STUDIES ON THE BIOSYNTHESIS OF BIS-LACTONE ANTIBIOTICS

A Thesis presented by

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Thanks are also due to Betty and Margaret for keeping my bench relatively tidy and to Dr. David Morris for Tea and Sympathy.

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Bures s/Yvette November 1979

CONTENTS

SUMMARY.

In this research it has been shown by incorporation experiments using synthetic, labelled precursors that the antibiotics ethisolide (1) and canadensolide (2) have closely related biosynthetic origins in the appropriately substituted citric acids derived from condensation of a fatty acid derived moiety with a TCA cycle acid (probably oxaloacetate). The most convincing evidence to date for this pathway comes from the efficient and specific incorporation by P.canadense of α-n-hexylitaconic acid into canadensolide (2) and the related metabolites.

Since α-n-butylitaconic acid was similarly incorporated into ethisolide (1), the key step in the biosynthesis of the latter must be the rearrangement of an itaconic acid derivativ whereby the C_3 unit on the α-position of the fatty acid becomes attached to the β-position thus disproving the originally proposed biosynthesis via a β-keto acid.

The pattern of labelling of avenaciolide (4) derived from [2,3-$^{13}C_2$] succinic acid has been found to show intact incorporation into the C_3 unit as well as incorporation via $^{13}CH_3{}^{13}CO_2H$ into the remainder of the molecule. This suggests a biosynthetic origin similar to that of ethisolide (1) and canadensolide (2).

The re-interpretation of C.D. data obtained for butenolides has enabled the absolute configuration of the new minor metabolite decumbic acid (40), isolated in the cours of the present work from P.decumbens, to be assigned as 5S. These C.D. results have also enabled the assignment of the absolute configurations of dihydrocanadensolide (3) and

canadensic acid (60) as (2S,3R,4R) and (2S,3R) respectively. Reduction of canadensolide (2) to dihydrocanadensolide (3) has shown it to have the same absolute configuration as dihydrocanadensolide viz. (2S,3R,4R).

Incorporation studies using [2-$^{13}CD_3$] acetate with P.canadense have enabled the stereochemistry of the reduction of crotonate to butyrate in fatty acid biosynthesis to be elucidated, with delivery of hydrogen at C-2 from the si face i.e. the hydrogen adopts the pro-S configuration. The retention or loss of deuterium at C-4 of ethisolide (1), canadensolide (2) and avenaciolide (4) derived from [2-$^{13}CD_3$] acetate, has been found to be completely in accord with the predicted result deduced from the known configuration at C-4 of these metabolites and the known configuration of the deuterium atom in the fatty acid precursors.

Chapter 1

Introduction

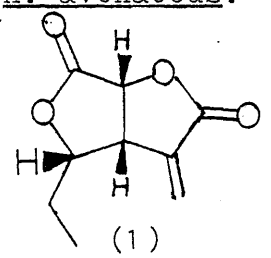
(i) Fungal Metabolites and Fungi.

(ii) Natural Products derived from Tricarboxylic acid Cycle Intermediates.

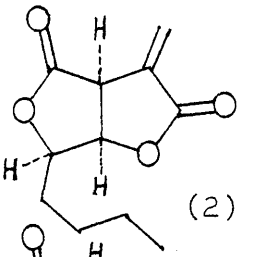
INTRODUCTION.

(i) Fungal Metabolites and Fungi

The research work to be discussed in this thesis is concerned with the biosynthesis of four fungal antibiotics which are related in structure. These compounds are; ethisolide (1), produced by *P. decumbens*; canadensolide (2) and dihydrocanadensolide (3) which are produced by *P. canadense* and finally avenaciolide (4), a metabolite of *A. avenaceus*.



(1)



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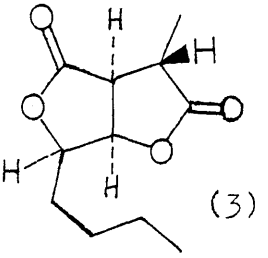

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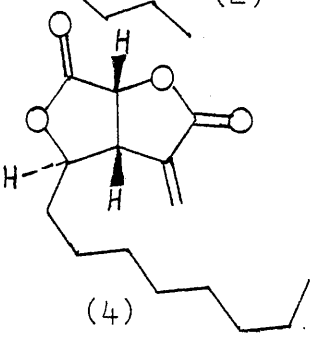


(4)

This introduction involves a brief discussion of the fungi and considers something of their role in nature and commercial importance.

Fungi are known[1] to have existed on earth for a very long time and their fossil record extends as far back as the Devonian and Pre-cambrian eras. The earliest written records of fungi are of their destructive ability and there are biblical accounts of fungal disease, including one (Leviticus 14 34-48) which gives practical instructions for the removal of 'leprosy in a house', thought to be a reference to dry rot.

The Romans held fungi to be great delicacies and they identified several different types, including 'boleti' (Amanita caesaria), 'fungi suilli' (Boletus edulis) and truffles (Tuber spp.) as well as the common field mushroom (Agaricus campestris). In the middle ages, some of the fungi and mushrooms with medicinal properties were classified as were some curiosities such as the 'fungus stone' (Polyporus tuberaster). Ergot of Rye was also known of at this time, but the connection between the infected rye and St.Anthony's Fire - Ergotism, was not made until much later.

The origin of the fungi was a great problem for the Greeks and Romans, and it was believed that poisonous fungi came from 'vipers' breath' while truffles were thought to be produced by lightning. The widely held view that fungi had no seeds, and were derived from organic matter persisted until the late nineteenth century, when purified fungal strains became available for study.

Today, fungi are classified[2] as members of the Thallophyta, a division of the plant kingdom, but they differ from other plants because they possess no chlorophyll and so are unable to use light energy to synthesise carbon compounds from carbon dioxide and water. The fungi obtain these basic organic materials such as sugar and starch, either from dead plant or animal tissue when they are called Saprophytes, or from living plants when they are said to be Parasites. Occasionally, originally parasitic fungi will evolve to a state of equilibrium with the host plant whereby both will derive benefit, and this condition, called Symbiosis, is typified by the lichens (algae and fungus) and also by the 'fungus roots' of many trees.

Fungi are the chief source of disease in plants and cause heavy losses in stored seed of all kinds[3], especially

when it is stored or shipped in bulk. Examples of fungal diseases are Chesnut Blight, which has destroyed chestnut trees in N.America and, of course, Dutch Elm disease, which has caused the death of European and N.American Elm trees. However, not all fungi are harmful[4] and a few of them have been exploited for human benefit in one way or another. Some fungi are grown commercially to produce drugs like the Penicillins and Cephalosporins, chemicals like organic acids, and enzymes and vitamins. In the food industry, they are used to produce cheese and yoghurt as well as beer and wine, and they are used in scientific research, giving information about the ways that genes produce enzymes and about biochemical pathways in living cells.

The molecules whose biosyntheses are studied here are produced as secondary metabolites by their respective fungi, as are the majority of fungal products. Secondary metabolites are produced also by higher plants and are defined as molecules which play no obvious role in the metabolism of the organism that produces them. The biosynthetic pathways by which secondary metabolites are produced are broadly understood but little is known about the reason for their production.

An early attempt[5] to explain the production of secondary metabolites used the apparent fact that their production was stimulated by an excess of carbohydrate, which saturated the oxidative enzymes of the organism, resulting in the accumulation of incompletely oxidised products like the organic acids. This explanation of the phenomenon took no account of the vast array of chemical structures which are produced by a few enzymic reactions, and implied that the process of secondary metabolism has little effect on the organism in its natural environment.

The fact that some insects show a great deal of specificity for certain food plants was linked[6] to the presence or absence of plant secondary metabolites, since the basic food requirements for all insects (essential amino acids, vitamins, sterols and minerals) are similar and occur in all living cells. These secondary metabolites occur sporadically and have large differences in chemical structure and are therefore unlikely to be of nutritional importance to the insect in the same sense as primary metabolites. In this theory it was assumed that, in early evolution, plants produced chemicals as protection against the insects, and that the insects responded to the chemical control of the plant, a host preference arising when a given insect species, by genetic selection overcomes the repellant effect of the plants chemical defences and gains a new source of food. Further evolution of the insects resulted in the former chemical repellant becoming an attractant which induces feeding.

It seems reasonable therefore to consider a similar plant - fungi evolutionary relationship in which fungal secondary metabolism plays a significant role. Formation of some kind of secondary metabolite is a feature of most micro-organisms, and an organism can survive quite effectively even when it devotes a very high proportion of its synthetic ability to production of secondary metabolites eg. it has been found[7] that P.decumbens will routinely produce large amounts of citric acid (ca. 7.5g) and ethisolide (1) (ca. 1.5g) per litre of culture medium. It must be supposed that this capacity for biosynthesis of secondary metabolites confers some selective benefit to organisms in the course of their evolution. However, such is the variety found in secondary metabolites that there is no single property

which can be found common to all of them.

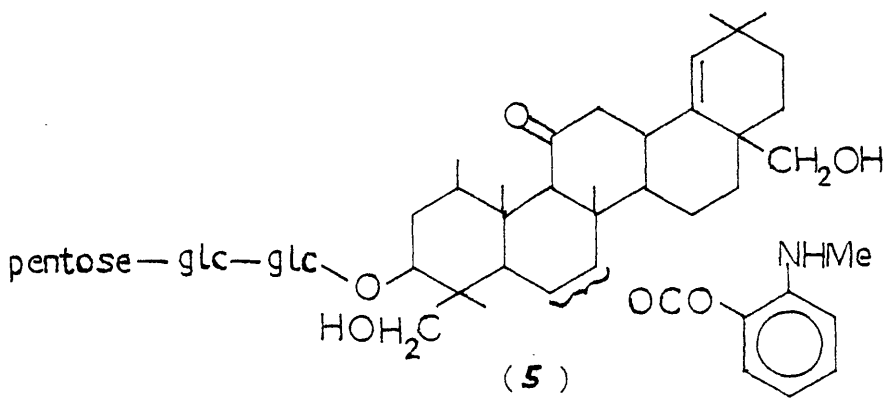
In an attempt to explain the occurrence of all types of secondary metabolites, Bu'Lock[3] has listed four general features common to the process of secondary metabolism namely; the conversion of normal substrates into important intermediates of general metabolism using standard mechanisms, followed by the assembly of these intermediates in an unusual manner by means of a combination of standard general mechanisms with a selection from a relatively small number of special mechanisms. These special mechanisms are peculiar to secondary metabolism, although they are not unrelated to the general mechanisms†. Also, secondary metabolic activity appears, or is intensified, in adaptive response to nutritive conditions unfavourable for cell multiplication. On the basis of these common features, Bu'Lock proposed that the selective advantage of secondary metabolism is that it serves to maintain the mechanisms essential to cell multiplication in 'working order' when the local environment makes cell multiplication impossible. Conditions like these could be envisaged when a plant releases chemicals to repel the attack of a micro-organism.

It is quite well accepted that cellular enzymes will break down when they are deprived of their substrates and hence, when the integrated biosynthesis of new cell material is prevented, a general breakdown of all these synthetic mechanisms would follow. This has two consequences for the organism, the first being that the uptake of nutrients will cease, leaving these nutrients available to any competitors of the organism. The other consequence is that normal functioning of the organism can only be restored after extensive

† It may be noted that these features have direct relevance in the present work. For example, it was found useful to consider the biosynthesis of a number of anti-biotics to be closely linked to primary metabolism.

renewal of enzymes and co-enzymes. The capacity for secondary metabolism enables the organism to resist this situation. Limited de novo synthesis of a few special enzymes will be enough to allow a number of general synthetic mechanisms to continue, thus preventing the breakdown of these enzymes and continuing the uptake of nutrients. The lack of general intrinsic properties of secondary metabolites is explained using the idea that it is the activity of biosynthesis rather than the nature of the metabolites produced that is of value to the organism. An example of the de novo synthesis of a special enzyme is provided by the ability of a wide range of penicillin and cephalosporin resistant bacteria to produce the penicillinase enzyme[9] in natural conditions.

An example of the close inter-relationship between plant chemical defences and the fungal enzyme systems of secondary metabolism is provided by the resistance of oats (Avena sativa) to the fungus responsible for the 'take-all' disease, Ophibolus graminis. The compound responsible for this resistance was found to be avenacin (5)[10].

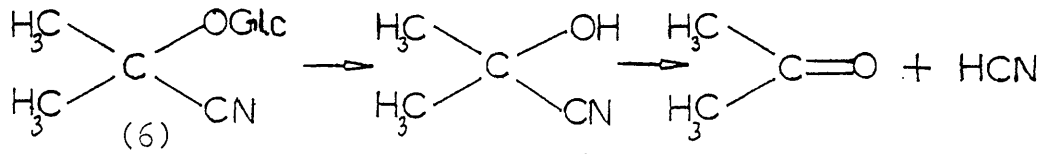


(5)

Both the pentose unit and the N-methyl anthranilic acid moiety are necessary for inhibition of O.graminis, but the fungus O.graminis var. avenacinae is able to successfully attack Avena sativa because it has the ability to produce the enzyme avenacinase which catalyses the removal of the

pentose unit.

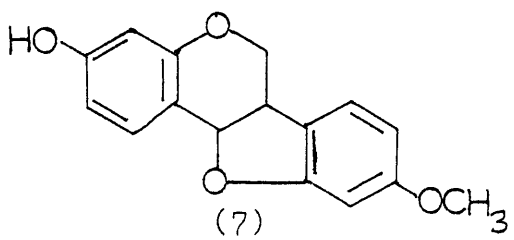
Plants have the ability to inhibit fungal attack by storing toxins within healthy tissue as inactive glycosides; the active toxin being released, after fungal invasion, by hydrolysis or oxidation. In damaged shoots and leaves of the Birdsfoot Trefoil plants, the post-inhibitin linamarin (6) is hydrolysed to produce cyanide (fig 1).



(fig 1)

Many fungi will induce the cyanide production, but only Stemphilium loti tolerates the cyanide produced[11]. It does this by synthesis of the enzyme formamide hydro-lyase, which converts the cyanide to formamide.

Plants are capable also of responding to fungal attack by producing anti-fungal substances or phytoalexins by de novo synthesis. However, the fungi themselves have evolved enzymes which will render these anti-fungal agents inactive. For example, the sweet clover phytoalexin medicarpin (7) is detoxified[12] by Botrytis cinerea by



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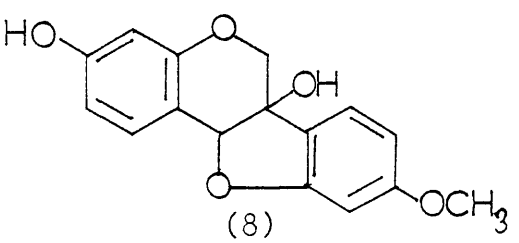
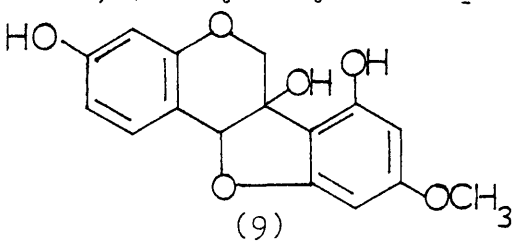


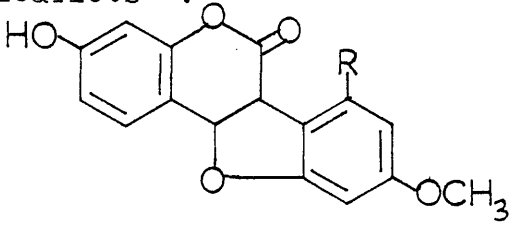
(8)

hydroxylation to give the weakly active 6a-hydroxy-medicarpin (8), while Colletrichum coffeanum converts the phytoalexin to the inactive 6a, 7-dihydroxymedicarpin (9).



(9)

A similar oxidative process is thought to be the source of 3-hydroxy-9- methoxycoumestan (10) and trifoliol (3,7-dihydroxy-9-methoxycoumestan) (11) found in infected white clover leaflets[13].

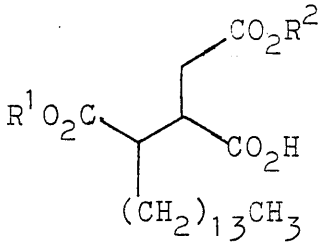


(10) R=H

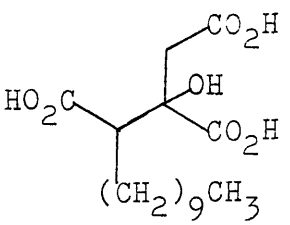
(11) R=OH

Secondary metabolites may also be produced by fungi which induce disease in the host plant. These metabolites, phytotoxins[14,15,16], are produced only once the organism is established within the host plant. Their production has a direct benefit to the fungus in addition to that discussed above since the dead host tissue resulting from the induced infection becomes a source of nutrients for the invading micro-organism.

TABLE 1



(12) R^1 or R^2 = CH_3



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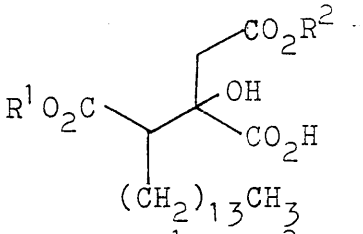
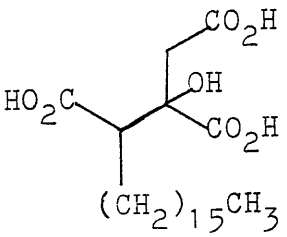


(14) R_1^1 or R_2^2 = CH_3
(15) R^1 or R^2 = H



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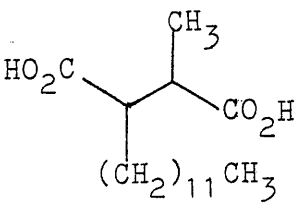
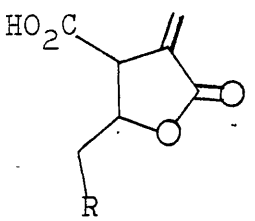
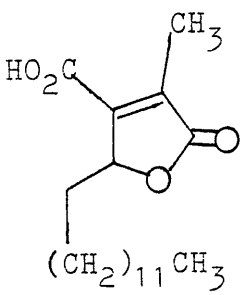


(17)

(18) R = $(CH_2)_9CH_3$



(19) R = $(CH_2)_{11}CH_3$



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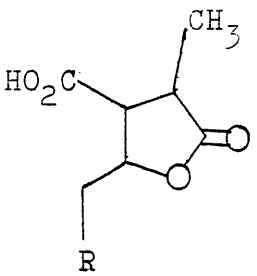
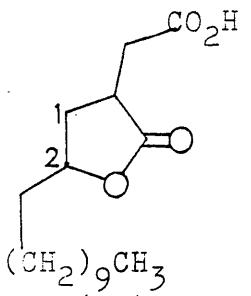
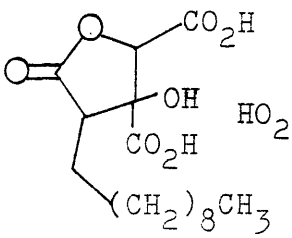


(21) R = $(CH_2)_9CH_3$
(22) R = $(CH_2)_{11}CH_3$



(23)
(24) $\Delta^{1,2}$



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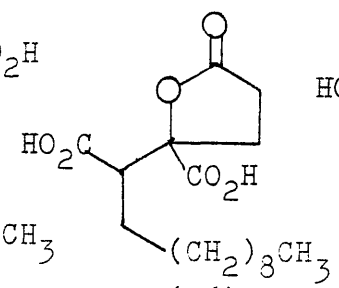

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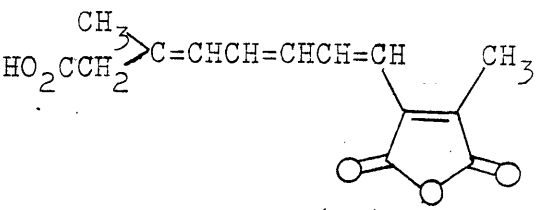

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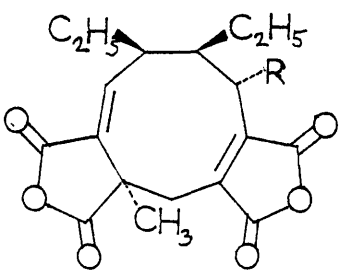
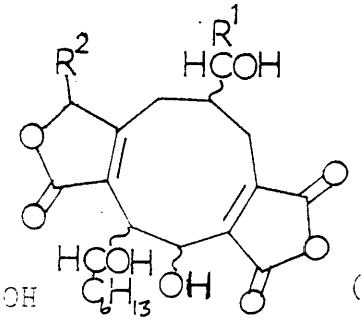
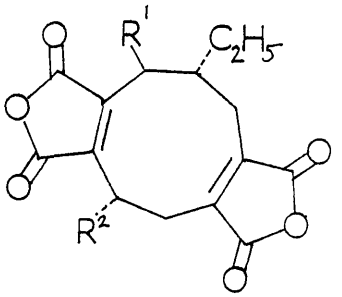
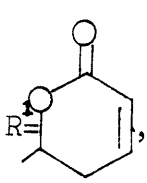


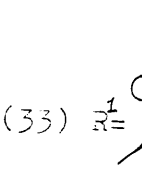
(28) R = OH
(29) R = H

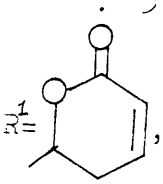




(30) R^1 = H, R^2 = C_3H_8
(31) R^1 = C_3H_8, R^2 = H

(32) 



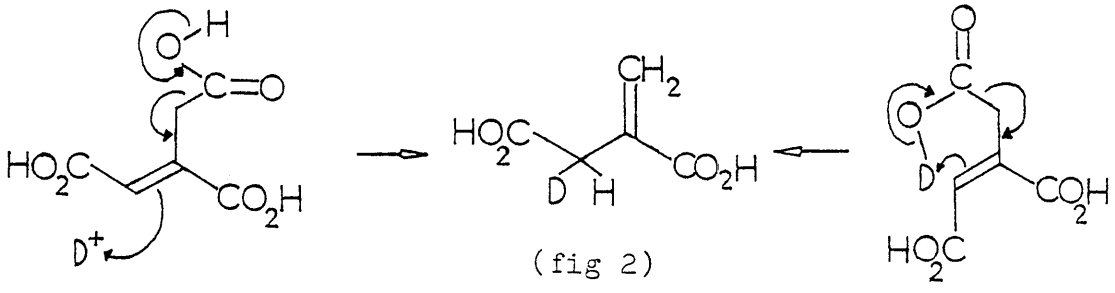
(33) 

(ii) Natural Products derived from Tricarboxylic acid Cycle Intermediates

Canadensolide (2) and dihydrocanadensolide (3) may be grouped with a number of other natural products which appear to be the products of condensation of the keto-group of oxalo-acetic acid, a tricarboxylic acid cycle (TCA cycle) metabolite, with the α-methylene group of a fatty acid[17]. Some examples of this type of metabolite are given in TABLE 1.

