of little consequence as this is lost when dehydration and decarboxylation take place to form the alkylitaconic acid (Fig. 4).

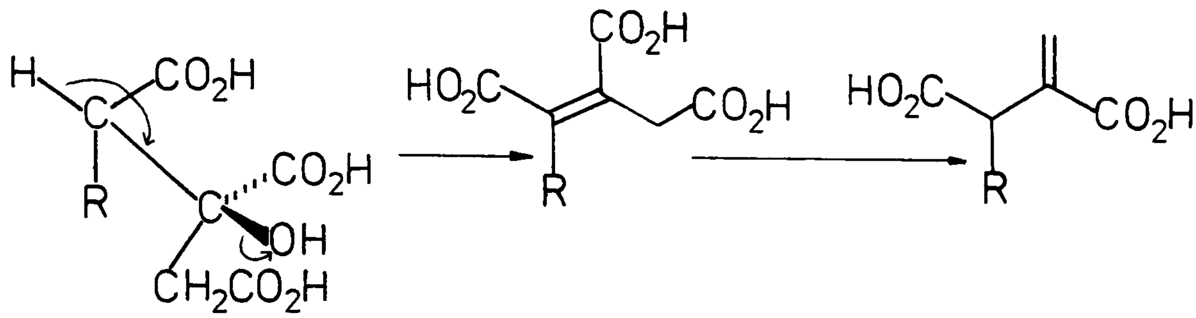
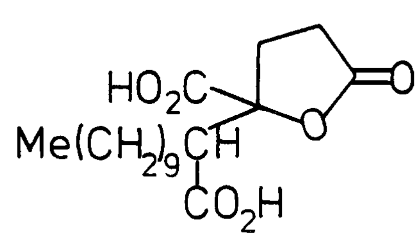


Fig. 4

The synthesis of another fungal acid, namely spiculisporic acid (24) from *P. spiculisporum*, arising by condensation of lauryl CoA with an alternative T.C.A. intermediate has also been demonstrated using a cell free system.[48]



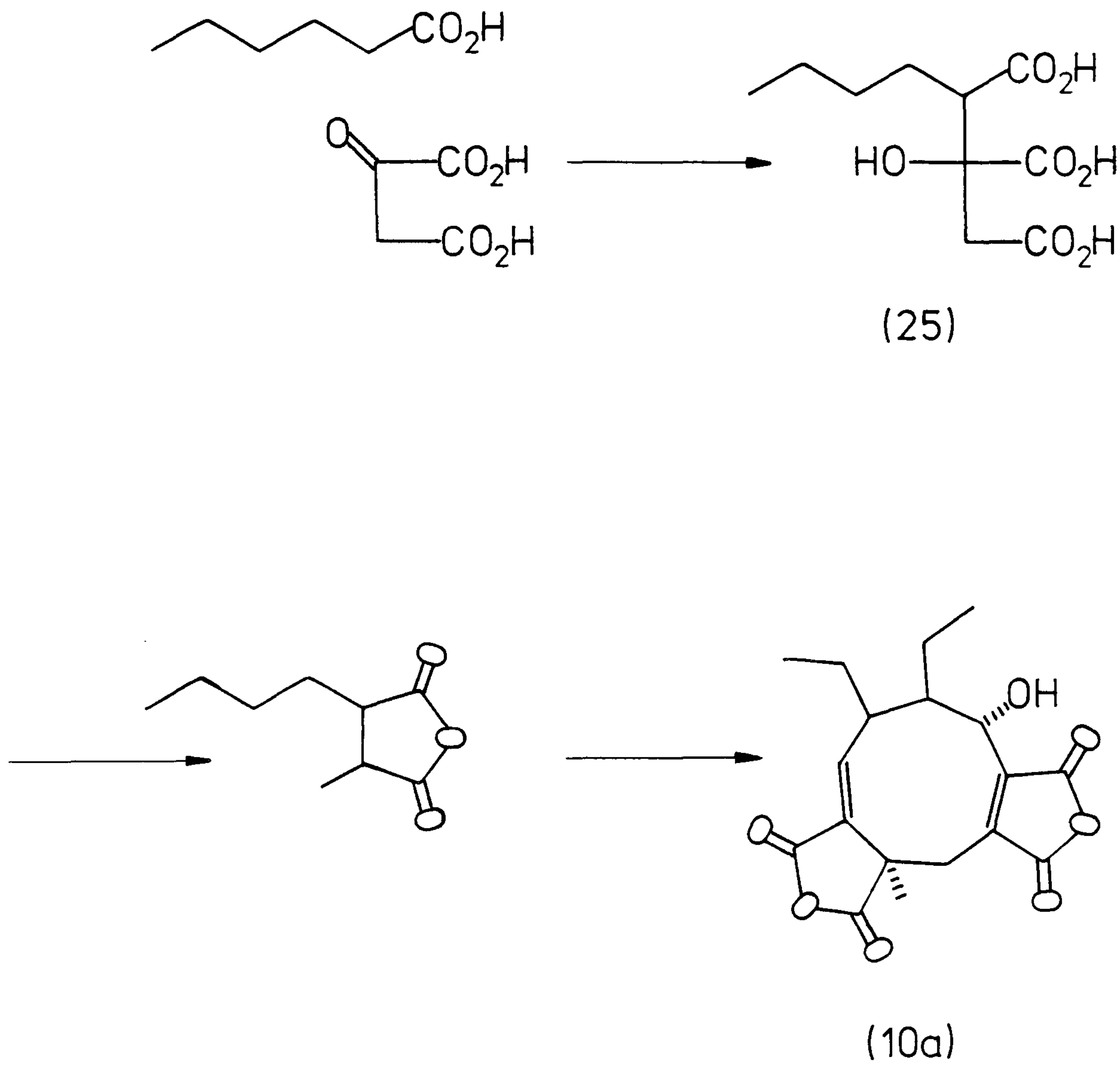
(24)

(v) Biosynthesis of Fungal Metabolites

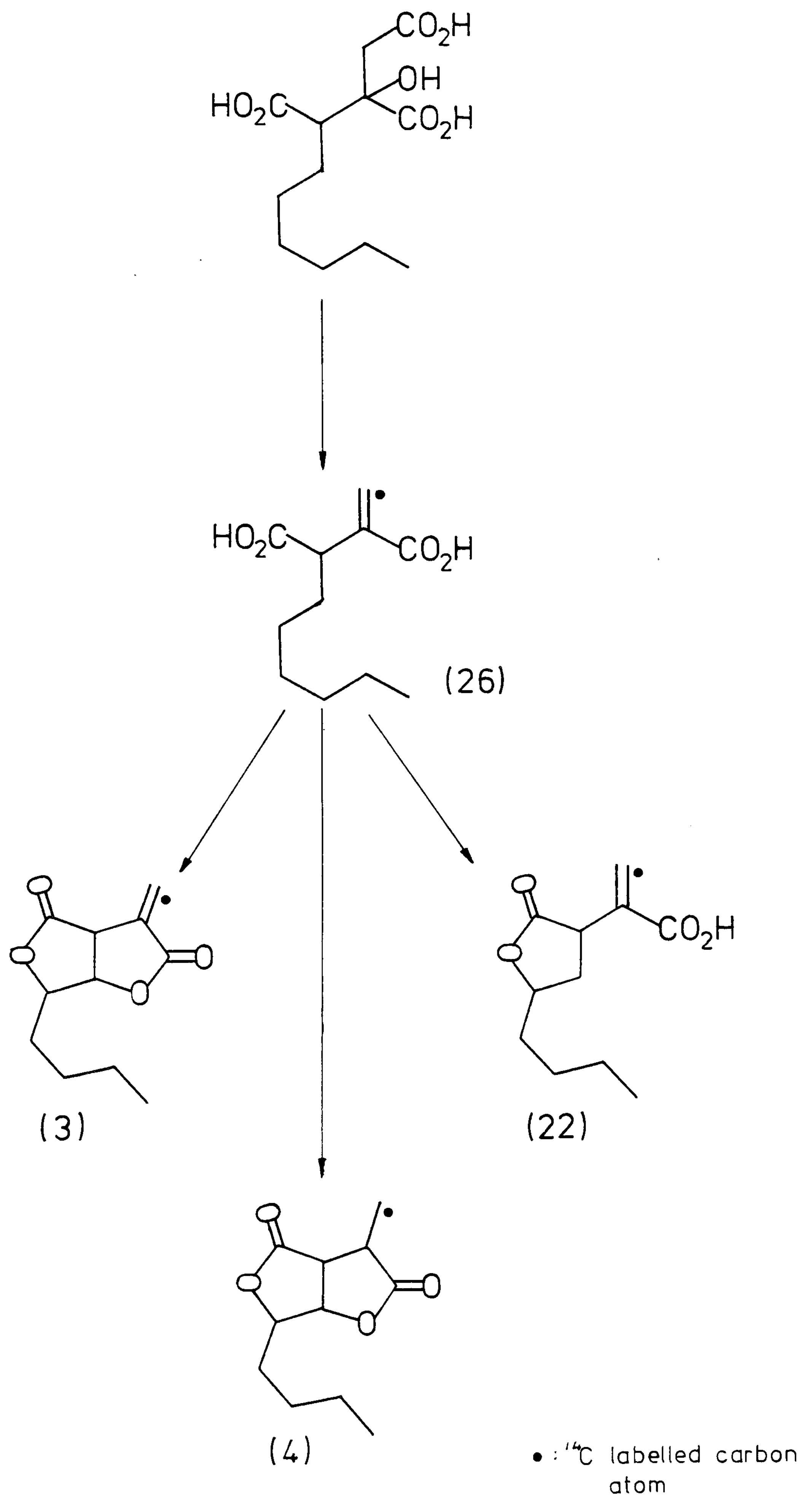
Canadensolide (3) and dihydrocanadensolide (4) were isolated by McCorkindale *et al.* from *P. canadense*[7] and dihydrocanadensolide (4) was also isolated from cultures of *Aspergillus indicus* by Birch and coworkers.[49] Birch noted the possible intermediacy of an alkylcitric acid arising from condensation of octanoic acid and oxaloacetate or pyruvate. Degradation of a sample obtained from feeding radiolabelled acetate showed that most of the activity was in the alkyl chain and this further supported the hypothesis. Both Birch and McCorkindale noted the structural and possibly biosynthetic similarities between these bislactones and the $C_9$ precursor of glauconic acid (10a) which had been shown to arise biogenetically from an alkylcitric acid intermediate (25) by extensive labelling studies[50] (Scheme 6).

In the case of the major metabolites of *P. canadense*, McCorkindale demonstrated the probable intermediacy of an alkylcitric acid by extensive incorporation studies using both C-14 and C-13 labelled acetates and succinates as well as the corresponding C-14 labelled alkylitaconic acid, which would almost certainly arise from dehydration and decarboxylation of the alkylcitric acid, thought to be an intermediate in the metabolites' synthesis. (Scheme 7). The [$^{14}$C-methylene]-hexylitaconic acid (26) was incorporated into canadensolide (3), dihydrocanadensolide (4) and canadensic acid (22) at 20.6%, 11.9% and 33.6% respectively.[47,51]

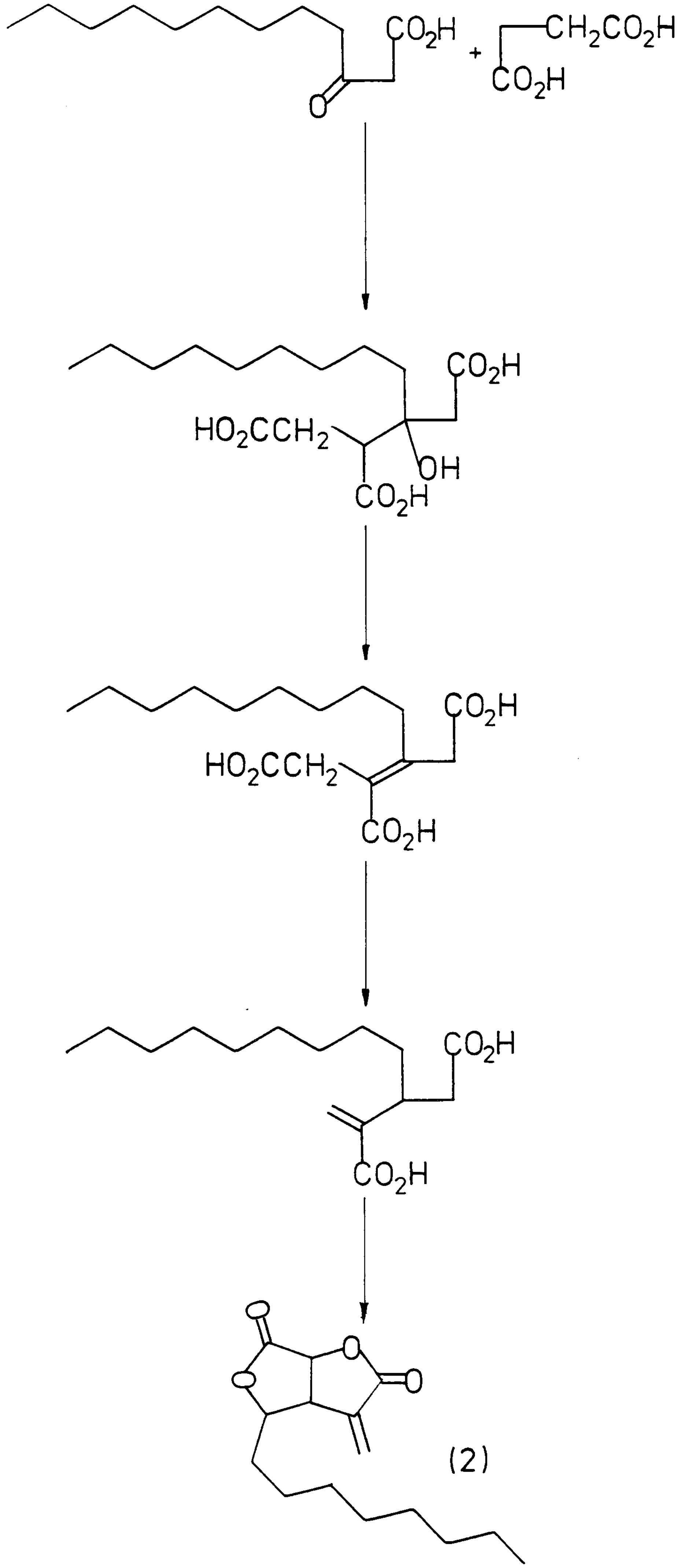
The two type B lactones, ethisolide (1) and avenaciolide (2) presented more of a biosynthetic problem. A $C_3$ unit is linked to the $\beta$-position of a fatty acid chain and it was suggested by Turner[52] that condensation of a $\beta$-keto acid moeity with succinic acid resulted in the formation of an intermediate which could be cyclised to the bislactone (Scheme 8). This was supported by a $^{13}$C n.m.r. study by




Scheme 6


CO2H
OH
HO2C
CO2H
HO2C
CO2H
(26)
CO2H
(3)
(22)
(4)
• : 14C labelled carbon atom

Scheme 7



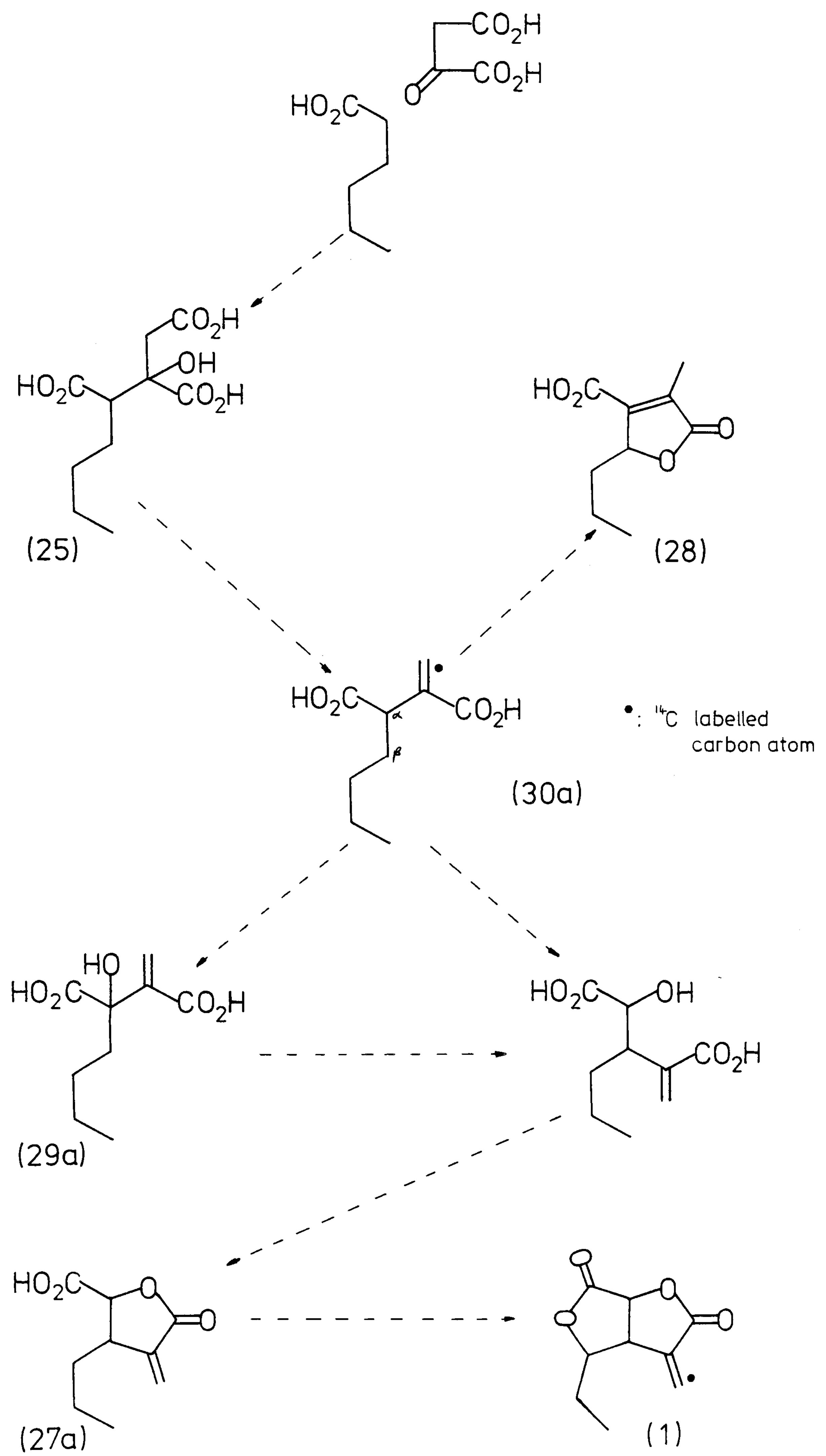
(2)

Scheme 8

Tanabe[53] who observed the incorporation of C-13 acetate into avenaciolide (2) in the predicted manner. It should be noted, however, that there was no evidence for the intermediacy of the postulated intermediates.

McCorkindale,[47] who had earlier noted the similarities between avenaciolide (2) and two *P. canadense* metabolites, canadensolide (3) and dihydrocanadensolide (4), conducted a series of incorporation experiments using singly and doubly labelled C-13 acetate and doubly labelled C-13 succinate. The $^{13}C$ n.m.r. spectra obtained from the derived ethisolide (1) and avenaciolide (2) demonstrated the similarity in the incorporation pattern of acetate and succinate into both the type A metabolites of *P. canadense* and the type B metabolites of *P. decumbens* and *A. avenaceus*. Furthermore, the isolation of a number of minor metabolites of *P. decumbens* provided clues as to the biogenetic origin of the bislactone itself. One of these metabolites, ethisic acid (27a) has the type B structure, but more interestingly, the other three metabolites have the type A structure. Decumbic acid (28) is isomeric with ethisic acid (27a) and is probably derived from *n*-butylcitric acid (25) which was itself isolated from cultures of *P. decumbens* along with *n*-butylhydroxyitaconic acid (29a). An alternative scheme (Scheme 9) was proposed which involved the rearrangement of the $C_3$ unit originally attached to the α-position of a fatty acid becoming attached to the β-position.[47] An analogous enzymatic rearrangement was already known.[14]

The bacterium *Clostridium barkeri* developed two new enzymes when grown on nicotinic acid. One, methylitaconate isomerase, catalysed the conversion of methylitaconic acid to dimethyl maleic acid (Fig. 5) and, of even more significance, the second enzyme, α-methyleneglutarate mutase, catalysed the interconversion of α-methyleneglutaric acid with methylitaconic acid (Fig. 6). A



HO2C
CO2H
OH
O
α
β
•: 14C labelled carbon atom
HO
(25)
(28)
(30a)
(29a)
(27a)
(1)


Scheme 9

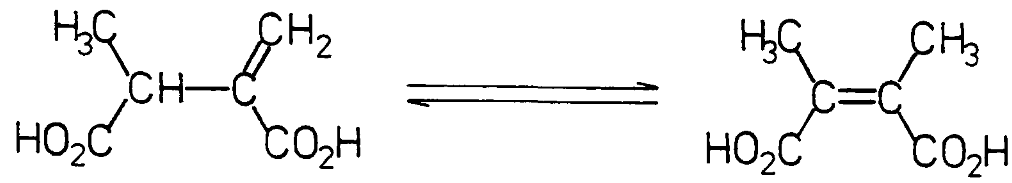


Fig. 5

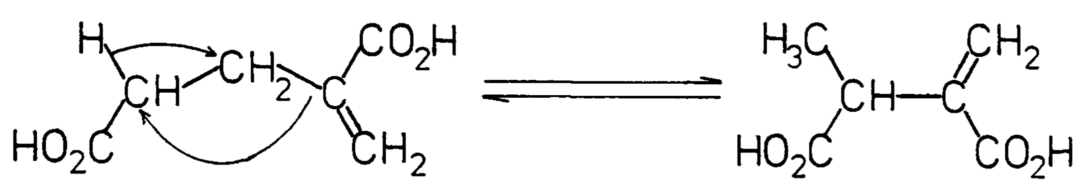


Fig. 6

number of these $B_{12}$-coenzyme dependent enzymatic rearrangements have been observed in other systems[54,55] and can be summarised in the general form shown (Fig. 7). The stereochemical aspects of these

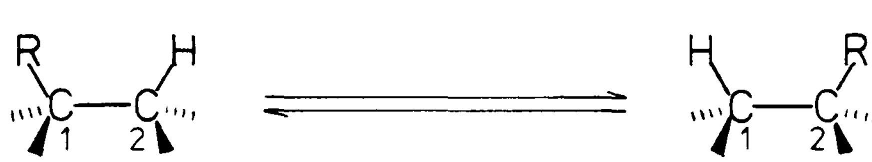


Fig. 7

$B_{12}$-coenzyme catalysed rearrangements is the subject of a recent article by Retey.[56]

The proposed scheme for the biosynthesis of the type B lactones (Scheme 9) involved, in the case of ethisolide (1), formation of *n*-butylcitric acid (25) which would give, by dehydration and decarboxylation, *n*-butylitaconic acid (30a). Synthetic [$^{14}$C-methylene]-*n*-butylitaconic acid was fed to cultures of *P. decumbens* and an incorporation of 10.4% was found, with all of the activity retained in the terminal methylene group of ethisolide (1).[47] Similarly, the corresponding [$^{14}$C-methylene]-*n*-decylitaconic acid was specifically incorporated into avenaciolide (2) by *A. avenaceus*. The level of incorporation being 7.7%.[57]

It seems clear that both types of lactone do arise *via* a common biogenetic pathway and the major part of this present work is devoted to establishing the acyclic and monolactonic intermediates occurring after the rearrangement in *P. decumbens* and *A. avenaceus* and the possible monolactonic intermediates in the biosynthesis of the *P. canadense* metabolites.

# CHAPTER 2

## Discussion

### 1. Biosynthesis of the Type B Bislactones: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with *P. decumbens* and *A. avenaceus*

(i) Potential Biosynthetic Intermediates

(ii) Synthesis of α-[$^{14}$C-methylene]-β-*n*-alkylglutaric acids and Incorporation Studies using *P. decumbens* and *A. avenaceus*

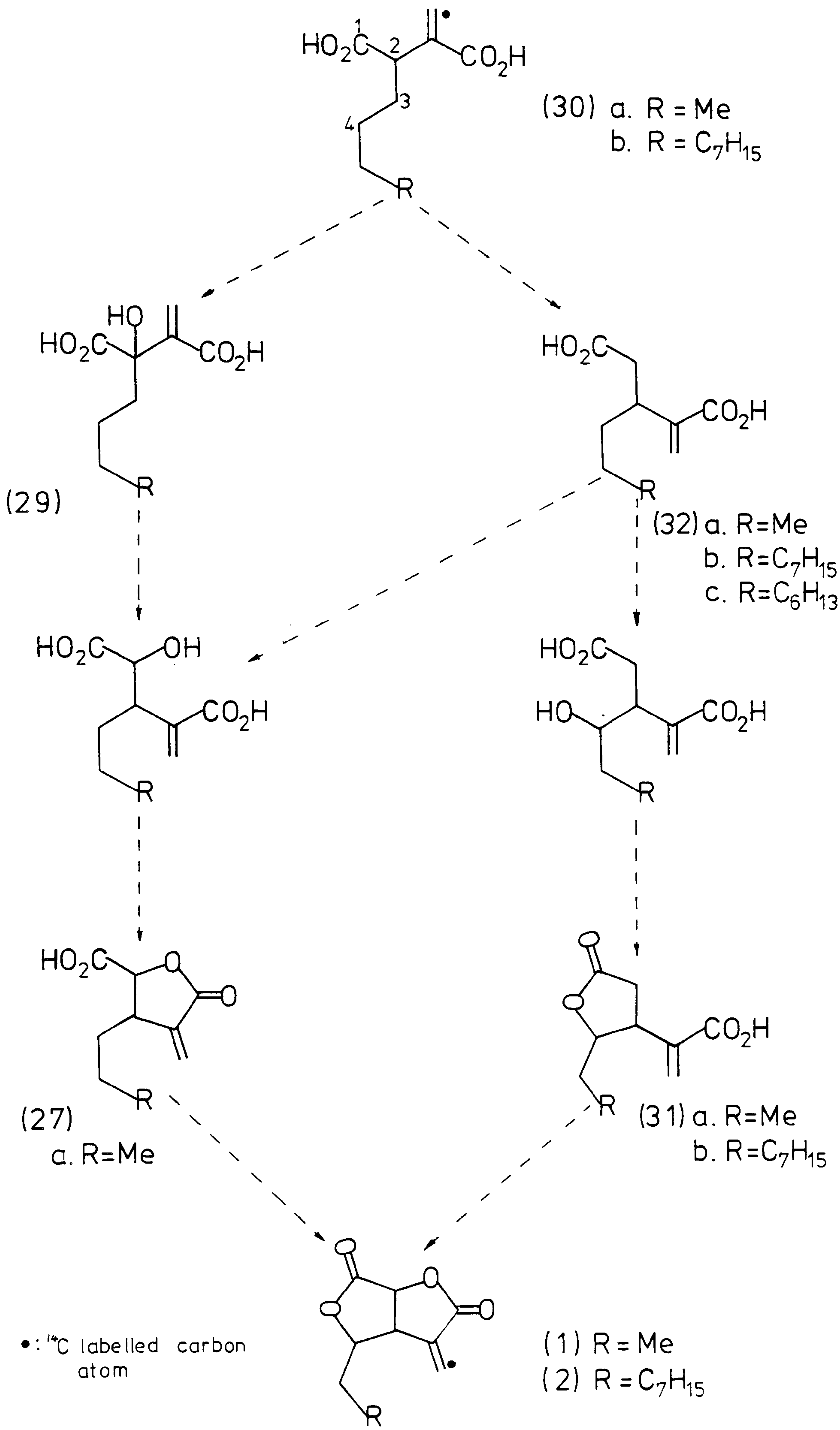
(iii) Synthesis of β-(1-Hydroxyalkyl)-α-[$^{14}$C-methylene]-glutaric acid lactones and Incorporation Studies using *P. decumbens* and *A. avenaceus*

Biosynthesis of the Type B Bislactones: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with *P. decumbens* and *A. avenaceus*

(i) Potential Biosynthetic Intermediates

In the introduction, it has been shown that a number of precursors in the biogenesis of the two type B lactones, ethisolide (1) and avenaciolide (2), were suggested by the isolation and characterisation of a number of significant minor metabolites, extensive $^{13}C$ n.m.r. studies using labelled acetate and succinate and by the efficient, intact incorporation of a synthetic sample of [$^{14}C$-methylene]-*n*-butylitaconic acid (30a). There was, however, still considerable scope for a number of possible pathways following the rearrangement of the alkylitaconic acid itself. The most immediate problem being at what stage, before or after hydroxylation at the C-2 position, did the alkylitaconic acid rearrange and what, therefore, was its immediate successor.

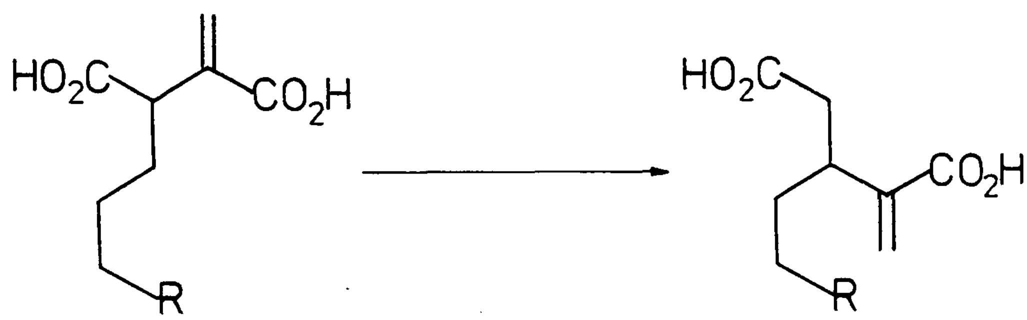
Furthermore, the formation of the final bislactone structure required two hydroxylations of the carbon skeleton and the order in which they occurred would give rise to two possible isomeric monolactonic precursors (Scheme 10). If hydroxylation at C-2 occurs first then the product following lactonisation would be the known minor metabolite of *P. decumbens*, ethisic acid (27a). However, if hydroxylation occurs firstly at the C-4 position of the fatty acid derived portion of the carbon skeleton, then the isomeric monolactone (31a) would result. Obviously what was required to clarify this situation was a number of incorporation studies using radiolabelled samples of these potential precursors. The simplest compounds to synthesise were the $\alpha$-methylene-$\beta$-*n*-alkylglutaric acids (32) as outlined below.


(30) a. R = Me
b. R = $C_7H_{15}$
(29)
(32) a. R=Me
b. R=$C_7H_{15}$
c. R=$C_6H_{13}$
(27)
a. R=Me
(31) a. R=Me
b. R=$C_7H_{15}$
•: $^{14}C$ labelled carbon atom
(1) R = Me
(2) R = $C_7H_{15}$

Scheme 10

(ii) <u>Synthesis of α-[$^{14}$C-methylene]-β-*n*-alkylglutaric acids (32) and Incorporation Studies using *P. decumbens* and *A. avenaceus*</u>

The first potential biosynthetic precursors to be synthesised were the acyclic type B compounds, the α-methylene-β-*n*-alkylglutaric acids (32) which could result biogenetically by the $B_{12}$-coenzyme catalysed rearrangement from the appropriate alkylitaconic acid (30). (Fig. 8).

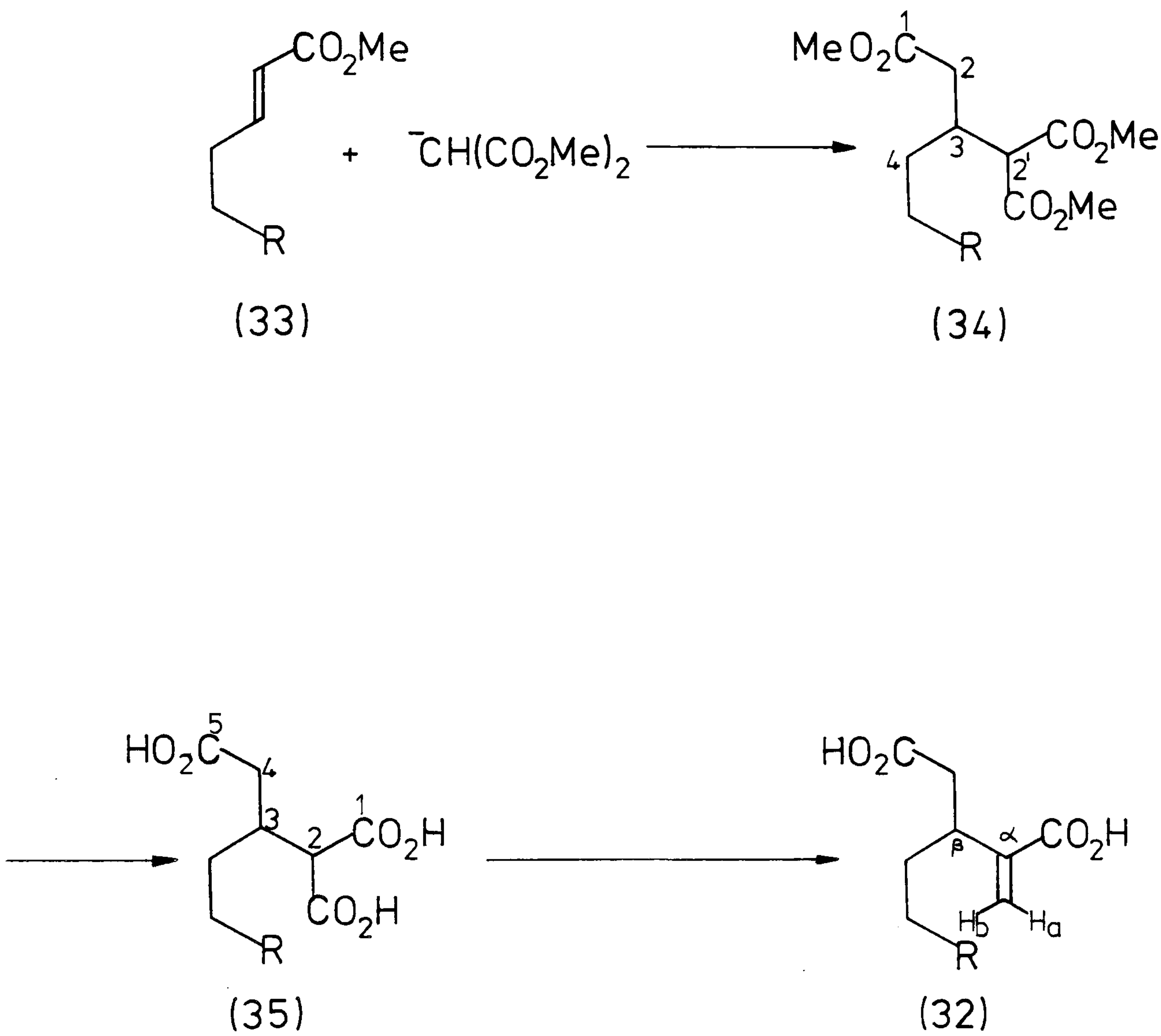


(30) (32)

R = Me, $C_7H_{15}$, $C_6H_{13}$

Fig. 8

The synthesis is outlined in Scheme 11. The appropriate α,β-unsaturated methyl ester (33), prepared by the Knoevenagel procedure and esterification,[58] underwent a Michael addition with dimethyl malonate, a variation of the condensation of ethyl crotonate with diethyl malonate,[59] to give the trimethyl ester (34) which was efficiently converted to the 2-carboxy-3-*n*-alkylglutaric acid (35) by treatment with aqueous sodium hydroxide. The conversion of the triacid (35) to the desired α-methylene-β-*n*-alkylglutaric acid (32) was effected by the methylenation procedure using the triacid (35) and either aqueous formaldehyde and 40% aqueous dimethylamine[60] or aqueous formaldehyde and diethylamine.[61] An essential feature of the methylenation procedure is that the carbon atom of the exo-methylene group is derived from formaldehyde. Therefore, by using [$^{14}$C]-formaldehyde at this stage, the corresponding α-[$^{14}$C-methylene]-β-*n*-alkylglutaric acids (32a) and (32b) were obtained for use in incorporation studies with *P. decumbens* and *A. avenaceus*.




a. R = Me
b. R = $C_7H_{15}$
c. R = $C_6H_{13}$

<u>Scheme 11</u>

Time/incorporation studies using [1-$^{14}$C]-sodium acetate on the production of ethisolide (1) from *P. decumbens* and avenaciolide (2) from *A. avenaceus* had shown that 24 hr. pulsed inoculations on the seventh, eighth and ninth days of growth, followed by extraction on the fourteenth day gave the highest incorporation of acetate into ethisolide (1)[62] and that 12 hr. pulsed inoculations on the seventh, eighth and ninth days of growth followed by extraction after acidification on the fourteenth day gave the highest incorporation of acetate into avenaciolide (2).[63]

These results were used in the present incorporation studies involving *P. decumbens* and *A. avenaceus*. α-[$^{14}$C-Methylene]-β-*n*-propylglutaric acid (32a) was fed in three 24 hr. pulses to cultures of *P. decumbens* and the ethisolide (1) recovered was found to contain 26% of the administered radioactivity. Likewise, α-[$^{14}$C-methylene]-β-*n*-nonylglutaric acid (32b) was fed in five 12 hr. pulses to cultures of *A. avenaceus* giving avenaciolide (2) which contained 10.6% of the administered radioactivity. In both cases, degradation of the labelled bislactone, by ozonolysis to give formaldehyde which was trapped and counted as its dimedone derivative, showed that the radioactivity was specifically incorporated in the terminal methylene carbon.

This clearly showed that the two type B lactones, ethisolide (1) and avenaciolide (2) are biosynthesised *via* the α-methylene-β-*n*-alkyl-glutaric acids (32a, 32b) which result from rearrangement of the corresponding *n*-alkylitaconic acid (30) without prior hydroxylation. This conclusively ruled out the intermediacy of the minor metabolite *n*-butylhydroxyitaconic acid (29a) in the biosynthesis of ethisolide (1) and the corresponding *n*-decyl analogue in the biosynthesis of avenaciolide (2). This result is in complete accord with the incorporation of the tritiated itaconic acid (36) into ethisolide (1) with complete retention of tritium[64] (Fig. 9).

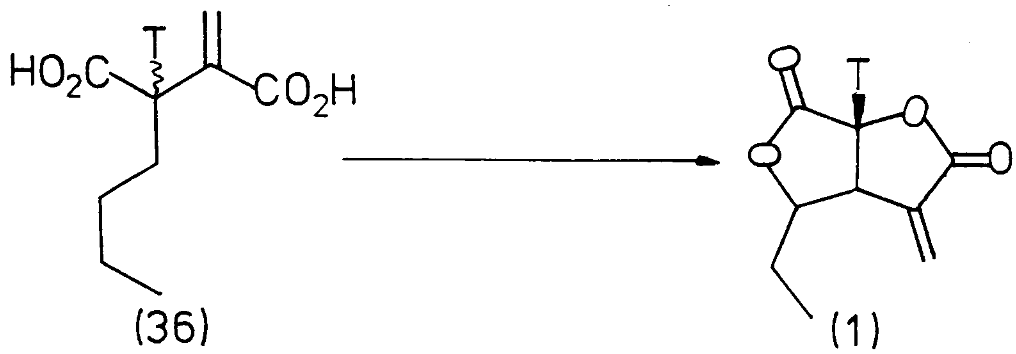


Fig. 9

Moreover, the observed values for the incorporation and dilution of the type B lactone precursors (Tables 1 and 2) clearly support the proposed acetate → itaconate (30) → glutarate (32) pathway (Scheme 10) by following the accepted pattern of increasing per cent incorporation and decreasing dilution value the closer the precursor is to the product.[65]

In an experiment to establish the substrate specificity of the enzymes responsible for the lactonisation of these acyclic precursors (32), it was decided to feed a sample of α-[$^{14}$C-methylene]-β-*n*-octylglutaric acid (32c) to cultures of *A. avenaceus* to investigate whether or not this shorter chain homologue could be utilised (as mentioned previously, the enzyme decylcitrate synthase was found to be relatively non-specific for the fatty acid moeity and a range of shorter chain substrates could be used[45,46]).

The crude avenaciolide (2) obtained proved difficult to crystallise and further preparative t.l.c. was necessary. Crystallisation to constant activity gave colourless plates m.p. 52-53°C, different from the two known m.p.s of avenaciolide[6] (avenaciolide has a double m.p. 49-50°C and 54-56°C). This was homogeneous by t.l.c., but g.l.c. separated two components, the second corresponded to avenaciolide (2), $R_t$ 3.6 min., the first component, $R_t$ 2.5 min., was

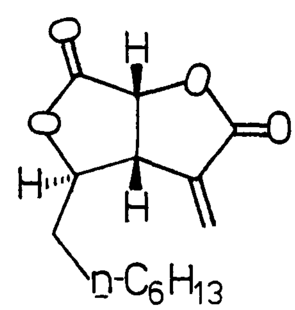
| Precursor Fed | Incorporation (%) | Dilution Values |
|---|---|---|
| $[^{14}C]$-Succinate[62] | 5.5 | $2.9 \times 10^4$ |
| $[^{14}C]$-Acetate[62] | 14 | $4.4 \times 10^3$ |
| $[^{14}C$-Methylene]-*n*-butylitaconate[47] | 10.4 | 47.2 |
| α-$[^{14}C$-Methylene]-β-*n*-propylglutarate | 26 | 40.5 |

Table 1. Per Cent Incorporation and Dilution Values for Ethisolide (1) Precursors.

| Precursor Fed | Incorporation (%) | Dilution Values |
|---|---|---|
| $[^{14}C]$-Acetate[63] | 2.5 | $1.4 \times 10^3$ |
| $[^{14}C$-Methylene]-*n*-decylitaconate[57] | 7.7 | 32 |
| α-$[^{14}C$-Methylene]-β-*n*-nonylglutarate | 10.6 | 13.4 |

Table 2. Per Cent Incorporation and Dilution Values for Avenaciolide (2) Precursors.

approximately 3% of the total mixture. The observed difference in retention times under the specified conditions was approximately equal to that observed in a standard hydrocarbon mixture ($C_{17}$ to $C_{15}$) between each of the components, the lower molecular weight hydrocarbon having the shortest retention time. It seemed probable therefore, that the minor component in the "avenaciolide" mixture was a shorter chain homologue - *nor*-avenaciolide (2a) into which the precursor (32c)



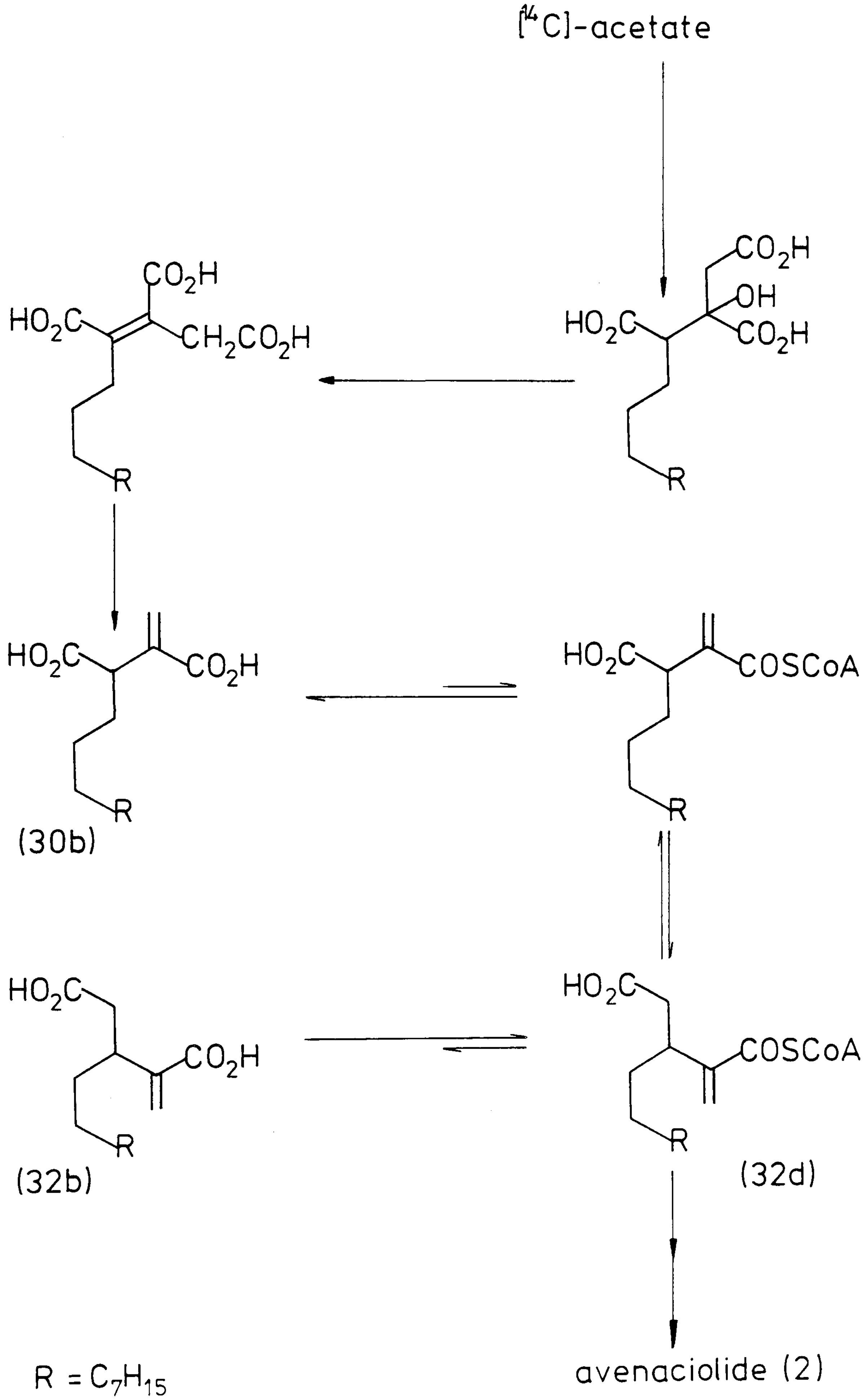
(2a)

is incorporated. The incorporation from α-[$^{14}$C-methylene]-β-*n*-octylglutaric acid (32c) was 6.2% of the administered radioactivity, a level of incorporation far in excess of that of any possible degradation products, *i.e.* acetate, succinate, *etc.* This indicates that the homologue (32c) was efficiently utilised as a substrate by *A. avenaceus* and thus supports the observation of non-specificity in other systems.

Having established that the α-methylene-β-*n*-alkylglutaric acids (32) are efficient precursors, it must also be borne in mind that this does not prove that they are genuine intermediates on the pathway in question. One criterion, conversion to the product, has been shown, but the other important criterion, that the compound is itself formed by the pathway, has not. If the intermediate in question is consumed as quickly as it is formed then direct isolation is not possible.

In this case evidence may be obtained using the technique of *in vivo* isotope trapping.[65] By feeding [$^{14}C$]-acetate to cultures of *A. avenaceus* (*P. decumbens* produces large amounts of citric acid which would complicate the isolation and purification of the acidic intermediates) along with a quantity of non-labelled $\alpha$-methylene-$\beta$-*n*-nonylglutaric acid (32b) it should be possible to reisolate and analyse the glutaric acid (32b) to find if any radioactivity is present. If this is the case then it would be considered positive proof that the $\alpha$-methylene-$\beta$-*n*-alkylglutaric acids (32) are true intermediates on the type B lactone pathway. On carrying out the experiment, it was found that both $\alpha$-methylene-$\beta$-*n*-nonylglutaric acid (32b) and *n*-decylitaconic acid (30b) were obtained and furthermore, the radioactivity was contained exclusively in the itaconic acid (30b). The level of activity isolated was *ca.* 1.3% of the total activity administered as [$^{14}C$]-acetate. A sample of avenaciolide (2) was also obtained, this contained 2.7% of the administered activity - a value typical of the incorporation level expected when feeding [$^{14}C$]-acetate to *A. avenaceus* (*cf.* Table 2). Hence the pathway from acetate to avenaciolide (2) is proceeding as normal and the non-incorporation of acetate into $\alpha$-methylene-$\beta$-*n*-nonylglutaric acid (32b) is not due to blocking of the main pathway by feedback inhibition. In such a case, the avenaciolide (2) obtained would be expected to contain little or no activity.

These results seem to rule out $\alpha$-methylene-$\beta$-*n*-nonylglutaric acid (32b) as an intermediate. However, the biosynthesis may be represented as in Scheme 12. From this it may be noted that whereas the itaconate (30b) lies on the main pathway, the glutarate (32b), although incorporated very well, enters the pathway after conversion to its coenzyme A ester (32d). This step could well be subject to feedback inhibition in the presence of excess glutarate (32b) and conversion of endogenous ester (32d) (which will be labelled from acetate) to the

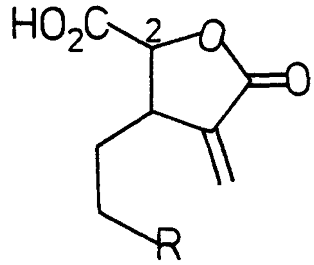

[14C]-acetate
HO2C
CO2H
OH
CO2H
CH2CO2H
R
COSCoA
(30b)
(32b)
(32d)
avenaciolide (2)
R = C7H15

Scheme 12

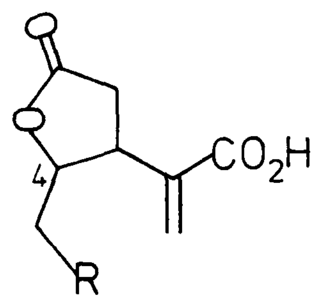
free acid may be completely suppressed. Hence although the free α-methylene-β-*n*-nonylglutaric acid (32b) strictly speaking does not lie on the main pathway, its ester (32d) probably does.

(iii) Synthesis of β-(1-Hydroxyalkyl)-α-[$^{14}$C-methylene]-glutaric acid lactones (31) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

Two types of monolactonic intermediate are possible by lactonisation following hydroxylation at either C-2 or C-4, the ethisic acid type (27) or its isomer (31) respectively.



