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STEREOCHEMICAL ASPECTS OF THE
BIOSYNTHESIS OF SOME BISLACTONE
ANTIBIOTICS.

THESIS

Submitted for the degree of
MASTER OF SCIENCE (RESEARCH)

to

The University of Glasgow

by

DJAMEL EDDINE KHELIFI

September, 1988.

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To my family
and my late father.

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September, 1988.

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SUMMARY

Part 1 is concerned with biosynthetic studies on the alkylcitric acid metabolites of the fungi *P. decumbens* and *P. canadense* using deuterium labelled acetate, succinate and fumarate, with particular regard to the terminal methylene hydrogens. In the antibiotic ethisolide obtained from *P. decumbens*, label from acetate appeared as expected mainly in the hexanoic acid derived part of the molecule i.e. mainly in the methyl group (C-6) and a smaller amount at (C-4). There was however, consistently, a much smaller amount in the oxaloacetate derived part of the molecule in both terminal methylene positions.

Since acetate would be expected to give mono-deutero oxaloacetate by the Krebs Cycle via succinate and fumarate, one explanation would be operation of the glyoxylate cycle to afford a dideutero-oxaloacetate. However, this was ruled out by feedings with d_4 -succinate and d_2 -fumarate which gave ethisolide and the minor metabolite ethisic acid, with deuterium enrichment in both terminal methylene hydrogens. Another significant result was that label from d_4 succinate appeared in only one of the two terminal methylene positions of canadensic acid. Since the latter has an unrearranged skeleton, these results seem to reflect the mechanism of the rearrangement involved in the biosynthesis of ethisolide and ethisic acid.

The discovery in the present work of a method of

selective esterification of the minor acidic broth metabolite in the presence of large amounts of citric acid has led to the isolation of the key intermediate butylitaconic acid as its methyl ester for the first time and opens the way to its biosynthetic investigation.

Part II deals with the attempted development of a shorter and cheaper synthesis of deuterated alkylitaconic acids. A number of potentially useful Meldrum's acid derivatives were obtained using dry catalysed condensation methods. The alkylidene compound obtained from ethyl pyruvate, rather than deuterium exchange or alkylation reactions underwent undesired Michael addition reactions (with Meldrum's acid or itself). An attempt to block these side reactions using PhSH gave a vinyl sulphide byproduct, whose formation and possible uses were studied.

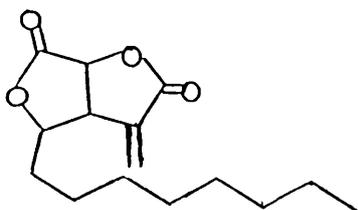
The key biosynthetic intermediate butylitaconic acid has been prepared and converted to its dimethyl ester under a variety of conditions. Hydrolysis was achieved but attempts to convert the ester to its α -hydroxy analogue (isolated from some fermentations) using several conditions have been unsuccessful. On the other hand bromination-dehydrobromination of the same ester afforded a potentially useful unsaturated compound together with the allylic bromides which could open the way to alkylitaconic acids.

INTRODUCTION

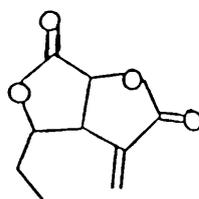
1. General Introduction
2. Secondary Metabolites Derived from Fatty Acids and Intermediates of the Tricarboxylic Acid Cycle.
3. Stereochemical Aspects of the Biosynthesis of Some Fungal Metabolites.
4. New Synthetic Routes to Deuterated Alkylitaconic Acids.

1. General Introduction.

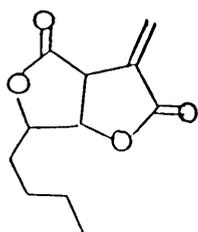
The work to be discussed in this thesis is concerned with the biosynthetic pathways to some bislactone antibiotics namely avenaciolide (1), ethisolide (2) and canadensolide (3). These can all formally be derived from a fatty acid to which a C-3 unit is linked either at the α -position, i.e. type A lactones, or the β -position, i.e. type B lactones.



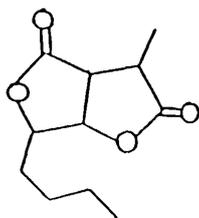
(1)



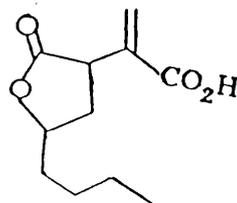
(2)



(3)



(4)

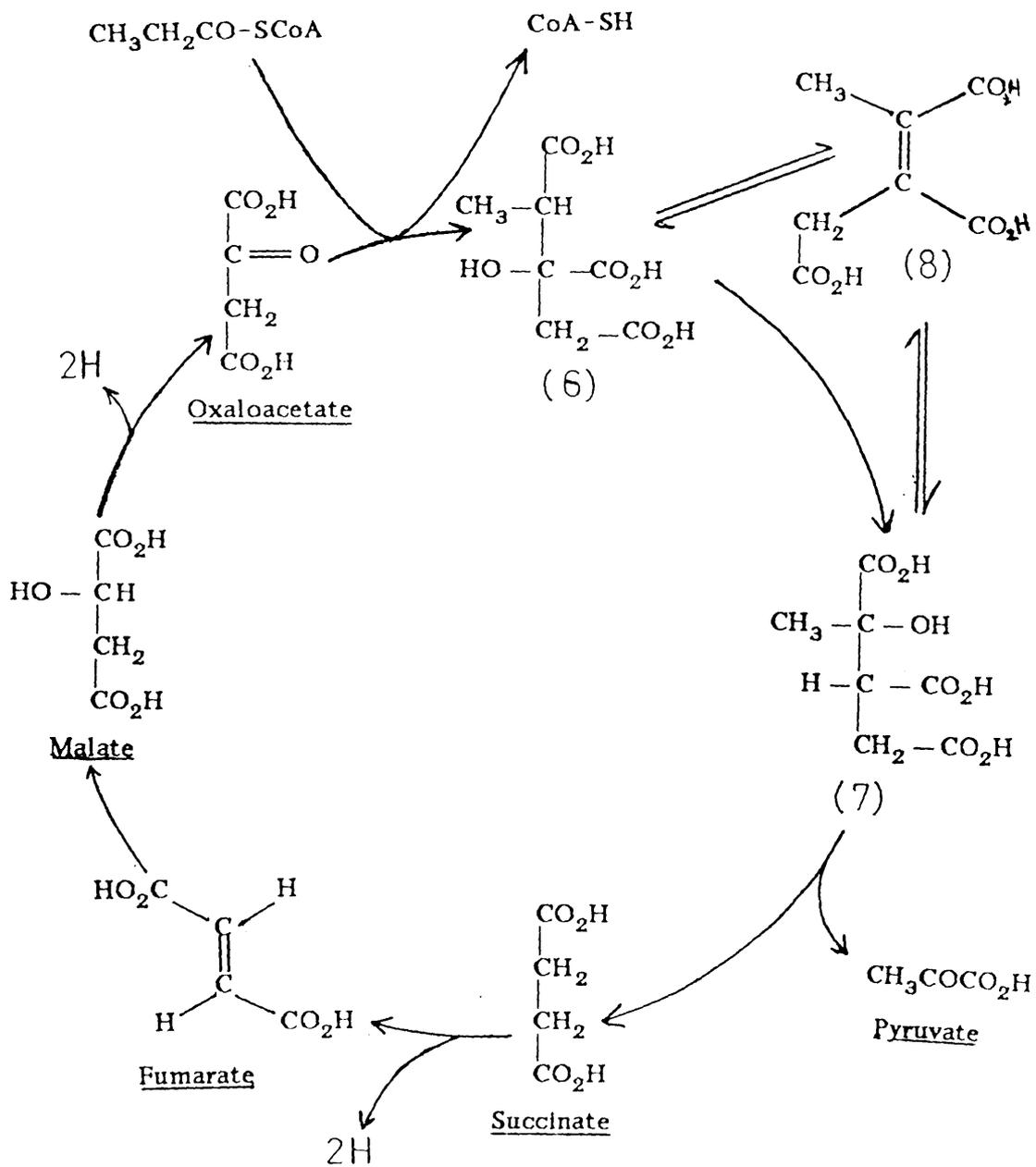


(5)

The fungal metabolites canadensolide(3) dihydrocanadensolide (4), canadensic acid (5) first isolated from *P. canadense*¹ in 1968, and the antibiotics (1) and (2) from *A. avenaceus*² and *P. decumbens*³ respectively, have been shown by a series of incorporation studies to have considerable similarities in biosynthetic origin⁴ as discussed below.

2. Secondary metabolites derived from fatty acids and intermediates of the Tricarboxylic Acid Cycle.

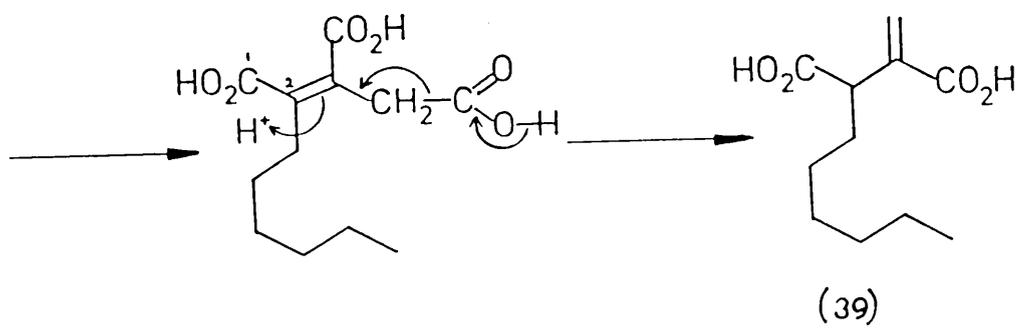
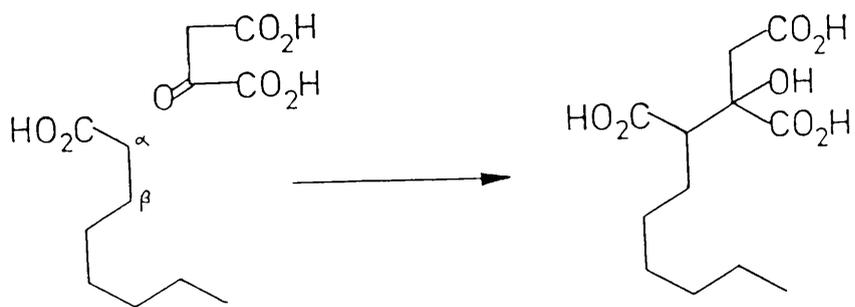
One of the vital primary metabolic processes of a vast range of living organisms is the condensation of acetyl CoA with oxaloacetic acid (an intermediate in the T.C.A. Cycle, cf. Scheme 1a) to produce citric acid, the enzyme responsible for this condensation is citrate synthase. At least two other citrate synthases responsible for the formation of alkylcitric acid secondary metabolite have been isolated and investigated. The first, methylcitrate synthase, has been shown to condense propionyl-CoA with oxaloacetate to produce (-) methyl citric acid along with other alkylated T.C.A. Cycle intermediates formed by other alkylcitrate specific enzymes.⁵ Another catalyses, the formation of (-) decylcitric acid isolated from cultures of Penicillium Spiculisporum⁶ and although the original claim that (+) decylcitric acid was also produced has since been corrected,⁷ the enzyme catalysing the condensation between lauryl-CoA and oxaloacetate was isolated and purified.⁸ Studies on decylcitrate synthase⁹ showed that the enzyme, while very specific for the C₄ diacid species was relatively non-specific with respect to the fatty acid moiety used and that a range of shorter chain substrates could be utilised. Another alkylcitric acid, namely n-butylcitric acid, has been isolated from cultures of P. decumbens¹⁰ and this further supported the intermediacy



Modified T. C. A. Cycle

Scheme 1a

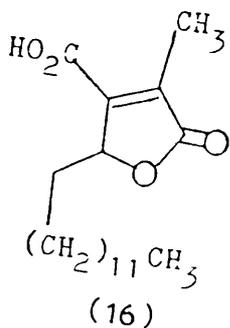
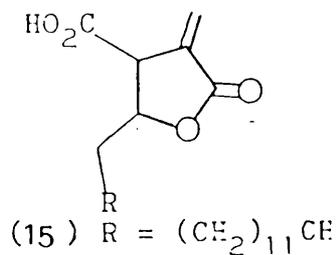
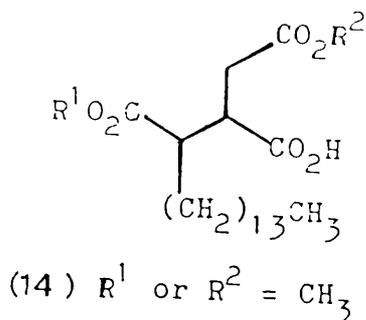
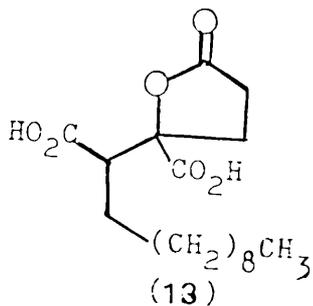
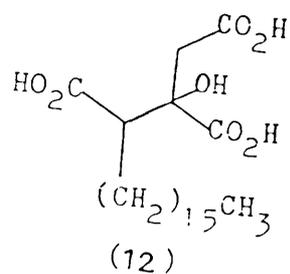
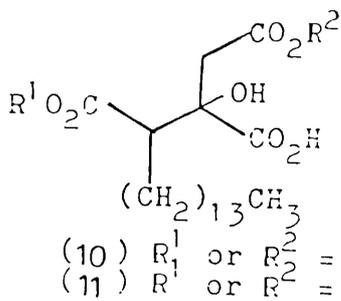
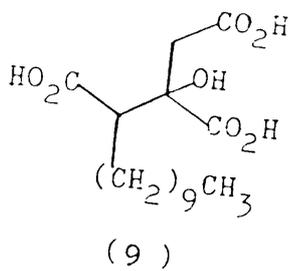
of an alkylcitric acid derived intermediate (discussed later) in the biosynthesis of the type B lactones avenaciolide (1) and ethisolide (2). The natural products canadensolide (3) and dihydrocanadensolide (4) again may be regarded as a consequence of the condensation of the keto-group of oxaloacetic acid with the α -methylene group of fatty acid¹¹ (Scheme 2). This type of compounds (Table 1) are found in nature, examples being decylcitric acid (9)^{7,10} caperatic and non-caperatic acids (10)¹² and (11)¹³ and agaricic acid (12)¹⁴. Spiculisporic acid (13)¹⁵ is evidently produced by condensation of oxo-glutaric acid and the fatty acid followed by cyclisation while rangiformic acid (14)¹² probably results from de-hydroxylation of (10), perhaps by an elimination-reduction as it occurs in the interconversion of malic acid and succinic acid in the T.C.A. cycle. In many secondary metabolites of this type e.g. protolichesterinic acid (15)¹² and lichesterinic acid (16)¹² the condensation products have undergone lactonisation using a hydroxyl group at the β -position of the fatty acid. Some other natural products may arise by a similar pathway but with decarboxylation step as an additional modification, as it occurs in the biosynthesis of protolichesterinic acid (15) and itaconic acid (17). This process was established for the biosynthesis of (17) using cell-free extracts of *A. terreus*^{16,17} by using D₂O experiments which showed that decarboxylation involved also the migration of the double bond. (Figure 1).



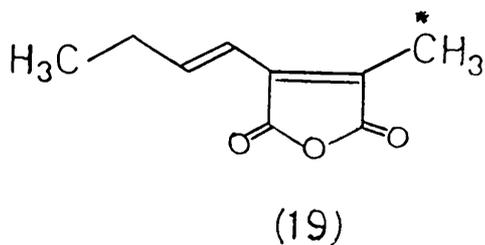
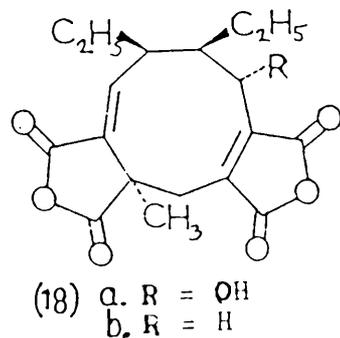
→ → (3) and (4)

Scheme 2

Table 1.



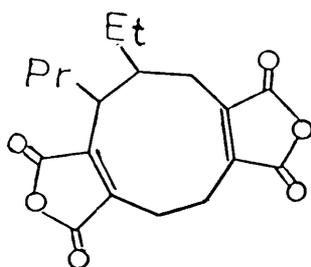
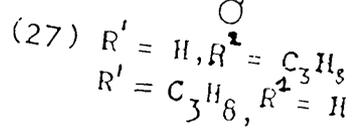
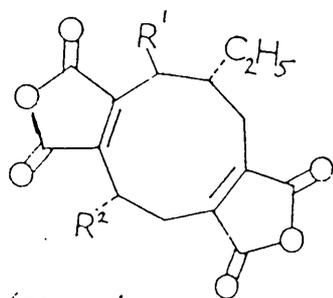
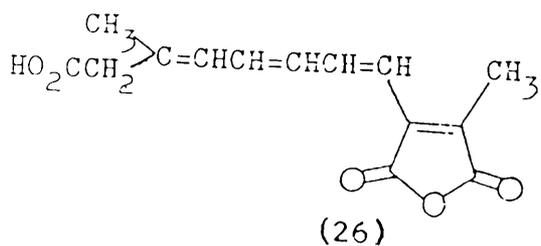
see Fig. 1.
 (17)



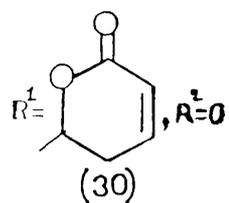
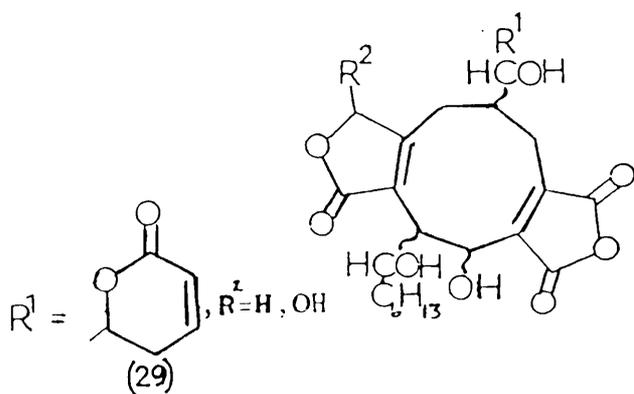
see Table 2.

(20) to (25)

Table 1.



(28)



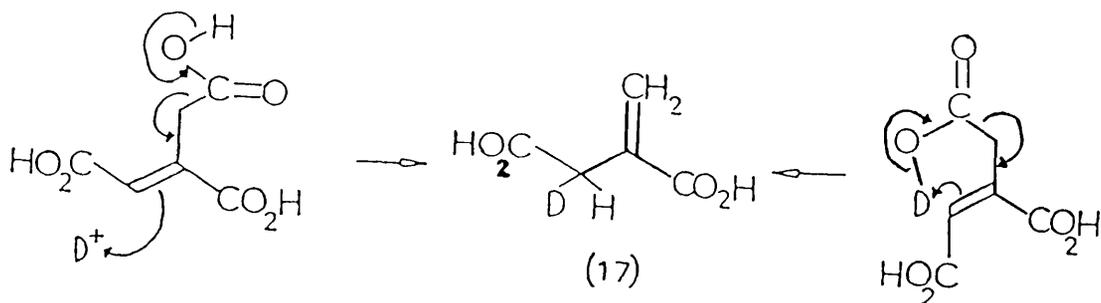
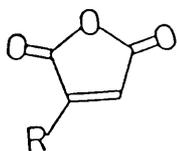


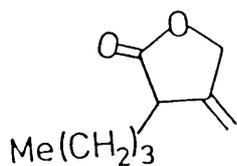
Figure 1.

Type A metabolites also include simple anhydrides and the dimeric anhydrides known as nonadrides, such as glauconic and glaucanic acid (18a) and (18b) ¹⁸. It was found that the labelled anhydride (19) was incorporated into (18a) (50.8%) and (18b) (4.3%) in Penicillium purpurogenum ¹⁸ and base-catalysed dimerisation of (19) gave an epimer of (18b) in 5% yield. There are more examples of metabolites (Table 2) which may be listed under the class of the condensation of fatty acids with T.C.A. intermediates, ¹¹ such as a number of citraconic anhydride derivatives (20a-20d) from Aspergillus Wentii ¹⁹ two γ -butyrolactones (21) and (22) from Hypoxylon serpens ²⁰, the disubstituted anhydride (23) from cultures of Paecilomyces variotti, and namely acaranoic (24) and acarenoic (25) acids which have been revised to δ -lactones. ²² The biosynthesis of itaconitin (26), acetate and malonate were incorporated ²³ into C-1 to C-9 and also into C-13, showing this portion to be derived from a fatty acid (Figure 2).

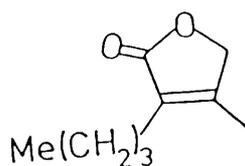
Table 2.



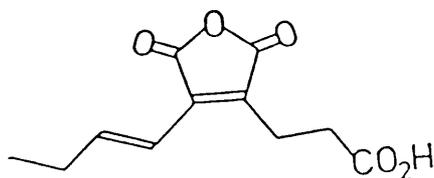
- (20) a. $R = (CH_2)_{16} CH_2OAc$
 b. $R = (CH_2)_{14} CHOAc.Me$
 c. $R = (CH_2)_{14} CHOH.Me$
 d. $R = (CH_2)_{14} CO.Me$



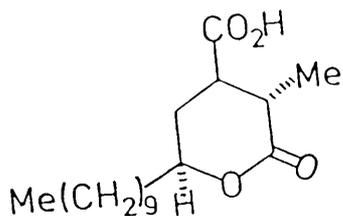
(21)



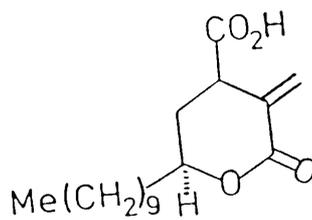
(22)



(23)



(24)



(25)

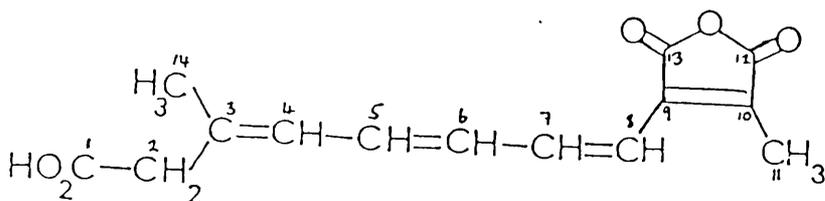


Figure 2.

Studies with [$1-^{14}\text{C}$] pyruvic acid [$1,5-^{14}\text{C}_2$] citric acid and [$2,3-^{14}\text{C}_2$] succinic acid, and [$6-^{14}\text{C}$] glucose incorporation suggested that the TCA cycle was a poor source of carbon atoms for the C_3 unit, and it was derived from the carboxylation of phosphoenol pyruvate (Figure 3) respectively.



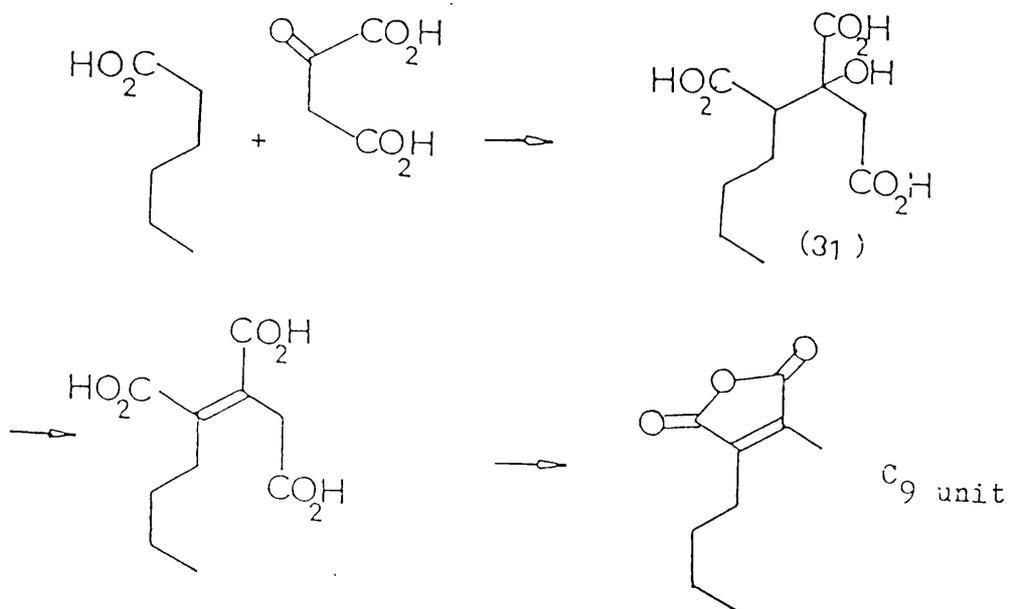
Figure 3.

The natural products with a characteristic C-9 ring, such

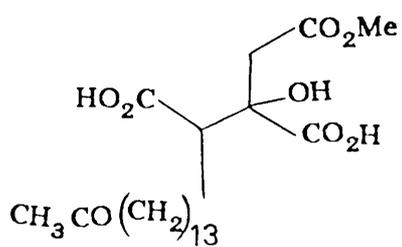
as byssochlamic acid (27)²⁴ and its isomer heveadride (28)²⁵, the acids (18a) and (18b) and the rubratoxins A and B (29)²⁶ and (30)²⁷ respectively can be grouped with the lichen acids on the basis of their biosynthesis. These were proposed²⁸ to arise by dimerisation of a unit (C-9 in the case of glauconic acid) which could be derived from an alkylcitric acid e.g. (31) (see Scheme 3) formed by the condensation of the α -methylene group of hexanoic acid (Scheme 3).

One way in which the cis aconitic acid could undergo the final transformation to the proven intermediate, the unsaturated C-9 anhydride (19) would be via decarboxylation to butylitaconic acid. Recently,²⁹ butyl itaconic acid labelled with deuterium in the terminal methylene group was fed to cultures producing glauconic (18a) and glaucanic (18b) acids and no deuterium was detected in the metabolites. Hence, desaturation may occur prior to decarboxylation.

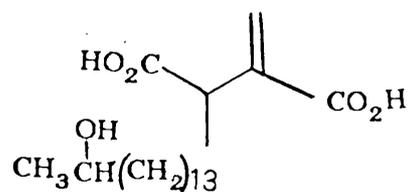
The compounds (31) and (32) from Usnea meridensis³⁰ and Usnea aliphatica³¹ respectively are possible biosynthesis precursors of the *A. wentii* anhydride e.g. (19), (34) and of the murolic acids and constipatic acid e.g. (35)³² and (36)³². α -Methylene butyrolacetone (37)³², the aglycone of the plant product tuliposide A, is biosynthesised from acetate and pyruvate and not from a C-4 T.C.A. intermediate as expected.³³ The type B metabolites avenaciolide (1) and ethisolide (2) are included in the present study and the biosynthesis details of which will be discussed later.



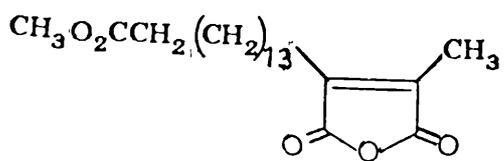
Scheme 3.