The simplest condensation of this type, between acetic acid and oxalo-acetic acid to give citric acid, is one of the reactions of the TCA cycle and a key intermediate in the biosynthesis of these metabolites is predicted to be the appropriately substituted alkyl citric acid. This latter type of compound is found in nature, examples being decyl-citric acid (13)[18,19], caperatic[20] and nor-caperatic[21] acids (14) and (15), and agaricic acid(16)[22]. Spiculisporic acid (26)[23] is evidently produced by condensation of oxo-glutaric acid and the fatty acid followed by cyclisation while rangiformic acid (12)[20] probably results from de-hydroxylation of (14), perhaps by an elimination-reduction procedure similar to that used in the interconversion of malic acid and succinic acid in the TCA cycle. In many secondary metabolites of this type(nephrosterinic acid (18)[20], proto-lichesterinic acid (19)[20], lichesterinic acid (20)[20], nephrosteranic acid (21)[20], nephromopsic acid (22)[20], aracanoic and aracenoic acids (23) and (24)[24], and minio-luteic acid (25)[25]) the simple condensation products have undergone lactonisation using a hydroxyl group at the β position of the fatty acid. The origin of this hydroxyl group has not been established but, although it could conceivably be a residual β-oxygen from the polyketide biosynthesis of the fatty acid chain, it seems more likely

to be produced by hydroxylation of the fatty acid chain. A number of natural products, such as protolichesterinic acid (19), may arise by a similar pathway but with a decarboxylation step as an additional modification. The process involved could be analogous to that established for the biosynthesis of itaconic acid using cell-free extracts of A.terreus[26,27] . Experiments using D_2O showed that decarboxylation involved also the migration of the double bond. (fig 2).



(fig 2)

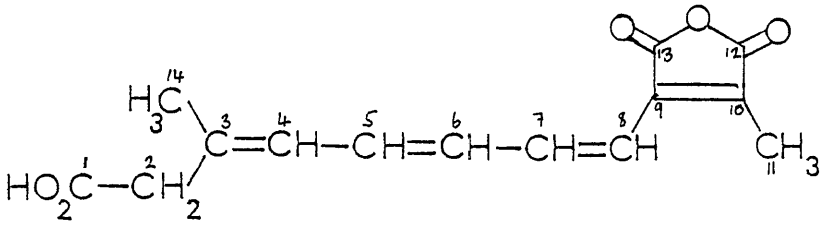
Experimental evidence for the biosynthesis of a metabolite from a fatty acid and a TCA cycle intermediate has been obtained in only a few cases. An enzymic synthesis of spiculisporic acid (26) from lauryl Co-enzyme A and oxoglutaric acid has been demonstrated[28], and the enzyme which catalyses the synthesis of (-)-decyl-citric acid (13) in P.spiculisporum has been isolated[29] and found also to catalyse the condensation of shorter fatty acid CoA derivatives with oxalo-acetic acid. It had been reported[30] that both (+) and (-)-decyl-citric acids (13) were metabolites of P.spiculisporum, but a later study showed that the true metabolite was (-)-decyl-citric acid, the'(+)' acid being a mixture of (-)-decyl-citric acid and (+)-isocitric acid.[31]

It was found[32] that [1-^{14}C] acetate was incorporated into (+)-protolichesterinic acid (19) by C.islandica, mainly into the fatty acid part of the molecule. Later biosynthetic work[33] showed [1,4-$^{14}C_2$] succinic acid to be incorporated at least partly into the C_3 unit of (+)-

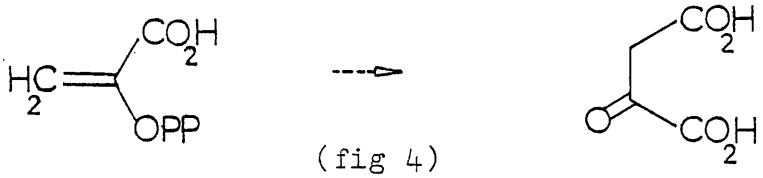
protolichesterinic acid (19) (0.001% - summer only). Although the 'seasonal' aspect of the incorporation suggested that the biosynthesis is a minor metabolic pathway of C.islandica, these results supported the hypothesis that the aliphatic lichen acids have common precursors close to the fatty acids and also to the TCA cycle.

In the biosynthesis of itaconitin (27), acetate and malonate were incorporated[34] into C-1 to C-9 and also into C-13, showing this portion to be derived from a fatty acid (fig 3).

(fig 3)



However, although the C_3 unit (C-10, C-11 and C-12) could be predicted to come from a TCA cycle metabolite, very poor incorporations of [1-^{14}C] pyruvic acid, [1,5-$^{14}C_2$] citric acid and [2,3-$^{14}C_2$] succinic acid were found[34]. This suggested that the TCA cycle was a poor source of carbon atoms for the C_3 unit. [6-^{14}C] glucose was better incorporated, although it gave unequal labelling of C-10 and C-11, and it was suggested that the C_3 unit was derived from the carboxylation of phospho-enol pyruvate (fig 4).

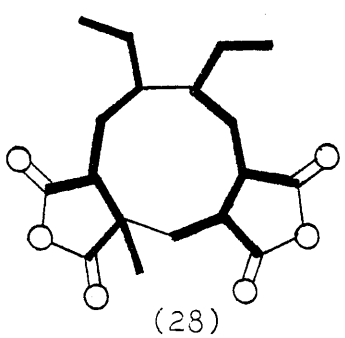


(fig 4)

The Nonadrides, natural products with a characteristic C_9 ring, such as glauconic acid (28)[35], glaucanic acid (29)[36], byssochlamic acid (30)[37] and its isomer heveadride (31)[38] and the rubratoxins A and B, (32)[39] and (33)[40] repectively, can be grouped with the lichen acids on the basis of their

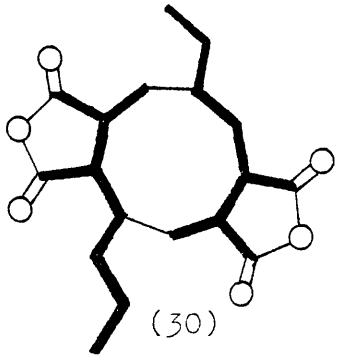
biosynthesis.

A feature of glauconic acid (28) and byssochlamic acid (30) is that each contains two C_9 units of identical skeleton (fig 5)



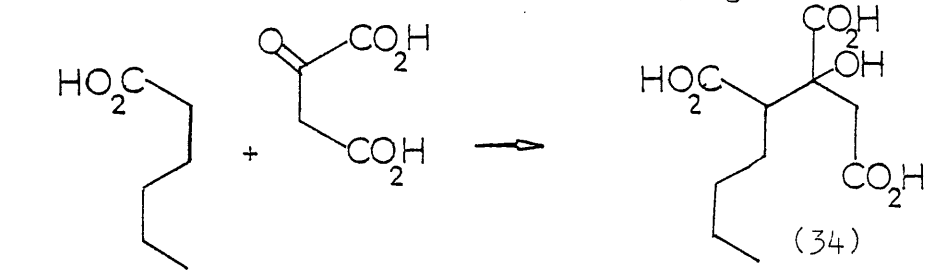
(28)

(fig 5)

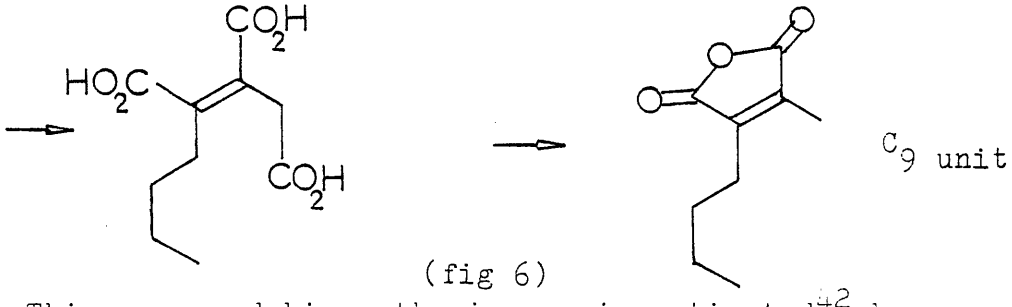


(30)

and it was proposed[41] that these acids arose by dimerisation of a C_9 unit which could be derived from n-butylcitric acid (34) formed by the condensation of the α-methylene group of hexanoic acid with oxalo-acetic acid (fig 6).



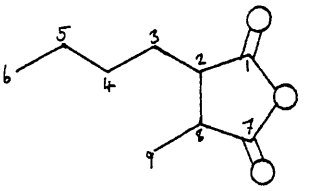
(34)

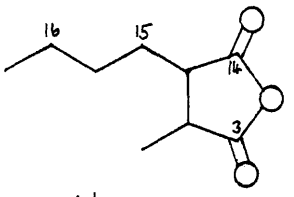


C_9 unit

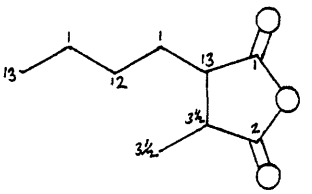
(fig 6)

This proposed biosynthesis was investigated[42] by feeding experiments on P. purpurogenum, and degradation of the glauconic acid (28) isolated resulted in the percentage distribution of activity in each C_9 unit illustrated in (fig 7). This activity distribution in the C_6 chain agreed with its being produced, via the fatty acid pathway, from one acetyl and two malonyl units. In the C_3 unit, C-8 and C-9 were found to have activity from the methyl group of





(fig 7)

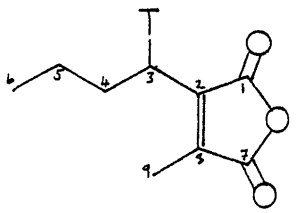


[1-^{14}C] acetate [2-^{14}C] acetate

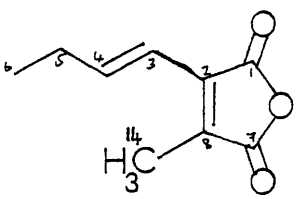
acetate while C-7 incorporated activity from both the methyl and the carboxyl of acetate. [2-^{14}C] pyruvic acid was incorporated into C_8 (8%) and C_9 (1.5%) which showed that, although [2-^{14}C] pyruvate is a good precursor for [1-^{14}C] acetate, the pyruvate can be incorporated without degradation to acetate†, presumably via oxalo-acetate, and without going through a symmetrical C_4 intermediate such as succinate.

The fact that the C_3 unit was derived from a C_4 acid was clearly illustrated by the incorporation of [2,3-$^{14}C_2$] succinate into C-8 and C-9 (55% of total)[42].

Support for the dimerisation step of the proposed biosynthesis was obtained using synthetic, labelled, C_9 precursors[43]. Thus the anhydride (35), labelled with tritium at C-3, afforded glauconic acid (28) (0.25% total incorporation) having half the total tritium activity located at



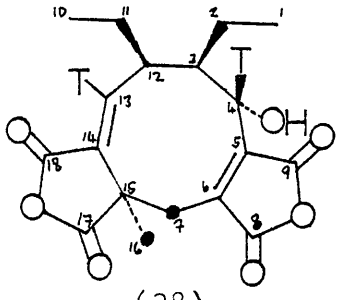
(35)



(36)

C-13 and half at C-4 (fig 8). Also, glauconic acid (28) isolated from P.purpurogenum grown in the presence of the anhydride (36), labelled as shown, retained 50.8% of the

† 1-^{14}C acetate was not found to be incorporated into C-8 or C-9.



(28)

(fig 8)

^{14}C ex. (36)

total activity fed, 97.5% of this being located at C-7 and C-16 (fig 8).

This is a short summary of the biosynthetic knowledge available on these metabolites at the time this work was commenced. There have been two directly relevant results published since then, and these can be found in Appendix 1.

In the present work, strong evidence is presented that canadensolide (2) and dihydrocanadensolide (3) are produced by condensation of the α-methylene group of octanoic acid with the carbonyl group of oxalo-acetic acid to give n-hexyl-citric acid, followed by hydroxylations and lactonisation.

The metabolites listed in TABLE 1 apparently arise by the condensation of a C_3-C_5 unit at the α-position of a fatty acid and are classified as TYPE A metabolites. However, both ethisolide (1) and avenaciolide (4) which are classified as TYPE B metabolites, differ from all these in having a C_3 unit attached to the β-position of a fatty acid chain. As indicated later one biogenetic theory, involving condensation of a β-keto-acid with succinic acid, has proved unsatisfactory and part of this thesis is devoted to establishing the true course of the biosynthesis.

Chapter 2

Discussion:

1. Metabolites of P.decumbens.

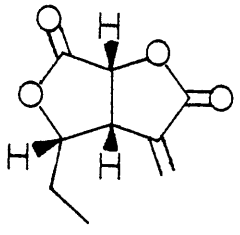
   (i) Previously Known Metabolites

   (ii) Decumbic Acid: A New Minor Metabolite

Metabolites of P.decumbens

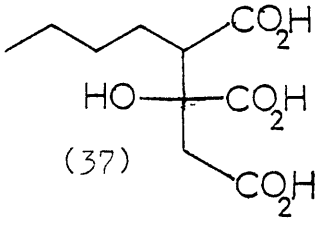
(i) Previously Known Metabolites

The antibiotic ethisolide (1) was first isolated[44] from P.decumbens in 1971. It can be formally derived from a fatty acid to which a C_3 unit is attached at the β-position and is thus a Type B metabolite as classified earlier.

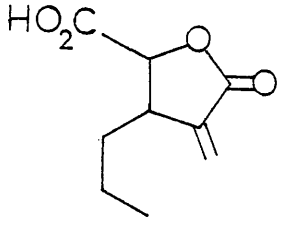


(1)

Extensive studies of the fungal broth of P.decumbens[45] led to the isolation and identification of citric acid (present in substantial quantities) and two minor metabolites viz. n-butylcitric acid (37), a Type A metabolite, and the Type B metabolite ethisic acid (38) which were characterised as their methyl esters.

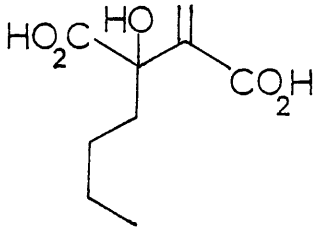


(37)



(38)

In later fermentations[46], the organism afforded smaller quantities of n-butylcitric acid (37), producing instead the Type A minor metabolite α-(OH)-α-n-butylitaconic acid (39), again characterised as the methyl ester.

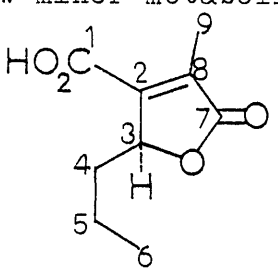


(39)

The significance of these metabolites in relation to the biosynthesis of ethisolide (1) will be discussed later.

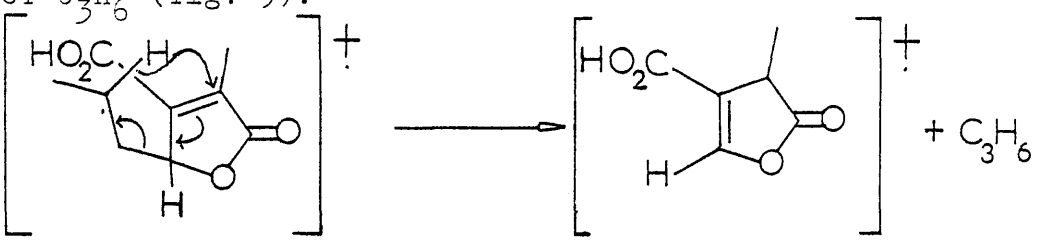
(ii) Decumbic Acid: a new Minor Metabolite.

In an attempt to isolate the minor metabolites (37), (38) and (39) as acids, and so eventually obtain biosynthetically labelled precursors for use in incorporation studies, further cultures of P.decumbens were investigated. These, in addition to ethisolide (1) and citric acid, gave a small quantity of the new minor metabolite decumbic acid (40).



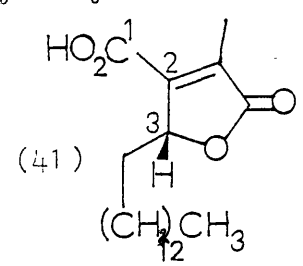
(40)

The I.R. spectrum of decumbic acid (40) showed characteristic peaks at 1780 cm^{-1} (butenolide) and at 3500-2400 cm^{-1} and 1710 cm^{-1} (acid) while the long-range coupling between the C-9 methyl group and the C-3 hydrogen atom (J=2Hz.) was visible in the ^{1}H-N.M.R. spectrum. The Mass Spectrum of decumbic acid showed a molecular ion at m/e 184 and the base peak at m/e 140 could be produced by McLafferty rearrangement and the loss of C_3H_6 (fig. 9).



(fig. 9)

The C.D. curve of decumbic acid (40) (fig. 10) was very similar to the mirror image of the curve found for (+)-lichesterinic acid (41)[47] and decumbic acid can thus be assigned the same 3S configuration as isocanadensic acid (42) and hydroxyisocanadensic acid (43) (Table 2).



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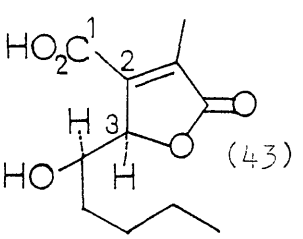
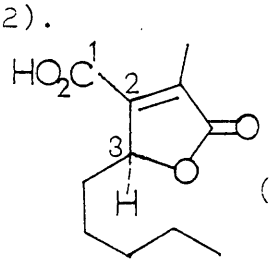
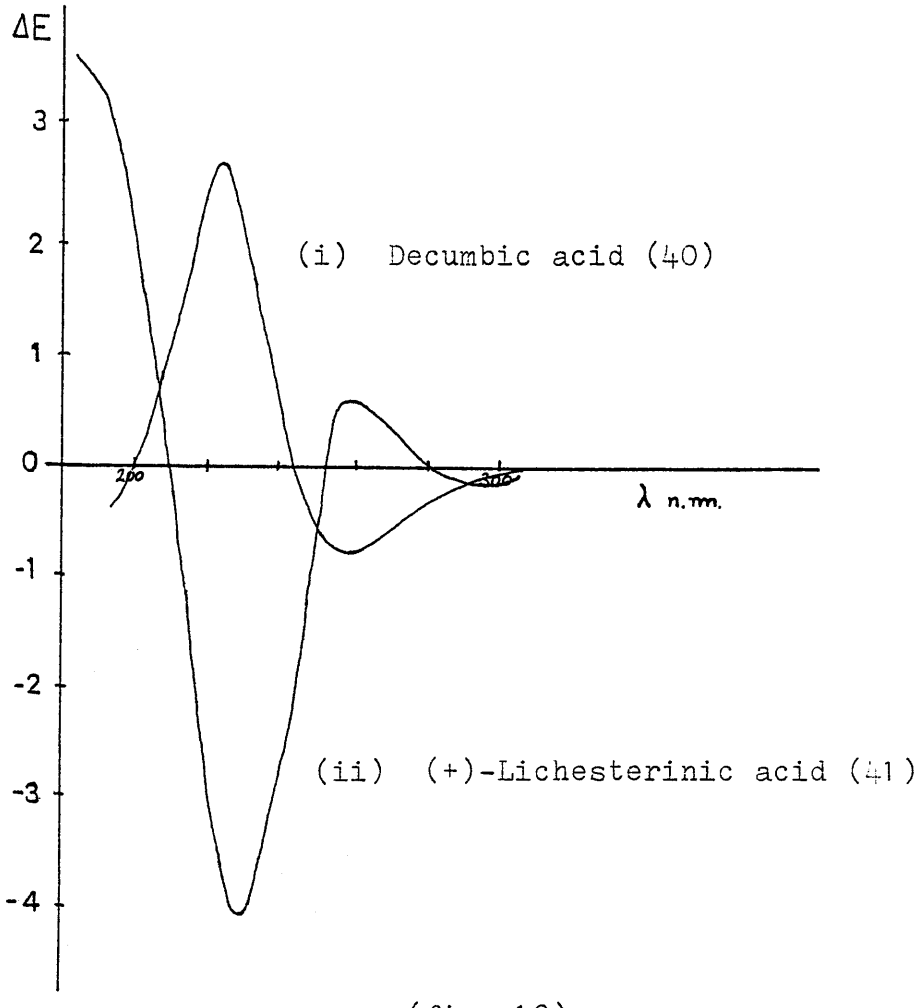


(43)



(42)



(fig. 10)

TABLE 2

Compound	Config. at C-3	λn.m./ΔE
(40)	S	300/0; 256/-0.79; 223/+2.76
(41)	R	292/-0.11; 258/+0.56; 230/-4.13; 188/+3.86.
(42) Me-ester	S	283/+0.35; 275/+0.32; 228/+3.45; 198/-1.75.
(43) Me-ester	S	330/0; 275inf./+1.15; 243/+4.02; 208/-6.31.