(27)

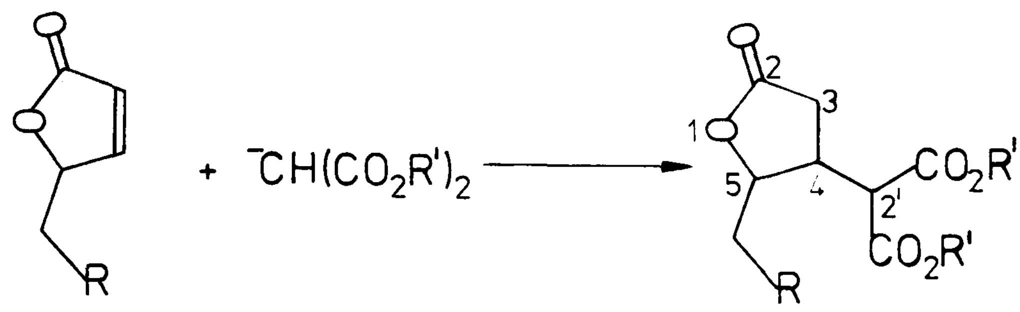


(31)

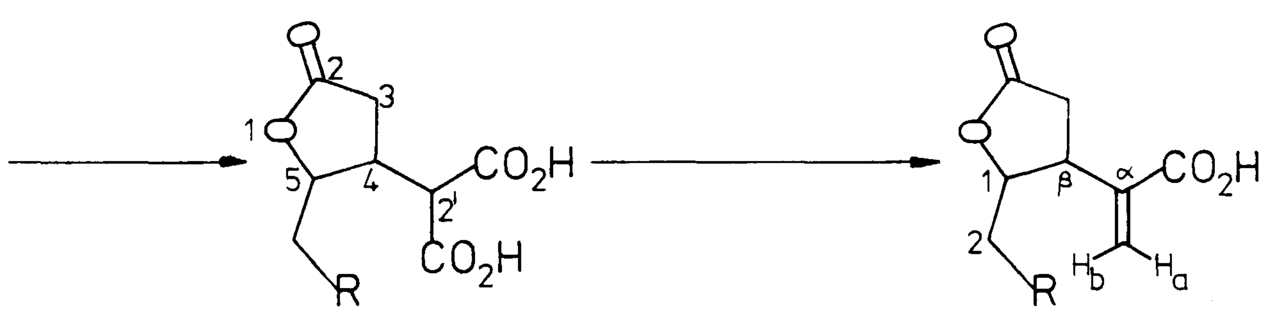
a. R = Me , b. R = $C_7H_{15}$

It seemed likely that only one type would be the normal, main path intermediate, but it was desirable to synthesise and feed both types in order to establish this as so. Following the success of the synthetic route to the α-methylene-β-*n*-alkylglutaric acids (32), an analogous route to the lactone acids (31) was envisaged (Scheme 13) involving Michael addition of malonic ester to the appropriate butenolide (37) followed by hydrolysis and methylenation to afford the desired lactone acid (31).

Initially what was required, therefore, was an efficient route to the butenolides (37). The first synthetic route tried was that of Kurono *et al.*[66] involving alkylation of the appropriate β-keto sulphoxide (38) followed by reduction and lactonisation to the desired butenolide (37) (Fig. 10).



(37) (44)



(46) (31)

a. R = Me
b. R = $C_7H_{15}$

R' = Me, Bzl.

Scheme 13

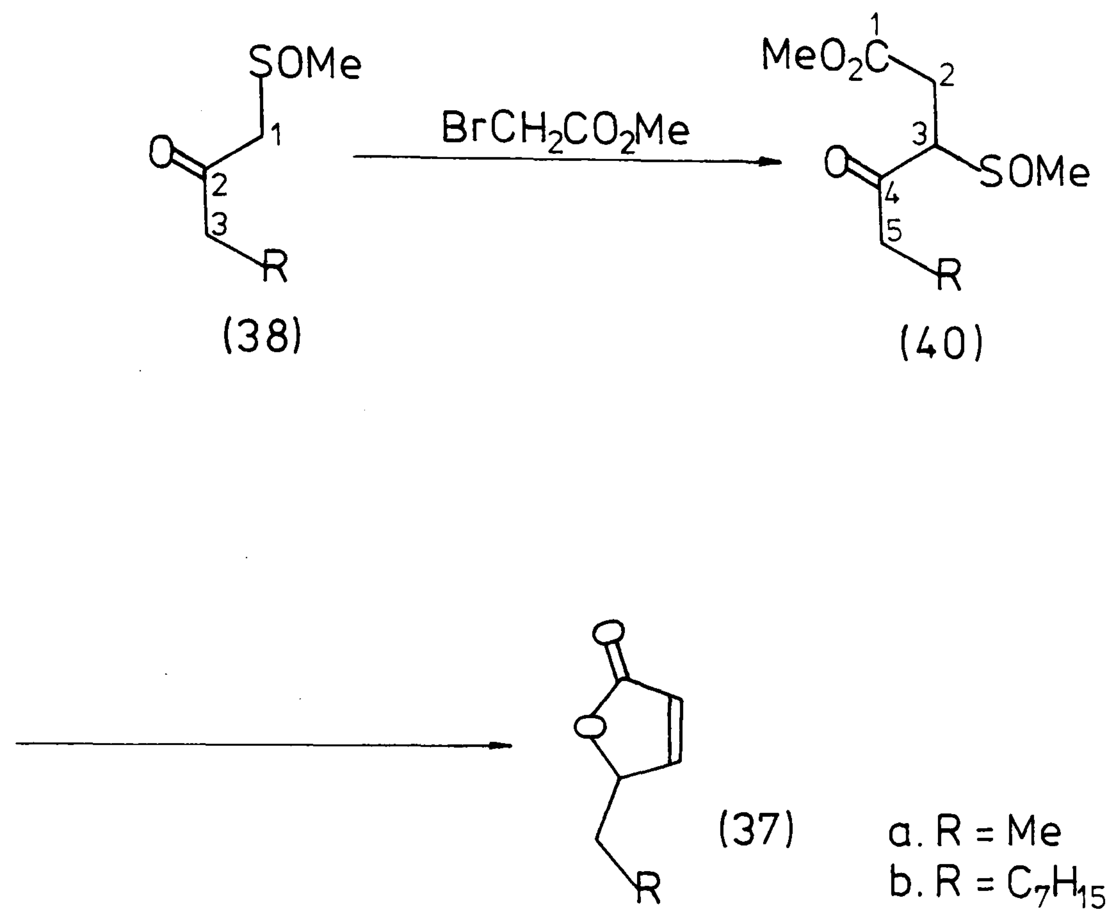
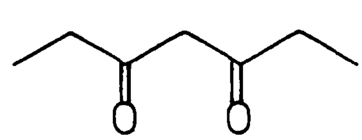
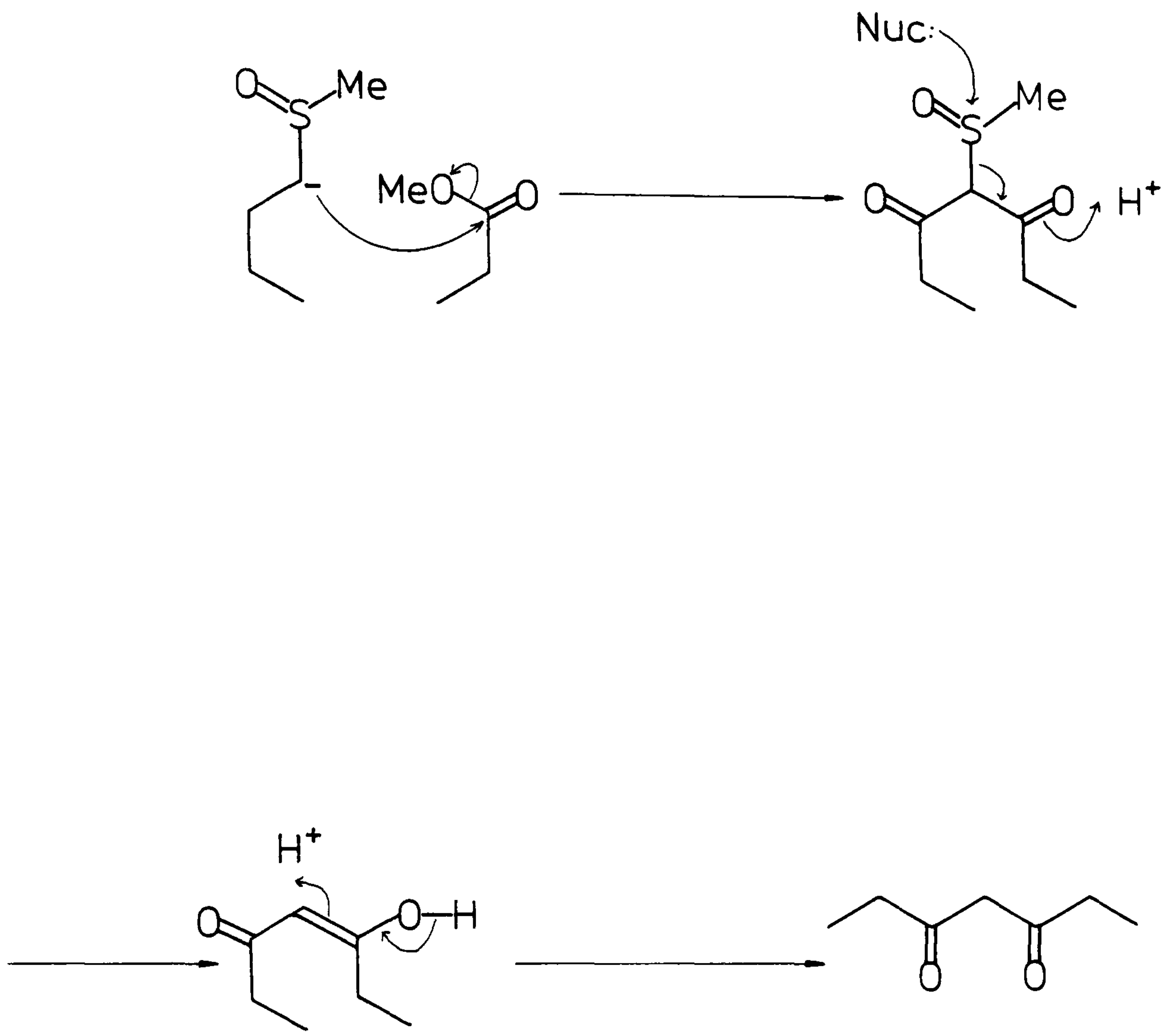



Fig. 10

The long chain β-keto sulphoxide (38b) was successfully prepared by the method of Corey and Chaykovsky,[67] but repeated attempts to prepare the shorter chain analogue (38a) failed, only small amounts of 3,5-diketoheptane (39) being detected (Scheme 14).



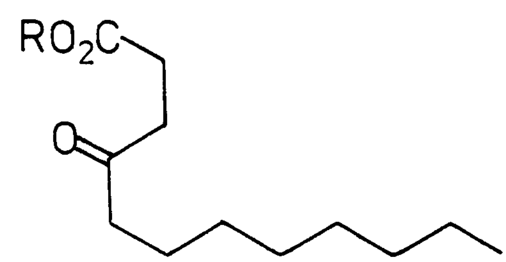
(39)




(39)

Scheme 14

Alkylation of the β-keto sulphoxide (38b) afforded the methylsulphonyl keto ester (40b), reduction of which, with sodium borohydride at 0°C, gave not the corresponding hydroxy compound, but a product consisting almost entirely of ethyl 4-oxododecanoate (41a).




(41) a. R=Et
b. R=H

This almost certainly arose by subsequent elimination of sulphoxide following reduction of the ketone, the ethyl ester being formed by transesterification[68] (the reduction solvent being ethanol) (Fig. 11).

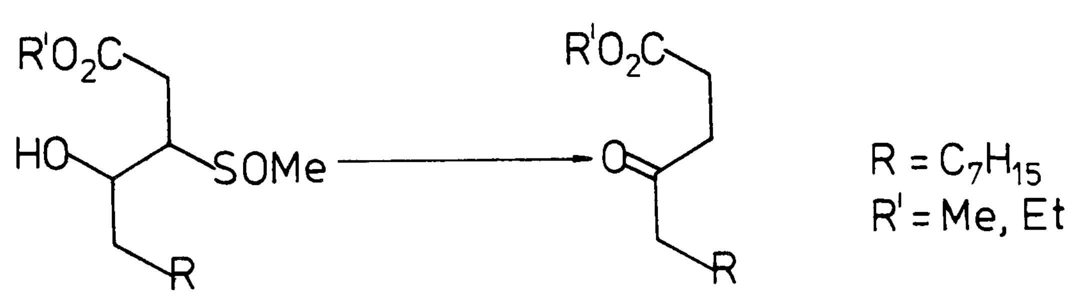
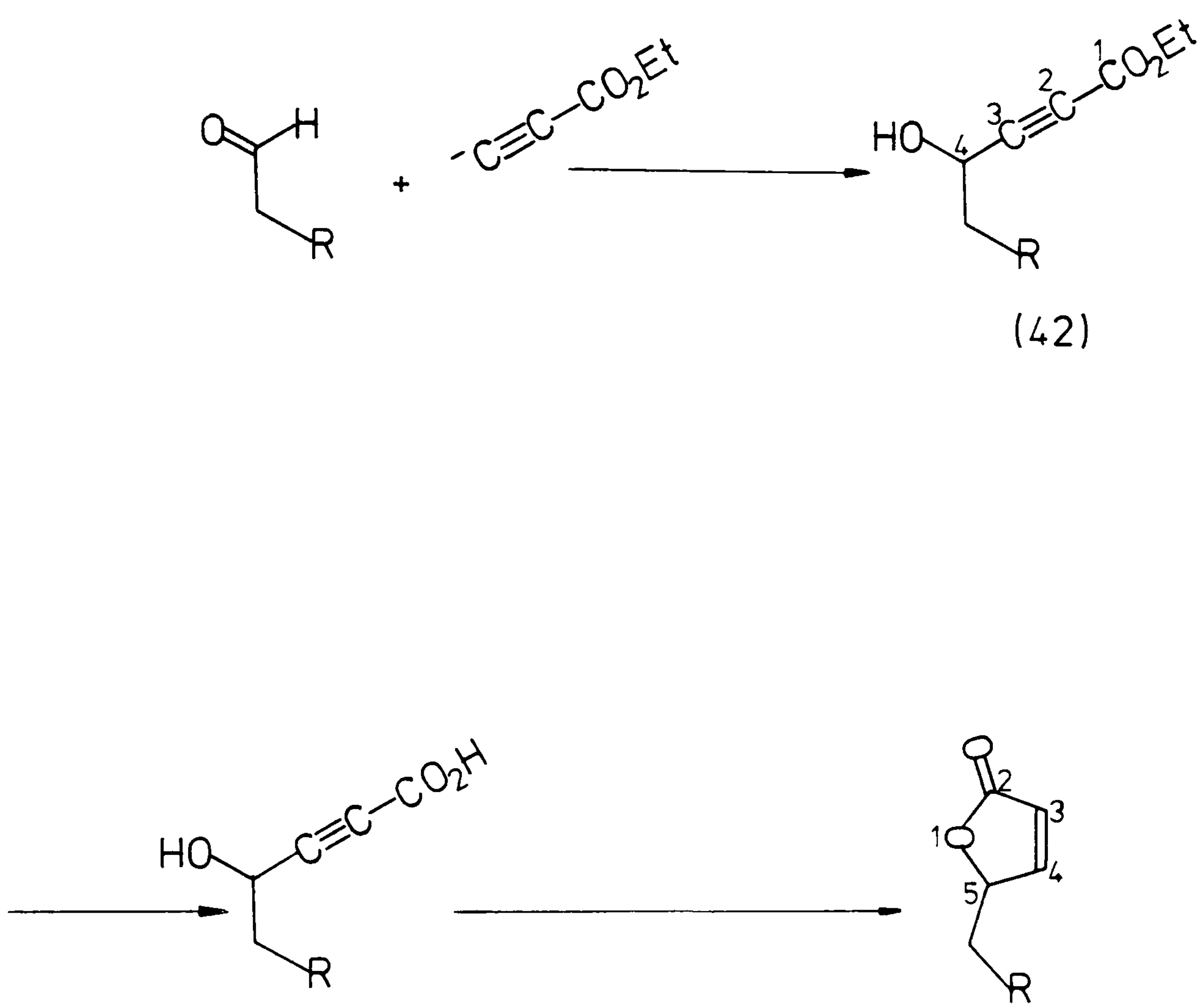



Fig. 11

Attempts to lactonise[69,70] either the keto ester (41a) or its hydrolysis product, the keto acid (41b) by prolonged heating with or without acid catalysts such as polyphosphoric acid, trifluoroacetic acid, trifluoroacetic anhydride or *p*-toluenesulphonic acid in benzene or xylene were all unsuccessful and the route was not investigated further.

Another synthetic route (Scheme 15) to the butenolides (37) was



(42)

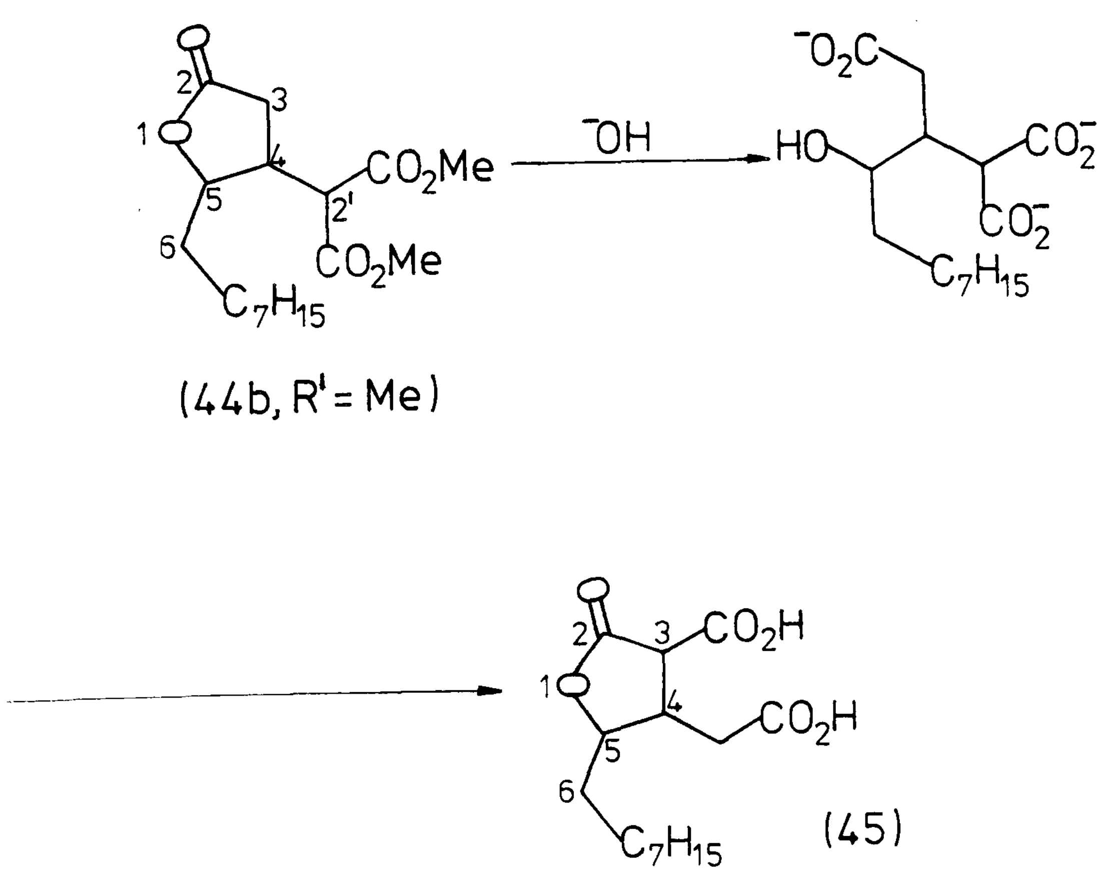
(43) (37)

a. R = Me
b. R = $C_7H_{15}$

Scheme 15

based on that of Schlessinger *et al.*[71] who had successfully prepared the desired butenolide (37b) by the reaction of the lithium salt of ethyl propiolate with *n*-nonanal at -70°C to give the hydroxy ester (42b). Hydrolysis to the corresponding acid (43b), followed by a reduction/lactonisation step using 5% palladium/$BaSO_4$ and quinoline afforded the butenolide (37b) in good yield. Similarly, propanal afforded the other butenolide (37a), but in rather lower yield.

Having successfully prepared the two butenolides (37), the next stage of the synthesis (Scheme 13), the Michael addition, was attempted. Treatment of the butenolide (37b) with dimethyl sodiomalonate in THF gave the dimethyl ester lactone (44b, R' = Me) in 41% yield. It was realised that treatment of the dimethyl ester (44b, R' = Me) with aqueous KOH was not only likely to hydrolyse the two ester groups to carboxylic acids, but could also open up the lactone ring, thus leading to a rearranged diacid lactone (45) on acidification (Fig. 12).



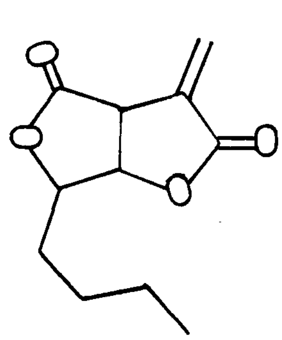

(44b, R' = Me)

(45)

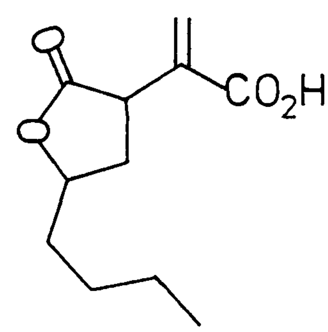
Fig. 12

However it was hoped that nevertheless a workable yield of the unrearranged lactone (46b) might be obtained. In the event a crystalline product was obtained which appeared to be a single compound from its $^{1}H$ and $^{13}C$ n.m.r. spectra.

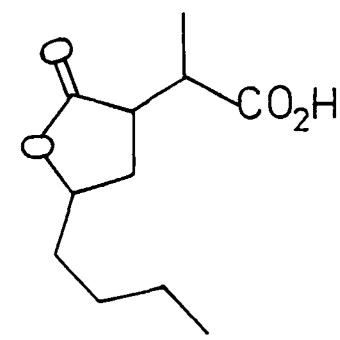
Unfortunately, the desired diacid lactone (46b) would almost certainly exhibit very similar spectroscopic properties to the isomer (45) and structural assignment of the product of basic hydrolysis was not possible at this stage. However, it was thought that at least a tentative assignment of the diacid lactone structure could be made by conversion to the terminal methylene compound and comparing its i.r. spectrum with those of three model compounds, canadensolide (3), canadensic acid (22) and dihydrocanadensic acid (47) (Fig. 13). The i.r. spectrum of the methylenation product exhibited two strong bands at 1770 and 1720 $cm^{-1}$ due to the carbonyl stretch of the lactone and the carboxylic acid function respectively and also a weak band at 1630 $cm^{-1}$ assigned to the olefinic stretching mode. The lactone carbonyl stretch in the three model compounds appears at 1770 $cm^{-1}$, but the exo-methylene stretch at 1630 $cm^{-1}$ in the spectrum of canadensolide (3) is weak, whereas in canadensic acid the olefinic stretch at 1630 $cm^{-1}$ is strong. This suggests that the exo-methylene function is fused to the lactone ring *i.e.* as in structure (48) rather than structure (31b). This structural assignment is further supported by the position of the acid carbonyl stretching frequency. In the spectrum of canadensic acid (22), the carboxylic acid is $\alpha$ to the methylene group and the band appears at 1700 $cm^{-1}$, whereas in dihydrocanadensic acid (47) there is no methylene group $\alpha$ to the carboxylic acid and the band in the i.r. spectrum appears at 1720 $cm^{-1}$ as it does in the methylenation product. This points to the structure of the methylenation product being (48) rather than the desired product (31b) and, therefore, the structure of the precursor diacid lactone



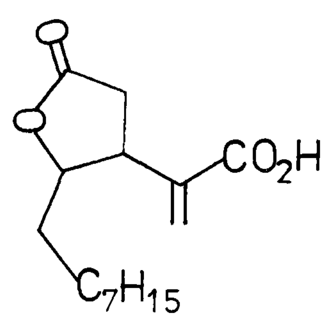
(3)



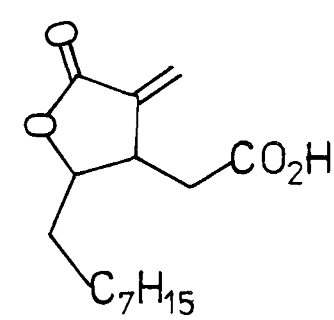
(22)



(47)



(31b)



(48)

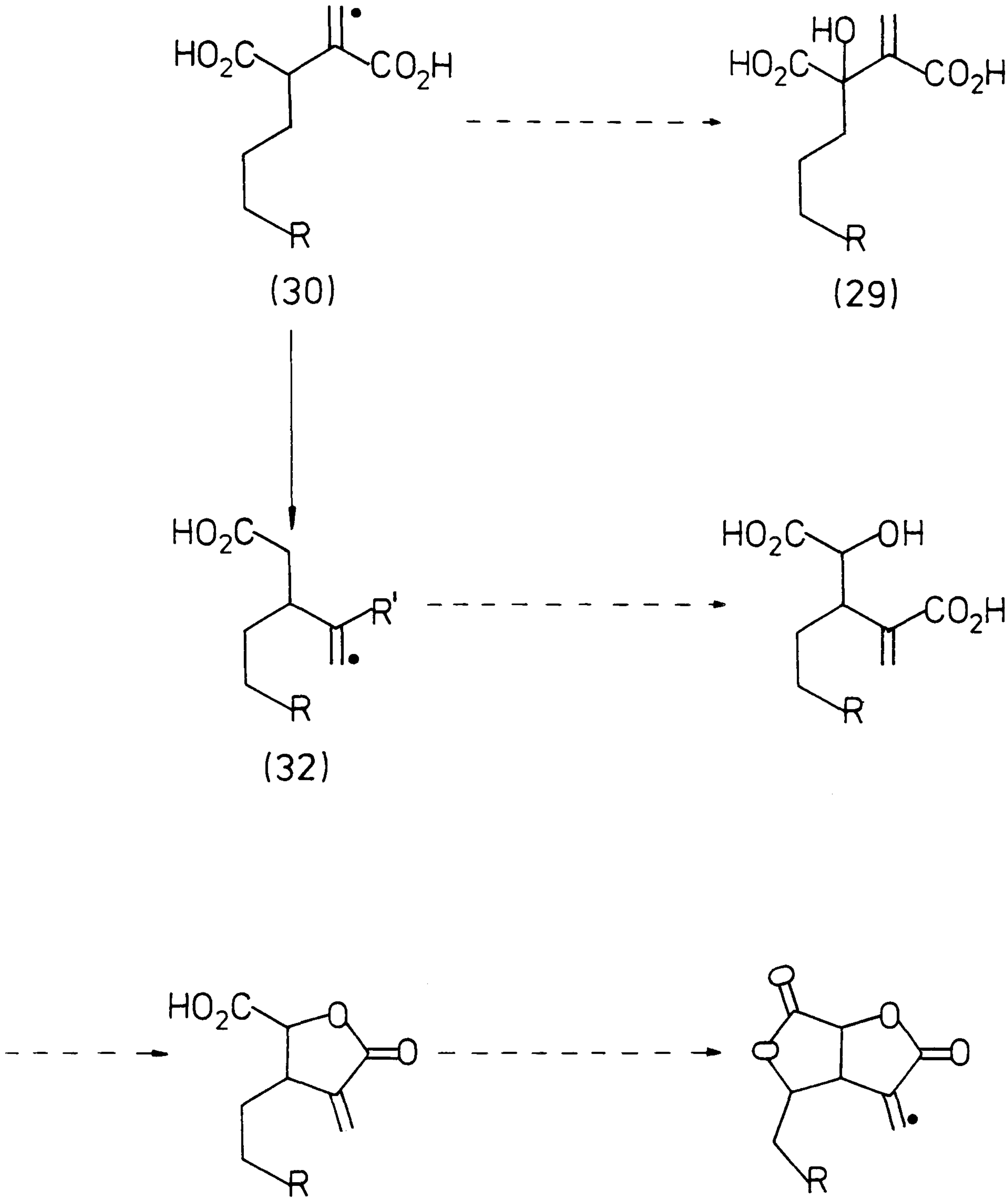
Fig. 13

can be assigned as (45). These findings were proved correct by the subsequent successful synthesis of the diacid lactone (46b) and the exo-methylene compound (31b) as described below.

In order to avoid hydrolysis of the lactone ring and its subsequent rearrangement, dibenzyl sodiomalonate was substituted for dimethyl sodiomalonate in the Michael addition step (Scheme 13) giving the corresponding dibenzyl ester lactones (44, R' = Bzl) in 27% yield. Hydrogenolysis over palladium/charcoal afforded the diacid lactones (46) in good yield, but the normal methylenation procedure using diethylamine and aqueous formaldehyde[61] proved largely ineffective, giving either a low yield of the desired product in one case (46a) or essentially unreacted diacid lactone (46b) in the other. An alternative decarboxylative methylenation procedure, developed by Johnson,[72] involved treating the carboxylactone with a mixture of diethylamine, formaldehyde, sodium acetate and acetic acid. Johnson also mentioned that this acidic reagent was sometimes necessary where the normal methylenation procedure had failed. In this case, treatment of the diacid lactones (46) with the reagent afforded the desired lactone acids (31) in over 70% yield. By using [$^{14}$C]-formaldehyde, the two $\beta$-(1-hydroxyalkyl)-$\alpha$-[$^{14}$C-methylene]-glutaric acid lactones (31) were likewise obtained for incorporation studies.

$\beta$-(1-Hydroxy-*n*-propyl)-$\alpha$-[$^{14}$C-methylene]-glutaric acid lactone (31a) was fed in three 24 h pulses to cultures of *P. decumbens* and the ethisolide (1) recovered was found to contain less than 0.03% of the administered radioactivity. Moreover, $\beta$-(1-hydroxy-*n*-nonyl)-$\alpha$-[$^{14}$C-methylene]-glutaric acid lactone (31b) was fed in five 12 h pulses to cultures of *A. avenaceus* giving avenaciolide (2) which contained less than 0.3% of the administered radioactivity. This indicated that the lactone acids (31) were, at best, only poor precursors of the corresponding bislactones and this increased the likelihood that in each case the ethisic acid type of monolactone (27) is the correct precursor. The original proposed biosynthetic pathway (Scheme 10) could now be modified to take account of the preceding results (Scheme 16). After rearrangement of the alkylitaconic acid (30) to the corresponding α-methylene-β-*n*-alkylglutaric acid (32) hydroxylation and lactonisation occur at C-2 to produce the ethisic acid type of monolactone (27) which would then hydroxylate and lactonise to form the corresponding bislactones (1) and (2).

Although the evidence for this biosynthetic pathway is now substantial, what was required for final confirmation was the efficient incorporation of the ethisic acid type monolactones (27) in order to prove their role as intermediates.




(27)

(1) R = Me
(2) R = $C_7H_{15}$

R = Me, $C_7H_{15}$
R' = $CO_2H$, COSCoA

• : $^{14}$C labelled carbon atom

Scheme 16

# CHAPTER 3

## Discussion

## 2. Attempted Syntheses of Ethisic Acid

(i) Previous Biosynthetic and Synthetic Attempts

(ii) Attempted Syntheses *via* Halo-esters

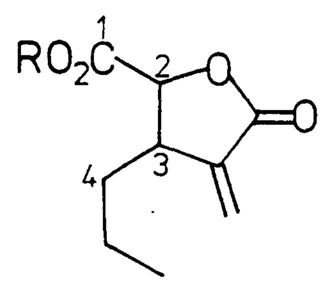
(iii) Attempted Syntheses *via* Hydroxy-esters

(iv) *Via* 2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde

## 2. Attempted Syntheses of Ethisic Acid (27a)

### (i) Previous Biosynthetic and Synthetic Attempts

Concurrent with the synthesis of the lactone acids (31), a number of attempts were made to synthesise one of the C-2 lactonised potential intermediates for incorporation studies, namely ethisic acid (27a). This had been isolated as one of a number of minor

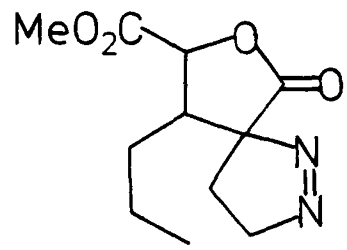


(27a) R = H
(49) R = Me

metabolites of *P. decumbens* and therefore seemed more likely to be the immediate precursor to ethisolide (1).

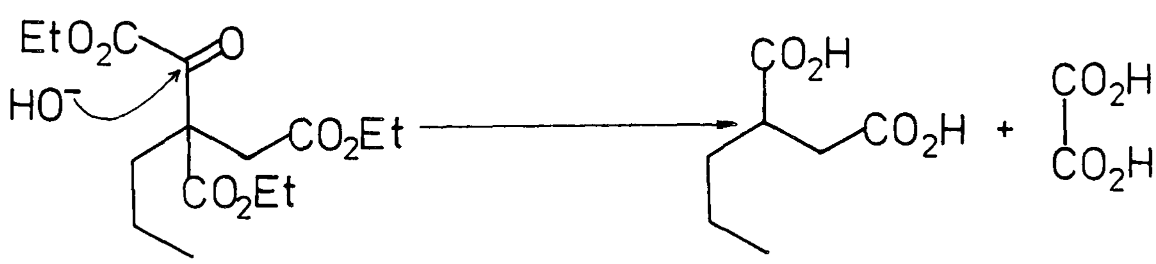
Initially, attempts were made to obtain a sample of ethisic acid (27a) biosynthetically labelled for incorporation studies by either treatment of the broth extract from *P. decumbens* with diazomethane[73] or similar treatment of the dried mycelium.[74] In both cases a small quantity of methyl ethisate (49) was isolated along with the pyrazoline (50). Later attempts to isolate the free acid (27a) resulted in very little or no ethisic acid (27a) and the isolation of a new minor metabolite, decumbic acid (28).[75]

As all attempts to obtain biosynthetically labelled ethisic acid (27a) had failed, Johnston tried to obtain a sample by synthesis.[76] His first attempt, based on a synthesis of avenaciolide (2) by Schlessinger,[77] involved trapping of the ester enolate produced by Michael addition of *tert.* butyl-α-lithio-α-thiomethylpropionate (51)



(50)

to ethyl *trans* hex-2-enoate by iodine to give the iodo-ester (52) which could be lactonised and converted to ethisic acid (27a) (Scheme 17). Unfortunately, the Michael addition reaction gave only starting material. He also attempted to form the lactone skeleton (53) by an alternative route (Scheme 18). However, although the keto-triester (54) was obtained, basic decarboxylation conditions gave only *n*-propyl succinic acid (55) (Fig. 14) and attempts at

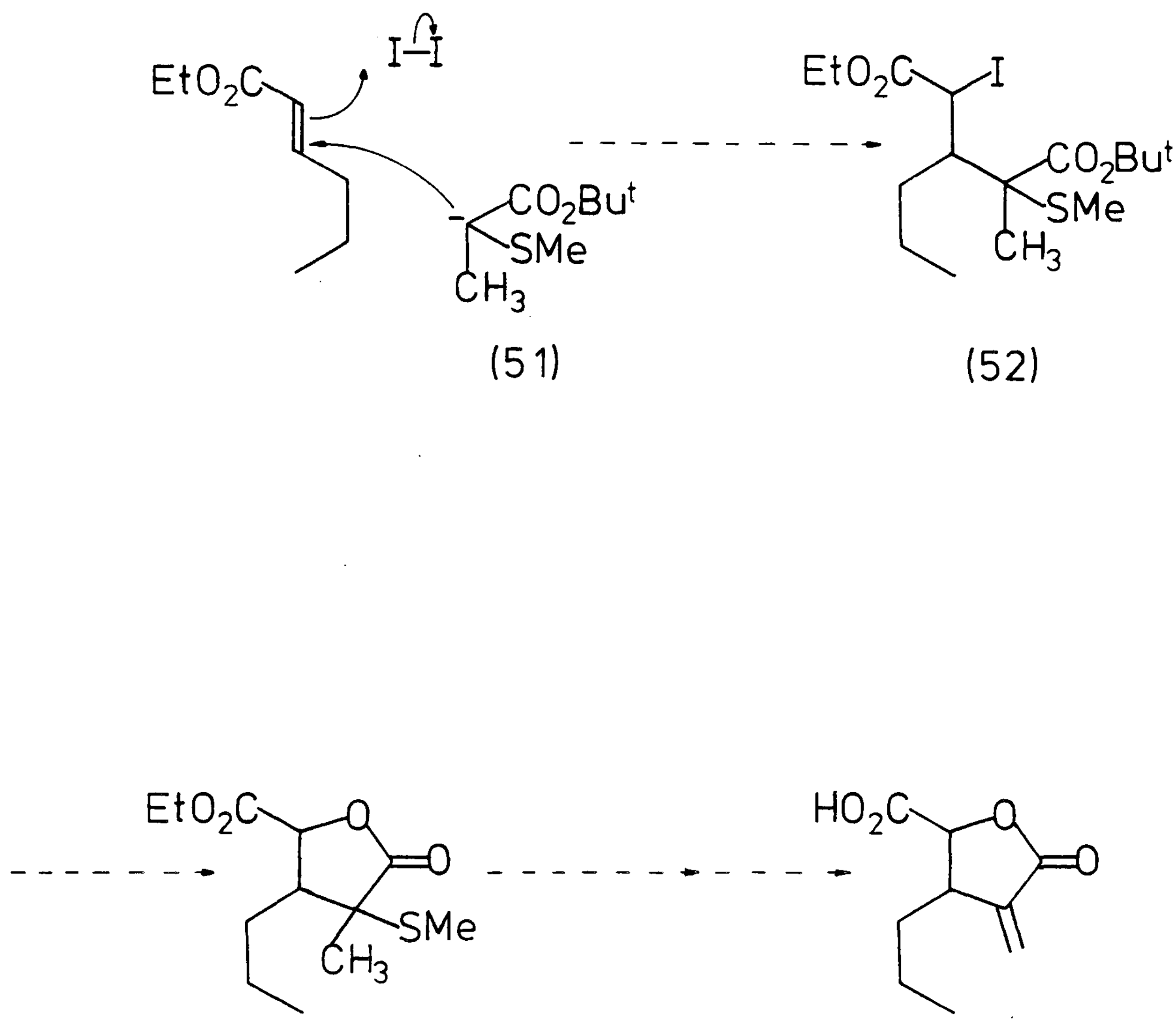


(54) (55)

Fig. 14

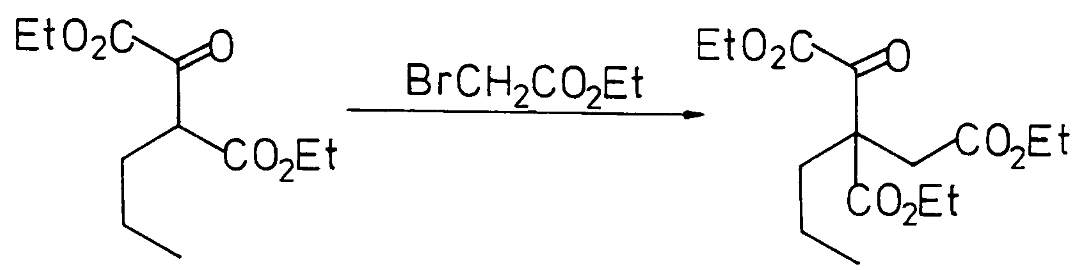
acidic decarboxylation gave only a small amount of the lactone ester (56) after treatment with diazomethane.

In this present work, it was hoped that a fresh approach to this elusive lactone (27a) would meet with more success.



(51) (52) (27a)

Scheme 17

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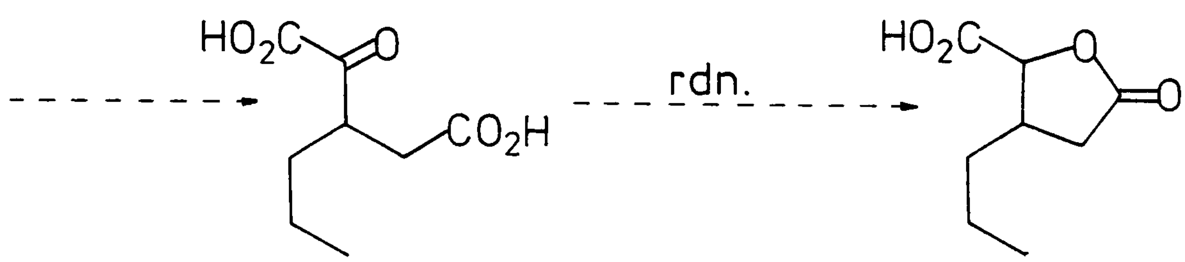


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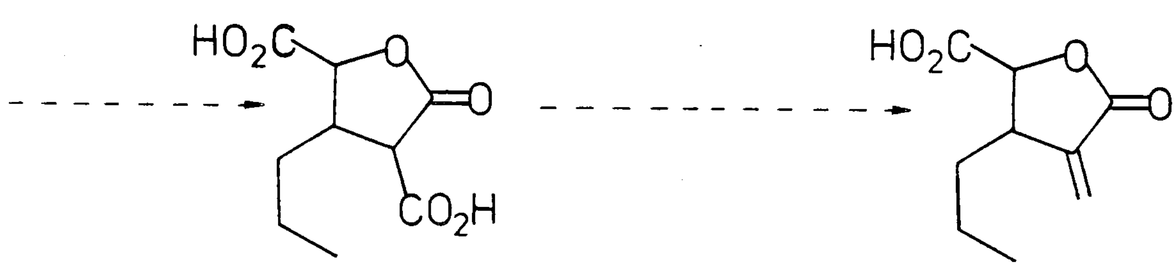
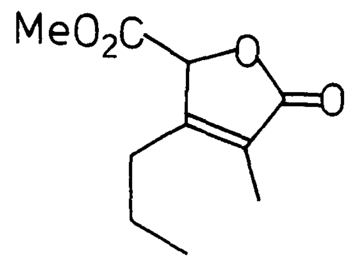



(27a)

<u>Scheme 18</u>



(56)

(ii) Attempted Syntheses *via* Halo-esters (57)

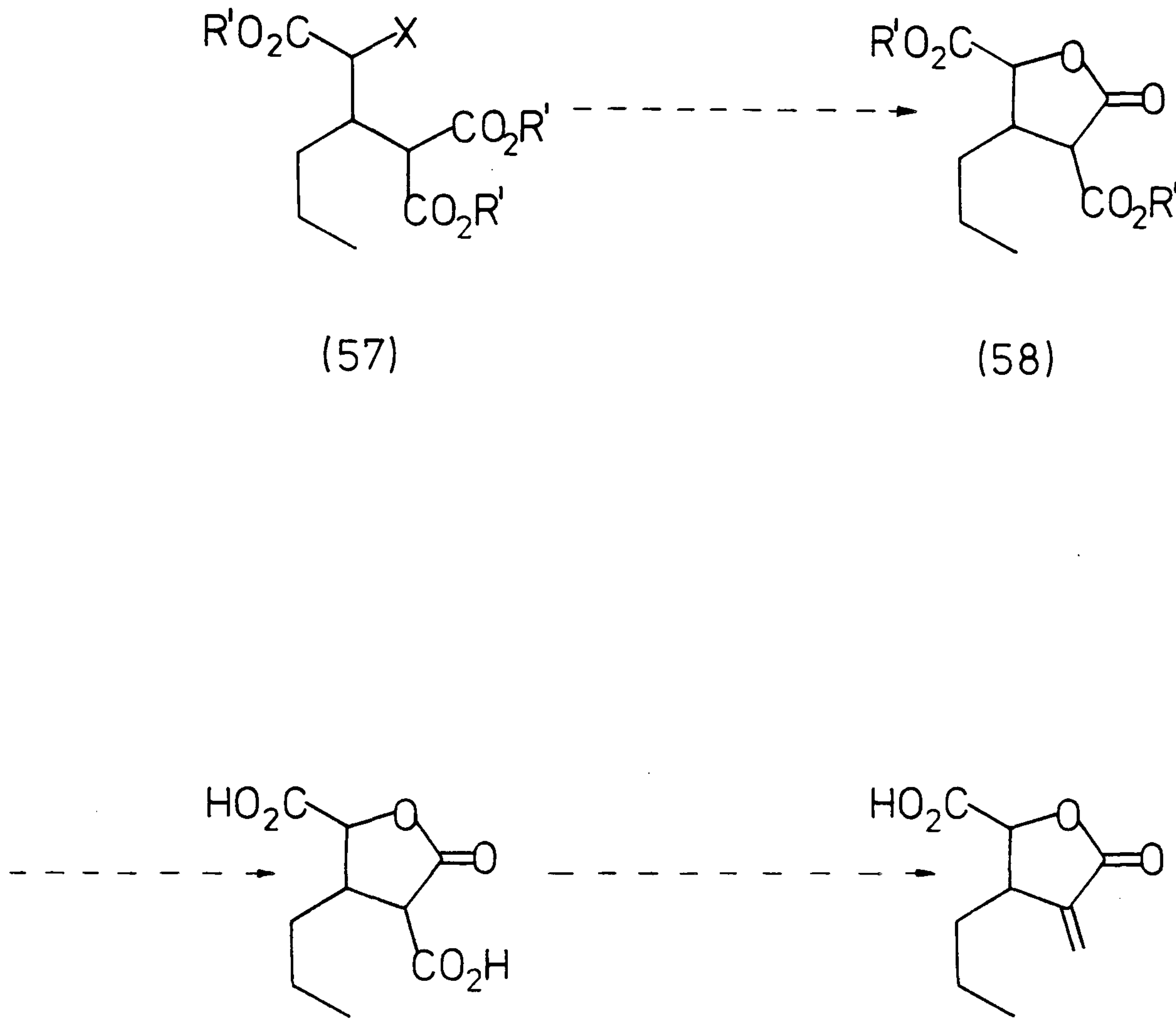
The first synthetic route tried is shown in Scheme 19. Lactonisation of the halogenated triester (57) would yield the diester lactone (58), the terminal methylene function would then be introduced by hydrolysis to the diacid lactone (59) followed by methylenation to give ethisic acid (27a). The first target therefore, was to synthesise the halogenated triester (57).

Ethyl *trans* hex-2-enoate was brominated in methylene chloride to give ethyl 2,3-dibromohexanoate (60) in high yield. It was expected that the β-bromo substituent would be more reactive to nucleophilic substitution than that α to the ester function and it was also hoped that even if elimination of the β-bromo atom occurred (Fig. 15), Michael addition of dimethyl sodiomalonate might still give the desired bromo triester (57). However, only unreacted material or

(60) (61)

Fig. 15

the elimination product (61) was obtained. An alternative method, using methyl 2-carbomethoxyhex-2-enoate (62) and ethyl chloroacetate was also unsuccessful (Fig. 16), work up affording an inseparable mixture of mostly unidentifiable components.