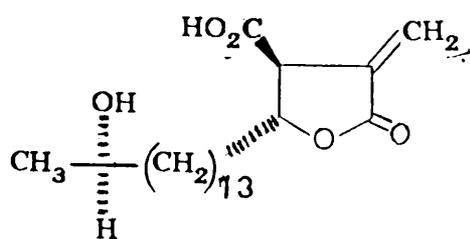
(32)



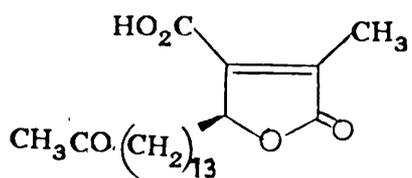
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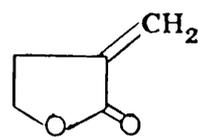
(34)



(35)



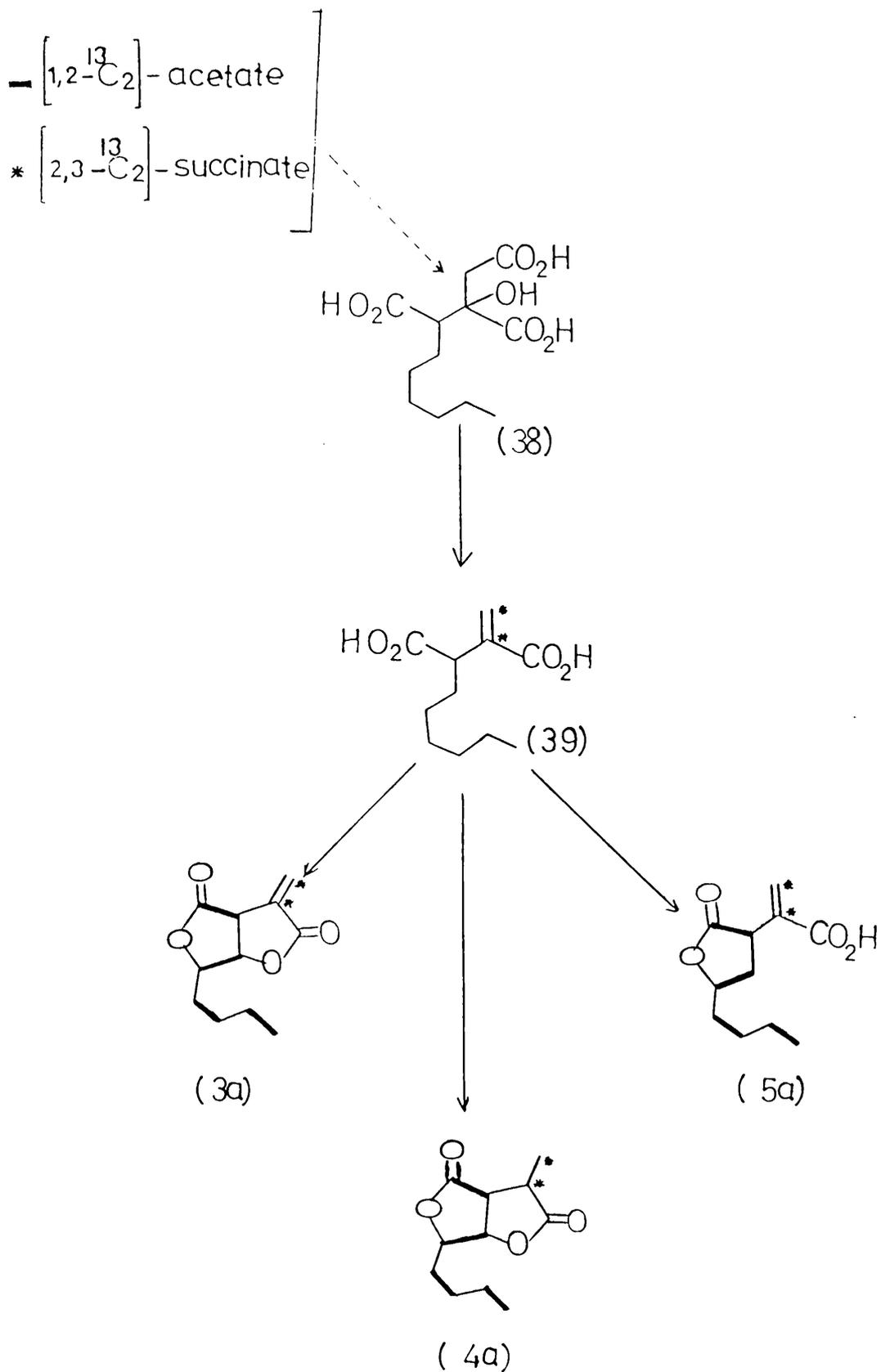
(36)



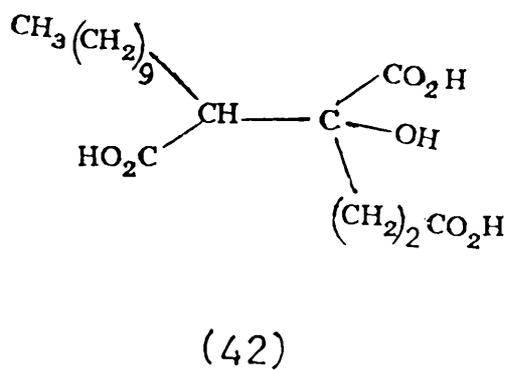
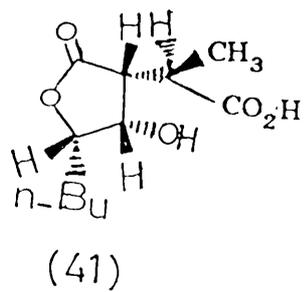
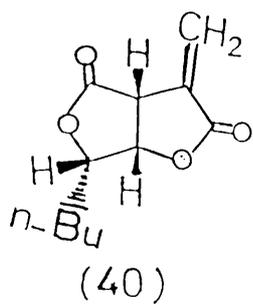
(37)

3. Stereochemical aspects of the biosynthesis of some fungal metabolites.

Evidence that the *P. canadense* metabolites canadensolide (3) dihydrocanadensolide (4) and canadensic acid (5) arise by condensation of the carbonyl group of oxaloacetate with the α -methylene group of a fatty acid (i.e. via an alkylcitric acid intermediate) was proved by McCorkindale⁴ who has demonstrated incorporation of ^{13}C -labelled precursors as shown in Scheme 4. $[1,2-^{13}\text{C}_2]$ acetate and $[2,3-^{13}\text{C}_2]$ succinate are incorporated into the fatty acid moiety and the C-3 unit respectively. However, significant label from $[2,3-^{13}\text{C}_2]$ succinate is also incorporated as $[1,2-^{13}\text{C}_2]$ succinate into the C-3-unit and as $[1,2-^{13}\text{C}_2]$ acetate into the fatty acid due to randomisation of label via the T.C.A. cycle. The results of these labelling studies suggested the intermediacy of hexyl-citric acid (38) and hexyl-itaconic acid (39). The intermediacy of these has not been demonstrated directly but synthetic ^{14}C -labelled (39) was efficiently incorporated into (3a) to (5a) by *P. canadense*. The stereochemistry of canadensolide has been defined as in (40)³⁴ and the compound has been isolated from *Penicillium avenicola* and *Aspergillus strametii*³⁵ and from *A. tamaris*.³⁶ Under controlled pH conditions the hydroxy-acid (41) derived from dihydrocanadensolide (4) has been isolated to the exclusion of the bis-lactone from the culture medium of *P. canadense*³⁷. Similarly, 4-hydroxy-4,5-dicarboxy



Scheme (4)



Pentadecanoic acid (42), the ringopened form of spiculisporic acid can be isolated from *P. spiculisporum*.³⁸ Spiculisporic acid is biosynthesised in *P. fariculosuk* by condensation of lauric acid, derived from acetate, with α -ketoglutaric acid, a T.C.A. cycle intermediate as expected.³⁹

Avenaciolide (1) and ethisolide (2) can be derived formally by condensation of C_3 -unit with the β -position of a fatty acid chain and it has been suggested that this type of compound may be formed by condensation of succinic acid with a β -keto acid. This theory is supported by Tanabe⁴⁰ who has demonstrated the expected labelling pattern with avenaciolide formed from $[1-^{13}C]$ and $[2-^{13}C]$ acetate in *A. avenaceus*⁴⁰ and by McCorkindale who has demonstrated incorporation of ^{13}C -labelled precursors into the *P. decumbens*⁴ metabolites. He has reported⁴ that ethisolide (2) formed from $[1-^{13}C]$ and $[2-^{13}C]$ acetate and $[2,3-^{13}C_2]$ succinate in the latter fungus. However, during the latter study four co-metabolites of ethisolide (2) were isolated, ethisic acid (43) which has the same skeleton as ethisolide, and compounds (44), (45) and (46) with the 'normal' skeleton. Since the α -hydrogen atom of (47) is retained in ethisolide, (45) is not an intermediate and in (43) at least rearrangement must have occurred without prior hydroxylation at the γ -position. The logical deduction is that the rearrangement⁴¹ is probably of (47) to (47a) (Scheme 5).

It has been found that $[\text{CH}_2 - {}^{14}\text{C}]$ butylitaconic acid (47) was incorporated (10.4%) into ethisolide (2) with activity specifically in the terminal methylene group⁴ as determined by ozonolysis. Similarly, the corresponding $[\text{CH}_2 - {}^{14}\text{C}]$ -n-decylitaconic acid was specifically incorporated into avenaciolide (1) by A. avenaceus.⁴² The level of incorporation being 7.7%. In addition a sample of α - $[\text{CH}_2 - {}^{14}\text{C}]$ -glutarate was incorporated (26%) by P. decumbens into ethisolide with the label all in the terminal methylene group and β -nonyl- α - $[\text{CH}_2 - {}^{14}\text{C}]$ -glutarate incorporated (11%) by A. avenaceus into avenaciolide(1) again with the activity in the terminal methylene group. This was found^{to be} even a better precursor to ethisolide than the itaconic acid. And according to these findings, McCorkindale has proposed a common pathway (Scheme 5) for the P. decumbens metabolites in which the early stages of the biosynthesis are 'typical' (cf. canadensolide) and the ethisolide skeleton arises by rearrangement of an alkylitaconic acid derivative (47). This is exemplified by the B_{12} -coenzyme/ α -methylene glutarate mutase interconversion of methyl itaconic acid and α -methylene glutaric acid⁴³ (Figure 4).

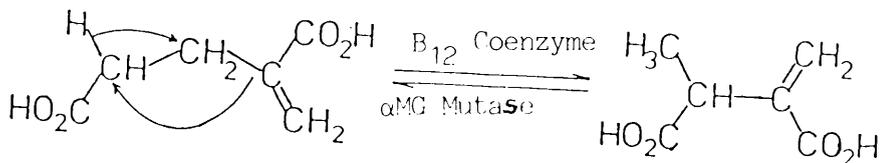
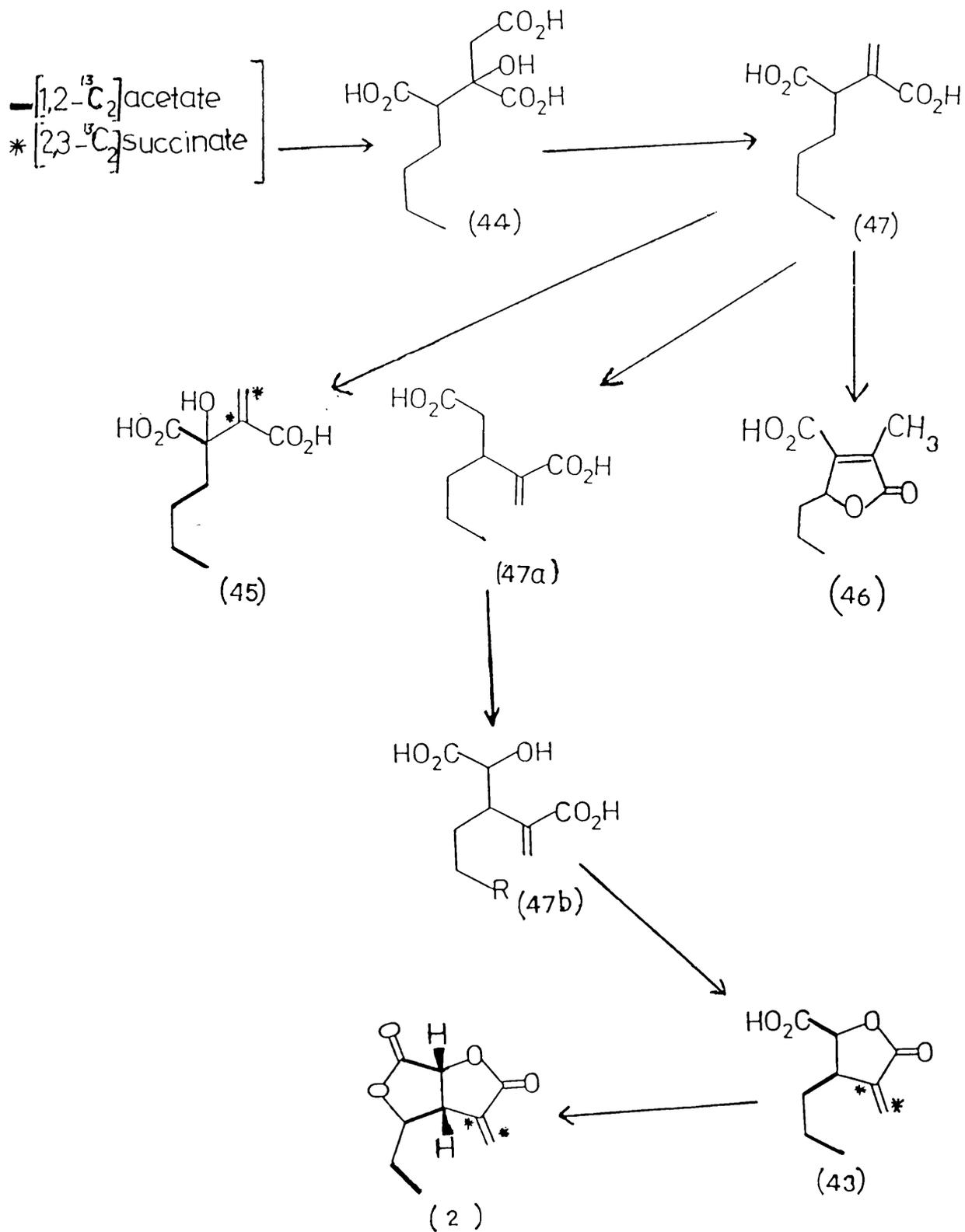


Figure 4.

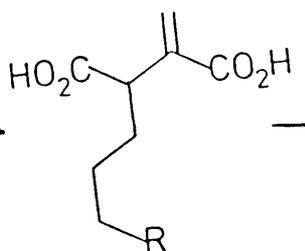
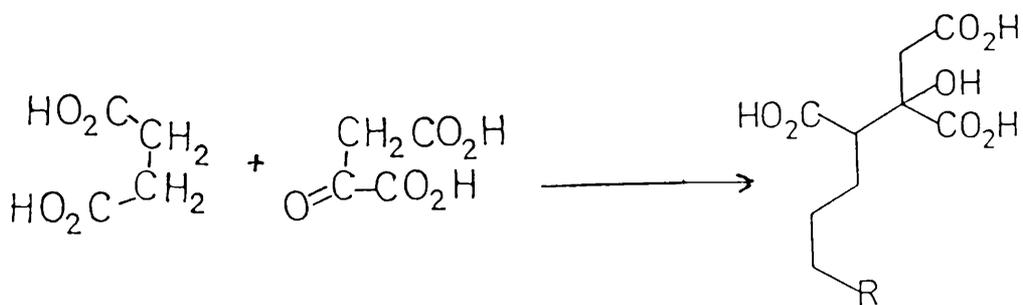


Scheme (5)

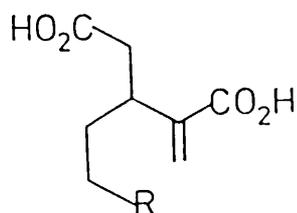
The alternative scheme proposed for the type B metabolites (Scheme 5) involved the rearrangement of the C₃-unit originally attached to the α -position becoming attached to the β -positions. This rearrangement merits a detailed examination and a contribution is made in the present work.

The stereochemistry of some apparently related coenzyme-B₁₂ dependent enzymatic rearrangements, e.g. the glutamate mutase reaction and the methylmalonyl-CoA mutase reaction have been studied and contrasting results obtained. McCorkindale et. al.,⁴¹ firstly showed that the appropriate alkylitaconic acid, e.g. (47) labelled with [H-3] at the α -position and with [c-14] at the terminal methylene carbon, afforded ethisolide (2) or avenaciolide (1) with the same tritium/carbon ratio as the administered itaconic acid (47). This suggests retention of the α -hydrogen atom.

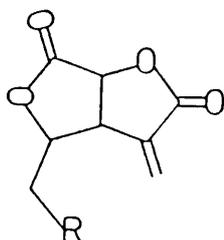
The presumed methyleneglutaric acid intermediates can not be detected in culture fluids and it is not therefore possible to study the labelling pattern of these intermediates directly.⁴¹ However, by studying the samples of (1) and (2) produced from feeding variously labelled acids (47) or (48) (Scheme 6), conclusions could be drawn for most of stereochemical changes involved. Of major importance in interpreting the results of the feedings of stereospecifically deuterated and optically active samples of (47) is to distinguish whether incorporation of, or retention of



(47) $\text{R}=\text{Me}$
 (48) $\text{R}=\text{C}_7\text{H}_{15}$



(47a) $\text{R}=\text{Me}$
 (48a) $\text{R}=\text{C}_7\text{H}_{15}$



(2) $\text{R}=\text{Me}$
 (1) $\text{R}=\text{C}_7\text{H}_{15}$

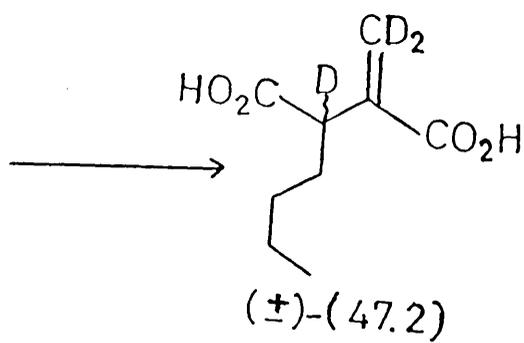
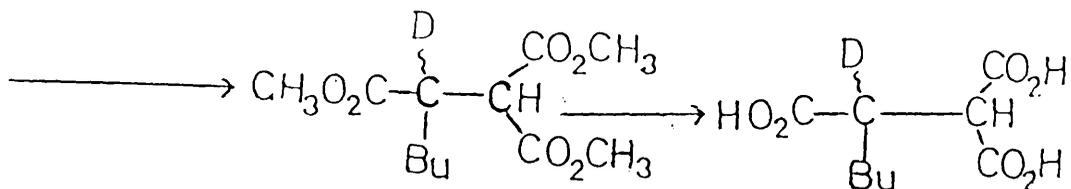
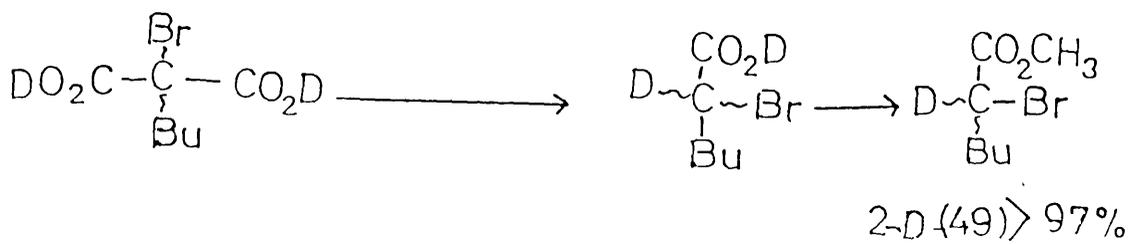
Scheme 6

deuterium is from the enantiomer present in minor amounts (e.g. 50%) or from that present in major amounts [d_2]-formaldehyde, generated from commercially available (but very expensive) labelled paraformaldehyde was used to prepare the deuterated acid (47.1).⁴¹ This was efficiently incorporated by *P. decumbens* into (2) labelled only in the terminal methylene group as shown by deuterium n.m.r. Hence labelling of the terminal methylene group can be used as an internal reference check of deuterium retention from elsewhere in the molecule.

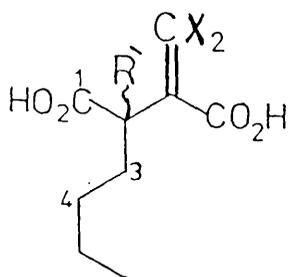
The presence of tritium at the 2' position in the ethisolide sample obtained from [$\alpha - ^3H$, methylene - ^{14}C] (47) was not proved since no degradative methods had been established. The ethisolide (2) was therefore obtained from (47) labelled with 2H at the α -position (i.e. 4'). Under the conditions used to prepare (47) substantial loss of 2H were observed both at the malonation and saponification stages (Scheme 7).

The required racemic deuterated (47) was then obtained with d- enrichment at C-2 about 60% of that at either of the terminal methylene positions.

This was administered to cultures of *P. decumbens*⁴¹ and gave ethisolide showing 2H enrichment at C-2 again of ca. 60% of that of either of the terminal methylene positions.⁴¹ This indicates complete retention of the 2H atom at (C-2), confirming the study with tritium labelled material.



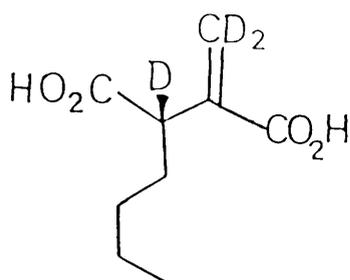
Scheme (7)



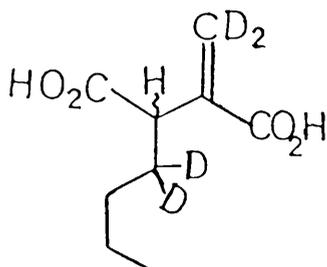
(47)

- 47.1 $\tilde{R} = (2RS) - 2^{-1}H ; X = D$
 .2 $\tilde{R} = (2RS) - 2^{-2}H ; X = D$
 .3 $\tilde{R} = (2R) - 2^{-1}H ; X = H$
 .4 $\tilde{R} = (2R) - 2^{-2}H ; X = H$
 .5 $\tilde{R} = (2R) - 2^{-3}H ; X = D$

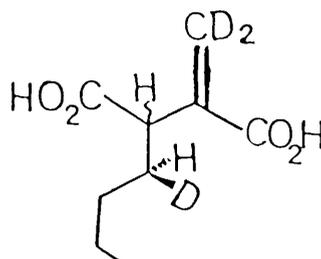
It had been reported⁴¹ that the biosynthesis of ethisolide (2) involves butylcitric acid formed by condensation of hexanoic acid and oxaloacetate. This has been proved using various deuterated hexanoic acids, e.g. with [2,2,3,3-d₄]-hexanoic acid, it was found that no deuterium from C-2 of the hexanoic acid was retained at C-2 of ethisolide (2).⁴¹ Deuterium was, however retained at C-3 of ethisolide (Figure 5) since this corresponds to a position derived from the carbonyl group of acetate, incorporation of the hexanoic must have been intact rather than via break down to acetate units. It may be noted that the retention of deuterium from the 3- position of hexanoic acid directly disproves the involvement of 3-oxohexanoic acid suggested as an intermediate in the first biogenesis expounded by Turner *et. al.*^{40,44}



(47.5)



(47.6)



(47.7)

The stereochemistry of the rearrangement proposed in ethisolide(2) was also studied by feeding various alkylitaconic acids (as shown above) labelled with ²H at C-2 or C-3 and in each case, as an internal standard, with H² in the terminal methylene group.

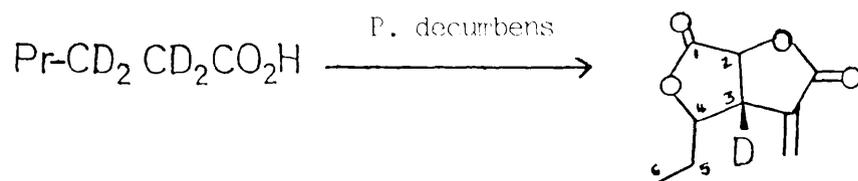


Figure 5.

Clear results were obtained for (47.2) and (47.6) which gave ethisolides showing retention of one ^2H atom from C-2 and one ^2H atom from C-3 respectively. On the other hand, 2/3 and 1/2 respectively of the chiral ^2H label was retained from (47.5) and (47.7) respectively. This can be rationalised in terms of the rearrangement proceeding partly with retention of configuration and partly with inversion of configuration of these centres.

It is well known that in the T.C.A. cycle, dehydration of citric acid gives cis aconitic acid by loss of a proton from the oxaloacetate - derived methylene group.⁴⁵ However, the assumed alkylitaconic acid in ethisolide biosynthesis would have to be formed by loss of a hydrogen atom from C-2 of the chain derived from hexanoate. This was shown by the studies with hexanoate described earlier and also by feeding deuterioacetate doubly labelled with C-13 at either C-1 or C-2. ^{13}C n.m.r. confirmed the absence of deuterium at C-2 in the resulting ethisolide and also showed the presence of deuterium at C-4 and C-6 in a ratio of 0.5 to 3 in keeping with a pronounced starter effect. The deuterium n.m.r. spectrum of ethisolide derived from deuterioacetate itself also showed this and in addition seemed to have lower but significant deuterium content in each of the terminal methylene positions.⁴¹ This was unexpected since acetate incorporation was presumed to occur via the T.C.A. cycle, i.e. via fumarate, [3S-3d]-L-malic acid, and hence [3S-3d]-oxaloacetate. It was expected that the subsequent steps

e.g. the aconitate decarboxylase type reactions,⁴⁶ would be stereospecific leading to only one of the terminal methylene hydrogens being labelled with deuterium. The incorporation of deuterium from acetate, succinate and fumarate into the terminal methylene group of ethisolide (2) and the minor metabolites of *P. decumbens* have been studied in the present work. The results of these and of similar studies are thought to have important implications in the biosynthesis of ethisolide (2) and other alkylcitric acid metabolites having an acrylic acid unit in the molecule.

4. New Synthetic Routes to Deuterated Alkylitaconic Acids.

The importance of alkylitaconic acids in the biosynthesis of various antibiotics and related lactones has already been discussed. In the case of ethisolide (2) and avenaciolide (1), investigation of the skeletal rearrangement required synthesis of variously deuterated samples of the alkylitaconic acids. The current synthetic pathways to the itaconic acids, an example of which is given in (Scheme 25) are very long, involving many steps, and not high yielding overall. Another serious drawback in this synthesis is very high cost of commercial deuterated formaldehyde. Finally, recent work⁴⁷ at the University of Tokyo has established that hexylitaconic acid (39) has powerful root growth stimulating properties and hence the alkylitaconic acids and related compounds have potential biological interest and use. Clearly a shorter, simpler synthetic route to these could be of economic importance in this respect.

DISCUSSION

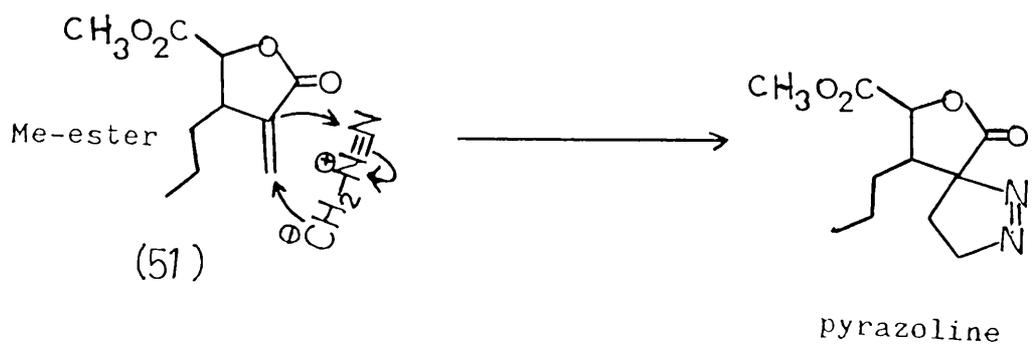
PART 1.

The biosynthesis of the terminal methylene group in metabolites of *P. decumbens* and *P. canadense*.

The first objective of this current work was to substantiate the evidence already presented for the biosynthesis of bislactone antibiotics like ethisolide (2) and canadensolide (3), based on extensive ^{13}C n.m.r and ^2H n.m.r studies using labelled acetate, succinate and alkylitaconic acids and thereby to lend support to the proposed biosynthetic pathway (Scheme 5).

In particular the unexpected findings in preliminary feedings that deuterium from $\text{d}_3\text{-NaOAc}$ or $\text{d}_4\text{-succinate}$ was incorporated into both terminal methylene hydrogens of ethisolide required examination in more detail.

It was hoped to confirm these results and examine the pattern of incorporation into the minor metabolites particularly ethisic acid (43) which has the same rearranged skeleton as ethisolide (2) and the reported⁴ metabolite α -butyl- α -hydroxyitaconic acid (45) which has a terminal methylene group as part of a C_3 acrylic acid grouping which has not undergone migration to the adjacent carbon atom. Esters are much easier to separate than acids, but diazomethane is well known to react with $\alpha\beta$ -unsaturated esters and lactones to give pyrazolines⁴⁸ (Scheme 8), and so alternative methods of esterification were sought. Having established that methanolic HCl (generated by adding SOCl_2 to methanol) gave



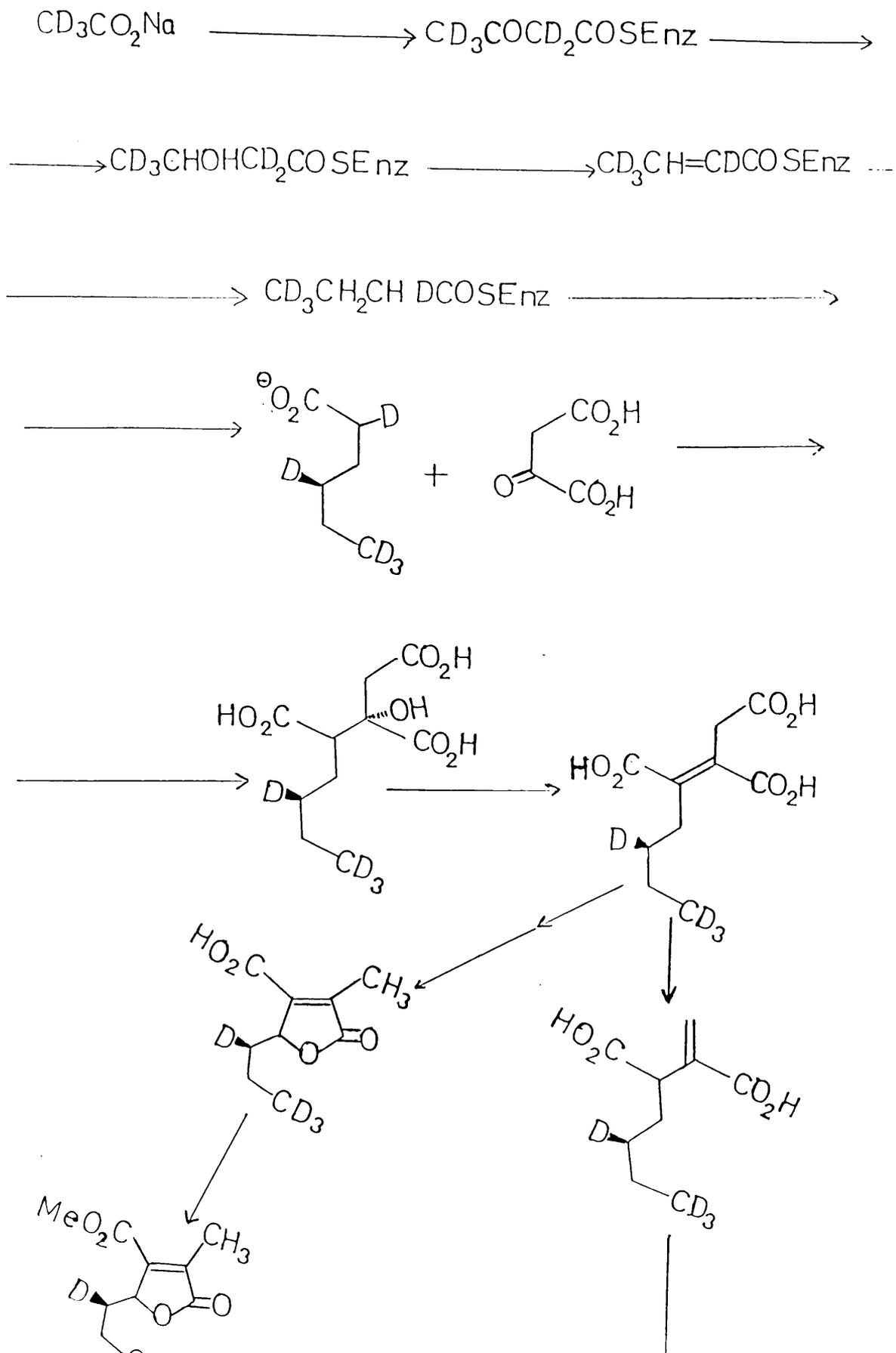
Scheme 8.