The isolation of decumbic acid (40) from P.decumbens was important because of its structural similarity to the P.canadense metabolites isocanadensic acid (42) and hydroxy-isocanadensic acid (43). These molecules have the same 3S absolute configuration and their structures suggest that they may have similar biosynthetic origins. The proposal that the metabolites of P.decumbens and P.canadense have similar biosyntheses will be discussed later with respect to ethisolide (1) and canadensolide (2).

Chapter 3

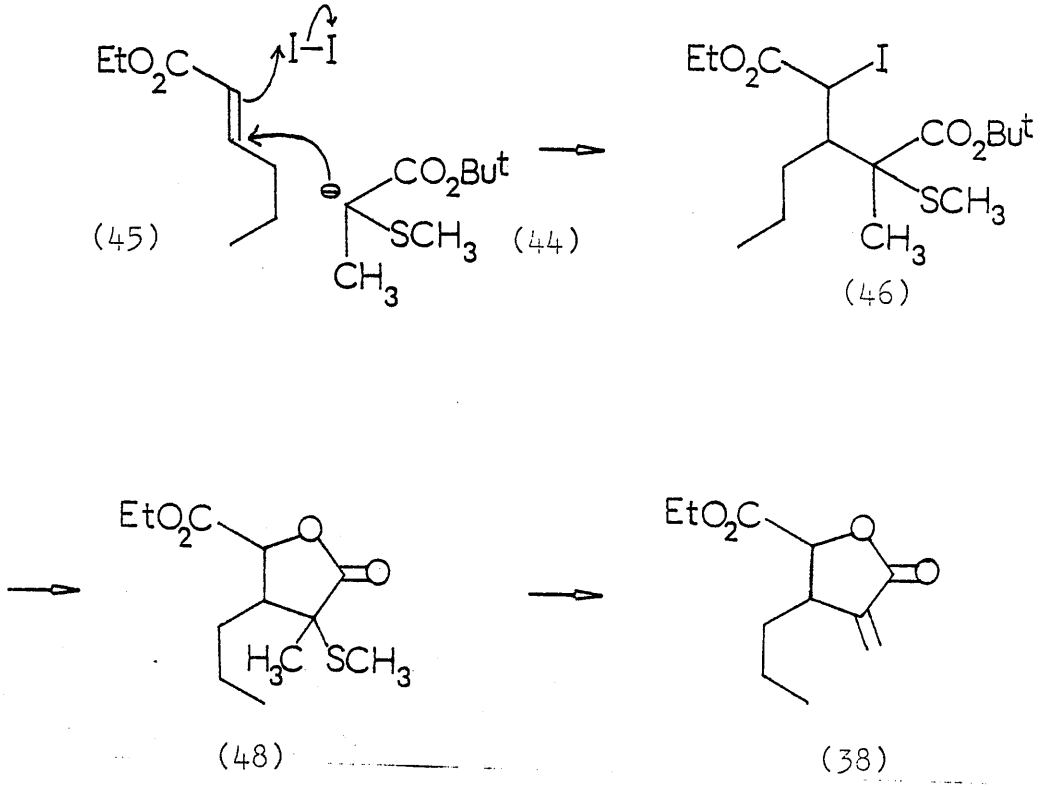
Discussion:

2. Ethisolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with P.decumbens.

   (i) Attempted Syntheses of Ethisic Acid

   (ii) Synthesis of [1-^{14}C]-2,3-dicarboxyhept-1-ene and Incorporation Studies using P.decumbens

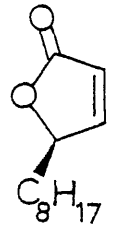
SCHEME 1


EtO2C
I–I
CO2But
SCH3
CH3
(45)
(44)
EtO2C
I
CO2But
SCH3
CH3
(46)
EtO2C
H3C SCH3
(48)
EtO2C
(38)

Ethisolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with P.decumbens.

(i) Attempted Syntheses of Ethisic acid (38).

The synthesis of this minor metabolite of P.decumbens was attempted in order to characterise the molecule fully and to obtain specifically labelled material for incorporation studies with P.decumbens. The first synthetic route tried is illustrated in Scheme 1. The reaction, which involved the Michael addition of tert.butyl-α-lithio-α-thiomethylpropionate (44) to ethyl (trans)hex-2-enoate (45) followed by the trapping of the resulting ester enolate with iodine to give the iodo-ester (46), is a variation of an elegant synthesis[48] of (dl)-avenaciolide (4). This synthesis used as receptor the butenolide (47), and the condensation was claimed to be a general process.

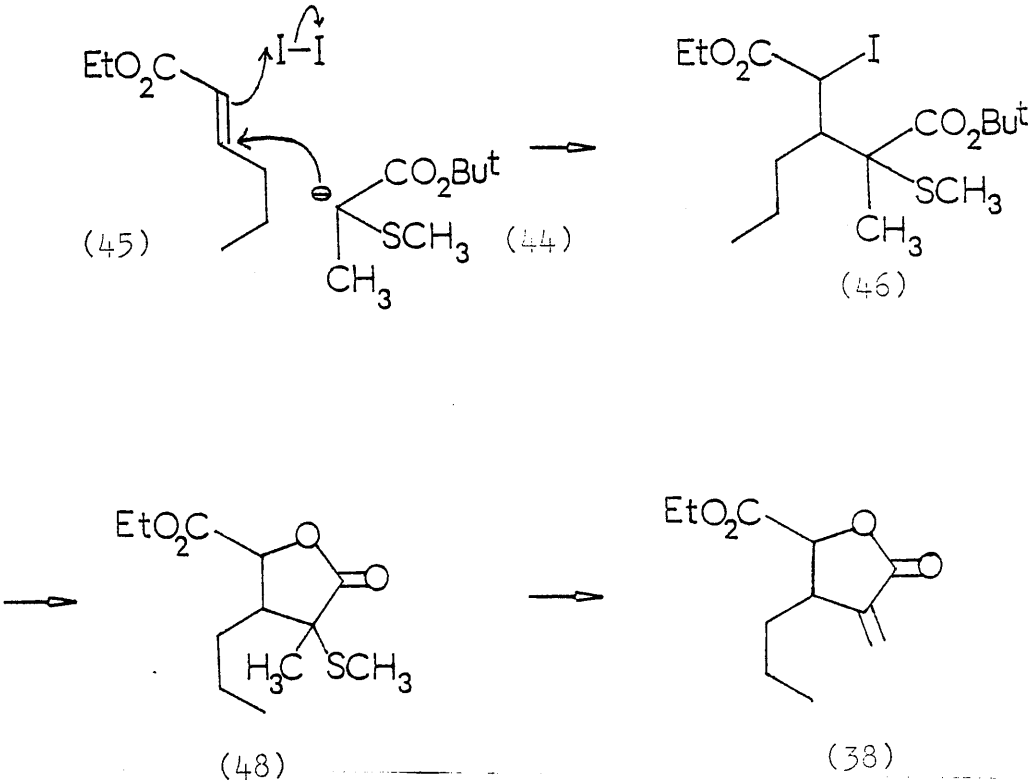


(47)

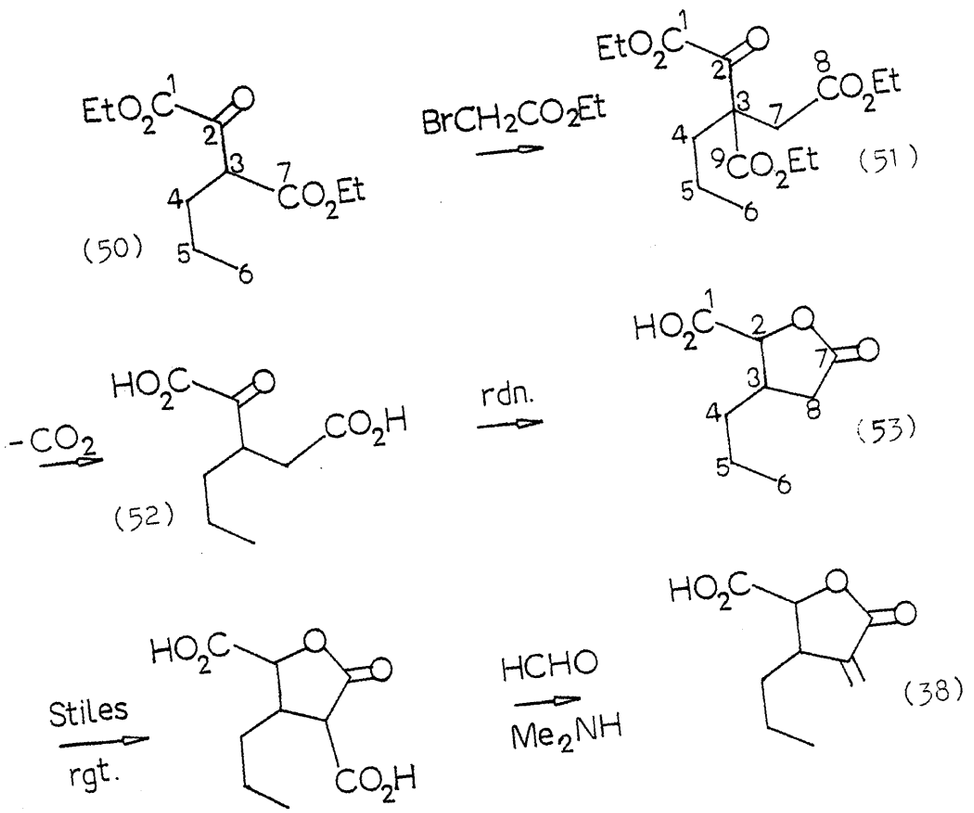
Hydrolysis of the iodo-ester (46) would be expected to give the lactone (48), the exo-methylene function being introduced by pyrolysis of the sulphoxide of (48)[49]. However, the Michael addition reaction gave only starting material and a compound thought to be ethyl (cis)hex-2-enoate, and further attempts to reach (48) without isolating (46), although yielding some lactonic material (identified by I.R. absorption at 1785 cm^{-1}) gave principally starting material and the synthetic route was abandoned as a result.

Another synthetic route (Scheme 2) to ethisic acid (38) which enabled the introduction of a labelled carbon at the exo-methylene position was also attempted. As can be seen, the route involves construction of the lactone skeleton

SCHEME 1

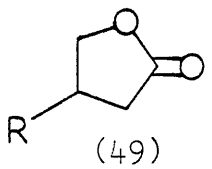

EtO2C
I–I
CO2But
SCH3
CH3
(45)
(44)
EtO2C
I
CO2But
SCH3
CH3
(46)
EtO2C
H3C
SCH3
(48)
EtO2C
(38)

SCHEME 2

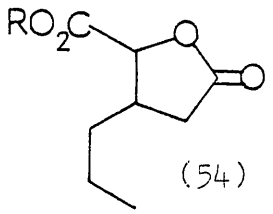

EtO2C
CO2Et
(50)
BrCH2CO2Et
CO2Et
(51)
HO2C
CO2H
(52)
-CO2
rdn.
(53)
Stiles
rgt.
HCHO
Me2NH
(38)

and introduction, at the end, of the exo-methylene group.

A number of ways of converting lactones of type (49), via their enolates, into the corresponding ∝-methylene lactones have been described.[50] However these methods cannot successfully be used in the sequence indicated in Scheme 2 because C-2 and C-8 of lactone (53) are enolisable.



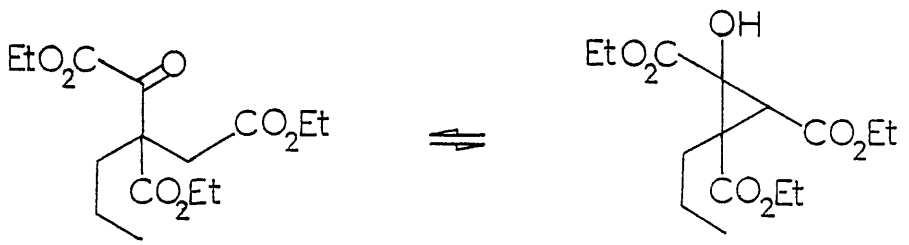
(49)



(54)

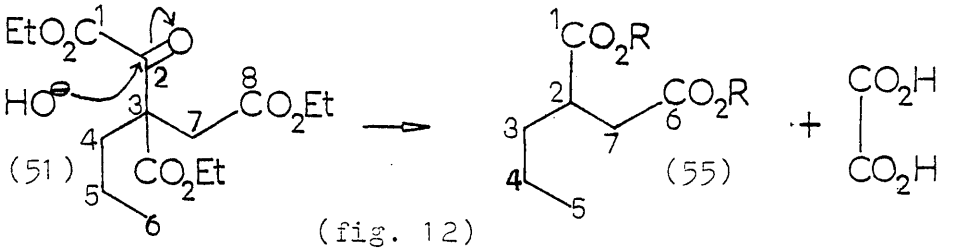
Carboxylation of lactones of type (54) using Stile's reagent (magnesium methyl carbonate)[51], followed by methylenation of the resulting carboxylactone using aqueous formaldehyde and dimethylamine[52] has been used to introduce the exo-methylene function in syntheses of (dl)-avenaciolide[53] and 4-iso-avenaciolide[54] and this became therefore the method of choice in the present case.

Ethyl 3-carbethoxy-2-oxo-hexanoate (50) was prepared by condensation of the enolate of ethyl pentanoate and diethyl oxalate, and the reaction of the enolate of (50) and ethyl bromoacetate gave ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51). These compounds were completely characterised, although the methylene protons on C-7 of (51) gave a broad multiplet in the ^{1}H-N.M.R. spectrum instead of the AB quartet expected because they are adjacent to the asymetric centre at C-3. This was explained by invoking the possibility of ring-chain tautomerism existing in the molecule[55,56] (fig 11).



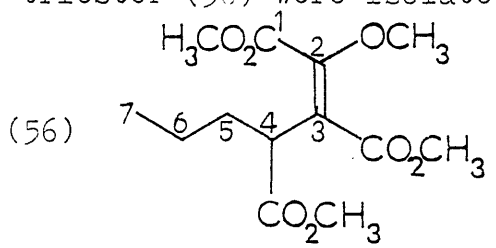
(fig. 11)

The next step in the synthesis is β-decarbethoxylation of the triester (51) to give the keto-diacid (52). Treatment of (51) with 3M-aqueous potassium hydroxide (fig. 12) gave only n-propyl succinic acid (55, R=H) which was identified by ^{13}C-N.M.R. and characterised as its dimethyl ester. The production of (55) requires that the hydroxide ion acts as a nucleophile, attacking the 2-keto group of (51) (fig. 12).



(fig. 12)

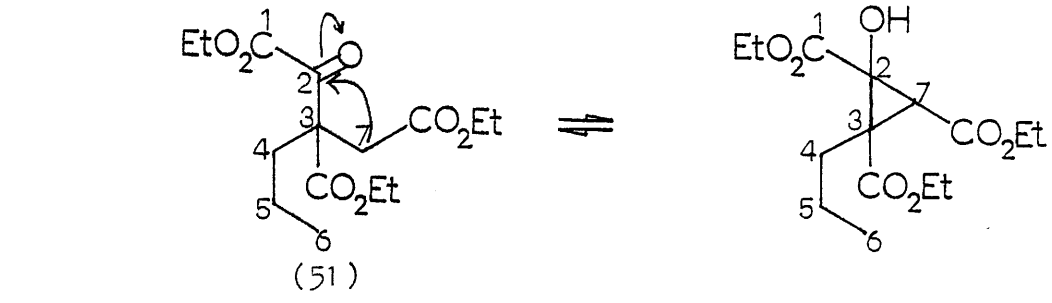
In an attempt to avoid this nucleophilic attack, the tri-ester (51) was treated with four equivalents of 1M-aqueous potassium hydroxide. After treatment of the reaction mixture with diazomethane, dimethyl n-propyl succinic acid (55, R=CH_3) and the methoxy-triester (56) were isolated.



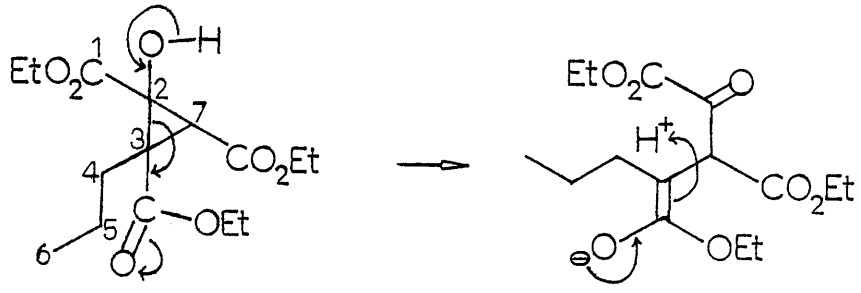
The production of (56) is rationalised once more by using the possibility of ring-chain tautomerism[55,56] (Scheme 3). The important feature here is the formation of the ring between C-2 and C-7 of (51) and its re-opening between C-2 and C-3. This leads to the observed carbon skeleton and (56) is then produced by hydrolysis and methylation.

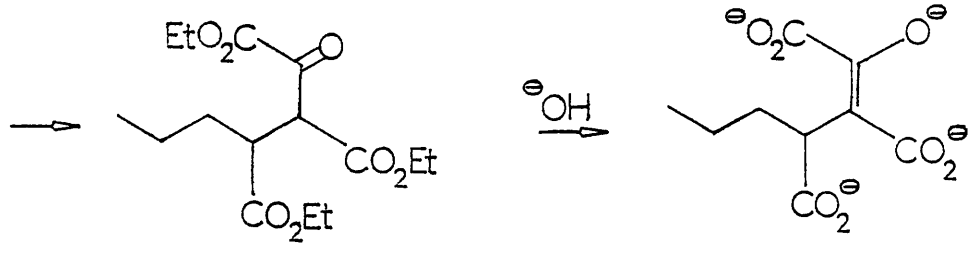
The tri-ester (51) was thus found to be extremely labile in basic conditions and an acidic decarboxylation of (51) was therefore attempted. The expected product of acidic decarbethoxylation is the butenolide (57). This is because the initially produced γ-keto acid is known to readily equilibrate with the butenolide in acidic conditions[56] and hydrogenation would

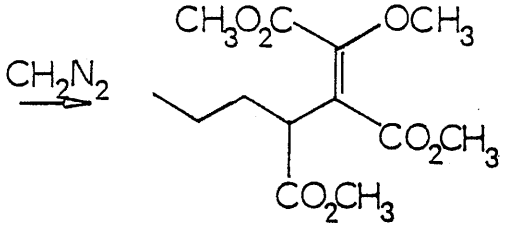
SCHEME 3



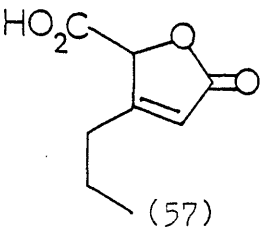
(51)







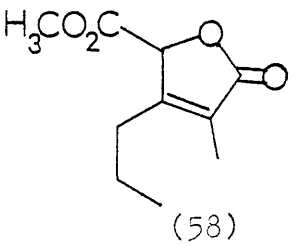
(56)



(57)

give the desired lactone skeleton.

Acid treatment of the tri-ester (51), followed by reaction of the crude product with diazomethane gave the butenolide (58) in 13.8% yield. This is isomeric with the methyl ester of ethisic acid (38) and is produced by C-methylation of a butenolide of type (57) by diazomethane (Scheme 3a)[117].



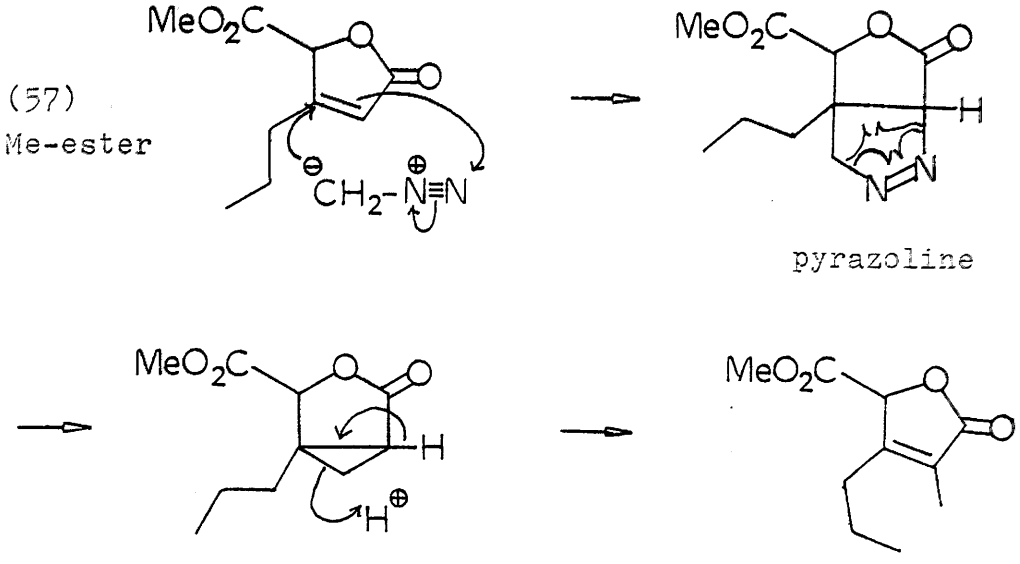
(58)

In attempt to avoid the C-methylation found when diazomethane is used, the initial acidic product was esterified using $SOCl_2$/methanol but this reaction gave only a complex mixture of esters (by ^{1}H-N.M.R.) and the method was abandoned.