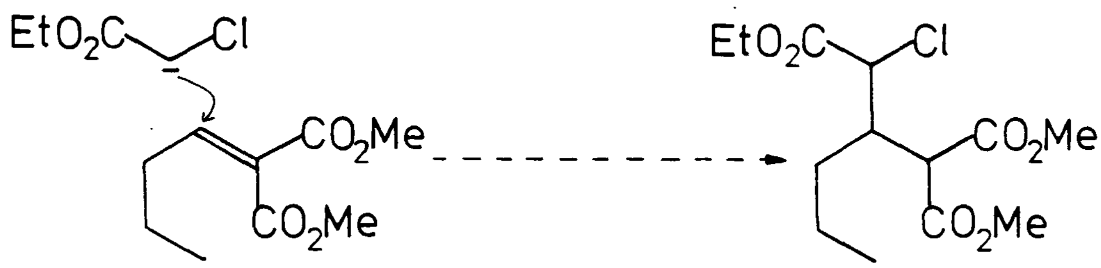


(57) (58)

(59) (27a)

R' = Me, Et.     X = Halogen

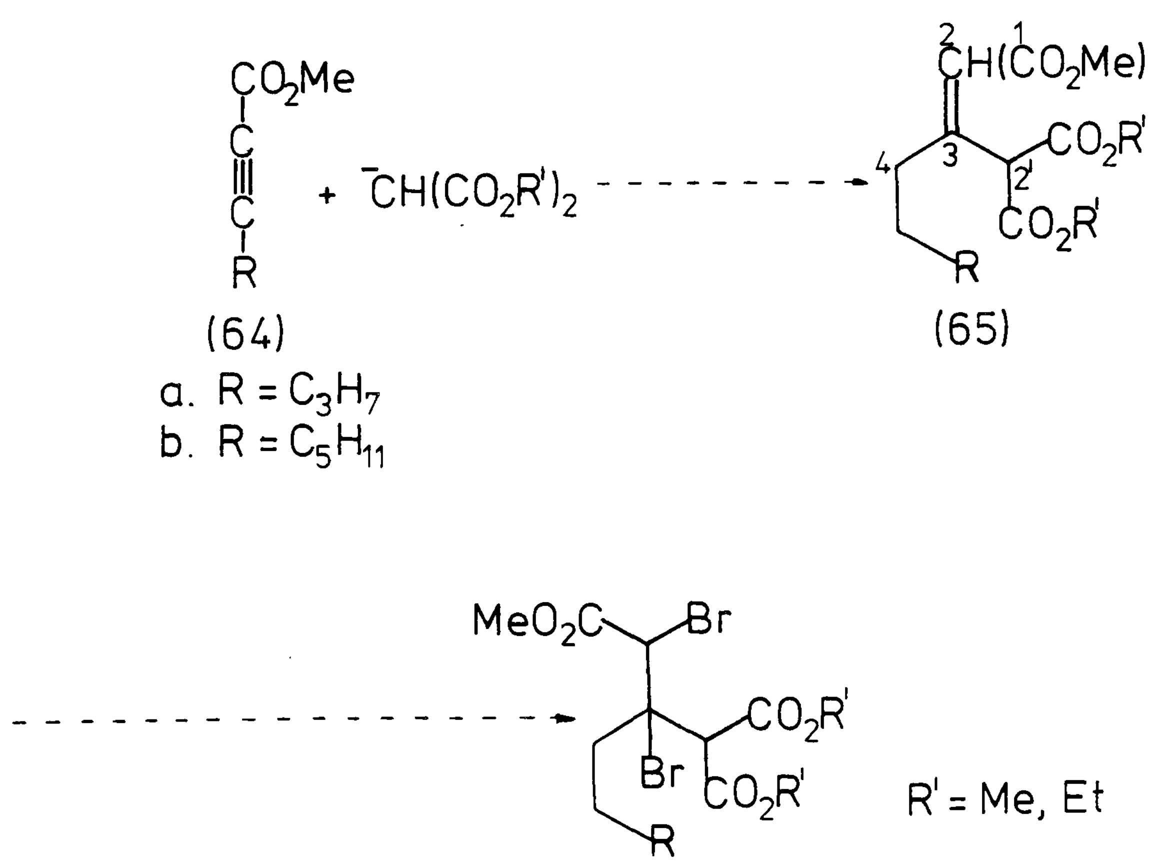
Scheme 19



(62) (57)

Fig. 16

In order to circumvent this problem, an attempt to produce the alternative halogenated triester (63) *via* the Michael addition of dimethyl sodiomalonate to the alkyne ester (64), followed by bromination was carried out (Fig. 17). A sample of methyl 2-octynoate



(64)

a. $R = C_3H_7$

b. $R = C_5H_{11}$

(65)

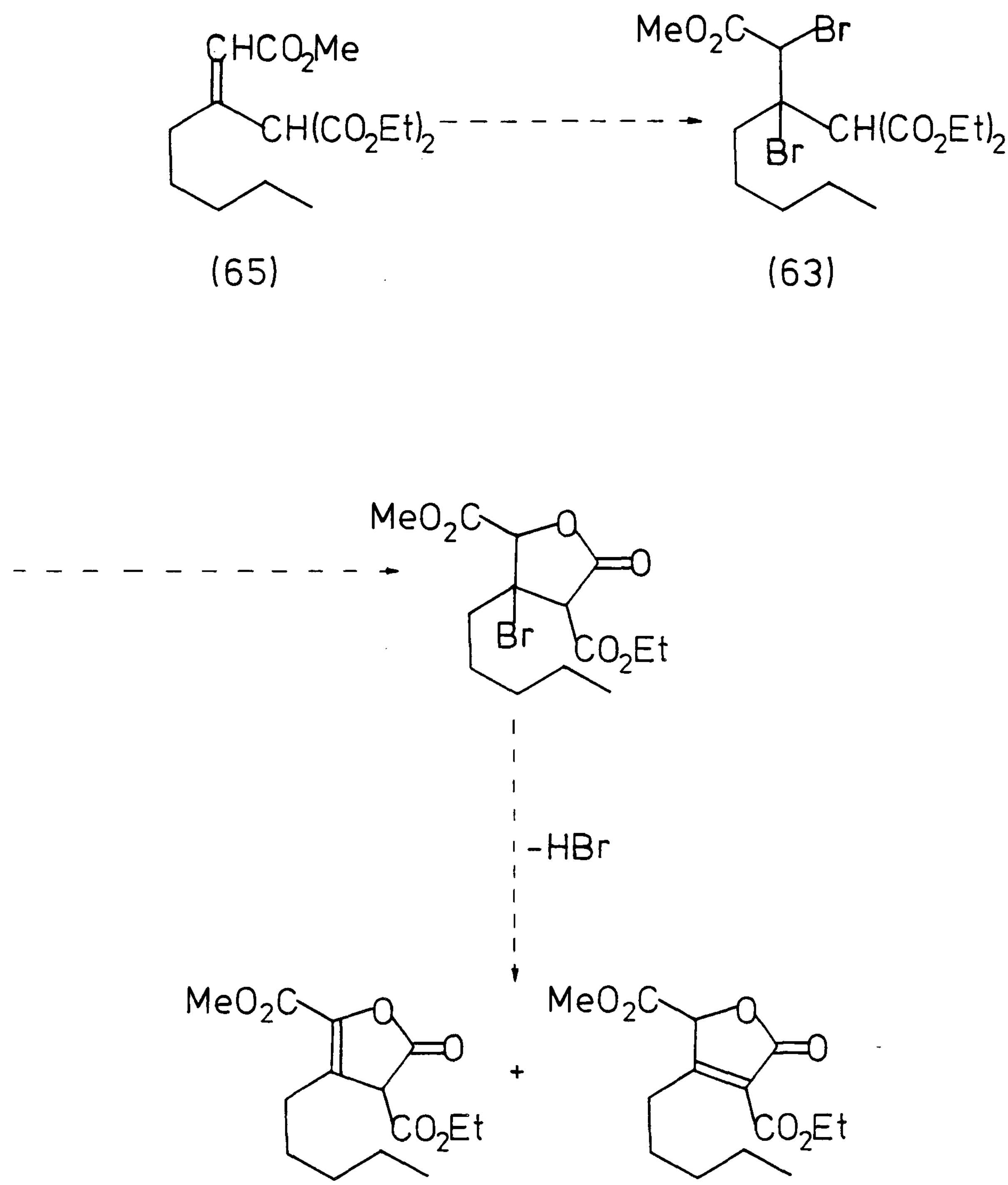
(63)

$R' = Me, Et$

Fig. 17

(64b) was available and this was used as a model to react with diethyl and dimethyl sodiomalonate. In both cases, the product obtained did not appear to correspond to the desired unsaturated triester (65), the expected doublet at *ca.* 3.6$\delta$ in the n.m.r. spectrum for H-2' being absent. Preparative t.l.c. failed to separate the components present and their structures remained to be determined at the time. The route was not abandoned immediately however. Bromination of the diethyl malonate derived product in acetic acid, followed by heating at 120°C, led to an oil which exhibited bands in its i.r. spectrum at 1800, 1780, 1740 and 1640 cm$^{-1}$. This tended to suggest that part of the bromination product consisted of $\beta,\gamma$ and $\alpha,\beta$ unsaturated lactone esters (66) formed by a bromination/lactonisation/dehydrobromination sequence (Scheme 20). Unfortunately, the bromination products proved to be inseparable and the route was not investigated further. However, the identity of the original Michael addition product(s) was at least partially resolved in the following way.

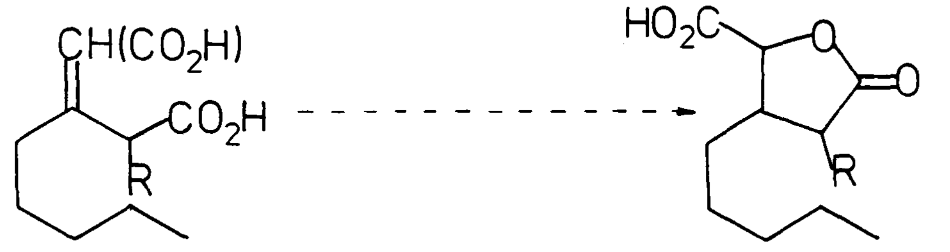
Hydrolysis of the dimethyl malonate derived product gave a solid which was almost certainly a mixture in view of its wide m.p. (80-100°C) and spectroscopic properties, but if any unsaturated triacid (67) or its decarboxylation product, the diacid (68) were present then it was thought that lactonisation using a strong acid *e.g.* $H_2SO_4$ should be possible.[78,79] (Fig. 18). Treatment with conc. $H_2SO_4$ led, however, to the isolation of an unsaturated diacid (69) and not the desired carboxylactone. Returning to the identification of the original Michael addition product(s), the presence of the isomeric unsaturated triester and/or diester (70) would at least account for the observed absence of the characteristic H-2' doublet in its n.m.r. spectrum.




(65) (63)

(66)

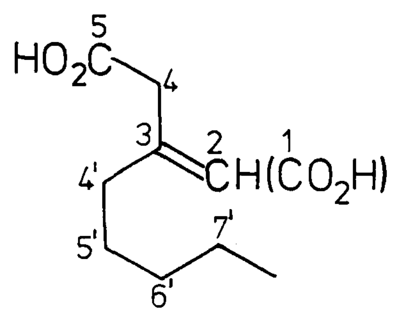
Scheme 20



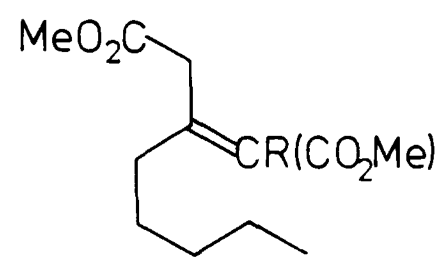
(67) R = $CO_2H$
(68) R = H

R = H, $CO_2H$

Fig. 18



(69)



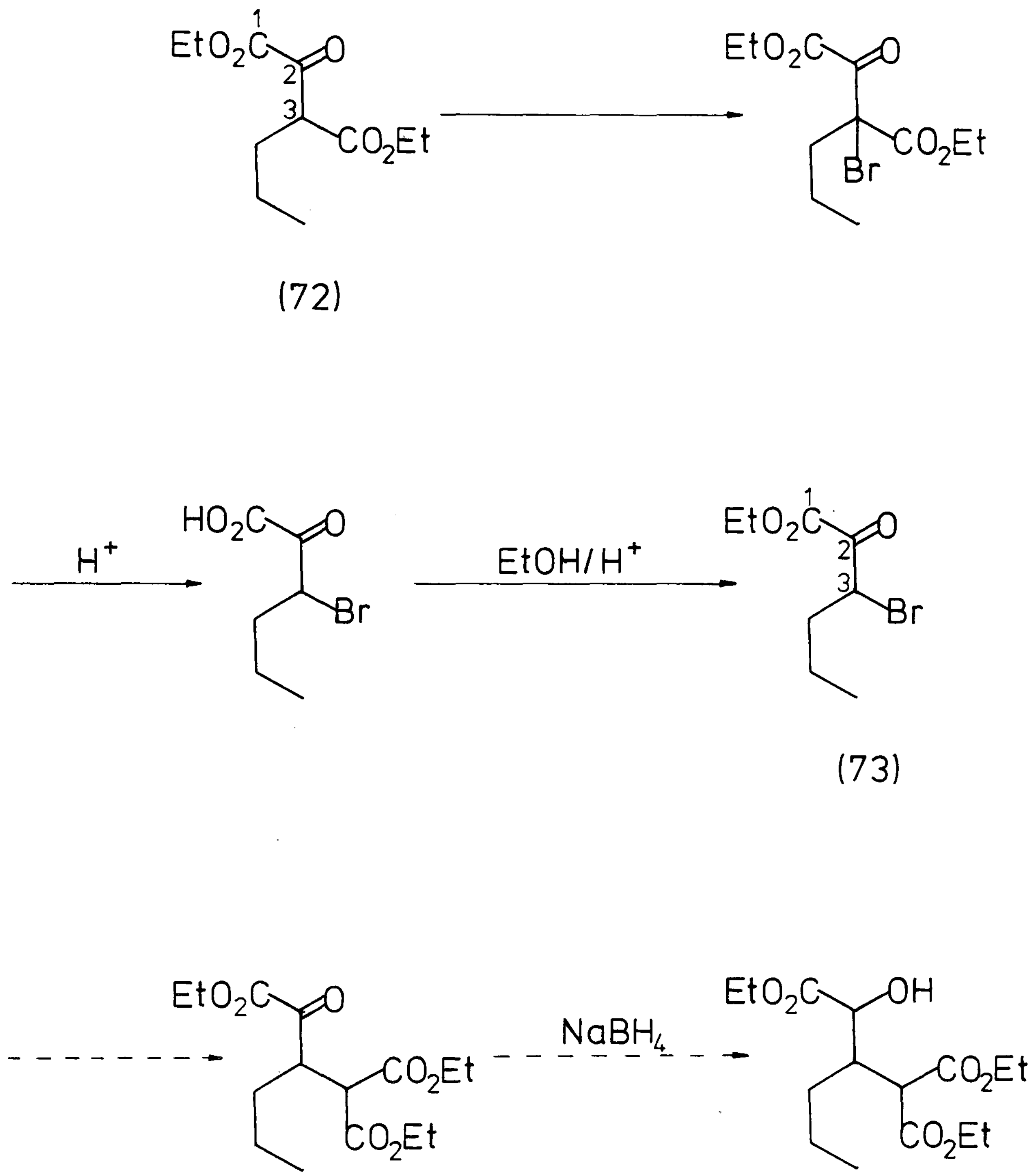
(70) R = $CO_2Me$, H

(iii) Attempted Syntheses *via* Hydroxy-esters (57, X = OH)

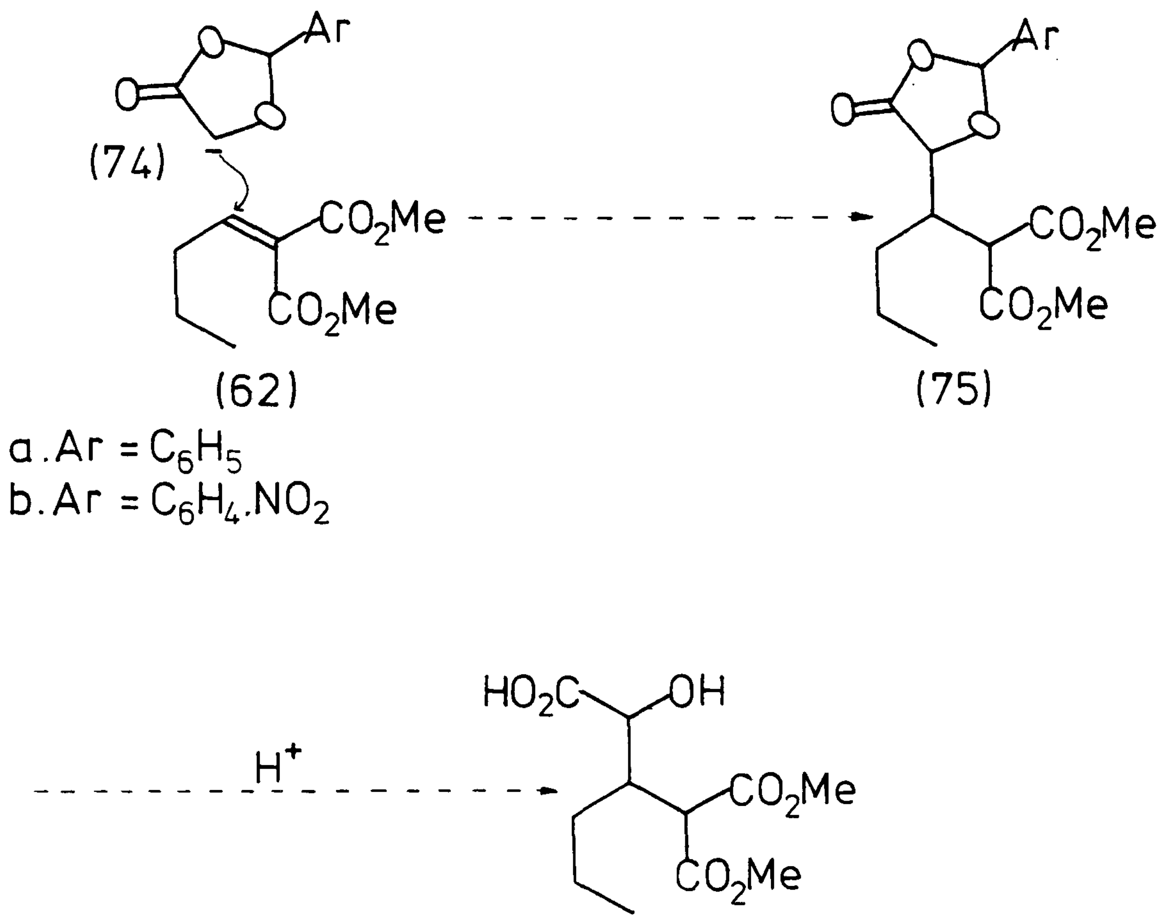
As the syntheses of the halogenated triesters (57, 63) were unsuccessful, it was decided to modify the route by attempting to prepare the corresponding hydroxylated compound (57, X = OH) which should be capable of lactonisation, *etc.* as previously envisaged in Scheme 19. The synthetic route tried is shown in Scheme 21 and bears some similarities to Johnston's unsuccessful route *via* the keto-ester (54) (Scheme 18) which failed because of the need to carry out a decarboxylative hydrolysis on the keto-ester (54) prior to reduction and lactonisation. The new route (Scheme 21) avoids this by the synthesis of an alternative keto-ester (71) which need only be reduced prior to lactonisation.

Ethyl 3-carboethoxy-2-oxohexanoate (72) was successfully converted to the desired ethyl 3-bromo-2-oxohexanoate (73) by bromination, hydrolysis and decarboxylation followed by esterification. However, treatment of the bromo keto-ester (73) with diethyl sodiomalonate gave a complex mixture of esters (t.l.c., n.m.r. - multiplicity of $-OCH_2-$ peaks *ca.* 4.3$\delta$), none of which corresponded to the desired keto-triester (71) [n.m.r. - absence of characteristic $-\underline{C}\underline{H}(CO_2Et)_2$ doublet *ca.* 3.5$\delta$] and the reaction was not investigated further.

An ambitious new route to the hydroxy-triester (57, X = OH), in which all of the functionality is introduced in one step was attempted (Fig. 19). This involved addition of the anion of a 2-aryl-1,3-dioxolan-4-one (74) to methyl 2-carbomethoxyhex-2-enoate (62) to form the dioxolanone diester (75).




Scheme 21




(57, X = OH)

Fig. 19

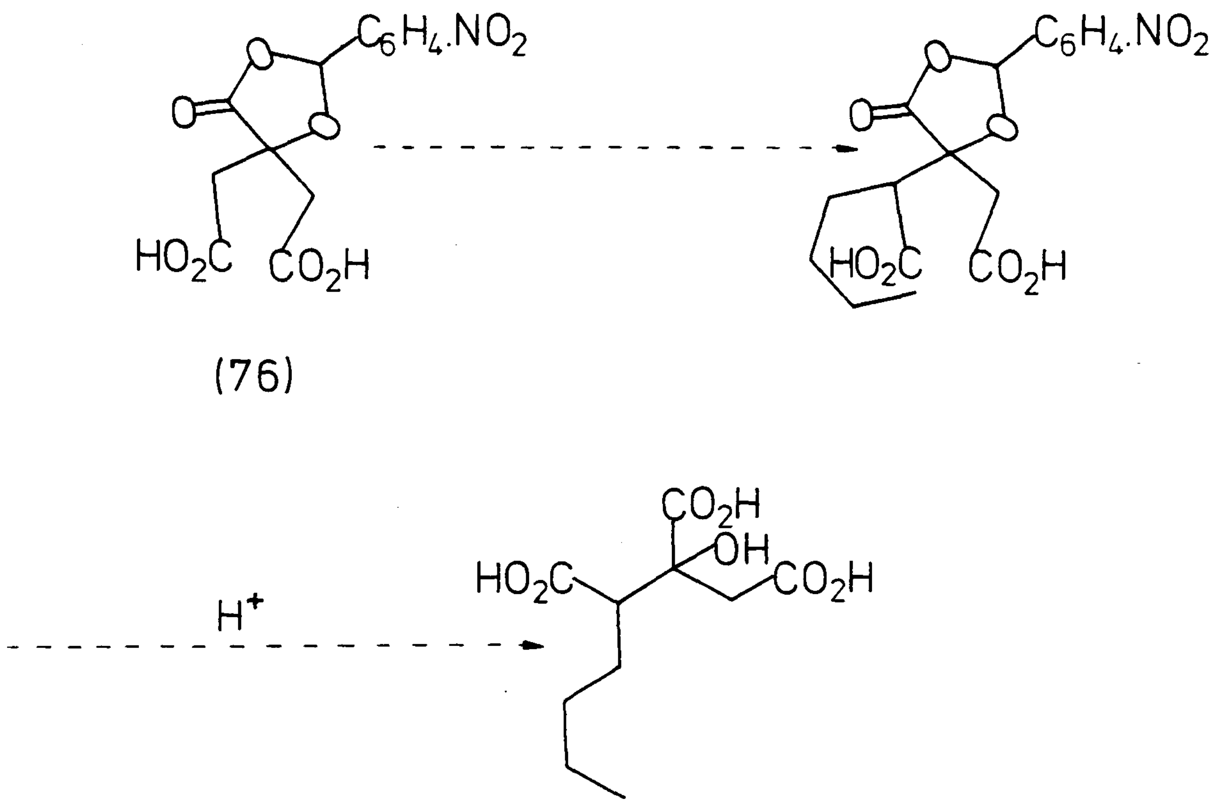
The desired hydroxy-ester species (57, X = OH) would then be liberated by treatment of the dioxolanone (75) with dilute acid.

Attempted preparation of the parent 2-phenyl-1,3-dioxolan-4-one (74a) from glycollic acid and benzaldehyde by the aid of azeotropic distillation[80] was unsuccessful. Similarly, attempts by other workers[81] to prepare alternative, stable 2-subst.-1,3-dioxolan-4-ones have also failed. However, it was thought likely that the stability of the dioxolanone ring system could be increased by the substitution of a more powerful electron withdrawing group than a phenyl group alone. As predicted, 2-*p*-nitrophenyl-1,3-dioxolan-4-one (74b) was successfully prepared as a stable, crystalline solid from glycollic acid and *p*-nitrobenzaldehyde.

Unfortunately, treatment of this 1,3-dioxolan-4-one (74b) with aprotic base (LDA) produced a greenish black oil which, after stirring with the unsaturated diester (62) and standard work up, gave mainly tarry material lacking the characteristic i.r. absorption of 1,3-dioxolan-4-ones. Furthermore, an attempt to carboxylate the dioxolanone (74b) $\alpha$ to the carbonyl group in order to make the remaining proton more easily removed by base again led to tars and decomposition of the starting material. None of the desired product could be detected (i.r.) and the reaction was not investigated further.

A previous synthesis in these laboratories of alkylcitric acids[82] led to 9:1 mixtures of diastereoisomers in which the isomer considered most likely to be useful as a precursor for incorporation experiments with both the type A and type B lactones was in the smaller amount. An alternative synthesis was therefore desirable and the possibility of alkylating a protected form of citric acid was one route considered. As glycollic acid had successfully been converted to a stable 1,3-dioxolan-4-one by treatment with *p*-nitrobenzaldehyde it was hoped that citric acid would behave similarly and the resulting compound (76) could be alkylated by treatment with base (three equivalents) and *n*-butyl bromide to give *n*-butylcitric acid (25) (Fig. 20).

However, although the new citrate dioxolanone (76) was prepared, the alkylation was unsuccessful and this possible route to citric acids was not examined further.



(76)

(25)

Fig. 20

(iv) *Via* 2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde (77)

As all attempts to prepare the lactone skeleton had up until now been unsuccessful, a new synthetic route was proposed in which 2,3-*O*-isopropylidene-aldehydo-D-glyceraldehyde (77) would be converted to the diester (78) *via* the alcohol (79) by alkylation of the precursor (80). Cleavage of the acetonide and hydrolysis of the diester would then give the lactone (81) which could be converted to ethisic acid (27a) by oxidation to the diacid lactone (59) followed by methylenation (Scheme 22). A feature of this synthetic route is that the absolute configuration at C-2 in the ethisic acid (27a) produced could be selected by choosing the appropriate chiral glyceraldehyde (77). Both the R- and S - 2,3-*O*-isopropylidine-aldehydo-glyceraldehydes (77) were available by literature methods, the S-isomer could be conveniently prepared from vitamin C (ascorbic acid) by the method of Jung and Shaw[83] and the more widely used R-isomer was synthesised by oxidation of the protected D-mannitol, 1,2:5,6-di-*O*-isopropylidene-D-mannitol (82).[84] The absolute stereochemistry of natural ethisic acid (27a) is not known, but if it is an intermediate in the biosynthesis of ethisolide (1), then the isomer involved will be expected to have the same stereochemistry as ethisolide (1) *i.e.* (2R, 3R) (Fig. 21). In which case, the correct stereochemistry at

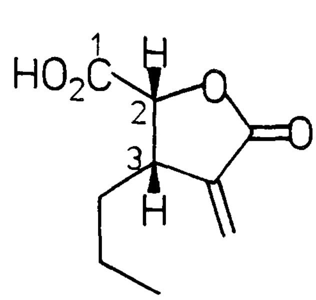
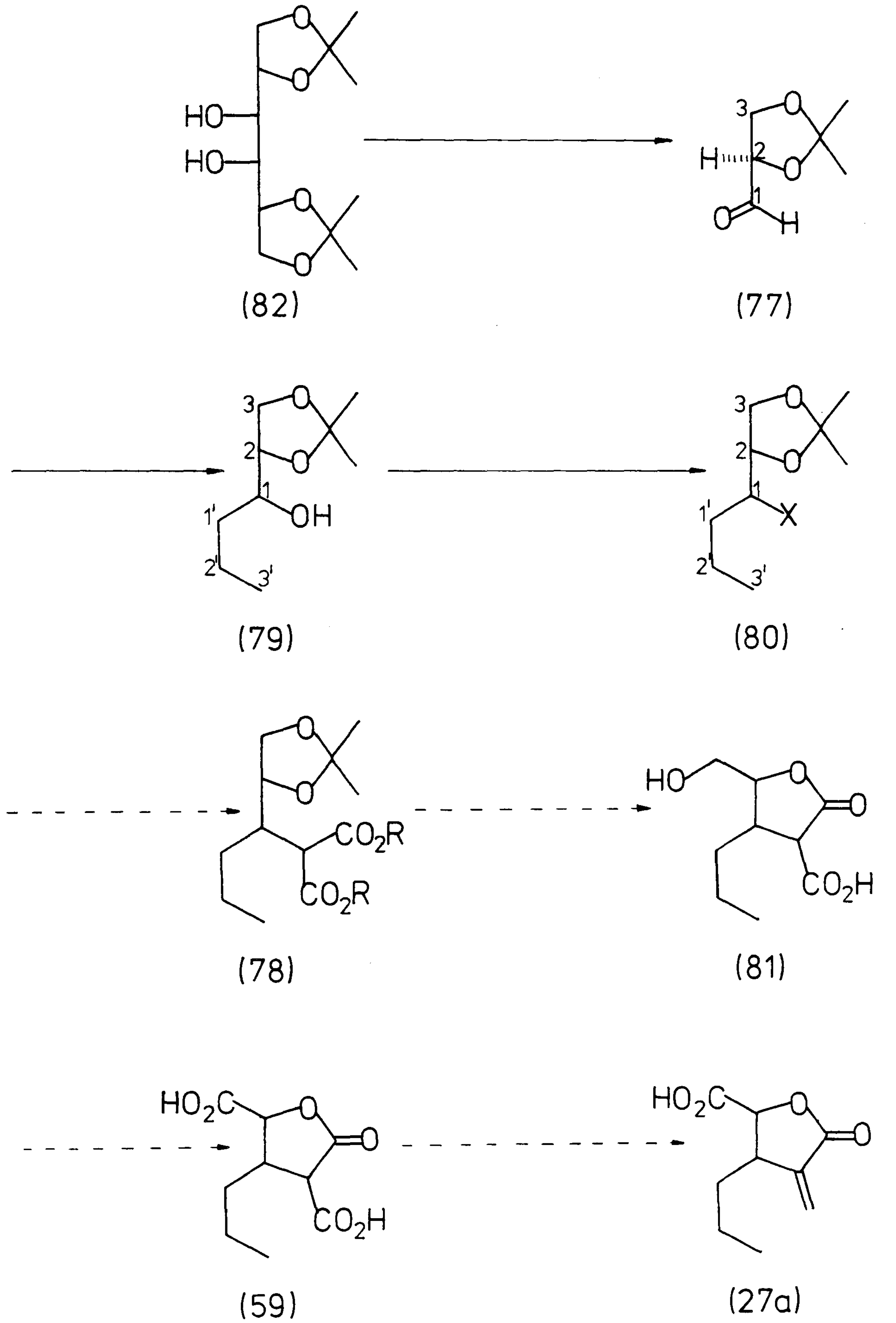


Fig. 21



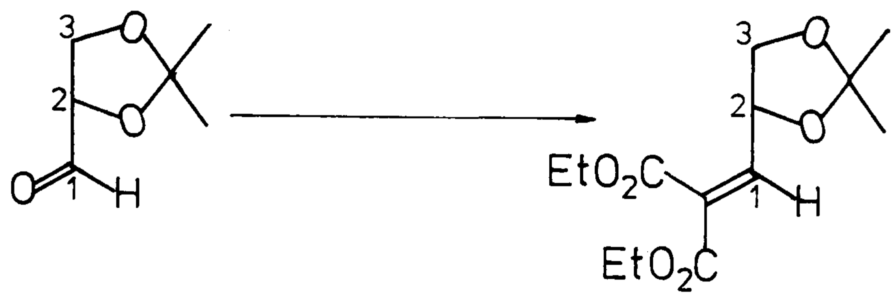

R = Me, Bzl., H

X = OMs, OTs, OBs, Br

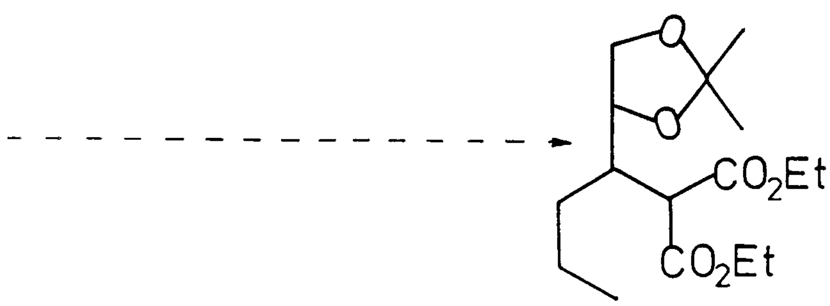
<u>Scheme 22</u>

C-2 would be achieved by using the S-glyceraldehyde (77). However, it should be borne in mind that the ethisic acid (27a) isolated may be the isomer not utilised by *P. decumbens* in which case the R-glyceraldehyde (77) would produce the correct C-2 stereochemistry. For incorporation studies therefore, it would be desirable to have synthetic samples of both the ethisic acid (27a) C-2 isomers. The R-glyceraldehyde (77) was easier to synthesise and so it was used to assess the feasibility of the proposed synthetic route (Scheme 22). The Grignard reaction had been investigated by a number of workers[85,86,87] and the desired alcohol (79) was obtained in 62% yield. This was efficiently converted to both the *p*-toluenesulphonate (80, X = OTs) and the *p*-bromobenzenesulphonate (80, X = OBs) by the normal procedure,[88] but treatment with dibenzyl sodiomalonate in THF or DMF gave only unreacted sulphonate (80). Likewise, a sample of the methanesulphonate (80, X = OMs), which was prepared in 96% yield by the method of Crossland *et al.*,[89] gave only unreacted material on treatment with dimethyl sodiomalonate in DMF or in benzene containing 18-crown-6. In order to get round this problem an alternative method to the diester (78, R = Et) was attempted (Fig. 22). It was hoped that the glyceraldehyde (77) would form the corresponding olefinic diester (82) on treatment with ethoxymagnesiummalonic ester[90] and distillation. This would then yield the sought after diester (78) by conjugate 1,4-addition of *n*-propylMgBr in a similar manner to Yoshikoshi's synthesis of canadensolide.[91] The n.m.r. spectrum revealed a doublet *ca.* 6.95$\delta$ assigned to H-1 and a quartet *ca.* 4.9$\delta$ assigned to H-2 - evidence that the unsaturated diester (82) had been formed. However, this constituted only *ca.* 28% (by integration) of a mixture of esters (multiplicity of signals at *ca.* 4.3$\delta$) which could not be separated and the reaction was not investigated further.

Returning to the alcohol (79), it was thought that conversion



(77) (82)



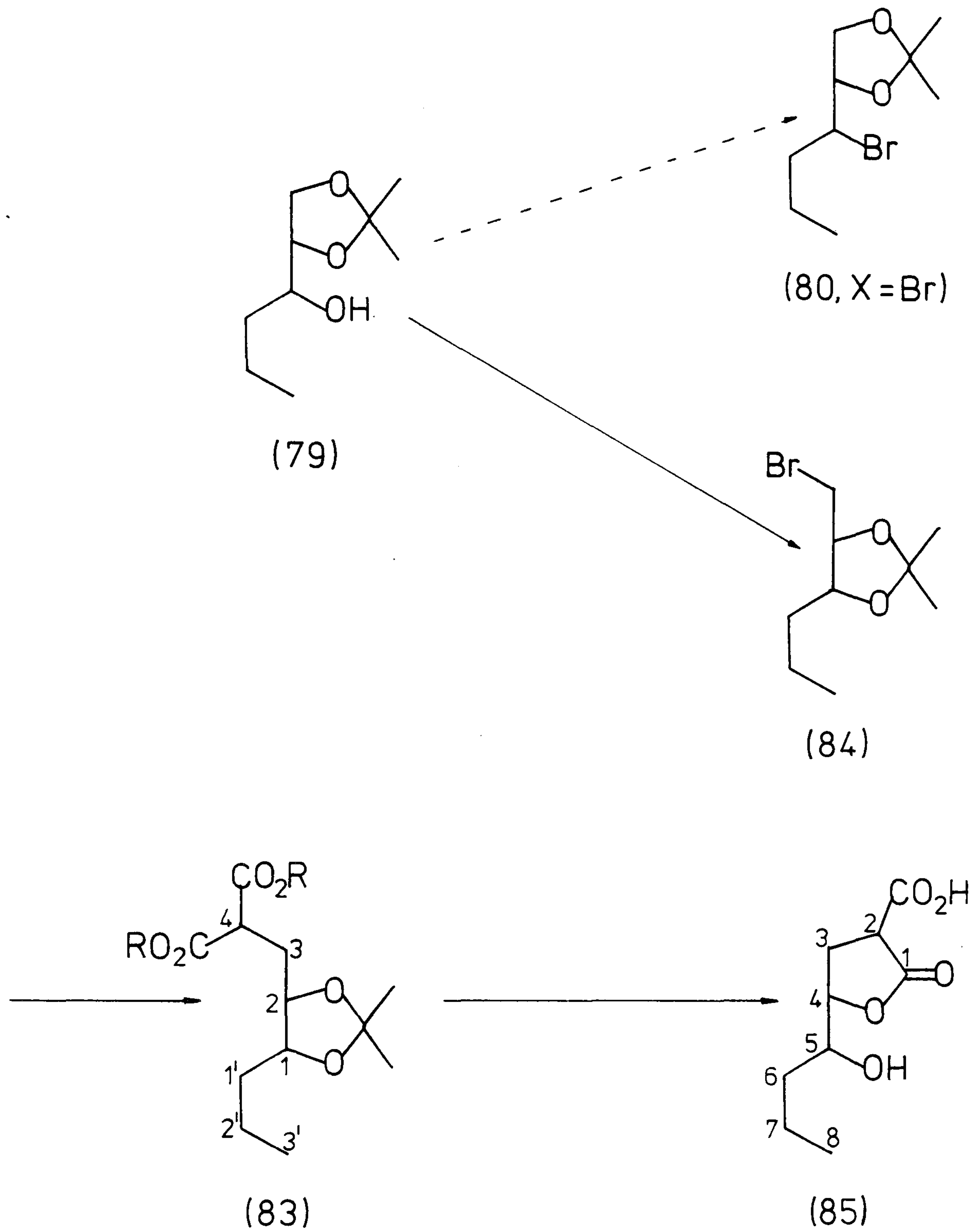
(78, R = Et)

Fig. 22

to the bromide (80, X = Br) would increase the reactivity towards alkylation and the diester (78) would thus be obtained.

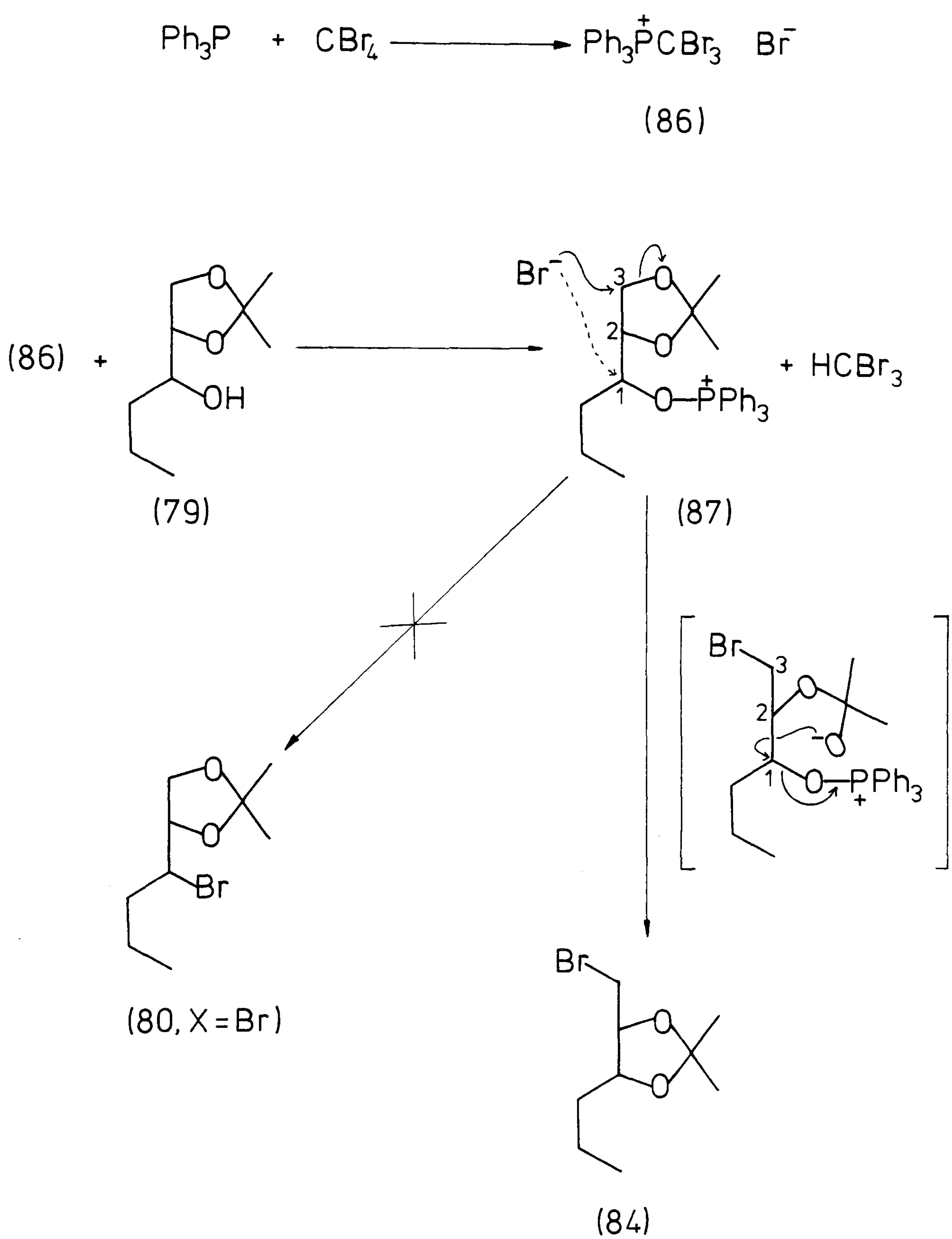
Treatment of the alcohol (79) with carbon tetrabromide and triphenylphosphine,[92,93] the reaction proceeding under neutral, anhydrous conditions - thus ensuring the stability of the protecting group, gave a labile oil which was immediately treated with dibenzyl sodiomalonate in DMF to give the corresponding dibenzyl ester. Unfortunately, and this was not known at the time, the dibenzyl ester obtained was not of structure (78, R = Bzl), but was the isomer (83, R = Bzl) formed by the reaction of dibenzyl sodiomalonate with the rearranged bromide (84), unwittingly produced on treatment of the alcohol (79) with carbon tetrabromide and triphenylphosphine. The actual structures of the subsequent products were not revealed until the dibenzyl ester (83, R = Bzl) was converted to the lactone (85) [not (81)] *via* the diacid (83, R = H) by treatment with dilute HCl (Scheme 23). The $^{13}C$ n.m.r. off resonance decoupled spectrum of the lactone (85) revealed a characteristic doublet which was assigned to C-5, the secondary alcohol carbon. Had the structure of the compound been that of the desired lactone (81), then a triplet would have been observed at this part of the spectrum. Unfortunately, this rearrangement was not discovered earlier because of the inherent similarities in the proton n.m.r. and i.r. spectra between the desired compounds [(80, X = Br), (78, R = Bzl, H)] and the actual isomeric compounds produced [(84), (83, R = Bzl, H)]. Why the rearranged bromide (84) was produced rather than (80, X = Br) is not entirely clear, but is presumably for steric reasons. The reaction mechanism has been postulated to be of the Arbusov type[92,94] (Scheme 24) involving the initial formation of the phosphonium salt (86) from triphenylphosphine and carbon tetrabromide. Subsequent reaction with the alcohol (79) producing bromoform and the intermediate compound (87) which should readily yield the bromide (80, X = Br) and $Ph_3P{=}O$ by nucleophilic attack of $Br^-$ on C-1. However, if nucleophilic attack takes place at C-3, then the acetonide would open and the oxygen anion formed could conceivably attack C-1, expelling $Ph_3P{=}O$ and forming the rearranged product (84).

In order to get round the difficulty of preparing the diester (78), a modified route to ethisic acid (27a) was undertaken. Based on a number of elegant chiral syntheses of bislactones by Anderson and Fraser-Reid[35,36,37] using "diacetone glucose", the proposed route is shown in Scheme 25. In which the alcohol (79) is oxidised to the ketone (88) using pyridinium dichromate (PDC) in DMF[95] followed by a Wittig condensation to the olefin (89) and reduction to the

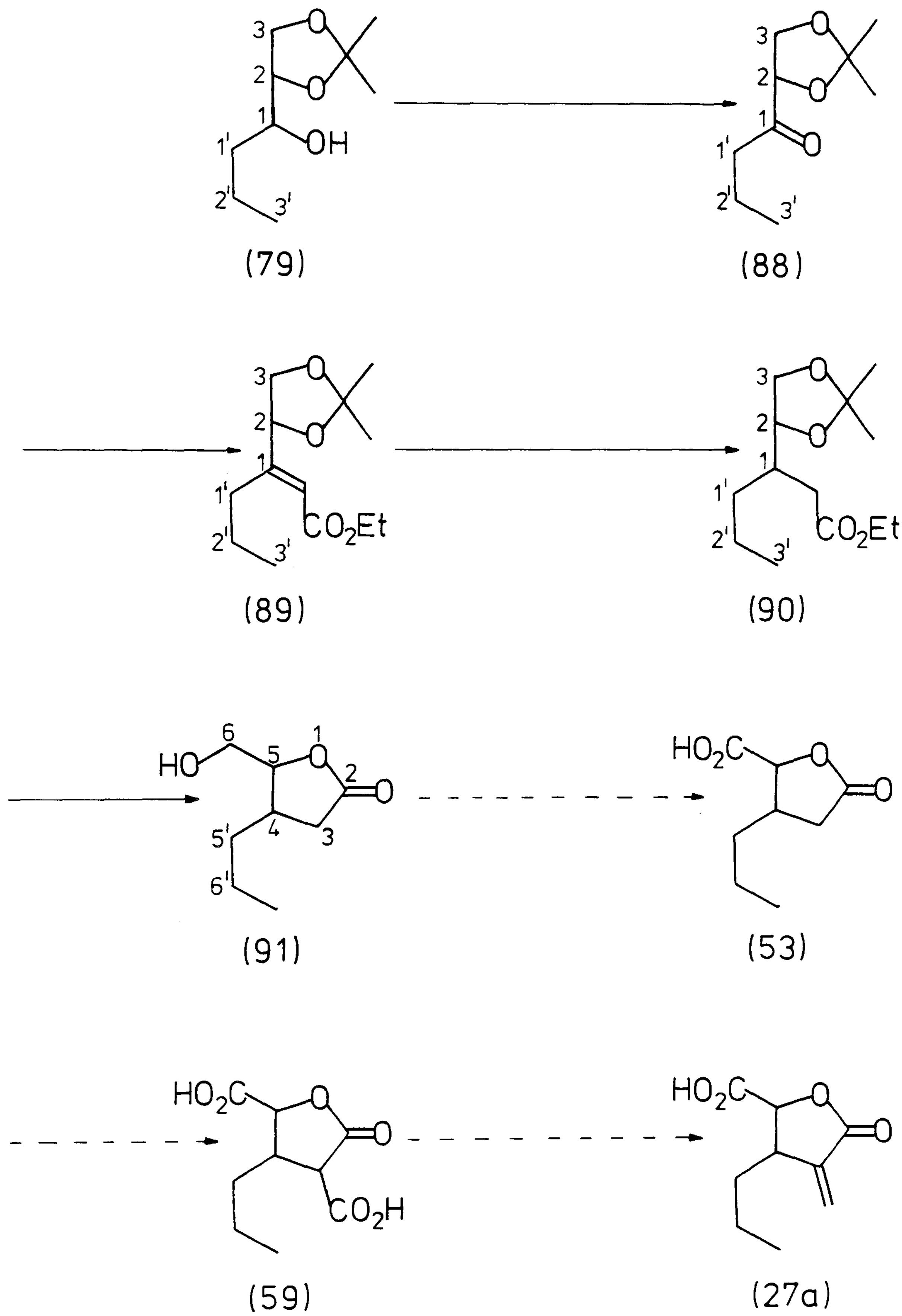



R = Bzl., H

Scheme 23



Scheme 24




Scheme 25

saturated ester (90). Hydrolysis of the ester (90) and deprotection of the diol function should lead to the lactone alcohol (91) which would be converted to ethisic acid (27a) by oxidation to the acid lactone (53) and carboxylation using Stiles' reagent[96] to give the diacid lactone (59) followed by methylenation to the desired compound.

As anticipated, the alcohol (79) was successfully converted to the ketone (88) in high yield using PDC in DMF and the Wittig reaction, which had been successfully reported for a similar system,[97] using the modified reagent, triethyl phosphonoacetate,[98] gave the desired olefinic ester (89) as an oil. Subsequent reduction over palladium/charcoal gave a product which, although apparently having the correct structure (90) from the i.r., $^{1}H$ n.m.r. and mass spectral data of a fresh sample, had a $^{13}C$ n.m.r. spectrum containing a significantly higher number of peaks than could be accounted for, even when doubling of peaks owing to the presence of diastereoisomers was considered. As the $^{13}C$ n.m.r. spectrum had been obtained some weeks after the product was prepared, the possibility that decomposition had subsequently taken place was thought to be a reasonable explanation. This was confirmed by obtaining a second $^{1}H$ n.m.r. spectrum of the product and this was now considerably different from the first, with one of the signals at *ca.* 2.1δ immediately recognisable as acetone - presumably produced by cleavage of the acetonide. Prior to the discovery of the instability of the ester (90), and some time after its preparation, it had been treated with 70% acetic acid in an attempt to form the lactone alcohol (91). The product obtained had bands in its i.r. spectrum at 3440, 1770 and 1730 $cm^{-1}$ suggesting formation of lactone alcohol (91) and the presence of a saturated ester. Distillation gave a colourless oil whose i.r. spectrum contained two significant bands at 3440 and 1770 $cm^{-1}$ - indicative of the desired lactone alcohol (91). However, treatment of this alcohol with PDC in

DMF gave, not the lactone acid (53), but essentially unchanged lactone alcohol.

Although lack of time has prevented this synthesis reaching a successful conclusion, there is every hope that this route, with its elegant incorporation of stereochemistry, will in the very near future provide the first successful synthesis of ethisic acid (27a) and its long chain analogue.