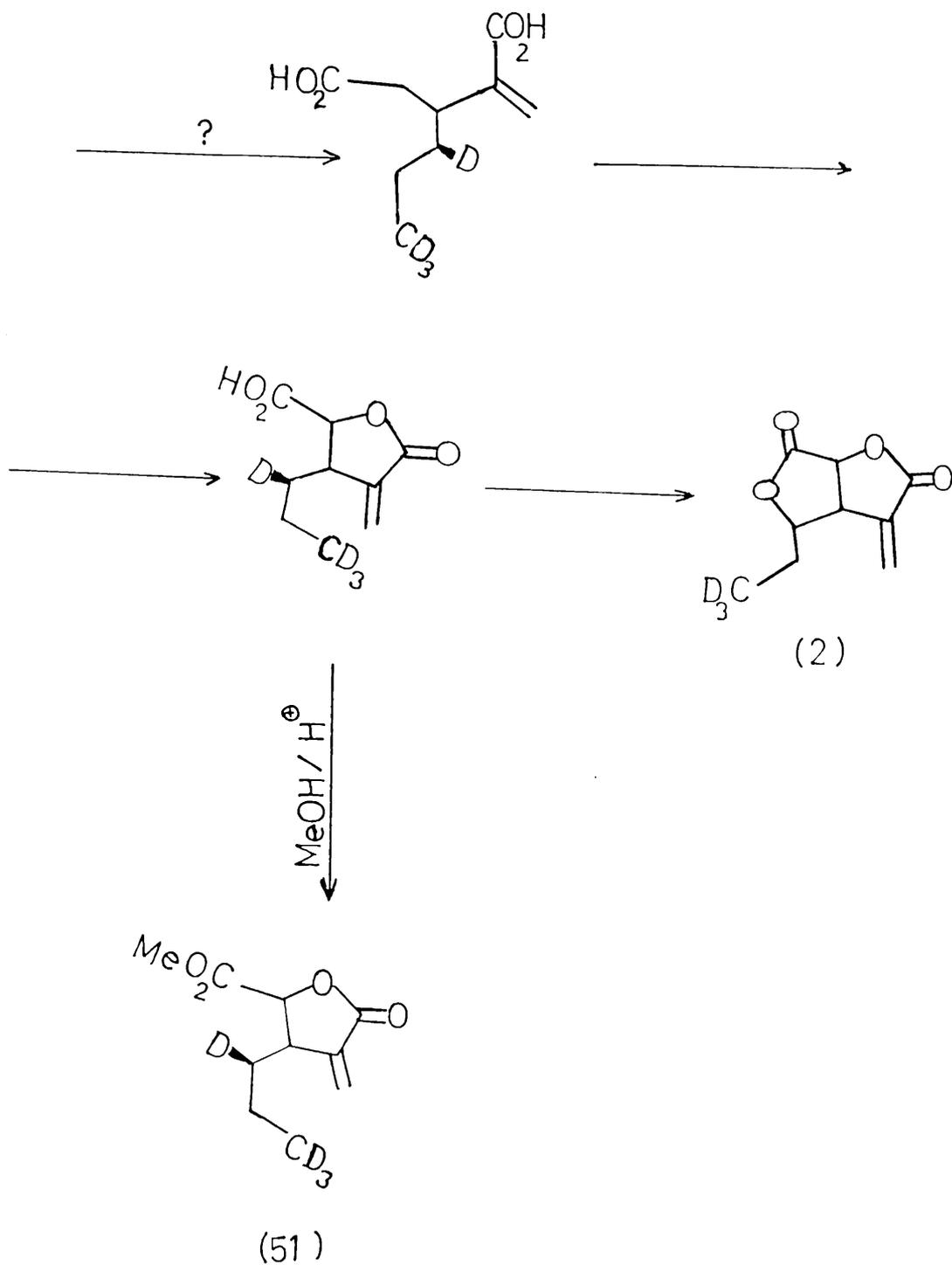
the ester (53) of α -butylitaconic acid in excellent yield, it was decided to use this method of esterification in the isolation of the methyl esters of the minor acidic metabolites.

d_3 -Acetate was fed to surface cultures of *P. decumbens* and after incubation, extraction of the culture fluid with ethyl acetate gave substantial quantities of ethisolide (2). The 2H n.m.r showed major peaks at $\delta 4.67$ and 1.06 indicating the main deuterium enrichment to be at C-4 and C-6 confirming the results found by previous⁴¹ workers. Weaker peaks also appeared at $\delta 6.63$ and 5.90 showing that a small but significant amount of deuterium from d_3 -acetate is incorporated into both terminal methylene positions confirming previous results. As discussed previously, this incorporation is evidently via the Krebs cycle, the special feature of interest being that although monodeutero precursors (malic acid and oxaloacetate) are involved the terminal methylene positions are equally labelled in the ethisolide (2) (Scheme 9).

After evaporation to dryness the mother liquor was treated with thionyl chloride in methanol at 0° to give a mixture of esters. Treatment of the mixture with ether gave crystalline trimethyl citrate (50) (the major product). Silica gel column chromatography of the ether solution gave a further quantity of ethyl citrate (50) and the remaining ester was separated by preparative t.l.c was therefore separated by preparative t.l.c using as solvent a mixture of ether and ethyl acetate. The column was eluted with a mixture of acetone: ether (1:1) and gave three main fractions, the

Scheme 9.





Scheme 9.

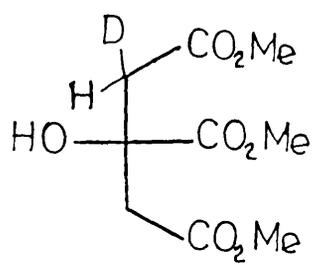
most polar of which showed a strong resonance at δ 3.75 and 3.27 in the ^1H n.m.r. This could correspond to the presence of a methyl ether function possibly formed by addition of methanol to a terminal methylene function, but this could not be obtained sufficiently pure to be characterised.

The second fraction showed signals corresponding to an alkyl group and an ester methoxyl group together with one proton fine doublets at δ 6.15, 5.52 and at δ 4.93.

This was identified as methyl ethisate (51), the low field signals corresponding to the terminal methylene protons (singlets) and H-2 doublet respectively, comparison being made with an authentic sample.

The third fraction again showed signals corresponding to an alkyl function and an ester methoxyl group together with a three proton doublet at δ 2.2 ($J = 3\text{Hz}$) and a one proton multiplet at δ 5.15. This was identified as methyl decumbate (52), the vinyl methyl group coupled to H-3 giving rise to the characteristic features indicated. The mass spectrum of the trimethyl citrate (50) showed considerable enrichment of deuterium but only of 1 atom as might be expected from its derivations via malate as discussed in the introduction.

The ^2H n.m.r spectra of the methyl ethisate and methyl decumbate each showed a strong signal at ca. 0.92 ppm



(50)

corresponding to the starter methyl group together with a weak signal at ca. 1.4 ppm corresponding to a methylene group (C-4). These results were very similar to those previously obtained for ethisolide and further substantiate the proposed pathway (Scheme 9).

Unfortunately, it had been hoped that α -butyl- α -hydroxyitaconic acid (45) might be isolated along with the above metabolites but this could not be detected. Its non-appearance could be due to variations in the metabolites produced or to the procedure of acid catalysed esterification used in the work up. This result cast some doubt on the desirability of the previous esterification method, so it was decided to carry out the esterification under basic conditions. The mixture of the acids, obtained from complete evaporation of the aqueous phase from 9 day old unfed surface cultures of *P. decumbens*, was esterified by treating with Me_2SO_4 and aqueous NaHCO_3 at 60° for 3 days followed by treatment with NaOAc to destroy any excess of Me_2SO_4 . Under these conditions only a small amount of mixed esters was obtained but it was found that trimethyl citrate was absent from the mixture. Extensive preparative t.l.c gave methyl decumbate (52) (characteristic methyl doublet at $\delta 2.2$) together with a new olefinic ester.

This showed a three proton triplet ($J = 6.75\text{Hz}$) in the ^1H n.m.r at 0.9 ppm for the methyl group attached to the CH_2 - in a butyl group, two methoxyl three proton singlets

peaks at δ 3.68 and 3.51. The two vinyl protons resonated at δ 6.34 and 5.75 each as one proton singlets; in addition to a triplet peak at δ 3.51 ($J = 9\text{Hz}$) corresponding to H-2. This was identified as dimethyl α -n-butylitaconate (53) a product which had previously been obtained by synthesis (as described later) and spectra of the natural and synthetic material were identical. This represents the first detection and isolation of this key intermediate.

The results of the above reaction seemed to suggest that selective esterification of the minor metabolites had occurred under these conditions. But the yield of minor metabolites was somewhat low. In order to check whether selective esterification does occur under these conditions, the reaction was repeated with a mixture of citric acid and itaconic acid. This afforded after work-up essentially pure dimethyl itaconate (54) in 38% yield uncontaminated by trimethyl citrate. The alkaline mother liquors, however afforded some partially esterified material namely a crystalline monomethyl ester (55) of citric acid (28% yield) and a mixture of monoesters (56) and (57) of itaconic acid (36% yield). When the reaction was carried out in the absence of citric acid, dimethyl itaconate (54) was obtained in 49% yield. This investigation reveals that the mixture is only partially esterified, and butylitaconic acid (47) and (46) appear to be preferentially esterified under these conditions, offering a convenient method of isolation in the

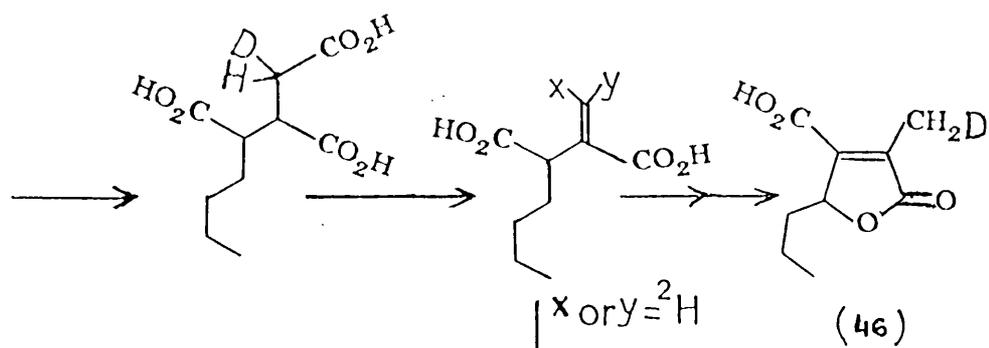
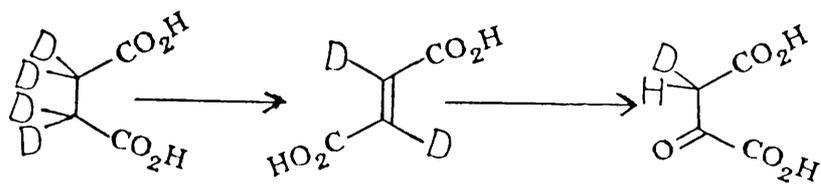
presence of large quantities of citric acid.

The incorporation of deuterium into both terminal methylene positions of ethisolide (2) and ethisic acid (43) was of particular interest. It would be expected that acetate incorporation occurs via the T.C.A cycle, via fumarate, [3S-3d]-L-malic acid and hence [3S-3d]-oxaloacetate.

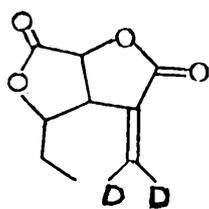
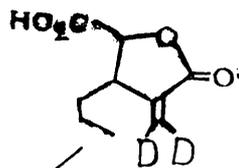
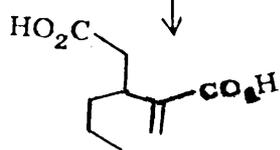
It might also be expected that the subsequent steps involving an aconitate decarboxylase reaction⁴⁶ would be stereospecific leading to only one of the terminal methylene hydrogens being labelled with deuterium. It seemed possible that acetate might be incorporated predominantly via [3,3-d₂]-L-malic acid formed by the glyoxylate cycle which in fact bypasses fumarate and succinate (Scheme 1b).

It was therefore important to test the incorporation of deuterium from deuterated samples of fumarate and succinate into ethisolide (2) and ethisic acid (43) and if possible into butylitaconic acid⁽⁴⁷⁾. d₄-Succinic acid was fed to surface cultures of *P. decumbens*. After incubation and the subsequent extraction with ethyl acetate substantial quantities of ethisolide (2) were obtained. The ²H n.m.r spectrum showed prominent peaks at 6.54 and 5.90 ppm indicating the incorporation of deuterium into both terminal methylene positions (Scheme 10).

A similar result was obtained upon feeding fumarate-d₂ to *P. decumbens*. Deuterium enrichments obtained were somewhat lower than in the foregoing experiment but from the



$\text{X or Y} = {}^2\text{H}$



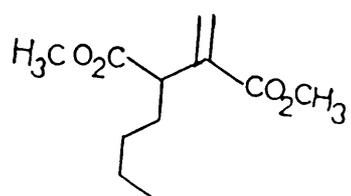
(2)

^2H n.m.r spectrum it was quite apparent that both of the terminal methylene hydrogens at C-9 had been enriched.

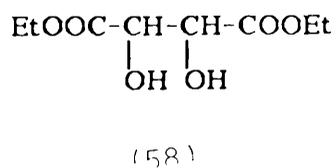
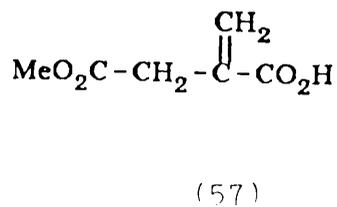
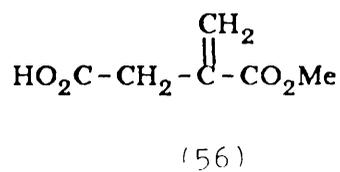
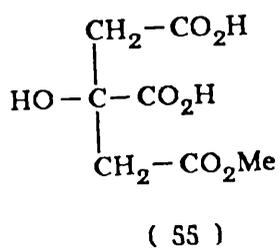
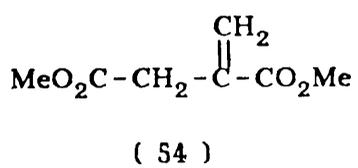
Since succinate and fumarate would be expected to give $[\text{d}_1]$ - rather than $[\text{d}_2]$ -oxaloacetate, the glyoxylate pathway is not necessarily involved and the randomisation of the label within the terminal methylene group must have another explanation.

Isolation of the minor acidic components from the d_4 -succinate feeding experiment was attempted under conditions similar to those used previously with Me_2SO_4 and aqueous NaHCO_3 (although the subsequent treatment with sodium acetate was omitted in this case). The crude mixture of esters was isolated by extraction with ethylacetate, preparative t.l.c giving methyl decumbate and methyl ethisate. The ^2H n.m.r spectrum of the methyl decumbate (52) showed evidence of deuterium incorporation into the vinyl methyl group as expected. The spectrum of methyl ethisate (51) showed strong signals at 6.34 and 5.63 ppm indicating as for ethisolides, incorporation of deuterium into both terminal methylene positions (Scheme 10).

It had been hoped that it might be possible to isolate the ester of butylitaconic acid (53) from this feeding experiment. However the residual esters formed a complex mixture in which diethyl tartrate (58) appeared to be a major constituent. Tartaric acid is a constituent of the medium and the ester may have been formed during work-up by



(53)



transesterification from ethyl acetate catalysed by H_2SO_4 formed from residual Me_2SO_4 . Although there appeared to be compounds present containing terminal methylene functions these were not obtained sufficiently pure to identify.

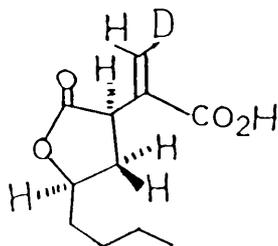
It was interesting that one fraction which showed a pair of terminal methylene peaks at δ 5.78 and 6.37 was found to have deuterium enrichment at both positions. Owing to lack of time these mixtures were not investigated further. A future investigation using μ lc or hplc might be rewarding.

Although it has not been possible as yet to examine the pattern of incorporation of deuterium from $[d_4]$ -succinate or $[d_2]$ -fumarate into butylitaconic acid or its elusive hydroxy analogue (45) in the fungus *P. decumbens*, relevant results have been obtained from similar incorporation studies into canadensic acid (5) produced by *P. canadense*. This has the alkylitaconic acid skeleton and a parallel biosynthetic scheme seems most likely. Results of similar feedings could be a pointer as to whether the randomisation of label in the case of ethisolide occurs before or after the formation of the alkylitaconic acid intermediate.

The terminal methylene protons of canadensic acid appear in the nmr as discrete singlets at 5.75 and 6.59 ppm. The 2H nmr spectrum of canadensic acid (5) obtained from feeding $[d_4]$ -succinic acid to *P. canadense* showed a peak

only at 6.58 ppm indicating that only one of the terminal methylene positions had been enriched. Recent work has established that the proton corresponding to the signal at $\delta 5.75$ is trans to the carboxyl group as it shows a Nuclear Overhauser effect with the methine proton at C-2 i.e. it must be on the same side of the double bond as the proton at C-2.⁴⁹

However the enriched signal appeared at $\delta 6.58$ and so it must be concluded that the deuterium atom is cis to the carboxyl group (see Fig.6).



(5)

Figure 6.

This result gives rise to a number of interesting conclusions regarding the stereochemistry involved in the biosynthesis, both of canadensic acid (5) and also of ethisolide (2).

The proven stereochemical character of fumarate hydratase (Scheme 11) leads directly to the assignment of

malate, derived from succinate-d₄ then from fumarate-d₂, as [3S,2S-2,3d₂] malate.⁵⁰ The specifically labelled malate would be expected to continue along the pathway described in Scheme 11, until it reaches the alkylitaconic acid. At this stage there are two concerted mechanisms available for the decarboxylation step⁴⁶ to give hexylitaconic acid.

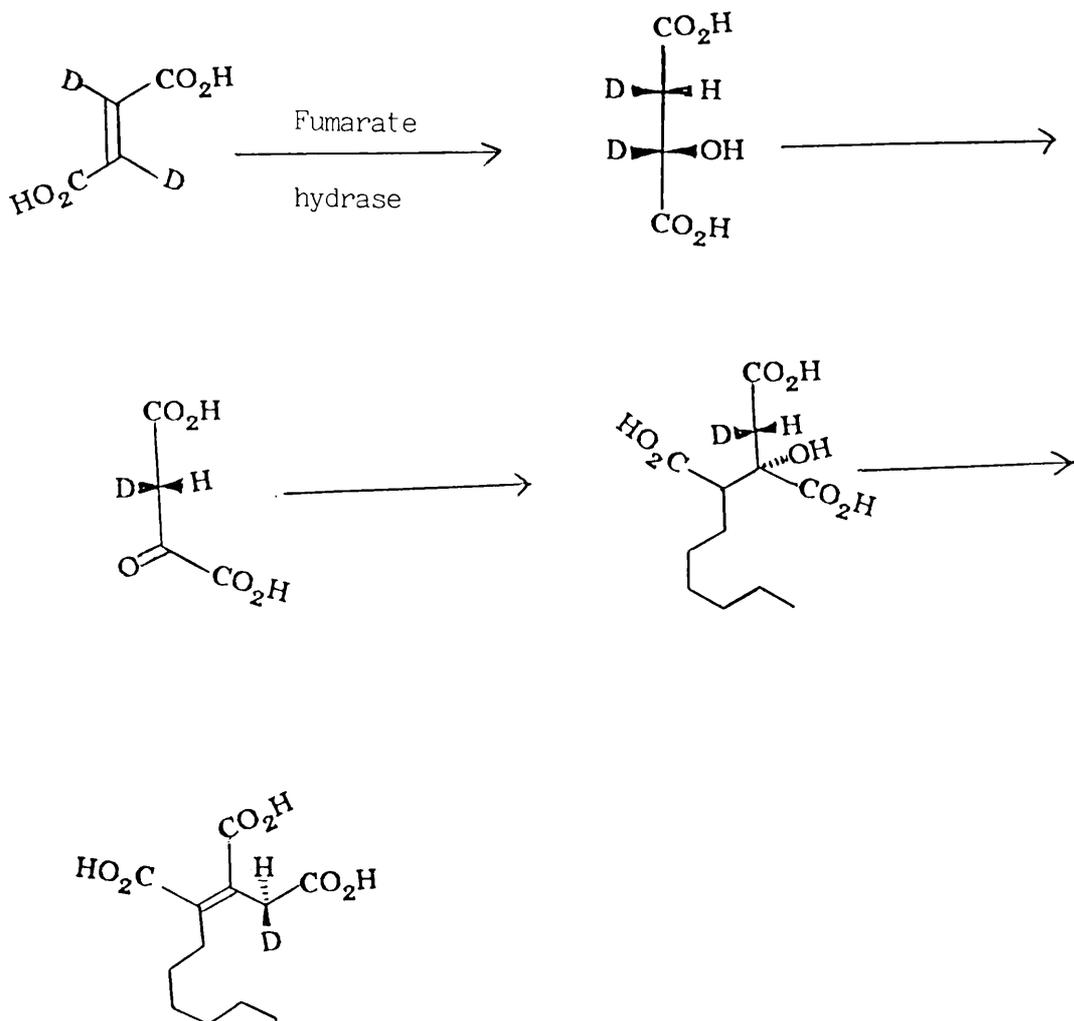
In mechanism 1, the carbon dioxide is lost from the opposite side of the molecule to which the proton is added, thereby resulting in the deuterium being trans to the CO₂H group in (39).

In mechanism 2, the carbon dioxide is lost from the same side of the molecule as the proton is added, giving a cis relationship between the deuterium atom and (CO₂H) group.

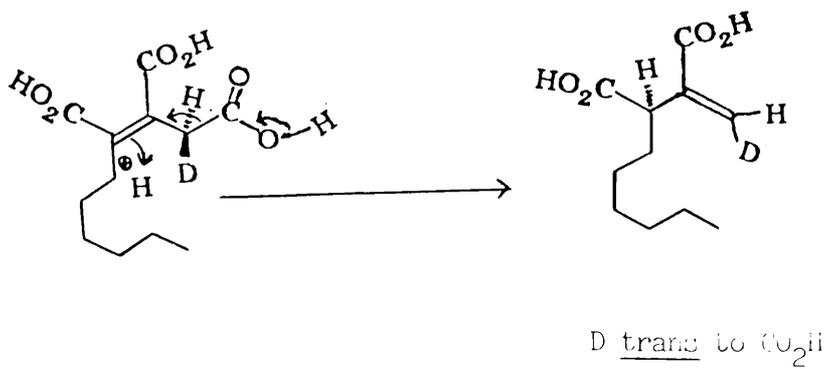
As the enriched proton in the present experiment appears cis to the CO₂H group, it may be presumed to be in the same configuration in the hexylitaconic acid intermediates, thus leading support for mechanism 2.

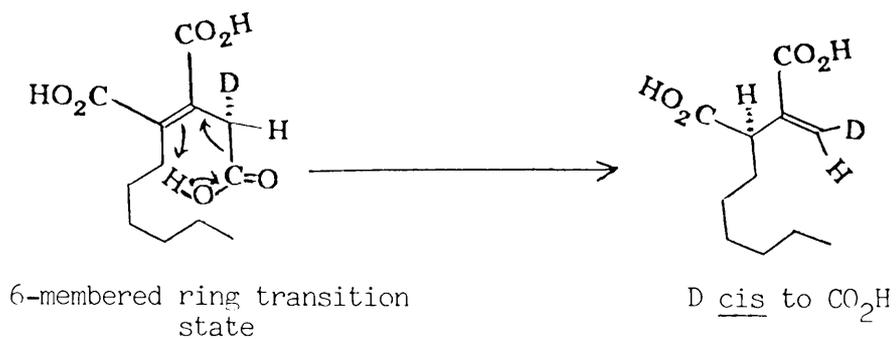
Although direct proof is still lacking that only one of the vinyl hydrogens in butylitaconic acid (47) is derived from succinate or fumarate, if the result for canadensic acid also applies to butylitaconic acid (47) it must be concluded that the randomisation of the deuterium label occurs during the rearrangement to α -methylene- β -propylglutaric acid (47a). A possible way in which this might occur would involve a radical intermediate (59) formed

Scheme 11.

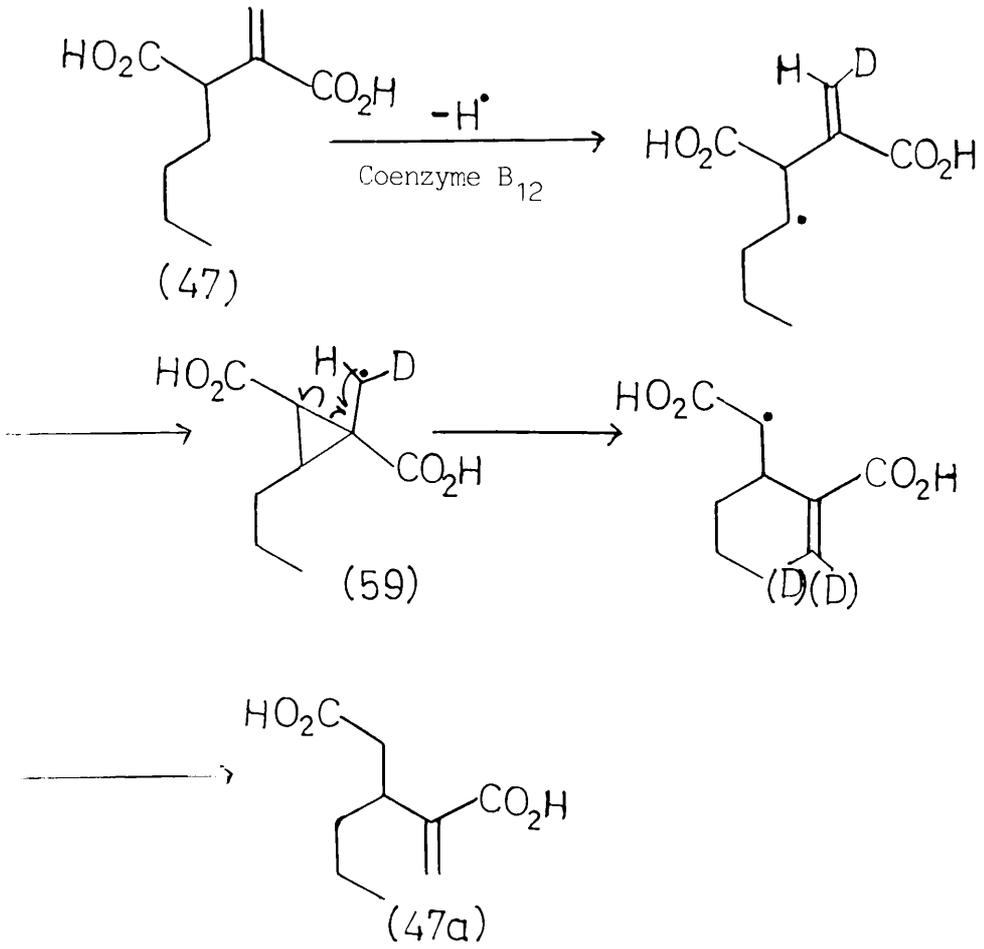


Mechanism I



Mechanism IIScheme 11.

as in scheme 12 in which rotation about the single bond would result in an equal distribution of label in the two terminal methylene positions of the rearranged product.