Decarboxylations of geminal diesters, β-keto esters and α-cyano esters have been reported [57,58,59] using excess sodium chloride in aqueous dimethyl sulphoxide, and this method was applied to triester (51), but as before, complex mixtures of esters were produced which were not investigated further.

◆ These syntheses are outlined later in Appendix 2.

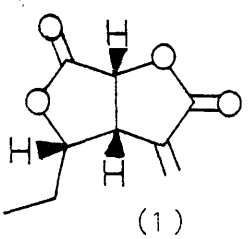
SCHEME 3a



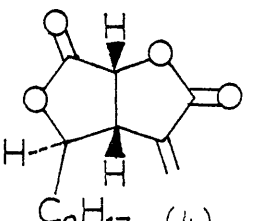
(58)

(ii) Synthesis of [1-^{14}C] -2,3-dicarboxyhept-1-ene (59) and Incorporation Studies with P.decumbens.

It was suggested[44] that ethisolide (1) and the related Type B metabolite avenaciolide (4) might be formed by condensation of succinic acid with a β-keto acid. This



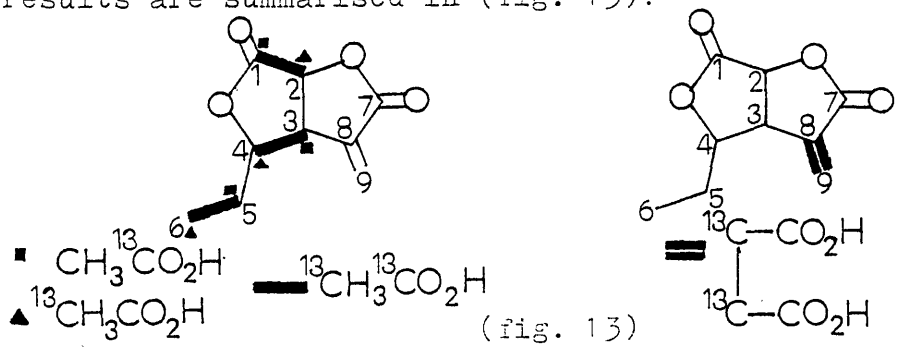
(1)



C_8H_{17} (4)

suggestion was supported by the incorporation of [2-^{13}C] acetate into the fatty acid part of avenaciolide (4) in the predicted manner[60]. A detectable incorporation of [2-^{13}C] acetate into the C_3 unit of avenaciolide was also noticed which could arise by conversion of [2-^{13}C] acetate into ^{13}C-labelled succinate via the Krebs' Cycle (Scheme 4), the labelled succinate then being incorporated into the C_3 unit of (4).

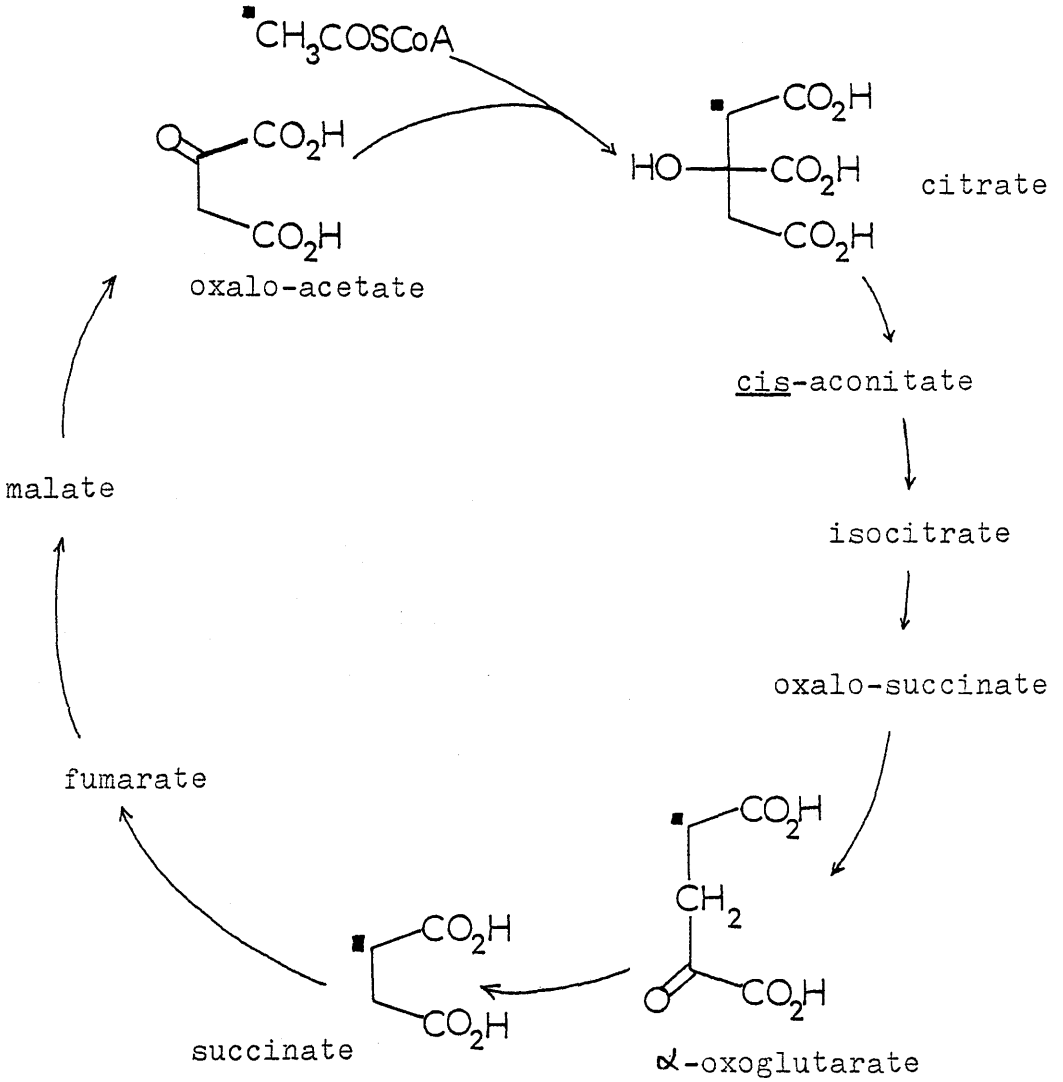
Incorporation studies on P.decumbens using [1-^{13}C] ,[2-^{13}C] and [1,2-$^{13}C_2$] labelled acetate[61] clearly showed C-1 to C-6 of ethisolide to be derived via the acetate-malonate pathway, while the intact incorporation of [2,3-$^{13}C_2$] succinate, synthesised from [1,2-$^{13}C_2$] ethylene dibromide showed C-7, 8 and 9 to originate in a C_4 acid such as oxaloacetate[61]. These results are summarised in (fig. 13).

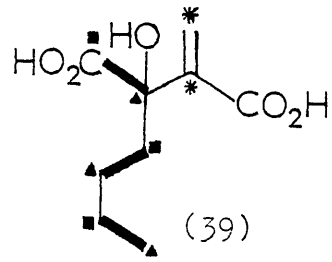


(fig. 13)

The clearly observed incorporation of acetate and succinate into α-HO-α-n-butylitaconic acid (59, fig. 14)[62] showed that very closely related biosynthetic processes lead to the Type A and Type B compounds produced by P.decumbens and we therefore

SCHEME 4


*CH3COSCoA
CO2H
CO2H
oxalo-acetate
CO2H
HO
CO2H
CO2H
citrate
cis-aconitate
isocitrate
oxalo-succinate
CO2H
CH2
CO2H
α-oxoglutarate
CO2H
CO2H
succinate
fumarate
malate



(39)

* [2,3-$^{13}C_2$] succinate

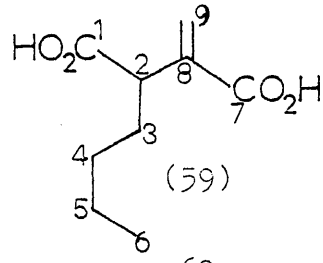
▪ [1-^{13}C] acetate

▲ [2-^{13}C] acetate

▬ [1,2-$^{13}C_2$] acetate

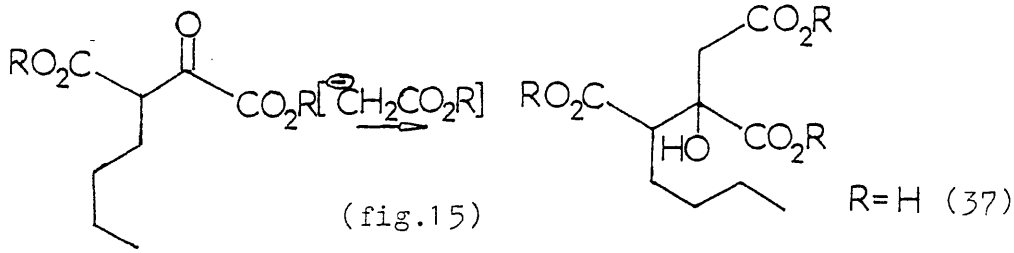
(fig. 14)

proposed that ethisolide (1) is biosynthesised via a Type A precursor. This hypothesis would involve a rearrangement whereby the C_3 unit, originally attached to the α-position of the fatty acid moiety in the Type A precursor, becomes attached to the β-position. In order to prove this hypothesis, the intact incorporation of a synthetic, labelled Type A precursor into ethisolide (1) by P.decumbens must be shown. The proposed biosynthetic intermediate chosen for this study was 2,3-dicarboxyhept-1-ene (α-n-butylitaconic acid) (59).



(59)

Previous synthetic work[62] was directed towards n-butyl-citric acid (37) another Type A metabolite of P.decumbens. However, although the reaction using the α-anion of ethyl acetate (fig 15) gave n-butyl citric acid (37), the product

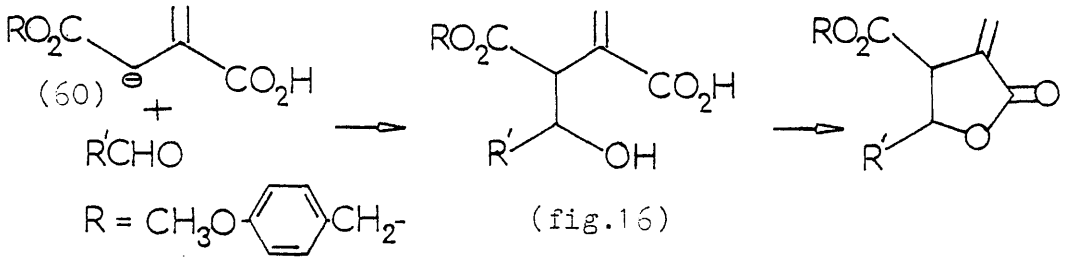


(fig.15)

was an inseparable erythro-threo mixture with the natural material constituting only 10% of the synthetic material. The synthetic n-butyl citric acid was therefore not wholly satisfactory for incorporation studies. However, 2,3-dicarboxy-hept-1-ene (59) has only one assymetric centre, offers the possibility of introducing a labelled carbon atom at the exo-

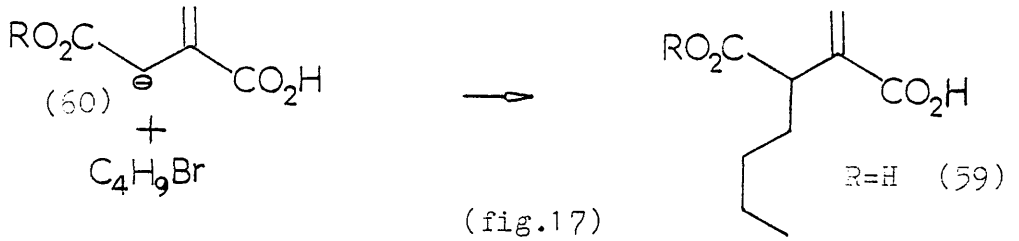
methylene position and was therefore a biosynthetically interesting molecule.

The first synthetic route attempted was an adaptation of a method used in a synthesis of canadensolide (2)[63]. Reaction of the anion of the 4-methoxybenzyl ester of itaconic acid (60) with a series of aldehydes gave a direct route to α-methylene lactones (fig.16) and it was hoped that, by using 1-bromo-



(fig.16)

butane as the receptor in the reaction, the desired skeleton could be achieved directly (fig.17).

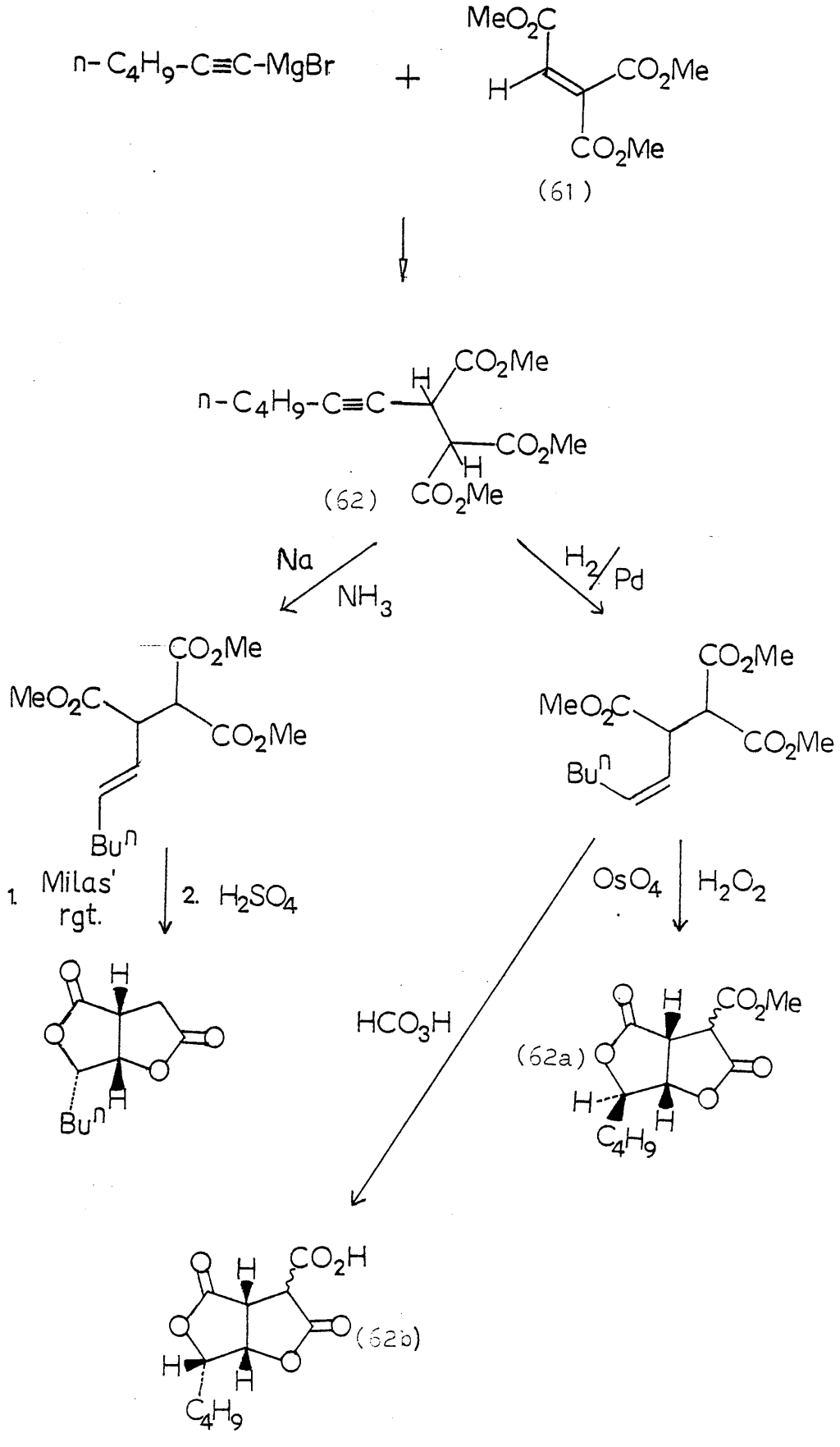


(fig.17)

4-Methoxybenzyl itaconate (60) was prepared using the method of Baker[64] but reaction of its α-anion with 1-bromo-butane at -78°C gave only unreacted (60) on work-up. Hexa-methylphosphoramide has been used [65] in the high yield alkylation of aliphatic acids, but a similar experiment using (60) at 0°C gave only 4-methoxybenzyl alcohol, showing that hydrolysis of starting material had occurred . The synthesis was not investigated further.

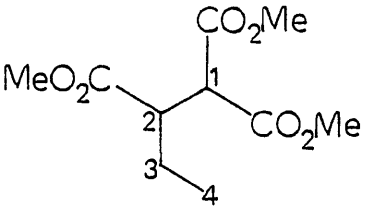
Further synthetic studies of canadensolide and related dilactones[66] used, as the initial step, the 1,4 Michael addition of 1-hexynyl magnesium bromide with 1,1,2-tricarbo-methoxyethene (61) (Scheme 5). Cis and trans hydrogenation of the triple bond in (62) led to alkenes, which after cis and trans dihydroxylation and lactonisation gave the dilactones

SCHEME 5


n-C4H9-C≡C-MgBr
MeO2C
CO2Me
H
CO2Me
(61)
n-C4H9-C≡C
H
CO2Me
CO2Me
H
CO2Me
(62)
Na
NH3
H2/Pd
CO2Me
MeO2C
CO2Me
Bun
1. Milas' rgt.
2. H2SO4
OsO4
H2O2
HCO3H
H
Bun
CO2Me
(62a)
H
C4H9
CO2H
(62b)

(62a and b) and the exo-methylene group was introduced using a Mannich reaction.

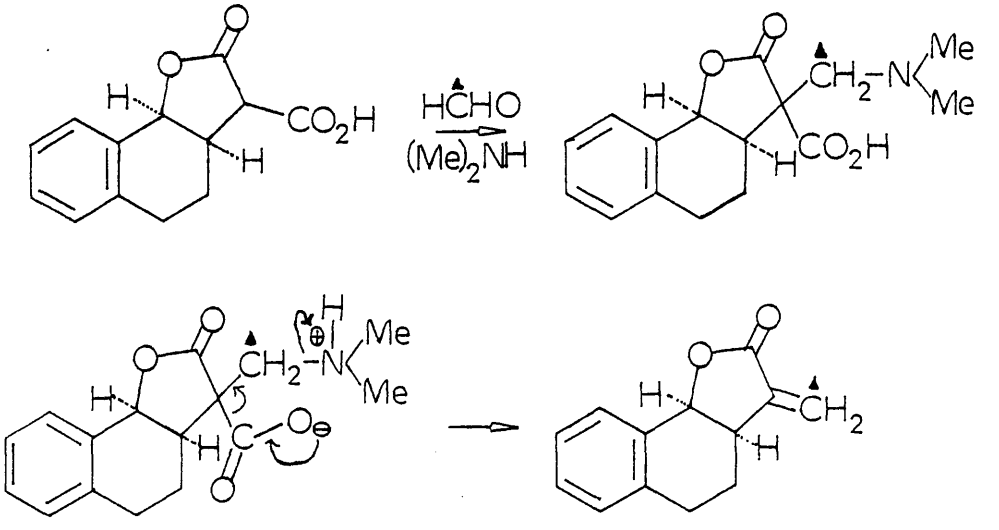
The above reactions were adapted to the synthesis of ∝-n-butylitaconic acid (59). 1-Butynylmagnesium bromide was prepared [66] by a Grignard exchange reaction in tetrahydrofuran with ethylmagnesium bromide. Reaction of this Grignard mixture with (61) prepared by the method of House[67] in the presence of Cu(1)Cl (Scheme 6) gave 1,1,2-tricarbomethoxy-butane (63) as the sole product.