# CHAPTER 4

## 3. Biosynthesis of the Type A Bislactones: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with *P. canadense*

(i) Metabolites of *P. canadense*

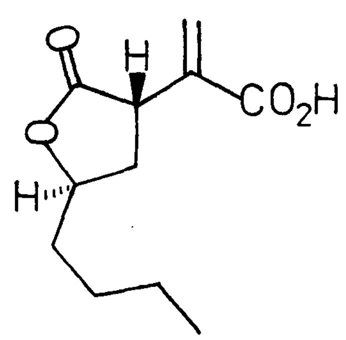
(ii) Synthesis of Isocanadensic acid and Protoisocanadensic acid

(iii) Synthesis of Canadensic acid and the $C_9$ Homologue relevant to Ethisolide Biosynthesis and Attempted Syntheses of Dihydroisocanadensic acid

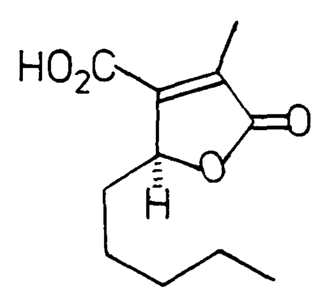
## 3. Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with *P. canadense*

### (i) Metabolites of *P. canadense*

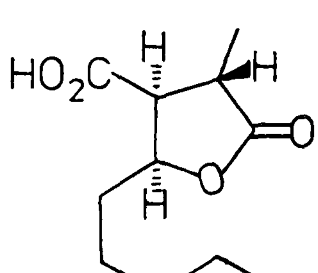
Apart from canadensolide (3) and dihydrocanadensolide (4), McCorkindale and coworkers isolated a number of other related metabolites from cultures of *P. canadense*.[47] The four minor metabolites isolated all had the type A lactone structure and the biosynthetic relationship between all of the metabolites of *P. canadense* forms part of this present work. The proposed biosynthesis of canadensolide (3), dihydrocanadensolide (4) and the other monolactonic compounds canadensic acid (22), isocanadensic acid (92), dihydroisocanadensic acid (93) and hydroxyisocanadensic acid (94) is *via* the *n*-hexylcitric



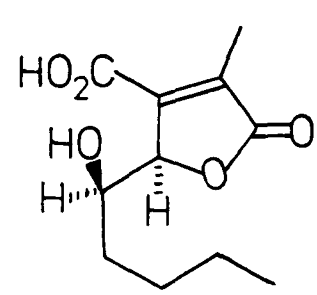
(22)



(92)



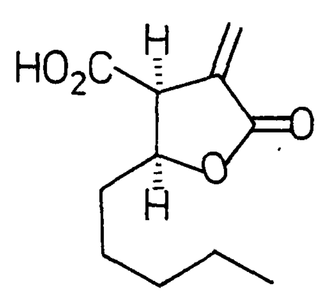
(93)



(94)

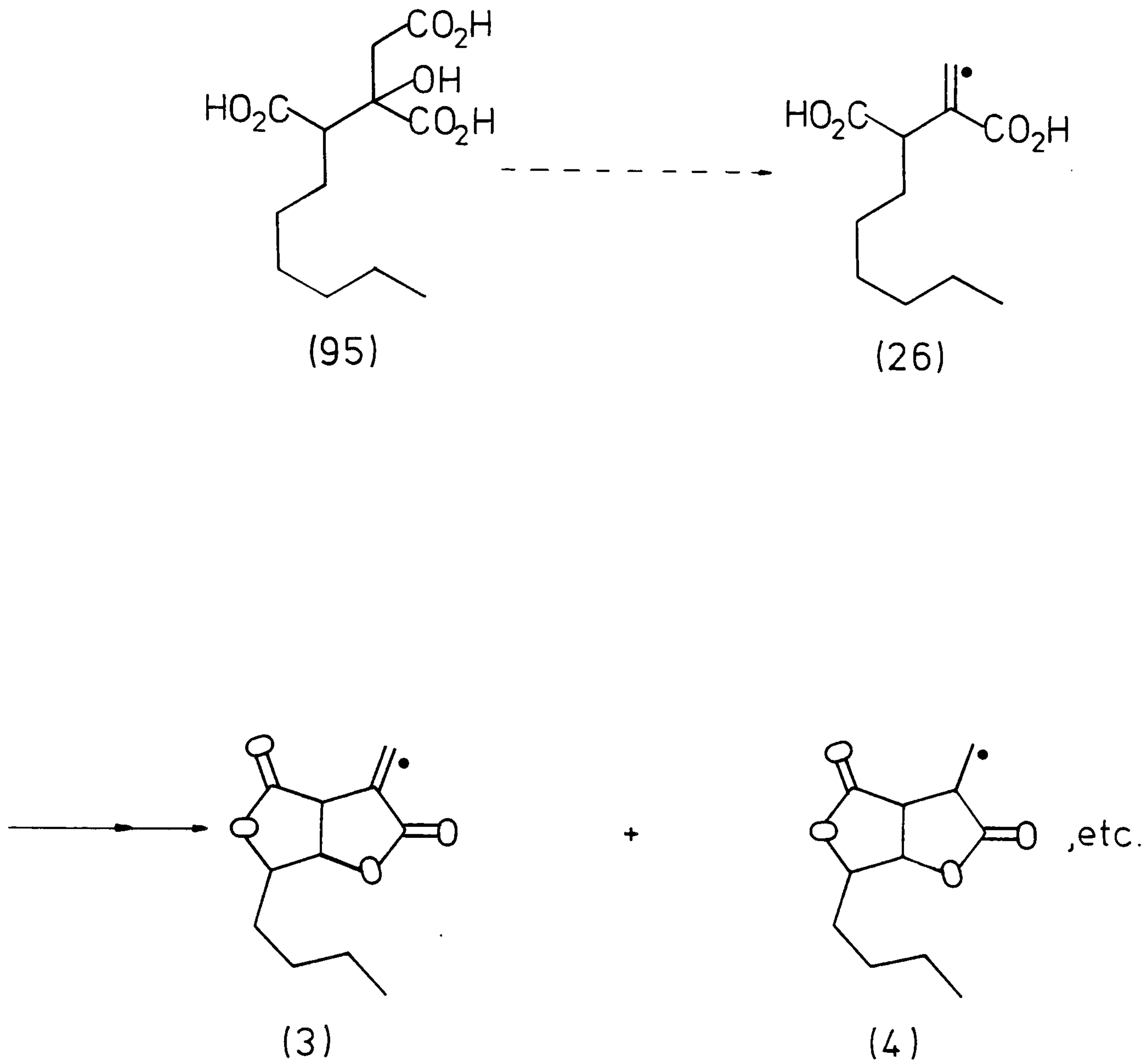
acid (95) formed by condensation of octanoic acid with oxaloacetate (Scheme 26). Extensive labelling studies using radiolabelled or $^{13}C$-labelled precursors confirmed a biosynthetic pathway as in Scheme 26 and the successful synthesis and incorporation of *n*-hexylitaconic acid (26) further supported this pathway.[47,51] However, the details of the interrelationships between the six known *P. canadense* metabolites are largely unknown.

Some preliminary work established that dihydrocanadensolide (4) was not the product of a biological reduction on canadensolide (3) by feeding a radiolabelled sample of (3) and it was also established that canadensic acid (22) was not the biogenetic precursor to canadensolide by a similar incorporation experiment.[99] This suggested that the key monolactonic precursor was rather the isomeric lactone acid - protoisocanadensic acid (96) and the later stages of the



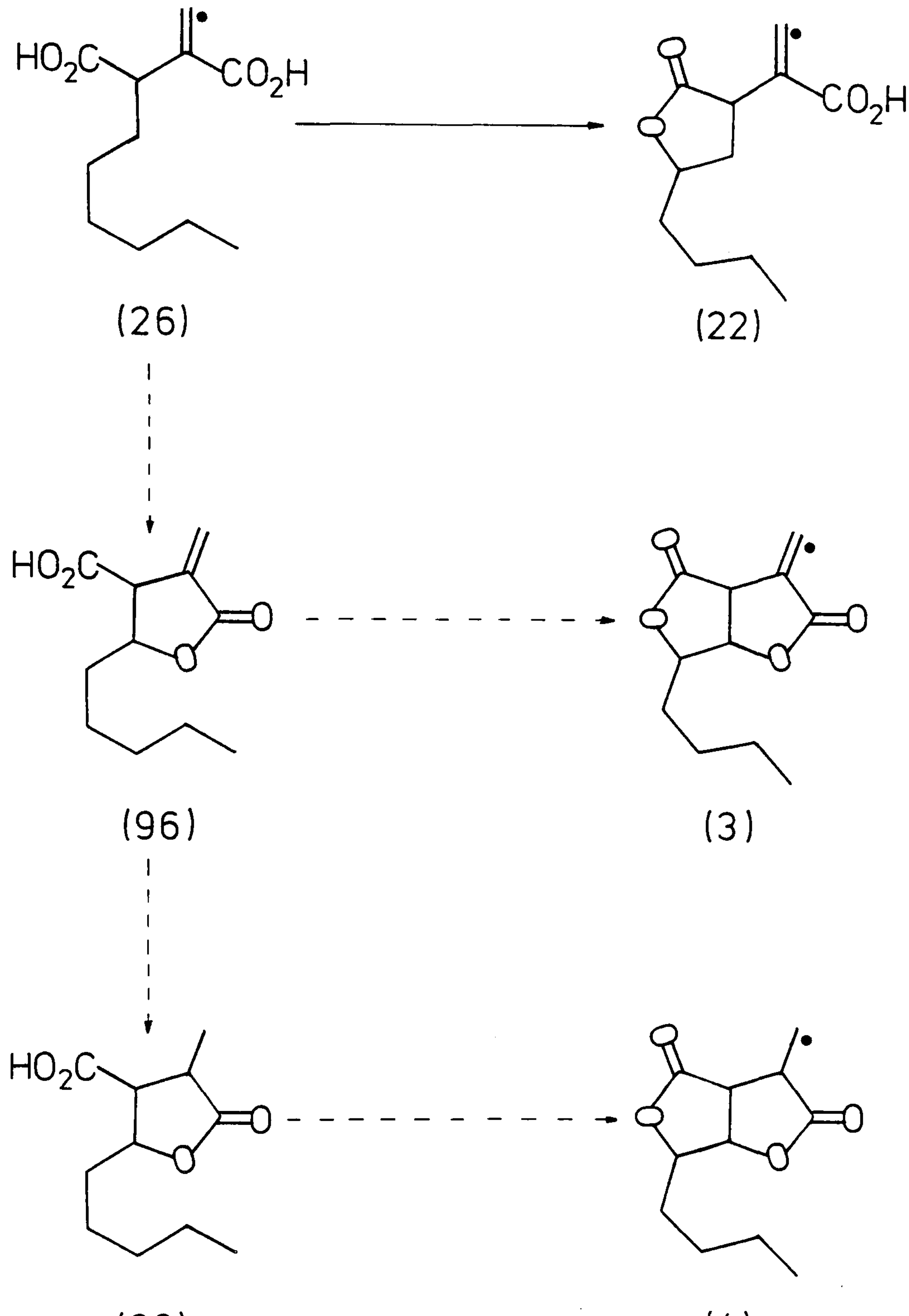

(96)

biosynthesis of a number of *P. canadense* metabolites was proposed to be as in Scheme 27. It can be seen, therefore, that a significant step in establishing this pathway would be the efficient incorporation of a labelled sample of protoisocanadensic acid (96) into canadensolide (3) and dihydrocanadensolide (4). The syntheses of protoisocanadensic acid (96) and two other metabolites, isocanadensic acid (92 ) and canadensic acid (22) are now described.




•: $^{14}C$ labelled carbon atom

Scheme 26




• : $^{14}C$ labelled carbon atom

Scheme 27

(ii) Synthesis of Isocanadensic Acid (92) and Protoisocanadensic Acid (96)

The first synthetic route towards protoisocanadensic acid (96) is outlined in Scheme 28. The triester (97), obtained from the reaction of diethyl malonate and ethyl 2-bromocaprylate, would be brominated to produce the bromo-triester (98). The dehydrobromination of similar bromo-triesters has been investigated previously in these laboratories[100] and the reaction was found to give mixtures of tri- and tetra-substituted olefins, with the tri-substituted olefin being the major product. The reaction presumably involves initial formation of the tetra-substituted olefin and base catalysed isomerisation to the thermodynamically more stable isomer (Fig. 23).

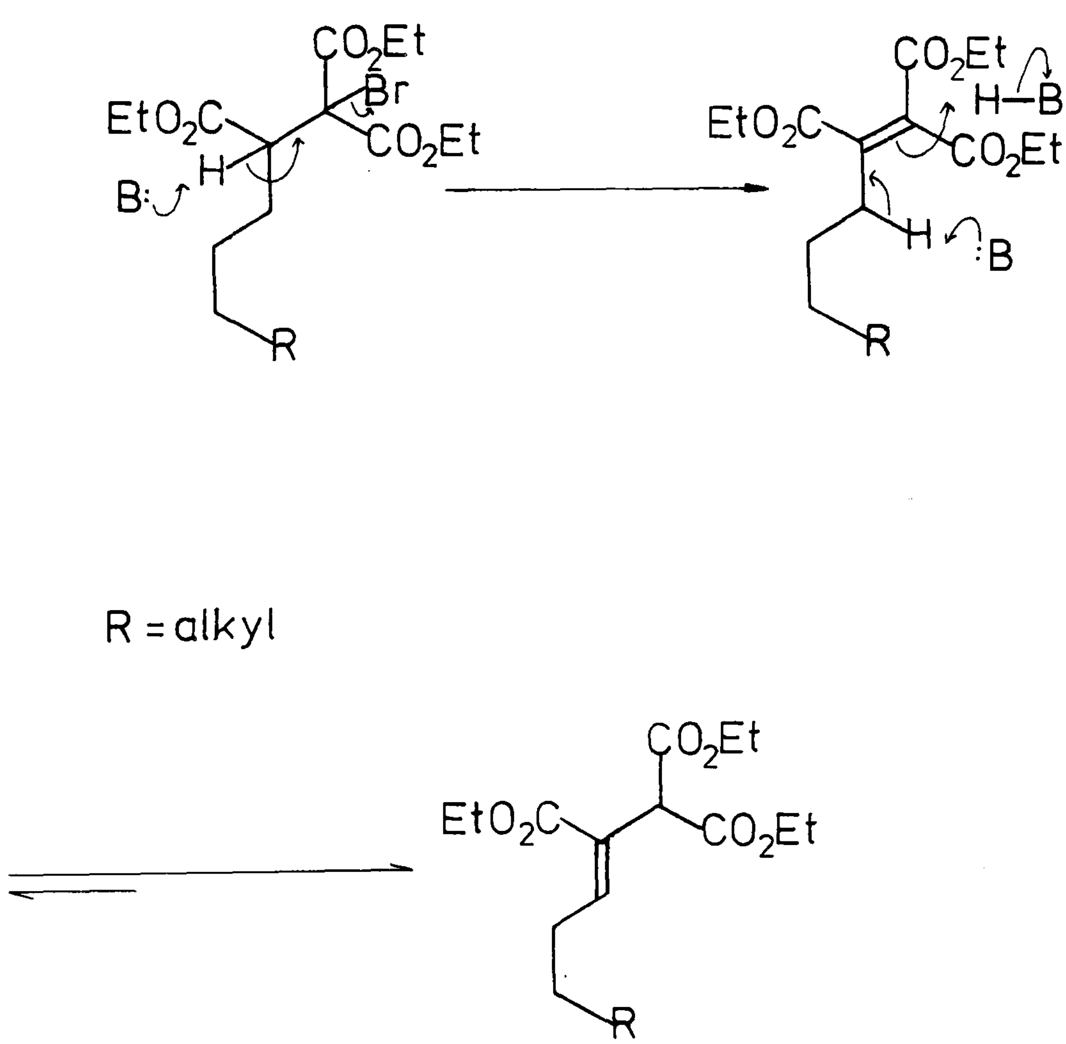
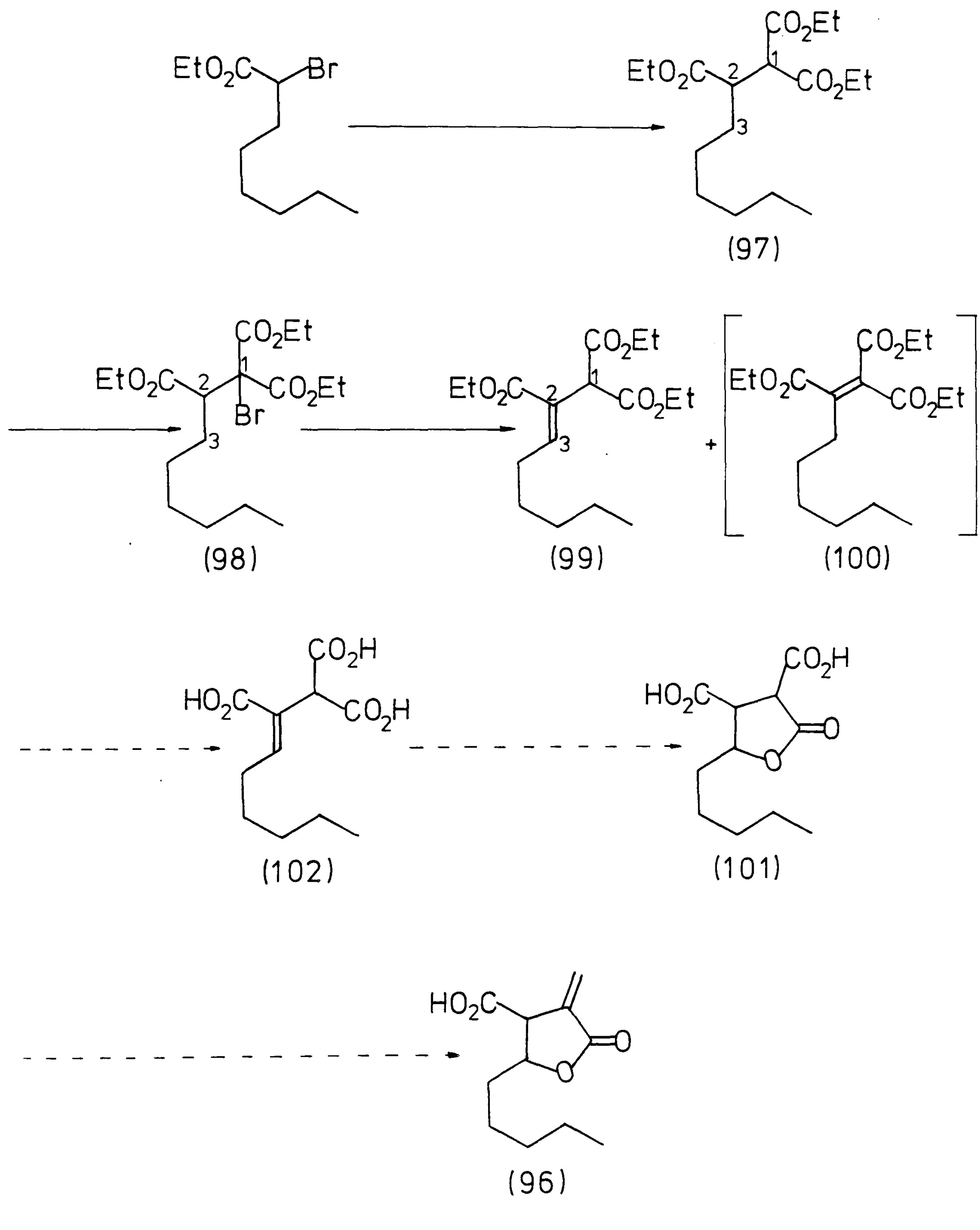


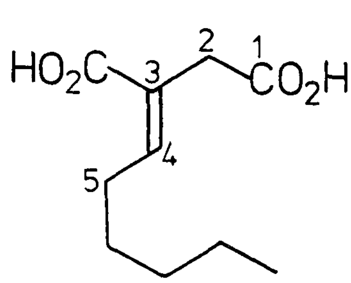

Fig. 23



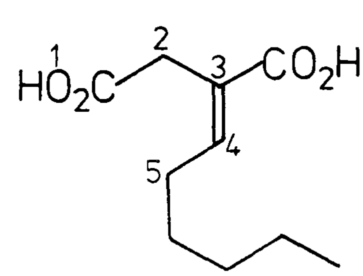

Scheme 28

Dehydrobromination of the bromo-triester (98) should give a mixture of unsaturated triesters (99, 100) and the diacid lactone (101) would then be obtained following hydrolysis of (99) to the unsaturated triacid (102). Protoisocanadensic acid (96) would then be produced by methylenation of the diacid lactone (101).

1,1,2-Tricarboethoxyoctane (97) was successfully prepared and a bromination/dehydrobromination sequence was used to produce the desired 1,1,2-tricarboethoxyoct-2-ene (99) along with the isomeric tetra-substituted olefin (100) which could be separated using preparative t.l.c. Furthermore, treatment of the tetra-substituted olefin (100) with sodium ethoxide resulted in isomerisation to the desired olefinic ester (99) (*cf*. Fig. 23, B = ethoxide). Hydrolysis of the olefinic ester (99) with aqueous sodium hydroxide did not, however, produce the unsaturated triacid (102), the decarboxylated compound, (Z)-3-carboxynon-3-enoic acid (103) was obtained. This was assigned the (Z) configuration by comparison of the spectral data with a sample of 3-carboxynon-3-enoic acid (104) prepared from diethyl succinate and *n*-hexanal.[101]



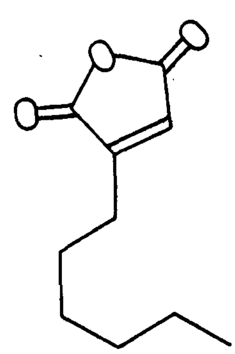

(103)




(104)

The H-4 proton appeared at δ6.28 (t) in the n.m.r. spectrum of (103) and the H-5 protons appeared at δ2.63 (m), the corresponding signals in the spectrum of (104) appearing at δ7.2 (t) and δ2.2 (m), the observed chemical shift differences being attributed to the deshielding effect of the carboxylic acid and the structures of (103) and (104) were assigned (Z) and (E) respectively.

The unsaturated diacid (103) could still be usable if lactonisation and carboxylation could be achieved to form the desired diacid lactone (101). Unfortunately however, attempts to lactonise the 3-enoic acids (103, 104) by acid catalysis in the usual manner[102,103] all failed. One lactonisation attempt, using p.t.s.a. in benzene, gave a product whose i.r. spectrum exhibited bands at 1850 and 1785 $cm^{-1}$ and this was probably the anhydride (105).



(105)

Likewise, attempts to prepare the unsaturated lactone (106) *via* an iodolactonisation reaction[104] also failed (Fig. 24).

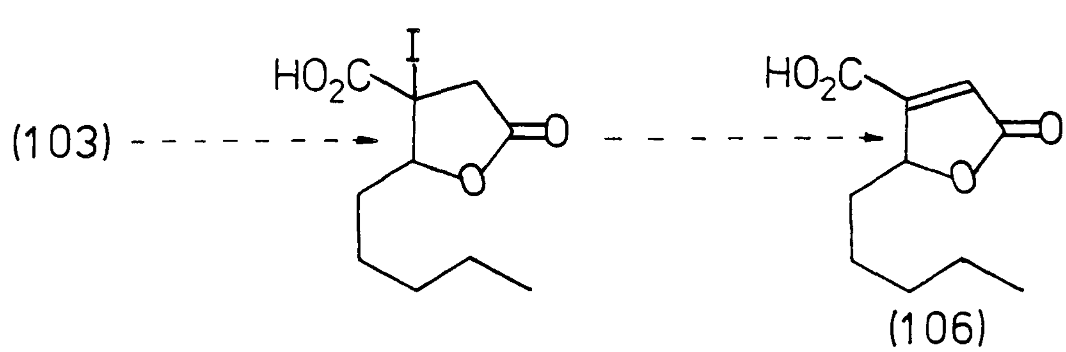


Fig. 24

Returning to the unsaturated triester (99), it was considered that bromination would give a suitable precursor for lactonisation (Fig. 25).

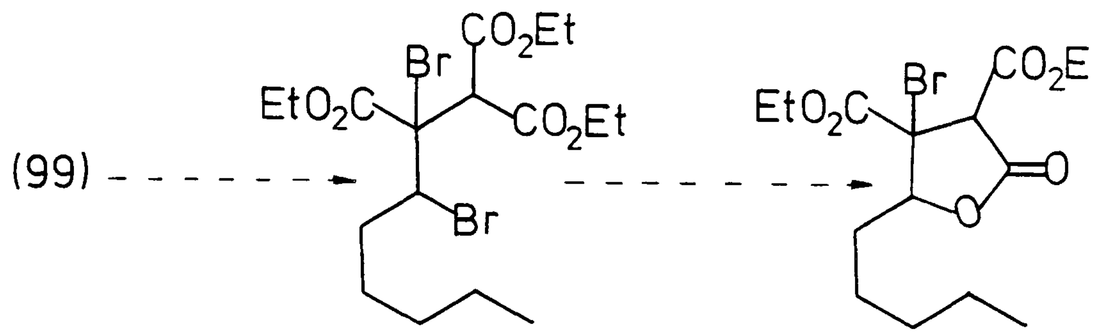
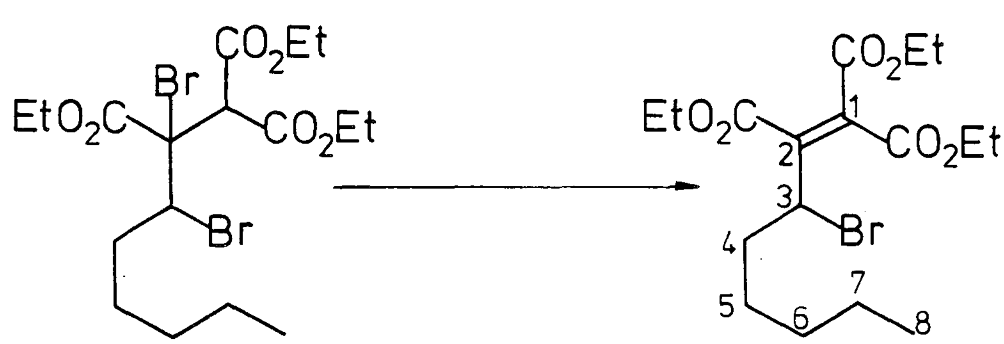


Fig. 25

However, bromination yielded the unsaturated bromo-ester (107) not the dibromo-ester (108) although this was presumably the initial product prior to a dehydrobromination step (Fig. 26).



(108) (107)

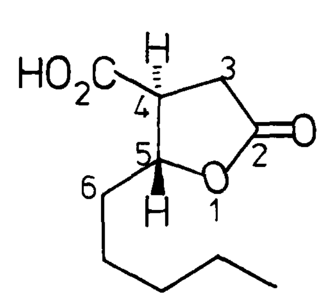
Fig. 26

The product of the bromination reaction was assigned structure (107) on the basis of its $^{13}C$ n.m.r. off resonance decoupled spectrum, which exhibited two singlets at *ca*. 146 and 128 p.p.m. (the two olefinic carbon atoms) and a doublet, J = 59 Hz at *ca*. 46 p.p.m. (-CH-Br) and its mass spectrum (parent m/e 406/408). Again, attempted lactonisation (heat, acid) of this bromo-ester (107) failed and the reaction was not investigated further.

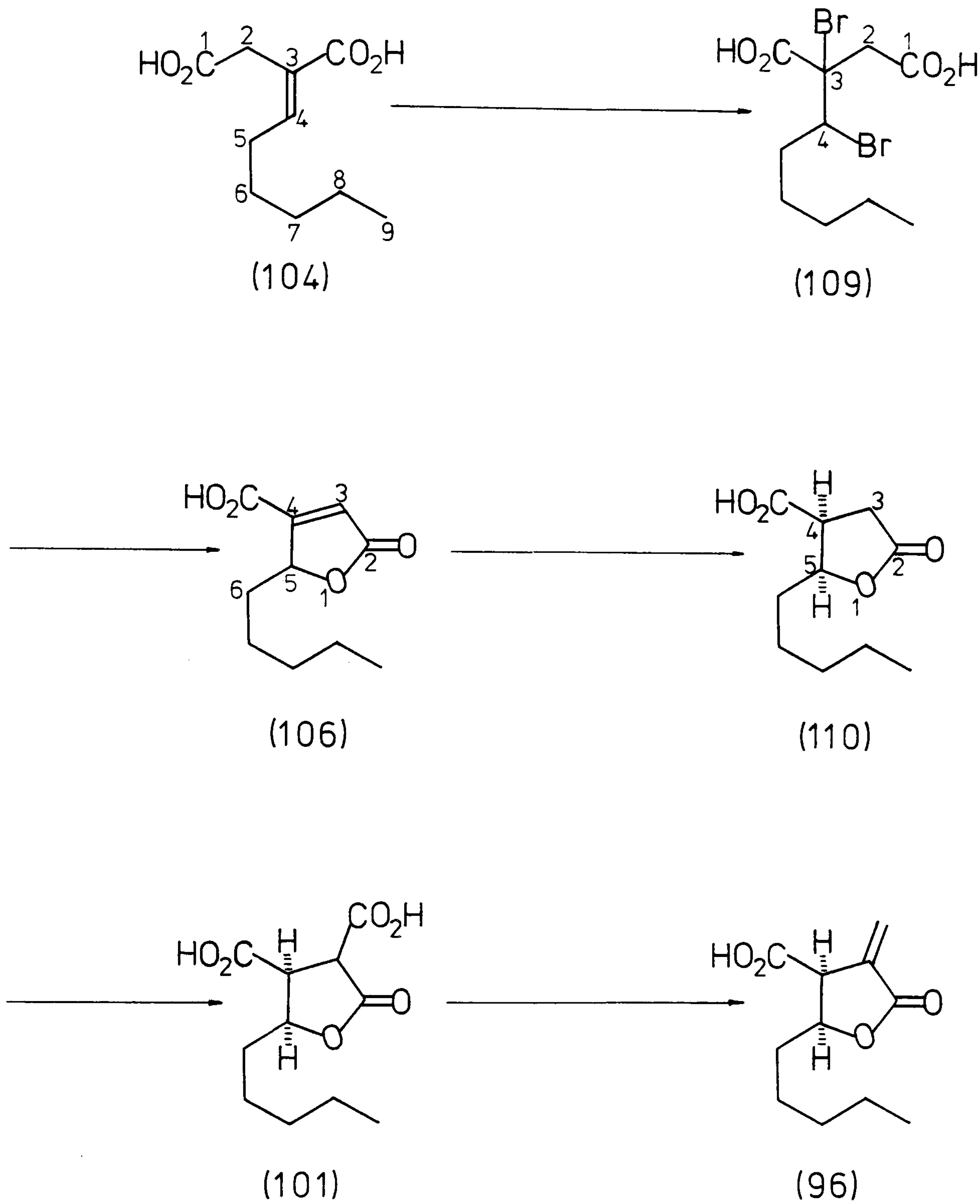
A modified route to protoisocanadensic acid (96) was envisaged (Scheme 29), which retained the diacid lactone (101) as the key intermediate. The unsaturated lactone, 5-*n*-pentylaconic acid (106), was obtained by treatment of the dibromo diacid (109) with aqueous sodium hydroxide[101] and this was successfully reduced from the least hindered side using a rhodium/alumina catalyst to the desired *cis* carboxy lactone (110).

Attempted reduction of (106) using palladium catalysts gave only unreacted material and the use of Adam's catalyst ($PtO_2$) gave mainly unreacted (106) and some hydrogenolysis products.

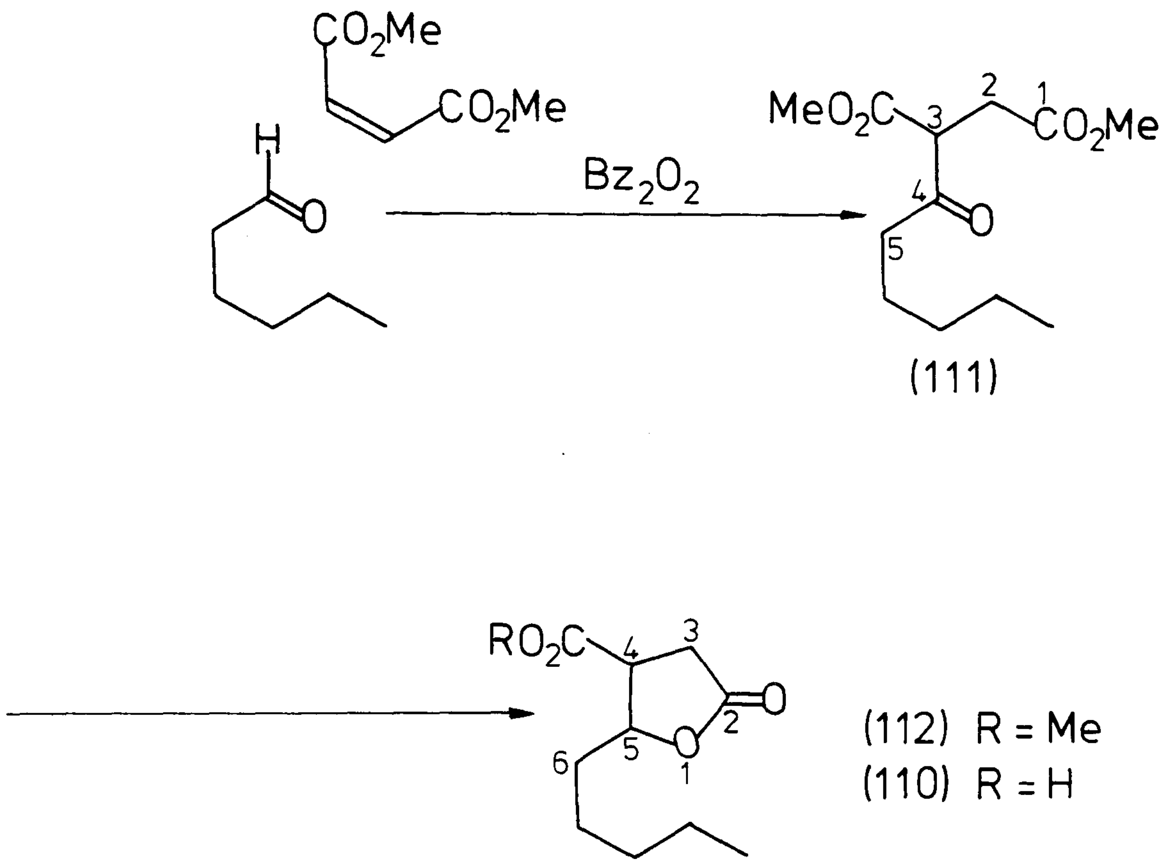
A more convenient route to the *cis* lactone (110), based on a synthesis of protolichesterinic acid (5a)[105] was also successful. This involved free-radical addition of *n*-hexanal to dimethyl maleate to yield the keto diester (111) which was reduced and lactonised by treatment with sodium borohydride to the methyl ester (112). Hydrolysis then afforded the same *cis* acid lactone (110) (Fig. 27) by fractional crystallisation from benzene. It was found however, that if a large excess of sodium borohydride was used then the reduction product was the hydroxy lactone (113), not the lactone methyl ester (112). In this case, oxidation using PDC afforded a sample of the *trans* acid lactone (114).




(114)



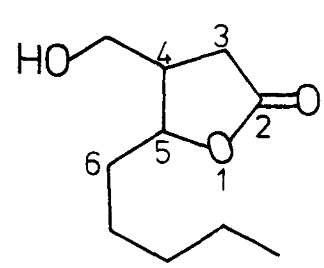
Scheme 29




(111)

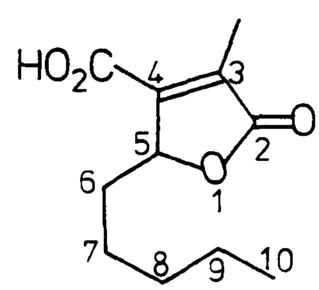
(112) R = Me
(110) R = H

Fig. 27




(113)

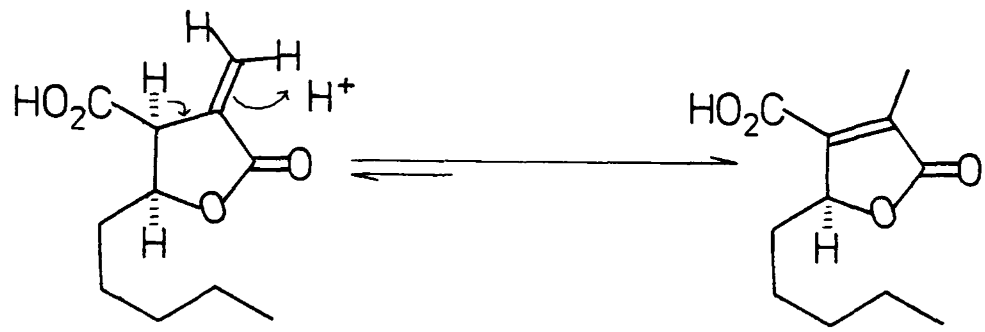
Having successfully obtained the pure *cis* acid lactone (110), the final two steps to protoisocanadensic acid (96) were attempted following the lines of Johnson's synthesis of protolichesterinic acid (5a).[105] Treatment of the acid lactone (110) with Stiles' reagent[96] afforded the diacid lactone (101) as a colourless, viscous oil which resisted all attempts to produce crystalline material. Reaction of this crude material with diethylamine and aqueous formaldehyde gave, not the expected exo-methylene compound (96), but the isomeric product, isocanadensic acid (92) with a small amount (*ca*. 10%) of the desired product (96). Likewise, treatment of




(92)

the diacid lactone (101) with the acetate buffered methylenation reagent[72] afforded only isocanadensic acid (92).

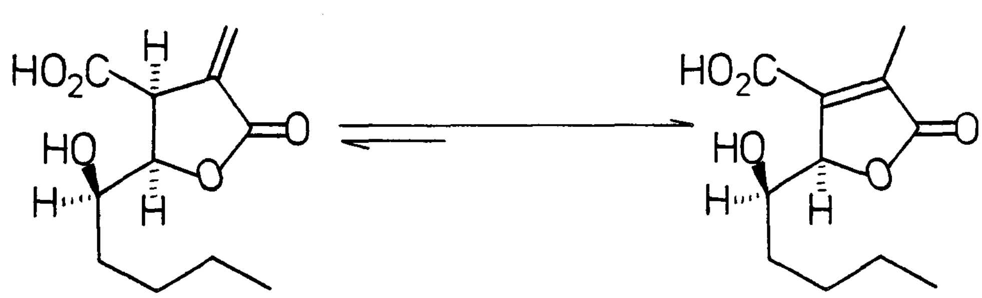
Reaction of the diacid lactone (101) with Mannich reagents will produce the exo-methylene lactone (96) as the initial product, although this must have then isomerised to the seemingly more stable endo-methylene compound (92) (Fig. 28). This observation could call into question the inclusion of isocanadensic acid (92) as a genuine metabolite of *P. canadense* and almost certainly that of hydroxyisocanadensic acid (94) as well. On the basis of the experimental evidence, it seems quite possible that both isocanadensic acid (92) and hydroxyisocanadensic acid (94) are actually more stable artefacts,



(96) (92)

Fig. 28

produced during isolation of the *P. canadense* metabolites, of the genuine precursors, protoisocanadensic acid (96) and hydroxyproto-isocanadensic acid (115) respectively. If this is correct, then



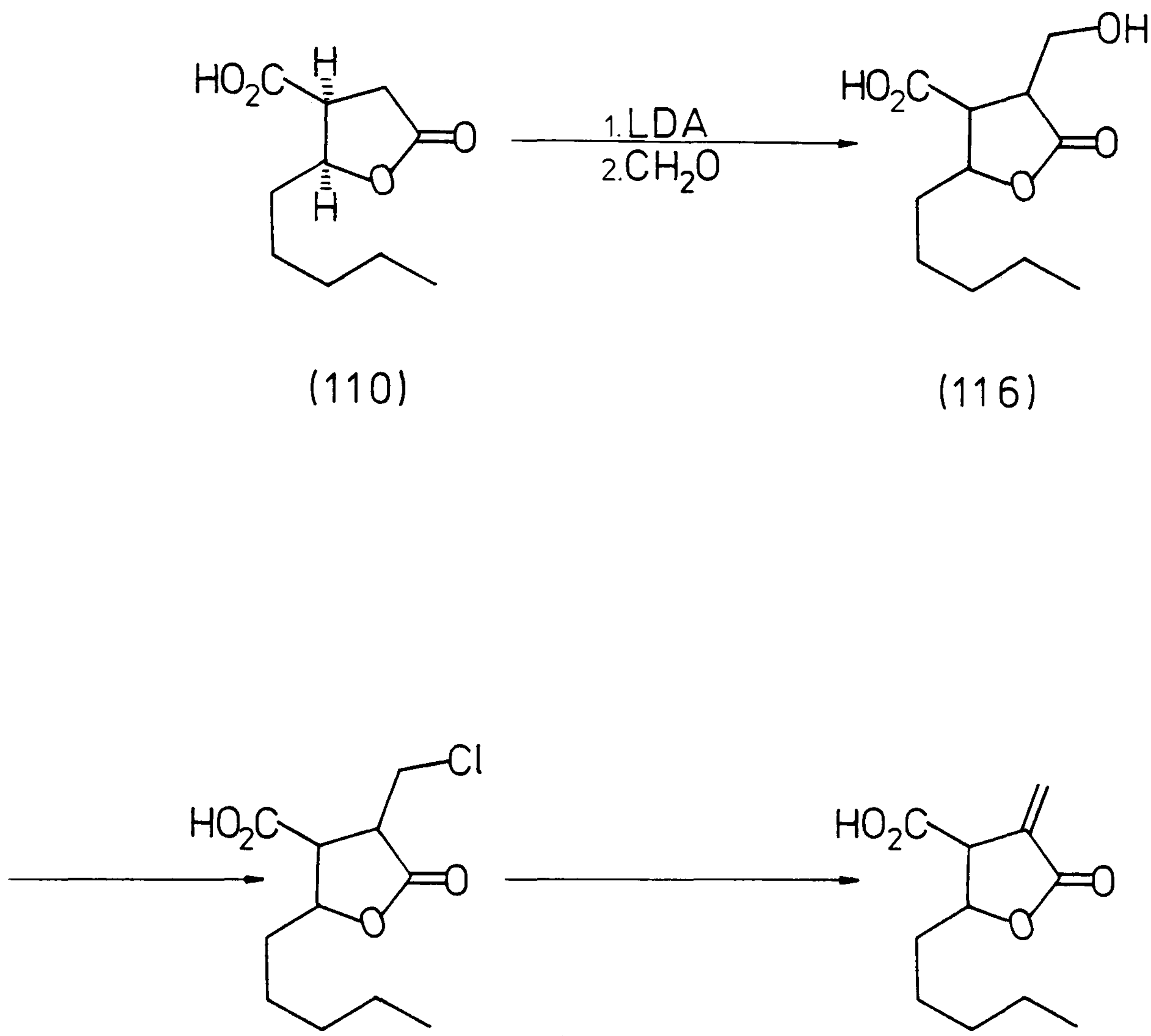
(115) (94)

the biosynthetic pathway is simplified and the proposed scheme (Scheme 27) is further supported. However, the apparent facility of isomerisation to isocanadensic acid (92) is liable to make the methylenation of the diacid lactone (101) to protoisocanadensic acid (96) more difficult.

An alternative approach to methylenation was made based on a protolichesterinic acid (5a) synthesis by Schlessinger.[106] The proposed route (Scheme 30) involves treating the dianion of the *cis* acid lactone (110) with gaseous formaldehyde to form the primary alcohol (116), conversion to the chloride (117) using $PCl_5$ and reaction with NaH or KH at room temperature should give the desired exo-methylene lactone (96).

The preparation of the alcohol (116) presented an immediate problem when the formaldehyde gas polymerised at the low reaction temperature and contaminated the product with paraformaldehyde. Nevertheless, in order to test the feasibility of the subsequent steps, the crude alcohol (116) was allowed to react with $PCl_5$ and the resulting oil treated with NaH in dry THF to give, after preparative t.l.c., a homogeneous oil containing isocanadensic acid (92) and *ca.* 20% protoisocanadensic acid (96). The two lactones were identified by the characteristic doublet at 2.2$\delta$ ($R_2C{=}CR{-}\underline{CH_3}$) and the two exo-methylene signals at 6.0$\delta$ and 6.45$\delta$ in the n.m.r. spectrum. Since further chromatography failed to separate the two lactones, the search for alternative methods of methylenation was continued.

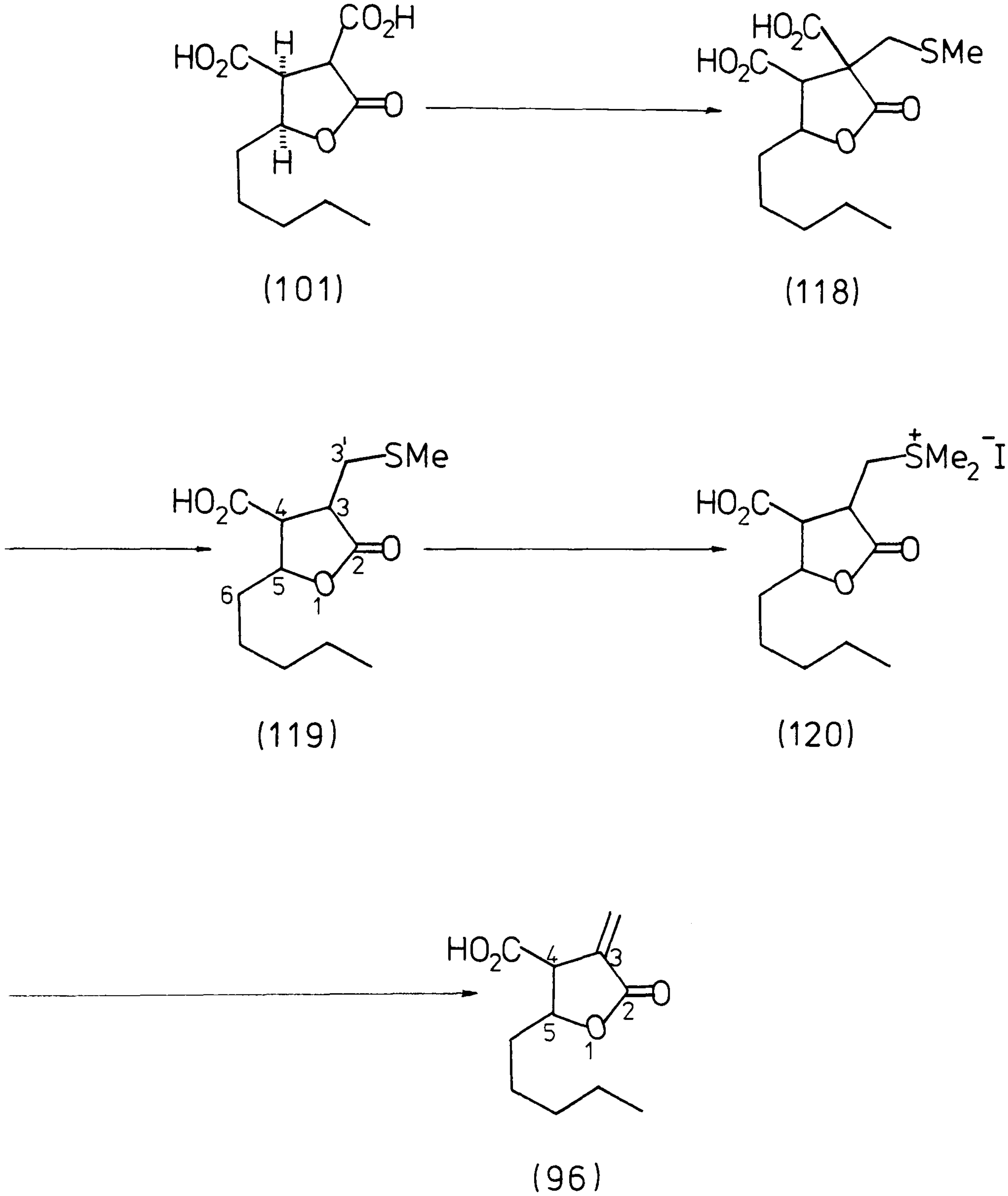
Thankfully, a successful methylenation procedure was found, based on the method of Ronald.[107] The new route (Scheme 31) involved construction of the exo-methylene function in a protected, stable form which would readily yield the methylene group under very mild conditions. Treatment of the diacid lactone (101) with three equivalents of LDA followed by iodomethyl methyl sulphide (generated *in situ* from chloromethyl methyl sulphide and anhydrous lithium iodide) and HMPA afforded the crude diacid sulphide (118). This was decarboxylated by heating in xylene and chromatographed to give the (methylthio)-methyl lactone (119) which was efficiently converted to protoisocanadensic acid (96) by heating in excess methyl iodide to give the sulphonium salt (120) followed by mild base catalysed β-elimination using dilute aqueous $NaHCO_3$. The protoisocanadensic acid (96) produced was approximately




(110) (116)

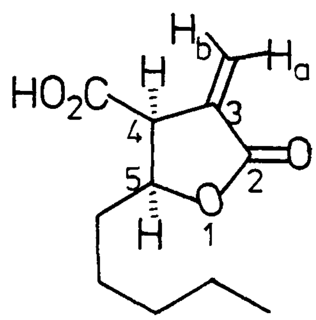
(117) (96)

Scheme 30

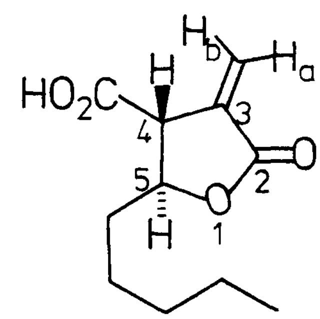



Scheme 31

95% pure (by n.m.r.), the impurity being a small amount of iso-canadensic acid (92). The n.m.r. spectrum indicated that a 60:40 mixture of *cis* and *trans* protoisocanadensic acids had been formed (Fig. 29). In both isomers the $H_a$ and $H_5$ protons appeared at *ca.*



Cis



Trans

Fig. 29

6.4δ and *ca.* 4.7δ respectively, but there were distinct differences in the chemical shifts and couplings of the $H_b$ and $H_4$ protons for the two isomers. The *cis* isomer $H_b$ proton appeared as a sharp doublet, J = 2 Hz, centred at *ca.* 5.9δ and its associated $H_4$ proton appeared as a double triplet, J = 7.5, 2 Hz, centred at *ca.* 4.03δ. The *trans* isomer $H_b$ proton appeared as a sharp doublet, J = 3 Hz, at *ca.* 6.02δ and its $H_4$ proton appeared as a double triplet, J = 5.5, 3 Hz, centred at *ca.* 3.63δ. The assignment of the isomers was made by comparison of the chemical shift and splitting pattern of the *cis* fused ring protons of canadensolide (3). Although the synthesis had used only the *cis* lactone acid (110), epimerisation had probably taken place when the diacid lactone (101) was treated with LDA to form the trianion.

An alternative method of unmasking the methylene function by pyrolytic elimination of the lactone sulphoxide (121)[108,109] (formed by treatment of the sulphide (119) with sodium metaperiodate[110]) gave, as before, a mixture of the two isomeric methylene lactones (Fig. 30).