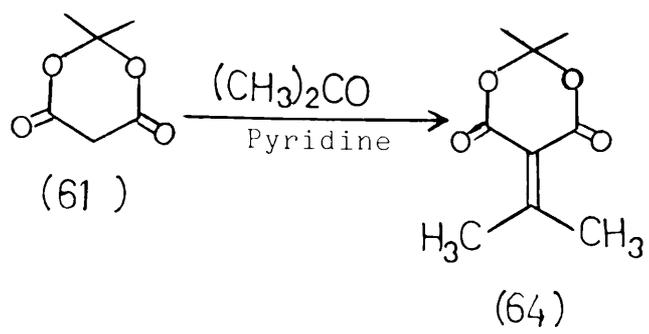
Scheme 12.

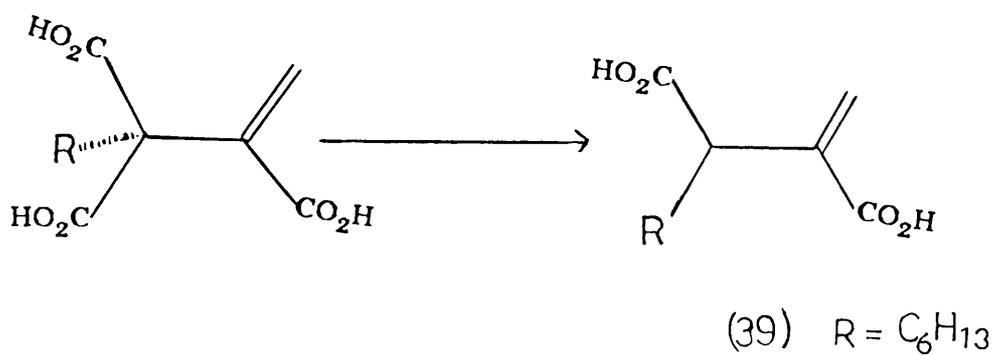
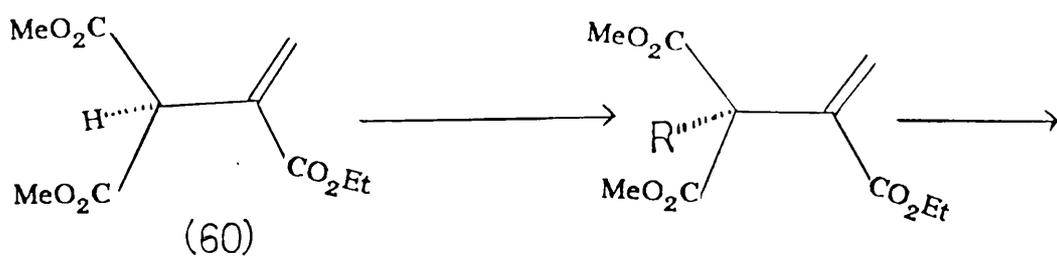
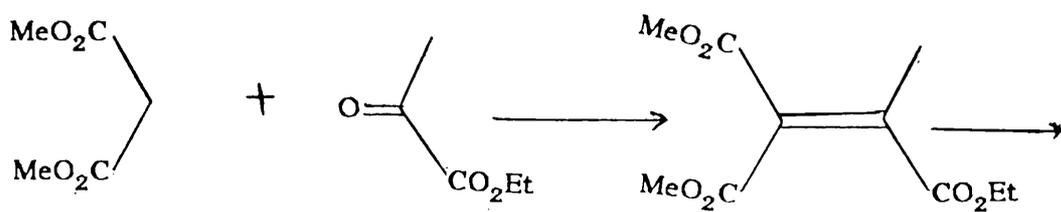
DISCUSSION

PART II.

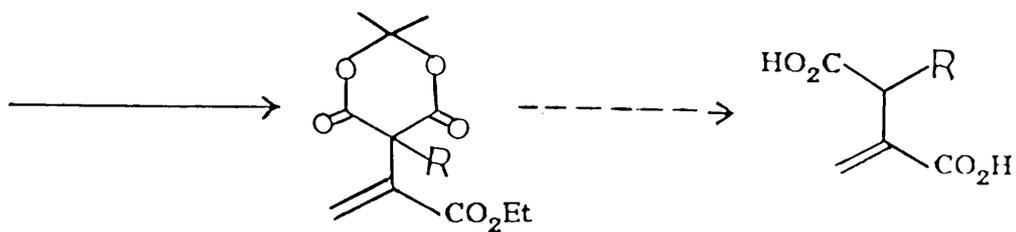
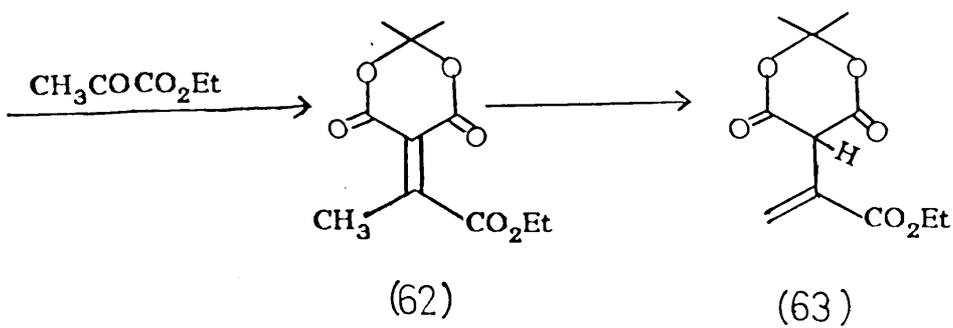
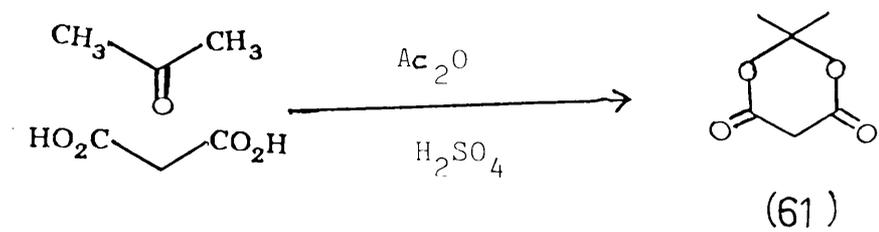
Attempted synthesis of potential intermediates in the
biosynthesis of ethisolide (2)

The aim of this present synthetic work was to establish a new, shorter and more highly yielding synthesis of alkylitaconic acids (hexylitaconic and butylitaconic acids in particular), than those already available and to extend this to the synthesis of other intermediates on the alkylcitric acid pathway. In the course of this work, it was also hoped that an easy and relatively cheap means of specifically labelling the desired compounds could be found. One previous attempt⁵¹ to prepare deuterium labelled alkylitaconic acids by the route outlined in (Scheme 13) appeared to be promising, since the deuterated triester (60) was obtained with introduction of the label from the relatively cheap source D₂O. However, the alkylation of this was unsuccessful. In the proposed new route (Scheme 14), it was hoped that a Meldrum's acid analogue (63) of this could be prepared and that the much greater reactivity of its methine hydrogen would allow the alkylation to occur. Meldrum's acid (61) (2,2-dimethyl-1,3-dioxan-4,6-dione) is a good source of malonate carbanion⁵² and an acidity (pK = 4.83) very close to that of acetic acid, which is used with acetate as a catalyst in Knoevenagel and related condensations. For these reasons, the Meldrum's acid (61) is readily made by the reaction of malonic acid and acetone following the modified Meldrum method.⁵³ The attempted condensation⁵⁴ between (61) and ethyl pyruvate at





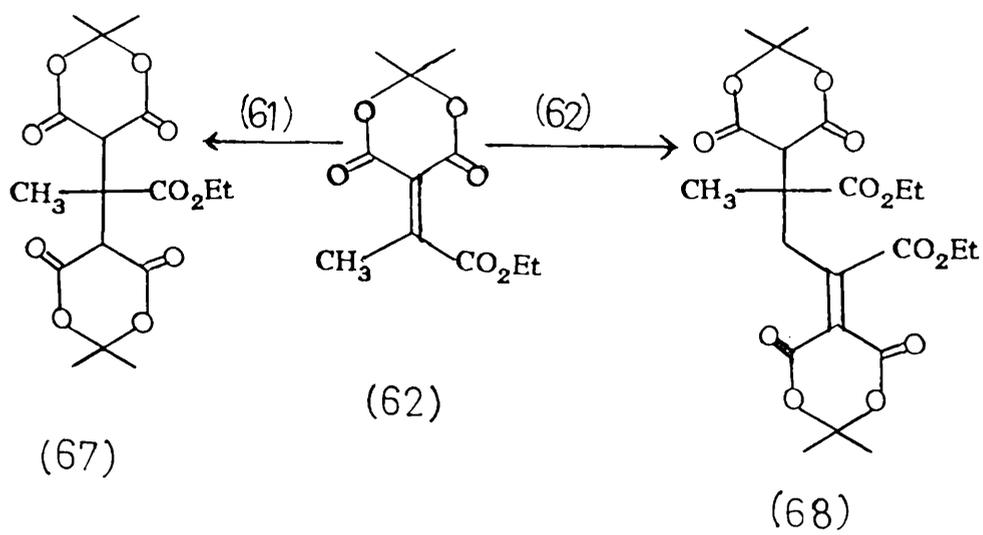
Scheme 13.



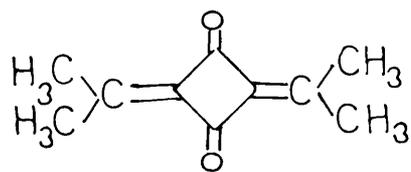
Scheme 14

room temperature in the presence of pyridine and molecular sieve 4A⁵⁵ led to a complex mixture, inseparable by preparative t.l.c or silica gel columns using several solvent systems. Under the same conditions, acetone condensed smoothly with Meldrum's acid (61) producing the isopropylidene isopropylidenemalonate (64).^{54,55} The ¹H n.m.r gave sharp singlets at δ 2.52 and 1.72 indicating the presence of the vinyl methyl groups and the isopropylidene methyl groups (i.e. -O-C(CH₃)₂-O-) respectively. The mass spectrum showed no ion corresponding to the molecular ion. The highest ion at m/e 164, corresponding to C₁₀H₁₂O₈ can be readily explained since the pyrolysis of Meldrum's acid derivatives has long been known to give ketenes.^{56,57} The mechanism presumably involves homolysis of a C-O bond, followed by cleavage of acetone and carbon dioxide, leads to the ketene m/e 82 (cf 65), which gives by dimerisation⁵⁷ the ion at m/e 164 (cf 66).

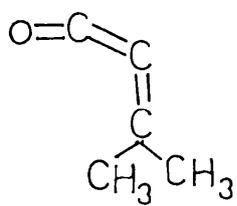
Meldrum's acid (61) can also be condensed with ketones and aldehydes⁵⁸ using neutral alumina as base. The condensation reaction of (61) and ethyl pyruvate on neutral alumina at room temperature for various times, furnished a number of products. The first of these was a crystalline ether-insoluble product m.p. 140-141^o deduced to be the 2:1 condensation product C₁₇H₂₂O₁₀ (67) (Scheme 15). This structure was assigned on the basis of the mass spectrum including the ions at m/e 242 corresponding to loss of



Scheme 15.



(66) m/e 164



(65) m/e 82

Meldrum's acid (61) and at m/e 227 corresponding to a further loss of a methyl group. In the IR, it exhibited strong C=O bands at 1785 and 1747 cm^{-1} due to the dioxandione system and a peak at 1727 cm^{-1} due to the carbethoxyl group. The latter also showed a quartet and a triplet at δ 4.20 and 1.25 respectively in the ^1H n.m.r. The ^1H n.m.r data for this compound and a number of related Meldrum's acid derivatives are summarised in Table 3.

The chemical shift for the 12 gem-dimethyl protons seems to correspond exactly to that in Meldrum's acid itself (δ 1.80). The remaining methyl resonance at δ 2.05 seems quite far downfield for a methyl group β to a carbethoxyl function (normally ca. δ 1.2). Evidently there is considerable deshielding by the two dioxandione systems. The methine protons appeared as a broad signal at δ 4.49. The ^{13}C n.m.r data for this compound and a number of related Meldrum's acid derivatives are listed and compared in Table 4. Multiplicities were established using off resonance decoupling and for (67) peaks of the expected multiplicity were found for the gem-dimethyl grouping, the acetal carbon (δ 105.3) and carbethoxyl grouping. The special features of the spectrum of this compound are at δ 25.4 (the central methyl group), δ 49.1 (the central quaternary carbon atom) and a broad signal at δ 51.0 (the methine carbon atoms).

After removal of the above product, the ether solution afforded after standing overnight at 0°C a crystalline

product m.p. 117 - 118^o from ethanol, deduced to have structure (68) (Scheme 15). The molecular formula C₂₂H₂₈O₁₂ was deduced from microanalyses and mass spectroscopy which gave a molecular ion at m/e 484 and a recognisable cracking pattern. The IR spectrum apart from dioxandione absorption at 1785, 1750 cm⁻¹ exhibited as well, a strong band at 1720 cm⁻¹ and a strong C=C stretching frequency at 1620 cm⁻¹ due to the αβ unsaturated ester. The ¹H n.m.r spectrum (cf Table 3) showed the expected peaks for the dioxandione and carbethoxyl methyl groups. The central methyl group appeared at δ1.49, perhaps as for (67) showing a degree of deshielding by the adjacent dioxandione system (one in this case). The allylic methylene hydrogens were non-equivalent appearing as an AB quartet at δ4.18 and 3.60. The hydrogens in one of the ethoxyl methylene groups were also non-equivalent appearing as an AB system further split into quartets (as revealed in a 200MHz ¹H n.m.r spectrum). These non-equivalences are probably due to the proximity of the central chiral carbon. The ¹³C n.m.r spectrum (Table 4) was somewhat complex but interpretation was greatly simplified by obtaining high resolution spectra and manipulation of the phase using DEPT (Distortionless Enhancement by Polarisation Transfer).⁵⁹ This displayed CH₂ carbons negatively, concealed quaternary carbons, displayed CH carbons only etc. All the resonances were present for a 5-monosubstituted 1,3-dioxan-4,6-dione system, including the methine carbon (51.06 ppm) and carbonyl carbons (163.96 and 163.47 ppm). The unsaturated dioxandione system

Table 3. ^1H NMR data for some Meldrum's acid derivatives.

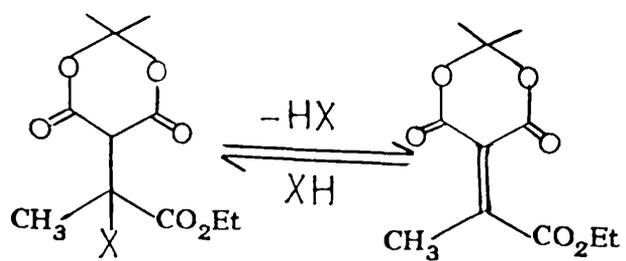
Compound :	(61)	(64)	(67)	(68)	(62)	(70)
$\text{CH}_3\text{CH}_2\text{O}$			1.25t	1.36t, 1.33t	1.35t	1.3t
$(\text{CH}_3)_2\text{C}$ (sat ring)	1.8, 1.8		1.8, 1.8	1.90, 1.89		1.77, 1.77
$(\text{CH}_3)_2\text{C}$ (unsat rg)		1.72, 1.72		1.72, 1.72	1.75, 1.75	
$\text{CH}_3\text{-C-CO}_2\text{Et}$			2.05	1.49		
$\text{CH}_3\text{C=}$		2.52			2.60	*
$\text{-C-CH}_2\text{C=}$				4.18, 3.60 (ABq J=12.6)		
$\text{-OCH}_2\text{Me}$			4.2q	4.35, 4.00 (dq) and 4.28q	4.40q	4.25q
-COCHR-CO	3.63s		4.49br	4.79s		

[* Also 7.35m (Ar-H) and 7.36 obscured (-S-CH=)]

Table 4. ^{13}C NMR data for some Meldrum's acid derivatives.

Compound:	(61)	(67)	(68)	(62)	(70)
$\underline{\text{C}}\text{H}_3\text{CH}_2\text{O}$		13.67	13.69, 13.64	13.74	13.96
$(\underline{\text{C}}\text{H}_3)_2\text{C}$	27.54	28.27, 27.28	28.63, 28.05 27.47, 25.33	27.5, 27.5	28.1, 27.5
$\underline{\text{C}}\text{H}_3\text{-C-CO}_2\text{Et}$		25.4	18.91		
$\underline{\text{C}}\text{H}_3\text{C=}$				19.9	
$-\underline{\text{C}}\text{H}_2\text{-C=}$			36.53		
$\text{Me-}\underline{\text{C}}\text{-CO}_2\text{Et}$		49.61	48.22		
$-\text{CO-}\underline{\text{C}}\text{H-CO-}$	36.20	51.0	51.06		51.45
$-\text{O}\underline{\text{C}}\text{H}_2\text{-CH}_3$		62.36	62.99, 62.34	62.6	61.66
$-\text{O-}\underline{\text{C}}\text{Me}_2\text{-O}$	106.34	105.3	105.56 105.37	105.1	105.1
$=\underline{\text{C}}\text{-CO}_2\text{Et}$			113.00	116.1	116.7
$=\underline{\text{C}}(\text{CO})_2$			119.67	128.6	*
unsat ring C=O			160.72 159.91	159.9	
sat ring C=O	163.19	164.64 163.43	163.96 163.47		164.51
$=\text{C-}\underline{\text{C}}\text{O}_2\text{Et}$			169.02	167.9	164.04
$-\text{C-}\underline{\text{C}}\text{O}_2\text{Et}$		171.5	173.17		

[* Also $\text{Ar}\underline{\text{C}}\text{H}$ at 128.7, 129.5 and 131.7
 $\text{Ar-}\underline{\text{C}}\text{-S}$ at 135.7 and $=\underline{\text{C}}\text{HS-}$ at 153.17]



(69) a. $\text{x} = \text{OCH}_3$
b. $\text{x} = \text{sPh}$

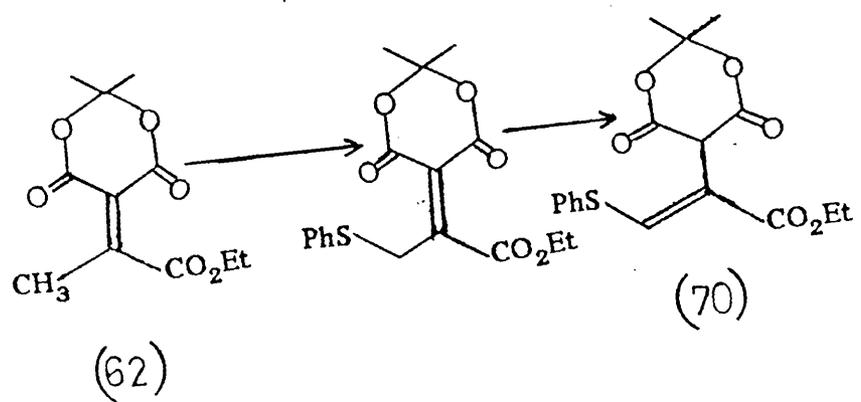
(62)

present showed carbonyl resonances at 160.72 and 159.91 ppm and the olefinic carbons appeared at 119.67 and 113.00 ppm. The central methyl and methylene groups appeared at 18.91 and 36.53 ppm respectively while the saturated and unsaturated carbethoxyl carbons appeared at 173.17 and 169.02 respectively.

It is evident that the compounds (67) and (68) are formed by Michael addition of the required alkylidene compound (62) with (61) and itself respectively (Scheme 15). Under these conditions there was none of the alkylidene compound (62) itself obtained. The condensation reaction of Meldrum's acid (61) and ethyl pyruvate was also attempted in presence of florisil as a base. Unfortunately a mixture of unreacted starting materials and the dimer compound (68) was obtained under these milder conditions. In order to prevent the dimerisation of the desired ester (62), attempts were made to trap (62) by addition of a nucleophile to give the adduct (69) from which (62) might subsequently be regenerated. The sodium salt of (61) was prepared using NaHCO_3 in distilled water and its ^1H nmr spectrum showed sharp singlets at δ 3.04 and 1.73 of integrated intensities 1:6 confirming the formation of the sodium salt of (61), which was allowed to react in methanol⁶⁰ with ethyl pyruvate. However, after treatment with 1N HCl to promote loss of methanol from any (69a, X = OCH_3) formed. Unfortunately the major product proved to be the dimer (68).

Condensation of (61) with ethyl pyruvate using alumina was carried out in the presence of benzene thiol in an attempt

to prepare (69b, X = SPh). At short reaction times, unreacted starting materials were present. When the reaction was allowed to proceed for 7 days a crystalline product was obtained. Although this was a sulphur containing compound it was finally deduced to be the enolsulphide diketodioxan derivative (70) (Scheme 16). The assigned structure of (70) was consistent with microanalytical figures corresponding to the molecular formula $C_{17}H_{18}O_6S$ and a mass spectral molecular ion at m/e 350. The I.R spectrum possessed sharp C=O stretching bands at 1795, 1740 and 1700cm^{-1} and exhibited a strong C=C stretching band at 1580cm^{-1} . The ^1H nmr spectrum (Table 3) showed the expected features for the dioxandione system, the methine proton appearing at $\delta 4.15$. The vinyl proton was concealed beneath the envelope of the aryl protons at ca. $\delta 7.35$, as deduced from ^{13}C nmr spectra. In an O.R.D. spectrum a doublet at $\delta 153.17$ was assigned to the -S-CH= carbon. The residual C-H coupling (108.6Hz) was just larger than that of the aryl carbons bearing hydrogen (105, 103.9 and 103.5 Hz respectively). Since the residual coupling is proportional to the difference in the ^1H chemical shift from the irradiating frequency (TMS). It can be deduced that the ^1H signals will be in approximately the same region. (In the same spectrum the residual couplings for the ethoxyl signals at 61.66 and 13.96 were 63.3 and 18.6 Hz respectively). The other features of the ^{13}C nmr spectrum (Table 4) were as expected from the previously obtained data.

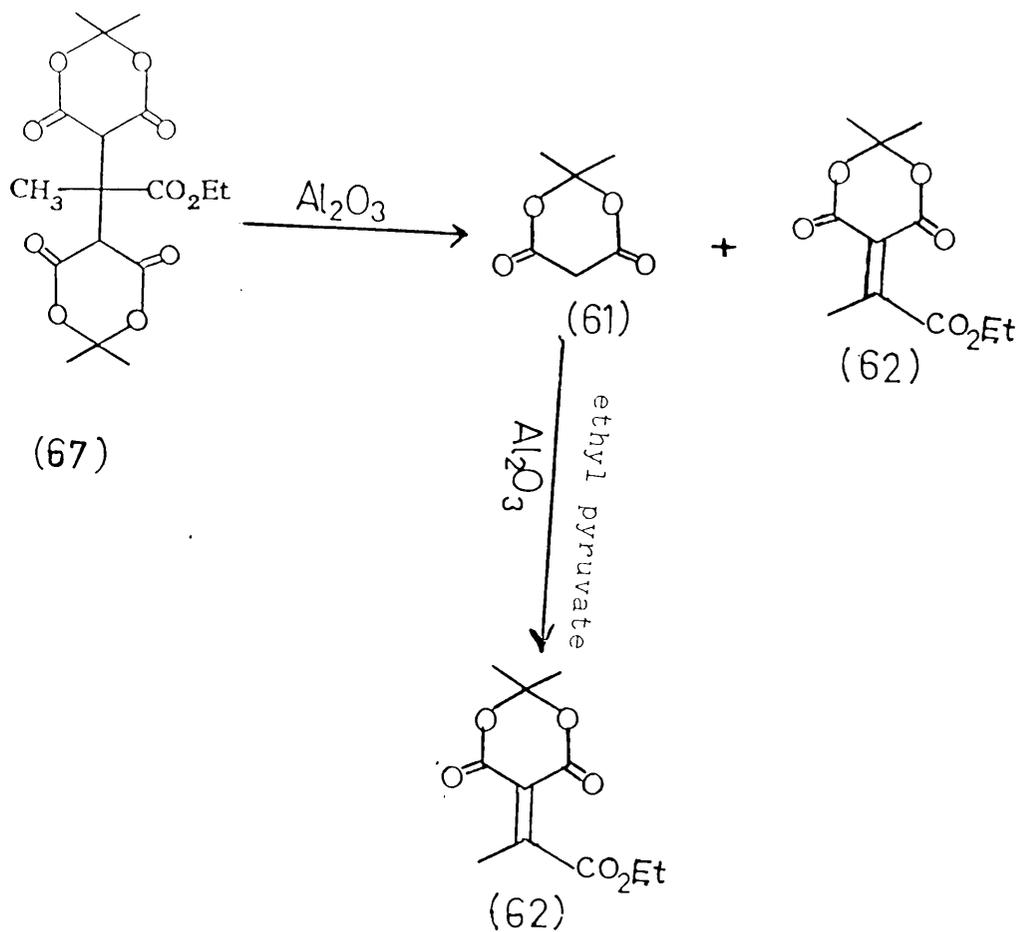


Scheme 16

After removal of the above product the ether solution afforded after evaporation a mixture of starting materials and diphenyl disulphide which gradually separated as white crystals. In addition, the ^1H nmr spectrum of the mixture showed a singlet at $\delta 2.60$ corresponding to the vinyl methyl group in the desired alkylidene compound (62) although this was not known at the time. The mode of formation and chemistry of this sulphur compound (70) was studied briefly and this is discussed later.

An attempt was next made to carry out a reaction between ethyl pyruvate and an excess of the 2:1 condensation compound (67) in the presence of alumina as base hoping that the desired ester (62) and Meldrum's acid would be generated by a reverse Michael reaction and that the latter might condense with ethyl pyruvate to give a further amount of (62) (Scheme 17). After work-up, the ^1H nmr spectrum confirmed that no reaction took place and the starting materials being recovered unchanged.

The foregoing studies seemed to indicate that the desired ester had been formed in the various reactions using alumina, but that the product was susceptible to base catalysed Michael addition reactions giving various products. It was therefore of interest to see whether condensation occurred under mild acidic conditions. A mixture of Meldrum's acid (61) and ethyl pyruvate were carefully melted and absorbed on silicic acid, and when the reaction was worked up after overnight stirring at room temperature, t.l.c and



Scheme 17

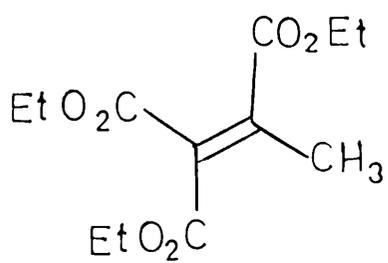
the ^1H nmr spectrum indicated the presence of a substantial quantity of the vinyl methyl compound (62) and the compounds (67) and (68), in addition to unreacted starting materials. The reaction was allowed to proceed for 7 days, and the byproducts (67) and (68) were removed as before. The desired alkylidene compound (62) (Scheme 14), was obtained as a colourless oil in 32% yield after column chromatography followed by distillation. The structure (62) was confirmed by microanalysis and the mass spectrum including the molecular ion of m/e 242 and a peak at m/e 227 (corresponding to loss of a methyl group). The ^1H nmr (Table 3) included sharp singlets at δ 2.60 and 1.75 indicating the presence of the vinyl methyl group and the isopropylidene methyl groups respectively, comparable to those found for the isopropylidene compound (64). The IR spectrum showed peaks for the dioxandione $\text{C}=\text{O}$ groups at 1770, 1750 (br) cm^{-1} and also bands at 1720 and 1630 cm^{-1} possibly corresponding to the $\text{C}=\text{O}$ and $\text{C}=\text{C}$ of the unsaturated ester system. The ^{13}C nmr spectrum (Table 4) showed peaks of the expected chemical shift and multiplicities confirmed by an O.R.D. spectrum, for this structure (62). The vinyl methyl signal appeared at δ 19.9 and the chemical shift of the carbonyl groups in the dioxandione system and the carbethoxyl function were both in keeping with unsaturated carbonyls rather than 'saturated' carbonyls (cf Table 4). This reaction has been carried out under various conditions and some samples were thought, from signals in the ^1H nmr to contain traces of the

methylidene isomer (63). This is characterised by 1H singlets at δ 6.5, 5.9 and 4.65. The ratio of the peaks characteristic of the two isomers suggest the product to be a ca. 5:2 mixture in favour of the vinyl methyl compound (62)

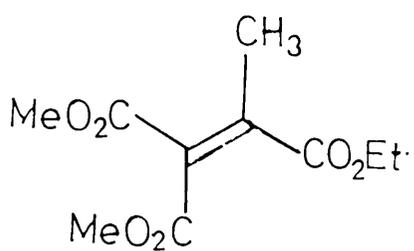
After distillation however, only the vinyl methyl compound (62) appeared to be present.

In an attempt to obtain (62) in good overall yield, the above reaction was carried out in the presence of anhydrous zinc chloride and acetic anhydride in benzene as a solvent. Indeed the compound (62) was detected by the ^1H n.m.r, but could not be isolated without decomposition occurring. Unfortunately and unexpectedly monoethyl itaconate (71) (Scheme 18) was obtained as a major product. The isolation and structural elucidation of this compound produced in another reaction is discussed below.