(63)

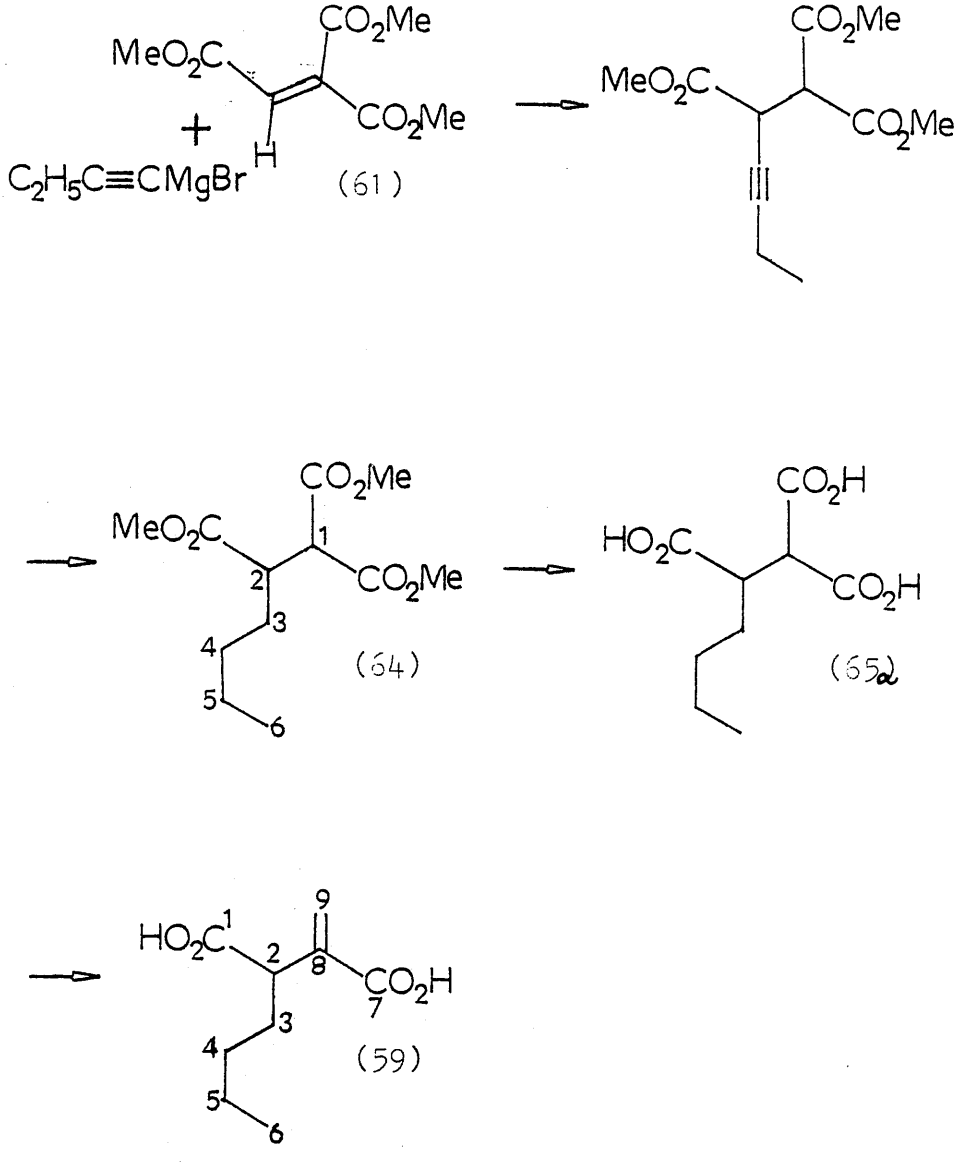
The isolation of this compound was of great importance because, although the desired addition had not taken place, it showed that 1,4 saturated alkyl Grignard addition was possible, thus enabling the direct synthesis of 1,1,2-tricarbomethoxy hexane (64) from (61) and n-butylmagnesium bromide. After base hydrolysis of the triester to the corresponding triacid (65), the exo-methylene moiety was introduced by Mannich reaction[52] using formaldehyde and aqueous dimethylamine giving ∝-n-butylitaconic acid (59).

The mechanism of the Mannich reaction has been studied[68] using the system in (fig.18), and it was proposed that a



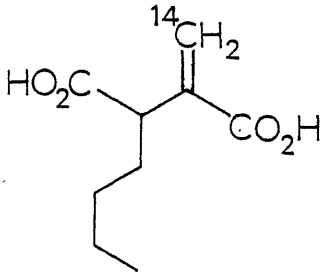
(fig.18)

SCHEME 6


MeO2C
CO2Me
CO2Me
H
C2H5C≡CMgBr
(61)
MeO2C
CO2Me
CO2Me
MeO2C
CO2Me
CO2Me
1
2
3
4
5
6
(64)
HO2C
CO2H
CO2H
(65a)
HO2C
1
2
9
8
CO2H
7
3
4
5
6
(59)

concerted rather than stepwise decarboxylation-elimination mechanism was operating. This was proposed on the basis of the observed speed of exo-methylene lactone formation.

The important feature of the above, from the point of view of this study, is that the carbon atom of the exo-methylene group is derived from formaldehyde. By using [^{14}C] formaldehyde in the last stage of the reaction, [1-^{14}C]-2,3-dicarboxyhept-1-ene (fig.19) was obtained for use in incorporation studies with P.decumbens.



(fig.19)

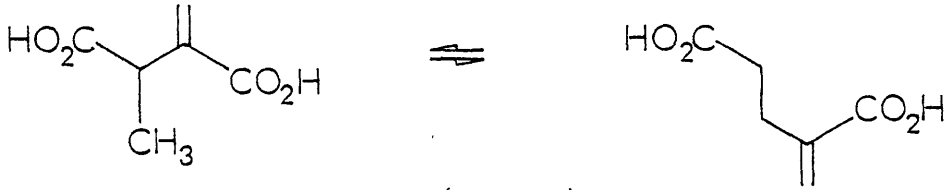
Time/incorporation studies for the production of ethisolide by P.decumbens had previously been carried out using [1-^{14}C] sodium acetate[69]. These results were used in the present work, [1-^{14}C]-2,3-dicarboxyhept-1-ene (fig.19) being administered to P.decumbens in three pulses at 24hr. intervals in order to minimise its degradation by the organism.

The ethisolide recovered from the broth contained 10.4% of the administered radioactivity while citric acid, recovered as its trimethyl ester, was found to be almost inactive. Ozonolysis of the labelled ethisolide to give formaldehyde, isolated and analysed as its dimedone derivative, showed that the radioactivity was located entirely in the terminal methylene carbon.

These results illustrated clearly that ethisolide (1) is indeed biosynthesised via the Type A precursor α-n-butyl-itaconic acid (59). In addition, the fact that the citric acid contained no activity shows that the synthetic precursor is not degraded to any great extent by the organism.

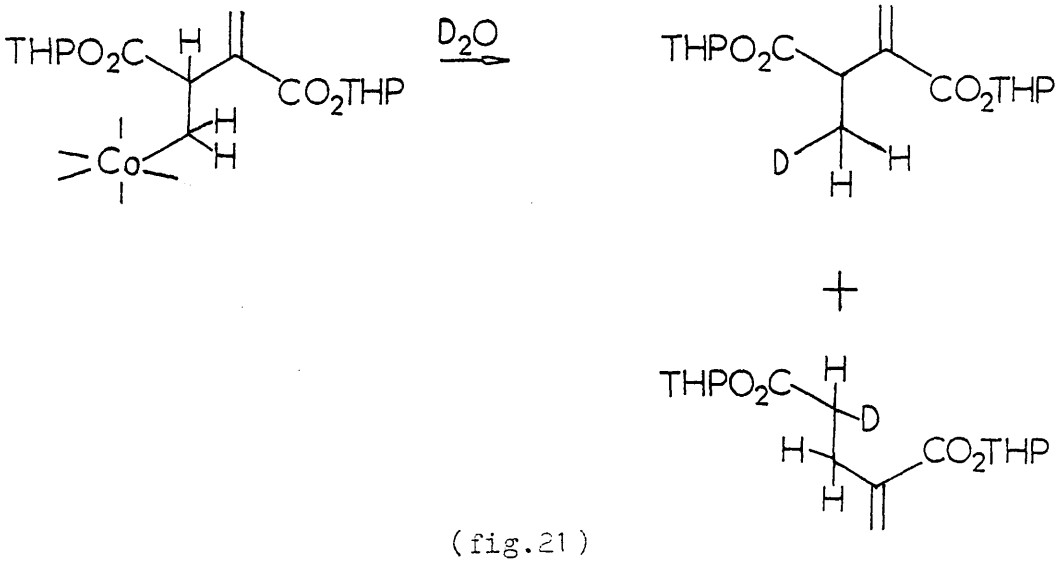
A biosynthesis for ethisolide (1) can now be proposed starting from the condensation between a fatty acid and oxalo-acetate (Scheme 7) to give the n-butylcitric acid (37) which is converted as described earlier into the corresponding itaconic acid (59). The key step in the biosynthesis is the re-arrangement, occurring either at (59) or (39), whereby the C_3 unit on the ∝-position of the fatty acid becomes attached to the β-position as in (59a) and (39a).

There is a related rearrangement already known in a natural system. Two enzymes have been isolated from the bacterium Clostridium barkeri, which catalyse the interconversion of methyl itaconic acid and ∝-methylene glutaric acid[69] (fig.20).



(fig.20)

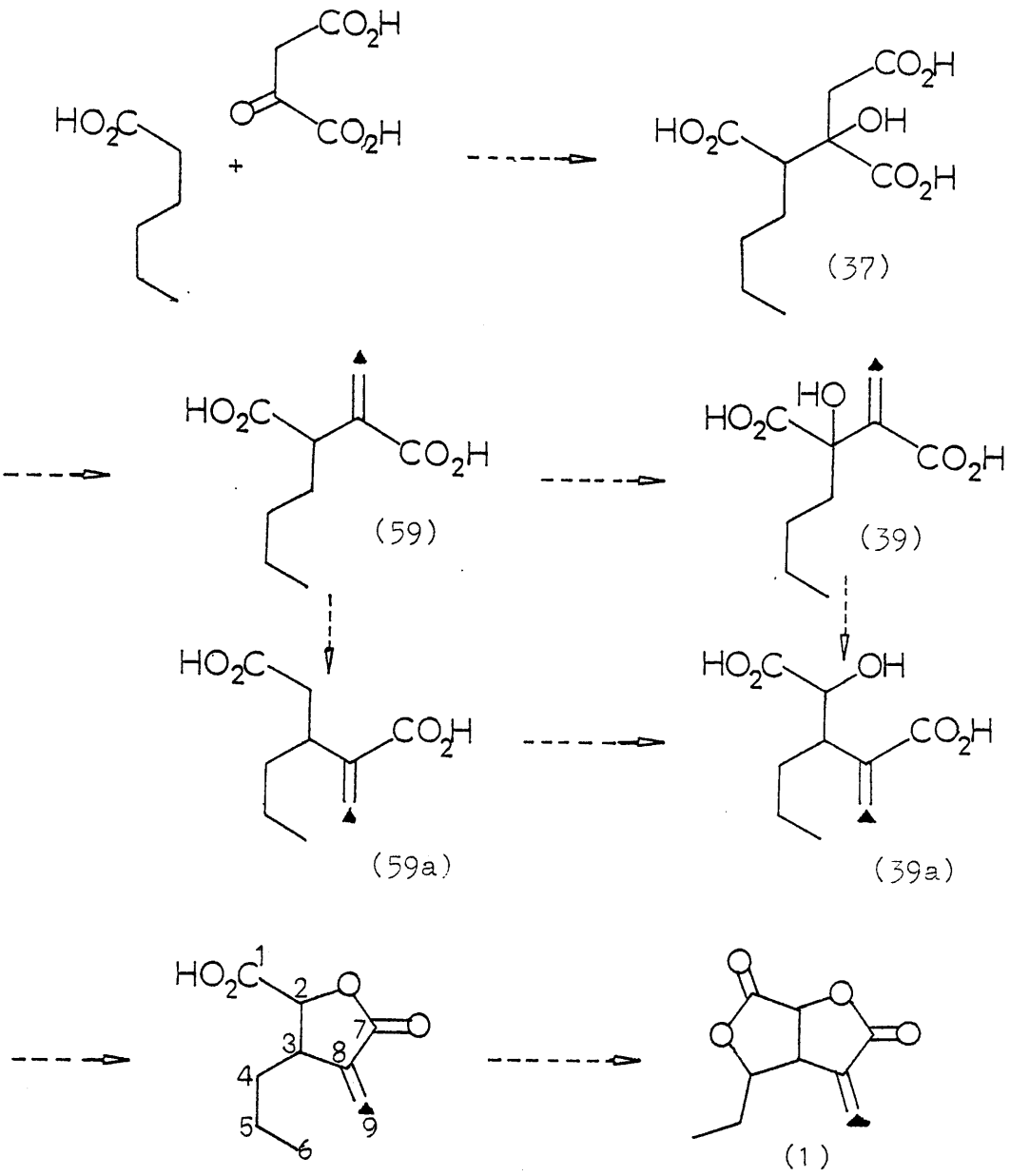
These enzymes are dependant on a B_{12} co-enzyme, and the re-arrangement has been reproduced photochemically *in vitro* using an alkyl cobalamine derivative[70]. Later experiments using D_2O in the work-up[71] showed that a simple C-shell re-arrangement occurred, the C_3 unit being the only migrating group (fig.21).



(fig.21)

rearranged product

SCHEME 7

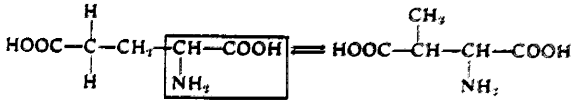
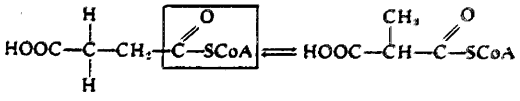
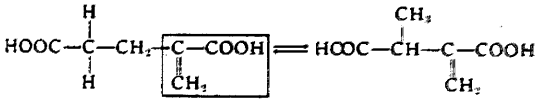
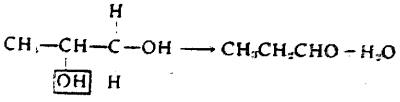
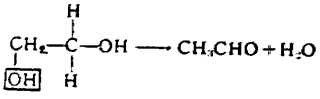
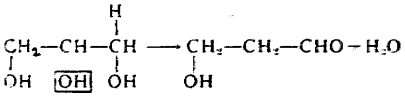
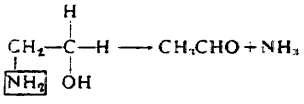
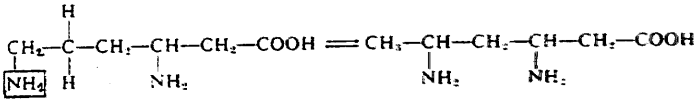
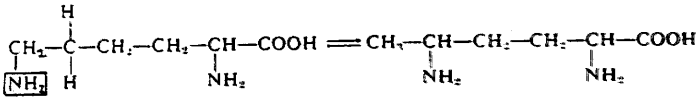
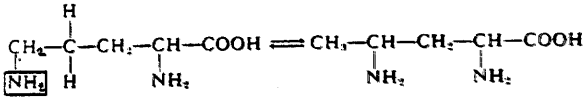


▲ ^{14}C labelled carbon atom

In natural systems there are several other examples of B_{12}-dependant rearrangements and these have been discussed in some detail[118]. These reactions are essentially hydrogen transfer processes with concomitant cleavage of a C-C, C-O or C-N bond and are summarised in (Table 3). The migrating group that is replaced by hydrogen is enclosed by a rectangle.

Our experiments cannot of course tell at which point, (59) or (39), the rearrangement occurs in the biosynthesis of ethisolide, but the earlier isolation of (39) from P.decumbens[46] does hint that the rearrangement may occur after hydroxylation. The final steps of the biosynthesis are quite predictable; lactonisation to give ethisic acid (38), followed by hydroxylation at C-4 and lactonisation leading to ethisolide (1).

TABLE 3

Enzyme	Reaction catalysed
Glutamate mutase	
Methylmalonyl-CoA mutase	
α-Methylene-glutarate mutase	
Diol dehydratase	
Diol dehydratase	
Glycerol dehydratase	
Ethanolamine deaminase	
L-β-Lysine mutase	
D-α-Lysine mutase	
Ornithine mutase	

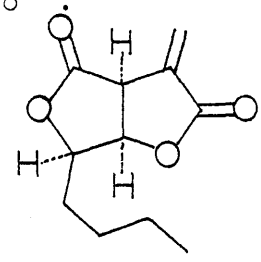
Chapter 4

Discussion:

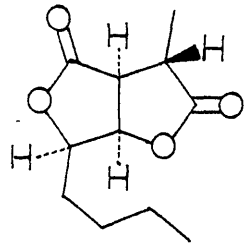
3. Canadensolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with P.canadense.

Canadensolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with P.canadense.

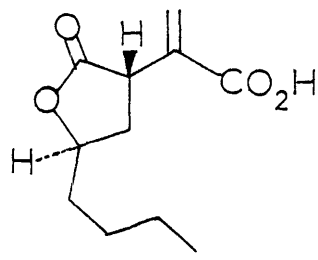
The fungal anti-biotic canadensolide (2), the related anti-ulcer compound dihydrocanadensolide (3) and the metabolite canadensic acid (60) were first isolated from P.canadense in 1968[72].



(2)

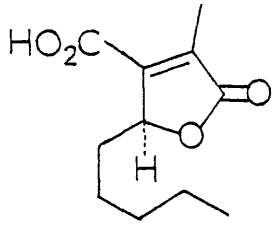


(3)

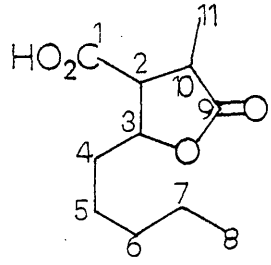


(60)

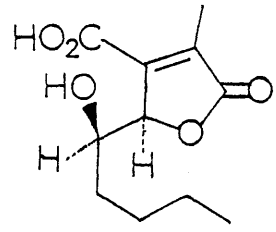
A number of minor metabolites have also been isolated[72], especially from immature cultures of P.canadense. These compounds, isocanadensic acid (61), dihydro-isocanadensic acid (62) and hydroxy-isocanadensic acid (63)



(61)

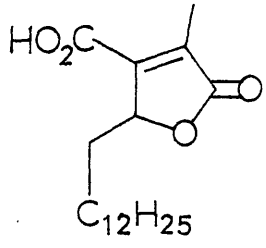


(62)

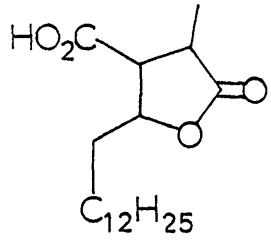


(63)

have structures closely related to the lichen acids e.g. lichesterinic acid (20) and nephromopsic acid (22).



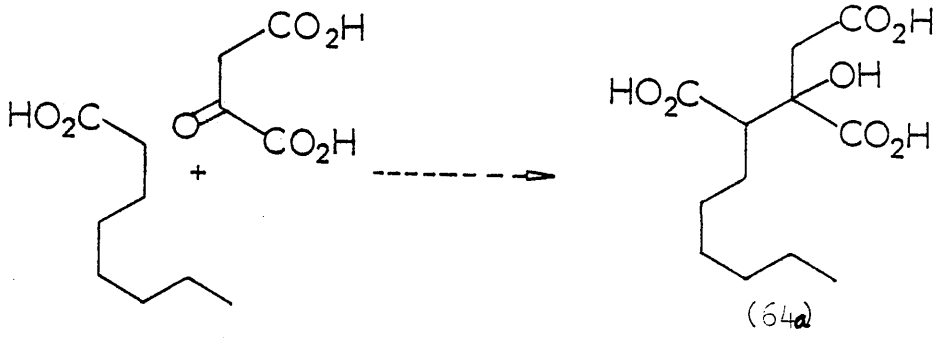
(20)



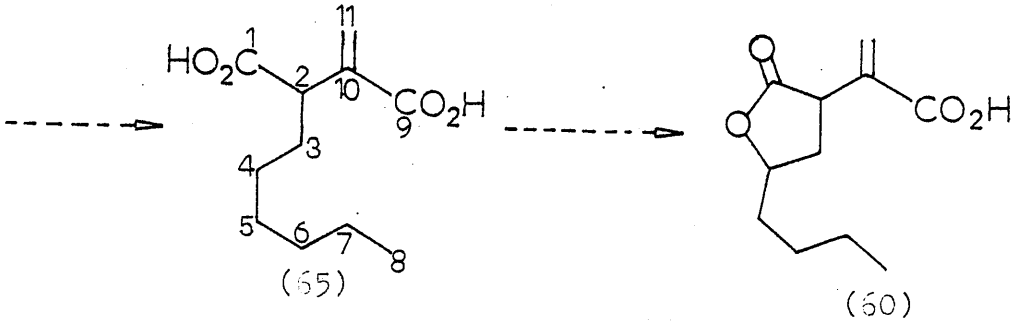
(22)

The metabolites of P.canadense are Type A lactones (c.f. page 14) and the proposed biosynthesis is via the condensation of an octanoic acid derivative and oxalo-acetic acid to give n-hexylcitric acid (64a) (Scheme 3). Evidence for this biosynthesis has been obtained from extensive incorporation

SCHEME 8

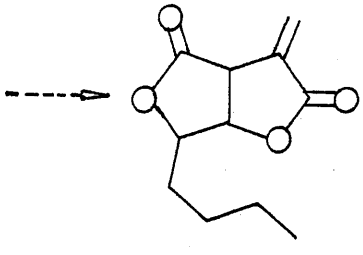


(64a)



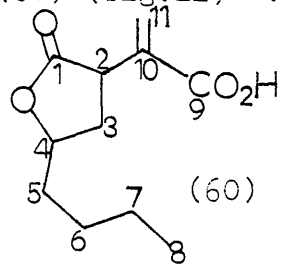
(65)

(60)



(2)

studies on P.canadense using both radio- and ^{13}C-labelled precursors. [2-^{14}C] labelled acetate was incorporated fairly specifically into the fatty acid derived position (C-1 to C-8) of canadensic acid (60) (fig.22)[73].

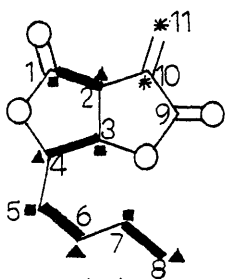


(fig.22) (60)

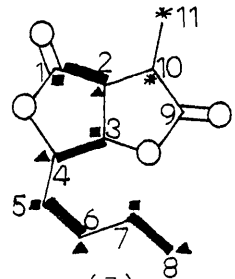
Kuhn-Roth oxidation showed, by comparing the activity of C-7 and C-8 with that of C-10 and C-11, that only 2-3% of the label was found in the C_3 unit as opposed to 20-30% in the fatty acid carbons. Incorporation studies on P.canadense using ^{3}H-labelled succinic acid[73] gave canadensic acid (60) which contained 18% of its activity in the terminal methylene group. This of course implies that there has been a degree of randomisation of label but these results are in accord with biosynthesis of canadensic acid as in (Scheme 8).