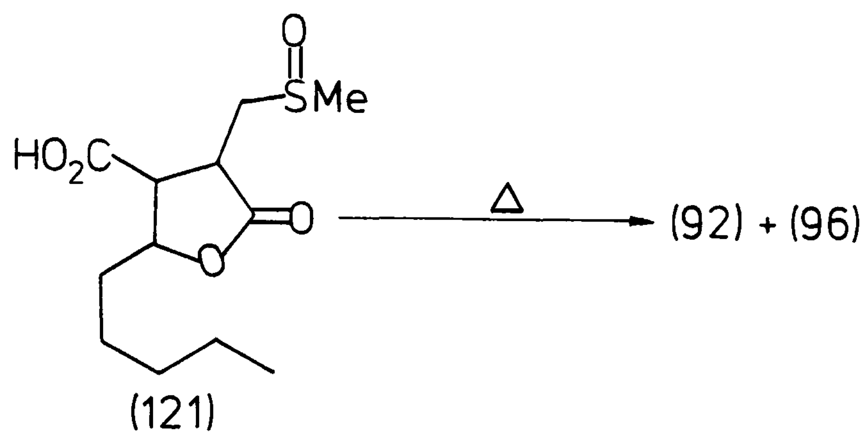
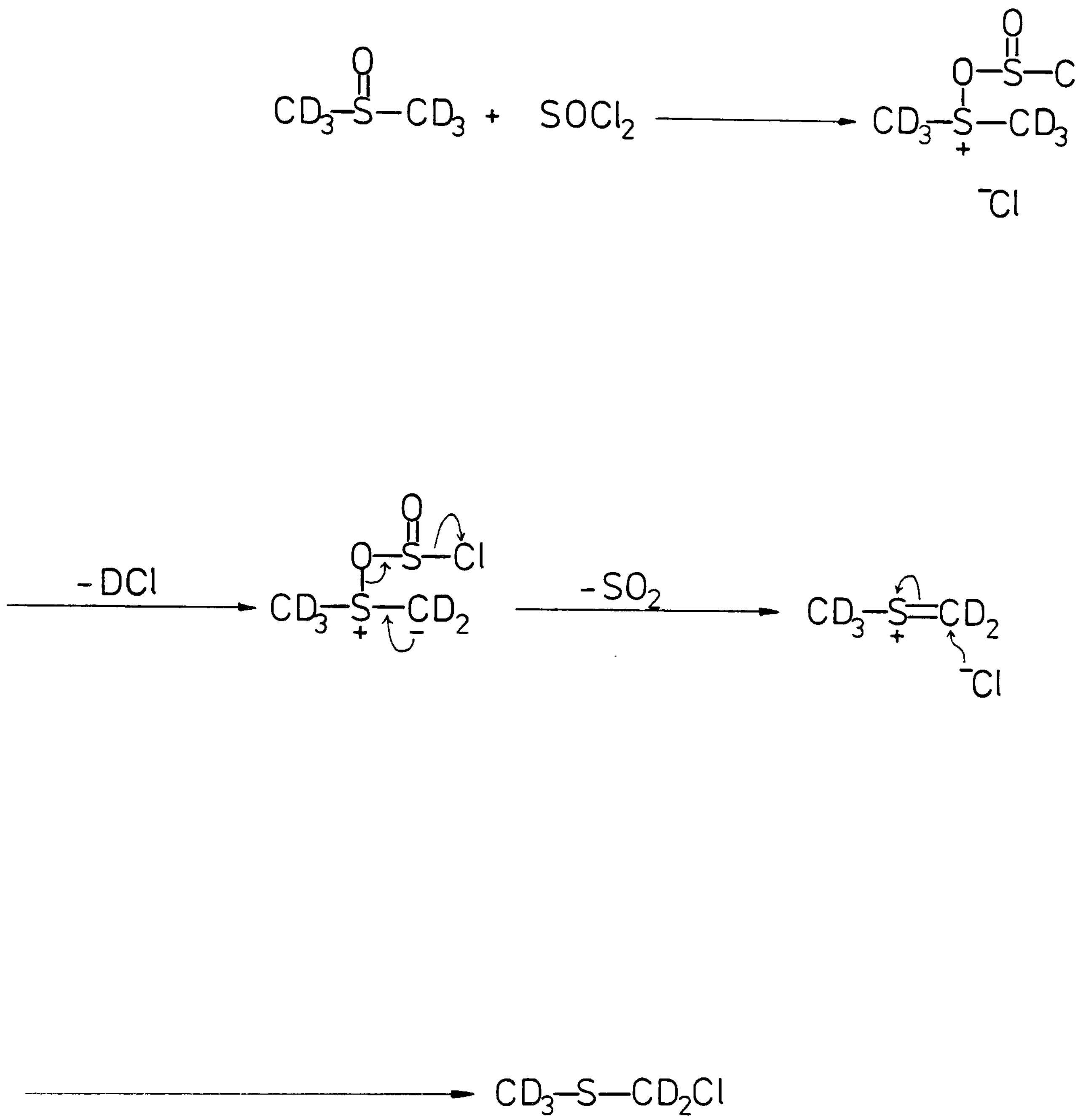


Fig. 30

Having achieved an efficient synthesis of protoisocanadensic acid (96), albeit as a mixture of diastereoisomers what was now required was the synthesis of a labelled sample for incorporation studies using cultures of *P. canadense* in order to test the validity of the proposed biosynthetic pathway of canadensolide (3) and dihydrocanadensolide (4) (Scheme 27). Obviously [$^{14}$C]-formaldehyde would be no use in this synthesis as the exo-methylene carbon is derived from the chloromethyl methyl sulphide alkylating reagent, which is not available commercially either labelled with $^{14}$C or stable isotopes. However, an efficient synthesis of chloromethyl methyl sulphide, by treating dimethyl sulphoxide with thionyl chloride, was known[111] and by using $D_6$-dimethyl sulphoxide, readily available as an n.m.r. solvent, a sample of $D_5$-chloromethyl methyl sulphide was prepared in 84% yield. The sulphide is formed by a Pummerer rearrangement and the mechanism is shown in Figure 31.

Repeating the protoisocanadensic acid (96) synthesis (Scheme 31) using $D_5$-chloromethyl methyl sulphide afforded a sample of $D_2$-protoisocanadensic acid (Fig. 32) although in rather low yield, mainly due to incomplete alkylation giving a quantity of acid lactone (110) as the major impurity.



($D_5$-chloromethyl methyl sulphide)

Fig. 31

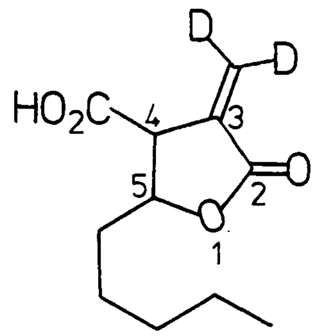
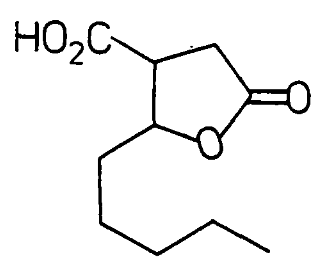


Fig. 32



(110)

When using stable isotopes, a fairly large quantity of material (*ca*. 250 mg) is desirable for feeding in order to ensure that sufficient incorporation into the product will take place to be detectable. However, in the present case there was no time in which to prepare more material and it was decided to use the 50 mg available (as a mixture of epimers and slightly contaminated with $D_2$-isocanadensic acid) in an incorporation experiment.

Previous time/incorporation studies on *P. canadense* using [$^{14}$C]-acetate had been carried out[112] and the results were used in the present experiment, the impure $D_2$-protoisocanadensic acid (Fig. 32) being fed in three 24 hr. pulses. Canadensolide (3), dihydrocanadensolide

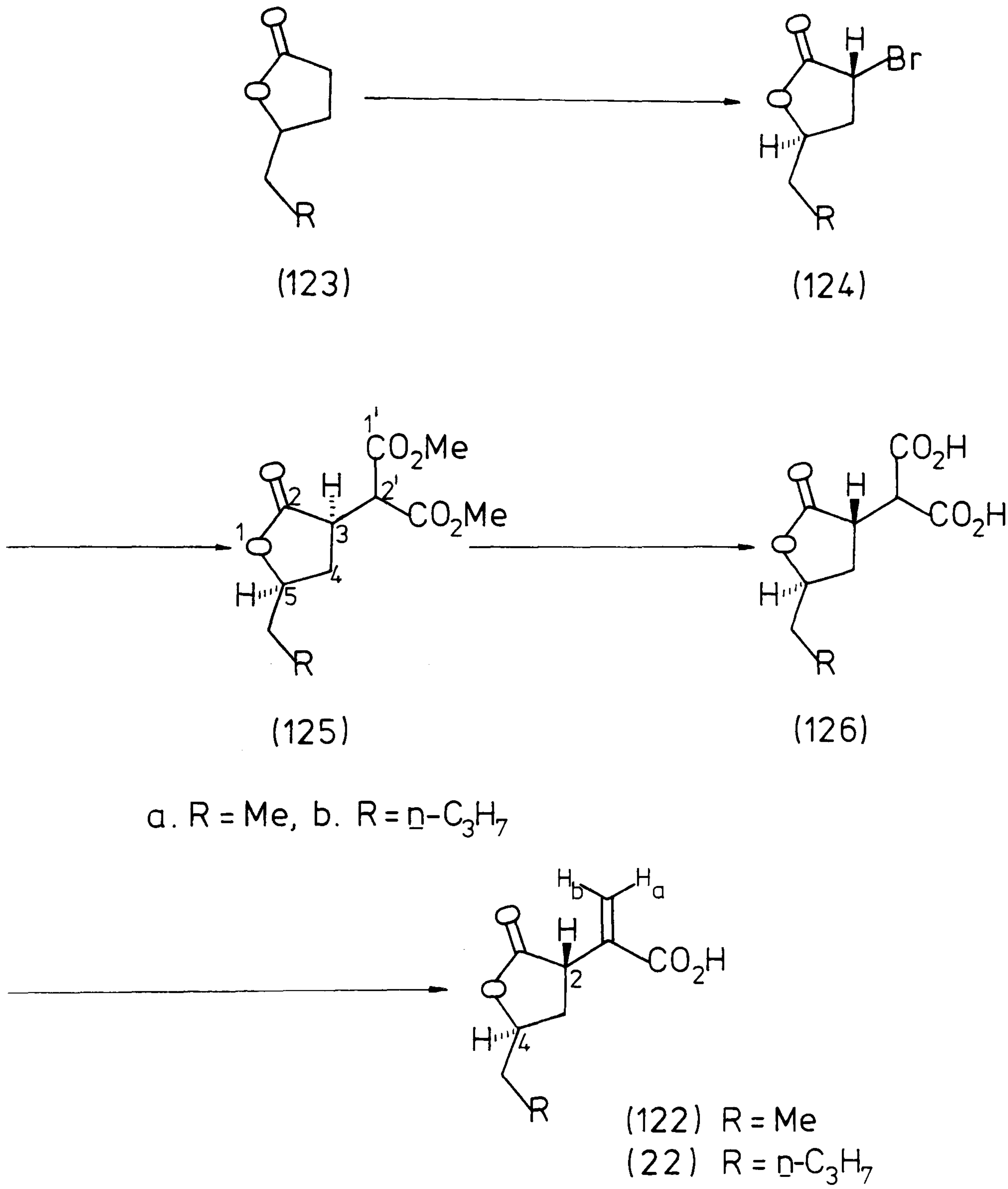
(4) and canadensic acid (22) were recovered from the broth and, if the proposed biosynthetic route (Scheme 27) was correct, canadensolide (3) and dihydrocanadensolide (4) should contain deuterium. High resolution mass spectrometry on a sample of the dihydrocanadensolide (4) showed that very little, if any, deuterium was present and the result of this incorporation experiment can only be described as inconclusive. This is, perhaps, not too surprising considering the small amount of labelled precursor fed and the relative insensitivity of the detection method for stable isotopes as opposed to that for radiolabelled compounds. A sample of [$^{14}$C]-dimethyl sulphoxide was subsequently obtained, but due to lack of time this has not, as yet, been used to prepare a sample of [$^{14}$C-methylene]-protoisocanadensic acid (96) for more extensive incorporation studies.

(iii) Synthesis of Canadensic Acid (22) and the $C_9$ Homologue (122) relevant to Ethisolide Biosynthesis and Attempted Syntheses of Dihydroisocanadensic Acid (93)

Although canadensic acid (22) is not a precursor to canadensolide (3) or a likely precursor to dihydrocanadensolide (4)[99] and is therefore not of interest for incorporation studies, it has not up until now been available other than from natural sources and its total synthesis would at least unambiguously confirm its structure.

An efficient four step synthesis of (±) canadensic acid (22) and its $C_9$ homologue (122) was carried out by α-bromination of the appropriate lactone[113] (123) followed by alkylation of the resultant bromo lactones (124) with dimethyl sodiomalonate to give the lactone diesters (125). Hydrolysis with aqueous sodium hydroxide afforded the diacid lactones (126) which were converted to the corresponding exo-methylene lactones (22 or 122) by treatment with diethylamine and aqueous formaldehyde (Scheme 32). In this case, the use of the acetate buffered methylenation reagent[72] gave very little of the desired compound. The spectral data of the racemic canadensic acid (22) obtained was identical in every respect (apart from optical rotation) with a sample of natural (-) canadensic acid (22). A small quantity (*ca.* 5%) of the *cis* isomer was present, but this was no more than is found in natural (-) canadensic acid (22) under the usual conditions of isolation.

This exceeded the expectations of the synthesis in producing the racemate of canadensic acid since canadensic acid readily epimerises to give a 50:50 mixture of diastereoisomers[114] (Fig. 33). The evidence that canadensic acid (22) is *trans* with respect to the substituents at C-2 and C-4 rests on the following. Esterification following reduction of canadensic acid (22) gives a dihydro derivative (127, $R_t$ 29.0 min.) while the mixture of diastereoisomeric canadensic acids (22) and (128)



H
Br
R
(123)
(124)
1'
$CO_2Me$
H
2
2'
$CO_2Me$
1
3
4
5
H
R
(125)
$CO_2H$
H
$CO_2H$
H
R
(126)
a. R = Me, b. R = n-$C_3H_7$
$H_b$
$H_a$
H
$CO_2H$
2
H
4
R
(122) R = Me
(22) R = n-$C_3H_7$


Scheme 32

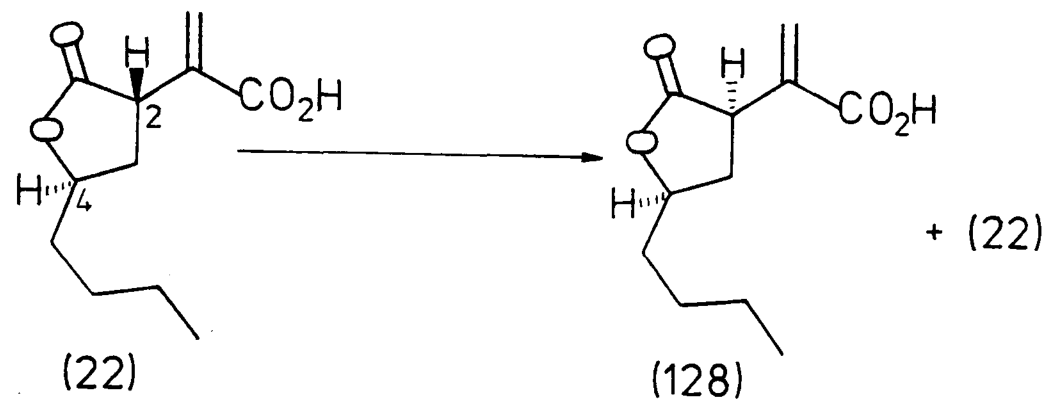
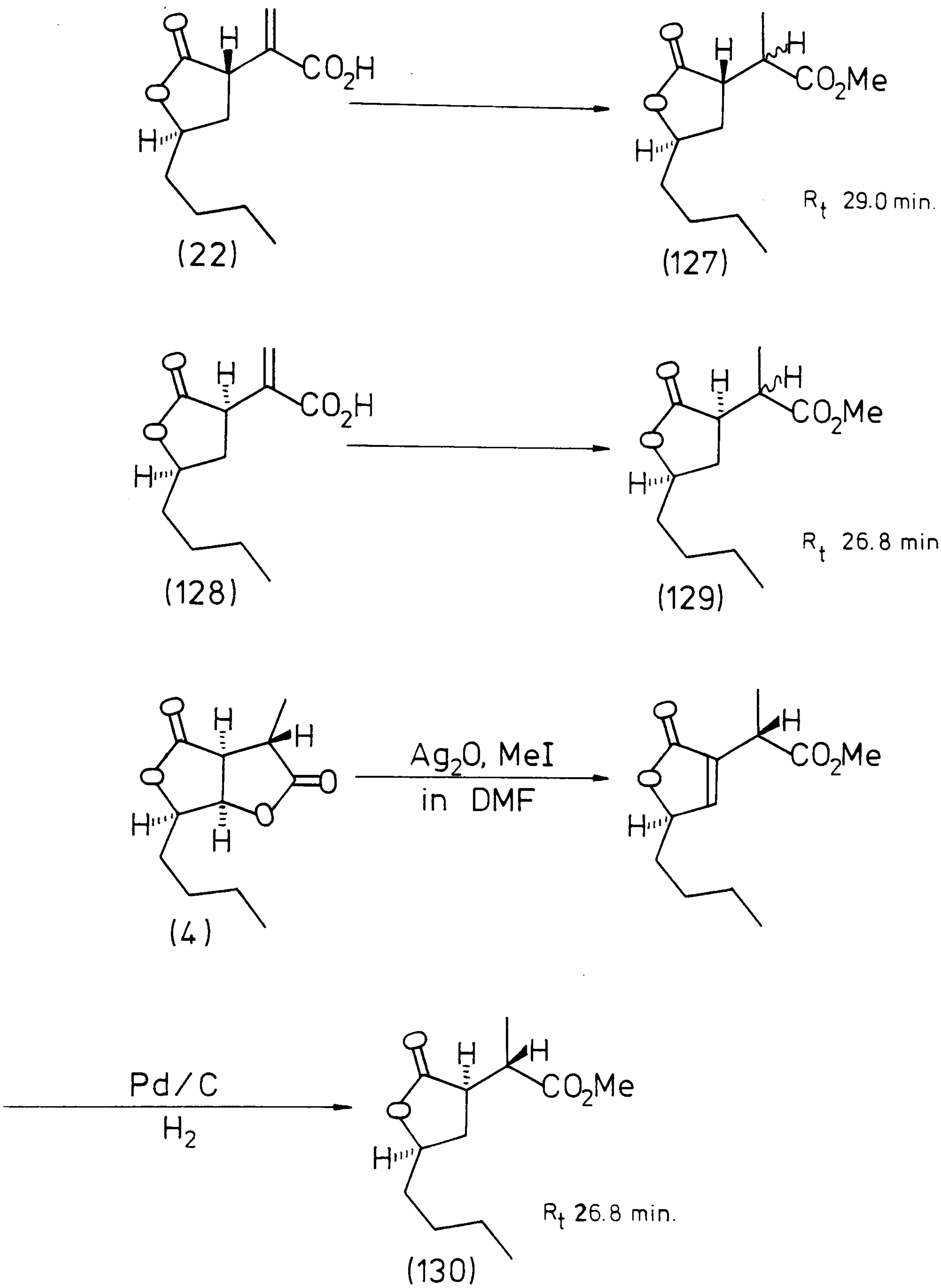


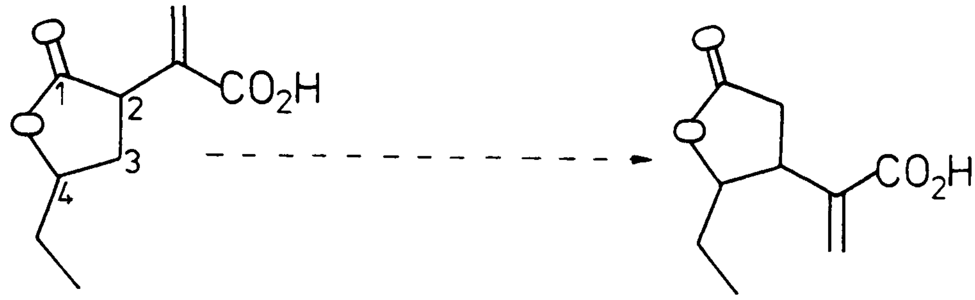
Fig. 33

gave dihydro derivatives (127, $R_t$ 29.0 min.) and (129, $R_t$ 26.8 min.). The *cis* methyl dihydrocanadensate (130, $R_t$ 26.8 min.) was obtained independently from dihydrocanadensolide (4) as shown[115] (Scheme 33). The fact that only the *trans* isomer of canadensic acid (22) is obtained by the above synthesis might arise if the stereochemistry proceeded as shown in Scheme 32, *i.e.* formation of a *trans* bromo compound (124) would be expected and subsequent displacement of the bromo group would be expected to occur with inversion of configuration. Therefore, in order to end up with *trans* C-2/C-4 stereochemistry, epimerisation at C-2 would have to occur either before or during hydrolysis to give the diacid (126).

Similarly, the $C_9$ homologue (122) consisted mainly of the one isomer (*trans*?) from its $^1H$ n.m.r. spectrum, although a mixture of *cis* and *trans* isomers had been expected. The $C_9$ homologue (122) was synthesised in order to test the substrate specificity of the enzyme responsible for the $B_{12}$-coenzyme catalysed rearrangement of *n*-butylitaconic acid (30a) to α-methylene-β-*n*-propylglutaric acid (32a) in cultures of *P. decumbens*. It was of interest to know if rearrangement would take place after hydroxylation and lactonisation at C-4 (Fig. 34). However, the non-incorporation of the lactone (31a) into ethisolide (1) by cultures of *P. decumbens* makes the incorporation of this acid (122) unlikely.


$CO_2H$
(22)
H
$CO_2Me$
$R_t$ 29.0 min.
(127)
$CO_2H$
(128)
$CO_2Me$
$R_t$ 26.8 min.
(129)
(4)
$Ag_2O$, MeI
in DMF
$CO_2Me$
Pd/C
$H_2$
$CO_2Me$
$R_t$ 26.8 min.
(130)

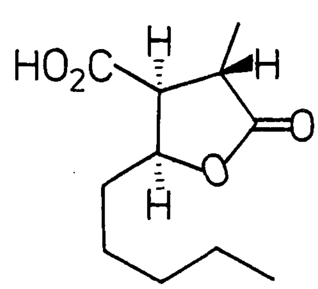
Scheme 33




(122) (31a)

Fig. 34

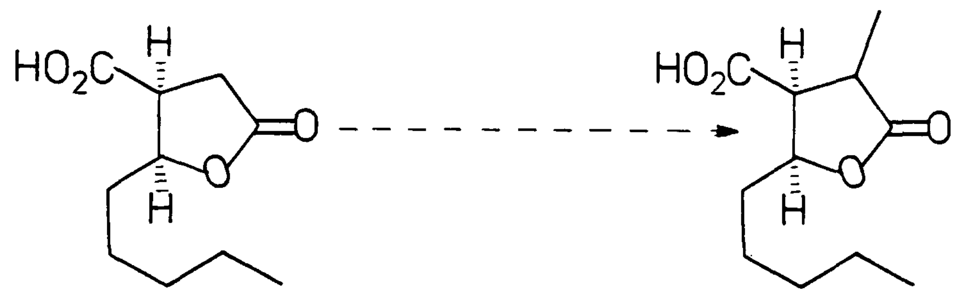
Dihydroisocanadensic acid (93) is, on the other hand, one of the proposed intermediates in the biosynthesis of dihydrocanadensolide (4) (Scheme 27) and the synthesis of a labelled sample would be desirable for incorporation studies. A previous attempt to effect




(93)

the desired *trans* reduction of isocanadensic acid (92) using zinc dust and acetic acid was unsuccessful and the method was discontinued.[116]

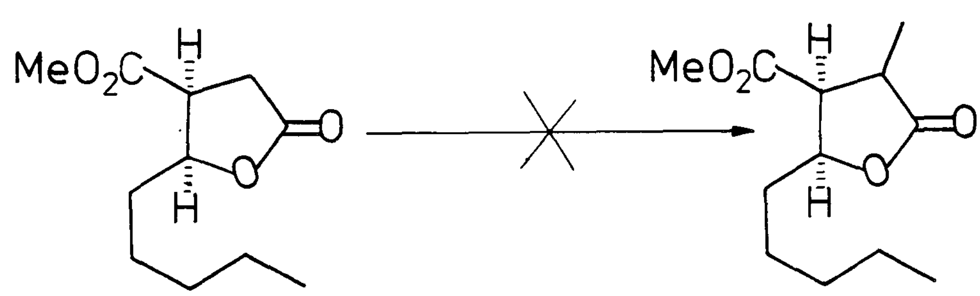
One method for its synthesis would be by reduction of proto-isocanadensic acid (96), but as the synthesis proved to be rather long and complex a more direct route was wanted. The most obvious route would be by direct methylation of the *cis* lactone acid (110) (Fig. 35).




(110) (93)

Fig. 35

A number of methods for the α-alkylation of γ-butyrolactone enolates have been reported,[117,118,119] involving the use of lithium diisopropylamide, the appropriate alkyl iodide and, in some cases,[118,119] HMPA. However, attempted alkylation of the *cis* lactone methyl ester (131) under similar conditions to those above gave only unreacted material (Fig. 36) and this method was not investigated further.




(131)

Fig. 36

A second α-methylation method, involving the alkylation of the O-silylated lactone enolates[108] (132, 133) (Fig. 37) also failed, when the reported method for the preparation of the O-silylated compounds[120] afforded only unreacted lactone acid (110) or lactone methyl ester (131).

Another possible synthesis would be by alkylation of the diacid lactone (101) followed by decarboxylation (*cf.* Scheme 31), but due to lack of time this was not pursued further.

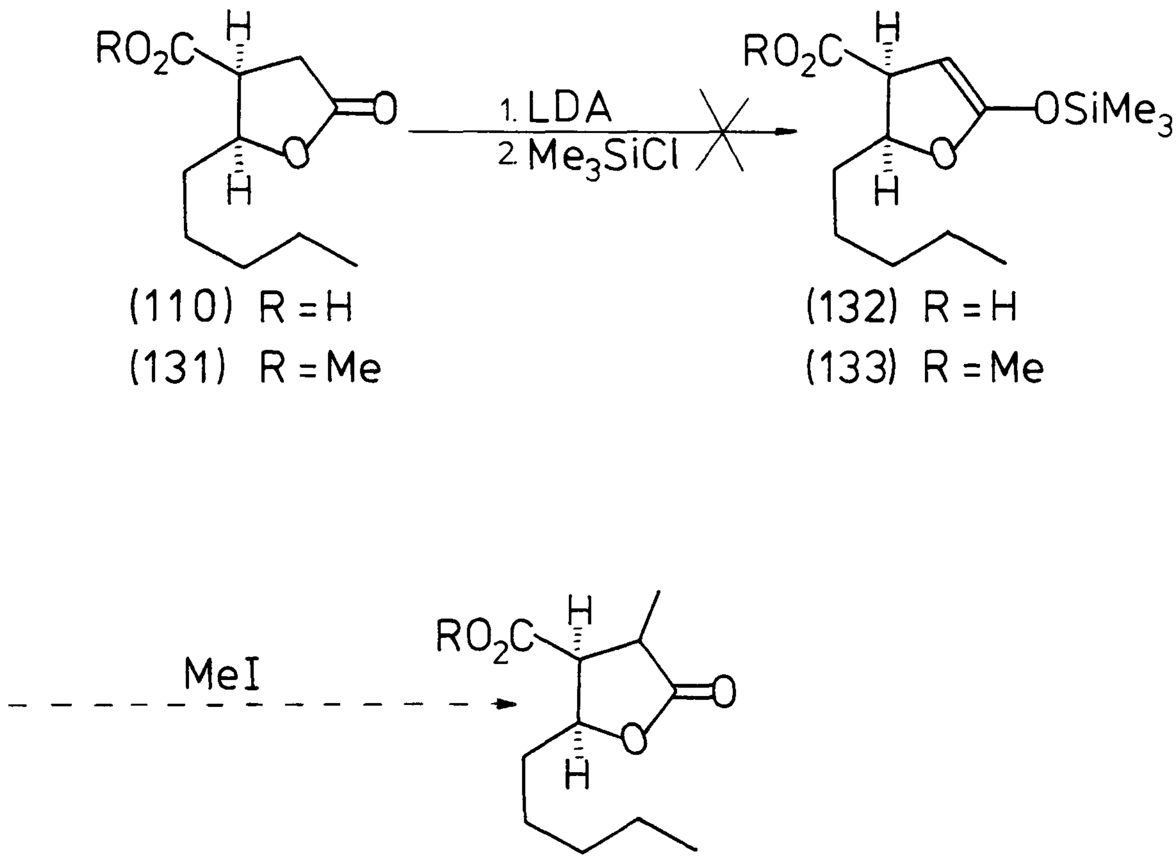



Fig. 37

# CHAPTER 5

## Experimental Section

I General

II Biosynthesis of the Type B Bislactones

(a) Synthesis of α-[$^{14}$C-methylene]-β-*n*-alkylglutaric acids (32) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

(b) Synthesis of β-(1-Hydroxyalkyl)-α-[$^{14}$C-methylene]-glutaric acid lactones (31) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

III Attempted Syntheses of Ethisic Acid (27a)

(a) Attempted Syntheses *via* Halo-esters

(b) Attempted Syntheses *via* Hydroxy-esters

(c) *Via* 2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde

IV Biosynthesis of the Type A Bislactones

(a) Synthesis of Isocanadensic acid (92) and Protoisocanadensic acid (96)

(b) Synthesis of Canadensic acid (22) and the $C_9$ Homologue (122) relevant to Ethisolide Biosynthesis and Attempted Syntheses of Dihydroisocanadensic acid (93)

## I. General

The three cultures studied in this thesis are *Penicillium decumbens* (3903 OOT), *Penicillium canadense* (Commonwealth Mycological Institute No. 95493) and *Aspergillus avenaceus* (AGG 4558). The fungi were subcultured on to 2% malt agar slants and thence to agar seed bottles prior to inoculating Roux surface culture bottles containing culture medium (200 ml) which had previously been sterilised (0.5 h with steam at 117°C and 12 p.s.i.).

*P. decumbens* and *A. avenaceus* were grown on a culture medium (Czapek-Dox + 0.1% yeast extract) containing glucose (50 g), $NaNO_3$ (2 g), KCl (1 g), $MgSO_4.7H_2O$ (1 g), $K_2HPO_4$ (0.5 g), $FeSO_4.7H_2O$ (0.01 g) and yeast extract (0.1 g) per litre of distilled water, while *P. canadense* was grown on a culture medium containing glucose (50 g), ammonium tartrate (2.8 g), $K_2HPO_4$ (5 g), $MgSO_4.7H_2O$ (1 g), NaCl (1 g), yeast extract (0.5 g), $FeSO_4.7H_2O$ (0.1 g), $ZnSO_4.7H_2O$ (0.05 g), $CuSO_4.5H_2O$ (0.015 g), $MnSO_4$(0.01 g) and $NaMoO_4$(0.01 g) per litre of distilled water.

The cultures were allowed to grow undisturbed at 25°C and 70% relative humidity, artificial illumination being provided by fluorescent tubes for eight hours per day.

Thanks and recognition are due to Mrs. Pearl Tait and her staff of the Mycology Unit who prepared all of the cultures used in this work.

Radioactive assays were carried out using a Panax Radiochromatogram Scanner System RTLS-1A and a Phillips Liquid Scintillation Counter. Samples were weighed on metal foil, transferred to Packard scintillation vials and dissolved in toluene scintillation solution (*ca.* 15 ml) or, where necessary, in Packard Insta-Gel emulsifier. The scintillation solution contained 2,5-diphenyloxazole (4 g) and 1,4-bis-2(4-methyl-5-phenyloxazalyl)-benzene (0.1 g) per litre of toluene. The samples were crystallised until constant activity was achieved and counted sufficiently long to achieve an error of less than 3%.

$^{13}C$ N.m.r. spectra were recorded using a Varian XL-100 F.T. spectrometer and, unless otherwise stated, the spectra were determined in $CDCl_3$ solutions containing tetramethylsilane as an internal reference.

$^{1}H$ N.m.r. spectra were recorded using Varian T-60, XL-100 and Perkin-Elmer R32 spectrometers using, unless otherwise stated, $CDCl_3$ solutions with tetramethylsilane as an internal reference.

I.r. spectra were recorded using Perkin-Elmer 197, 257, 580 and Pye Unicam SP3-300 spectrophotometers. Mass spectra were recorded on a G.E.C.-A.E.I. M.S. 12 spectrometer (low resolution) and a G.E.C.-A.E.I. M.S. 902 spectrometer (high resolution).

Melting points were recorded on a Reichert hot-stage apparatus and are uncorrected.

Unless otherwise stated, all organic extracts were washed with satd. aqueous NaCl and dried over anhyd. $Na_2SO_4$ or anhyd. $MgSO_4$ prior to evaporation. Light petroleum refers to the fraction boiling between 60-80°C unless otherwise stated.

## Abbreviations

I.R. : s strong; m medium; w weak; br broad.

N.M.R. : s singlet; d doublet; t triplet; q quartet;
m multiplet; br broad.

## Experimental Section

## II Biosynthesis of the Type B Bislactones

(a) Synthesis of $\alpha$-[$^{14}$C-methylene]-$\beta$-*n*-alkylglutaric acids (32) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

(b) Synthesis of $\beta$-(1-Hydroxyalkyl)-$\alpha$-[$^{14}$C-methylene]- glutaric acid lactones (31) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

(a) Synthesis of α-[$^{14}$C-methylene]-β-*n*-alkylglutaric acids (32) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

Methyl *trans* hex-2-enoate (33a)

Redistilled *n*-butanal (144 g) was added over 1 h to a cooled (4°C) mixture of malonic acid (100 g), methanol (135 ml) and pyridine (85 ml). The mixture was then gently warmed to dissolve the malonic acid and stirred at room temperature for 2 h. After refluxing for a further 3 h, the mixture was acidified with conc. HCl to pH 1 and extracted thoroughly with ether. The resulting crude product was esterified by refluxing with methanol (80 ml) containing conc. $H_2SO_4$ (6 ml) overnight. Ether and water were then added and the ether layer separated to give the ester (33a), b.p. 56-58°C/13 mm, (80 g, 31%), (Lit.[121] b.p. 32°C/0.2 mm).

I.R. $\nu_{max}$ (film), 1720 (s, ester C=O); 1660 (m, C=C); 980 (m, *trans* -CH=CH-) cm$^{-1}$.

N.M.R. (60 MHz) δ, 0.9 (3H, br t, H-6); 2.1 (4H, br s, H-4 and H-5); 3.7 (3H, s, $-OCH_3$); 5.8 (1H, d, $J$ = 15 Hz, H-2); 7.0 (1H, d-t, $J$ = 15 Hz, 7 Hz, H-3).

Methyl *trans* dodec-2-enoate (33b)

The above procedure was repeated using *n*-decanal (100 g), malonic acid (32 g), methanol (50 ml) and pyridine (28 ml) to give the crude acid which was esterified as before by heating in methanol (50 ml) and conc. $H_2SO_4$ (2 ml) to give methyl *trans* dodec-2-enoate (33b), b.p. 70-75°C/0.02 mm (Kugelrohr), (44 g, 33%), (Lit.[122] b.p. 89-91°C/0.63 mm).

I.R. $\nu_{max}$ ($CHCl_3$), 1720 (s, ester C=O); 1660 (m, C=C); 987 (m, -CH=CH-) cm$^{-1}$

N.M.R. (90 MHz) δ, 0.84 (3H, br t, $-CH_3$); 1.25 (14H, br s, $-CH_2-$); 2.15 (2H, m, H-4); 3.71 (3H, s, $-OCH_3$); 5.78 (1H, d-t, $J$ = 15 Hz, 1.5 Hz, H-2); 6.94 (1H, d-t, $J$ = 15 Hz, 7 Hz, H-3).

Methyl *trans* undec-2-enoate (33c)

The above procedure was repeated using *n*-nonanal (25 g), malonic acid (9.2 g), methanol ( 13.5 ml) and pyridine (7.8 ml). The residue was heated with methanol (20 ml) and conc. $H_2SO_4$ (1 ml) and extracted as before to give methyl *trans* undec-2-enoate (33c), b.p. 90°C/0.4 mm, (13.8 g, 40%), (Lit.[123] b.p. 79°C/0.15 mm).

I.R. $\nu_{max}$ (film), 1730 (s, ester C=O); 1660 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.87 (3H, br t, $-CH_3$); 1.28 (12H, br s, $-CH_2-$); 2.18 (2H, m, H-4); 3.72 (3H, s, $-OCH_3$); 5.81 (1H, d-t, $J$ = 15 Hz, 1.5 Hz, H-2); 6.97 (1H, d-t, $J$ = 15 Hz, 7 Hz, H-3).

Methyl 3-(dicarbomethoxymethyl)hexanoate (34a)

Methyl *trans* hex-2-enoate (33a) (9.65 g) in dry DMF (10 ml) was added dropwise to a solution of dimethyl sodiomalonate [prepared from sodium hydride (2.01 g) and dimethyl malonate (10 ml) in dry DMF (20 ml)] and the solution stirred and heated under reflux overnight. The solution was evaporated and the residue taken up in water and extracted with ether to give an oil (15 g). Distillation gave *methyl 3-(dicarbomethoxymethyl)hexanoate* (34a) b.p. ~120°C/0.8 mm, (8 g, 41%).

I.R. $\nu_{max}$ (film), 1735 (s, ester C=O) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, m, $-CH_3$); 1.4 (4H, m, H-4 and H-5); 2.4-2.6 (3H, m, H-2 and H-3); 3.66 (4H, s, H-2' and $-OCH_3$); 3.78 (6H, s, $-OCH_3$).

Analysis Found: C, 55.56; H, 7.76. $C_{12}H_{20}O_6$ requires C, 55.37; H, 7.74%.

Methyl 3-(dicarbomethoxymethyl)undecanoate (34c)

Methyl *trans* undec-2-enoate (33c) (13.7 g) in dry DMF (50 ml) was added to a solution of dimethyl sodiomalonate [prepared from sodium hydride (1.68 g) and dimethyl malonate (9.25 g) in dry DMF (50 ml)] and the mixture heated to 60°C for 12 h. After cooling, the usual workup afforded *methyl 3-(dicarbomethoxymethyl)undecanoate* (34c) as an oil (16.7 g, 73%) b.p. 150°C/0.03 mm (kugelrohr).

I.R. $\nu_{max}$ ($CCl_4$), 1745, 1740 (s, ester C=0) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.87 (3H, br t, $-CH_3$); 1.2-1.6 (14H, br s, H-4 to H-10); 2.52 (3H, m, H-2 and H-3); 3.6 (1H, d, $J$ = 7 Hz, H-2'); 3.68 (3H, s, $-OCH_3$); 3.74 (6H, s, $-OCH_3$).

Mass Spectrum m/e (relative abundance) $M^+$ 330 (1), 299 (10, M - $OCH_3$), 257 (6, M - $CH_2CO_2Me$), 225 (5), 217 (5), 199 (25, $CH_3C_9H_{18}CO_2Me$), 132 (100, $CH_2(CO_2Me)_2$).

Mass Measurement Found: $M^+$ 330.2034. $C_{17}H_{30}O_6$ requires 330.2042.

2-Carboxy-3-*n*-propylglutaric acid (35a)

The trimethyl ester (34a) (1.58 g) and 1M aqueous sodium hydroxide (32.3 ml) were stirred overnight at room temperature. The mixture was then heated at 70°C for 3 h and after cooling, acidified at 0°C with dilute HCl, saturated with solid NaCl and thoroughly extracted with ethyl acetate to give *2-carboxy-3-n-propylglutaric acid* (35a) (0.93 g, 66%), m.p. 122-124°C from ethyl acetate/light petroleum.

I.R. $\nu_{max}$ (nujol), 3300-2400 (s, $-CO_2H$); 1710 (s, acid C=0) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$ ($C_5D_5N$), 0.9 (3H, br t, $-CH_3$); 1.8 (4H, m, $-CH_2-$); 3.2 (3H, m, H-3 and H-4); 4.4 (1H, d, $J$ = 6 Hz, H-2); 12.6 (3H, s, $-CO_2H$).

Analysis Found: C, 49.66; H, 6.42. $C_9H_{14}O_6$ requires C, 49.54; H, 6.42%.

2-Carboxy-3-*n*-nonylglutaric acid (35b)

Methyl *trans* dodec-2-enoate (33b) (13.23 g) in dry DMF (20 ml) was added dropwise to a suspension of dimethyl sodiomalonate [prepared from sodium hydride (1.48 g) and dimethyl malonate (7.2 ml) in dry DMF (50 ml)] and the mixture heated to 70°C overnight. The solvent was removed under reduced pressure and the residue taken up in water and thoroughly extracted with ether to give the trimethyl ester (34b) as a red oil (16.4 g, 76%) (i.r. showed no band at 1660 $cm^{-1}$).

A portion of the crude triester (34b) (9.7 g) was treated with 1M aqueous NaOH (140 ml) at room temperature overnight, then heated to 100°C for 3 h. After cooling, the solution was extracted with ether to give unreacted ester (3 g). The aqueous layer was acidified with conc. HCl and extracted with ether to give a semi-solid oil which crystallised from ethyl acetate/light petroleum (b.p. 100-120°C) to give *2-carboxy-3-n-nonylglutaric acid* (35b) (3.9 g, 66%), m.p. 115-117°C.

I.R. $\nu_{max}$ (nujol), 3600-2400 (s, $-CO_2H$); 1700 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($C_5D_5N$), 0.8 (3H, br t, $-CH_3$); 1.15-2.0 (16H, br m, $-CH_2-$); 3.16 (3H, br m, H-3 and H-4); 4.2 (1H, d, $J$ = 5 Hz, H-2); 8.5 (3H, s, $-CO_2H$).

Analysis Found: C, 60.14; H, 8.8. $C_{15}H_{26}O_6$ requires C, 59.60; H, 8.66%.

Mass Spectrum m/e (relative abundance) $M^+$ 302 (1), 284 (3, M - $H_2O$), 240 (26, M - 18 - 44), 199 (100, M - $CH(CO_2H)_2$).

2-Carboxy-3-*n*-octylglutaric acid (35c)

The triester (34c) (8.07 g) in aqueous 1M NaOH (150 ml) was stirred at room temperature overnight. After heating at 100°C for 6 h, the mixture was cooled and worked up in the usual manner to give an oil (6.4 g). Crystallisation gave *2-carboxy-3-n-octylglutaric acid* (35c) (3.6 g, 51%) m.p. 99-101°C from ethyl acetate/light petroleum.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1710 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.87 (3H, br t, $-CH_3$); 1.2-1.6 (15H, br s, H-3 and $-CH_2-$); 2.56 (2H, m, H-4); 3.67 (1H, d, $J$ = 6 Hz, H-2); 10.7 (3H, br s, $-CO_2H$).

Analysis Found: C, 58.26; H, 8.34. $C_{14}H_{24}O_6$ requires C, 58.32; H, 8.39%.

α-Methylene-β-*n*-propylglutaric acid (32a)

A solution of 2-carboxy-3-*n*-propylglutaric acid (35a) (0.68 g) in methanol (2 ml) was treated with 40% aqueous dimethylamine (5 ml), stirred at -10°C for 10 mins. and 37% aqueous formaldehyde (10 ml) in methanol (2 ml) was added dropwise. The mixture was stirred overnight at room temperature, refluxed for 2 h and evaporated at reduced pressure. The residue was taken up in water, acidified at 0°C with dilute HCl, saturated with solid NaCl and thoroughly extracted with ethyl acetate. Removal of the solvent gave *α-methylene-β-n-propylglutaric acid* (32a) (0.28 g, 66%), m.p. 55-57°C from ethyl acetate/light petroleum.

I.R. $\nu_{max}$ (nujol), 3300-2400 (s, $-CO_2H$); 1700 (s, acid C=O); 1620 (m, C=C); 920 (m, $C=CH_2$) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, m, $-CH_3$); 1.5 (4H, m, $-CH_2-$); 2.6 (2H, d, $J$ = 7 Hz, $-\underline{CH_2}CO_2H$); 3.0 (1H, m, H-β); 5.8 (1H, s, $H_b$); 6.5 (1H, s, $H_a$); 11.2 (2H, s, $-CO_2H$).

Analysis Found: C, 57.91; H, 7.6. $C_9H_{14}O_4$ requires C, 58.06; H, 7.53%.

Mass Spectrum m/e (relative abundance) $M^+$ 186 (4), 168 (20, M - $H_2O$), 143 (31, M - $C_3H_7$), 140 (47, M - 18 - 28), 127 (73, M - $CH_2CO_2H$), 109 (63), 98 (100).

Mass measurement Found: 186.0886. $C_9H_{14}O_4$ requires 186.0892 a.m.u.

α-Methylene-β-*n*-nonylglutaric acid (32b)

2-Carboxy-3-*n*-nonylglutaric acid (35b) (0.456 g) in methanol (2 ml) at -20 °C was treated with 40% dimethylamine (2 ml) and methanol (2 ml) and the solution stirred for 30 mins. 37% Aqueous formaldehyde (2 ml) in methanol (2 ml) was then added dropwise and the mixture stirred at room temperature overnight. After heating to 85°C for 1 h, the solvent was removed under reduced pressure and the residue was taken up in water and acidified at 0°C with dilute HCl, saturated with solid NaCl, and thoroughly extracted with ethyl acetate to give *α-methylene-β-n-nonylglutaric acid* (32b) as an off-white solid (340 mg, 83%), m.p. 46-48 °C from light petroleum(b.p. 40-60°C).

I.R. $\nu_{max}$ (nujol), 3500-2400 (m, $-CO_2H$); 1695 (s, acid C=O); 1625 (m, C=C); 920 (m, $C=CH_2$) $cm^{-1}$.

N.M.R. (60 MHz) δ, 0.9 (3H, br t, $-CH_3$); 1.25 (16H, m, $-CH_2-$); 2.56 (2H, br d, $-\underline{CH_2}CO_2H$); 2.99 (1H, m, H-β); 5.69 (1H, s, $H_b$); 6.40 (1H, s, $H_a$); 11.5 (2H, s, $-CO_2H$).

Analysis Found: C, 66.95; H, 9.67. $C_{15}H_{26}O_4$ requires C, 66.64; H, 9.69%.

Mass Spectrum m/e (relative abundance) $M^+$ 270 (20). 252 (100, M - $H_2O$), 234 (32, M - 36), 224 (34, M - 18 - 28).

α-Methylene-β-*n*-octylglutaric acid (32c)

2-Carboxy-3-*n*-octylglutaric acid (35c) (562 mg) in methanol (3 ml) was treated at -10°C with diethylamine (0.4 ml), stirred for 30 mins. and 37% aqueous formaldehyde (0.9 ml) added. The mixture was warmed to room temperature and stirred overnight. After refluxing for 1 h, the mixture was cooled, the solvent evaporated and the residue taken up in water, acidified with dilute HCl and extracted with ether to give *α-methylene-β-n-octylglutaric acid* (32c) (260 mg, 52%) m.p. 58-59.5°C from light petroleum (b.p. 40-60°C).

I.R. $\nu_{max}$ (KBr), 3500-2400 (s, $-CO_2H$); 1690 (br s, acid C=O); 1622 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.87 (3H, br t, $-CH_3$); 1.2-1.7 (14H, br s, $-CH_2-$); 2.59 (2H, d, $J$ = 7 Hz, $-\underline{CH}_2CO_2H$); 3.04 (1H, m, H-$\beta$); 5.71 (1H, s, $H_b$); 6.41 (1H, s, $H_a$); 12.0 (2H, s, $-CO_2H$).

Analysis Found: C, 65.61; H, 10.09. $C_{14}H_{24}O_4$ requires C, 65.60; H, 9.43%.

α-[$^{14}$C-Methylene]-β-*n*-propylglutaric acid (32a)

To a stirred solution containing 2-carboxy-3-*n*-propylglutaric acid (35a) (75 mg), methanol (2.5 ml) and 40% aqueous dimethylamine (0.34 ml) under nitrogen, at -10°C, was added [$^{14}$C]-formaldehyde (500 μCi) in distilled water (1 ml). After stirring at -10°C for 30 mins., 37% aqueous formaldehyde (0.06 ml) in methanol (1 ml) was added and the mixture stirred at room temperature overnight. The mixture was now refluxed and stirred for 1 h. After evaporation, the residue was acidified with dilute aqueous HCl, saturated with solid NaCl and thoroughly extracted with ethyl acetate, to give an oil (70 mg). The oil was diluted with a sample of pure α-methylene-β-*n*-propylglutaric acid (25 mg). Preparative t.l.c. (EtOAc) and several crystallisations from light petroleum (b.p. 40-60°C) then giving α-[$^{14}$*C-methylene*]-β-*n-propylglutaric acid* (32a) $R_f$ 0.75 (23 mg, $1.02 \times 10^6$ dpm/mg), m.p. 58-59°C.

α-[$^{14}$C-Methylene]-β-*n*-nonylglutaric acid (32b)

To a stirred solution containing 2-carboxy-3-*n*-nonylglutaric acid (35b) (87 mg), methanol (3 ml) and a solution of dimethylamine [40% aqueous (0.3 ml)] in methanol (1 ml) under argon, at -10°C, was added [$^{14}$C]-formaldehyde (∿250 μCi) in distilled water (1 ml). After stirring at -10°C for 30 mins., 37% aqueous formaldehyde (0.07 ml) in methanol (1.5 ml) was added and the mixture stirred at room temperature overnight. The mixture was now refluxed and stirred for 1 h. After evaporation, the residue was taken up in distilled water, acidified with dilute HCl, saturated with solid NaCl and thoroughly extracted with ethyl acetate to give an oil which crystallised from ethyl acetate/light petroleum (b.p. 100-120°C) (4x) to give a white solid (40 mg, m.p. 115.5-117°C) consisting (m.p., t.l.c.) of unreacted triacid (35b). The combined mother liquors were diluted with a sample of pure α-methylene-β-*n*-nonylglutaric acid (41 mg). Crystallisation from light petroleum (b.p. 40-60°C) gave *α-[$^{14}$C-methylene]-β-n-nonylglutaric acid* (32b) (53 mg, $5.1882 \times 10^5$ dpm/mg), m.p. 46-48°C.

α-[$^{14}$C-Methylene]-β-*n*-octylglutaric acid (32c)

2-Carboxy-3-*n*-octylglutaric acid (35c) (52 mg) in methanol (0.5 ml) was treated at -10°C with diethylamine (40 μl), stirred for 30 mins. and [$^{14}$C]-formaldehyde (250 μCi) in distilled water (1 ml) added. After stirring for an additional 30 mins., 37% aqueous formaldehyde (80 μl) was added. The mixture was stirred overnight at room temperature, refluxed for 1 h and evaporated at reduced pressure. The residue was taken up in water, acidified at 0°C with dilute HCl and extracted with ether to give an oil (46 mg). The oil was diluted with a sample of pure α-methylene-β-*n*-octylglutaric acid (21 mg) and several crystallisations from light petroleum (b.p. 40-60°C) gave *α-[$^{14}$C-methylene]-β-n-octylglutaric acid* (32c) (32.2 mg, $7.627 \times 10^4$ dpm/mg) m.p. 58-59.5°C.