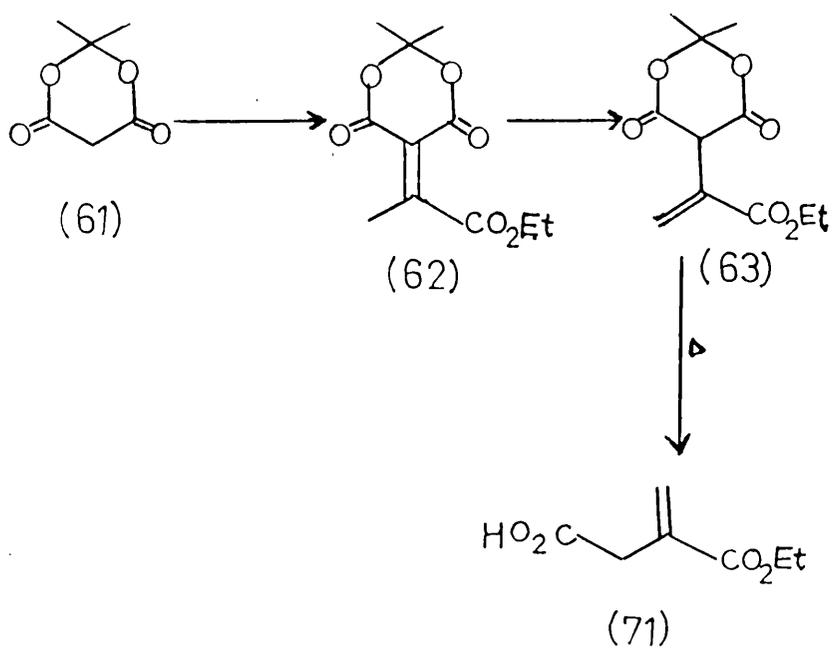
It has been reported that the methylation ⁶¹ of diethyl 3-carboethoxyitaconate (72) and the alkylation of 2-carboethoxy-1,1-dicarbomethoxypropene ⁵¹ (73) indicated a necessity to isomerise prior to attempting the alkylation. It was hoped that isomerisation of (62) to (63) might be effected by pyridine, however, the ^1H n.m.r spectrum of (62) in d_5 -pyridine showed that it had immediately and quantitatively dimerised to (68) (Scheme 19). Significantly, the vinyl methyl peak at δ 2.60 had virtually disappeared implying the product to be essentially the dimer (68) which is characterised by 1H singlets at δ 4.80, 3.68 and 3.52, and 3H singlets



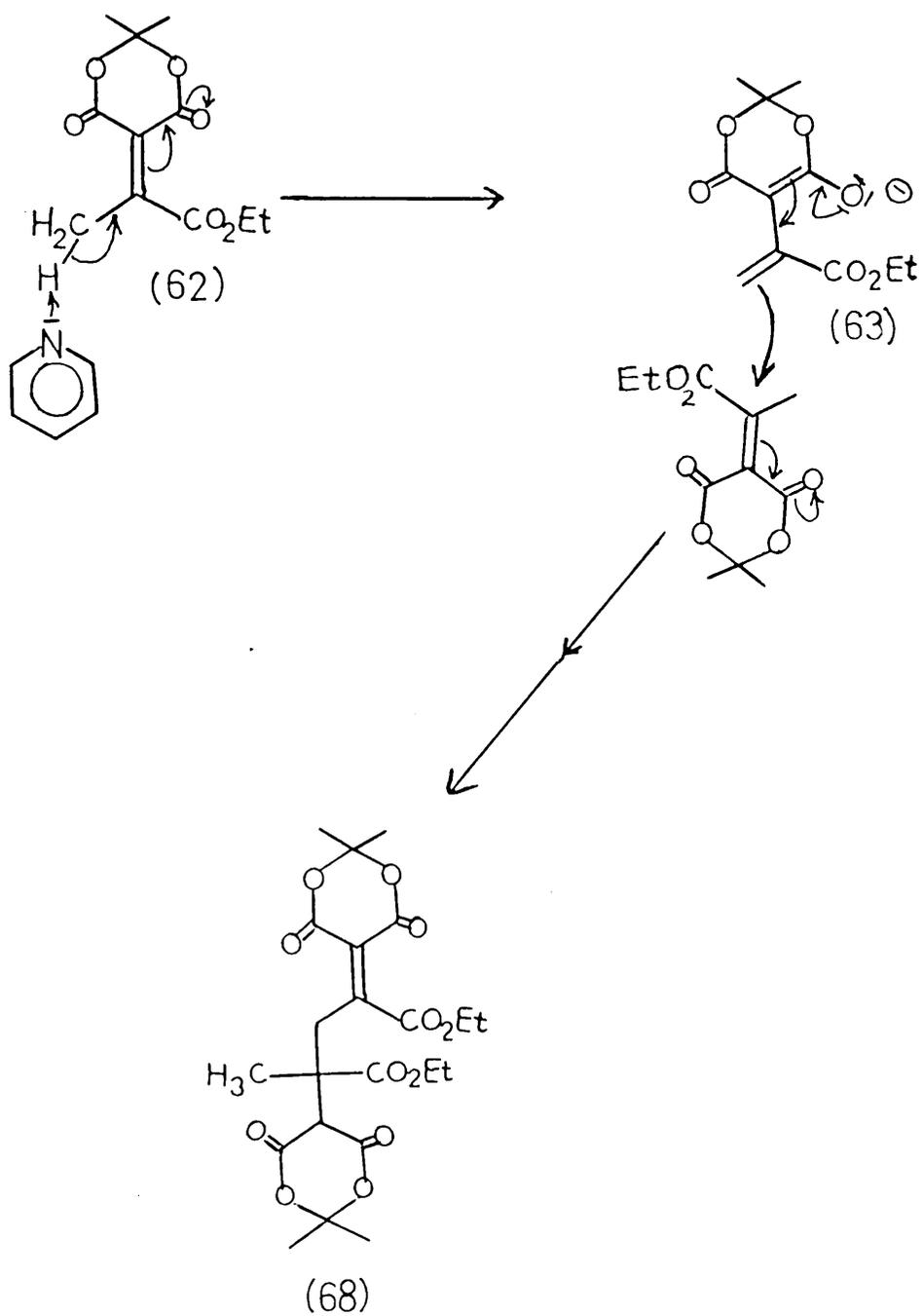
(72)



(73)



Scheme 18



Scheme 10

at δ 1.9 and 1.49. This was identical with the spectrum of the dimer (68) in d_5 -pyridine.

When (62) was treated with K_2CO_3 in dry acetone, although the anion was evidently formed, recovery from the K_2CO_3 using 1N HCl afforded unchanged (62). It was decided to repeat the experiment, but this time using iodohexane in an attempt to bring about alkylation. After 3 days at room temperature a new methylenide compound was formed but this again appears to be 1-monoethyl itaconate (71). The structure was indicated by the 1H n.m.r spectrum which shows a quartet and a triplet at δ 4.2 and 1.3 respectively indicating the presence of the carbethoxyl group and a sharp singlet at δ 3.31 corresponding to the methylene group. The two vinyl protons gave singlets at δ 6.3 and 5.7. Comparison with the spectra of itaconic acid (available) and dimethyl itaconate (54) (prepared as described in Part 1) leave little doubt that the ester is 1-monoethyl itaconate (71) presumably formed by attack at the heterocyclic ring and decarboxylation perhaps catalysed by iodide ion generated in the reaction. A similar product was obtained when activated Amberlyst and BuBr were used at 40° .

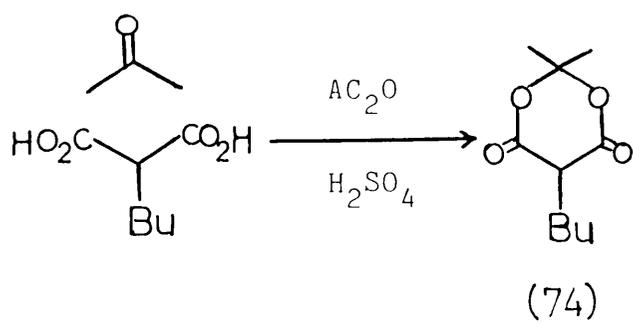
Alkylation was also attempted using silver carbonate and benzene with butyl bromide as alkylating agent. At room temperature no reaction occurred and when the reaction mixture was refluxed a complex mixture was obtained. The ester (62) was also treated with silver oxide and butyl bromide in

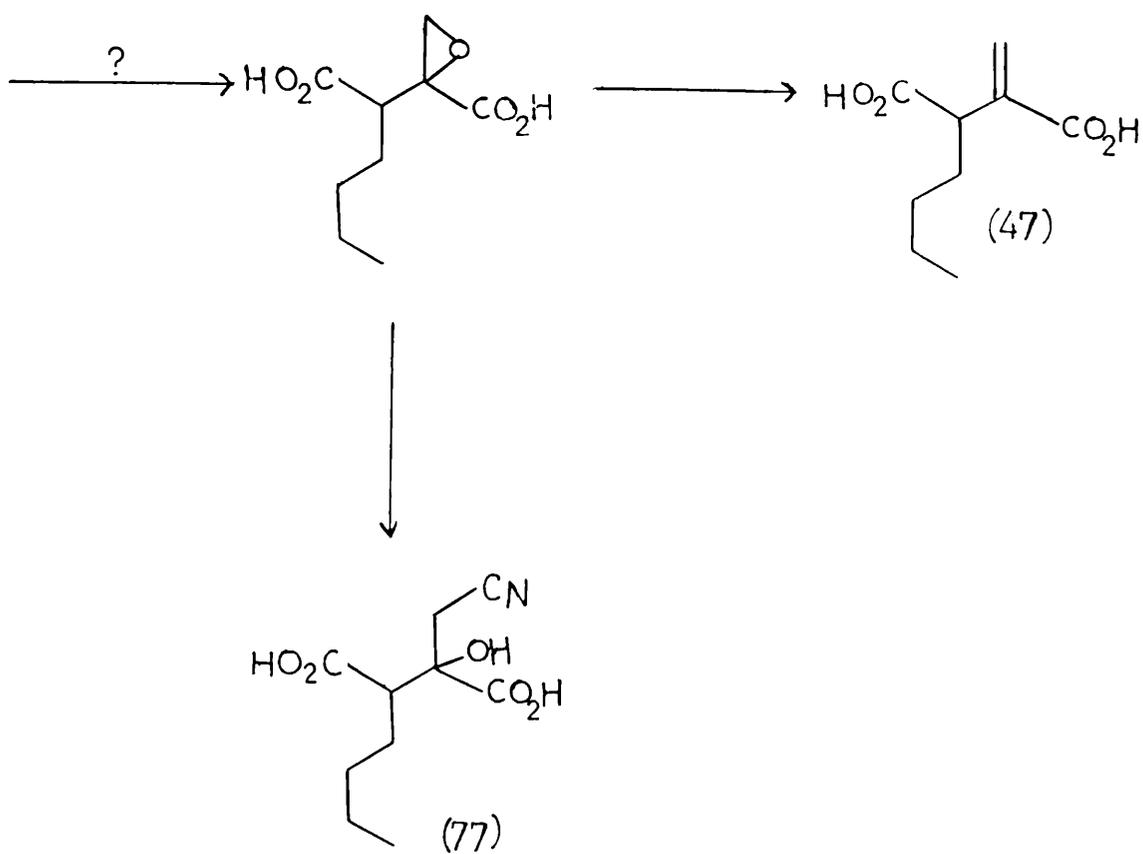
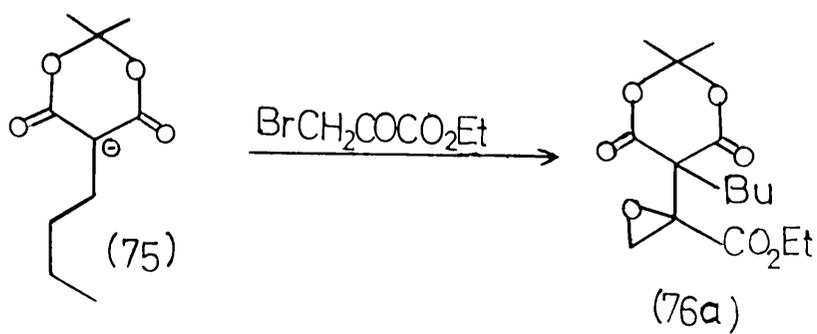
an effort to effect isomerisation and then alkylation. Again there was no indication of any alkylation having taken place.

In view of the difficulties found in introducing the butyl group, the potential of the butyl derivative of Meldrum's acid (74) (which was readily synthesised from butylmalonic acid)⁶² was explored briefly. It was hoped that condensation of this with ethyl bromopyruvate might produce the epoxyester (76a) of the corresponding bromohydrin rather than the ketonic alkylation product (76b). The former type of product might have led to the itaconic or citric acid derivatives (47) or (77) (Scheme 20). However, under various conditions [e.g. with silicic acid or with $\text{Ac}_2\text{O}/\text{DMF}$ or with the preformed sodium salt (75)] no useful products were obtained. In view of the successful synthesis of the alkylidene compound (62), it was hoped that an analogous condensation might be carried out with diethyl 2-methyloxaloacetate. This could afford the alkylcitric or alkylitaconic acid e.g. (6) or (79) (Scheme 21).

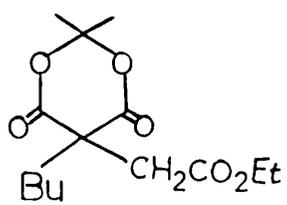
In preliminary experiments using silicic acid no reaction was observed but further study using more vigorous conditions could be useful [self condensation would be less likely to be a problem than in the case of (62)].

It should also be noted that ethyl bromopyruvate underwent condensation with Meldrum's acid (61) itself using silicic acid giving products analogous to those

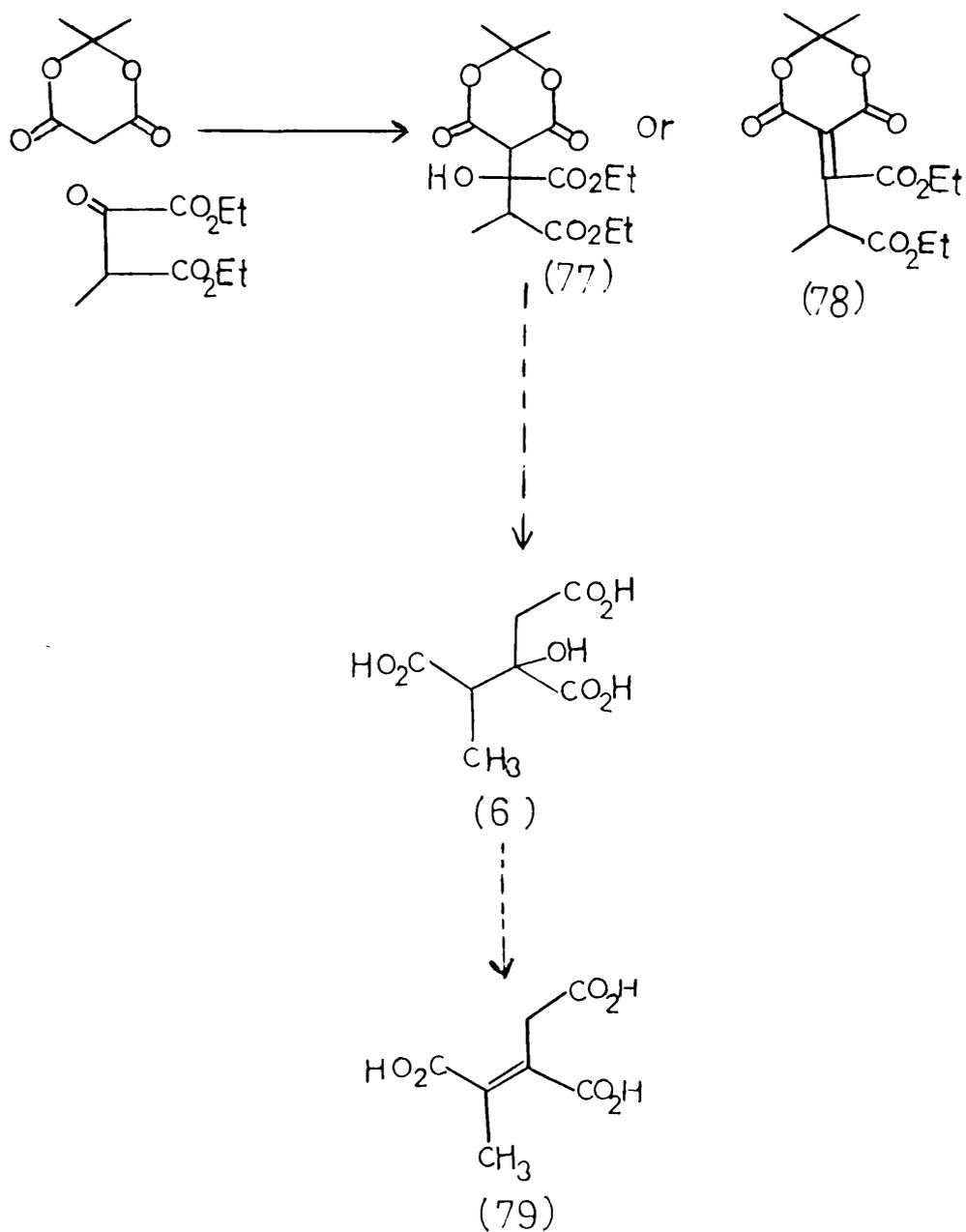




Scheme 20.



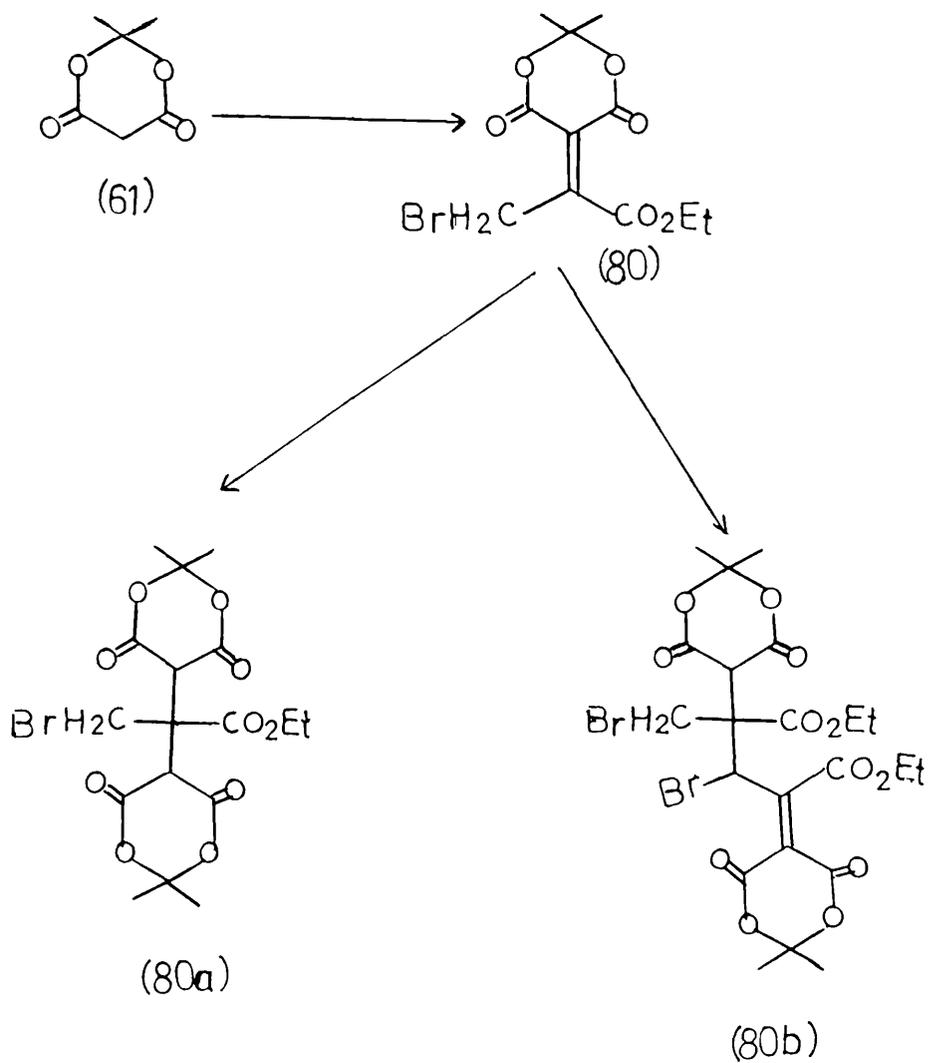
(76b)



Scheme 21

reported earlier for ethyl pyruvate. The most interesting of these was the bromo alkylidene compound (80) (Scheme 22), obtained as an oil in 76% yield after distillation. The structure was confirmed by elemental analysis, mass spectroscopy (molecular ion at m/e 322 as expected for $C_{11}H_{13}O_6Br$), and from the infra-red bands at 1768, 1752 cm^{-1} for the dioxandione carbonyl groups, 1720 cm^{-1} for the unsaturated ester grouping and 1635 cm^{-1} for the C=C absorption. The 1H n.m.r spectrum showed the expected resonances together with a sharp singlet at 4.82 indicating the presence of the allylic bromomethylene group ($BrCH_2-C=$).

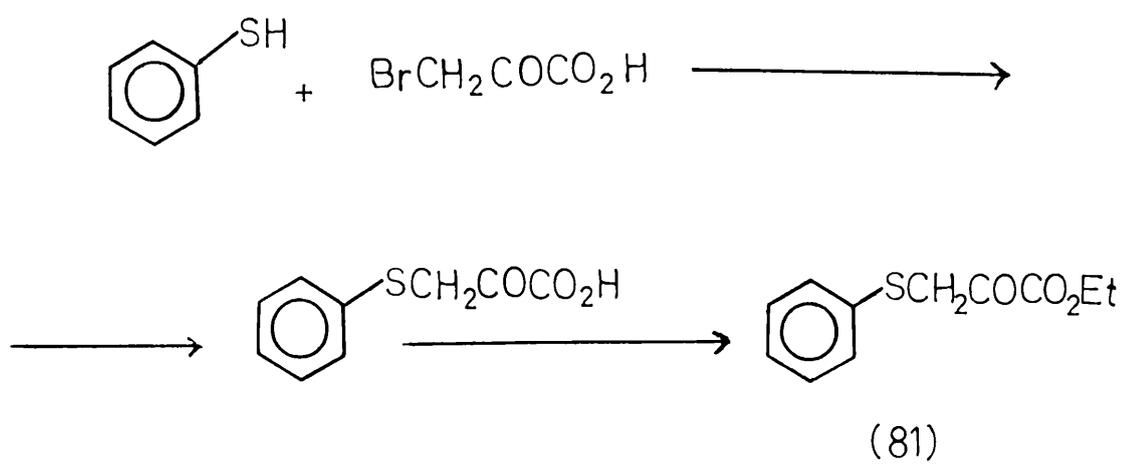
The formation of the vinyl sulphide (70) from an attempt to prepare the thioether (69b) was mentioned earlier. Some work was carried out to investigate its mode of formation and possible use. It was thought that the product might be formed by sulphenylation of the vinyl ester (62) followed by tautomerism to the vinyl sulphide, which could be formed from benzene thiol by aerial oxidation in situ under the basic conditions⁶³ since air was not rigorously excluded. Indeed diphenyl disulphide was detected amongst the products of the reaction. However, when Meldrum's acid (61) and ethyl pyruvate were allowed to react in the presence of alumina and diphenyl disulphide this compound was not detected in the products although the alkylidene ester (62) was obtained (t.l.c, n.m.r) contaminated by only traces of dimer (68). An alternative mode of formation seemed possible in which underwent sulphenylation to give the



Scheme 22

sulphide ester (70) which might react with Meldrum's acid (61) to afford, after tautomerisation, the vinyl sulphide (70). However, there appeared to be no reaction between ethyl pyruvate and diphenyl disulphide on alumina after a prolonged period. Possibly sulphenylation is effected in this reaction by PhS^\bullet radicals rather than by diphenyl disulphide. Such radicals are probably generated in the oxidative process leading from PhSH to PhSSPh , (Scheme 16). An attempt was also made to provide structural proof for the vinylsulphide (70) by an independent synthesis. Namely via condensation of Meldrum's acid (61) with ethyl 3-phenylthio-2-ketopropanoate (81). This ester was synthesised from bromopyruvic acid in 47% yield by reaction with benzenethiol and alkali followed by esterification using ethanolic HCl (Scheme 23). This compound has not been reported previously.

The sample so obtained was characterised by elemental analysis and mass spectroscopy. The carbonyl absorption in the IR appeared at 1720 cm^{-1} . The ^1H and ^{13}C n.m.r spectra showed the expected peaks for the aryl protons, the thiomethylene grouping ($-\text{SCH}_2$) giving a 2H singlet at $\delta 3.7$ and a carbon resonance at 40.29. However, in the ^1H n.m.r spectrum the signals corresponding to the ethoxyl grouping were somewhat diffuse, as was the ^{13}C signal for the ethoxyl methyl grouping. Initially, it was considered that the ^1H n.m.r spectrum could be accounted for by the presence of enolic tautomers but a number of unaccountable peaks in the



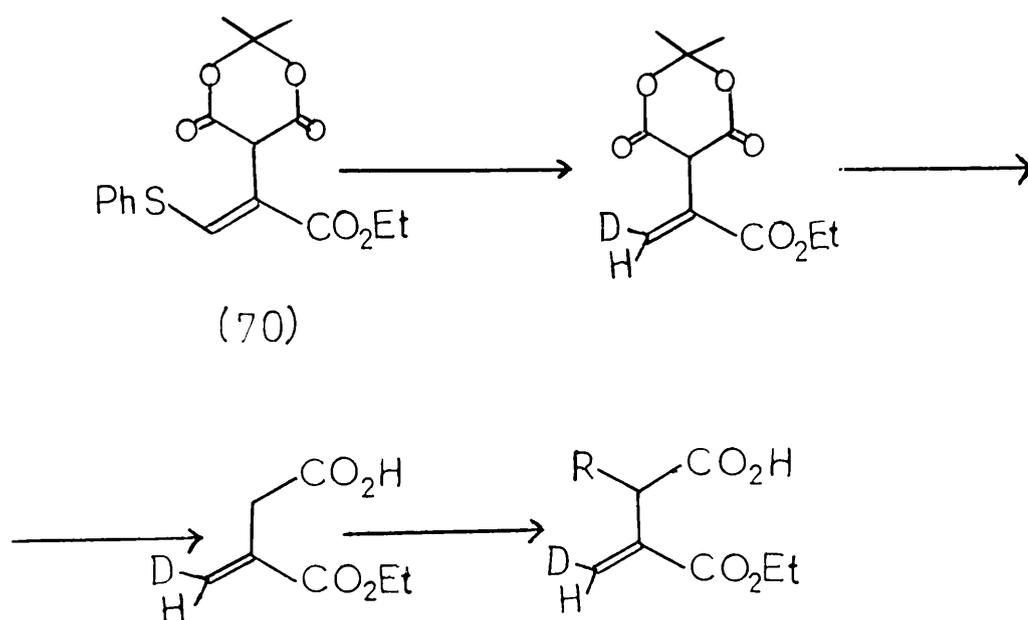
Scheme 23.

^{13}C n.m.r spectrum led to the conclusion that another compound, must form a substantial part of the sample. This sample of the thioester (81) was recovered unchanged (^1H n.m.r) after attempted reaction of this with Meldrum's acid, using silicic acid. As yet rigorous purification and condensations under more forcing conditions have not been attempted. Two features of the vinyl sulphide structure (70) suggested that it might prove a valuable intermediate. Firstly it seemed possible that the methine hydrogen on the dioxandione ring would be reactive enough to allow alkylation. Indeed it appeared to be quite acedic since it was readily exchanged by D_2O . However attempted alkylation using methyl iodide and silver oxide in acetonitrile gave a complex mixture which was not investigated further.

A second feature which could prove valuable is the vinyl sulphide grouping. If this could be reductively removed with Raney nickel, by using deuterated catalyst it might be possible to generate an itaconic acid derivative with a terminal methylene grouping deuterated at only one of the positions (Scheme 24).

The desirability of such a compound in studies of the biosynthesis of ethisolide (2) and avenaciolide (1) is discussed in Part 1 of the Discussion of this thesis.