Incorporation studies on P.canadense using [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] acetate[74] clearly showed C-1 to C-8 of canadensolide (2), dihydrocanadensolide (3) and canadensic acid (60) to be derived via the acetate-malonate pathway, while the intact incorporation of [2,3-$^{13}C_2$] succinate[74] showed C-9, C-10 and C-11 to originate in a C_4 acid, such as oxaloacetate.

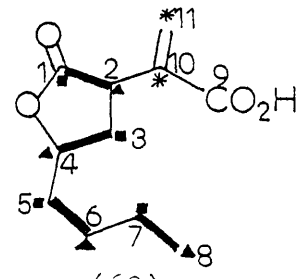
These results also confirm a biosynthesis as in (Scheme 8) and they are summarised in (fig.23). In order to verify that



(2)



(3)



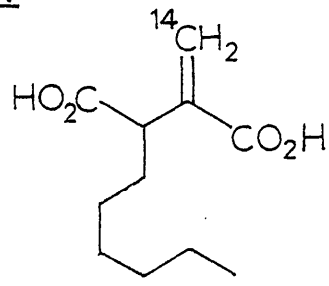
(60)

■ [1-^{13}C] acetate ▲ [2-^{13}C] acetate ▬ [1,2-$^{13}C_2$] acetate
* [2,3-$^{13}C_2$] succinate

(fig.23)

the metabolites of P.canadense are indeed biosynthesised as in (Scheme 8), the synthesis of the key intermediate 2,3-dicarboxynon-1-ene (∝-n-hexylitaconic acid) (65) was studied with a view to feeding a suitably labelled sample to P.canadense. ∝-n-Hexylitaconic acid (65) was chosen for reasons which have already been discussed (pages 23-24).

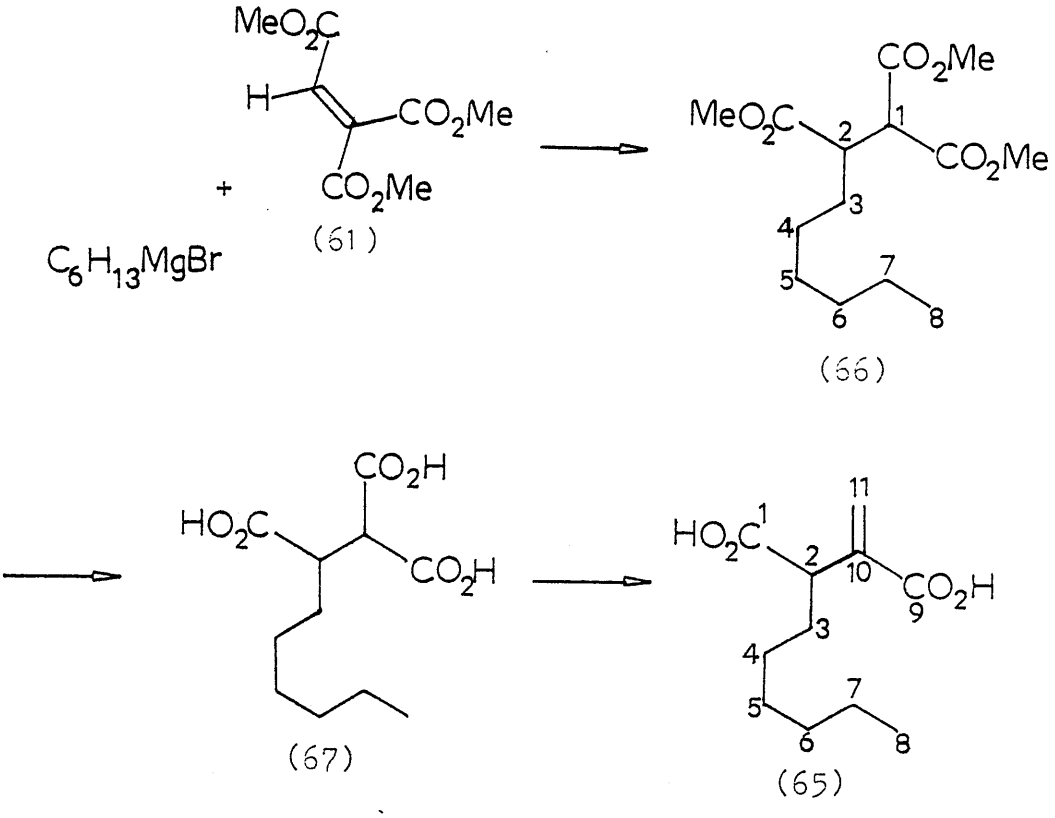
The molecule was synthesised using a modification of the ∝-n-butylitaconic acid (59) preparation via 1,1,2-tricarbomethoxyethene (61) and n-hexyl magnesium bromide (Scheme 9). The initial Cu(1)Cl catalysed 1,4 addition reaction between (61) and n-hexyl magnesium bromide was found to occur more easily in ether than in the previously used tetrahydrofuran despite the poorer solubility of (61) in ether. Hydrolysis of the resulting 1,1,2-tricarbomethoxyoctane (66) afforded the corresponding triacid (67) which, on treatment with aqueous formaldehyde in aqueous dimethylamine gave ∝-n-hexylitaconic acid (65). The use of ^{14}C formaldehyde in the Mannich reaction, the last step of the synthesis, gave [1-^{14}C]-2,3-dicarboxynon-1-ene (fig.24) suitable for use in incorporation studies with P.canadense.



(fig.24)

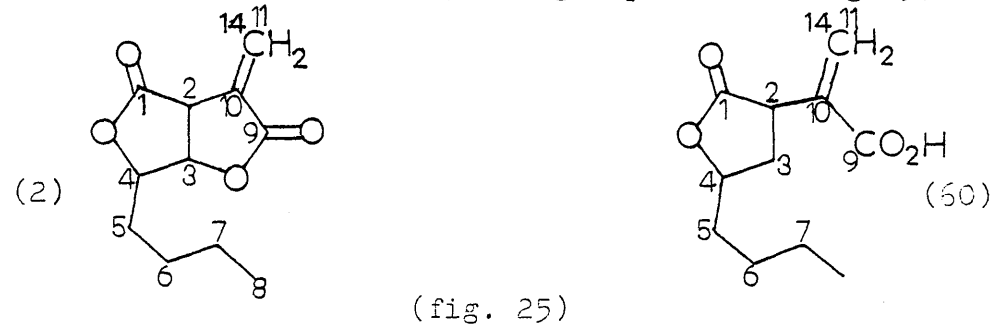
Time/incorporation studies for the production of canadensolide (2), dihydrocanadensolide (3) and canadensic acid (60) by P.canadense had previously been carried out[75] using [1-^{14}C] sodium acetate. These results were used in the present work and [1-^{14}C] -2,3-dicarboxynon-1-ene (fig.24) was administered to cultures of P.canadense in three pulses at 24hr. intervals so that, if there were any degradation

SCHEME 9


MeO2C
H
CO2Me
CO2Me
+
C6H13MgBr
(61)
CO2Me
MeO2C
2
1
CO2Me
3
4
5
7
6
8
(66)
CO2H
HO2C
CO2H
(67)
11
HO2C
1
2
10
CO2H
9
3
4
5
7
6
8
(65)

of the precursor by the organism a pool of labelled precursor at the optimum time for metabolite production would be maintained.

Canadensolide (2), dihydrocanadensolide (3) and canadensic acid (60) isolated from the P.canadense contained 20.6%, 11.9% and 33.6% of the radioactivity administered respectively. Ozonolysis of canadensolide (2) and of canadensic acid (60) in each case gave formaldehyde, isolated and counted as its dimedone derivative, containing all the activity. Hence the activity in these metabolites is located entirely in the exocyclic methylene group (C-11) (fig.25).



(fig. 25)

∝-n-Hexylitaconic acid (65) is thus efficiently and specifically incorporated into canadensolide (2) and canadensic acid (60) and the high level of incorporation into dihydrocanadensolide (3) can also be assumed to be specific. This is compelling evidence that this acid (65) is a true precursor of all three metabolites as suggested in Scheme 3.

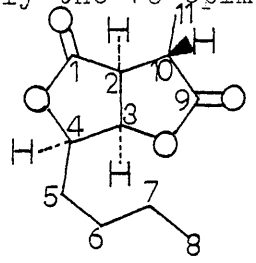
This experiment also gave access to biosynthetically labelled [11-^{14}C] canadensolide and [11-^{14}C] canadensic acid (fig.25) which were used in an attempt to clarify the later stages of canadensolide and dihydrocanadensolide in P.canadense.

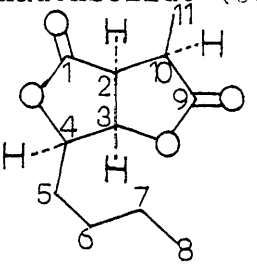
[11-^{14}C] canadensolide was administered in three 24hr. pulses to cultures of P.canadense and the dihydrocanadensolide isolated was found to have an incorporation of 0.86% of the ^{14}C activity administered. Although there would appear to be a link between canadensolide (2) and dihydrocanadensolide (3)

on the basis of this result, the incorporation is extremely small compared to the ca. 20% expected on the basis of the previous results and another explanation of the observed result must be considered.

In the purification of canadensolide, dihydrocanadensolide tends to be a persistent impurity and samples apparently pure according to m.p. are revealed by ^{1}H-N.M.R. to contain 5-10% of this impurity. If, in the present case, the ^{14}C canadensolide fed contained as little as 1% of ^{14}C dihydro-canadensolide (undetectable by m.p. or ^{1}H-N.M.R.) this would result in the observed 'incorporation'. It may be said that, at best, conversion of canadensolide to dihydrocanaden-solide is probably a very minor metabolic pathway of P.canadense. It is indeed possible that the biosynthesis of dihydrocanadensolide (3) could proceed via hydroxylation at C-4 and lactonisation of dihydro-isocanadensic acid (62) already known as a minor metabolite of P.canadense.

Moreover in chemical hydrogenation of canadensolide, reduction occurs from the least hindered side to give exclusively the 10-epimer of dihydrocanadensolide (68)[76].

(3) 

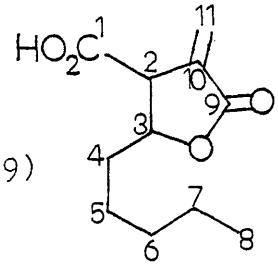
 (68)

It therefore seems not only possible but likely that the biosynthesis of dihydrocanadensolide does not involve reduction of canadensolide. The production of canadensic acid along with canadensolide raises the possibility that hydroxyl-ation of ∝-n-hexylitaconic acid (65) at C-4 and lactonisation to give canadensic acid might be the first steps on the route to canadensolide.

P.canadense to which [11-^{14}C] -canadensic acid (fig.25)

had been administered, was found in this particular fermentation to produce neglgible amounts of dihydrocanadensolide (3). Canadensolide (2) was, however, obtained but was found to contain no ^{14}C activity, suggesting that the canadensic acid (60) is not a precursor of canadensolide (2). If this is so, a hydroxylation at C-3 rather than at C-4 of the ∝-n-hexylitaconic acid (65) could be the first stage on the route to canadensolide and a possible intermediate would be the acid (69) isomeric with canadensic acid (60).

(69)



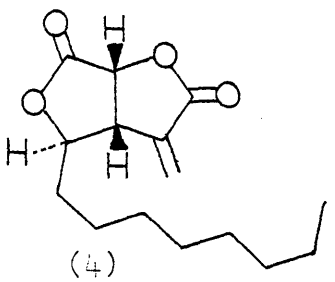
Chapter 5

Discussion:

4. Avenaciolide: Incorporation Studies using A.avenaceus and Attempted Synthesis of Potential Biosynthetic Intermediates.

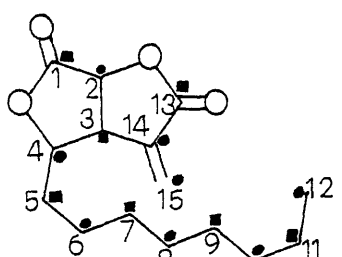
Avenaciolide: Incorporation Studies using A.avenaceus and Attempted Synthesis of Potential Biosynthetic Intermediates.

The fungal antibiotic avenaciolide (4), was first isolated from A.avenaceus in 1963[77].



(4)

It was proposed that this Type B metabolite, closely related structurally to ethisolide (1), is biosynthesised from a β-keto acid and succinic acid [77] (Scheme 10). Supporting incorporation experiments using [1-^{13}C] and [2-^{13}C] acetate on A.avenaceus[60] gave avenaciolide (4) showing a high degree of incorporation into the fatty acid portion. There was also however a detectable incorporation into the C_3 unit (fig.26)



■ [1-^{13}C] acetate

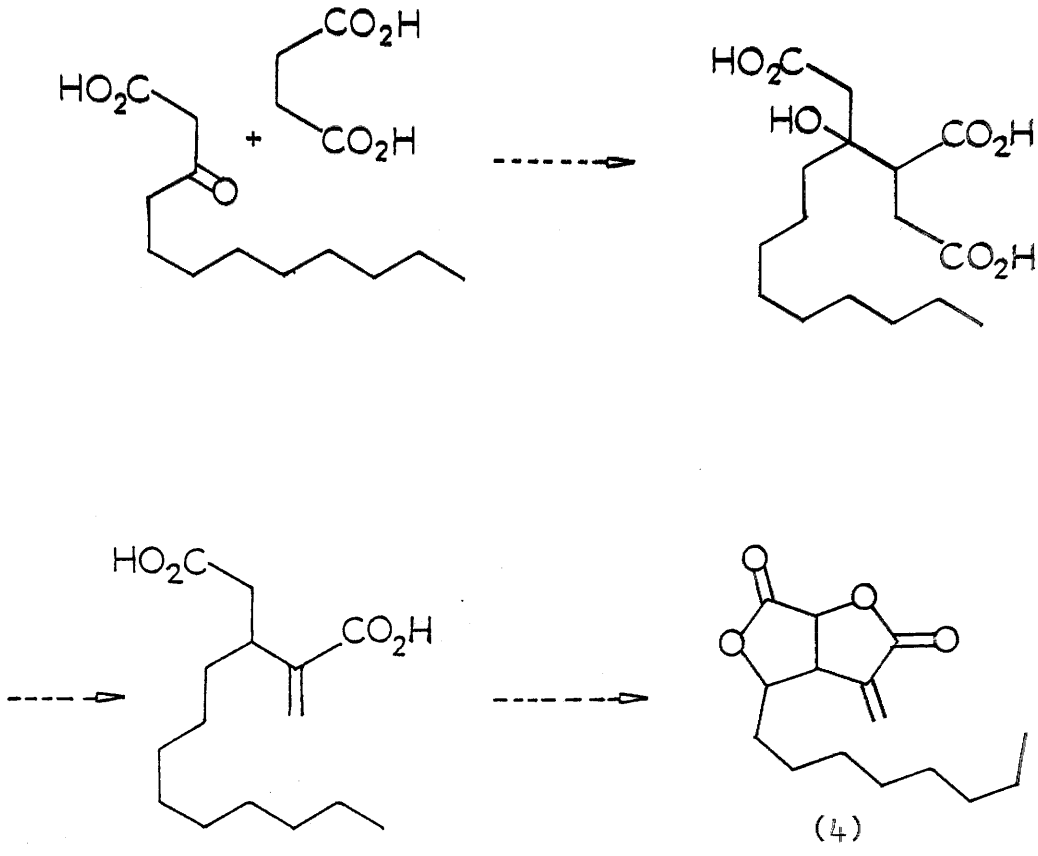
● [2-^{13}C] acetate

(fig.26)

particularly noticeable as a pair of weak doublets associated with C-14 and C-15. This was attributed to incorporation into succinate via the TCA cycle (Scheme 4). Incorporation of acetate into succinate would give singly labelled succinate as indicated in Scheme 4 and this would be expected to give some enrichment of all three carbons in the C_3 unit. Indeed the published spectra show evidence of a modest enhancement of the appropriate signals although this is not commented on[78,79]. The pairs of doublets could only arise via 2,3-doubly labelled succinate which would imply the operation of a second turn† of the Krebs cycle involving accidental condensation of

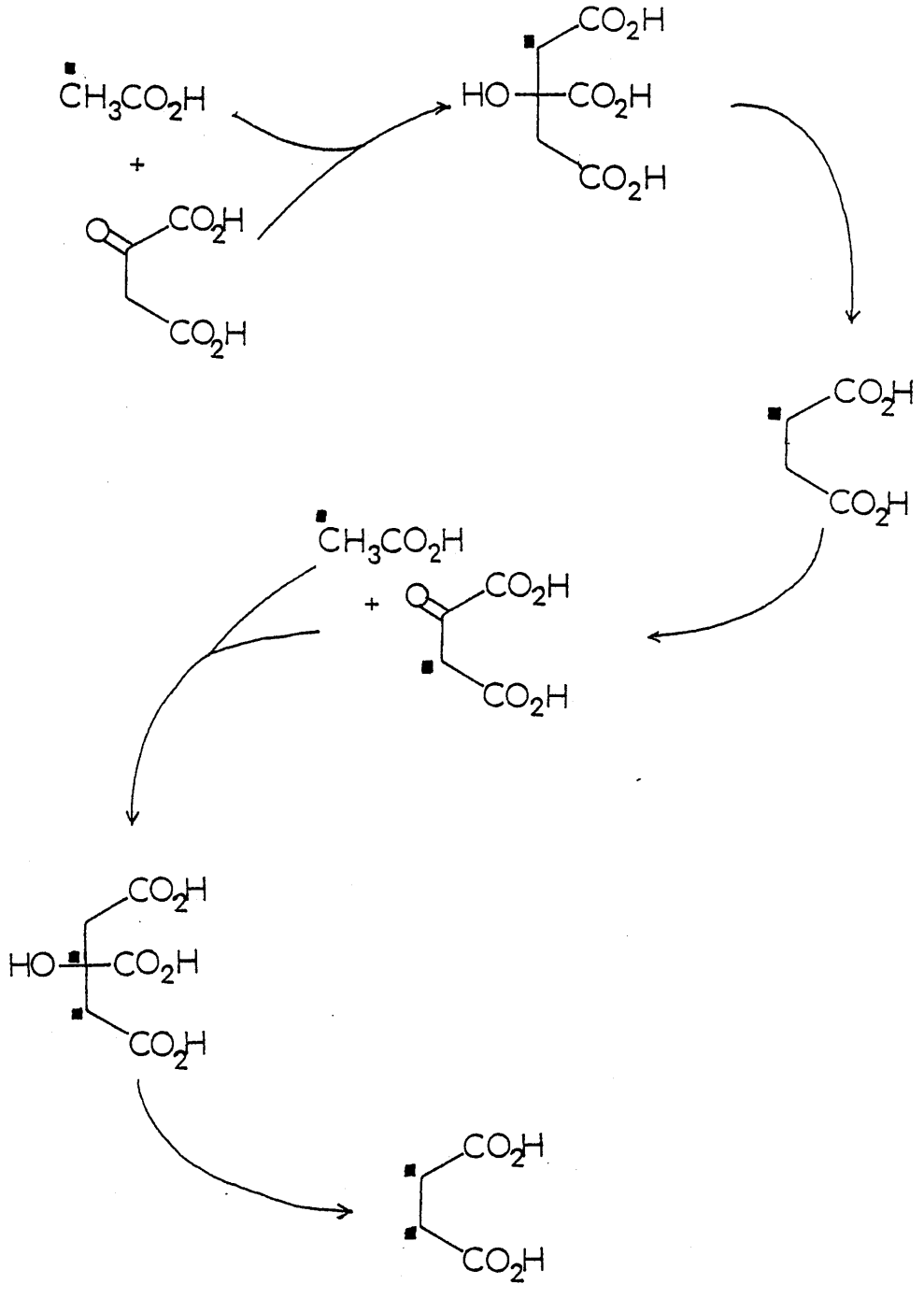
† see Scheme 10a

SCHEME 10



(4)

SCHEME 10a

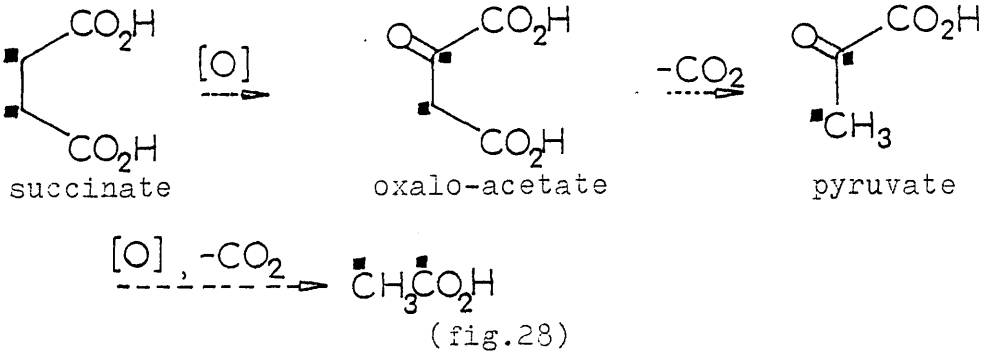


■ ^{13}C label

[2-^{13}C] acetate molecules with [2-^{13}C] succinate molecules to give doubly labelled citric acid and subsequently [2,3-$^{13}C_2$] succinate. The apparent level of incorporation of doubly labelled succinate compared with singly labelled succinate (arising respectively from two and one turns of the Krebs cycle) seem somewhat surprising and in the present work it was decided to seek more direct evidence of the involvement of succinic acid in the C_3 unit by feeding the [2,3-$^{13}C_2$] labelled acid.