<u>Incorporation of [$^{14}$C]-acetate into Ethisolide by cultures of *P. decumbens*</u>

[$^{14}$C]-Acetate (20 μCi) as its sodium salt was fed, in two 24 h pulses, on the 8th and 9th days after inoculation, to two Roux bottles containing cultures of *P. decumbens*. On the 14th day after inoculation, the aqueous broth was separated by decantation and continuously extracted with ethyl acetate for 48 h, after which, the ethyl acetate was washed with brine, dried and evaporated to give ethisolide (1) (590 mg, $1.39 \times 10^4$ dpm/mg), m.p. 122-122.5°C from ethanol (Lit.[5] m.p. 122-123°C). The total activity isolated as [$^{14}$C]-ethisolide was $8.201 \times 10^6$ dpm, giving an incorporation of 18.4% from [$^{14}$C]-acetate.

<u>Incorporation of α-[$^{14}$C-Methylene]-β-*n*-propylglutaric acid (32a) into Ethisolide (1) by cultures of *P. decumbens*</u>

(i) α-[$^{14}$C-Methylene]-β-*n*-propylglutaric acid (10.7 mg, 4.91 μCi) as its sodium salt was sterilised and fed, in three 24 h pulses, on the 7th, 8th and 9th days after inoculation, to three Roux bottles containing cultures of *P. decumbens*. On the 14th day after inoculation, the aqueous broth was separated by decantation and continuously extracted with ethyl acetate for 72 h, after which, the ethyl acetate was extracted with satd. aqueous $NaHCO_3$, washed with brine, dried and evaporated to give ethisolide (1) (540 mg, 5335 dpm/mg), m.p. 122-123°C from ethanol (Lit.[5] m.p. 122-123°C). The total activity isolated as [$^{14}$C]-ethisolide was $2.89 \times 10^6$ dpm, giving an incorporation of 26% from α-[$^{14}$C-methylene]-β-*n*-propylglutaric acid;dilution 194.6.

(ii) α-[$^{14}$C-Methylene]-β-*n*-propylglutaric acid (35.4 mg, 1.905 μCi) as its sodium salt was fed, in two 24 h pulses, on the 8th and 9th days after inoculation, to two Roux bottles containing cultures of *P. decumbens*. On the 15th day after inoculation the aqueous broth was separated and extracted as above to give ethisolide (1) (280 mg, 3015 dpm/mg), m.p. 122-123°C from ethanol (Lit.[5] m.p. 122-123°C). The total activity isolated as [$^{14}$C]-ethisolide was $8.442 \times 10^5$ dpm, giving an incorporation of 20% from α-[$^{14}$C-methylene]-β-*n*-propylglutaric acid; dilution 40.5.

Distribution of label in [$^{14}$C]-Ethisolide biosynthesised from α-[$^{14}$C-methylene]-β-*n*-propylglutaric acid (32a)

(i) The above [$^{14}$C]-ethisolide (87 mg, 5277 dpm/mg) was dissolved in glacial acetic acid (15 ml) and a slow stream of ozonised oxygen was passed through the solution for 1.5 h at room temperature. 2N HCl (6 ml) was added and the solution left for 2 h. After the addition of glacial acetic acid (6 ml), the solution was left overnight whilst nitrogen flushed the apparatus into a trap containing dimedone (134 mg), piperidine (1 ml) and ethanol (12 ml). The solution was then steam distilled and once *ca*. 50 ml of distillate had collected, the dimedone solution was boiled for 15 mins. and allowed to cool giving methylene-bis-dimedone (86.8 mg, 62%, 3024 dpm/mg), m.p. 192-193°C from ethanol/water (Lit.[124] m.p. 191-191.5°C). The [$^{14}$C]-ethisolide used had an activity of $9.6127 \times 10^5$ dpm/mmole. This corresponds to 92.3% of the activity of the [$^{14}$C]-ethisolide being located in the terminal methylene carbon.

(ii) [$^{14}$C]-Ethisolide (73.7 mg, 5277 dpm/mg) was treated as above and the formaldehyde trapped in a solution of dimedone (118 mg), piperidine (1 ml) and ethanol (12 ml). This yielded methylene-bis-dimedone (63 mg, 53%, 3212.5 dpm/mg), m.p. 192-192.5°C from ethanol/water (Lit.[124] m.p. 191-191.5°C). The [$^{14}$C]-ethisolide used had an activity of $9.6127 \times 10^5$ dpm/mmole. This corresponds to 97.2% of the activity of the [$^{14}$C]-ethisolide being located in the terminal methylene carbon.

Incorporation of $\alpha$-[$^{14}$C-Methylene]-$\beta$-*n*-nonylglutaric acid (32b) into Avenaciolide (2) by cultures of *A. avenaceus*

$\alpha$-[$^{14}$C-Methylene]-$\beta$-*n*-nonylglutaric acid (53 mg, 12.5 $\mu$Ci) as its sodium salt was sterilised and fed, in five 12 h pulses, on the 5th, 6th and 7th days after inoculation, to three Roux bottles containing cultures of *A. avenaceus*. On the 14th day after inoculation, the aqueous broth was separated by decantation, acidified to pH 3 with conc. HCl and continuously extracted with ethyl acetate for 48 h. The organic phase was dried and evaporated to give a dark oil, which was taken up in chloroform, filtered and evaporated to give a red oil (0.72 g). Preparative t.l.c. ($CHCl_3$) gave avenaciolide (3) $R_f$ 0.3 (96.7 mg, 39689 dpm/mg), m.p. 54-55°C from ether/light petroleum (b.p. 40-60°C) (Lit.[6] m.p. 54-56°C). The total activity isolated as [$^{14}$C]-avenaciolide was $2.94 \times 10^6$ dpm, giving an incorporation of 10.6% from $\alpha$-[$^{14}$C-methylene]-$\beta$-*n*-nonylglutaric acid; dilution 13.39.

Distribution of label in [$^{14}$C]-Avenaciolide biosynthesised from $\alpha$-[$^{14}$C-methylene]-$\beta$-*n*-nonylglutaric acid (32b)

(i) The above [$^{14}$C]-avenaciolide (17.53 mg, $3.9 \times 10^4$ dpm/mg) and cold avenaciolide (25.62 mg) were dissolved in glacial acetic acid (10 ml) and a slow stream of ozonised oxygen was passed through the solution for 2 h at room temperature. 2N HCl (6 ml) was added and the solution left for 2 h. After the addition of glacial acetic acid (6 ml), the solution was left overnight whilst argon flushed the apparatus into a trap containing dimedone (45.5 mg), piperidine (0.7 ml) and ethanol (10 ml). The solution was then steam distilled and once *ca.* 50 ml of distillate had collected, the dimedone solution was boiled for 15 mins. and allowed to cool giving methylene-bis-dimedone (27.4 mg, 58%, 13250 dpm/mg), m.p. 190-191°C from ethanol/water (Lit.[124] m.p. 191-191.5°C). The diluted [$^{14}$C]-avenaciolide used had an activity of $4.2 \times 10^6$ dpm/mmole. This corresponds to 92% of the activity of the [$^{14}$C]-avenaciolide being located in the terminal methylene carbon.

(ii) [$^{14}$C]-Avenaciolide (21.3 mg, $3.9 \times 10^4$ dpm/mg) and cold avenaciolide (53.39 mg) were treated as above and the formaldehyde trapped in a solution of dimedone (80.6 mg), piperidine (0.6 ml) and ethanol (10 ml). This yielded methylene-bis-dimedone (20 mg, 24%, 9391 dpm/mg), m.p. 190-191°C from ethanol/water (Lit.[124] m.p. 191-191.5°C). The diluted [$^{14}$C]-avenaciolide used had an activity of $2.95 \times 10^6$ dpm/mmole. This corresponds to 93% of the activity of the [$^{14}$C]-avenaciolide being located in the terminal methylene carbon.

Incorporation of α-[$^{14}$C-Methylene]-β-*n*-octylglutaric acid (32c) into *nor*-Avenaciolide (2a) by cultures of *A. avenaceus*

α-[$^{14}$C-Methylene]-β-*n*-octylglutaric acid (26.9 mg, 0.9 μCi) as its sodium salt was fed, in five 12 h pulses, on the 7th, 8th and 9th days after inoculation, to three Roux bottles containing cultures of *A. avenaceus*. On the 14th day, the aqueous broth was separated and extracted as before to give a red oil (0.9 g). Preparative t.l.c. ($CHCl_3$) gave an oil, $R_f$ 0.3 (140 mg) which, after further preparative t.l.c. and several crystallisations from ether/light petroleum (b.p. 40-60°C), gave colourless plates (73.4 mg, 1694 dpm/mg), m.p. 52-53°C. The total activity isolated was $1.24 \times 10^5$ dpm, giving an incorporation of 6.2% from α-[$^{14}$C-methylene]-β-*n*-octylglutaric acid. G.l.c. (1% OV-1, 6 ft column at 190°C, nitrogen 22 p.s.i.): $R_t$ 3.6 min. (avenaciolide), $R_t$ 2.5 min. (? *nor*-avenaciolide), ratio *ca*. 40:1.

<u>Trapping experiment designed to detect *n*-Decylitaconic acid (30b) and α-Methylene-β-*n*-propylglutaric acid (32b) in *A. avenaceus*</u>

[$^{14}$C]-Acetate (60 μCi) and α-methylene-β-*n*-propylglutaric acid (32b) (500 mg) were fed in five 12 h pulses, on the 7th, 8th and 9th days after inoculation, to three Roux bottles containing cultures of *A. avenaceus*. On the 10th day after inoculation, the aqueous broth was separated and extracted as before. The organic phase was evaporated to a convenient volume (*ca.* 250 ml) and extracted with satd. aqueous $NaHCO_3$. The organic layer gave, after evaporation and preparative t.l.c. ($CHCl_3$), avenaciolide (2) $R_f$ 0.3 (37 mg, $9.78 \times 10^4$ dpm/mg) m.p. 50-50.5°C from ether/light petroleum (b.p. 40-60°C) (Lit.[6] m.p. 49-50°C). The total activity isolated as [$^{14}$C]-avenaciolide (2) was $3.62 \times 10^6$ dpm, giving an incorporation of 2.7% from [$^{14}$C]-acetate. The aqueous $NaHCO_3$ layer was acidified with dilute HCl and extracted with ethyl acetate to give an oily solid which was dissolved in chloroform and filtered to give crude (32b) (*ca.* 400 mg). Extensive preparative t.l.c. [light petroleum (b.p. 40-60°C):ether:acetic acid, 28:12:1] of a portion (185 mg) separated two components. The first, $R_f$ 0.31, was diluted with a pure sample of *n*-decylitaconic acid (30b) (73 mg) and crystallised to constant activity, giving [$^{14}$C]-*n*-decylitaconic acid (54 mg, $1.384 \times 10^4$ dpm/mg) m.p. 76-77.5°C (Lit.[125] m.p. 77-78°C). The activity isolated as [$^{14}$C]-*n*-decylitaconic acid (30b) was $7.47 \times 10^5$ dpm giving a total incorporation of 1.21% from [$^{14}$C]-acetate (taking into account that only a portion of the crude acid mixture was chromatographed). The second component, $R_f$ 0.37, corresponded to α-methylene-β-*n*-nonyl-glutaric acid (32b) (110 mg, 10.8 dpm/mg) m.p. 48-49°C from light petroleum. The activity isolated as α-methylene-β-*n*-nonylglutaric acid (32b) was 1188 dpm (2561 dpm, taking into account that only a portion of the acid was chromatographed). Hence the incorporation from [$^{14}$C]-acetate was less than 0.002%.

(b) Synthesis of β-(1-Hydroxyalkyl)-α-[$^{14}$C-methylene]-glutaric acid lactones (31) and Incorporation Studies using *P. decumbens* and *A. avenaceus*

Methyl Nonanoate

*n*-Nonanoic acid (45.3 g) was refluxed overnight in methanol (150 ml) containing $H_2SO_4$ (6 ml). After cooling, the mixture was poured into water (250 ml) and extracted with ether to give methyl nonanoate as a pale yellow oil (43 g, 87%) $R_f$ 0.34 (20% ethyl acetate/ light petroleum).

I.R. $\nu_{max}$ (film), 1750 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) δ, 0.86 (3H, br t, $-CH_3$); 1.27 (10H, br s, H-4 to H-8); 1.6 (2H, m, H-3); 2.29 (2H, br t, H-2); 3.67 (3H, s, $-OCH_3$).

This was used in the subsequent reaction without further purification.

1-(Methylsulphonyl)-2-decanone (38b)

Dry dimethyl sulphoxide (125 ml) was added to 50% sodium hydride dispersion in oil (12.3 g) which had been washed 3 times with light petroleum and the mixture stirred at 65°C under argon until evolution of $H_2$ ceased. After cooling in an ice bath, dry THF (125 ml) was added, followed by methyl nonanoate (21.5 g) and the solution stirred at room temperature for a further 30 mins. This was then poured into water (750 ml) and acidified to pH 3 with conc. HCl and extracted with chloroform to give 1-*(methylsulphonyl)-2-decanone* (38b) as colourless crystals (24 g, 88%), m.p. 70.5-71.5°C from ethyl acetate.

I.R. $\nu_{max}$ ($CCl_4$), 1710 (s, C=O); 1090 (s, S=O) $cm^{-1}$.

N.M.R. (90 MHz) δ, 0.82 (3H, br t, H-10); 1.23 (10H, br s, H-5 to H-9); 1.56 (2H, m, H-4); 2.55 (2H, t, $J$ = 7 Hz, H-3); 2.62 (3H, s, $S-CH_3$); 3.72 (2H, q, $J$ = 14 Hz, H-1).

Analysis Found: C, 60.29; H, 10.22; S, 14.4. $C_{11}H_{22}O_2S$ requires C, 60.51; H, 10.16; S, 14.68%.

Attempted formation of 1-(Methylsulphonyl)-2-butanone (38a)

Under similar conditions to those described for the formation of 1-(methylsulphonyl)-2-decanone (38b), methyl propiolate did not form any of the desired product. N.m.r. (90 MHz) contained no 3H singlet at *ca.* 2.5$\delta$, but showed signals at $\delta$, 1.1 (3H, t, $J$ = 7 Hz); 2.72 (2H, q, $J$ = 7 Hz); 4.37 (1H, s). [(39)]. The product was not investigated further.

Methyl-(3-methylsulphonyl)-4-oxododecanoate (40b)

(i) 1-(Methylsulphonyl)-2-decanone (38b) (10.5 g) in dry THF (40 ml) was added to a suspension of sodium hydride in THF (50 ml) under argon (50% sodium hydride dispersion in oil (2.31 g) was used and washed 3 times with light petroleum). After the evolution of $H_2$ ceased, the solution was cooled to 0°C and a solution of methyl bromoacetate (11.3 g) in dry THF (20 ml) added. After stirring at room temperature overnight, the mixture was extracted with chloroform to give *methyl-(3-methylsulphonyl)-4-oxododecanoate* (40b) as colourless crystals (4.6 g, 33%), m.p. 73-74°C from ethanol.

I.R. $\nu_{max}$ ($CHCl_3$), 1735 (s, ester C=O); 1705 (s, C=O); 1290 (s, S=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.84 (3H, br t, H-12); 1.26 (10H, br s, H-7 to H-11); 1.6 (2H, m, H-6); 2.46 (3H, s, S-$CH_3$); 2.7 (4H, m, H-5 and H-2); 3.7 (3H, s, -$OCH_3$); 4.3 (1H, d-d, $J$ = 5Hz, 10 Hz, H-3).

Analysis Found: C, 58.16; H, 8.79; S, 10.93. $C_{14}H_{26}O_4S$ requires C, 57.90; H, 9.02; S, 11.03%

(ii) If the above procedure was repeated without cooling to 0°C before the methyl bromoacetate was added, then the above product (40b) was not obtained. Instead a red oil was produced which contained no 3H singlet at *ca.* 2.46$\delta$, but contained $\delta$ 6.87 (q, $J$ = 16 Hz, ? olefinic H). This product was not investigated further.

Treatment of the Sulphoxide (40b) with Sodium Borohydride

The sulphoxide (40b) (2.87 g) in ethanol (20 ml) at 0°C was added dropwise to an ice-cold solution of sodium borohydride (0.11 g) in ethanol (20 ml) and the mixture stirred overnight. After diluting with water (25 ml), the solution was thoroughly extracted with chloroform to give a pale yellow oil (1.85 g). Distillation afforded ethyl 4-oxododecanoate (41a) (1.46 g, 58%), b.p. 95-98°C/0.1 mm (Lit.[126] b.p. 145-146°C/4 mm).

N.M.R. (60 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.35 (15H, br s, $-OCCH_3$ and H-6 to H-11); 2.2-2.8 (6H, m, H-2 to H-5); 4.16 (2H, q, $J$ = 7 Hz, $-OCH_2-$).

A number of attempts were made to lactonise the ethyl 4-oxododecanoate (41a) produced, all gave unreacted material *e.g.* by distillation at atmospheric or reduced pressure with or without the presence of phosphoric acid, or by refluxing in benzene or xylene with *p*-toluenesulphonic acid. The reaction was not investigated further.

4-Oxododecanoic acid (41b)

Ethyl 4-oxododecanoate (41a) (0.76 g) was treated with 1M aqueous sodium hydroxide (12 ml) and stirred at room temperature overnight followed by heating to 60°C for 1.5 h. After cooling, the mixture was extracted with ether and the aqueous portion acidified with dilute HCl and extracted with ether to give 4-oxododecanoic acid (41b) as colourless crystals (0.62 g, 92%), m.p. 79-80°C (Lit.[127] m.p. 79°C) from light petroleum (b.p. 40-60°C).

I.R. $\nu_{max}$ (KBr), 3500-2500 (s, $-CO_2H$); 1702 (s, C=O); 1696 (s, acid C=O) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.3 (12H, br s, H-6 to H-11); 2.3-2.8 (6H, m, H-2 to H-5); 9.3 (1H, br s, $-CO_2H$).

A number of attempts were made to lactonise 4-oxododecanoic acid (41b) *e.g.* by refluxing in benzene or xylene with *p*-toluenesulphonic acid, by refluxing with trifluoroacetic acid or trifluoroacetic acid/trifluoroacetic anhydride, by heating with acetic anhydride/acetyl chloride to 80°C or by heating in the presence of polyphosphoric acid at 140°C. All gave essentially unchanged keto acid (by spectral data) and the reaction was not investigated further.

## Ethyl Propiolate

To a solution of propiolic acid (28.4 g) in dry ethanol (90 ml) at 0°C was added, dropwise over 10 minutes, sulphuric acid (9 g). The mixture was stirred at room temperature for 51 h and then extracted with ether, washed with satd. aqueous $NaHCO_3$ and dried to give ethyl propiolate (21.7 g, 55%), b.p. 118°C (Lit.[128] b.p. 119°C). I.R. $\nu_{max}$ (film), 3280 (s br, -C≡C-H); 1710 (s, ester C=O) $cm^{-1}$. N.M.R. (90 MHz) $\delta$, 1.28 (3H, t, $J$ = 7 Hz, $-OCCH_3$); 2.83 (1H, s, -C≡C-H); 4.24 (2H, q, $J$ = 7 Hz, $-OCH_2-$).

## Ethyl 4-Hydroxy-2-hexynoate (42a)

To a solution of lithium diisopropylamide (0.014M) in dry THF (20 ml) at -74°C was added redistilled ethyl propiolate (1.387 g). The resulting yellow solution was stirred at -70°C for 70 minutes. Redistilled propanal (0.84 g) was added dropwise at -70°C and the reaction stirred at this temperature for 2 h followed by stirring at -20°C for a further 2 h. The mixture was then recooled to -70°C and quenched with 6M HCl(aq). Extraction with ether gave *ethyl 4-hydroxy-2-hexynoate* (42a) as a yellow oil (2.2 g, 99%), b.p. 70-75°C/0.09 mm (kugelrohr).

I.R. $\nu_{max}$ (film), 3390 (s, -OH); 2220 (s, -C≡C-); 1695 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) δ, 0.99 (3H, t, *J* = 7 Hz, H-6); 1.26 (3H, t, *J* = 7 Hz, -OCC$H_3$); 1.76 (2H, quintet, *J* = 7 Hz, H-5); 2.54 (1H, br s, -OH); 4.23 (2H, q, *J* = 7 Hz, -OC$H_2$-); 4.43 (1H, t, *J* = 7 Hz, H-4).

Analysis Found: C, 61.24; H, 7.41. $C_8H_{12}O_3$ requires C, 61.52; H, 7.74%.

Ethyl 4-Hydroxy-2-dodecynoate[71] (42b)

To a solution of lithium diisopropylamide (0.0105M) in dry THF (20 ml) at -74°C was added redistilled ethyl propiolate (1.033 g). The resulting yellow solution was stirred at -70°C for 70 mins. then *n*-nonanal (1.5 g) was added and the solution warmed to -30°C and stirred for a further 2 h. After recooling to -70°C, the reaction was quenched with 6 M HCl(aq). Ether extraction gave ethyl 4-hydroxy-2-dodecynoate (42b) (2.4 g, 95%).

I.R. $\nu_{max}$ (film), 3600-3100 (s, -OH); 2240 (m,-C≡C-); 1715 (s, ester C=O) $cm^{-1}$.

N. M.R. (90 MHz) δ, 0.87 (3H, br t, -C$H_3$); 1.28-1.65 (17H, br m, -C$H_2$- and -OCC$H_3$); 3.4 (1H, s, -OH); 4.16 (2H, q, *J* = 7 Hz, -OC$H_2$-); 4.37 (1H, m, H-4).

4-Hydroxy-2-hexynoic acid (43a)

20% Aqueous KOH (3.4 ml) was added dropwise to ethyl 4-hydroxy-2-hexynoate (42a) (1.52 g) in methanol (10 ml) at 0°C. After stirring at room temperature for 1 h, the mixture was poured into ice/water (20 ml) and extracted with ether. The aqueous portion was acidified with conc. HCl at 0°C and extracted with methylene chloride to give 4-*hydroxy*-2-*hexynoic acid* (43a) (500 mg, 40%) as a low melting solid, m.p. 35-42°C.

I.R. $\nu_{max}$ (film), 3600-2400 (br s, $-CO_2H$); 2230 (m, -C≡C-); 1700 (br s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 1.0 (3H, t, $J$ = 7 Hz, H-6); 1.78 (2H, quintet, $J$ = 7 Hz, H-5); 4.45 (1H, $J$ = 7 Hz, H-4); 5.74 (2H, br s, -OH and $-CO_2H$).

Mass Measurement Found: $M^+$ 128.0484. $C_6H_8O_3$ requires 128.0473.

4-Hydroxy-2-dodecynoic acid (43b)

20% Aqueous KOH (3.3 ml) was added dropwise to ethyl 4-hydroxy-2-dodecynoate (42b) (2.4 g) in methanol (15 ml) at 0°C and stirred at room temperature for 1.5 h. After pouring onto ice/water and extracting with ether, the aqueous layer was acidified with conc. HCl at 0°C and extracted with methylene chloride to give 4-hydroxy-2-dodecynoic acid (43b) (880 mg, 42%), m.p. 64-66°C (Lit.[71] m.p. 64-66°C) from ether/light petroleum (b.p. 40-60°C).

5-Ethyl-2(5H)-furanone (37a)

4-Hydroxy-2-hexynoic acid (43a) (722 mg) in methanol (15 ml) was hydrogenated over 5% $Pd/BaSO_4$ (10.1 mg) and quinoline (9.4 μl) at room temperature and pressure until one equivalent of $H_2$ was taken up. The solution was filtered and evaporated. The residue, in ether (15 ml), was washed successively with conc. HCl (1.5 ml), water and satd. $NaHCO_3$, evaporation giving an orange oil (377 mg). Preparative t.l.c. [light petroleum (b.p. 40-60°C):ether:acetic acid, 28:12:1] gave the butenolide (37a) as an oil ($R_f$ 0.4, 200 mg, 32%), b.p. 39-42°C/0.3 mm (Lit.[129] b.p. 99-100°C/19 mm).

I.R. $\nu_{max}$ (film), 1750 (s, lactone C=O); 1630 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.97 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.75 (2H, m, $-CH_2-$); 5.02 (1H, m, H-5); 6.14 (1H, d-d, $J$ = 6 Hz, 2 Hz, H-3); 7.46 (1H, d-d $J$ = 6 Hz, 2 Hz, H-4).

5-*n*-Octyl-2(5H)-furanone[71](37b)

4-Hydroxy-2-dodecynoic acid (43b) (330 mg) in methanol (15 ml) was hydrogenated over 5% Pd/$BaSO_4$ (9 mg) and quinoline (8.4 $\mu$l) at room temperature and pressure until one equivalent of $H_2$ was taken up. The solution was filtered, evaporated and the residue taken up in ether (10 ml) and washed successively with conc. HCl (1 ml), water and saturated $NaHCO_3$ to give 5-*n*-octyl-2(5H)-furanone (37b) as a pale yellow oil (263 mg, 86%).

I.R. $\nu_{max}$ (film), 1750 (s, unsatd. C=O); 1600 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.85 (3H, br t, $-CH_3$); 1.27 (12H, br s, $-CH_2-$); 1.68 (2H, br s, H-6); 5.04 (1H, m, H-5); 6.07 (1H, d-d, $J$ = 7 Hz, 2 Hz, H-3); 7.43 (1H, d-d, $J$ = 5 Hz, 1.5 Hz, H-4).

Dimethyl ester Lactone (44b, R' = Me)

5-*n*-Octyl-2(5H)-furanone (37b) (100 mg) in dry THF (6 ml) was added to a solution of dimethyl sodiomalonate [prepared from sodium hydride (15 mg) and dimethyl malonate (80 mg) in dry THF (15 ml)] and the mixture refluxed overnight under argon. After cooling, the solvent was evaporated off and the residue taken up in water, acidified with dilute HCl and extracted with ether to give an oil which was dissolved in chloroform and filtered to give a yellow oil (190 mg). Preparative t.l.c. (20% ethyl acetate/light petroleum) gave the *dimethyl ester lactone* (44b, R' = Me) as an oil, $R_f$ 0.25 (68 mg, 41%).

I.R. $\nu_{max}$ (film), 1775 (s, lactone C=O); 1750 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.85 (3H, br t, $-CH_3$); 1.27 (12H, br s, $-CH_2-$); 1.53 (2H, m, H-6); 2.7 (3H, m, H-3 and H-4); 3.54 (1H, d, $J$ = 7 Hz, H-2'); 3.77 (6H, s, $-OCH_3$); 4.3 (1H, m, H-5).

Mass Measurement Found: $M^+$ 328.1872. $C_{17}H_{28}O_6$ requires 328.1886.

Diacid Lactone (45)

1M Aqueous KOH (0.6 ml) was added to the dimethyl ester lactone (44b, R' = Me) (68 mg) in methanol (1.6 ml) at 0°C and the mixture stirred at room temperature for 2.5 h. Water (4 ml) was then added and the solution extracted with ether. The aqueous portion was acidified at 0°C with dilute HCl and extracted with methylene chloride to give a colourless solid (54 mg). Crystallisation from acetone gave the *diacid lactone* (45) as colourless crystals (25 mg, 40%) m.p. 138-140°C.

I.R. $\nu_{max}$ (KBr), 1760 (s, lactone C=O); 1740 (s); 1690 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.83 (3H, br t, $-CH_3$); 1.28 (12H, br s, $-CH_2-$); 1.5 (2H, m, H-6); 2.6 (2H, m, $-\underline{CH_2}CO_2H$); 3.36 (1H, m, H-4); 3.55 (1H, d, $J$ = 9 Hz, H-3); 4.76 (1H, m, H-5); 11.2 (2H, s, $-CO_2H$).

$^{13}C$-N.M.R. $\delta$ ($D_6$-acetone), 172.8 (s, lactone C=O); 171.8, 169.1 (both s, $CO_2H$); 82.1 (d, $J_{res}$ 68.7 Hz, -CH-O-); 51.9 (d, $J_{res}$ 46 Hz, C-3); 40.0 (d, $J_{res}$ 45 Hz, C-4); 33.1 (t, $-\underline{C}H_2CO_2H$); 32.5 (t); 28.29 (t, $J_{res}$ *ca.* 15 Hz, C-10); 26.64 (t, $J_{res}$ *ca.* 15 Hz, C-11); 23.25 (t, $J_{res}$ *ca.* 15 Hz, C-12); 14.34 (q, $J_{res}$ *ca.* 10 Hz, $-CH_3$); C-7 to C-9 obscured by solvent.

Mass Measurement Found: $M^+$ 300.1568. $C_{15}H_{24}O_6$ requires 300.1573.

The diacid lactone (45) (52 mg) in methanol (1 ml) was treated with 40% aqueous dimethylamine (0.2 ml), stirred at -20°C for 20 mins. and 37% aqueous formaldehyde (0.04 ml) in methanol (1 ml) added. After stirring overnight at room temperature, the solution was refluxed for 2 h and evaporated under reduced pressure. The residue was taken up in water, acidified at 0°C with dilute HCl, saturated with solid NaCl and extracted with ether to give the methylene lactone (48) as an oil (6 mg, 13.5%), t.l.c. (50% methanol/chloroform), $R_f$ 0.5 (yellow with $KMnO_4$).

I.R. $\nu_{max}$ ($CHCl_3$), 1770 (s, lactone C=O); 1720 (s, acid C=O); 1630 (w, C=C) $cm^{-1}$.

Dibenzyl malonate[130]

A mixture of malonic acid (52 g), benzyl alcohol (119 g) and conc. $H_2SO_4$ (1 ml) was heated at 120°C for 2 h. After cooling, ether (400 ml) was added and the solution washed successively with water, satd. $NaHCO_3$ and brine to give the diester, b.p. 172°C/0.1-0.3 mm (Lit.[131] 188°C/0.2 mm), (52 g, 37%).

N.M.R. (90 MHz) $\delta$, 3.43 (2H, s); 5.15 (4H, s); 7.3 (10H, s).

Dibenzyl ester Lactone (44a, R' = Bzl)

5-Ethyl-2(5H)-furanone (37a) (90 mg) was added to a solution of dibenzyl sodiomalonate [prepared from sodium hydride (31 mg) and dibenzyl malonate (349 mg) in dry THF (18 ml)] and the mixture refluxed for 2 h. After cooling, water and a few drops of dilute HCl were added. Extraction with ether followed by preparative t.l.c. (20% ethyl acetate/light petroleum), (eluted 2 times) gave the *dibenzyl ester lactone* (44a, R' = Bzl) as an oil, ($R_f$ 0.31, 80 mg, 25%).

I.R. $\nu_{max}$ ($CCl_4$), 1790 (s, lactone C=O); 1775, 1740 (s, ester C=O); 700 (m, aromatic C-H) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, t, $J$ = 8 Hz, $-CH_3$); 1.56 (2H, m, $-CH_2-$); 2.65 (3H, m, H-3 and H-4); 3.55 (1H, d, $J$ = 7 Hz, H-2'); 4.23 (1H, m, H-5); 5.14 (4H, s, $-OCH_2-$); 7.3 (10H, s, aromatic H).

Mass Spectrum m/e (relative abundance) $M^+$ 396 (1), 305 (17, $M - C_7H_7$), 199 (100, $M - C_7H_7 - C_7H_6O$), 180 (27).

Mass Measurement Found: $M^+$ 396.1567. $C_{23}H_{24}O_6$ requires 396.15727.

Dibenzyl ester Lactone (44b, R' = Bzl)

5-*n*-Octyl-2(5H)-furanone (37b) (170 mg) was added to a solution of dibenzyl sodiomalonate [prepared from sodium hydride (30 mg) and dibenzyl malonate (350 mg) in dry THF (25 ml)] and the mixture stirred at 60°C, under argon, for 2 h. After cooling, water and a few drops of dilute HCl were added. Extraction with methylene chloride followed by preparative t.l.c. (10% ethyl acetate/light petroleum), (eluted 2 times) gave the *dibenzyl ester lactone* (44b, R' = Bzl) as an oil, ($R_f$ 0.29, 112 mg, 27%).

I.R. $\nu_{max}$ ($CCl_4$), 1790 (s, lactone C=O); 1760, 1740 (s, ester C=O); 700 (m, aromatic C-H) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.83 (3H, br t, $-CH_3$); 1.22 (12H, br s, $-CH_2-$); 1.5 (2H, m, H-6); 2.7 (3H, m, H-3 and H-4); 3.56 (1H, d, $J$ = 7 Hz, H-2'); 4.28 (1H, m, H-5); 5.14 (4H, s, $-OCH_2-$); 7.29 (10H, s, aromatic H).

Mass Spectrum m/e (relative abundance) $M^+$ 480 (1), 389 (10, M - $C_7H_7$), 283 (100, M - $C_7H_7$ - $C_7H_6O$), 180 (29).

Mass Measurement Found: $M^+$ 480.2513. $C_{29}H_{36}O_6$ requires 480.25117.

Diacid Lactone (46a)

The dibenzyl ester lactone (44a, R' = Bzl) (200 mg) in ethyl acetate (9 ml) was hydrogenated over 10% Pd/charcoal (73 mg) at room temperature and pressure until the uptake of $H_2$ ceased. The solution was filtered and evaporated to give the *diacid lactone* (46a) (95 mg, 87%) m.p. 136-138°C from acetone.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1770 (s, lactone C=O); 1720 (br s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone). 0.95 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.7 (2H, m, $-CH_2-$); 2.75 (3H, m, H-3 and H-4); 3.7 (1H, d, $J$ = 7 Hz, H-2'); 4.4 (1H, m, H-5); 10.4 (2H, br s, $-CO_2H$).

Mass Spectrum m/e (relative abundance) 187 (10, M - $C_2H_5$), 169 (20, M - $C_2H_5$ - $H_2O$), 143 (90, M - $C_2H_5$ - $CO_2$), 125 (80, 143 - $H_2O$), 113 (100, M - $CH(CO_2H)_2$), 97 (90).

$^{13}C$ N.M.R. $\delta$ ($D_6$-acetone), 176.25 (s, lactone C=O), 169.68 (s, 2 x $CO_2H$); 84.85 (d, $J_{res}$ 51.8 Hz, -CH-O); 54.29 (d, $J_{res}$ 38.8 Hz, C-2'); 39.55 (d, $J_{res}$ 30.9 Hz, C-4); 32.94 (t, $J_{res}$ 30 Hz, C-3); 28.69 (t, $J_{res}$ *ca.* 15 Hz, C-6); 9.9 (q, $J_{res}$ *ca.* 10 Hz, $-CH_3$).

Mass Measurement Found; $M^+$-$C_2H_5$ 187.0232. $C_7H_7O_6$ requires 187.0241.

Diacid Lactone (46b)

The dibenzyl ester lactone (44b, R' = Bzl) (100 mg) in ethyl acetate (2 ml) was hydrogenated over 10% Pd/charcoal (38 mg) at room temperature and pressure until the uptake of $H_2$ ceased. The solution was filtered and evaporated to give the *diacid lactone* (46b) (34 mg, 55%) m.p. 127-129°C from acetone.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1770-1690 (br s, lactone and acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.86 (3H, br t, $-CH_3$); 1.3 (12H, br s, $-CH_2-$); 1.65 (2H, m, H-6); 2.7 (3H, m, H-3 and H-4); 3.55 (1H, d, $J$ = 7 Hz, H-2'); 4.43 (1H, m, H-5); 6.33 (2H, br s, $-CO_2H$).

Mass Spectrum m/e (relative abundance) 257 (1, M - $C_3H_7$), 238 (10, M - $CO_2$ - $H_2O$), 228 (8), 197 (30, M - $CH(CO_2H)_2$), 143 (100, M - $C_8H_{17}$ - $CO_2$)

Analysis Found: C, 59.71; H, 7.95. $C_{15}H_{24}O_6$ requires C, 59.9; H, 8.05%.

β-(1-Hydroxy-*n*-propyl)-α-methyleneglutaric acid Lactone (31a)

The diacid lactone (46a) (10.1 mg) in acetate buffered formylation mixture[72] (50 μl) was shaken at room temperature for 5 mins., followed by heating at 100°C (steam bath) for 10 mins. After standing overnight at room temperature, the mixture was diluted with water and extracted with ether to give the *β-(1-hydroxy-n-propyl)-α-methyleneglutaric acid lactone* (31a) as colourless crystals (6 mg, 70%)

m.p. 90-92 °C.

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2400 (s, $-CO_2H$); 1775 (s, lactone C=O); 1698 (s, acid C=O); 1625 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.99 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.7 (2H, m, $J$ = 7 Hz, $-CH_2-$); 2.71 (2H, m, $-CH_2CO_2-$); 3.16 (1H, m, H-$\beta$); 4.4 (1H, q, $J$ = 7 Hz, H-1); 5.83 (1H, s, $H_b$); 6.48 (1H, s, $H_a$); 7.8 (1H, s, $-CO_2H$).

Mass Spectrum m/e (relative abundance) $M^+$ 184 (1), 166 (1, M - $H_2O$), 155 (60, M - $C_2H_5$), 127 (30, M - $CH_3CH_2\overset{+}{C}=O$), 126 (100, M - $CH_3CH_2CHO$), 109 (32).

Mass Measurement Found: $M^+$ 184.0731. $C_9H_{12}O_4$ requires 184.0734.

Treatment of the diacid lactone (46a) with diethylamine and 37% aqueous formaldehyde at room temperature for 7 days gave, after workup and preparative t.l.c., a small amount (25% yield) of the desired terminal methylene compound (31a).

## $\beta$-(1-Hydroxy-*n*-nonyl)-$\alpha$-methyleneglutaric acid Lactone (31b)

The diacid lactone (46b) (98 mg) in acetate buffered formylation mixture[72] (0.44 ml) was shaken at room temperature for 5 mins., followed by heating at 100°C (steam bath) for 10 mins. After standing overnight at room temperature, the mixture was diluted with water and extracted with ether to give the *$\beta$-(1-hydroxy-n-nonyl)-$\alpha$-methyleneglutaric acid lactone* (31b) as colourless crystals (65 mg, 74%) m.p. 97.5-100°C from benzene/light petroleum (b.p. 40-60°C).

I.R. $\nu_{max}$ (KBr), 3500-2500 (m, $-CO_2H$); 1765 (s, lactone C=O); 1690 (s, acid C=O); 1625 (s, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.89 (3H, br t, $-CH_3$); 1.29 (12H, br s, $-CH_2-$); 1.6 (2H, m, H-2); 2.78 (2H, m, $-CH_2CO_2-$); 3.2 (1H, m, H-$\beta$); 4.5 (1H, m, H-1); 5.85 (1H, s, $H_b$); 6.5 (1H, s, $H_a$); 10.2 (1H, s, $-CO_2H$).

Analysis Found: C, 67.0; H, 8.90. $C_{15}H_{24}O_4$ requires C, 67.16; H, 8.95%.

Mass Spectrum m/e (relative abundance) $M^+$ 268 (9), 179 (20), 155 (34, M - $C_8H_{17}$), 135 (40), 126 (100, M - $C_8H_{17}CHO$).

Treatment of the diacid lactone (46b) with diethylamine and 37% aqueous formaldehyde at room temperature for 10 days gave only unreacted lactone (46b) (m.p., n.m.r., i.r.).

β-(1-Hydroxy-*n*-propyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31a)

The diacid lactone (46a) (17.4 mg) was treated with [$^{14}$C]-formaldehyde (14 μl, 250 μCi) and a solution of diethylamine (10 μl), acetic acid (40 μl) and sodium acetate (1.1 mg) and the mixture shaken for 5 mins. at room temperature. 37% Aqueous formaldehyde (25 μl) was then added and the mixture shaken for an additional 5 mins. at room temperature, followed by heating at 100°C (steam bath) for 10 mins. After standing at room temperature overnight, the usual workup gave β-*(1-hydroxy*-n-*propyl)*-α-[$^{14}$*C-methylene*]*-glutaric acid lactone* (31a) as a crystalline oil (9.6 mg, 65%, $5.74 \times 10^6$ dpm/mg). Identical (n.m.r., t.l.c.) with a pure sample of β-(1-hydroxy-*n*-propyl)-α-methyleneglutaric acid lactone (31a).

Treatment of diacid lactone (46a) (51 mg) with diethylamine (0.2 ml), [$^{14}$C]-formaldehyde (250 μCi, 20 ml) and 37% aqueous formaldehyde (0.1 ml) gave only unreacted diacid lactone (46a) (n.m.r., m.p.).

β-(1-Hydroxy-*n*-nonyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31b)

The diacid lactone (46b) (25.2 mg) was treated with [$^{14}$C]-formaldehyde (14 μl, 250 μCi) and a solution of diethylamine (10 μl), acetic acid (40 μl) and sodium acetate (1.1 mg) and the mixture shaken for 5 mins. at room temperature. 37% Aqueous formaldehyde (25 μl) was then added and the mixture shaken for an additional 5 mins. at room temperature, followed by heating at 100°C (steam bath) for 10 mins.

After standing at room temperature overnight, the usual workup gave the crude lactone (31b) as colourless crystals (18.6 mg, 82%). This was diluted with pure β-(1-hydroxy-*n*-nonyl)-α-methyleneglutaric acid lactone (31b) (28 mg) and crystallised repeatedly from benzene/light petroleum (b.p. 40-60°C) giving *β-(1-hydroxy-n-nonyl)-α-[$^{14}$C-methylene]-glutaric acid lactone* (31b) (31 mg, $2.802 \times 10^5$ dpm/mg) m.p. 99-100°C.

<u>Incorporation of β-(1-Hydroxy-*n*-propyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31a) into Ethisolide (1) by cultures of *P. decumbens*</u>

β-(1-Hydroxy-*n*-propyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31a) (4.8 mg, 12.41 μCi) as its sodium salt was fed, in three 24 h pulses, on the 7th, 8th and 9th days after inoculation, to three Roux bottles containing cultures of *P. decumbens*. On the 14th day after inoculation, the aqueous broth was separated by decantation and continuously extracted with ethyl acetate for 48 h, after which the ethyl acetate was washed with brine, dried and evaporated to give ethisolide (1) (590 mg, 13.6 dpm/mg), m.p. 122-122.5°C from ethanol (Lit.[5] m.p. 122-123°C). The total activity isolated as [$^{14}$C]-ethisolide was 8024 dpm, giving an incorporation of 0.03% from β-(1-hydroxy-*n*-propyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31a).

<u>Incorporation of β-(1-Hydroxy-*n*-nonyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31b) into Avenaciolide (2) by cultures of *A. avenaceus*</u>

β-(1-Hydroxy-*n*-nonyl)-α-[$^{14}$C-methylene]-glutaric acid lactone (31b) (12.4 mg, 1.56 μCi) as its sodium salt was fed, in five 12 h pulses, on the 7th, 8th and 9th days after inoculation, to three Roux bottles containing cultures of *A. avenaceus*. On the 14th day after inoculation, the aqueous broth was separated by decantation, acidified with conc. HCl and continuously extracted with ethyl acetate for 48 h. The organic phase was dried and evaporated to give a dark oil, which was taken up in chloroform, filtered and evaporated to give a red oil (0.8 g). Preparative t.l.c. ($CHCl_3$) gave avenaciolide (2) $R_f$ 0.3 (105 mg, 106 dpm/mg), m.p. 50-50.5 C from ether/light petroleum (b.p. 40-60°C), (Lit.[6] m.p. 49-50°C). The total activity isolated as [$^{14}C$]-avenaciolide was 11130 dpm, giving an incorporation of 0.3% from β-(1-hydroxy-*n*-nonyl)-α-[$^{14}C$-methylene]-glutaric acid lactone (31b).

## Experimental Section

## III Attempted Syntheses of Ethisic Acid (27a)

(a) Attempted Syntheses *via* Halo-esters

(b) Attempted Syntheses *via* Hydroxy-esters

(c) *Via* 2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde

## (a) Attempted Syntheses *via* Halo-esters

### Ethyl 2,3-Dibromohexanoate (60)

Bromine (18.7 g) was added dropwise to a solution of ethyl *trans* hex-2-enoate[58] (15.4 g) in dry methylene chloride (20 ml). After the addition was complete (2 h), the solution was stirred at room temperature for an additional 2 h. The solvent was removed under reduced pressure and the resultant oil distilled to give *ethyl 2,3-dibromohexanoate* (60) as a pale yellow oil (26 g, 78%), b.p. 74-83°C/ 0.6-0.7 mm.

I.R. $\nu_{max}$ (film), 1735 (s, C=O) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 1.0 (3H, br t, H-6); 1.3 (3H, t, $J$ = 7 Hz, $-OCCH_3$); 1.4-2.4 (4H, m, H-4 and H-5); 4.3 (2H, q, $J$ = 7 Hz, $-OCH_2-$); 4.3-4.4 (2H, m, H-2 and H-3).

Analysis Found: C, 31.66; H, 4.78; Br, 52.58. $C_8H_{14}Br_2O_2$ requires C, 31.81; H, 4.67; Br, 52.9%.

### Treatment of Ethyl 2,3-dibromohexanoate (60) with Dimethyl sodiomalonate

(a) Ethyl 2,3-dibromohexanoate (60) (10.14 g) in dry ether (40 ml) was added dropwise to a suspension of dimethyl sodiomalonate [prepared from sodium (0.77 g), methanol (1.07 g) and dimethyl malonate (4.44 g) in anhydrous ether (200 ml)] and the mixture stirred at room temperature for 4 h. After work up the unreacted ester (60) was recovered unchanged.

(b) Ethyl 2,3-dibromohexanoate (60) (10.06 g) and one equivalent of dimethyl sodiomalonate in dry ether (200 ml) was refluxed for 40 h The solvent was changed for dry benzene (200 ml) and refluxed for an additional 24 h. As before, unreacted ester (60) was recovered unchanged.

Ethyl 2-bromohex-2-enoate[132] (61)

Dimethyl sodiomalonate was prepared from sodium hydride (0.303 g) and dimethyl malonate (1.75 g) in dry DMF (15 ml) under argon. This was added, dropwise over 1 h, to ethyl 2,3-dibromohexanoate (60) (4.01 g) in dry DMF (10 ml). The mixture was stirred at 100°C overnight and, after cooling, the solvent was removed under reduced pressure, the residue taken up in water and extracted with ether to give a yellow oil (4 g). Preparative t.l.c. (20% ethyl acetate/light petroleum) of a portion (170 mg) afforded ethyl 2-bromohex-2-enoate (61), as an oil ($R_f$ 0.7, 110 mg, 64%).

I.R. $\nu_{max}$ (film), 1720 (s, ester C=O); 1620 (m, C=C) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 1.0 (3H, br t, H-6); 1.4 (3H, t, $J$ = 7 Hz, $-OCCH_3$); 1.45 (2H, m, H-5); 2.5 (2H, d-t, $J$ = 7 Hz, H-4); 4.3 (2H, q, $J$ = 7 Hz, $-OCH_2-$); 6.8 (1H, t, $J$ = 7 Hz, H-3).

Analysis Found: C, 43.5; H, 5.72; Br, 36.5. $C_8H_{13}BrO_2$ requires C, 43.46; H, 5.92; Br, 36.2%.

Treatment of a portion of the above bromo ester (61) with one equivalent of diethyl sodiomalonate in DMF at 100°C, gave only unreacted bromo ester (61). This reaction was not investigated further.

Methyl 2-carbomethoxyhex-2-enoate[133] (62)

A mixture of *n*-butanal (48.36 g), dimethyl malonate (44.48 g) and acetic anhydride (51.5 g) was refluxed for 22 h. The mixture was then distilled up to a temperature of 140°C to remove excess *n*-butanal and acetic anhydride together with some acetic acid. Fractional distillation of the residue gave the dimethyl ester (62) as a colourless oil, (44 g, 70%) b.p. 72-74°C/0.5 mm (Lit.[133] b.p. 96°C/3 mm).

I.R. $\nu_{max}$ (film), 2870 (m, $-OCH_3$); 1735 (s, ester C=O); 1640 (m, C=C) $cm^{-1}$

N.M.R. (60 MHz) $\delta$, 0.95 (3H, br t, H-6); 1.5 (2H, m, H-5); 2.3 (2H, m, H-4); 3.76 (3H, s, $-OCH_3$); 3.83 (3H, s, $-OCH_3$); 7.0 (1H, t, $J$ = 7 Hz, H-3).

Treatment of Methyl 2-carbomethoxyhex-2-enoate (62) with Ethyl chloroacetate

A mixture of methyl 2-carbomethoxyhex-2-enoate (62) (18.54 g) and ethyl chloroacetate (12.5 g) was added, over 2 h, to powdered sodium (2.35 g) in dry ether (250 ml) at 0°C and then stirred at room temperature for 50 h. After the addition of wet ether the mixture was acidified with dilute AcOH and extracted with ether to give a pale yellow oil (21 g). Preparative t.l.c. (20% ethyl acetate/light petroleum) of a portion (170 mg) produced a number of components, the major one ($R_f$ 0.3) was an oily mixture (50 mg). (-$OCH_3$ peaks *e.g.* 3.7, 3.75, 3.8, 3.83 and olefinic peaks *e.g.* 6.76, 6.95$\delta$.)

A portion of the above oil (88 mg) in ethyl acetate (5 ml) was hydrogenated over 20% palladium/charcoal (81 mg) at room temperature and pressure until the uptake of hydrogen ceased. The catalyst was removed by filtration and the solution evaporated to give a clear oil (80 mg) whose n.m.r. spectrum (*e.g.* $\delta$3.7, 3.75, 3.8) was essentially the same as before hydrogenation. This product was not investigated further.

Treatment of Methyl 2-octynoate (64 b) with dialkyl malonates

(i) Methyl 2-octynoate[134] (64b) (3 g) in dry ether (10 ml) was added to a suspension of dimethyl sodiomalonate [prepared from sodium hydride (0.45 g) and dimethyl malonate (2.5 g) in dry ether (30 ml)] and the mixture refluxed for 3 h. After acidification (conc. AcOH) the ethereal layer was separated to give an oil (5.1 g). Preparative t.l.c. of a portion (200 mg) gave an oil ($R_f$ 0.65, 140 mg) [no doublet *ca.* 3.6$\delta$, expected for malonate -CH-, but signals at *e.g.* 5.9, 3.73, 3.68$\delta$; i.r. peaks at 1715 (s, unsatd. ester C=O); 1740 (s, ester C=O) and 1645 (C=C) $cm^{-1}$].