In Part I of this thesis efforts were made to isolate butylitaconic acid (47) and its hydroxy analogue (45) as their esters. Also in the foregoing work aimed at the

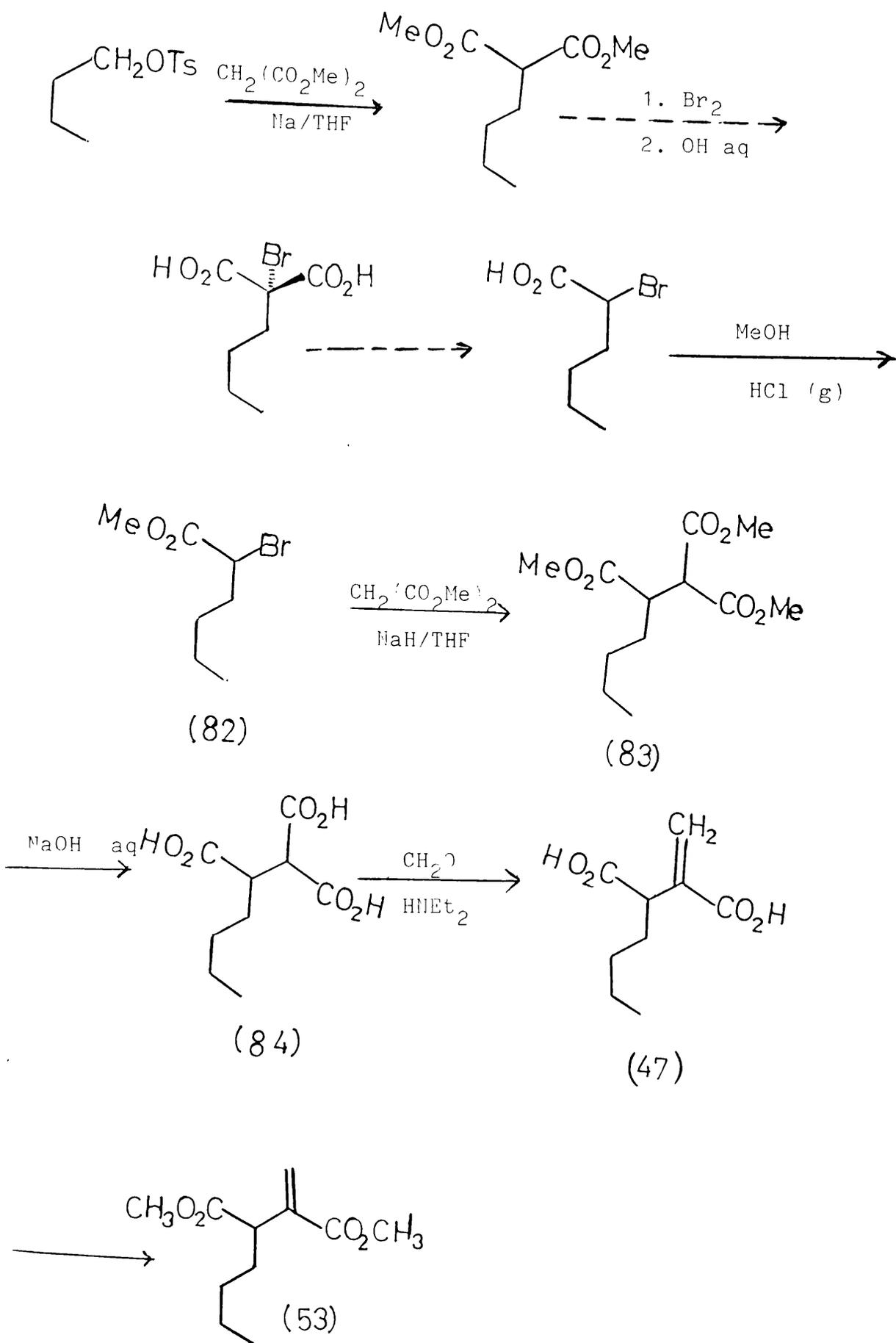


Scheme 24.

synthesis of alkylitaconic acids, the likely synthetic precursors would have been esters of these. It was therefore desirable to investigate the esterification of butylitaconic acid (47) and the stability of its ester (53) during hydrolysis. A number of other reactions of the ester (oxidation, bromination) were also studied.

Butylitaconic acid (47) was synthesised by a standard route⁶⁴ from the bromoester (82). Condensation of this with diethyl malonate using NaH in dry THF gave the triester (83). Hydrolysis in 1M aq NaOH at room temperature gave the corresponding triacid (84) in 54% yield. This reacted with aqueous formaldehyde in the presence of diethylamine to give butylitaconic acid (47) in low yield (24%). The various intermediates and the final product were identified by comparison with the spectra and samples of authentic material. (Scheme 25).

Butylitaconic acid (47) was esterified by refluxing with methanolic HCl generated by adding SOCl_2 to methanol. The desired ester (53) was obtained as a colourless oil in 92% yield. This was characterised as $\text{C}_{11}\text{H}_{18}\text{O}_4$ by microanalysis and mass spectroscopy. The ^1H n.m.r spectrum showed singlets at δ 3.78 and 3.68 indicating the presence of the two methoxyl groups and it also exhibited C=O absorption at 1740 and 1720 cm^{-1} and conjugated C=CH₂ absorption at 1660 cm^{-1} respectively. The ^{13}C n.m.r showed peaks of the appropriate multiplicity at 173.54 and 166.45 for saturated

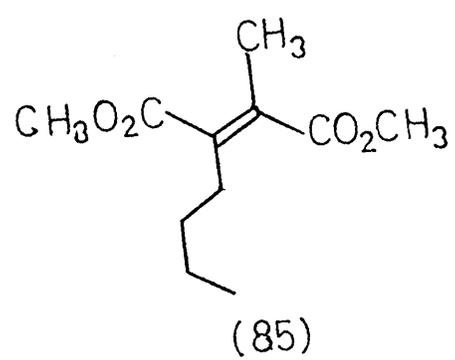


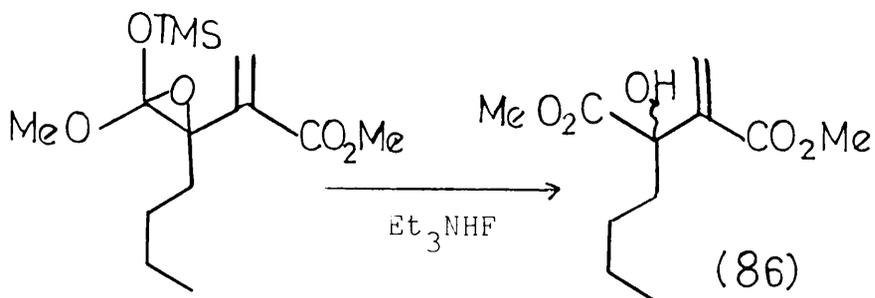
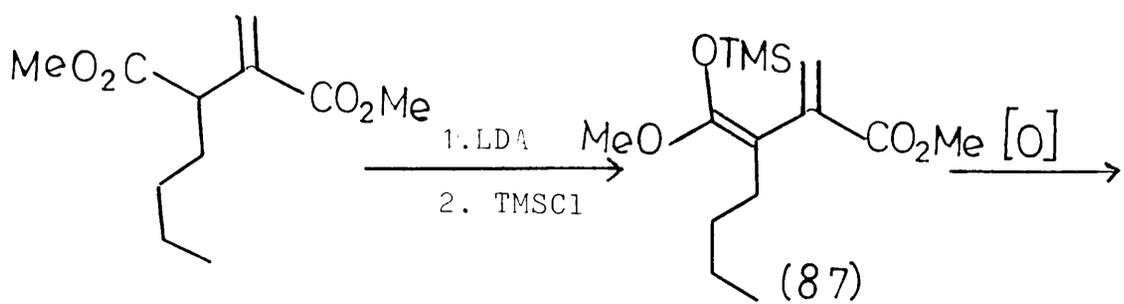
Scheme 25.

and unsaturated C=O groups respectively, at 138.39 and 124.75 for the terminal methylene carbons and at 46.40 for the methine carbon, together with appropriate peaks for the methoxyl groups and butyl groups.

In order to examine the stability of its ester to hydrolysis, (53) was treated with aqueous KOH at room temperature. Acidification afforded the corresponding acid (47) containing only a small amount (ca.5%) of double bond isomer (85), detectable from the ^1H n.m.r resonance at δ 2.35, corresponding to the vinyl methyl protons.

Now that (53) was available, it was possible to attempt and convert it to its α -hydroxy analogue (86) which had previously been isolated from some fermentations.⁴ The first attempt at hydroxylation of (53) was made using charcoal and triethylamine in ethyl acetate. These mild conditions were reportedly successful in the hydroxylation of Hagemann's esters.⁶⁵ However, in this case the diester (53) was recovered unchanged, as was observed after bubbling O_2 continuously through a solution of (53) in THF. An attempt was also made to carry out peracid oxidation⁶⁶ of the corresponding enolsilyl ether (87) in anhydrous conditions (Scheme 26). This failed to give the desired compound (86). It was later concluded that there might be steric hindrance to the formation of the anion required at C-2 in (53) using LDA. Hoping to prepare the enol silyl ether, the diester (53) was therefore treated with a less hindered base $[\text{EtNH}_2\text{-Li}]$ ⁶⁷ at room temperature, then followed by the addition of



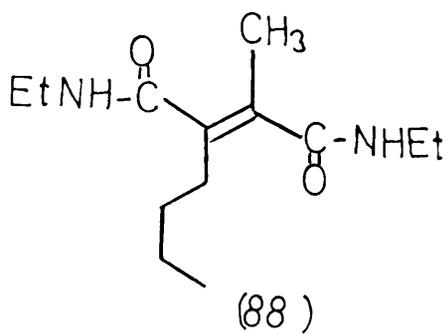
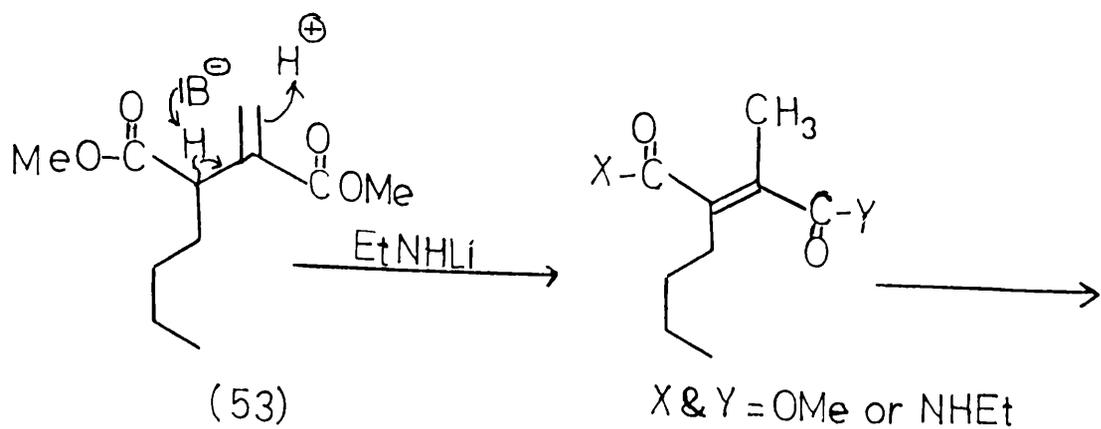


Scheme 26

TMSCl. Preparative tlc gave two products, neither of which contained silicon or retained the terminal methylene grouping. These were deduced to be the cis and trans isomers of the amide (88). Mass spectroscopy gave peaks including the molecular ion at m/e 240 as expected for $C_{13}H_{24}O_2N_2$ and a peak at m/e 184 corresponding to loss of the butyl group. It also exhibited IR absorption corresponding to the amide I, amide II and NH stretching bands of an acyclic secondary amide at 1690, 1510 and 3458 cm^{-1} respectively. From the 1H n.m.r spectrum it was evident that there were two NH groupings from a 2H multiplet at $\delta 5.75$ and appropriate signals were present for two $-NCH_2CH_3$ groupings. A three proton singlet at 3.4 was assigned to a vinyl methyl grouping and the remaining signals to the butyl group.

These compounds are evidently formed as described in (Scheme 27). The fact that the esters had been converted to amides would not have invalidated this experiment but complete isomerisation of the terminal methylene group to its vinyl isomer(s) ruled it out as a means of obtaining a derivative of α -butyl- α -hydroxyitaconic acid. As indicated in Part I the target for biosynthetic studies has now been altered to α -butylitaconic acid itself and the foregoing studies in esterification and hydrolysis were therefore extremely relevant.

Finally one other approach to the synthesis of (86) was made which could also afford alkylitaconic acids which are required as possible intermediates in the biosynthesis



Scheme 27.

of avenaciolide (1) and ethisolide (2). It was hoped that bromination followed by dehydrobromination of (53) would give the allylic bromo compound (89) which could serve to provide both types of compound as in scheme 28.

Bromination of the ester (53) gave a 9:4 mixture of the threo and erythro isomers of the required dibromo compound readily separated by preparative tlc.

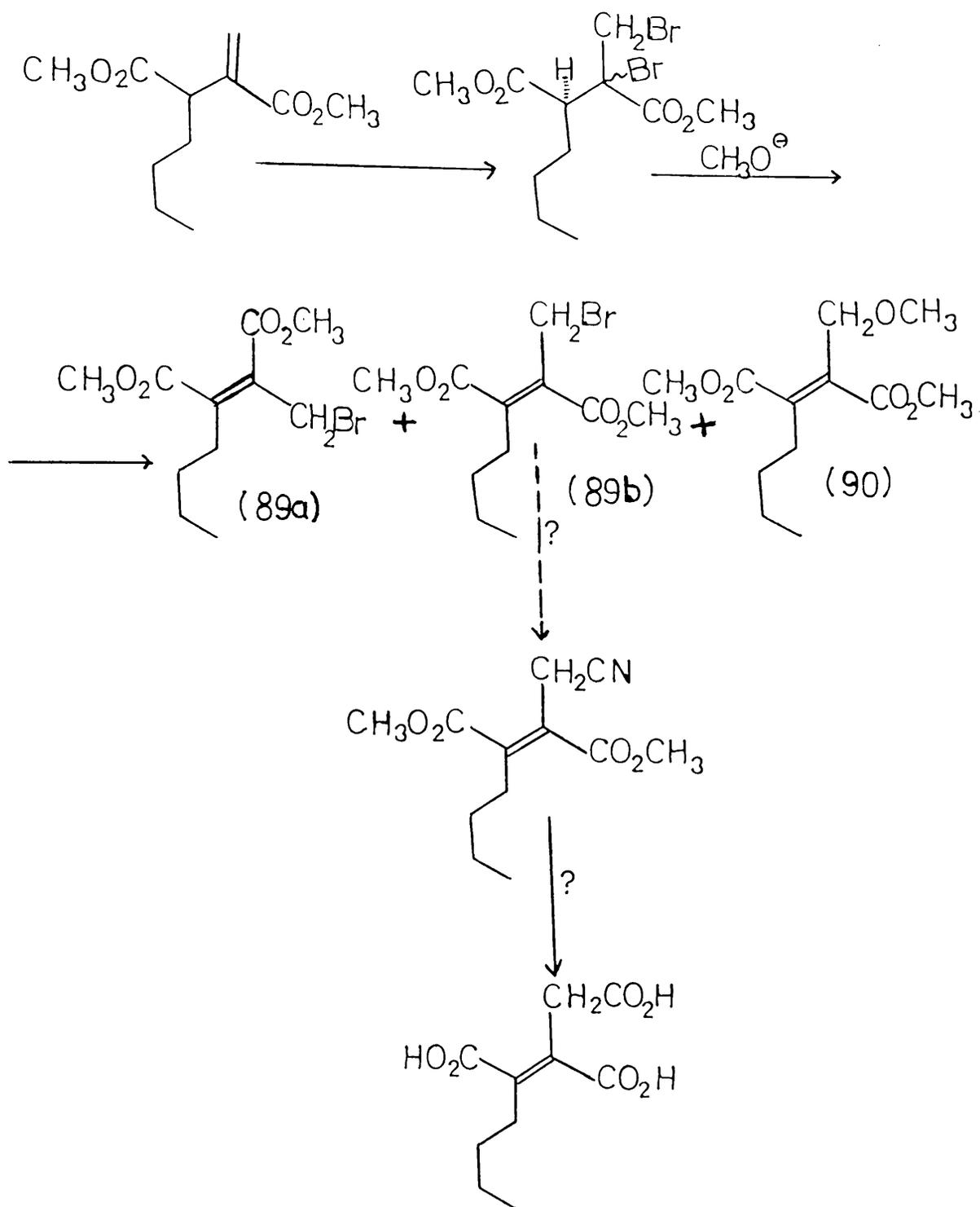
The mixture was characterised by elemental analysis. Each component gave the same IR (absence of double bond absorption) and mass spectrum but differed slightly in their ^1H n.m.r spectra. In these the absence of olefinic hydrogens was again evident and the bromomethylene groupings appeared as a singlet at 4.02 ppm and an AB quartet (H_A 4.06, H_B 4.18 ppm, J_{AB} 12 Hz) respectively. The methine protons appeared at 3.25 and 3.26 ppm respectively, but in the latter case having the appearance of the X part of an ABX system (4 peaks). The ^{13}C n.m.r spectrum of the first of these compounds was also in accord with its structure, assignments being made with the aid of ORD. The CH_2Br and $=\text{CBr}$ carbons appeared at 35.29 and 61.99 ppm respectively and one of the ester carbonyl groups was upfield of the other by ca 4 ppm, probably due to the halogen substituents on the α carbon.

Only one attempt has been made so far to dehydrobrominate the isomeric mixture of dibromo compounds. Treatment with sodium methoxide in refluxing methanol gave a mixture of products from which three compounds of interest were isolated

by preparative tlc.

The two isomeric allylic bromo compounds (89a) and (89b) were characterised by mass spectroscopy. IR and mass spectra were identical while only slight differences were apparent in the ^1H n.m.r spectrum e.g., in the chemical shifts of the methoxyl groups. The allylic CH_2Br protons in both cases appeared at 4.23 ppm. The remaining compound was obtained in 50% yield and was deduced to be the methoxy compound (90) from mass spectroscopy and ^1H n.m.r spectroscopy. In addition to the usual resonances for esters and butyl groups the allylic CH_2OMe group appeared as an AB quartet centred at 4.22 ppm while the ethereal methoxyl group appeared as a singlet at 3.38ppm.

The formation of the last compound seems to show that the allylic bromo group in (89a) and (89b) are readily replaceable. This therefore offers a route to alkylaconitic acids as in Scheme 28. Also the ester of α -butyl- α -hydroxy-itaconic acid (45) could be accessible from the allylic bromo compound as shown.



Scheme 28.

GENERAL EXPERIMENTAL.

1. General Experimental.

The cultures studied in this thesis are *Penicillium decumbens* (3903 OOT), and *Penicillium canadense* (Commonwealth Mycological Institute No. 95493). 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 was grown on a culture medium (Czapek-Dox + 0.1% yeast extract) containing glucose (50 g), NaNO_3 (2 g), KCl (1 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g), K_2HPO_4 (0.5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (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), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g), NaCl (1 g), yeast extract (0.5 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (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, Maureen, Joyce and Mary of the mycology unit who prepared all of the cultures used in this work.

^{13}C nmr 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.

^1H nmr spectra were recorded on Perkin Elmer R3290MHz spectrometer. Unless otherwise stated nmr were recorded with CDCl_3 as solvent and TMS as internal standard.

^2H nmr spectra were recorded by Dr. D. Rycroft and Mr. J. Gall with a BRUKER W.P. 200SY spectrometer. Unless otherwise stated nmr were recorded with CHCl_3 as solvent and TMS as internal standard. Melting points which are uncorrected, were determined on a Kofler hot stage apparatus. Micro-analysis were performed by Mrs. W. Harkness and her staff. Infrared spectra were recorded on a Perkin Elmer 225 spectrometer by Mr. G. McCulloch and his staff. Mass spectra were recorded by Mr. A. Ritchie with an AEI-GEC MS 9 mass spectrometer. Kieselgel GF₂₅₄ (MERCK) or HF₂₅₄ (MERCK) was used for preparative t.l.c; Kieselgel (MERCK) was used for analytical t.l.c. Analytical and preparative t.l.c plates were viewed under an ultra-violet lamp (254 or 350 nm). Analytical plates were developed by iodine vapour.

Light petroleum refers to the fraction having b.p. 60° - 80° . All solutions, unless otherwise stated, were dried over anhydrous MgSO_4 or Na_2SO_4 . The solvents used for chromatography are expressed in a volume ratio, e.g. ethylacetate - light petroleum (2:1).

ABBREVIATIONS.

The following abbreviations and symbols have been used in this thesis:-

br	broad
d	doublet
m	multiplet
q	quartet
s	singlet
t	triplet
i.r.	infra-red
u.v	untra-violet
n.m.r.	nuclear magnetic resonance
t.l.c	thin layer chromatography
Hz	Hertz
hr.	hour
min.	minute
lit.	literature
m.p.	melting point
m/e	mass to charge ratio
med.	medium
str.	strong
w	weak

EXPERIMENTAL

PART ONE.

Feeding of d_3 -acetic acid to surface cultures of *P. decumbens*.

D_3 -Sodium acetate prepared from d_3 -acetic acid was administered to ten Roux bottles containing seven day old surface cultures of *P. decumbens* on two consecutive days. Ten days after inoculation, the fungus was harvested. The aqueous broth was decanted off and extracted at end pH with EtOAc (3 x 50 ml). The ethyl acetate was dried over $MgSO_4$ and evaporated to give d-enriched ethisolide (2), which crystallised from EtOH in colourless needles (1.28 g), m.p. 121 - 122.5° [lit.⁶⁹m.p. 122-123°].

1H n.m.r. ($CDCl_3$) δ 6.49 (d, J3Hz, 1H, H-9a), 5.87 (d, J2Hz, 1H, H-9b), 5.14 (d, J7.2Hz, 1H, H-2), 4.70 (m, 1H, H-4), 4.06 (m, 1H, H-3), 1.70 (m, 1H, H-5a), 1.52 (m, 1H, H-5b), 1.04 (t, J6Hz, 3H, H-6).

2H n.m.r. ($CHCl_3$) δ 5.63 (w), 5.90 (w), 4.67 (w), 1.06 (str).

The extracted aqueous phase was concentrated using a Buchi-evaporator equipped with an acetone/'DRIKOLD' cooling bath on receiver flask, to give a mixture of acids (24 mg). The residue (12 g) was treated with dry methanol (115 ml) and thionyl chloride(13.75 ml) and the mixture was stirred at 0° for a period of 2 hrs. After stirring overnight at room temperature, the solution was refluxed at 50° for 3hrs and

the methanol evaporated under reduced pressure to give an oil. This was partitioned between CHCl_3 (150 ml) and water (100 ml). The aqueous phase was further extracted with CHCl_3 (2 x 75 ml) and the combined extracts were dried over Na_2SO_4 , filtered and the filtrate evaporated to a brown oil (7 g). On cooling and seeding most of the trimethyl citrate crystallised. After overnight standing at 0° the paste was triturated with ether (8 x 5 ml). The crude trimethyl citrate was dissolved in benzene (30 ml) and filtered through a short column of silica gel HF₂₅₄ (3 x 1.5 cm). Elution with $\text{EtOAc}:\text{C}_6\text{H}_6$ (1:1) separated trimethyl citrate from yellow impurities. Recrystallisation from $i\text{Pr}_2\text{O}$ gave d-enriched trimethyl citrate (50) (2.3 g, 33%) as white crystals m.p. $76-77^\circ$ [lit.⁷⁰ m.p. $75-76^\circ$].

^1H n.m.r (CDCl_3) δ 4.1 (s, 1H, OH), 3.82 (s, 3H, OCH_3), 3.7 (s, 6H, OCH_3), 2.85 (d, $J_{\text{O,CH}_2}$ 3Hz, 4H, CH_2)

M.S. m/e 235 (M^+ , $\text{C}_9\text{H}_{13}\text{O}_7$, 0.37%), 234 (M^+ , $\text{C}_9\text{H}_{14}\text{O}_7$), 203 ($\text{M}^+ - \text{OMe}$, 0.32%), 175 ($\text{M}^+ - \text{CO}_2\text{Me}$, 35.44%), 176 ($\text{M}^+ - \text{CO}_2\text{Me}$, 2.89%), 161 ($\text{M}^+ - \text{C}_3\text{H}_6\text{O}_2$, 2.05%), 101 (161- HCO_2Me , 100%), 74 ($\text{CH}_3\text{CO}_2\text{Me}$, 7.47%).

The ether solution containing the minor metabolites was evaporated with a stream of nitrogen and the residue dissolved in benzene. Initial isolation of these metabolites was achieved by column chromatography on silica gel HF₂₅₄ (30 g).

Elution with EtOAc:C₆H₆ (1:9) gave the mixture of the minor metabolites almost free from trimethyl citrate and impurities, which were retained on the column. The fractions were assayed by t.l.c using acetone:light petrol (1:4) and the appropriate fractions were combined and rechromatographed on neutral alumina. Elution with C₆H₆ gave an oil (222 mg), which was shown by t.l.c in acetone:light petrol (1:1) to be a mixture of four compounds. Preparative t.l.c in this solvent gave (i) further crystalline trimethyl citrate m.p. 76-77°, R_F 0.0 (22.5 mg). (ii) an unidentified compound R_F 0.25 (50 mg) [¹H n.m.r δ3.8 (s) 3.75 (s), 3.27 (s), 2.2 (m)]. (iii) deuterium enriched methyl ethisate (51) R_F 0.45 as a colourless oil (55 mg).

¹H n.m.r (CDCl₃) δ6.15 (d, J3Hz, H-9), 5.52 (d, J3Hz, H-9)
 4.93 (d, J8Hz, H-2), 3.77 (s, 3H, CO₂CH₃)
 3.20 (m, 1H, H-3), 1.1-1.8(m, 4H, CH₂),
 0.95 (t, J6Hz, 3H, -CCH₃)

²H n.m.r (CHCl₃) δ0.93 (str), ca. 1.3 (w).

identified by comparison with the spectra of an authentic sample and (iv) d-enriched methyl decumbate (52) R_F 0.55 (20 mg)

¹H n.m.r (CDCl₃) δ5.15 (m(br), 1H, H-3), 3.88(s, 3H, OCH₃)
 2.2 (d, J3Hz, 3H, C=CH₃), 1.40(m, 4H, C(CH₂)₂)
 0.95 (t, J7Hz, 3H, CCH₃)

²H n.m.r (CHCl₃) δ0.92 (str), ca. 1.3 (w)

Isolation of esters of α -n-butylitaconic acid (53) and decumbic acid (52) from unfed surface cultures of *P. decumbens*.

Twenty Roux bottles containing 9 day old surface cultures of *P. decumbens*, were harvested by decanting off the aqueous broth, which was extracted at end pH with CHCl_3 for 48 hrs. The chloroform solution was dried over MgSO_4 , filtered and the filtrate evaporated to give ethisolid (2) (2.64 g), in colourless needles m.p. $122-123^\circ$ from ethanol, identical (mp and ^1H n.m.r) to an authentic sample.

The extracted aqueous phase was concentrated using a Buchi evaporator equipped with an acetone/'DRIKOLD' cooling bath on receiver flask, to give a mixture of acids. This was treated with NaHCO_3 (50 g) in distilled water (30 ml) and Me_2SO_4 (40 ml), and left stirring at 60° for 3 days. The resulting solution was then treated with $\text{NaOAc} \cdot 3\text{H}_2\text{O}$ (15 g) to destroy any unreacted Me_2SO_4 . After overnight stirring at room temperature, the reaction mixture was extracted with EtOAc (2 x 75 ml), dried over MgSO_4 , filtered and the filtrate evaporated to give an oil (300 mg). This was shown by t.l.c in acetone:light petrol (1:4) to be a mixture of two compounds. Preparative t.l.c on silica gel (HF_{254}) gave dimethyl α -n-butylitaconate (53) R_F 0.6 as an oil. This was identical (R_F & ^1H n.m.r.) with a sample prepared by esterification of (47). Also obtained was methyl decumbate (52), R_F 0.8 as a colourless oil. ^1H n.m.r (CDCl_3) identical with that of the ester obtained earlier.

Feeding of d_4 -succinic acid to surface cultures of
P. decumbens.

d_4 -Succinic acid (600 mg) converted to its sodium salt in sterile water, was administered to 10 Roux bottles containing 7 day old surface cultures of *P. decumbens* on 2 consecutive days. Ten days after inoculation the cultures were harvested by decanting off the aqueous broth, which was extracted at end pH with EtOAc for 48 hrs. The ethyl acetate was dried over $MgSO_4$ and evaporated to give ethyl succinate (2) (1.89 g) m.p. 121-122° from ethanol [lit.⁶⁹ m.p. 122-123°].

1H n.m.r ($CDCl_3$) as described for previously obtained samples.

2H n.m.r ($CHCl_3$) δ 6.54 (str), 5.90 (str), 1.04 (med).

The extracted aqueous phase was concentrated using a Buchi evaporator equipped with an acetone/'DRIKOLD' cooling bath on receiver flask to give the crude acids (7.3 g). This was treated with a mixture of Me_2SO_4 (45 ml) and $NaHCO_3$ (30 g) in distilled water (15 ml) and left stirring at 60° for 3 days. The solution was extracted with EtOAc (5 x 100 ml), dried over $MgSO_4$, filtered and the filtrate evaporated between water and chloroform (1:4), and the combined extracts were dried over $MgSO_4$ and evaporated to a brown oil (5.72 g). This was shown by t.l.c. in ethyl acetate:light petrol (1:2) to be a mixture of at least five

compounds. Preparative t.l.c in the same solvent gave (i) fraction 1, R_F 0.2 consisting mainly of diethyl tartrate (58) [^1H n.m.r. peaks at δ 4.58 (s, 2H, CHOH); 4.35 (q, J 5.78Hz, OCH_2 -) 3.5 (m(br), 2H, OH), 1.35 (t, J 5.78, 3H, OCH_2CH_3)] together with traces of olefinic compounds (terminal methylene peaks as doublets ca $J = 1.5\text{Hz}$, at δ 5.78 and 6.37 and vinyl methyl as d $J = 2\text{Hz}$ at δ 2.14).

^2H n.m.r: peaks at 1.35 and 4.32 (corresponding nat. abund. ^2H in ethyl tartrate).

δ 3.2 (traces of d_4 -succinic acid) and as strong as these peaks at 5.83 and 6.45.