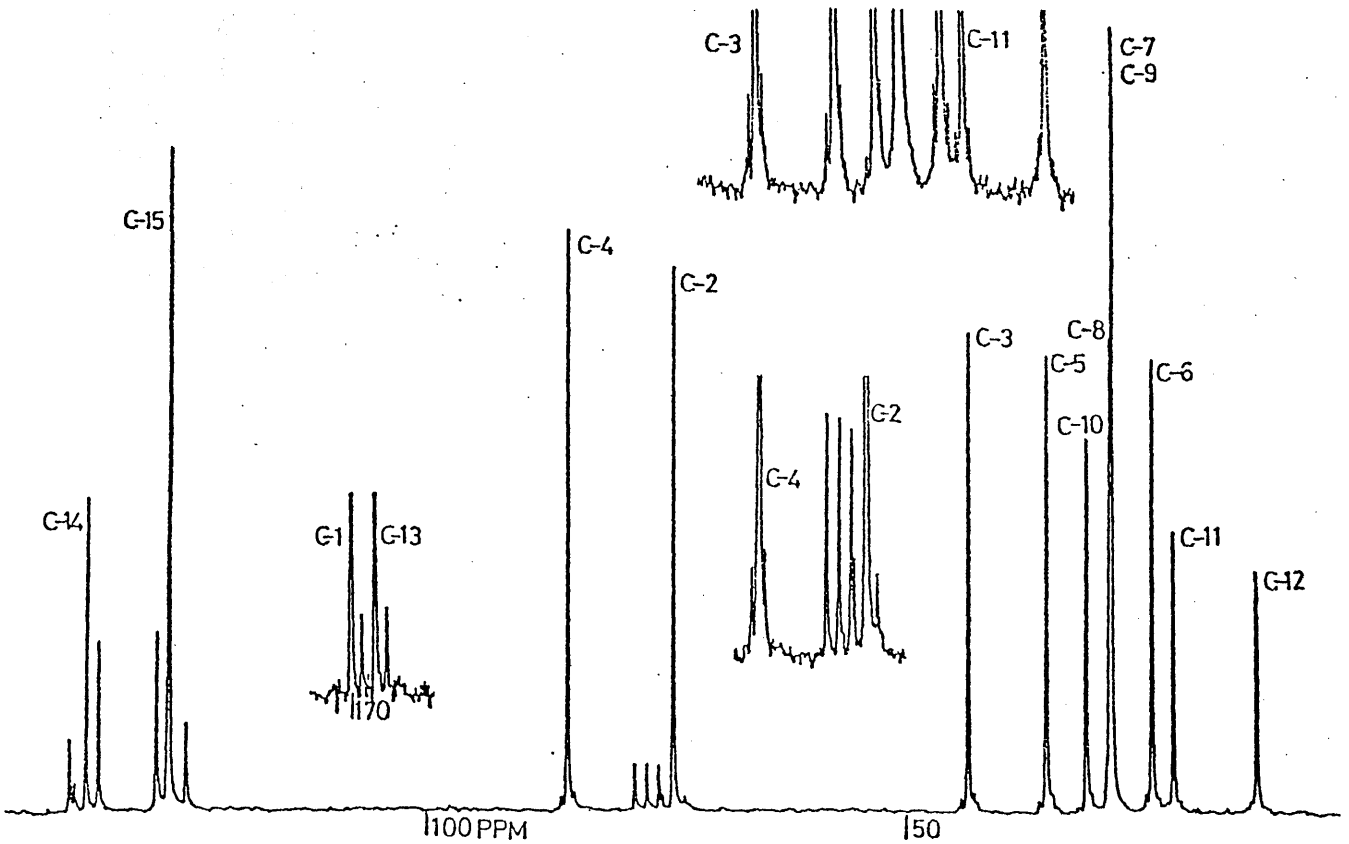
Time/incorporation studies on A.avenaceus were first carried out using [1-^{14}C] sodium acetate and it was found that 24hr. pulsed inoculations on the seventh, eighth and ninth days of growth followed by extraction of the broth after acidification on the fourteenth day of growth gave the highest incorporation of acetate into avenaciolide (4).

This result was used in an incorporation study on A.avenaceus with [2,3-$^{13}C_2$] succinate and an intact incorporation into C-14 and C-15 of avenaciolide was found. Also clearly visible in the ^{13}C-N.M.R. spectrum (fig.27) was incorporation of ^{13}C label into the fatty acid moiety via [1,2-$^{13}C_2$] acetate. This incorporation probably comes from acetate derived from succinate (fig.28) and is similar to

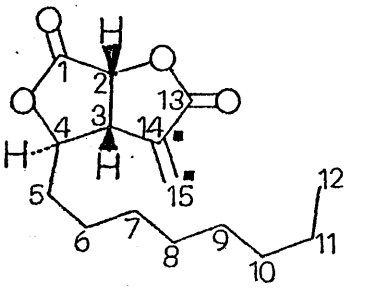


(fig.28)

that noticed in [2,3-$^{13}C_2$] succinate incorporations into ethisolide (1)[61] and canadensolide (2) and canadensic acid (60)[62].



(4)



■ [2,3-$^{13}C_2$] succinate

(fig. 27)

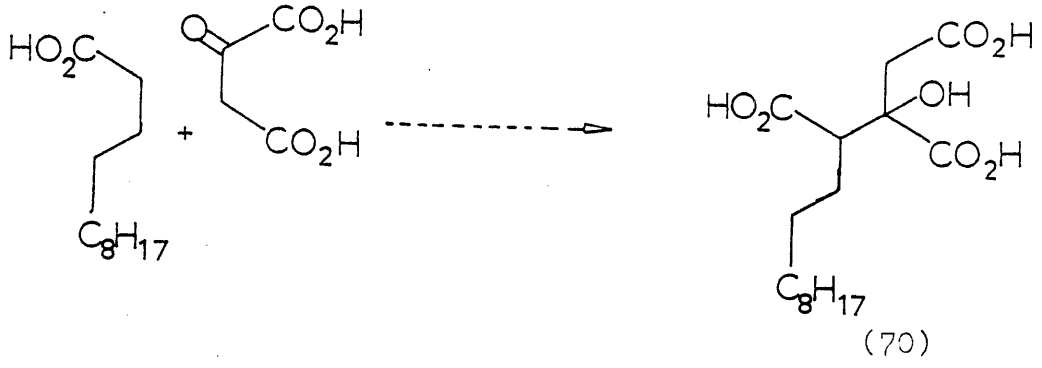
This result is complementary to the [1-^{13}C] and [2-^{13}C] acetate incorporations already described, showing that avenaciolide (4) is derived biosynthetically from a fatty acid derivative and a TCA cycle acid (probably oxalo-acetate). In view of the results described earlier for ethisolide (1) it seems probable that avenaciolide (4) is formed via an alkylcitric acid intermediate with subsequent re-arrangement of the C_3 unit from the α to the β position of the fatty acid (Scheme 11).

The key intermediate is n-decylcitric acid (70) and the re-arrangement might be either of α-OH-n-decylitaconic acid (71) or of α-n-decylitaconic acid (2,3-dicarboxyundec-1-ene) (72). In order to test this proposal, it is necessary to synthesise n-decylcitric acid (70) or, more conveniently, one of the derived itaconic acids (71) or (72) and show that it is incorporated intact into avenaciolide (4) by cultures of A.avenaceus.

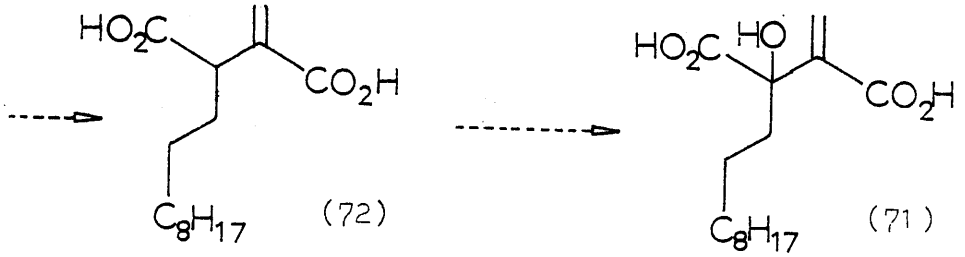
For the reasons already discussed (page 23-24) 2,3-dicarboxyundec-1-ene (α-n-decylitaconic acid) (72) was chosen for synthesis. The route followed was a modification of that already used earlier for α-n-butylitaconic acid (59) and α-n-hexylitaconic acid (65) and involved copper (I) catalysed 1,4 Michael addition of n-decyl magnesium bromide to 1,1,2-tricarbomethoxyethene (61) (Scheme 12). Hydrolysis of the resulting 1,1,2-tricarbomethoxydodecane (73) would give the corresponding triacid which, on treatment with aqueous formaldehyde in dimethylamine would give the desired 2,3-dicarboxyundec-1-ene (72). The use of ^{14}C formaldehyde in the final step of the synthesis would give [1-^{14}C]-2,3-dicarboxyundec-1-ene for incorporation studies with A.avenaceus.

The desired n-decyl magnesium bromide could not be prepared using metallic magnesium and 1-bromodecane in anhyd.

SCHEME 11

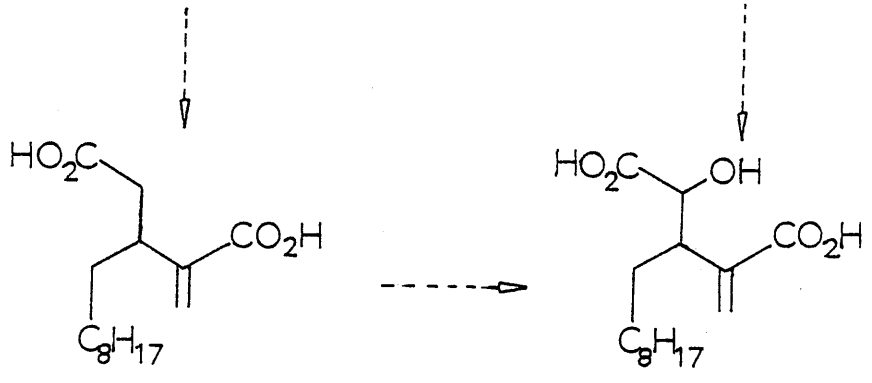


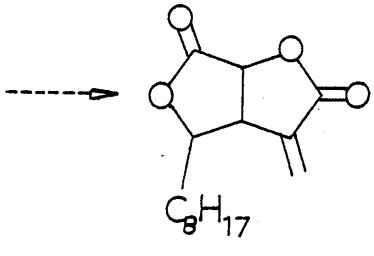
(70)



(72)

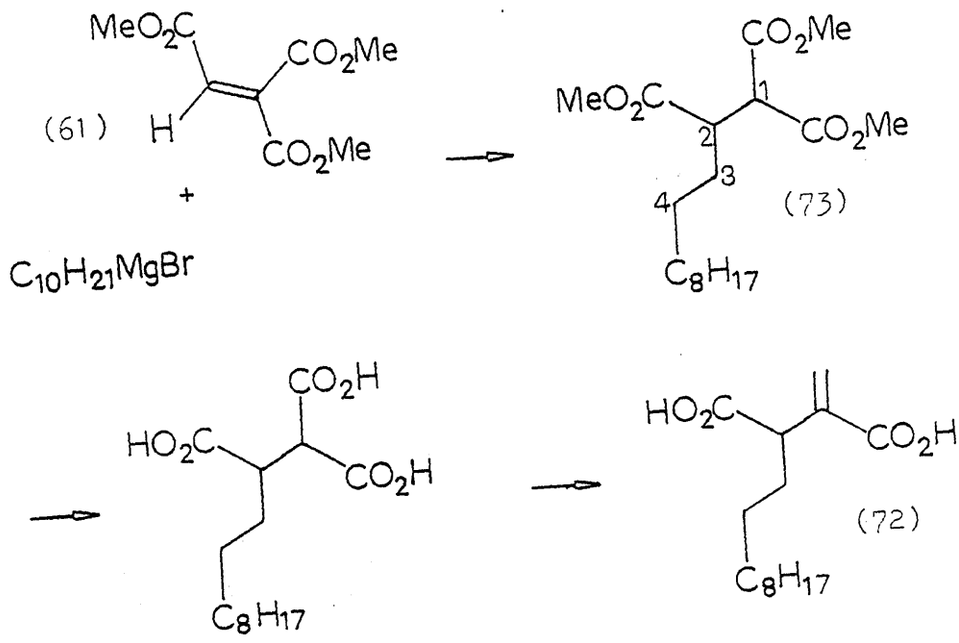
(71)



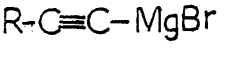


(4)

SCHEME 12


MeO2C
CO2Me
(61)
H
CO2Me
+
C10H21MgBr
CO2Me
MeO2C
1
2
3
4
CO2Me
(73)
C8H17
CO2H
HO2C
CO2H
C8H17
HO2C
CO2H
(72)
C8H17

tetrahydrofuran. Activation of the magnesium using ethylene bromide[30] did enable the Grignard reagent to be produced in anhydrous tetrahydrofuran, but the resulting addition reaction was unsuccessful. It had been already noted in the related synthesis of n-hexylitaconic acid (65) (page 31) that ether was a more suitable solvent for the addition, and when the reaction was repeated using these conditions, 1,1,2-tricarbomethoxydodecane (73) was isolated in low yield (ca.7%). Attempts to maximise the yield by heating gave only polar products. The greater steric hindrance found with the 1-bromodecane is the probable cause of the low yields in this reaction. A more attractive method of synthesis of (73) might be to use 1-decynyl magnesium bromide (74) in the Michael addition[66], thus reducing the steric hindrance, but lack of time prevented further studies on this system.



(74)

$R = C_8H_{17}$

Chapter 6

Discussion:

5. Stereochemistry

   (i) Avenaciolide and Ethisolide

   (ii) *P.canadense* Metabolites

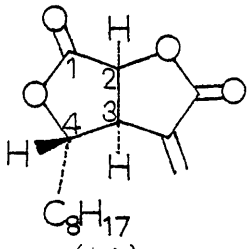
      (a) Canadensolide and Dihydrocanadensolide

      (b) Canadensic Acid

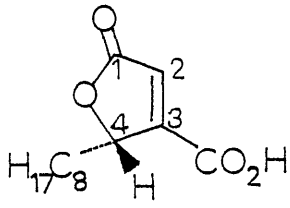
An exact knowledge of the stereochemistry of the metabolites studied here is essential for the interpretation [2-^{13}C, 2-$^{2}H_3$] acetate incorporation results discussed later.

(i) Avenaciolide and Ethisolide

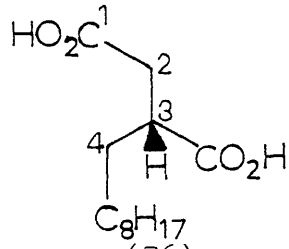
The initial assignment of cis relative stereochemistry to avenaciolide (4) was made on the basis of ^{1}H-N.M.R. coupling constants[77] . Degradation to the butenolide (75), followed by hydrogenation (assumed to occur at the least hindered face) gave R-(+)-nonylsuccinic acid (76).



(4A)



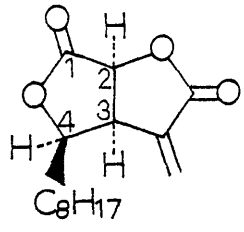
(75)



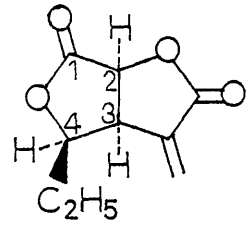
(76)

These two results enabled the absolute stereochemistry of avenaciolide to be given as (2S,3S, 4S) (4A).

The later isolation from A.avenaceus of a minor metabolite 4-iso-avenaciolide (77), whose absolute stereochemistry was given as (2S,3S,4R) (77A) by comparison of ^{1}H-N.M.R. coupling constants with those of avenaciolide (4)[44], enabled the absolute stereochemistry of the closely related metabolite ethisolide (1), isolated at the same time from P.decumbens[44], to be given as (2S,3S,4R) (1A).



(77A)

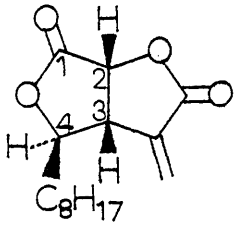


(1A)

This assignment was made on the basis of similarities in the ^{1}H-N.M.R. spectra of (77) and (1) and their optical rotations (viz. $[\alpha]_D$ = -154° for 4-iso-avenaciolide and $[\alpha]_D$ = -214° for

ethisolide).

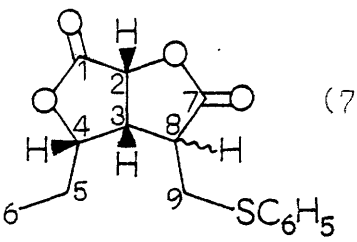
However, in 1975, two similar stereospecific syntheses of natural avenaciolide from D-glucose were reported[81,82] which necessitated revision of the absolute stereochemistry of natural avenaciolide to (2R,3R,4R) (4).



(4)

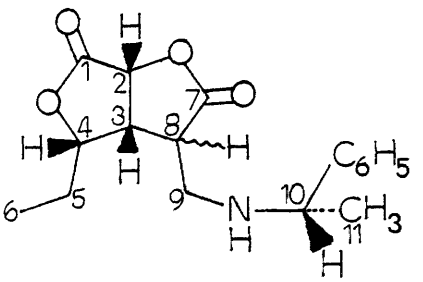
The evidence advanced for the stereochemistry of 4-iso-avenaciolide (77) only established that a different stereochemistry existed at C-4 relative to C-2 and C-3 as compared with avenaciolide (4). The absolute stereochemistry, and that of ethisolide (1) remained to be determined. Accordingly, in the present work, experiments were initiated towards an X-ray crystallographic determination of the absolute stereochemistry via a suitable derivative.

An initial experiment showed that it was possible to add thiophenol to the exo-methylene group of ethisolide under slightly basic conditions (pH 9)[83] to give 9-(thiophenyl)-8,9-dihydroethisolide (78).



(78)

This discovery was used in the addition of (S)-α-phenylethylamine to ethisolide (1)[84] giving 9-((S)-α-phenylethylamino)-8,9-dihydroethisolide (79).



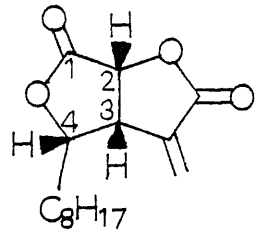
(79)

This showed the expected spectroscopic characteristics e.g. I.R. absorptions at 1770 cm^{-1} and 1750 cm^{-1} (saturated lactone) and at 3500 cm^{-1} and 3400cm^{-1} (N-H).

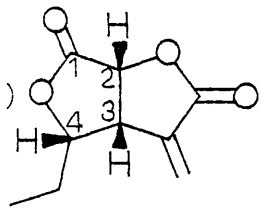
It cannot be assumed that addition would proceed exclusively from the least hindered face since addition of diazomethane to ethisolide is known to give a mixture of pyrazolines[61]. However, the product (79) crystallised spontaneously with a sharp, stable m.p. 224°C and there was no obvious doubling of signals in the ^{1}H-N.M.R. or I.R. spectrum.

Before the configuration at C-8 could be established and the X-ray analysis initiated, a stereospecific synthesis♦ of natural 4-<u>iso</u>-avenaciolide (77) from <u>D</u>-glucose was reported[85] establishing the absolute stereochemistry as (2R,3R,4S) (77).

(77)



(1)



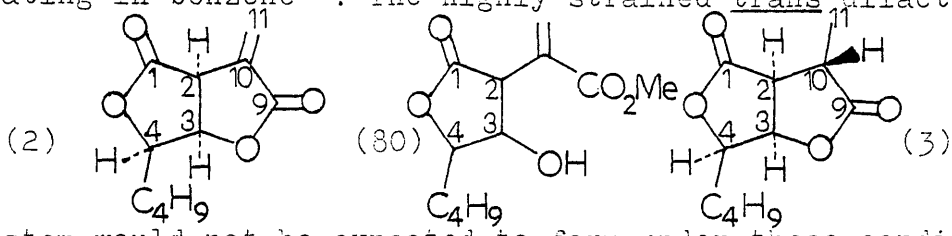
Since, as pointed out previously, the relative and absolute configurations of 4-<u>iso</u>-avenaciolide (77) and ethisolide are almost certainly the same, the absolute stereochemistry of ethisolide can be assigned as (2R,3R,4S) (1).

♦ These syntheses are outlined later in Appendix 2.

(ii) P.canadense Metabolites

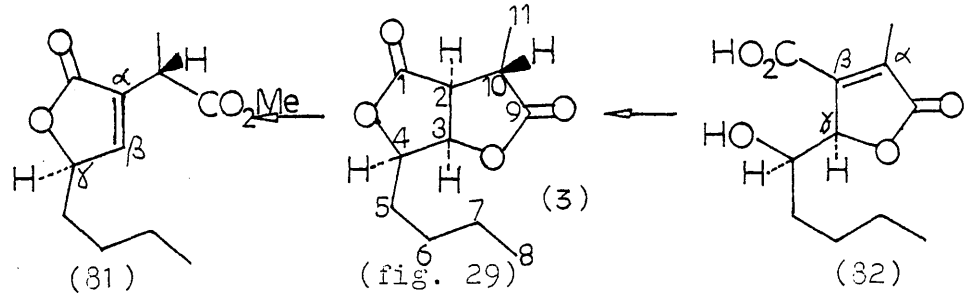
(a) Canadensolide and Dihydrocanadensolide

The original assignment of the relative C-3/C-4 stereochemistry of canadensolide (2)(trans) was made on the basis of ^{1}H-N.M.R. coupling constants. The cis relationship of the bridgehead hydrogens was confirmed by the observation that the hydroxy-ester (80), obtained by methanolysis of canadensolide (2), readily re-cyclised to canadensolide on heating in benzene[86]. The highly strained trans dilactone



system would not be expected to form under these conditions. Similar relative stereochemistry was deduced for dihydrocanadensolide (3) from ^{1}H-N.M.R. coupling constants at the same time.