A portion of the above oil (2.04 g) was suspended in aqueous

1M NaOH (35 ml) and the mixture stirred at room temperature overnight. After heating to 80°C for a further 3 h the basic mixture was acidified (dilute HCl) and extracted with ethyl acetate to give a solid (1.25 g) m.p. 80-100°C (ethyl acetate/light petroleum, b.p. 100-120°C). N.m.r. signals ($C_5D_5N$) at *e.g.* 4.2 (s), 4.3 (s) and 2.4 (m), 3.0 (m) $\delta$.

(ii) Similarly, methyl 2-octynoate (64b) (3.2 g) was treated with one equivalent of diethyl sodiomalonate in DMF giving, after work up, an oil (5.7 g). As before, this appeared to be a mixture [no doublet *ca.* 3.6 , expected for malonate -CH-; i.r. peaks at 1740-1710 (br, s) and 1640 (C=C) $cm^{-1}$].

A portion of the above oil (1.15 g) was treated with bromine (0.2 ml) and acetic acid (16 ml) at 120°C. Work up afforded an oil (800 mg) which appeared to be a mixture [*e.g.* three $-OCH_3$ peaks *ca.* 3.7 ; i.r. peaks at *ca.* 1800, 1780, 1740 and 1640 $cm^{-1}$]. Preparative t.l.c. failed to separate the components and the reaction was not investigated further.

3-*n*-Pentylglutaconic acid (69)

A portion of the above solid [from (i)] (91 mg) was treated with approx. 20 x its weight of conc. $H_2SO_4$ and the mixture stirred at room temperature for 2 h. This was then poured onto ice, saturated with solid NaCl and extracted with ether to give 3-n-*pentylglutaconic acid* (69) as a white solid, m.p. 110-112°C from ether (40 mg).

I.R. $\nu_{max}$ (nujol), 3500-2400 (s, $-CO_2H$); 1710 (s, acid C=O); 1690 (s, unsatd. acid C=O); 1640 (s, C=C) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$ ($C_5D_5N$), 0.9 (3H, br t, $-CH_3$); 1.3 (6H, m, H-5' to H-7'); 2.4 (2H, br t, H-4'); 4.25 (2H, s, H-4); 6.2 (1H, s, olefinic H); 9.7 (2H, s, $-CO_2H$).

Analysis Found: C, 60.08; H, 8.13. $C_{10}H_{16}O_4$ requires C, 59.98; H, 8.05%.

Mass Spectrum m/e (relative abundance) $M^+$ 200 (3), 182 (28, M - $H_2O$), 156 (20, M - $CO_2$), 154 (36, M - $H_2O$ - 28), 129 (28, M - $C_5H_{11}$), 95 (100).

(b) Attempted Syntheses *via* Hydroxy-esters

Ethyl 3-Bromo-2-oxohexanoate (73)

Ethyl 3-carboethoxy-2-oxohexanoate[135] (72) (19.5 g) in dry carbon tetrachloride (50 ml) was treated with one equivalent of bromine (2.19 ml) at room temperature. Evaporation of the solvent gave the bromo keto diester as an oil (24.8 g). A portion of this oil (7 g) in 3M aqueous HCl (40 ml) and dioxan (40 ml) was refluxed for 5 h. The usual work up afforded crude 3-bromo-2-oxohexanoic acid as an oil (6 g). (The preparation was repeated to give further bromo keto acid.) The crude 3-bromo-2-oxohexanoic acid (8.3 g) in dry ethanol (20 ml) and dry benzene (50 ml) containing conc. $H_2SO_4$ (2 ml) was refluxed for 20 h. After cooling, the mixture was poured into water (150 ml) and the benzene layer separated and washed successively with satd. aqueous $NaHCO_3$ and water to give ethyl 3-bromo-2-oxohexanoate (73) as a colourless oil (5.2 g, 55%), b.p. 40-45°C/0.1 mm (Lit.[136] b.p. 74-78°C/0.6 mm).

I.R. $\nu_{max}$ (film), 1725 (br s, ester and ketone C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.96 (3H, br t, $-CH_3$); 1.38 (5H, br t, $J$ = 7 Hz, $-OCCH_3$ and H-5); 1.96 (2H, m, H-4); 4.39 (2H, q, $J$ = 7 Hz, $-OCH_2-$); 4.95 (1H, t, $J$ = 6 Hz, H-3).

Treatment of Ethyl 3-Bromo-2-oxohexanoate (73) with diethyl malonate

Ethyl 3-bromo-2-oxohexanoate (73) (3.82 g) in dry DMF was added to diethyl sodiomalonate [prepared from sodium hydride (406 mg) and diethyl malonate (2.72 g) in dry DMF (15 ml)] under argon and the mixture heated to 70°C for 2 h. After cooling, the mixture was poured into water (350 ml) and extracted with ether to give an oil (5.4 g). Distillation removed diethyl malonate, leaving a residue (3.2 g) with a high b.p. [I.R. $\nu_{max}$ (film), 3500 (m); 1730 (br s); 1615 (br m) $cm^{-1}$;

N.M.R. (90 MHz) *e.g.* δ, 1.3 (m); 4.3 (m), no doublet at *ca.* 3.5 -C<u>H</u>($CO_2Et$)$_2$].

A portion of the above product (505 mg) in aqueous 1M NaOH was stirred at room temperature for 48 h. Acidification and extraction with ether gave a viscous oil [N.M.R. (90 MHz) *e.g.* δ ($CDCl_3$/$D_6$-acetone), 2.3 (br m); 7.84 (br s, -$CO_2H$)]. This reaction was not investigated further.

<u>2-*p*-Nitrophenyl-1,3-Dioxolan-4-one (74b)</u>

*p*-Nitrobenzaldehyde (15.1 g), glycollic acid (7.6 g) and a few mgs of p.t.s.a., in dry diglyme (75 ml) and dry benzene (125 ml) were refluxed, with azeotropic removal of water, in a Dean and Stark apparatus for 24 h. After which, a further portion of glycollic acid (3.8 g) was added and heating continued for a further 24 h. After cooling, the solvent was removed and the residue taken up in chloroform and washed with water. Evaporation gave an oil which was taken up in ether and washed with water to give 2-p-*nitrophenyl*-1,3-*dioxolan*-4-*one* (74b) (5.75 g, 28%) m.p. 95-95.5°C from benzene.

<u>I.R.</u> $\nu_{max}$ (KBr), 1810 (s, dioxolan-4-one C=O); 1620 (m, aromatic C-H); 1540, 1350 (s, -$NO_2$) $cm^{-1}$.

<u>N.M.R.</u> (90 MHz) δ, 4.46 (2H, s, H-5); 6.59 (1H, s, H-2); 7.96 (4H, q, $J$ = 9 Hz, aromatic H).

<u>Analysis</u> Found: C, 51.89; H, 3.52; N, 6.76. $C_9H_7NO_5$ requires C, 51.68; H, 3.37; N, 6.70%.

Numerous attempts were made to synthesise the parent aromatic dioxolan-4-one (74a) using benzaldehyde and glycollic acid, all of these were unsuccessful, yielding only unreacted materials or (in a similar procedure to the one above) the product was detected (i.r.), but could not be isolated without decomposition occurring.

Attempted Condensation of the Dioxolan-4-one (74b) with Methyl 2 - Carbomethoxyhex-2-enoate (62)

The dioxolan-4-one (74b) (836 mg) in dry THF (6 ml) was added to a solution of lithium diisopropylamide (1 equivalent) in dry THF (10 ml) at -70°C (a green/black viscous oil was produced). Methyl 2-carbomethoxyhex-2-enoate (62) (752 mg) in dry THF (10 ml) was then added. After warming to room temperature and stirring overnight, the usual work up gave a dark viscous oil [I.R. $\nu_{max}$ (film) no band at *ca*. 1810 $cm^{-1}$]. The reaction was not investigated further.

Attempted Carboxylation of the Dioxolan-4-one (74b)

The dioxolan-4-one (74b) (0.5 g) in Stiles' reagent[96] (60 ml) was heated at 90°C while a slow stream of nitrogen passed through the mixture. The mixture quickly turned to a black tar and work up afforded no product identifiable as dioxolan-4-one (i.r.). The reaction was not investigated further.

Citrate Dioxolan-4-one (76)

*p*-Nitrobenzaldehyde (15.74 g), citric acid monohydrate (9.6 g) and a few mgs of p.t.s.a. in dry diglyme (75 ml) and dry benzene (150 mg) were refluxed, with azeotropic removal of water, in a Dean and Stark apparatus for 24 h. After which, a further portion of citric acid (9.6 g) was added and heating continued for a further 24 h. After cooling, the solvent was removed and the residue taken up in chloroform and washed with water. Evaporation gave an oil which was taken up in ether and extracted with saturated aqueous $NaHCO_3$. The aqueous layer was acidified with saturated aqueous citric acid and extracted with methylene chloride to give the *citrate dioxolan-4-one* (76) as colourless crystals (4.7 g, 16%) m.p. 174-176°C (decomp.) from chloroform.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1805 (s, dioxolan-4-one C=O); 1615 (m, aromatic C-H); 1528, 1354 (s, $-NO_2$) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 3.06, 3.13 (4H, both s, $-C\underline{H}_2CO_2H$); 6.76 (1H, s, H-2); 8.03 (4H, q, $J$ = 9 Hz, aromatic H); 8.5 (2H, s, $-CO_2H$).

Analysis Found: C, 47.79; H, 3.43; N, 4.29. $C_{13}H_{11}NO_9$ requires C, 48; H, 3.38; N, 4.3%.

Attempted formation of *n*-Butylcitric acid (25)

The citrate dioxolan-4-one (76) (500 mg) in dry THF (15 ml) at -70°C, under argon, was treated with butyl lithium (3 equivalents) giving a clear yellow solution. Redistilled *n*-butyl bromide (0.5 ml) was then added giving a greenish slurry, which was warmed to room temperature and stirred overnight. Extraction with methylene chloride gave a brown oil (150 mg) [N.M.R. (90 MHz) *e.g.* $\delta$, 0.9 (br t); 3.55 (m); 7.6, 8.15 (m); no singlet at *ca.* 6.7; I.R., no band *ca.* 1805 $cm^{-1}$]. The reaction was not investigated further.

(c) *Via* 2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde

1,2:5,6-di-*O*-Isopropylidene-D-mannitol[137] (82)

Anhydrous $ZnCl_2$ (270 g) in dry acetone (1350 ml) was allowed to stand overnight in a stoppered flask. This was then decanted onto D-mannitol (170 g) in a 3-necked flask, stirred vigorously for 2 h, filtered and the filtrate added rapidly to potassium carbonate (340 g) in water (340 ml) covered with ether (1350 ml). After stirring for 1 h, the acetone/ether layer was decanted and dried to give 1,2:5,6-di-*O*-isopropylidene-D-mannitol (82) as colourless crystals (63 g, 44%) m.p. 117-120°C from *n*-butyl ether (Lit.[137] m.p. 119-120°C).

2,3-*O*-Isopropylidene-aldehydo-D-glyceraldehyde[84] (77)

1,2:5,6-di-*O*-Isopropylidene-D-mannitol (82) (10 g) in dry benzene (200 ml) was treated with freshly prepared lead tetraacetate[138] (18 g) over 30 mins. and the mixture stirred vigorously for 3 h. This was then filtered and evaporated to yield a syrup which was coevaporated with four portions of $CCl_4$ (20 ml) to remove acetic acid giving 2,3-*O*-isopropylidene-aldehydo-D-glyceraldehyde (77) as a labile syrup (8.28 g, 83%).

N.M.R. (90 MHz) $\delta$, 1.38 (3H, s, $-CH_3$); 1.44 (3H, s, $-CH_3$); 4.13 (2H, m, H-3); 4.35 (1H, m, H-2); 9.67 (1H, d, $J$ = 2 Hz, -CH=O).
This was used immediately without further purification.

Acetonide alcohol (79)

Magnesium turnings (4.6 g) were reacted, under argon, with redistilled *n*-propyl bromide (23.3 g) in dry ether (75 ml) to give a solution of the alkyl magnesium bromide. 2,3-*O*-Isopropylidene-aldehydo-D-glyceraldehyde (77) (8.2 g) in dry ether (30 ml) was added dropwise at room temperature and stirring continued for a further 3 h. The mixture was then poured onto ammonium chloride (30 g) in iced water (100 ml), extracted with ether and washed with brine to give an oil (8 g). Distillation gave the *acetonide alcohol* (79) as an oil (6.9 g, 62%) b.p. 72-75°C/0.8 mm.

I.R. $\nu_{max}$ (film), 3450 (br s, -OH); 1070 (s, C-O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, H-3'); 1.3 (4H, m, H-1' and H-2'); 1.32 (3H, s, $-CH_3$); 1.4 (3H, s, $-CH_3$); 2.0 (1H, br s, -OH); 3.9 (4H, m, H-1 to H-3).

Mass Spectrum m/e (relative abundance) 159 (50, $M^+ - CH_3$), 131 (10, $M - CH_3 - CO$), 101 (100, $M - C_3H_5O_2$).

Analysis Found: C, 61.90; H, 10.28. $C_9H_{18}O_3$ requires C, 62.04; H, 10.41%.

Acetonide alcohol *p*-Toluenesulphonate (80, X = OTs)

The alcohol (79) (200 mg) in dry pyridine (2 ml) was cooled to -5°C and *p*-toluenesulphonyl chloride (241 mg) added and the mixture stirred overnight. Iced water was then added and the product extracted with chloroform, washed with water and dried to give an oil (195 mg). Preparative t.l.c. (2% methanol/benzene) gave the p-*toluenesulphonate* (80, X = OTs) as an oil, ($R_f$ 0.6, 130 mg, 35%).

I.R. $\nu_{max}$ (film), 1600 (m, aromatic C-H); 1190, 1180 (s, $-SO_2-O-$); 1070 (s, -C-O-); 890 (s, *p*-disubst. benzene) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.8 (3H, br t, H-3'); 1.25 (6H, s, $-C(CH_3)_2$); 1.5 (4H, m, H-1' and H-2'); 2.4 (3H, s, aryl-$CH_3$); 3.94 (3H, m, H-2 and H-3); 4.6 (1H, m, H-1); 7.54 (4H, q, $J$ = 8 Hz, aryl C-H).

Analysis Found: C, 58.23; H, 7.48; S, 9.42. $C_{16}H_{24}O_5S$ requires C, 58.51; H, 7.36; S, 9.76%.

Acetonide alcohol *p*-Bromobenzenesulphonate (80, X = OBs)

Under similar conditions to those above, the alcohol (79) (200 mg) and *p*-bromobenzenesulphonyl chloride (323 mg) gave the p-*bromobenzenesulphonate* (80, X = OBs) as an oil ($R_f$ 0.5, 210 mg, 46%).

N.M.R. (90 MHz) δ, 0.82 (3H, br t, H-3'); 1.25 (6H, s, $-C(CH_3)_2$); 1.5 (4H, m, H-1' and H-2'); 4.0 (3H, m, H-2 and H-3); 4.7 (1H, m, H-1); 7.71 (4H, q, *J* = 9 Hz, aryl C-H).

Analysis Found: C, 46.04; H, 5.52; Br, 20.59; S, 8.43. $C_{15}H_{21}BrO_5S$ requires C, 45.81; H, 5.38; Br. 20.32; S, 8.15%.

Treatment of the Tosylate (80, X = OTs) and Brosylate (80, X = OBs) with dibenzyl malonate

(i) The tosylate (80, X = OTs) (106 mg) was refluxed overnight with 1.2 equivalents of dibenzyl sodiomalonate in dry THF. After work up, an oil (230 mg) was isolated, which consisted essentially (t.l.c., spectral data) of unreacted (80, X = OTs).

(ii) The brosylate (80, X = OBs) (192 mg) was heated at 100°C for 14 h with 1.2 equivalents of dibenzyl sodiomalonate in dry DMF. Standard work up afforded an oil (220 mg), which again consisted essentially (t.l.c., spectral data) of unreacted (80, X = OBs).

Acetonide alcohol Methanesulphonate (80, X = OMs)

The alcohol (79) (104 mg) in dry methylene chloride (3.05 ml) containing dry triethylamine (92.5 mg) was treated with redistilled mesyl chloride (77 mg) at -10°C. After stirring for 20 mins., the reaction mixture was washed, successively, with water, saturated aqueous $NaHCO_3$ and brine and the organic portion dried and evaporated to give an oil. Preparative t.l.c. (20% ethyl acetate/light petroleum) afforded the *mesylate* (80, X = OMs) as an oil, $R_f$ 0.4 (145 mg, 96%).

<u>N.M.R.</u> (90 MHz) δ, 0.94 (3H, br t, H-3'); 1.28 (3H, s, $-CH_3$); 1.34 (3H, s, $-CH_3$); 1.55 (4H, m, H-1' and H-2'); 2.93 (3H, s, $-SCH_3$); 3.95 (3H, m, H-2 and H-3); 4.61 (1H, m, H-1).

<u>Mass Measurement</u> Found: $M^+ - CH_3$ 237.0798. $C_9H_{17}O_5S$ requires 237.0797.

<u>Treatment of the Mesylate (80, X = OMs) with Dimethyl sodiomalonate</u>

(i) The mesylate (80, X = OMs) (130 mg) in dry DMF (2 ml) was added to dimethyl sodiomalonate [prepared from sodium hydride (15 mg) and dimethyl malonate (82 mg) in dry DMF (5 ml)]. After heating at 95°C overnight, the usual work up gave an oil (115 mg). [N.M.R. (90 MHz) *e.g.* δ, 4.6 (m); 2.94 (s, $-SCH_3$): unchanged (80, X = OMs).]

(ii) Similarly, the mesylate (80, X = OMs) (115 mg) in dry benzene (2 ml) was added to a suspension of dimethyl sodiomalonate (1.2 equivalents) in dry benzene containing 18-crown-6 (12 mg). After refluxing for 120 h, the usual work up gave an oil (100 mg). [N.M.R. (90 MHz) *e.g.* δ, 4.55 (m); 2.87 (s, $-SCH_3$): unchanged (80, X = OMs).] This reaction was not investigated further.

<u>Treatment of the Acetonide glyceraldehyde (77) with Ethoxymagnesium-malonic ester</u>

2,3-*O*-Isopropylidene-aldehydo-glyceraldehyde (77) (2 g) in dry ether (5 ml) was added to ethoxymagnesiummalonic ester[90] [prepared from magnesium ethoxide (1.05 g) and diethyl malonate (2.94 g) in dry ether (30 ml)] and the mixture refluxed for 48 h. After cooling, the usual work up gave an oil (3.7 g) which, on distillation gave (2.1 g) b.p. 80-85°C/0.01 mm. [N.M.R. (90 MHz) *e.g.* δ, 4.18 (q, $J$ = 7 Hz, $-OCH_2-$); 4.91 (q, $J$ = 7 Hz, C=CH-C<u>H</u>-); 6.95 (d, $J$ = 7 Hz, C=C<u>H</u>-); I.R. $\nu_{max}$ (film), 1735 (s, ester C=O); 1645 (w, C=C) $cm^{-1}$.]

Acetonide Dibenzyl ester (83, R = Bzl)

The acetonide alcohol (79) (200 mg), carbon tetrabromide (518 mg), anhydrous triphenyl phosphine (382 mg) and dry benzene (1 ml) were heated at 80°C for 4 h. After cooling, the mixture was filtered and washed with ether to give an orange oil (372 mg). Column chromatography ($CHCl_3$) gave the bromide (84) as a labile colourless oil (210 mg). This was kept in the fridge and used immediately thereafter.

(i) The above crude bromide (84) (170 mg) was added to dibenzyl sodiomalonate [prepared from sodium hydride (20 mg) and dibenzyl malonate (245 mg) in dry DMF (10 ml)] and heated at 100°C overnight. The usual work up afforded an oil (400 mg) which after preparative t.l.c. (10% ethyl acetate/light petroleum) (eluted 2 times) gave the *acetonide dibenzyl ester* (83, R = Bzl) as an oil ($R_f$ 0.51, 95 mg, 30%).

I.R. $\nu_{max}$ (film), 1755, 1735 (s, ester C=0) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.87 (3H, br t, H-3'); 1.27 [6H, s, $-C(CH_3)_2$]; 1.4 (4H, m, H-1' and H-2'); 2.1 (2H, m, H-3); 3.7 (3H, m, H-1, H-2 and H-4); 5.13 (4H, d, aryl $-CH_2-$); 7.28 (10H, s, aryl C-H).

Mass Measurement Found: $M^+ - CH_3$ 425.1973. $C_{25}H_{29}O_6$ requires 425.1962.

(ii) When the above reaction was repeated using dry THF as solvent, only unreacted (84) (t.l.c., spectral data) was recovered.

Acetonide Diacid (83, R = H)

The dibenzyl ester (83, R = Bzl) (312.6 mg) in ethyl acetate (15 ml) was hydrogenated over 10% Pd/C (89 mg) at room temperature and pressure. After the uptake of $H_2$ ceased, the catalyst was removed by filtration to give the *acetonide diacid* (83, R = H) as colourless crystals (140 mg, 79%) m.p. 103-104°C from benzene.

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2400 (s, $-CO_2H$); 1720 (s, acid C=0) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.93 (3H, br t, H-3'); 1.35 [6H, s, $-C(CH_3)_2$]; 1.5 (4H, m, H-1' and H-2'); 2.2 (2H, m, H-3); 3.73 (3H, m, H-1, H-2 and H-4); 11.35 (2H, s, $-CO_2H$).

Analysis Found: C, 55.29; H, 7.53. $C_{12}H_{20}O_6$ requires C, 55.37, H, 7.74%.

Lactone acid (85)

The diacid (83, R = H) (90 mg) in dilute HCl (0.9 ml) [prepared from HCl (1 ml) and water (5 ml)] was heated at 50°C for 1 h. After cooling, the solution was saturated with solid NaCl and extracted with ether. The ethereal layer was washed with brine to give the *lactone acid* (85) as an oil (55 mg, 78%), b.p. 120-126°C/0.004 mm (kugelrohr).

I.R. $\nu_{max}$ ($CHCl_3$), 3500 (m, -OH); 3500-2400 (m, $-CO_2H$); 1780 (s, lactone C=O); 1720 (s sh, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, H-8); 1.5 (4H, m, H-6 and H-7); 2.5 (2H, m, H-3); 3.7 (2H, m, H-2 and H-5); 4.55 (1H, m, H-4); 6.35 (2H, s, -OH, $-CO_2H$).

$^{13}C$ N.M.R. $\delta$, 173.65, 173.04 (s, $-CO_2H$); 170.62, 170.23 (s, lactone C=O); 82.07 (d, $J_{res}$ 94 Hz, C-4); 73.06, 72.48 (d, $J_{res}$ 76.6 Hz, C-5); 46.65 (d, $J_{res}$ 73 Hz, C-2); 35.07, 34.63 (t, $J_{res}$ 29 Hz, C-6); 28.21, 27.77 (t, $J_{res}$ 56 Hz, C-3); 18.71 (t, $J_{res}$ 29 Hz, C-7); 13.86 (q, $J_{res}$ 19 Hz, $-CH_3$).

Mass Measurement Found: $M^+$ 202.0855. $C_9H_{14}O_5$ requires 202.0841.

Acetonide Ketone (88)

The alcohol (79) (8.45 g) was added to pyridinium dichromate[95] (150 g) in dry DMF (1000 ml) at 0°C. After stirring overnight at room temperature, the mixture is poured into water ( 5 litres) and thoroughly extracted with ether to give the *acetonide ketone* (88) as an oil (8.1 g, 97%) b.p. 52-56°C/0.5 mm.

I.R. $\nu_{max}$ (film), 1710 (s, C=O); 1068 (s, C-O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.91 (3H, t, $J$ = 7 Hz, H-3'); 1.38 (3H, s, $-CH_3$);

1.47 (3H, s, $-CH_3$); 1.58 (2H, m, H-2'); 2.58 (2H, t, $J$ = 7 Hz, H-1'); 4.2 (3H, m, H-2 and H-3).

Mass Measurement Found: $M^+ - CH_3$ 157.0871. $C_8H_{13}O_3$ requires 157.0864.

## Triethyl Phosphonoacetate

Triethyl phosphite (66.5 g) and ethyl bromoacetate (66.8 g) were heated at 110°C until no more ethyl bromide distilled over (∿ 20 mins.). Distillation of the residue gave triethyl phosphonoacetate (80 g, 89%) b.p. 80°C/0.2 mm (Lit.[139] 140°C/10 mm).

N.M.R. (90 MHz) $\delta$, 1.2 (3H, t, $J$ = 7 Hz, $CO_2CCH_3$); 1.26 (6H, t, $J$ = 7 Hz, $-OCCH_3$); 2.76, 3.0 (2H, s, $P-CH_2-$); 4.1 (6H, m, $-OCH_2-$).

## Acetonide ester (90)

The ketone (88) (7.8 g) was added to a solution of the sodium salt of triethyl phosphonoacetate [prepared from sodium hydride (1.09 g) and triethyl phosphonoacetate (10.15 g) in dry benzene (70 ml)] and stirred at room temperature overnight. After heating at 70°C for 20 mins., the mixture was cooled and water (70 ml) and ether (70 ml) added. The ethereal layer was separated and washed with water to give the crude unsaturated ester (89) as an oil (10.5 g, 96%). [I.R. $\nu_{max}$ (film), 1710 (s, ester C=O); 1640 (m, C=C); 1060 (s, C-O) $cm^{-1}$; N.M.R. (90 MHz) *e.g.* $\delta$, 2.2 (2H, m, H-1'); 5.7, 6.01 (s, -CH=C-); no triplet *ca.* 2.6 (ketone, H-1').]

The unsaturated ester (89) (10 g) in ethanol (300 ml) was hydrogenated over 10% Pd/charcoal (3 g) at room temperature and pressure until the uptake of $H_2$ ceased. The catalyst was filtered off to give the *saturated acetonide ester* (90) as an oil (8.3 g, 82%) b.p. 70-75°C/0.01 mm.

I.R. $\nu_{max}$ ($CCl_4$), 1738 (s, ester C=O); 1070 (m, C-O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, H-3'); 1.3 (11H, m, $C(CH_3)_2$, $-OCCH_3$ and H-2'); 1.8-2.5 (5H, m, H-1', H-1 and $-CH_2CO_2-$); 4.14 (5H, m, H-2, H-3 and $-OCH_2-$).

$^{13}C$ N.M.R. *e.g.* $\delta$, 172.84; 171.46; 108.69; 77.85; 68.55; 68.02; 66.58; 60.27; 60.17; 38.79; 38.56; 37.56; 36.60; 35.61; 34.94; 33.12; 31.29; 29.02; 26.46; 25.29; 20.12; 19.68; 14.30; 14.08.

Mass Measurement Found: $M^+ - CH_3$ 229.1440. $C_{12}H_{21}O_4$ requires 229.1440.

## Hydroxy Lactone (91)

Treatment of the diisopropylidene ester (90) (1.05 g) with 70% aqueous acetic acid (20 ml) at 70°C for 1 h, gave, after cooling and evaporation, an oil (356 mg). Distillation afforded the hydroxy lactone (91) as a colourless oil (147 mg, 22%), b.p. 115°C/0.25 mm (kugelrohr).

I.R. $\nu_{max}$ (film), 3440 (s, -OH); 1770 (s, lactone C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.4 (4H, m, H-5' and H-6'); 2.05-2.8 (3H, m, H-3 and H-4); 3.5 (1H, br s, exchangeable with $D_2O$, -OH); 3.8 (2H, br m, H-6); 4.2 (*ca.* $\frac{1}{2}$H, m, H-5); 4.53 (*ca.* $\frac{1}{2}$H, m, H-5).

Mass Measurement Found: $M^+$ 158.0950. $C_8H_{14}O_3$ requires 158.0943.

Attempted oxidation of this hydroxy lactone with PDC[95] in DMF afforded only essentially unreacted hydroxy lactone (91). [I.R. $\nu_{max}$ (film), *e.g.* 3440 (s, -OH); 1770 (s, lactone C=O) $cm^{-1}$.] Due to lack of time, this reaction has not yet been investigated further.

## Experimental Section

## IV Biosynthesis of the Type A Bislactones

(a) Synthesis of Isocanadensic acid (92) and Protoisocanadensic acid (96)

(b) Synthesis of Canadensic acid (22) and the $C_9$ Homologue relevant to Ethisolide Biosynthesis and Attempted Syntheses of Dihydroisocanadensic acid (93)

## (a) Synthesis of Isocanadensic acid (92) and Protoisocanadensic acid (96)

### 1,1,2-Tricarboethoxyoctane (97)

Ethyl 2-bromocaprylate (90 g) in dry ethanol (100 ml) was added to diethyl sodiomalonate [prepared from sodium (8.25 g) and diethyl malonate (57 g) in dry ethanol (130 ml)] and the mixture refluxed for 2 h. After removing the solvent under reduced pressure, the residue was treated with water and extracted with ether to give 1,1,2-*tricarboethoxyoctane* (97) as a pale yellow oil (75 g, 64%), b.p. 120-125°C/0.03 mm (kugelrohr).

I.R. $\nu_{max}$ (film), 1740 (s, ester C=0) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.8 (3H, br t, H-8); 1.4 (19H, m, H-3 to H-7 and $-OCCH_3$); 3.2 (1H, m, H-2); 3.73 (1H, d, $J$ = 10 Hz, H-1); 4.16 (6H, q, $J$ = 7 Hz, $-OCH_2-$).

Analysis Found: C, 61.52; H, 9.41. $C_{17}H_{30}O_6$ requires C, 61.80; H, 9.15%.

### 1,1,2-Tricarboxyoctane[140]

1M Aqueous NaOH (160 ml) was added to 1,1,2-tricarboethoxyoctane (97) (10.4 g) and the mixture stirred at 70-80°C for 24 h. After cooling, the solution was acidified with conc. HCl giving a colourless precipitate. The filtrate was saturated with solid NaCl and thoroughly extracted with ethyl acetate to give a further quantity of colourless solid. Crystallisation from ethyl acetate gave 1,1,2-tricarboxyoctane (5.04 g, 65%), m.p. 138-139°C (Lit.[140] 140-141°C).

I.R. $\nu_{max}$ (nujol), 3600-2400 (s, $-CO_2H$); 1700 (s, acid C=0); 1420 (m) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$ ($C_5D_5N$), 0.8 (3H, br t, H-8); 1.3-2.2 (10H, m, H-3 to H-7); 4.0 (1H, m, H-2); 4.6 (1H, d, $J$ = 10 Hz, H-1); 13.0 (3H, s, $-CO_2H$).

Analysis Found: C, 53.40; H, 7.20. $C_{11}H_{18}O_6$ requires C, 53.65; H, 7.31%.

1-Bromo-1,1,2-tricarboethoxyoctane (98)

Bromine (0.6 ml) in methylene chloride (5 ml) was added dropwise to 1,1,2-tricarboethoxyoctane (97) (5.43 g) in methylene chloride and the solution stirred at room temperature overnight. After refluxing for 3 h and cooling, the mixture was transferred to a separating funnel and washed successively with aqueous sodium metabisulphite, aqueous $NaHCO_3$ and brine to give a pale yellow oil (6.2 g). Preparative t.l.c. (20% ethyl acetate/light petroleum) of a portion (120 mg) gave *1-bromo-1,1,2-tricarboethoxyoctane* (98) as an oil ($R_f$ 0.6, 80 mg, 62%).

I.R. $\nu_{max}$ (film), 1740 (s, ester C=O) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, br t, H-8); 1.3 (19H, m, H-3 to H-7 and $-OCCH_3$); 3.3 (1H, m, H-2); 4.3 (6H, m, $-OCH_2-$).

Mass Spectrum m/e (relative abundance) $M^+$ 408/410 (1), 363/365 (14, M - $OC_2H_5$), 335/337 (5, M - $CO_2C_2H_5$), 329 (14, M - Br), 283 (45, M - Br - $C_2H_5OH$), 257 (30, M - Br - $C_2H_5OH$ - 26).

Mass Measurement Found: $M^+$ - OEt 365.0763 and 363.0797. $C_{15}H_{24}BrO_5$ requires 365.0787 and 363.0807.

1,1,2-Tricarboethoxyoct-2-ene (99)

1-Bromo-1,1,2-tricarboethoxyoctane (98) (6 g) was dissolved in pyridine (12.5 ml) and the mixture heated at 100°C for 2 h. After cooling, the mixture was poured onto ice and extracted with chloroform. The organic extract was washed successively with aqueous sodium metabisulphite, water and brine to give a red oil (3.6 g). Preparative t.l.c. of a portion of this oil (190 mg) using 20% ethyl acetate/petrol gave one major component ($R_f$ 0.62, 174 mg). Preparative t.l.c. of this band using 10% ethyl acetate/petrol and multiple elution (4 times) afforded two major components, one being *1,1,2-tricarboethoxyoct-2-ene* (99) ($R_f$ 0.51, 65 mg).

I.R. $\nu_{max}$ (film), 1735 (s, ester C=O); 1720 (s, unsatd. ester C=O); 1640 (w, C=C) $cm^{-1}$.

<u>N.M.R.</u> (90 MHz) δ, 0.86 (3H, br t, H-8); 1.26 (15H, m, H-5 to H-7 and $-OCCH_3$); 2.55 (2H, br t, *J* = 7 Hz, irr. 6.15 → m, H-4); 4.20 (6H, q, *J* = 7 Hz, $-OCH_2-$); 4.39 (1H, s, H-1); 6.15 (1H, t, *J* = 7 Hz, irr. 2.55 → s, H-3).

<u>Mass Spectrum</u> m/e (relative abundance) $M^+$ 328 (10), 283 (45, M - $OC_2H_5$), 282 (90, M - $C_2H_5OH$), 255 (100, M - $CO_2C_2H_5$), 209 (90, M - $C_2H_5OH$ - $CO_2C_2H_5$).

<u>Mass Measurement</u> Found: $M^+$ 328.1899. $C_{17}H_{28}O_6$ requires 328.1886.

The second component (100), $R_f$ 0.9 was an oil (35 mg) which lacked the characteristic triplet at *ca*. 6.15δ in the N.M.R. spectrum, but was similar in many other respects. After refluxing overnight in ethanol containing sodium ethoxide, the olefinic triplet at *ca*. 6.15δ of 1,1,2-tricarboethoxyoct-2-ene (99) appeared in the spectrum.

<u>(*Z*)-3-Carboxynon-3-enoic acid (103)</u>

1,1,2-Tricarboethoxyoct-2-ene (99) (490 mg) was treated with 1M aqueous sodium hydroxide (20 ml) and stirred at 80°C for 3 h. After cooling, the mixture was extracted with ether to remove any unreacted ester (15 mg) and the alkaline solution acidified with dilute HCl, saturated with solid NaCl and extracted with ethyl acetate to give a pale yellow oil (205 mg). This crystallised from benzene to give *(Z)-3-carboxynon-3-enoic acid* (103) as colourless crystals m.p. 118-121°C (170 mg, 58%).

<u>I.R.</u> $\nu_{max}$ (nujol), 3500-2400 (s, $-CO_2H$); 1710 (br s, acid C=O); 1645 (m, C=C) $cm^{-1}$.

<u>N.M.R.</u> (60 MHz) δ, 0.87 (3H, br t, H-9); 1.3 (6H, br s, H-6 to H-8); 2.63 (2H, m, H-5); 3.32 (2H, s, H-2); 6.28 (1H, t, *J* = 7 Hz, H-4); 11.6 (2H, s, $-CO_2H$).

<u>Analysis</u> Found: C, 59.88; H, 8.21. $C_{10}H_{16}O_4$ requires C, 59.98; H, 8.05%.

(*E*)-3-Carboxynon-3-enoic acid[101] (104)

Diethyl succinate (17.4 g) and *n*-hexanal (10 g), in dry *tert*-butanol (25 ml), were added over 15 mins. to a refluxing solution of potassium (3.9 g) in dry *tert*-butanol (80 ml), the mixture refluxed overnight, acidified with dilute HCl and the solvent evaporated. Ether (100 ml) was added and the mixture washed with water and then extracted with 10% aqueous sodium carbonate. The alkaline solution was acidified with conc. $H_2SO_4$, chilled and extracted with ether. After washing to neutrality with water the organic layer was evaporated to give a red oil which was heated at 80°C in 1M aqueous sodium hydroxide (180 ml) for 6 h. The solution was cooled to 0°C, acidified with dilute $H_2SO_4$ and ether added. The organic layer was washed to neutrality and evaporated to give an oil which when taken up in benzene and kept overnight gave (*E*)-3-carboxynon-3-enoic acid (104) as colourless needles m.p. 139-141°C (Lit.[101] 141-142°C) (10.8 g, 54%).

I.R. $\nu_{max}$ (nujol), 3500-2400 (s, $-CO_2H$); 1710 (s, acid C=O); 1696 (s., unsatd. acid C=O); 1650 (m, C=C) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, br t, H-9); 1.3 (6H, br s, H-6 to H-8); 2.2 (2H, m, H-5); 3.4 (2H, s, H-2); 7.2 (1H, t, $J$ = 7 Hz, H-4); 11.4 (2H, s, $-CO_2H$).

Attempted lactonisation of the 3-Carboxynon-3-enoic acids (103) and (104)

Treatment of either (*E*)- or (*Z*)-3-carboxynon-3-enoic acid (103) or (104) with acids such as trifluoroacetic at 60°C, conc. $H_2SO_4$ at room temperature, 50% $H_2SO_4/H_2O$ or 30% $H_2SO_4$/AcOH at reflux all led to essentially unchanged (from spectral data) unsaturated diacid (103) or (104). Treatment with *p*-toluenesulphonic acid in refluxing benzene resulted in the appearance of bands in the i.r. at 1850 $cm^{-1}$ and 1785 $cm^{-1}$. The reaction was not investigated further.

Treatment of (*Z*)-3-carboxynon-3-enoic acid (103) (122 mg) in

1M aqueous $NaHCO_3$ (3 ml) with potassium iodide (0.5 g) and iodine (0.25 g) in water (1.5 ml) gave, after standing in the dark for 11 days and normal work up, only unreacted diacid (103). The reaction was not investigated further.

3-Bromo-1,1,2-tricarboethoxyoct-1-ene (107)

1,1,2-Tricarboethoxyoct-2-ene (99) (422 mg) in dry methylene chloride (5 ml) was treated dropwise with bromine (1.1 equivalents) in methylene chloride whilst irradiated with a powerful bench lamp. After stirring at room temperature overnight, the mixture was refluxed for 6 h and the solvent evaporated to give an oil (531 mg). Preparative t.l.c. of a portion (307 mg) using 10% ethyl acetate/light petroleum and multiple elution (4 times) gave 3-*bromo*-1,1,2-*tricarboethoxyoct*-1-*ene* (107) as an oil ($R_f$ 0.58, 188 mg, 62%).

I.R. $\nu_{max}$ (film), 1725 (s, unsatd. ester C=O); 1625 (w, C=C) $cm^{-1}$.

N.M.R. (60 MHz) $\delta$, 0.9 (3H, br t, H-8); 1.3 (15H, br t, $J$ = 7 Hz, H-5 to H-7 and $-OCCH_3$); 2.03 (2H, m, H-4); 4.3 (6H, d-q, $J$ = 7 Hz, 2 Hz, $-OCH_2-$); 5.16 (1H, t, $J$ = 8 Hz, H-3).

$^{13}C$ N.M.R. $\delta$, 164.59 (s, C=O); 163.39 (s, C=O); 162.81 (s, C=O); 146.50 (s, C-2); 127.84 (s, C-1); 62.17 (t, $J_{res}$ 47 Hz, 3 x $-OCH_2-$); 46.20 (d, $J_{res}$ 59 Hz, -CHBr); 37.10 (t, $J_{res}$ *ca.* 15 Hz, C-4); 30.93 (t, $J_{res}$ *ca.* 15 Hz, C-5); 27.39 (t, $J_{res}$ *ca.* 14 Hz, C-6); 22.34 (t, $J_{res}$ *ca.* 14 Hz, C-7); 13.95 (q, $J_{res}$ *ca.* 10 Hz, 4 x $-CH_3$).

Mass Measurement Found: $M^+$ - OEt 363.0581 and 361.0585. $C_{15}H_{22}BrO_5$ requires 363.0631 and 363.0651.

The unsaturated bromo ester (107) (70 mg) was refluxed overnight in acetic acid (5 ml). Work up afforded a dark oil [N.M.R. (60 MHz), no triplet *ca.* 5.2 ; I.R., no band *ca.* 1760 $cm^{-1}$]. This reaction was not investigated further.

## 3-Carboxy-3,4-dibromononanoic acid[101] (109)

(*E*)-3-Carboxynon-3-enoic acid (104) (2 g), suspended in dry carbon tetrachloride (7 ml), and bromine (1 ml) were irradiated with a 254 nm ultraviolet lamp overnight. The solid was filtered off and crystallised from toluene to give 3-carboxy-3,4-dibromononanoic acid (109) as colourless needles m.p. 140-141°C (Lit[101] 140-141°C) (3.18 g, 88%).

I.R. $\nu_{max}$ (nujol), 3400-2400 (br s, $-CO_2H$); 1725 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.87 (3H, br t, H-9); 1.36 (6H, br s, H-6 to H-8); 2.1 (2H, m, H-5); 3.42 (2H, q, $J$ = 17 Hz, H-2); 4.54 (1H, d-d, $J_1 + J_2$ = 11 Hz, H-4); 11.2 (2H, s, $-CO_2H$).

$^{13}C$ N.M.R. $\delta$ ($D_6$-acetone), 170.46 (s, $-CO_2H$); 168.71 (s, $-CO_2H$); 65.77 (s, -CBr); 61.50 (d, $J_{res}$ 56.3 Hz, -CHBr); 44.97 (t, $J_{res}$ 38.2 Hz, C-2); 36.53 (t, $J_{res}$ 23.3 Hz, C-5); 31.24 (t, $J_{res}$ *ca.* 13 Hz, C-6); 27.99 (t, $J_{res}$ *ca.* 13 Hz, C-7); 22.82 (t, $J_{res}$ 14.6 Hz, C-8); 14.13 (q, $J_{res}$ 10.8 Hz, $-CH_3$).

## 5-*n*-Pentylaconic acid[101] (106)

3-Carboxy-3,4-dibromononanoic acid (109) (0.5 g) was stirred at room temperature for 30 minutes with 1M aqueous sodium hydroxide (4 ml), the mixture cooled to 0°C and acidified with dilute (3M) sulphuric acid. Extraction with ether followed by washing successively with water and brine gave 5-*n*-pentylaconic acid (106) as colourless crystals, m.p. 132-133°C (Lit.[101] 133-133.5°C) from chloroform (260 mg, 94%).

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2500 (m, $-CO_2H$); 1764 (s, unsatd. lactone C=O); 1715 (s, acid C=O); 1635 (w, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.87 (3H, br t, H-10); 1.32 (6H, br s, H-7 to H-9); 2.1 (2H, m, H-6); 5.25 (1H, m, H-5); 6.8 (1H, d, $J$ = 2 Hz, H-3); 10.1 (1H, s, $-CO_2H$).

4-Carboxy-5-*n*-pentyltetrahydrofuran-2-one (110)

5-*n*-Pentylaconic acid (106) (52 mg) in 1M aqueous $NaHCO_3$ (4 ml) was hydrogenated at room temperature and pressure over 5% rhodium/alumina (50 mg) until the uptake of $H_2$ ceased. After filtration through celite, the solution was acidified with dilute HCl, saturated with NaCl and extracted with methylene chloride to give *4-carboxy-5-n-pentyltetrahydrofuran-2-one* (110) (43 mg, 82%) as colourless plates, m.p. 95-98°C from benzene.

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2400 (s, $-CO_2H$); 1785 (s, lactone C=O); 1720 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.86 (3H, br t, $-CH_3$); 1.2-1.7 (8H, br m, H-6 to H-9); 2.8 (2H, m, H-3); 3.46 (1H, m, H-4); 4.67 (1H, m, H-5); 9.3 (1H, s, $-CO_2H$).

$^{13}C$ N.M.R. $\delta$, 175.74 (s, C=O); 175.59 (s, C=O); 80.63 (d, $J_{res}$ 61 Hz, -CH-O-); 44.25 (d, $J_{res}$ 42 Hz, C-4); 32.02 (t, $J_{res}$ *ca.* 5 Hz, C-3); 31.30 (t, $J_{res}$ 12.6 Hz, C-6); 31.23 (t, $J_{res}$ 13.5 Hz, C-7); 25.52 (t, $J_{res}$ 17.8 Hz, C-8); 22.42 (t, $J_{res}$ 15.4 Hz, C-9); 13.92 (q, $J_{res}$ 9.2 Hz, $-CH_3$).

Analysis Found: C, 60.09; H, 7.91. $C_{10}H_{16}O_4$ requires C, 59.98; H, 8.05%.

Attempted hydrogenation of 5-*n*-pentylaconic acid (106) over palladium or platinum catalysts led to the recovery of essentially unchanged (t.l.c., n.m.r.) unsaturated lactone (106).

Dimethyl Maleate[141]

Maleic acid (120 g) in dry methanol (195 ml) and dry benzene (350 ml), containing conc. $H_2SO_4$(12 ml) was refluxed for 22 h. After cooling, the mixture was poured into water ( 1 litre), the benzene layer separated, washed successively with saturated aqueous $NaHCO_3$ and water to give dimethyl maleate (85 g, 58%) b.p. 200-202°C (Lit.[141] 202°C).

N.M.R. (90 MHz) $\delta$, 3.72 (6H, s, $-OCH_3$); 6.17 (2H, s, olefinic H).

Methyl 3-Carbomethoxy-4-oxononoate (111)

Redistilled *n*-hexanal (19 g) and dimethyl maleate (18 g) were heated at 84° under nitrogen. Benzoyl peroxide (50 mg) was added, followed by a second portion of benzoyl peroxide (50 mg) after 4 h and a third portion (50 mg) after 16 h. After a further 24 h, distillation gave *methyl-3-carbomethoxy-4-oxononoate* (111) (19.7 g, 53%) b.p. 180°C/0.2 mm, $R_f$ 0.37 (20% ethyl acetate/light petroleum).

I.R. $\nu_{max}$ (film), 1735 (s br, ester and ketone C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.3 (4H, m, H-7 and H-8); 1.57 (2H, m, H-6); 2.55 (2H, d, $J$ = 7 Hz, H-2); 2.78 (2H, t, $J$ = 7 Hz, H-5); 3.63 (3H, s, $-OCH_3$); 3.7 (3H, s, $-OCH_3$); 3.84 (1H, t, $J$ = 7 Hz, H-3).

Mass Spectrum m/e (relative abundance) $M^+$ 244 (2), 213 (15, M - $OCH_3$), 188 (20, M - $C_4H_8$), 185 (15, M - $CO_2CH_3$), 156 (30, M - $C_4H_8$ - $CH_3OH$), 99 (100, $C_6H_{11}O$).

Mass Measurement Found: $M^+$ 244.1302. $C_{12}H_{20}O_5$ requires 244.1311.

Lactone Methyl ester (112)

Methyl 3-carbomethoxy-4-oxononoate (111) (22.7 g) in methanol (100 ml) was treated with sodium borohydride (1.2 g) at 0°C (added in small portions over *ca.* 1 h). After stirring for 1.5 h, the reaction was quenched with aqueous 6M HCl and stirred overnight. Ether extraction gave the *lactone methyl ester* (112) (16 g, 80%) b.p. 97-100°C/0.005 mm (kugelrohr).

I.R. $\nu_{max}$ ($CHCl_3$), 1780 (s, lactone C=O); 1740 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.4 (8H, m, H-6 to H-9); 2.64 (2H, m, H-3); 3.4 (1H, m, H-4); 3.72 (3H, s, $-OCH_3$); 4.5 (1H, m, H-5).

Mass Spectrum m/e (relative abundance) $M^+$ 214 (1), 196 (6, M - $H_2O$), 182 (20, M - $CH_3OH$), 154 (24, M - 60), 143 (60, M - $C_5H_{11}$), 115 (100, M - $C_5H_{11}\overset{+}{C}=O$).

Mass Measurement Found: $M^+$ 214.1205. $C_{11}H_{18}O_4$ requires 214.1205.

*Cis* Acid Lactone (110)

The crude lactone methyl ester (112) (20 g) in dioxan (100 ml) and aqueous 6M HCl (300 ml) was refluxed for 5 h. After cooling, the usual work up gave a semi-crystalline oil. Crystallisation from benzene gave the *cis* acid lactone (110) (8 g, 48%) m.p. 97-99°C. Identical in every respect with a sample of (110) prepared by reduction of 5-*n*-pentylaconic acid (106).

Hydroxy Lactone (113)

Methyl 3-carbomethoxy-4-oxononoate (111) (19.7 g) in methanol (95 ml) was treated with sodium borohydride (3.7 g) in methanol (47 ml) and water (47 ml) at 0°C. After stirring overnight, the mixture was acidified with aqueous 1M HCl, saturated with solid NaCl and extracted with ether to give an oil (10 g). [N.M.R. (90 MHz) *e.g.* $\delta$, 4.25 (m); 3.3 (br s, -OH); no signal at *ca.* 3.7 ($-OCH_3$); I.R. $\nu_{max}$ (film), 3450 (s, -OH); 1770 (s, lactone C=0) $cm^{-1}$.]

The above oil (10 g) was refluxed overnight in benzene (75 ml) containing a few crystals of p.t.s.a. The usual work up yielded an orange oil (8 g) essentially unchanged (t.l.c., spectral data) from unreacted material. This material (6.3 g) was treated with dioxan (80 ml) and aqueous 6M HCl (100 ml) and the product extracted with ether. The ethereal layer was extracted with saturated aqueous $NaHCO_3$ to give a small quantity of the known lactone acid (110). Identical (m.p., n.m.r., i.r.) with an authentic sample of the *cis* acid lactone (110). The non-acidic fraction gave the *hydroxy lactone* (113) (2 g) b.p. 115-125°C/0.005 mm (kugelrohr).

I.R. $\nu_{max}$ (film), 3450 (s, -OH); 1765 (s, lactone C=0) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.34 (8H, m, H-6 to H-9); 2.4 (3H, m, H-3 and H-4); 3.55 (3H, br d, $J$ = 4 Hz, $-C\underline{H}_2O\underline{H}$); 4.28 (1H, m, H-5).