(ii) Fraction 2, R_F 0.35 (43 mg) containing a mixture of (58) (^1H n.m.r as described above) and the monomethyl ester of itaconic acid [^1H n.m.r δ 6.34 (s, 1H, $\text{C}=\text{CH}$), 5.75 (s, 1H, $\text{C}=\text{CH}$), 3.8 (s, 3H, OCH_3), 3.42 (s, 2H $\text{C}=\text{CH}_2$)] with the ratio of 5:2 respectively.

(iii) Fraction 3, R_F 0.5 consisting of deuterium enriched methyl ethisate (51) (75 mg) as colourless oil.

^1H n.m.r (CDCl_3) δ 6.15 (d, J 3Hz, H- α), 5.52 (d, J 3Hz, H- α),
4.93 (d, J 8Hz, H-2), 3.77 (s, 3H, OCH_3),
3.20 (m, 1H, H-3), 1.1-1.8 (m, 4H, CCH_2),
0.95 (t, J 6Hz, 3H, CCH_3).

^2H n.m.r. (CHCl_3) δ 6.34 (str), 5.63 (str), 0.95 (w).

(iv) Fraction 4, R_F 0.55 consisting of d-enriched methyl decumbate (51) (55 mg). [Taking into account that only a portion of the mixture was chromatographed].

^1H n.m.r (CDCl₃) δ 5.15 (br m, 1H, H-3), 3.88 (s, 3H, OCH₃),
2.2 (d, J2Hz, 3H, C=CCH₃), 1.40 (m, 4H,
C(CH₃)₂), 0.95 (t, J7Hz, 3H, -CCH₃).

^2H n.m.r (CHCl₃) δ 2.58 (str), 2.2 (med).

Treatment of citric acid and itaconic acid with dimethyl sulphate.

To a mixture of citric acid (1g, 4.75mmol) itaconic acid (1g, 7.69mmol) and sodium bicarbonate (5.2 g, 0.06 mol) in distilled water (8 ml), was dropped in during 30 min. while stirring at room temperature, dimethyl sulphate (3.92 g, 0.03 mol) and the mixture was stirred at 60°C for four days. After cooling NaOAc.3H₂O (5 g) was added to destroy any unreacted Me₂SO₄ and stirring was continued for a period of 24 hrs. The reaction mixture was then extracted with EtOAc (3 x 50 ml) and the extract dried over MgSO₄, filtered and the filtrate evaporated to give dimethyl itaconate (54) (0.45 g, 38%).

^1H n.m.r (CDCl₃) δ 6.36 (s, 1H, C=CH), 5.69 (s, 1H, C=CH),
3.8 (s, 3H, OCH₃) 3.72 (s, 3H, OCH₃)
3.38 (s, 2H, CH₂)

This is identical with lit.⁷¹ data.

The aqueous phase was chilled and acidified with conc. HCl at 0°C, and then extracted with EtOAc (3 x 75 ml) dried with MgSO₄, filtered and the filtrate evaporated to give an oil (2.48 g). This was treated with CHCl₃ (10 ml) to give a crystalline ester (0.3 g, 28%) as white crystals m.p. 138-140°.

¹H n.m.r (CD₃OD) δ3.71 (s, 3H, OCH₃), 2.8 (d, J=4.09Hz, 4H, CH₂)

(This was thought to be a monomethyl ester of citric acid).

The chloroform solution was evaporated under reduced pressure to give a mixture of the monomethyl esters of itaconic acid (56) and (57) (0.4 g, 36%) as colourless oil.

¹H n.m.r (CDCl₃) Compound (56) characterised by 1H singlets at δ6.35 and 5.77, 3H singlets at δ3.8 and by 2H singlets at δ3.42
Compound (57) characterised by 1H singlets at δ6.48 and 5.86, 3H singlet at δ3.74 and by 2H singlet at δ3.38.

When the above reaction was repeated in the absence of citric acid, the yield of the dimethyl itaconate (54) obtained was (0.6 g, 49%).

Feeding d_2 -fumaric acid to surface cultures of *P. decumbens*

d_4 -Fumaric acid (600 mg) converted to its sodium salt in sterile water, was administered to 10 Roux bottles containing 7 day old surface cultures of *P. decumbens*, on 2 consecutive days. Ten days after inoculation the cultures were harvested by decanting off the aqueous broth which was extracted at end pH with EtOAc for 48 hrs. The ethyl acetate was dried over $MgSO_4$ and evaporated to give deuterium enriched ethylidide (2) (740 mg) as colourless needles m.p. 122-123° from ethanol [lit.⁶⁹m.p. 122-123°].

1H n.m.r ($CDCl_3$) as described for previously obtained samples.

2H n.m.r. ($CHCl_3$) δ 6.54 (str), 5.90 (str), 1.06 (med).

Feeding of d_4 -succinic acid to surface cultures of *P. canadense*

d_4 -Succinic acid (300 mg), prepared as the sodium salt was administered to 6 Roux bottles containing surface cultures of *P. canadense* in three pulses on days 7, 8 and 9 after inoculation. The cultures were then harvested on day 13 by decanting off the aqueous broth, which was then acidified to around pH2 and continually extracted with ethyl acetate for 48 hrs. The ethyl acetate was then dried over $MgSO_4$ and evaporated.

Two, one metre HF₂₅₄ Merck silica plates (0.75 mm thick) were loaded with the extract and eluted 4 times with 2:1 chloroform:light petroleum. The relevant bands were removed and extracted three times with cold chloroform. The band of R_F 0.29 gave deuterium enriched canadensic acid (5) m.p. 111-113^o (lit.¹ m.p. 113-114^o) after crystallisation from ether-hexane.

¹H n.m.r. (CDCl₃) δ6.56 (s, 1H, H-11), 5.97 (s, 1H, H-11(trans),
4.41 (m, 1H, H-4), 3.66 (dd, J8.7, 11.94Hz,
1H, H-2) 2.55 (m, 1H, H-3(α)), 1.99 (dt,
J10.32, 12.28Hz, 1H, H3(β)), 1.72 (m, 2H,
H-5), 1.37 (m, 4H, H-6, H-7) 0.90 (t,
J6.74Hz, 3H, H-8).

²H n.m.r (CHCl₃) δ6.58 (str), 0.952 (w),
No peak observed at δ ca. 5.97

The less polar products (probably canadensolide (3) and dihydrocanadensolide (4)) could not be obtained sufficiently pure to be characterised spectroscopically.

EXPERIMENTAL

PART TWO.

Preparation of Meldrum's Acid⁵³(61) .

To a suspension of powdered malonic acid (52 g, 0.5 mol) in acetic anhydride (60 ml, 0.6 mol) was added while stirring concentrated sulphuric acid (1.5 ml). Most of the malonic acid dissolved with spontaneous cooling to 20-25°. After allowing the reaction mixture to stand overnight at 0°, the resulting crystals were separated by filtration and washed with ice-water (3 x 50 ml), giving 2,2-dimethyl-4,6-dioxan-1,3-dione (61) (40 g, 56%) as white needles m.p. 94-95° from cold acetone [lit.⁵³m.p. 94-95°]

¹H n.m.r (CDCl₃) δ3.63 [s, 2H, CH₂(CO₂R)₂], 1.8 (s, 6H, CMe₂).

I.R. ν_{max} (KBr) 3000, 2930, 1790 1750 cm⁻¹.

¹³C n.m.r (CDCl₃) δ163.19 (s, CO), 106.34 (s), 36.20 (t), 27.54 (q).

M.S. m/e 144(M⁺), 129(M⁺-CH₃), 101(M⁺-CH₃-CO), 100(M⁺-CO₂), 72(M⁺-44-CO), 58(O=C=CHCO₂H).

Preparation of isopropylidene isopropylidenemalonate^{54,55} (64)

Meldrum's acid (61) (4 g, 0.027 mol) and acetone (2.04 ml, 0.028 mol) were stirred with pyridine (5.09 ml, 0.062 mol) and molecular sieve 4A (6 g) at room temperature for 2 days. Pyridine was removed under vacuum and the residue was extracted with ethyl acetate (3 x 30 ml). The molecular sieve was then filtered and the filtrate washed with brine (3 x 30 ml), dried with anhydrous Na₂SO₄ and evaporated. Crystallisation of the residue from aqueous methanol gave the acetyl compound (64) (3g, 59%) as colourless needles m.p. 74-75° [lit.⁵⁴ m.p. 74.5 - 76°].

¹H n.m.r (CDCl₃) 90MHz δ2.52 (s, 6H, =CMe₂) 1.72 (s, 6H, -CMe₂).

I.R. ν_{max} (KBr) 2950, 1732, 1650 (br), 1510, 1570 cm⁻¹

M.S. m/e 184(M⁺), 164(M⁺, 21.4%), 149(M⁺-CH₃, 11.2%)
136(M⁺-CO, 3.5%), 126(M⁺-(CH₃)₂CO, 0.6%).
121(M⁺-CH₃-CO, 16.1%), 108(M⁺-CO-CO, 48.6%),
93(M⁺-2CO-CH₃, 17.9%), 82(O=C=C=CMe₂, 2.66%).

Reaction of Meldrum's acid (61) with ethyl pyruvate using Pyridine.

A mixture of Meldrum's acid (5 g, 0.035 mol) and ethyl pyruvate (3.79 ml, 0.034 mol) in pyridine (7.142 ml 0.088 mol) was stirred in the presence of molecular sieve 4A (7.5 g) at room temperature for 5 days. Pyridine was evaporated under vacuum, and the residue (0.8 g) diluted with ethyl acetate (60 ml), molecular sieve 4A was filtered off, and the ethyl acetate solution washed with brine (3 x 30 ml). The organic phase was dried with sodium sulphate, and the solvent evaporated under reduced pressure to give an oily product, which was shown by t.l.c, ^1H n.m.r and I.R. spectra to be a complex mixture. Preparative t.l.c failed to separate the components and the reaction was not investigated further.

Dry alumina catalysed condensation of Meldrum's acid⁵⁸ (61) with ethyl pyruvate.

Meldrum's acid (61) (4.32 g, 0.03 mol) and ethyl pyruvate (3.48g, 0.029 mol) were carefully melted and absorbed on neutral alumina (12 g, 0.12 mol). The mixture was shaken for 5 hrs at room temperature, and a small sample was eluted with CH_2Cl_2 , the solution filtered and the filtrate evaporated to give an oil, which was mostly unreacted starting materials. The reaction mixture was therefore shaken for further six days, extracted with CH_2Cl_2

(3 x 30 ml) and alumina filtered off, CH_2Cl_2 evaporated to give an oil (7 g). This was treated with dry ether (25 ml) affording the 2:1 condensation product (67) (1.5 g, 13%) m.p. 140-141 $^\circ$. (Found C, 53.01; H, 5.82 $\text{C}_{17}\text{H}_{22}\text{O}_{10}$ requires C, 52.85; H, 5.67%).

^1H n.m.r. (CDCl_3) δ 4.49 (m, 1H, COCH), 4.20 (q, J9Hz; 2H, OCH_2CH_3), 2.05 (s, 3H, -CCMe), 1.80 (s, 12H, C-Me $_2$), 1.25 (t, J9Hz, 3H, OCH_2CH_3).

M.S. m/e 386 (M^+), 242 ($\text{M}^+ - \text{C}_6\text{H}_8\text{O}_4$), 227 (242- CH_3 , 1.05%), 198 (242- CO_2 , 0.45%), 197 (242-OEt, 2.47%), 185 (242-42- CH_3 , 5.42%), 157 (185-CO, 8.74%), 144 ($\text{M}^+ - 242$), 139 (157- H_2O , 11.8%), 101, 100 (5.42%).

I.R. ν_{max} (KBr) 3000, 2850, 1785, 1747, 1727 cm^{-1} .

^{13}C n.m.r. (CDCl_3) δ 171.51 (s), 164.67 (s), 163.43 (s), 105.30 (s), 62.36 (t), ca. 51.0 (br), 49.61 (s), 28.27 (q), 27.28 (q), 25.4 (q), 13.67 (q).

The ether solution was allowed to stand overnight at 0 $^\circ$ to give the dimer (68) of the alkylidene compound (62) as white solid (2 g, 14%) m.p. 117 $^\circ$ -118 $^\circ$ from ethanol. (Found C, 54.65; H, 6.02 $\text{C}_{22}\text{H}_{28}\text{O}_{12}$ requires C, 54.56; H, 5.78%).

^1H n.m.r (CDCl₃) δ 4.79 (s, 1H, -COCHCO-), 4.28 (q, J=7.18Hz, 2H, -OCH₂CH₃), 4.35 and 4.00 (dq, J = 7.20, 10.63Hz, 2H, OCH_AH_BCH₃), 4.18 and 3.60 (ABq, J = 12.63Hz, -C-CH₂C=), 1.90 and 1.89 (two s, each 3H, -CCH₃), 1.72 (s, 6H, -C(CH₃)₂), 1.49 (s, 3H, -C-CH₃), 1.36 and 1.33 (two t, J = 7.17Hz, each 3H, -OCH₂CH₃).

^1H n.m.r (d₅-pyridine) δ 4.3 (q, (br)), 3.68 (s), 3.35 (q, J9Hz), 1.78 (s), 1.68 (s), 1.2 (m, (br)).

I.R. ν_{max} ^{CCl₄} 3000, 1787 (br), 1735, 1720, 1620 cm⁻¹

^{13}C n.m.r (CDCl₃) 173.17 (s), 169.02 (s), 163.96 (s), 163.47 (s), 160.72 (s), 159.91 (s), 119.67 (s) 113 (s), 105.56 (s), 165.37 (s), 62.99 (t), 62.34 (t), 57.06 (d), 48.22 (s), 36.53 (t), 28.63 (q), 28.05 (q), 27.47 (q), 25.33 (q), 18.91 (q), 13.69 (q), 13.64 (q).

M.S. m/e 484 (M⁺), 324 (M⁺-2CH₃COCH₃-CO₂, 0.59%), 280 (324-CO₂), 252 (280 - CO, 1.63%), 251 (M⁺-CO₂Et), 237 (252-CH₃, 0.35%), 236 (251 - CH₃, 0.25%), 224 (252-CO, 0.49%), 209 (224 -CH₃, 1.53%), 179 (252-CO₂Et, 6.62%), 151 (M - CO₂Et, 1.78%), 135 (208 -CO₂Et, 18.4%), 129 (144 -CH₃, 0.35%), 105 (5.04%).

Preparation of the sodium salt of Meldrum's acid (61)

A mixture of Meldrum's acid (3.50 g, 0.024 mol), sodium hydrogen carbonate (2.04 g, 0.024 mol) and distilled water (10 ml) was stirred at room temperature for 45 minutes. The solution was then treated with acetone (2 x 10 ml), filtered and the filtrate evaporated to give the salt of Meldrum's acid (61) (4 g, 99%).

^1H n.m.r ($\text{d}_4\text{-MeOH}$) 90MHz, δ 3.04 (s, 1H, $\text{CH}(\text{CO})_2$), 1.73 (s, 6H - CMe_2)

Reaction of the Na salt of Meldrum's acid (61) with ethyl pyruvate in methanol.

The Na salt of Meldrum's acid (61) (3.72 g, 0.022 mol) was added dropwise in absolute methanol (50 ml) to distilled ethyl pyruvate (4ml, 0.036 mol) in absolute methanol (20 ml). Then ethyl pyruvate (4 ml, 0.036 mol) was added and the mixture was stirred at room temperature for 10 min. After removing the methanol in vacuo, the residue was made into a paste (slurry) in CH_2Cl_2 (30 ml) and acidified with 1N HCl. The CH_2Cl_2 solution was washed with brine (3 x 20 ml). The organic phase dried over MgSO_4 , filtered and evaporated to give a mixture (3.5 g) consisting (from ^1H n.m.r) of unreacted starting material and the dimer product (68) as a major component.

Reaction of Meldrum's acid (61) with ethyl pyruvate
using fl O rosil.

A mixture of Meldrum's acid (1.44 g, 0.01 mol), ethyl pyruvate (1.16 g, 0.01 mol) and fl O rosil (4 g, 0.04 mol) was shaken at room temperature for 24 hrs. Extraction with CH_2Cl_2 (3 x 30 ml) and the evaporation of the solvent under reduced pressure gave an oil (2.45 g), which was shown by the proton ^1H n.m.r to be a mixture of unreacted Meldrum's acid δ 3.63 (s), 1.8 (s), ethyl pyruvate δ 4.28 (q, 9Hz, CH_2), 2.45 (s, 3H), 1.35 (t, 3H, J9Hz) and the dimer (68) of the alkylidene ester (62). Treatment with ether allowed isolation of the latter (0.8 g, 30%). m.pt. 116-117 $^\circ\text{C}$. Found C, 54.62; H, 5.79 $\text{C}_{22}\text{H}_{28}\text{O}_{12}$ requires C, 54.56 ; H, 5.78%).

^1H n.m.r (CDCl_3) 90MHz δ 4.80 (s, 1H), 4.15 (q, 4H, CH_2),
 3.68 (s, 1H), 3.52 (s, 1H), 1.90 (s, 3H
 CH_3), 1.88 (s, 3H, CH_3), 1.71 (s, 6H, CH_3)
 1.49 (s, 3H, CH_3), 1.35 (t, 6H, CH_3)

I.R. ν_{max} (KBr) 3000, 1785, 1750 (br), 1620 (C=C) cm^{-1}

Treatment of the 2:1 condensation product (67) with ethyl pyruvate in the presence of alumina.

A mixture of 2:1 condensation product (67) (0.5 g, 1.3mmol), ethyl pyruvate (0.15 g, 1.29mmol) and alumina (0.5 g, 4.9mmol) was shaken at room temperature for 24 hrs. The reaction mixture was eluted with CH_2Cl_2 (3 x 30 ml), the solution filtered and the filtrate evaporated to give an oily product (0.6 g), which was shown by ^1H n.m.r spectrum to be unreacted starting materials.

Preparation of the alkylidene compound (62).

Meldrum's acid (4.3 g, 0.02 mol) and ethyl pyruvate (3.45 g, 0.03 mol) were carefully melted and absorbed on silicic acid (20 g, 0.12 mol) and the mixture was shaken at room temperature for seven days. The reaction mixture was eluted with CH_2Cl_2 (3 x 30 ml), the solution filtered and the filtrate evaporated to give an oil (5.2 g). This was treated with ether (2 x 25 ml) to give a precipitate of the 2:1 condensation product (0.82 g, 14%) m.p. 140-141 $^\circ$ which was filtered off. The ether solution was left at 0 $^\circ$ overnight to give the dimer (68) (1.08 g, 14%) m.p. 117-118 $^\circ$ as white crystals. The ether was then removed by evaporation under reduced pressure and the oil (3.45 g) produced was chromatographed rapidly on a silica gel column eluting with light petrol:dichloromethane using the ratios 9:1, 4:1 and 1:1. The fractions eluted by the (4:1)

mixture were combined and the solvents evaporated to give a yellow oil. Distillation in Kugelrohr at 120°/0.7 mm gave the alkylidene compound (62) as a colourless oil (2.5 g, 32%), Found C, 53.93; H, 5.78 $C_{11}H_{14}O_6$ requires C, 54.54; H, 5.78% .

1H n.m.r. ($CDCl_3$) δ 4.40 (q, J=8.25Hz, 2H, $-OCH_2$), 2.60 (s, 3H, $=C-CH_3$) 1.75 (s, 6H, $-CMe_2$), 1.35 (t, J=8.25Hz 3H, $-OCH_2CH_3$)

I.R. ν_{max} CCl_4 2990, 1770, 1746 (br), 1720, 1625 (C=C) cm^{-1}

M.S. m/e 242 (M^+), 227 (M^+-CH_3 , 2.3%), 213 (M^+-Et , 0.1%), 197 (M^+-OEt , 5.6%), 185 (M^+-CH_3-42 , 14.5%), 184 (M^+-CH_3)₂ 0.2%), 169 (M^+-73 , 0.9%), 156 (184-CO, 29.5%), 140 ($M^+-58-44$, 12.1%), 139 (157- H_2O , 30.8%), 138 (184-EtOH, 26.3%), 112 (156-CO₂, 15.7%), 111 (139-CO, 2.9%) 67(m/e 139-CO-CO₂, 100%).

^{13}C n.m.r ($CDCl_3$) 167.9 (s), 159.9 (s), 128.6 (s), 116.1 (s), 105.1 (s), 62.6 (t), 27.5 (q), 19.9 (q), 13.74 (q).

In the above reaction the crude product initially eluted from the silicic acid showed peaks in the n.m.r corresponding to three products isolated as described above. In addition three 1H singlets appeared, namely at δ 6.5, 5.91 and 4.65.

The Reaction of Meldrum's Acid (61) and ethyl pyruvate using $ZnCl_2$.

A mixture of anhydrous zinc chloride (7.5 g, 0.055 mol) and acetic anhydride (5.62 g, 0.055 mol) was stirred at room temperature overnight, then allowed to stand and the resulting clear solution decanted off. To this was then added Meldrum's acid (3.96 g, 0.0275 mol) and ethyl pyruvate (3.2 g, 0.0276 mol) and the mixture warmed on a boiling water bath for 5 minutes. After standing at room temperature the mixture was treated with benzene (20 ml) which was removed on the rotary evaporator. The residue was then washed with chloroform, filtered and the filtrate evaporated to give an oil (3 g). The resulting product was distilled in Kugelrohr giving ethyl itaconate (71) (1.5 g, 35%), b.p. $109-111^{\circ}C/0.6$ mm.

1H n.m.r. ($CDCl_3$) δ 6.3 (s, 1H, C=CH), 5.7 (s, 1H, C=CH), 4.2 (q, 2H, $-OCH_2CH_3$), 3.31 (s, 1H, CH-CO₂H), 1.3 (t, 3H, $-OCH_2CH_3$)

Attempted Condensation of the Meldrum's acid (61) with diethyl 2-methyloxaloacetate.

Meldrum's acid (5 g, 0.034 mol) and diethyl 2-methyloxaloacetate (7.021 g, 0.035 mol) were carefully melted and absorbed on silicic acid (8.34 g) and the mixture was shaken at room temperature for 3 days. The reaction

mixture was eluted with CHCl_3 (2 x 30 ml), the solution was filtered and the filtrate evaporated to give an oil (12.3 g) which was chromatographed rapidly on a column of silica gel HF₂₅₄ eluting with CHCl_3 to separate the product from orange impurities. This gave a yellow gum which was shown by t.l.c to be essentially unreacted starting materials.

(The ^1H n.m.r. of diethyl 2-methyloxaloacetate was characterised by 2H and 1H quartets at δ 4.25, 3H singlet at δ 1.88 and 3H triplets at δ 1.35).

Reaction of Meldrum's acid (61) with ethyl pyruvate using benzene thiol and alumina.

Meldrum's acid (61) (6 g, 0.042 mol) and ethyl pyruvate (4.83 g, 0.04 mol) were shaken with benzene thiol (4.58 g, 0.0416 mol) and alumina (17 g, 0.16 mol) in a sealed flask at room temperature for seven days. The reaction mixture was eluted with CH_2Cl_2 (3 x 30 ml) and alumina removed by filtration. The CH_2Cl_2 evaporated under reduced pressure to give an oil (10 g), which was mostly a crystalline product after standing overnight at 0°C . The mixture was treated with dry ether (25 ml) to give a white solid (4.75 g), which was chromatographed rapidly on a column of silica gel HF₂₅₄ eluting with ether to remove diphenyl disulphide and starting materials and then with CH_2Cl_2 (3 x 30 ml) to recover the product. The enolsulphide diketodioxan (70) was obtained as white crystals from

CH_2Cl_2 (4.5 g, 31%) m.p. 134-135°C (Found C, 58.17; H, 5.50

$\text{C}_{17}\text{H}_{18}\text{O}_6$ requires C, 58.28 ; H, 5.68%)

^1H n.m.r. (CDCl_3) δ 7.35 (m, 5H, aromatic H), 7.36 (s, 1H, CH=C), 4.25 (q, J8.66 Hz $-\text{OCH}_2\text{CH}_3$), 4.15 (s, 1H, exchangeable with D_2O $-(\text{CO})_2\text{CH}-$) 1.77 (s, 6H, $-\text{CMe}_2$), 1.3 (t, J8.66Hz, $-\text{OCH}_2\text{CH}_3$).

^{13}C n.m.r (CDCl_3) δ 164.51 (s, CO), 164.04 (s, CO), 153.17 (d), 135.70 (s), 131.70 (d), 129.50 (d), 128.70 (d), 116.70 (s), 105.10 (s), 61.66 (t), 51.45 (d), 28.1 (q), 27.5 (q), 13.96 (q).

I.R. ν_{max} (KBr) 3000, 2950, 1795 (br), 1740, 1700 1580 cm^{-1}

M.S. m/e 350 (M^+), 292 ($\text{M}^+ - (\text{CH}_3)_2\text{CO}$ 1%), 248 ($\text{M}^+ - (\text{CH}_3)_2\text{CO} - \text{CO}_2$, 36%), 220 (248-CO, 22%), 219 (292- CO_2Et , 6%), 202 (220- H_2O , 8%), 192 (220-CO, 08%), 191 (219-CO, 28%), 175 (220-OEt, 7%), 174 (202-CO, 16%), 147 (191- CO_2 or 220- CO_2Et , 100%), 146 (174-CO, 20%), 110 (PhSH, 13%), 109 (PhS $^+$, 16%), 77 (Ph $^+$, 30%).

The ether washings obtained above in the preparation

afforded an oil (4.8 g) from which crystals gradually separated. The ^1H n.m.r showed peaks corresponding to Meldrum's acid, ethyl pyruvate e.g. δ 4.28(q, J = 9Hz, OCH_2), 2.45 (s, CH_3), 1.35(t, J=9Hz, OCH_2CH_3). diphenyl disulphide e.g. δ 7.5, 7.25 (2m, 10H) and the alkylidene compound (62) e.g. δ 2.60 (s, CH_3) in the proportion 1:1:3:1.

Attempted preparation of (70).

(i) Using diphenyl disulphide and alumina.

Meldrum's acid (2g, 0.014 mol) ethyl pyruvate (1.612 g, 0.0138 mol) and diphenyl disulphide (3.032 g, 0.0139 mol) were carefully melted and absorbed on neutral alumina (5.66 g, 0.06 mol) and the mixture was shaken at room temperature for 3 days. This was eluted with CH_2Cl_2 (3 x 30 ml) filtered and the filtrate evaporated to give an oil (5.6 g), which was shown by t.l.c. in chloroform to be a mixture of three compounds. This was separated by preparative t.l.c on silica gel (HF_{254}) in the same solvent to give the alkylidene compound (62) R_F 0.3 (0.35 g, 10%); ethyl pyruvate R_F 0.7 and diphenyl disulphide R_F 0.8-0.9 m.p. 59 - 60 $^\circ$.

(ii) Using the thio ester (80) and silicic acid.

Meldrum's acid (61) (0.06 g, 0.4mmol) and the thio ester (81) (0.1 g, 0.45 mmol) were carefully melted and

absorbed on silicic acid (0.8 g,) and the mixture was shaken at room temperature for 8 days. The reaction mixture was eluted with CHCl_3 (3 x 10 ml), the solution filtered and the filtrate evaporated to give a gummy oil, which was shown by ^1H n.m.r to be a mixture of unreacted starting materials.

The Reaction of 2-Carboethoxy-1,1-dicarbomethoxy propene (73) with benzene thiol and alumina.

Benzene thiol (0.53 g, 4.8 mmol) and alumina (3 g, 0.03 mol) were shaken at room temperature for 24 hrs. After which time the ester (73) (1.1 g, 4.78 mmol) and alumina (2 g, 0.02 mol) were added and the mixture was shaken for a further four days, The reaction mixture was extracted with CH_2Cl_2 (3 x 30 ml), the solution filtered and the filtrate evaporated to give an oil (0.9 g) which was shown by t.l.c in CHCl_3 to be a mixture of two compounds. This was separated by preparative t.l.c in the same solvent to give the unreacted ester (73), R_F 0.4 and diphenyl disulphide R_F 0.65 m.p. 59-60 $^\circ$. No product was detected by ^1H n.m.r of the crude product.

Attempted methylation of the enolsulphide diketo dioxan (70).

To a suspension of silver oxide (0.33 g, 1.4 mmol) in a mixture of acetonitrile (0.5 ml, 0.09 mol) and methyl iodide (0.20 g, 1.2 mmol) was dropped in during one hour,

while stirring at 10° , a solution of the Meldrum's acid derivative (70) (0.5 g, 1.42 mmol) in acetonitrile (7 ml, 0.13 mol). After stirring four hours longer and allowing to stand overnight the silver-oxide - silver-iodide was filtered and washed with acetonitrile. The solvent was removed from the combined filtrates by distillation below 60° under reduced pressure to give an oil (301.4 mg). This was shown by t.l.c in light petrol:ethyl acetate 2:1 to be a mixture of several components. Extensive preparative t.l.c failed to separate them and the reaction was not investigated further.

Treatment of ethyl pyruvate with diphenyl disulphide and alumina.

A mixture of diphenyl disulphide (3g, 0.04 mol) ethyl pyruvate (1.5 g, 0.0136 mol) and alumina (5.61 g, 0.055 mol) was shaken at room temperature under nitrogen atmosphere for seven days. The reaction mixture was extracted with CH_2Cl_2 (2 x 25 ml), filtered and the filtrate evaporated under reduced pressure to give an oily product (7.5 g), which was shown by ^1H n.m.r and t.l.c in CHCl_3 to be a mixture of unreacted starting materials.

Preparation of ethyl 2-keto-3-(phenylthio)-propanoate.

Bromopyruvic acid (7 g, 0.04 mol) and sodium bicarbonate (3.52 g, 0.042 mol) in distilled water (75 ml), were

treated with benzene thiol (4.61 g, 0.042 mol) in 1N NaOH (100 ml) and stirred at 45°C for a period of 2hrs. After cooling at 0°C, the mixture was acidified with conc. HCl (8.33 ml) and then extracted with ether (3 x 10 ml). The solution was dried with anhydrous MgSO₄, filtered and the filtrate evaporated to give 2-keto-3-(phenylthio)propanoic acid (6.00g, 73%).

¹H n.m.r. (CDCl₃) δ7.3 (br, 5H, aromatic H), 4.07 (s, 2H, -SCH₂-), 3.44 (s, 1H, OH).

This was used immediately without further purification.

To the acid (2g, 0.01 mol) in dry ethanol (30 ml) while stirring, thionyl chloride (3.28 ml, 0.05 mol) was added dropwise at 0°C, for a period of 2 hrs. And after vigorous stirring at 20°C overnight, the reaction mixture was refluxed at 50°C for 3 hrs. The ethanol was evaporated under reduced pressure to give an oil (2.3 g), which was shown by t.l.c in chloroform-petroleum ether (1:1) to be a mixture of two compounds. Preparative t.l.c plates on silica gel HF₂₅₄ in the same solvent gave diphenyl disulphide (0.3 g) R_F 0.8 m.p. 59-60° [Lit.⁷²m.p. 59-60°] e.g. δ7.5 (m), 7.25 (m) and the ester (81) R_F 0.2, (1.45 g, 64%) b.p. 180°C/0.5 mm (Found C, 58.73; H, 5.12 C₁₁H₁₂O₃S requires C, 58.93; H, 5.35%).

¹H n.m.r (CDCl₃) δ7.30 (m, 5H, arom H), 4.00 (q, J = 7.8Hz, 2H, -OCH₂CH₃), 3.7 (s, 2H, -SCH₂-) 1.31(t, J = 7.8Hz, 3H, OCH₂CH₃).

^{13}C n.m.r. (CDCl_3) 123.1, 86.74 quaternary carbons 167.06, 166.89
144.9 135.2 at 63.11 and 40.29 CH_3 s at 13.6,
14.16 CH_2 s.

Signals in aryl region 132.1, 131.1, (130.1),
129.7, (129.3) (129.1) 128.7 (127.9).

M.S. m/e 224 (M^+), 179 (M^+-OEt), 151 ($\text{M}^+-\text{CO}_2\text{Et}$, 0.2%),
123 (M^+-PhSh_2 , 2.7%), 101 ($\text{M}^+-\text{COCO}_2\text{Et}$, 0.5%),
147 (M^+-Ph , 0.8%), 77 (Ph^+), 73 ($\text{M}^+-\text{PhSCH}_2\text{CO}$,
2.3%).

I.R. ν_{max} CCl_4 1720, 1700, 1600 cm^{-1} .

Attempted isomerisation of the alkylidene ester (62)

The ester (62) (0.3 g, 1.2 mmol) was treated with d_5 -pyridine (5 ml, 0.062 mol) and the mixture was allowed to stand at room temperature for 10 to 15 mins. with occasional shaking. The pyridine was then removed in vacuo to give the dimer (68), identified by comparison of the ^1H n.m.r spectrum of an authentic sample of the dimer (68) in d_5 -pyridine.

Attempted rearrangement and alkylation of the alkylidene ester (62).

(i) using potassium carbonate in acetone:

The ester (62) (0.69 g, 2.85 mmol) in acetone (20 ml) was treated with hexyliodide (0.62 g, 2.9 mmol). The solution was cooled in ice and anhydrous potassium carbonate

(0.4 g, 2.89 mmol) added and the mixture was then stirred at room temperature for 3 days. At the end of this time the mixture was filtered and the filter cake washed with diethyl ether (3 x 20 ml). The filtrate was concentrated and then taken up in further portion of ether (45 ml), which was subsequently washed with portions of brine (3 x 10 ml). The ether layer was then dried over sodium sulphate and concentrated to give an oil (247.5 mg), which was shown by t.l.c in chloroform:light petrol (1:1) to be a mixture of two compounds. Preparative t.l.c in the same solvent gave the mono ethyl itaconate (71) R_F 0.45 - 0.5 (0.2 g, 55%) and identical in its ^1H n.m.r spectrum to the sample obtained previously. There was also obtained a less polar component which was identified by comparison with literature spectra as hexanol.

(N.B. hexyl iodide ^1H n.m.r (CDCl_3) δ 3.38 (t, $\text{CH}_2\text{-I}$),
1.82 (m, CH_2). 1.31 (m, CH_2), 0.87 (m,
 CH_3).

hexanol ^1H n.m.r. (CDCl_3) δ 3.75 (m, CH_2OH), 1.14 (m,
8H, $-\text{C}(\text{CH}_2)_4-$), 0.92 (m, 3H, C-CH_3).

(ii) using amberlyst 15 in benzene

The ester (62) (0.4 g, 1.65 mmol) in redistilled benzene (10 ml) and activated amberlyst 15 (1 g) were stirred at 0°C under a stream of nitrogen for 30 mins. The reaction mixture was allowed to warm to room temperature and butyl

bromide (0.23 g, 1.39 mmol) was added dropwise. The solution was left stirring for four days, filtered and the filtrate evaporated under reduced pressure to give an oil (0.57 g). This was shown by ^1H n.m.r and I.R. spectra to be a mixture of the unreacted ester (62) and hexyl bromide.

(iii) when the above reaction mixture was refluxed at (40-50°C) the resulting product was ethyl itaconate (71) due to the decomposition of the ester (68).

(iv) the addition of alumina to the alkylidene ester (62) in benzene at room temperature led to the formation of the dimer (68) (^1H n.m.r. spectrum):

(v) using silver carbonate in benzene.

A mixture of the ester (62) (0.11 g, 0.45 mmol) in benzene (10 ml) silver carbonate (0.13 g, 0.47 mmol) and butyl bromide (0.055 g, 0.4 mmol) was stirred at room temperature for 24 hours. The reaction mixture was eluted with CHCl_3 (10 ml), the solution filtered and the filtrate evaporated under reduced pressure to give unreacted starting materials (^1H n.m.r spectrum). When the mixture was refluxed, or Ag_2O used instead of the silver carbonate the reaction led to a complex mixture. The reaction was not investigated further.

Reaction of Meldrum's Acid (61) with Ethyl Bromopyruvate
using Silicic Acid.

Meldrum's acid (1.44g, 0.01 moles) and ethyl bromopyruvate (1.95g, 0.01 moles) were carefully melted and absorbed on silicic acid (7g) and the mixture was shaken at room temperature for five days. The reaction mixture was eluted with CH_2Cl_2 (3 x 30ml), the solution filtered, and the filtrate evaporated to give an oil (3g). The resulting product was distilled in Kugelrohr to give the bromoalkylidene compound (80) (2.45g, 76%) as a colourless oil b.p. $120^\circ/\text{0.5mm}$. (Found C, 40.89; H, 4.21; Br 25.18 $\text{C}_{11}\text{H}_{13}\text{O}_6\text{Br}$ requires C, 41.12; H, 4.04; Br, 24.92%.)

^1H n.m.r (CDCl_3) δ 4.82 (s, 2H, $-\text{CH}_2\text{Br}$), 4.40 (q, $J=7\text{Hz}$; 2H, CH_2)
1.80 (s, 6H, CH_3), 1.35 (t, $J=7\text{Hz}$; 3H, CH_3)

IR $\nu_{\text{max}}^{\text{CCl}_4}$ 3050, 1768, 1752, 1720 cm^{-1}

M.S m/e 322 (M^+), 274, 265 ($\text{M}^+-\text{CO}-\text{Et}$, 0.1%), 264 ($\text{M}^+-\text{(CH}_3)_2\text{CO}$ -
0.2%), 227 ($\text{M}^+-\text{CH}_2\text{Br}$, 0.8%), 225, 220 ($\text{M}^+-\text{(CH}_3)_2\text{CO}-\text{CO}_2$ -
0.4%), 219 ($\text{M}^+-\text{(CH}_3)_2\text{CO}-\text{OEt}$, 0.4%), 218 ($320-\text{(CH}_3)_2\text{CO}-$
 CO_2 , 0.8%), 197 (3.7%), 192 (220^+-CO , 0.6%), 184 (M^+-
 $\text{HBr}-2\text{CO}$, 11.7%), 156 ($184^+-\text{C}_2\text{H}_4$, 12.2%), 139 (184^+-OEt ,
10.1%), 144 ($\text{C}_6\text{H}_8\text{O}_4$; 0.1%), 129 ($144-\text{CH}_3$, 2.2%),
100 ($144-\text{CO}_2$, 2.4%), 43 ($144-101$, 100%).

Preparation of isopropylidene butylmalonate⁶² (74)

To butylmalonic acid (5.76 g, 0.036 mol) in acetic anhydride (5 ml, 0.05 mol) was added while stirring **conc.** sulphuric acid (0.01 ml). Most of the butylmalonic acid dissolved with spontaneous cooling. To the resulting solution acetone (3.5 g, 0.049 mol) was added with cooling to 20°. After allowing the reaction mixture to stand at 0°C overnight, the resulting crystals were separated by adding ice-water (2 x 10 ml) and filtration gave isopropylidene butylmalonate (3 g, 42%) as white needles m.p. 58° from hexane [lit⁶² m.p. 58°].

¹H n.m.r (CDCl₃) δ3.50 (t, J = 4.5Hz, 1H), 2.25 (m, 2H, CH₂)
1.71 (s, 6H, CH₃), 1.40 (m, 4H, CH₂)
0.90 (t, J = 6.75Hz, 3H, CH₃).

Attempted condensation of isopropylidene butylmalonate (74) with ethyl bromopyruvate.

(i) using alumina

Isopropylidene butylmalonate (74) (0.62 g, 3.1 mmol) and ethyl bromopyruvate (0.60 g, 3.06 mmol) were carefully melted and absorbed on neutral alumina (1.264 g, 12.4 mmol) and the mixture was shaken at room temperature for a period of 5 days. The reaction mixture was eluted with CH₂Cl₂ (30 ml), the solution filtered and the filtrate evaporated to give an oil (1 g) which consisted essentially (t.l.c, EtOAc:Petrol

1:1, spectral data) of unreacted starting materials.

[^1H n.m.r spectrum for ethyl bromopyruvate showed peaks at δ 4.5 (q, 2H, OCH_2CH_3), 4.35 (s, 2H, CH_2Br), 1.4 (t, 3H, OCH_2CH_3)]

(ii) using DMF/ Ac_2O

Isopropylidenemalonate (74) (0.5 g, 2.5 mmol) and ethyl bromopyruvate (0.48 g, 2.7 mmol) were treated with a mixture of DMF: Ac_2O (2 ml) (1:1) and refluxed with vigorous stirring for 5 days. The solvent removed in vacuo to give an oil (1.693 g), which was shown by t.l.c in chloroform and petroleum ether (3:1) and ^1H n.m.r. to be a complex mixture. Preparative t.l.c failed to separate the components and the reaction was not investigated further.

(iii) The sodium salt of (74) (2g, 9 mmol) was prepared by dissolving isopropylidenemalonate (74) (1.80 g, 9 mmol) and sodium metal (0.28 g, 0.01 mol) in dry ethanol (10 ml). Then ethyl bromopyruvate (1.75 g, 8.97 mmol) in ethanol (5 ml) was added and the mixture was refluxed at 0°C for 2 days. The ethanol was evaporated to give an oil (3.5 g) which was shown by t.l.c in EtOAc:Hexane (1:1) and ^1H n.m.r to be a mixture of unreacted starting materials.

(iv) when the above reaction was repeated using d_6 -DMSO as solvent the work up afforded a brown oil, which was a mixture of several

components. Preparative t.l.c failed to separate them and the reaction was not investigated further.

Preparation of 1,1,2-Tricarbomethoxyhexane⁶⁴ (83).

To dimethyl malonate (0.76 g, 5.75 mmol) and the bromoester (82) (1 g, 4.78×10^{-2} mmol) in dry T.H.F (5 ml), was added sodiumhydride (140 mg, 5.8 mmol) in dry T.H.F (5 ml) at 0-5°C and the mixture was refluxed at 50°C for a period of 3 hrs. After cooling the mixture was poured into water (10 ml) and extracted with CHCl_3 (3 x 5 ml). The combined organic layers were dried over anhydrous MgSO_4 and CHCl_3 evaporated to give the tricarbomethoxy ester (83) (0.68 g, 70%).

^1H n.m.r (CDCl_3) δ 3.85 (s, 3H, CH_3), 3.75 (s, 6H, CH_3)
 3.33 (m, 2H, H-1, H-2), 1.55 (m, 2H, H-3),
 1.30 (m, 4H, H-4, H-5), 0.90 (t, J = 7Hz,
 3H, H-6).

I.R. $\nu_{\text{max}}^{\text{CCl}_4}$ 1755, 1740 cm^{-1} .

Preparation of 1,1,2-Tricarboxyhexane⁶⁴ (84).

1M aqueous sodium hydroxide (32 ml) was added to 1,1,2-tricarbomethoxyhexane (83) (1.65 g, 6.3 mmol) and the reaction mixture stirred overnight at room temperature. The solution was stirred at 70° for 3 hrs, allowed to cool and

saturated with solid NaCl. The reaction was thoroughly extracted with ethyl acetate and the extract dried and evaporated to give 1,1,2-tricarboxyhexane (0.74 g, 54%). m.p. 152 - 153° [lit.⁶⁴ m.p. 152 - 154°] from light petroleum (100 - 120°).

I.R. ν_{\max} (KBr) 3600 - 2300, 1700 (br) cm^{-1}

Preparation of α -n-butylitaconic acid⁶⁴ (47)

A mixture of 1,1,2-tricarboxyhexane (20 g, 0.09 mol) in diethylamine (30 ml) was treated with 37% aqueous formaldehyde (45 ml) and stirred at room temperature vigorously for a period of 1 hr. After standing overnight the solution was poured into brine and extracted with diethyl ether (3 x 25 ml) to remove diethylamine. The aqueous phase was then chilled and acidified with concentrated HCl at 0°C, and was then extracted with ether (3 x 25 ml). The combined organic phases were dried over MgSO_4 and evaporated at reduced pressure to give α -n-butylitaconic acid (2,3-dicarboxyhept-1-ene) (47) (4.07 g, 24%), m.p. 97 - 99° [lit m.p. 97-99°] from heptane.

(Found C, 58.02; H, 7.21 calc. for $\text{C}_9\text{H}_{14}\text{O}_4$ C, 58.06, H, 7.52%).

^1H n.m.r (CDCl_3) δ 6.35 (s, 1H, C=CH), 5.22 (s, 1H, C=CH), 3.40 (t(br), J7Hz, -C-CH), 1.83 (m, 2H, C-CH₂-), 1.35 (m, 4H, -C(CH₂)₂), 0.90 (t, J7Hz, 3H, CCH₃)

I.R. ν_{\max} (KBr) 3500 - 2300 cm^{-1} , 1690, 1630 cm^{-1}

M.S. m/e 186(M^+), 141($\text{M}^+ - \text{CO}_2\text{H}$, 23.1%), 143($\text{M}^+ - \text{Pr}$, 5.3%),
129($\text{M}^+ - n\text{-Bu}$, 40.9%), 130($\text{M}^+ - \text{CH}_3\text{CH}_2\text{CH}=\text{CH}_2$, 8%),

Esterification of α -n-butylitaconic acid (47)

To α -n-butylitaconic acid (47) (2.08 g, 0.011mol) in dry methanol (27 ml), was added dropwise while stirring, thionyl chloride (3.3 ml, 0.05 mol) and the mixture stirred at 0°C for a period of 2 hrs. After overnight stirring at room temperature the reaction mixture was refluxed at $45\text{-}50^\circ$ for a period of 3 hrs and the methanol was evaporated under reduced pressure to give a yellow oil. Short path distillation gave dimethyl α -n-butylitaconate (53) (2.2 g, 92%) as colourless oil b.p. $70^\circ/0.02$ mm (Found C, 61.70; H, 8.40 $\text{C}_{11}\text{H}_{18}\text{O}_4$ requires C, 61.68; H, 8.41%).

^1H n.m.r (CDCl_3) δ 6.34 (s, 1H, C=CH), 5.75 (s, 1H, C=CH),
3.78 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.51
(t, $J=9\text{Hz}$, 1H, H-2) 1.75 (m, 2H, CH_2 , H-3) 1.35 (m,
4H, CH_2 , H-4, H-5), 0.89 (t, $J=6.75\text{Hz}$, 3H, CH_3 , H-6).

I.R. $\nu_{\max}^{\text{CCl}_4}$ 1720, 1740, 1630 cm^{-1}

M.S. m/e 214(M^+), 183($\text{M}^+ - \text{OMe}$, 13.4%), 171($\text{M}^+ - n\text{-Pr}$, 5.5%),
158($\text{M}^+ - \text{CH}_3\text{CH}_2\text{CH}=\text{CH}_2$) 15.4%), 157($\text{M}^+ - n\text{-Bu}$, 90.2%),
155($\text{M}^+ - \text{CO}_2\text{Me}$, 66.1%), 126($\text{M}^+ - \text{C}_4\text{H}_8$, -MeOH, 41.9%),
98(126-CO, 16.9%), 95(126-OMe, 41.2%).

^{13}C n.m.r (CDCl_3) δ 173.54 (s), 166.45(s), 138.39(s),
 129.75(t, $\text{C}=\text{CH}_2$), 51.83(q), 51.68(q),
 46.40(d), 30.84(t), 29.47(t), 22.24(t),
 13.64 (q).

Hydrolysis of dimethyl α -n-butylitaconate (53)

A solution of KOH(0.51 g, 9.11 mmol) in distilled water (0.8 ml) was added to the diester () (0.5 g, 2.33 mmol). The reaction mixture was stirred for a period of 3 hrs and then allowed to stand overnight at room temperature. The reaction was thoroughly extracted with ether (3 x 10 ml) to remove unreacted ester and the aqueous phase was acidified at 0°C using conc. HCl, then saturated with solid KCl. The solution was washed with brine, extracted with ether and the extract dried and evaporated under reduced pressure to give α -n-butylitaconic acid (47) (0.4 g, 92%), m.p. $97-99^\circ$ from heptane. This was identical (m.p. ^1H n.m.r) with an authentic sample of the diacid prepared from tricarboxy-hexane except for traces of an impurity (5%) thought to be the vinyl methyl isomer (δ 2.3).

Attempted preparation of dimethyl α -hydroxy- α -n-butylitaconate (86).

(i) using charcoal⁶⁵

To the diester (53) (0.5 g, 2.33 mmol) in ethyl acetate (10 ml) was added activated charcoal (Darco G.60, 100-325 mesh powder) (2 g). The mixture was stirred at room

temperature in the presence of air for 3 days, after which time triethylamine (0.23 g, 2.3 mmol) was added. Stirring was continued at room temperature overnight and the solution filtered through a pad of silica gel HF₂₅₄, the filtrate evaporated under reduced pressure affording an oil (0.5 g). After distillation b.p. 80°/0.04 the unreacted ester (53) was recovered unchanged (¹H n.m.r.).

(ii) using L.D.A.⁶⁶

To diisopropylamine (0.05 g, 0.5 mmol) in dry T.H.F (1 ml) at -15°C (ice-methanol) under an atmosphere of nitrogen was added n-butyllithium (0.3 ml, 0.5 mmol) (2.6M in hexane). After cooling at -78°C (dry ice - acetone), the neat ester (53) (0.1 g, 0.467 mmol) was slowly added with stirring. When the addition was complete, the resulting mixture was stirred for an additional 10 min. at which time TMSCl (0.14 ml, 1.1 mmol) was rapidly added to the mixture. Stirring was continued for 3 hrs during which time the reaction mixture was gradually warmed to room temperature. Solvent was then removed in vacuo (Kuglrohr) and pentane ca.(10 ml) was then added to the residue. The solution was filtered and the filtrate was evaporated to give an oil (0.3 g). The resulting product in dry hexane (3 ml) was added to a solution of MCPBA (0.004 g, 0.02 mmol) in dry hexane (0.5 ml) under an atmosphere of nitrogen at (-15°C). After the addition was

complete ca. 5 mins, the resulting slurry was stirred for 30 mins at room temperature. The reaction mixture was then treated with triethylammonium fluoride (0.003 g, 0.02 mmol) then filtered and the filtrate diluted with ether (10 ml). This solution was then washed sequentially with 0.05N HCl (5 ml) and 5% aqueous sodium bicarbonate (2 x 5 ml). The organic layer was then dried using anhydrous magnesium sulfate. Filtration and solvent removal in vacuo gave unreacted starting materials.

(iii) using ethylamine and lithium.⁶⁷

Dry ethyl amine (5 ml, 0.07 mol) and lithium metal (300 mg) were allowed to stand at room temperature for a period of 2 hrs. The neat diester (100 mg, 0.46 mmol) in dry T.H.F. (1 ml) was added and the reaction mixture very quickly turned yellow and eventually brown. After reflux for 3 hrs the mixture was allowed to cool to room temperature and while stirring TMSCl (5 ml, 0.04 mol) was slowly added under an atmosphere of nitrogen. Stirring was continued at room temperature overnight and T.H.F. was evaporated. The residue was treated with distilled pentane (10 ml), filtered and the filtrate evaporated to give an oil. This was washed with water, extracted with CHCl_3 , dried over MgSO_4 and the chloroform evaporated affording an oil (0.10 g, 88%), which was shown by t.l.c in CHCl_3 -EtOAc (1:1) to be a mixture of two compounds (88) Preparative t.l.c gave isomer A, R_F 0.6 and isomer B, R_F 0.45.

Isomer A.

^1H n.m.r. (CDCl_3) δ 5.75 (m, 2H, -NH), 4.15 (q, J 6.75Hz, 2H, -N- $\underline{\text{CH}_2}$ - CH_3), 3.36 (q, J 9Hz, 2H, N- $\underline{\text{CH}_2}$ - CH_3), 3.4 (s, 3H, C= CH_3), 2.7 (t, J 7.87Hz, 2H, C= CH_2) 1.25 (t, J 9Hz, 3H, N CH_2 $\underline{\text{CH}_3}$), 1.2 (t, J 6.75Hz, 3H, N- CH_2 $\underline{\text{CH}_3}$), 1.19 (m, 4H, -C(CH_2) $_2$ -), 0.9 (t, J 5.62Hz, 3H, C- CH_3).

I.R. $\nu_{\text{max}}^{\text{CCl}_4}$ 3300 (-NH), 2960 (CH), 1690 (CO) 1510 cm^{-1}

M.S m/e 240 (M^+), 184 ($\text{M}^+ - \text{C}_4\text{H}_8$, 8.8%), 156 (184 - C_2H_4 , 2.6%), 212 ($\text{M}^+ - \text{CO}$, 0.7%), 168 ($\text{M}^+ - \text{CONHEt}$, 13.3%), 140 (168 - CO, 11.3%), 112 (156 - EtNH, 100%).

Isomer B.

^1H n.m.r. (CDCl_3) δ 5.75 (m, 2H, -NH), 4.15 (q, J 6.75Hz, 2H, -N- $\underline{\text{CH}_2}$ - CH_3), 3.36 (q, J 9Hz, 2H, N- $\underline{\text{CH}_2}$ - CH_3), 3.4 (s, 3H, C= CH_3), 2.7 (m, 2H, C= CH_2), 1.25 (t, J 9.00Hz, 3H, -N CH_2 $\underline{\text{CH}_3}$), 1.20 (t, J 6.75Hz, 3H, N CH_2 $\underline{\text{CH}_3}$), 1.19 (m, 4H, C(CH_2) $_2$), 0.9 (t, J 5.62Hz, 3H, C- CH_3).

M.S and I.R. spectra are identical with that of the above isomer.

The addition of bromine to the diester (53)

To a solution of the diester (53) (0.5 g, 2.3 mmol) in chloroform (3 ml), was added dropwise while stirring, bromine (0.37 g, 2.3 mmol) in chloroform (15 ml). The reaction mixture was stirred at 40° for a period of 2 hrs and then at room temperature overnight. The chloroform was evaporated under reduced pressure to give a reddish-oil. Short path distillation gave the dibromo compound (80) (0.82 g, 95%) as colourless oil b.p. 130/0.5mm (Found C, 35.18; H, 4.78, Br, 42.79, $C_{11}H_{18}O_4Br_2$ requires C, 35.11; H, 4.78, Br 42.55%). This was shown by t.l.c in $CHCl_3$:Petrol (1:1) to be separable into two components, R_F 0.5 and 0.35 in the ratio of 4:9 (by 1H n.m.r.). A sample of this was separated by preparative t.l.c.

Isomer A: R_F 0.5

1H n.m.r ($CDCl_3$) δ 4.02 (s, 2H, $-CH_2Br$), 3.85 (s, 3H, OCH_3)
 3.78 (s, 3H, OCH_3), 3.25 (m, 1H, C-CH), 1.75 (m,
 2H, CCH_2), 1.35 (m, 4H, $C(CH_2)_2$) 1.91 (t,
 J 6.75 Hz, 3H, C- CH_3).

I.R. $\nu_{max}^{CCl_4}$ 2958, 2930, 2865, 1740, 1430 cm^{-1}
 no peak at 1620 for (C=C).

M.S m/e 376 (M^+), 295 ($M^+ - Br$, 3.7%), 263 ($M^+ - HBr - OMe$, 26.4%),
 239 (295 - CO_2Me , 3.2%), 207 (263 - CO_2Me , 7.8%),
 183 ($M^+ - Br_2 - OMe$, 10.3%), 157 ($M^+ - Br_2 - Bu$, 73.2%),
 155 ($M^+ - Br_2 - CO_2Me$, 48.5%).

^{13}C n.m.r (CDCl₃) δ 172.47 (s, CO). 168.23(s, CO),
61.99(s, -C-Br), 53.65(q), 51.84(q),
50.11(d, CH), 35.29(t, CH₂Br), 29.77 (t), 29.47 (t),
22.36(t), 13.82(q).

Isomer B R_F 0.35

^1H n.m.r. (CDCl₃) δ 4.06 and 4.18 (ABq, J_{AB} 12Hz, CH₂Br), 3.85(s, 3H, OCH₃),
3.78 (s, 3H, OCH₃), 3.26 (m, 1H, -CH-),
1.75 (m, 2H, C-CH₂), 1.35 (m, 4H, -C(CH₂)₂),
1.91 (t, J6.75Hz, 3H, C-CH₃)

I.R and M.S spectra are identical with that of the above isomer.

Treatment of the dibromoester (89) with sodium methoxide

To a solution of the mixture of dibromoesters (89) (0.33 g, 0.88 mmol) in dry methanol, was added with vigorous stirring sodium methoxide (0.096 g, 1.7 mmol) in methanol (1 ml). The reaction mixture was refluxed at 50-60°C for 30 min, and then stirred at room temperature for 1 hr. After evaporation of the methanol, the semi-solid residue was extracted with CHCl₃ (10 ml) to give a colourless oil (0.25 g) which was shown by t.l.c in CHCl₃:Petroleum ether (3:1) to be a mixture of three compounds. Preparative t.l.c gave the methoxy compound (90) R_F 0.6, (0.11 g, 50%) as colourless oil b.p. 90°/0.02mm.

^1H n.m.r (CDCl₃) δ 4.18 and 4.31 (ABq, J=12Hz, CH₂OMe), 3.78(m, 6H, OCH₃), 3.38(s, 3H, OCH₃), 2.52(t, J=7.87Hz, 2H, C=CCH₂), 1.40(m, 4H, CH₂), 0.92(t, J=9Hz, 3H, CCH₃)

M.S. m/e 244(M⁺), 213(M⁺-OMe, 14.9%) 212(M⁺-MeOH, 22.4%)
199(M⁺-CH₂OMe, 16.5%), 180(M⁺-2MeOH, 24.8%),
169(M⁺-MeOH-C₃H₇, 23.19%).

I.R. ν_{max} ^{CCl₄} 2980, 1735(C=O), 1640(C=C), 1100(C-O) cm⁻¹

Preparative t.l.c also gave stereoisomers of the bromo-alkene ().

Isomer A. R_F 0.8 (0.09 g).

^1H n.m.r (CDCl₃) δ 4.23(s, 2H, -CH₂Br), 3.82(m, 6H, OCH₃)
2.5(m, 2H, =C-CH₂-C), 1.49(m, 4H, Me(CH₂)₂),
0.95(m, 3H, CCH₃). (some olefinic impurities were also apparent).

M.S. m/e 294(M⁺), 263(M⁺-OMe, 4.9%), 213(M⁺-Br, 9.5%),
199(M⁺-CH₂Br, 1.1%), 181(M⁺-HBr-MeO, 100%).
151(182-OMe, 24%), 123(182-CO₂Me, 3.3%).

I.R. ν_{max} ^{CCl₄} 2980, 1730(br, C=O), 1630(C=C), 1435 cm⁻¹

Isomer B. R_F 0.9 (0.05 g).

^1H n.m.r. (CDCl_3) δ 4.23(s, 2H, $\text{CH}_2\text{-Br}$), 3.88(s, 6H, OCH_3),
2.5(m, 2H, $=\text{C-CH}_2\text{-C}$), 1.49(m, 4H, $\text{Me}(\underline{\text{CH}_2})_2$),
0.90 (t, J 6.75Hz, 3H, CCH_3).

I.R. and M.S. spectra are identical to that of the above isomer.

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