<u>$^{13}C$ N.M.R.</u> $\delta$, 177.08 (s, C=O); 83.13 (d, $J_{res}$ 57.7 Hz, C-5); 62.74 (t, $J_{res}$ 47 Hz, $-CH_2OH$); 42.58 (d, $J_{res}$ 28.4 Hz, C-4); 35.18 (t, $J_{res}$ *ca.* 20 Hz, C-3); 31.65 (t, $J_{res}$ *ca.* 15 Hz, C-6); 31.50 (t, $J_{res}$ *ca.* 15 Hz, C-7); 25.14 (t, $J_{res}$ *ca.* 12 Hz, C-8); 22.52 (t, $J_{res}$ *ca.* 15 Hz, C-9); 13.98 (q, $J_{res}$ 9 Hz, $-CH_3$).

<u>Mass Measurement</u> Found: $M^+ - C_5H_{11}$ 115.0389. $C_5H_7O_3$ requires 115.0395.

## *Trans* Acid Lactone (114)

The hydroxy lactone (113) (0.875 g) in dry DMF (13 ml) was treated with PDC[95] (6.125 g) at 0°C and the mixture stirred at room temperature overnight. After pouring into water (100 ml), the solution was extracted with ether to give the trans *acid lactone* (114) (297 mg, 32%) as white plates, m.p. 83-84°C from benzene.

<u>I.R.</u> $\nu_{max}$ (KBr), 3500-2800 (s, $-CO_2H$); 1750 (s, lactone C=O); 1720 (s, acid C=O) $cm^{-1}$.

<u>N.M.R.</u> (90 MHz) $\delta$, 0.89 (3H, br t, $-CH_3$); 1.35 (6H, br s, H-7 to H-9); 1.72 (2H, br m, H-6); 2.92 (3H, m, H-3 and H-4); 4.64 (1H, m, H-5); 10.0 (1H, br s, $-CO_2H$).

<u>Analysis</u> Found: C, 59.91; H, 7.96. $C_{10}H_{16}O_4$ requires C, 59.98; H, 8.05%.

## Isocanadensic acid (92)

The *cis* acid lactone (110) (80 mg) in Stiles' reagent in DMF[96] (8 ml) was heated to 130°C under a slow stream of argon for 20 h. After cooling, the solution was poured into ether (40 ml), acidified with dilute HCl and the ethereal layer extracted with saturated aqueous $NaHCO_3$. The aqueous extract was acidified with dilute HCl and extracted with ether to give the diacid lactone (101) as an oil (100 mg). [N.M.R. *e.g.* $\delta$, 3.98 (1H, s); 4.3 (1H, m); 8.2 (2H, s, $-CO_2H$).]

The diacid lactone (101) (66 mg), 37% aqueous formaldehyde (0.3 ml) and diethylamine (0.1 ml) were stirred at room temperature for 36 h. Ether and brine, containing a few drops of HCl, were added and the ethereal layer separated and washed to give *isocanadensic acid* (92) (14 mg, 24%), m.p. 89-92°C from chloroform.

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2400 (s, $-CO_2H$); 1760 (s, lactone C=O); 1705 (s, acid C=O); 1605 (w, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.85 (3H, br t, H-10); 1.3 (8H, br s, H-6 to H-9); 2.19 (3H, d, $J$ = 2 Hz, $-CH_3$); 5.1 (1H, m, H-5); 8.1 (1H, br s, $-CO_2H$).

Mass Measurement Found: $M^+$ 212.1050. $C_{11}H_{16}O_4$ requires 212.1049.

Treatment of the *cis* diacid lactone (101) with the acetate buffered methylenation reagent[72] also gave isocanadensic acid (92) as part of a mixture (n.m.r.).

Attempted Preparation of Protoisocanadensic acid (96) *via* the Lactone Alcohol (116)

The *cis* acid lactone (110) (67 mg) in dry THF (2 ml) was added to lithium diisopropylamide (2.1 equivalents) in dry THF (10 ml) at -70°C. After stirring for 30 mins., the solution was warmed to -20°C and formaldehyde gas was bubbled in [prepared from paraformaldehyde (160 mg) heated to 180°C]. After stirring for an additional 1 h, the reaction was quenched with satd. aqueous $NH_4Cl$ and extracted with ether to give the crude alcohol (116) as a yellow oil (95 mg).

The above oil (95 mg) in dry ether (1.6 ml) was treated with $PCl_5$ (160 mg) at 0°C. After stirring at room temperature for 5.5h, the mixture was filtered into water (5 ml) and extracted with chloroform to give the crude chloride (117) as an oil (85 mg). [I.R. $\nu_{max}$ ($CHCl_3$), *e.g.* 3400-2400 (s, $-CO_2H$); 1780 (s, lactone C=O); 1715 (s, acid C=O) $cm^{-1}$.

The crude chloride (117) (83 mg) in dry THF (1.34 ml) was treated with sodium hydride (40 mg) and the suspension stirred at room temperature for 2.5 h. After adding water, the mixture was acidified with dilute HCl saturated with solid NaCl and extracted with ether to give an oil.

Preparative t.l.c. (20% methanol/chloroform) gave one major component $R_f$ 0.76, 43 mg. [I.R. $\nu_{max}$ ($CHCl_3$), *e.g.* 1760 (s, lactone C=O); 1710 (s, acid C=O) $cm^{-1}$; N.M.R. (90 MHz) *e.g.* $\delta$, 2.2 (d, $J$ = 2 Hz, olefinic $-CH_3$); 2.75 (m); 6.0, 6.45 (both s, $C=CH_2$).]

<u>(Methylthio)-Methyl Lactone acid (119)</u>

The diacid lactone (101) (403 mg) [prepared by treating the *cis* acid lactone (110) with Stiles' reagent[96]] in dry THF (5 ml) was added to a solution of lithium diisopropylamide (4.95 mM, 3 equivs.) in dry THF (15 ml) at -78°C. After warming to room temperature, HMPA (2.5 ml), followed by one equivalent of iodomethyl methyl sulphide [formed *in situ* from chloromethyl methyl sulphide (0.14 ml) and anhydrous LiI[142] (221 mg)] in dry THF (2.5 ml) was added and the mixture stirred for 1 h. The reaction was quenched with dilute HCl and extracted with methylene chloride to give the diacid lactone sulphide (118) as a viscous oil (275 mg, 65%). [N.M.R. (90 MHz) *e.g.* $\delta$, 2.12 (3H, s, -SMe); 4.6 (1H, m, -CH-O-); 8.78 (2H, s, $-CO_2H$).]

The above diacid lactone sulphide (118) (275 mg) in dry xylene (15 ml) was refluxed for 2 h. After cooling, the solvent was evaporated off and chromatography on silica gel gave the *(methylthio)-methyl lactone acid* (119) (190 mg, 81%), m.p. 115-117°C.

<u>I.R.</u> $\nu_{max}$ ($CHCl_3$), 3500-2400 (s, $-CO_2H$); 1775 (s, lactone C=O) 1712 (s, acid C=O) $cm^{-1}$.

<u>N.M.R.</u> (90 MHz) $\delta$, 0.88 (3H, br t, $-CH_3$); 1.2-1.7 (8H, m, H-6 to H-9); 2.11 (3H, s, $-SCH_3$); 2.7-3.7 (4H, m, H-3', H-3 and H-4); 4.6 (1H, m, H-5); 9.9 (1H, s, $-CO_2H$).

<u>Mass Measurement</u> Found: $M^+$ 260.1091. $C_{12}H_{20}O_4S$ requires 260.1082.

Protoisocanadensic acid (96)

The lactone sulphide (119) (130 mg) in methyl iodide (4 ml) was refluxed under argon for 40 h during which time a red solid separated out. After cooling, the solvent was evaporated off and the residue triturated with ether and dried under vacuum to give the sulphonium salt (120) as an orange solid. The sulphonium salt (120) in 5% aqueous $NaHCO_3$ (5 ml) covered with ether was stirred at room temperature for 4.5 h. The ether layer was removed and the aqueous portion saturated with solid NaCl, acidified at 0°C with 30% aqueous acetic acid and extracted with ether. The ether extract was washed with brine, dried and evaporated at 10°C to give *protoisocanadensic acid* (96) (50 mg, 47%) m.p. 59-63°C from chloroform.

I.R. $\nu_{max}$ ($CHCl_3$), 3500-2400 (s, $-CO_2H$); 1765 (s, lactone C=O); 1718 (s, acid C=O); 1660 (w, C=C); 1600 (w, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.88 (3H, br t, $-CH_3$); 1.34 (6H, m, H-7 to H-9); 1.67 (2H, m, H-6); 3.62 [*ca.* ½H, d-t, $J$ = 3 Hz, 5.5 Hz, irr. *ca.* 5.95$\delta$ → d, $J$ = 5.5 Hz, irr. *ca.* 4.7$\delta$ → br s, H-4 (*trans*)]; 4.03[*ca.* ½H, d-t, $J$ = 2 Hz, 7.5 Hz, irr. *ca.* 5.95$\delta$ → d, $J$ = 7.5 Hz, irr. *ca.* 4.7$\delta$ → br s, H-4 (*cis*)]; 4.72 (1H, m, H-5); 5.89 [*ca.* ½H, d, $J$ = 2 Hz, irr. *ca.* 6.4$\delta$ → s, $H_b$ (*cis*)]; 6.02 [*ca.* ½H, d, $J$ = 3 Hz, irr. *ca.* 6.4$\delta$ → s, $H_b$ (*trans*)]; 6.42 (t, $J$ = *ca.* 2 Hz, irr. *ca.* 5.95$\delta$ → s, $H_a$); 8.96 (1H, br s, $-CO_2H$).

Mass Spectrum m/e (relative abundance) $M^+$ 212 (6), 194 (10, M - $H_2O$), 168 (7, M - $CO_2H$), 141 (100, M - $C_5H_{11}$).

Mass Measurement Found: $M^+$ 212.1050. $C_{11}H_{16}O_4$ requires 212.1048.

Attempted formation of Protoisocanadensic acid (96) *via* the Lactone Sulphoxide (121)

The lactone sulphide (119) (28 mg) in methanol (0.4 ml) was added to sodium metaperiodate (23 mg) in water (0.5 ml) at 0°C and stirred for 15 h. Extraction with methylene chloride gave the lactone sulphoxide (121) as an oil (30 mg). [N.M.R. (90 MHz) *e.g.* $\delta$, 2.18 (d, $J$ = 2 Hz, C=C-C$\underline{H}_3$); 2.76 (m, -$SOCH_3$); 5.87, 6.4 (both s, C=C$\underline{H}_2$).]

The above oil (30 mg) was refluxed in toluene (2 ml) under nitrogen for 15 h. After cooling, the solvent was removed under reduced pressure to give an oil. Chromatography on silica gel afforded the isomeric lactones (92) and (96) (16 mg, 70%) as an oil. [N.M.R. (90 MHz) *e.g.* $\delta$, 2.18 (d, $J$ = 2 Hz, C=C-C$\underline{H}_3$); 5.88, 6.42 (both s, C=C$\underline{H}_2$).]

$D_5$-Chloromethyl methyl sulphide

Redistilled thionyl chloride (27 g) in dry methylene chloride (40 ml) was stirred at 35°C and $D_6$-dimethyl sulphoxide (17 g) in dry methylene chloride (40 ml) added dropwise over 2 h. The mixture was then refluxed for 1 h and distilled to give *$D_5$-chloromethyl methyl sulphide* as a colourless oil (17.4 g, 84%) b.p. 105-107°C [Lit.[111] b.p. 104-106°C (chloromethyl methyl sulphide)].

I.R. $\nu_{max}$ (film), 2130 (m); 1230 (m); 1045 (m); 935 (s); 710 (s) $cm^{-1}$.

$D_5$-(Methylthio)-methyl Lactone acid (119)

Using the same procedure described for the preparation of the (methylthio)-methyl lactone (119), the crude diacid lactone (400 mg) was alkylated using $D_5$-chloromethyl methyl sulphide (0.2 ml) and anhydrous LiI[142] (0.25 g) to give the crude $D_5$-diacid lactone sulphide (118) (479 mg). Refluxing in dry xylene (20 ml) gave, after work up, crude *$D_5$-(methylthio)-methyl lactone acid* (119) as an off-white solid

(394 mg). [I.R. $\nu_{max}$ ($CHCl_3$), 3500-2400 (s, $-CO_2H$); 1778 (s, lactone C=O); 1715 (s, acid C=O) $cm^{-1}$; N.M.R. (90 MHz) *e.g.* $\delta$, 0.9 ( br t, $-CH_3$); 2.8 (m); 3.5 (m).]

Mass Measurement Found: $M^+$ 265.1378. $C_{12}H_{15}D_5O_4S$ requires 265.1390.

Although contaminated ($^1H$ n.m.r.) with some acid lactone (110), this was used in the subsequent reaction without further purification.

$D_2$-Protoisocanadensic acid (96)

The crude $D_5$-lactone sulphide (119) (390 mg) in methyl iodide (7 ml) was refluxed under nitrogen for 45 h. After cooling, the solvent was evaporated and the residue triturated with ether and dried under vacuum to give the crude sulphonium salt (120). This was treated with 5% aqueous $NaHCO_3$ (10 ml) and ether, and stirred at room temperature for 4 h. The ether layer was removed and the aqueous portion saturated with solid NaCl, acidified with 30% aqueous acetic acid and extracted with ether to give *$D_2$-protoisocanadensic acid* (96) (54 mg, 17%) as a semi-crystalline oil.

I.R. $\nu_{max}$ ($CHCl_3$), 3400-2500 (s, $-CO_2H$); 1765 (s, lactone C=O); 1718 (s, acid C=O); 1630 (w, C=C) $cm^{-1}$.

N.M.R. (90 MHz) *e.g.* $\delta$, 0.83 (br t, $-CH_3$); 2.17 (d, $J$ = 2 Hz, isocanadensic acid, $C{=}C{-}CH_3$); 2.8 (m); 3.38 (m); 3.59 [*ca.* $\frac{1}{2}$H, d $J$ = 5.5 Hz, H-4 (*trans*)]; 3.99 [*ca.* $\frac{1}{2}$H, d, $J$ = 7.5 Hz, H-4 (*cis*)]; 4.7 (m); no signals at *ca.* 6.0 or *ca.* 6.4 .

Although contaminated with a small quantity (*ca.* 10%) of $D_2$-isocanadensic acid and the *cis* acid lactone (110) (*ca.* 25%) the product was used in a preliminary feeding experiment with *P. canadense*.

Administration of $D_2$-Protoisocanadensic acid (96) to cultures of *P. canadense*

The crude $D_2$-protoisocanadensic acid (96) (*ca.* 50 mg) as its sodium salt was fed, in three 24 h pulses, on the 8th, 9th and 10th days after inoculation, to five Roux bottles containing cultures of *P. canadense*. On the 12th day after inoculation, the aqueous broth was separated by decantation and acidified with conc. HCl to pH 2. Continuous extraction with ethyl acetate for 48 h yielded an oil (2.25 g), which after preparative t.l.c. (chloroform:light petroleum, 2:1) using multiple elution (4 times) afforded dihydrocanadensolide (4) ($R_f$ 0.5, 38.7 mg) m.p. 94-94.5°C (Lit.[7] m.p. 94-95°C) from ether/light petroleum (b.p. 40-60°C) and canadensolide (3) ($R_f$ 0.4, 183 mg) as a viscous oil (Lit.[7] m.p. 46-47.5°C). Preparative t.l.c. of an oily fraction ($R_f$ 0.1, 747 mg) using 2.5% acetic acid/chloroform, gave canadensic acid (22) ($R_f$ 0.4, 71 mg) m.p. 114-114.5°C (Lit.[7] m.p. 114-115°C) from benzene.

The sample of canadensolide (3) has been submitted for $^2H$-n.m.r.

(b) Synthesis of Canadensic acid (22) and the $C_9$ Homologue relevant to Ethisolide Biosynthesis and Attempted Syntheses of Dihydro-isocanadensic acid (93)

Lactone Dimethyl ester (125b)

γ-Octanoic lactone (123b) (14.23 g) and phosphorus tribromide (0.5 ml) were heated at 130°C and bromine (5 ml) added over 30-45 mins. The mixture was then heated at 150°C for 4 h, cooled and distilled to give the bromo lactone (124b) as a colourless oil (11.51 g, 52%) b.p. 140-150°C/0.02 mm (kugelrohr). This tended to decompose on standing and was therefore used immediately.

The crude bromo lactone (124b) (11.5 g) in dry THF (50 ml) was added to a suspension of dimethyl sodiomalonate [prepared from sodium hydride (1.25 g) and dimethyl malonate (6.88 g) in dry THF (100 ml)] under argon at room temperature. After heating overnight at 50°C, the mixture was cooled and diluted with ether and water. Separation of the ether layer, followed by evaporation gave the *lactone dimethyl ester* (125b) as an oil (6.4 g, 45%), b.p. 155-160°C/0.02 mm (kugelrohr).

I.R. $\nu_{max}$ ($CHCl_3$), 1770 (s, lactone C=O); 1755, 1740 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) δ, 0.89 (3H, br t, $-CH_3$); 1.3-1.9 (6H, m, H-6 to H-8); 2.45 (2H, m, H-4); 3.3 (1H, m, H-3); 3.75 (3H, s, $-OCH_3$); 3.78 (3H, s, $-OCH_3$); 3.87 (1H, d, $J$ = 7 Hz, H-2'); 4.45 (1H, m, H-5).

Mass Spectrum m/e (relative abundance) $M^+$ 272 (3), 241 (15, M - $OCH_3$), 215 (80, M - $C_4H_9$), 187 (45, M - $C_4H_9\overset{+}{C}{=}O$), 183 (40, M - $C_4H_9$ - $CH_3OH$), 155 (100, M - $C_4H_9\overset{+}{C}{=}O$ - $CH_3OH$).

Mass Measurement Found: $M^+$ 272.1256. $C_{13}H_{20}O_6$ requires 272.1260.

Lactone Dimethyl ester (125a)

γ-Hexanoic lactone (123a) (11.4 g) and phosphorus tribromide (0.5 ml) were heated at 130°C and bromine (5 ml) added over 30-45 mins. The mixture was then heated at 150°C for 4 h, cooled and distilled to give the bromo lactone (124a) as a colourless oil (11.6 g, 60%) b.p. 95-100°C/0.007 mm (kugelrohr). [ N.M.R. (90 MHz) *e.g.* δ, 0.99 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.76 (2H, m, H-6); 2.5 (2H, m, H-4); 4.5 (2H, m, H-3 and H-5)]. The bromo lactone tended to decompose on standing therefore it was used immediately.

The bromo lactone (124a) (11.6 g) in dry THF (50 ml) was added to a suspension of dimethyl sodiomalonate [prepared from sodium hydride (1.44 g) and dimethyl malonate (7.97 g) in dry THF (100 ml)] under argon at room temperature. After heating overnight at 50°C, the mixture was cooled and diluted with ether and water, and the ether layer separated to give the *lactone dimethyl ester* (125a) as an oil (10.1 g, 68%) b.p. 135-140°C/0.02 mm (kugelrohr).

I.R. $\nu_{max}$ ($CHCl_3$), 1775 (s, lactone C=O); 1755, 1740 (s, ester C=O) $cm^{-1}$.

N.M.R. (90 MHz) δ, 0.98 (3H, br t, $-CH_3$); 1.75 (2H, m, H-6); 2.1-2.6 (2H, m, H-4); 3.25 (1H, m, H-3); 3.73 (3H, s, $-OCH_3$); 3.78 (3H, s, $-OCH_3$); 3.82 (1H, d, $J$ = 7 Hz, H-2'); 4.4 (1H, m, H-5).

Mass Spectrum m/e (relative abundance) $M^+$ 244 (1), 215 (50, M - $C_2H_5$), 213 (20, M - $OCH_3$), 155 (100, M - $C_2H_5\overset{+}{C}{=}O$ - $CH_3OH$).

Mass Measurement Found: $M^+$ 244.0963. $C_{11}H_{16}O_6$ requires 244.0947.

Diacid Lactone (126b)

The lactone diester (125b) (3 g) was stirred at room temperature with aqueous 1M NaOH (40 ml) for 20 h. After extraction with ether, the aqueous solution was acidified with conc. HCl at 0°C, saturated with NaCl and extracted with ethyl acetate to give the *diacid lactone* (126b) (2.25 g, 83%) m.p. 125-127°C from ether/benzene.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1760 (s, lactone C=O); 1725, 1705 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.9 (3H, br t, $-CH_3$); 1.4-1.7 (6H, m, H-6 to H-8); 2.5 (2H, m, H-4); 3.35 (1H, m, H-3); 3.84 (1H, d, $J$ = 6 Hz, H-2'); 4.48 (1H, m, H-5); 10.0 (2H, s, $-CO_2H$).

Analysis Found: C, 54.24; H, 6.54. $C_{11}H_{16}O_6$ requires C, 54.09; H, 6.60%

Diacid Lactone (126a)

The lactone diester (125a) (5 g) was stirred at room temperature with aqueous 1M NaOH (80 ml) for 18 h. After extraction with ether, the aqueous solution was acidified with conc. HCl, saturated with NaCl and extracted with ethyl acetate to give the *diacid lactone* (126a) (3.8 g, 86%) m.p. 132-134°C from benzene.

I.R. $\nu_{max}$ (KBr), 3600-2500 (s, $-CO_2H$); 1755 (s, lactone C=O); 1725 (s, acid C=O) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.96 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.7 (2H, m, H-6); 2.5 (2H, m, H-4); 3.39 (1H, m, H-3); 3.86 (1H, d-d, $J$ = 6 Hz, 2Hz, H-2'); 4.48 (1H, m, H-5); 9.82 (2H, s, $-CO_2H$).

Analysis Found: C, 50.17; H, 5.27. $C_9H_{12}O_6$ requires C, 50.0; H, 5.59%.

(±)-Canadensic acid (22)

The diacid lactone (126b) (350 mg) was treated with diethylamine (0.2 ml) and 37% aqueous formaldehyde (0.6 ml) and the mixture stirred at room temperature for 60 h. Ether, followed by brine containing a few drops of HCl, was then added and the ethereal layer separated to give *(±)-canadensic acid* (22) (115 mg, 38%) m.p. 131-132.5°C from benzene.

I.R. $\nu_{max}$ ($CHCl_3$), 3500-2400 (s, $-CO_2H$); 1768 (s, lactone C=O); 1700 (s, acid C=O); 1630 (m, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$, 0.9 (3H, br t, $-CH_3$); 1.3-1.8 (6H, m, H-5 to H-7); 1.9-2.7 (2H, m, H-3); 3.69 (1H, d-d, H-2); 4.45 (1H, m, H-4); 5.97 (1H, s, $H_b$); 6.56 (1H, s, $H_a$); 10.3 (1H, br s, $-CO_2H$).

Analysis Found: C, 62.46; H, 7.27. $C_{11}H_{16}O_4$ requires C, 62.24; H, 7.59%

Treatment of the diacid lactone (126b) with the acetate buffered formylation reagent[72] resulted in only trace amounts of the desired compound (22) being found (n.m.r.).

Lactone $C_9$ Homologue (122)

The diacid lactone (126a) (500 mg) was treated with diethylamine (0.33 ml) and 37% aqueous formaldehyde (1 ml) and the mixture stirred at room temperature overnight. After the addition of brine (2 ml) containing a few drops of conc. HCl, extraction with ethyl acetate gave *lactone $C_9$ homologue* (122) as colourless crystals (240 mg, 56%) m.p. 125-127°C from benzene.

I.R. $\nu_{max}$ (KBr), 3600-2400 (s, $-CO_2H$); 1768 (s, lactone C=O); 1690 (s, acid C=O); 1632 (s, C=C) $cm^{-1}$.

N.M.R. (90 MHz) $\delta$ ($D_6$-acetone), 0.95 (3H, t, $J$ = 7 Hz, $-CH_3$); 1.7 (2H, m, H-5); 2.1-2.7 (2H, m, H-3); 3.77 (1H, d-d, H-2); 4.4 (1H, m, H-4); 5.88 (1H, s, $H_b$); 6.33 (1H, s, $H_a$); 8.48 (1H, br s, $-CO_2H$).

Analysis Found: C, 58.44; H, 6.29. $C_9H_{12}O_4$ requires C, 58.68; H, 6.56%

Attempted Preparation of Dihydroisocanadensic acid (93)

(i) Treatment of the *cis* lactone methyl ester (131) [prepared from the *cis* acid lactone (110) and diazomethane] (428 mg) with 1.2 equivalents of lithium diisopropylamide at -70°C in THF followed by the addition of methyl iodide (0.15 ml) and HMPA (405 mg) gave, after the usual work up, an orange oil. [N.M.R. (90 MHz) *e.g.* δ, 3.74 (s, $-OCH_3$); 4.58 (m); no signal at *ca.* 1.8; I.R. $\nu_{max}$ (film), 1775 (s, lactone C=O); 1735 (s, ester C=O) $cm^{-1}$.]

(ii) Treatment of the *cis* lactone methyl ester (131) (856 mg) or the *cis* acid lactone (110) (400 mg) with 1 equivalent and 2 equivalents respectively, of lithium diisopropylamide in THF at -70°C, followed by the addition of trimethylsilyl chloride gave, after non-aqueous work up,[120] an oil. In both cases, consisting essentially of unreacted material. [N.M.R. (90 MHz) *e.g.* δ(methyl ester); 3.72 (s, $-OCH_3$); 4.5 (m); no signal *ca.* 5-6 (olefinic H).] This reaction was not investigated further.

# CHAPTER 6

## Identification of *N*-Acetyl-6-hydroxytryptophan as a Natural Substrate from a Mutant of *Aspergillus nidulans*

(a) Introduction and Discussion

(b) Experimental Section

# Identification of *N*-Acetyl-6-hydroxytryptophan as a Natural Substrate of a Monophenolase Enzyme from a Mutant of *Aspergillus nidulans*

## (a) Introduction and Discussion

Many species of *Aspergillus* have a grey-brown pigment in the upper parts of the conidiophore, including the metulae and phialides.[143] Studies with conidiation mutants of *A. nidulans*[144] have used aconidial mutants, such as *brlA42*, in which these parts of the mycelium are exposed at the colony surface, to select "ivory" mutants lacking this pigment. These mutants fall into two groups, genetically distinguished as belonging to *ivoA* and *ivoB* loci. Mutants of the latter type are deficient in a specific phenol oxidase[145] and they also accumulate, at the time of conidiophore formation, a pigment precursor which is the subject of this work. The *ivoA* mutants possess the phenol oxidase and are assumed to be unable to synthesise the precursor.

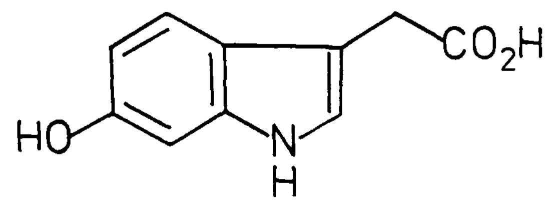
The *brlA42* mutants of *A. nidulans* are aconidial, but carry multiple tiers of pigmented metulae.[144] An *ivoB* derivative of this mutant[146,147] lacked the conidiophore pigment and was completely deficient in the monophenolase enzyme. Preliminary studies[148] showed that this strain, unlike the wild type or simple *brlA42* mutants, accumulated a compound soluble in water or methanol, but not in non-polar solvents, which after paper chromatography or silica gel t.l.c. could be detected by the ferric chloride - potassium ferricyanide reagent.[149] This compound also stained blue with Ehrlich's reagent and bright pink with diazotised sulphanilic acid which suggests the presence of a 6-hydroxyindole moeity.[150] This was supported by the u.v. and n.m.r. spectra of the purified metabolite which were very similar to those of 6-hydroxyindole acetic acid (134) (Tables 3 and 4).[151]

| | | | | | |
|---|---|---|---|---|---|
| *N*-Ac-6-HO-tryptophan (136) | | 235 | 267inf. | 276 | 295 |
| in $OH^-$ | *ca.* | 235 | 268 | 278inf. | 313 |
| 6-HO-indoleacetic acid[151] (134) | | 233 | 266inf. | 274 | 296 |
| in $OH^-$ | | 230 | 267 | 277inf. | 311 |

Table 3. U.v. data for 6-hydroxy indoles (136) and (134)

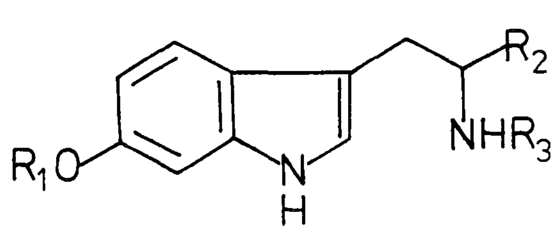
| | H-2 | H-4 | H-5 | H-7 |
|---|---|---|---|---|
| *N*-Ac-6-HO-tryptophan (136) | 6.91 | 7.36 | 6.59 | 6.74 |
| 6-HO-indoleacetic acid[151] (134) | 6.97 | 7.33 | 6.59 | 6.75 |

Table 4. Chemical shift values (δ) for aryl region of 6-hydroxy indoles (136) and (134).



(134)

The spectrum of the aryl proton region was particularly useful, showing the expected pattern of chemical shifts and ortho, meta and para coupling constants and being closely similar to this region in the spectrum of 6-hydroxytryptamine (135)( Fig. 38). The metabolite was deduced to be *N*-acetyl-6-hydroxytryptophan (136) from the remaining spectroscopic data and was characterised as its methyl ether methyl ester (137).



(135) $R_1 = R_2 = R_3 = H$

(136) $R_1 = H$, $R_2 = CO_2H$, $R_3 = COMe$

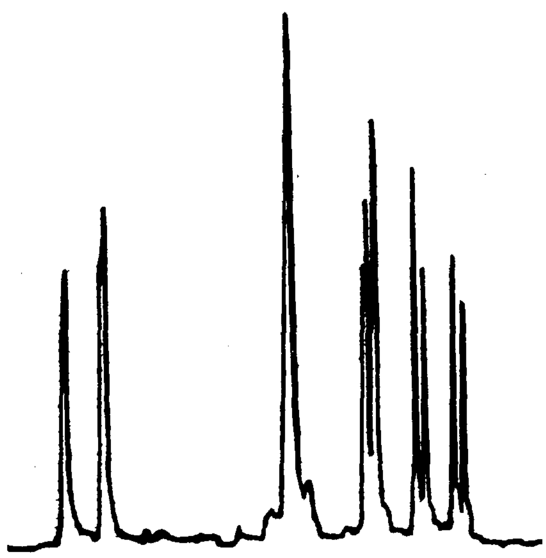
(137) $R_1 = Me$, $R_2 = CO_2Me$, $R_3 = COMe$

(138) $R_1 = Bzl$, $R_2 = CO_2H$, $R_3 = H$

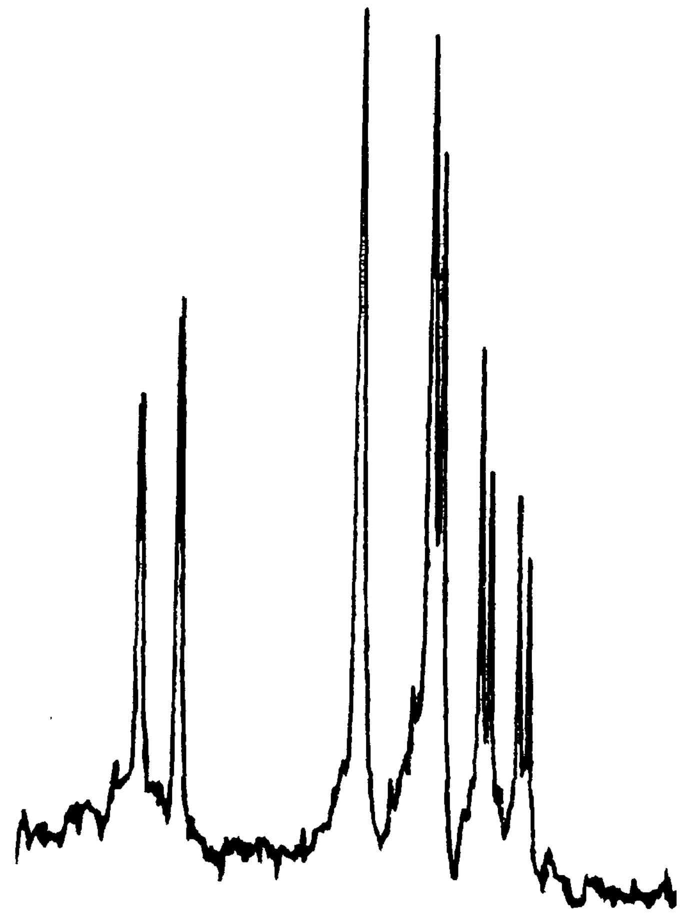
(141) $R_1 = R_3 = H$, $R_2 = CO_2H$

(142) $R_1 = H$, $R_2 = CO_2Me$, $R_3 = COMe$

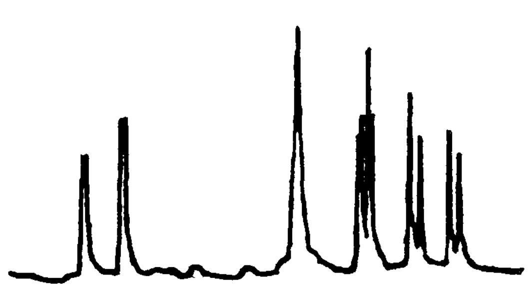
These structures were confirmed in this present work by the synthesis of (136) and (137) in racemic form from 6-benzyloxytryptophan (138). This compound has been used as an intermediate in the synthesis of analogues of the important tranquilliser reserpine (139) and we were fortunate to find a small sample of this available from



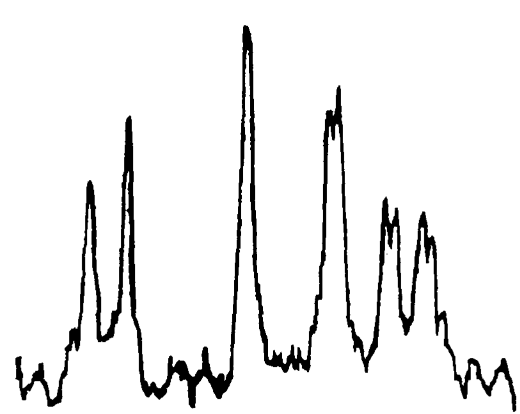
Synthetic (136)
100 MHz



Natural (136)
100 MHz



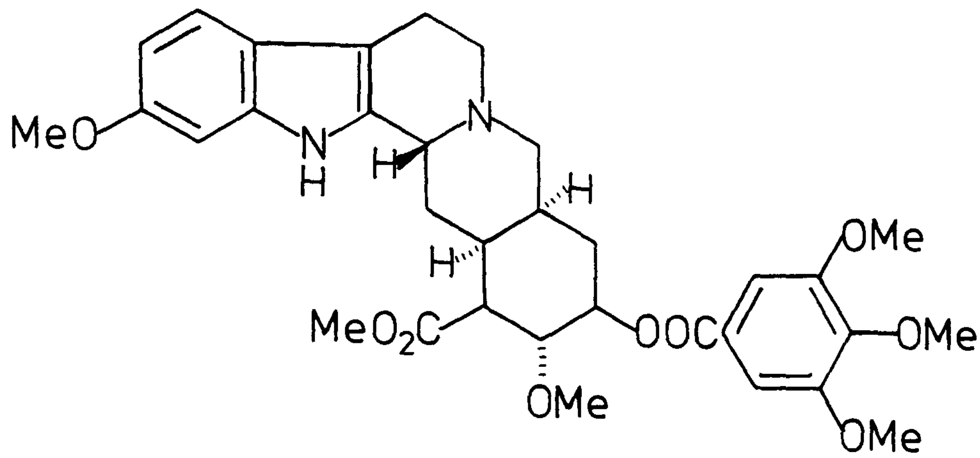
(142)
100 MHz



(135)
90 MHz

Fig. 38 $^1$H-n.m.r. spectra (aryl region) of 6-Hydroxyindoles

Sigma Chemical Co. (apparently their entire stock!).

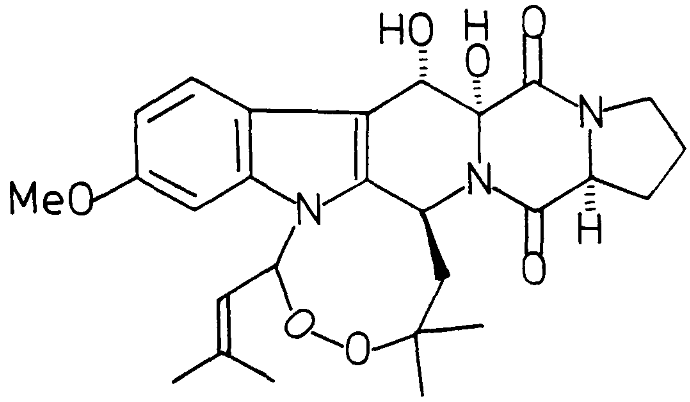


(139)

Selective acetylation of the amino function was effected by treatment of (138) with acetic anhydride in the presence of aqueous NaOH giving the *N*-acetyl derivative (138, $R_3$ = COMe) as a crystalline solid. Hydrogenolysis of this material (138, $R_3$ = COMe) using 10% Pd/C gave the desired *N*-acetyl-6-hydroxytryptophan (136) as an unstable colourless glass - the instability of this product was such that all manipulations had to be carried out at low temperature and in an inert atmosphere. Rapid treatment of (136) with an excess of ethereal diazomethane afforded the methyl ether methyl ester (137).

6-Oxygenated indoles are relatively uncommon as natural products. One recent example in fungal metabolism is verruculogen[152] (140).

Extracts of *ivoB* strains also showed the presence of traces of another phenolic compound which stained with the same reagents, but which was lost during the purification process. The $R_f$ of this compound on paper using butanol:acetic acid:water was 0.25 [*cf.* (136), $R_f$ 0.78] which is close to the published figure for 5-hydroxytryptophan. This suggests that this minor constituent may be 6-hydroxytryptophan (141).



(140)

Since the monophenolase enzyme is only produced at the time of conidiophore formation, enzyme preparations were obtained as crude extracts of an *ivoA brlA42* strain held at this stage of development. Both the natural and synthetic samples of (136) were rapidly oxidised by these preparations giving, where sufficient material was oxidised, brown products, suggesting comparison with the pigment found *in vivo*. In the present work a number of compounds related to (136) were also tested as substrates. A sample of the methyl ester (142) prepared from *N*-acetyl-6-benzyloxytryptophan (138, $R_3$ = COMe) by treatment with ethereal diazomethane followed by hydrogenolysis was utilised much more slowly (at least a hundred times) than the natural substrate (136), and commercial samples of 6-hydroxytryptamine (135) and 5-hydroxytryptophan were not oxidised at all.

## (b) Experimental Section

### Production and Isolation of *N*-Acetyl-6-hydroxytryptophan (136)[148]

Strain G841 of *Aspergillus nidulans* (full genotype *pabaA1; br1A42 uaY9 ivoB63*) was grown in Petri dishes on agar containing "complete medium"[147] [Czapek-Dox (250 ml), peptone (2 g), yeastrel (1 g), casein hydrolysate (5 ml), dextrose (10 g), vitamin soln. (1 ml), water (750ml), agar No. 3 Oxoid (12 g), J.A.P. trace elements[153] adjusted to pH 6 - 6.2 with 4N NaOH]. After 4 days at 37°C, the mycelial mat from 50 such cultures was dried and then ground several times with cold methanol. Evaporation of the combined extracts under reduced pressure gave an oil which was triturated with ethyl acetate to remove fats and other neutral impurities. A solution of the residue in water (30 ml) was satd. with ammonium sulphate and acidified to pH 2-3 with $H_3PO_4$. Extraction with ethyl acetate was followed by purification by t.l.c. using *n*-butanol:acetic acid:water (63:10:27) as eluent. The phenol was conveniently located on chromatograms by staining small areas with diazotised sulphanilic acid and was obtained as a colourless glass (2 - 5 mg).

I.R. $\nu_{max}$ (KBr), 3390 (br); 1710; 1620 (br); 1550 (sh) $cm^{-1}$.

The sample showed u.v. and n.m.r. spectra and t.l.c. behaviour identical to those of a sample of synthetic N-acetyl-6-hydroxy-DL-tryptophan (136). Fluorescence peaks measured in ethanol with a Baird Atomic Fluoripoint fluorimeter occurred at 410 nm (excitation 320 nm) at neutral or acid pH and at 440 nm (excitation 345 nm or less effectively 265 nm) in alkali.

### Methylation of the above Metabolite[154]

Treatment of the above phenolic acid (7 mg) in methanol with an excess of ethereal diazomethane gave an oil which after preparative t.l.c. (ethyl acetate) gave the *methyl ether methyl ester* (137), $R_f$ 0.3

(orange spot on development with $Ce^{4+}$ changing to green on heating).

U.V. $\lambda_{max}$ (EtOH), 225 nm ($\varepsilon$ 19,700); 275 (3,200); 294 (3,500).

I.R. $\nu_{max}$ ($CHCl_3$), 3480 (NH); 3440 (NH); 1740 (s, ester C=O); 1680 and 1550 (amide C=O); 1630; 1500; 1220 $cm^{-1}$.

N.M.R. (100 MHz) $\delta$, 1.96 (3H, s, $-COCH_3$); 3.28 (2H, d, $J$ = 5.3 Hz, $-C\underline{H}_2-CHN-$); 3.7 (3H, s, $-OCH_3$); 3.84 (3H, s, $-OCH_3$); 4.94 (1H, d-t, $J$ = 5.3 Hz, 8 Hz, $D_2O \rightarrow$ t, $J$ = 5.3 Hz, $-CH_2C\underline{H}NH-$); 6.02 (1H, br d, $J$ = 8 Hz, exchangeable with $D_2O$, NH); 6.79 (1H, d-d, $J$ = 1.9 Hz, 8.3 Hz, H-5); 6.83 (1H, s, H-2); 6.84 (1H, d, $J$ = 1.9 Hz, H-7); 7.38 (1H, d, $J$ = 8.3 Hz, H-4); 8.05 (1H, br s, exchangeable with $D_2O$, NH).

Mass Spectrum m/e 290 ($M^+$), 231 (M - 59), 216 (M - 59 - 15), 200, 189 (M - 59 - 42), 176, 174, 160 [base peak, $\beta$-(6-methoxyindolyl)$CH_2^+$], 145; with metastable ions corresponding to the transitions 290 $\rightarrow$ 231, 231 $\rightarrow$ 216, 189 $\rightarrow$ 174 and 160 $\rightarrow$ 145.

Mass Measurement Found: $M^+$ 290.12664. $C_{15}H_{15}N_2O_4$ requires 290.12663.

## 6-Hydroxytryptamine (135)

A sample of this was obtained from Sigma Chemical Company Ltd. as its creatinine sulphate complex.

N.M.R. (90 MHz) $\delta$ ($CD_3OD$, $H_2O$) *e.g.*, 6.71 (1H, d-d, $J$ = 2 Hz, 8.5 Hz, H-5); 6.90 (1H, d, $J$ = 2 Hz, H-7); 7.11 (1H, s, H-2); 7.36 (1H, d, $J$ = 8.5 Hz, H-4).

## *N*-Acetyl-6-benzyloxy-DL-tryptophan (138, $R_3$ = COMe)

6-Benzyloxy-DL-tryptophan (138) (100 mg) in 1M aqueous NaOH (2 ml) was treated with acetic anhydride (3 x 1.7 ml) with vigorous shaking after each addition. The mixture was then heated at *ca.* 40°C for 2 h and allowed to stand at room temperature overnight. The resulting ppt. was separated, and washed successively with cold $H_2O$ (2 x 3 ml), 1M aqueous HCl (3 ml) and cold $H_2O$ (2 x 3 ml). The crude product (138, $R_3$ = COMe) (81 mg) crystallised from methanol as a white microcrystalline solid, m.p. 206-208°C (Lit.[155] m.p. 208-210°C).

I.R. $\nu_{max}$ (nujol), 3405 (s, NH); 3335 (NH); 1725, 1715; 1650 (sh); 1635 (s); 1559; 1258 cm$^{-1}$.

N.M.R. (90 MHz) $\delta$, 1.9 (3H, s, NAc); 3.20 (2H, m, $-C\underline{H}_2CHN-$); 4.70 (1H, m, $-CH_2C\underline{H}N-$); 5.07 (2H, s, $-CH_2O-$); 6.77 (1H, d-d, $J$ = 2.2 Hz, 8.6 Hz, H-5); 6.92 (1H, d, $J$ = 2.2 Hz, H-7); 6.96 (1H, s, H-2); 7.28 - 7.45 (5H, m, Ph); 7.43 (1H, d, $J$ = 8.6 Hz, H-4).

High Resolution Mass Spectrum m/e (relative abundance) 352.1426 (15.6%, $M^+$, $C_{20}H_{20}N_2O_4$ requires 352.1423). 293.1049 (6.3%, M - $CH_3CONH_2$), 262.0929 (7.6%, M - $C_7H_6$), 261.0869 (16.1%, M - $C_7H_7$), 236.1064 [75.2%, β-(6-benzyloxyindolyl)$CH_2^+$], 202.0503 (12.9%, M - $C_7H_7$ - $CH_3CONH_2$), 146.0599 [22.5%, β-(6-hydroxyindolyl)$CH_2^+$], 117.0579 [7.4%, β-(6-hydroxy-indolyl)$CH_2^+$ - CHO].

*N*-Acetyl-6-hydroxy-DL-tryptophan (136)

A suspension of *N*-acetyl-6-benzyloxy-DL-tryptophan (10.4 mg) and 10% Pd/C (6.2 mg) in a mixture of ethanol (1 ml) and water (1 ml) was hydrogenated at room temperature and pressure until the uptake of $H_2$ ceased (2 h). After removal of the catalyst under nitrogen using glass paper, evaporation at <1 mm pressure gave the phenolic indole (136) as a colourless glass.

U.V. $\lambda_{max}$ (EtOH), 235 nm ($\varepsilon$ 22,000); 267 inf. (3,700); 276 (3,900); 295 (4,200).

U.V. $\lambda_{max}$ (EtOH, NaOH), *ca.* 235 ($\varepsilon$ 28,100); 268 inf. (5,000); 278 inf. (4,300); 313 (4,740).

I.R. $\nu_{max}$ (KBr), 3410 (s, NH); 1722; 1630 (s); 1555 cm$^{-1}$.

N.M.R. (100 MHz) $\delta$ ($CD_3OD$), 1.90 (3H, s, NAc); 3.0-3.42 (2H, m, $-C\underline{H}_2CHN-$); 4.63 (1H, m, $-CH_2C\underline{H}N-$); 6.28 (1H, m, NH); 6.59 (1H, d-d, $J$ = 2.2 Hz, 8.5 Hz, H-5); 6.74 (1H, d-d, $J$ = 0.6 Hz, 2.2 Hz, H-7);

6.91 (1H, br s, H-2); 6.97 (1H, m, indole NH); 7.36 (1H, d-d, *J* = 0.6 Hz, 8.5 Hz, H-4).

High Resolution Mass Spectrum m/e (relative abundance) 262.0953 (14%, $M^+$, $C_{13}H_{14}N_2O_4$ requires 262.0953), 203.0581 (19%, M - $CH_3CONH_2$), 146.0602 [100%, β-(6-hydroxyindolyl)$CH_2^+$], 133.0529 [4.8%, M - $CH_2$=C(NHAc)$CO_2$H], 117.0574 [2.6%, β-(6-hydroxyindolyl)$CH_2^+$ - CHO].

*N*-Acetyl-6-methoxy-DL-tryptophan methyl ester (137)

Methylation of the above phenolic acid (136) (5 mg) with ethereal diazomethane gave the methyl ether methyl ester (137), $R_f$ 0.3 (ethyl acetate) (orange spot on development with $Ce^{4+}$ changing to green on heating). I.r., u.v. and n.m.r. spectra were identical to those of a sample of the methyl ether methyl ester prepared from the metabolite.

Mass Measurement Found: $M^+$ 290.1276. $C_{15}H_{15}N_2O_4$ requires 290.1266.

*N*-Acetyl-6-hydroxy-DL-tryptophan methyl ester (142)

Similar catalytic reduction of N-acetyl-6-benzyloxy-DL-tryptophan methyl ester, $R_f$ 0.39 (chloroform:methanol, 9:1) (prepared using diazomethane) gave the methyl ester (142) $R_f$ 0.15 (chloroform:methanol, 9:1).

U.V. $\lambda_{max}$ (EtOH), 280 inf. (ε 1,900); 292 (2,200).

N.M.R. (100 MHz) δ, 1.91 (s, NAc); 1.99 (m, NH); 3.08, 3.21 and 4.68 (ABX system, *J* = 9.2 Hz, 4.4 Hz, 14.6 Hz, -$CH_2$CHN-); 3.65 (s, -$OCH_3$); 3.7 (m, NH); 6.59 (d-d, *J* = 2.1 Hz, 8.4 Hz, H-5); 6.73 (d-d, *J* = 0.6 Hz, 2.1 Hz, H-7); 6.89 (br s, H-2); 7.30 (d-d, *J* = 0.6 Hz, 8.4 Hz, H-4).

Preparation of the Monophenolase[148]

Cultures of strain AJC 7.46 (*ivoA1*; *ornB7 brlA42*) or, for the monophenolase-free control, strain G841, were grown as in the production of *N*-acetyl-6-hydroxytryptophan (136), but for only 2 days. The resulting mycelial mat was ground in a mortar with sand and tris-maleic buffer [trishydroxymethylaminomethane (24.2 g) and maleic acid (23.2 g) in water (1 litre)], pH 7.0 at 4°C. The crude extract was centrifuged for 5 mins. at 2500g and in some cases dialysed against the extraction buffer in the cold for 1 h. Enzyme activity was assayed by adding enzyme preparation (0.1 ml) to a mixture of 0.5M tris-maleic buffer, pH 7.0 (1.9 ml) and an aqueous solution (1 ml) containing hydroquinone monomethyl ether (25 mg) and catechol (0.2 mg). The formation of the brown oxidation product was followed as absorbance at 470 nm in a Unicam SP 1800 spectrophotometer.

Enzymatic Oxidation of (136)[148]

The oxidation of purified natural (136) on addition of monophenolase preparations could be followed polarographically and destruction of (136) could be monitored by chromatography using diazotised sulphanilic acid for detection. Control enzyme preparations from *ivoB* strains which lack monophenolase were inactive and (136) could still be detected on chromatograms of the mixture using diazotised sulphanilic acid.

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