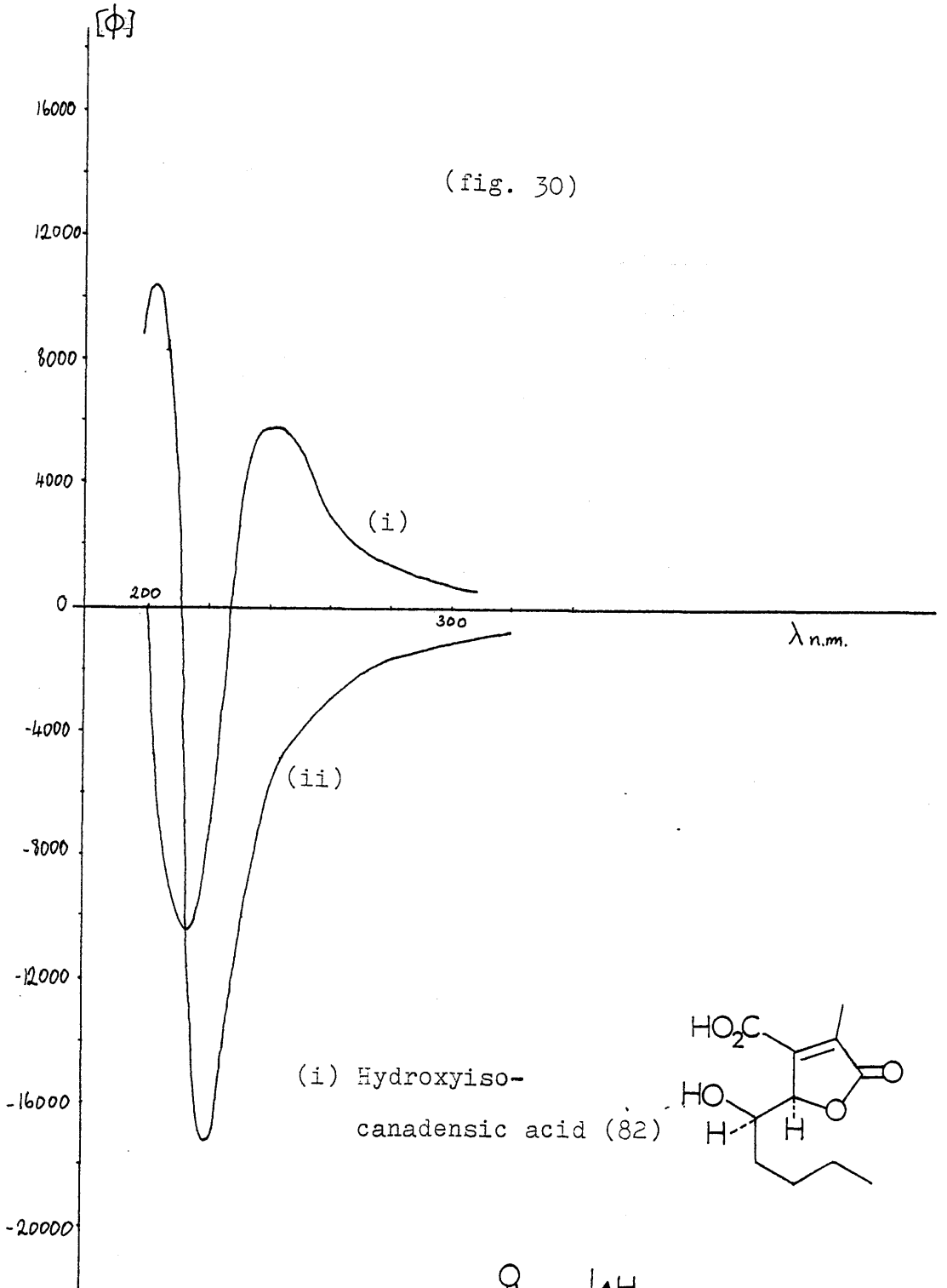
The C-3/C-4 relative stereochemistry of dihdrocanadensolide (3) and hence canadensolide (2) was reversed to cis on the basis of the O.R.D. curves of the two chemically related butenolides (81) and (82) whose γ-hydrogens correspond to H-4 and H-3 of dihydrocanadensolide (3) respectively (fig. 29)[86].



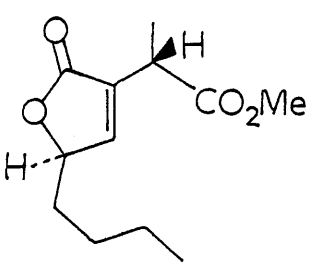
(fig. 29)

These butenolides gave O.R.D. curves (fig. 30) which appear to show Cotton Effects of opposite sign, and on these grounds H-3 and H-4 of dihydrocanadensolide were considered to be cis[86]. This was established unequivocally by independant work involving synthesis of the (±) epimers of .

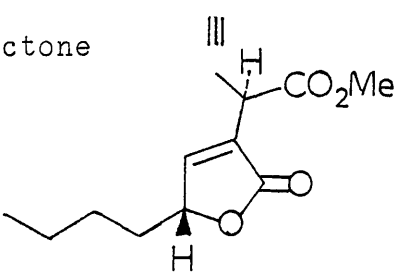
(fig. 30)



(i) Hydroxyiso-canadensic acid (82)



(ii) ester lactone (81)

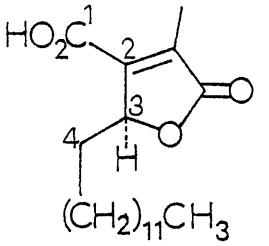


canadensolide (2) with H-4 respectively cis and trans to H-2 and H-3. The cis isomer was found to be identical spectroscopically to the natural material[66].

At the time of the present work however, there remained some doubts about the absolute configuration of canadensolide (2) and dihydrocanadensolide (3). Determination of the absolute configuration of the alcohol group in the methyl ester of (32) using the Horeau method with ∝-phenylbutyric anhydride[37] showed the absolute configuration to be R as indicated in (32). Since this is convertible into dihydrocanadensolide (3) this correctly gives the configuration of the latter as shown (fig.29, (3)). Unfortunately, more weight was given to an apparently conflicting result from C.D. studies as follows.

Boll[38] assigned the absolute stereochemistry of (-) lichesterinic acid (20) as 3S on the basis of the negative

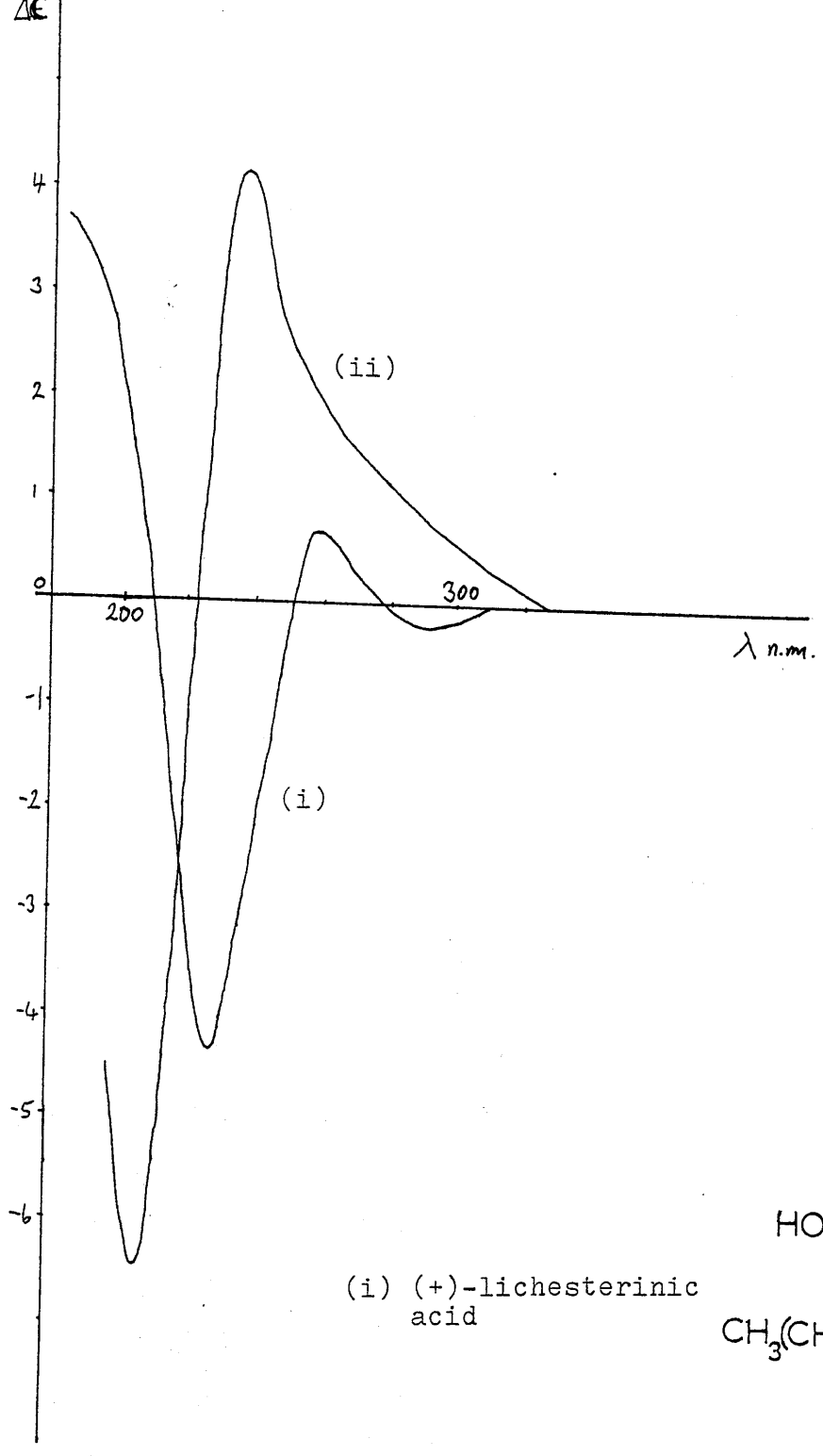
(20)



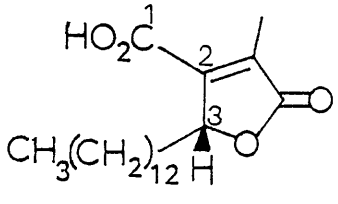
C.D. in the region 240-270 nm., by comparison with the C.D. shown by a number of simple butenolides of known absolute configuration (eg. tetronic acids). Since the methyl ester of butenolide (32) showed positive C.D. in this region (fig. 31(ii)), this was misinterpreted[36] as indicating (32) to have the opposite configuration to S-(-)-lichesterinic acid (20). If this were correct, dihydrocanadensolide (3) and, if the link between this and canadensolide is assumed, canadensolide (2) would have (2R,3S,4S) stereochemistry (the opposite to that shown in (2) and (3)).†

These C.D. results can however be reviewed in the light

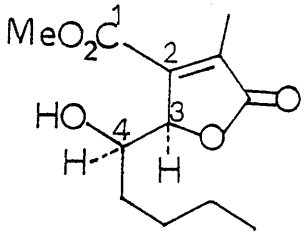
† This was communicated by N.J. McCorkindale to A. Yoshikoshi who quoted it in J. Org. Chem., (1975), 40, 1951.



(i) (+)-lichesterinic acid

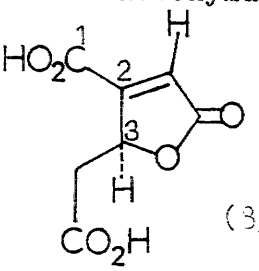


(ii) Methyl hydroxyiso-canadensic acid (82A)



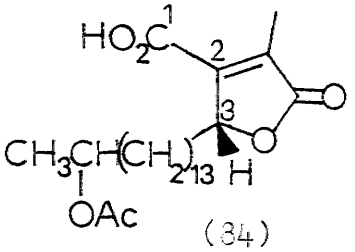
(fig. 31)

of more recent C.D. studies on lactones. Semi-empirical rules have been drawn up for the C.D. of butenolides based on qualitative MO-theory and it has been pointed out that the (-)-lichesterinic acid correlation made by Boll was fortuitously correct although a fumarate-type chromophore was compared to a butenolide chromophore[89]. An authentic model system is provided by the carboxymuconolactone (33) whose

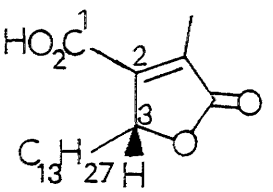


(33)

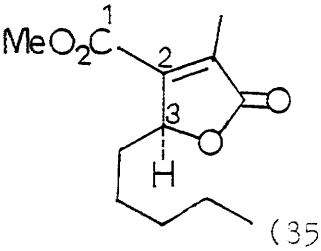
structure has been established by X-ray crystallography[90]. This has been recently used to assign the configurations of (+)-acetylisomurolic acid (34) and (+)-lichesterinic acid (20A).[91]



(34)

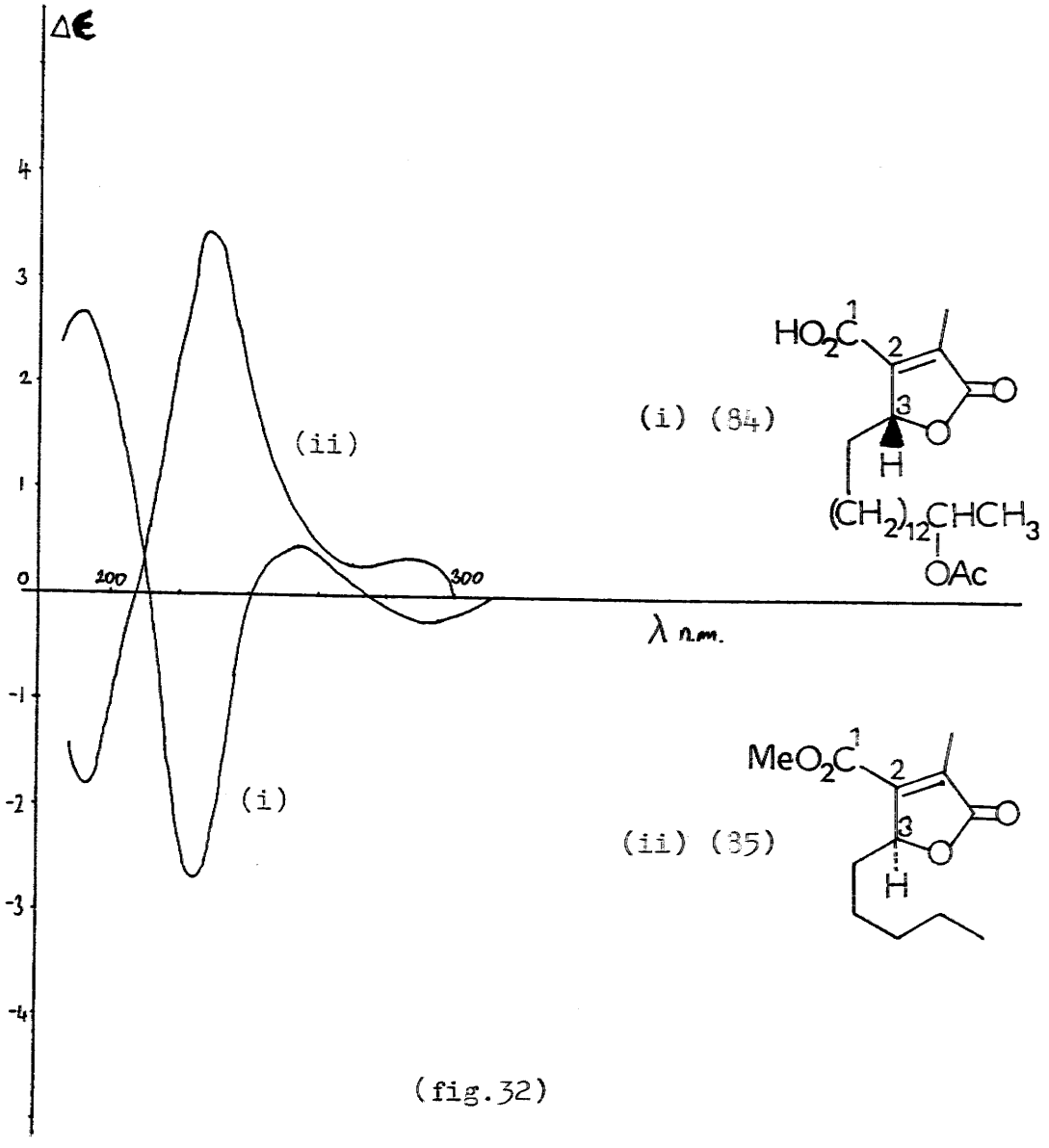


(20A)



(35)

The methyl ester of iso-canadensic acid (35), shows C.D. whose type is the mirror image of that shown by (+)-lichesterinic acid (20A) and (+)-acetylisomurolic acid (34) (figs. 31(i) and 32) and therefore has the S configuration at position 3. It may be noted however, that the curve does not cross the zero line in the region of 250nm but shows a minimum at 275nm which corresponds to a negative maximum. As already noted, the C.D. curve of the methyl hydroxyisocanadensate (fig.31(ii)) is positive in this region and it shows a slight inflection rather than a minimum at 275nm. It does, however, appear to be similar to that of methyl isocanadensate (35) in resembling the mirror image of the (+)-lichesterinic acid (20A) curve (fig.31(i)) and indicates the S configuration at position 3. These results are summarised in Table 4, and it is possible



(fig.32)

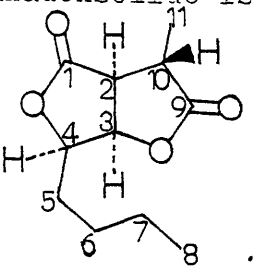
TABLE 4

C.D. of Carboxylactones

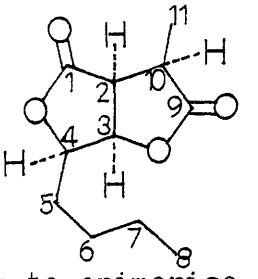
Compound	Config. at C-3	λ n.m./ΔE
(83)	S	315/+0.308; 264/-0.23; 218/+2.48; 190/-2.50
(84)	R	292/-0.07; 256/+0.36; 223/-2.53; 188/+2.58
(20A)	R	292/-0.11; 258/+0.58; 230/-4.15; 188/+3.68
(85)	S	283/+0.35; 275/+0.32; 228/+3.45; 198/-1.75
(82A)	S	330/0; 275inf./+1.15; 243/+4.02; 208/-6.31

that the C.D. curves of the acids will give a closer match in shape to those of (84) and (20A).

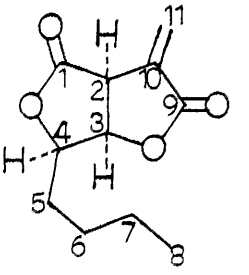
From the foregoing results, it may be deduced that the configuration of dihydrocanadensolide is (2S,3R,4R) (3). In

(3) 

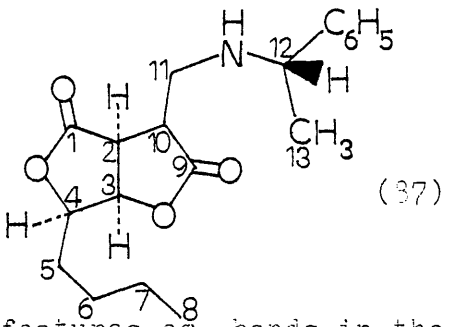
order to extend deductions to the absolute stereochemistry of canadensolide itself, it was necessary that there should be no doubt about the chemical conversion of this into dihydrocanadensolide which had been carried out but without, unfortunately comparing the optical properties of the product with those of the natural material[86]. Hydrogenation of canadensolide (2) using H_2/Pd, expected to occur at the less hindered face of the molecule, gave 10-epi-dihydrocanadensolide (86) as previously reported[86].

(86) 

However, attempts to epimerise the C-10 centre were unsuccessful and only polar products were obtained. A new and improved method of interconversion proved to be reduction of canadensolide with zinc in glacial acetic acid which gave dihydrocanadensolide as the sole product directly, and this was identical in all respects including optical rotation with the natural material. Hence canadensolide (2) has the same absolute stereochemistry as dihydrocanadensolide (i.e. 2S,3R,4R).

(2) 

Before rationalisation of the C.D. spectra was achieved, it was intended to obtain conclusive evidence for the absolute stereochemistry of canadensolide (2) by X-ray crystallography. In order to obtain a derivative in which a centre of known absolute configuration was incorporated, (S)-α-phenylethylamine was added to the exo-cyclic methylene group of canadensolide (2) to give 11-((S)-α-phenylethylamino)-10,11-dihydrocanadensolide[84] (87). This showed the expected



(87)

spectroscopic features eg. bands in the I.R. at 1770 cm^{-1} and 1750 cm^{-1} (saturated lactones) and at 3500 cm^{-1} and 3400 cm^{-1} (N-H).

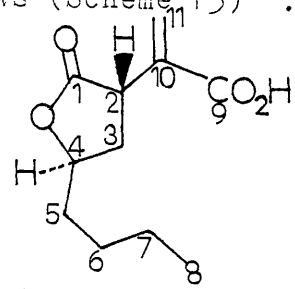
It cannot be assumed that addition would proceed exclusively from the least hindered side since addition of diazomethane is known to give a mixture of pyrazolines (1:1)[86]. However the product (87) appeared to be homogeneous from TLC, and from the sharpness and constancy of the m.p. and also from the lack of obvious doubling of peaks in the I.R. or ^{1}H-N.M.R. spectra.

However, before the configuration at C-10 could be established and the X-ray analysis initiated, a stereospecific synthesis of natural canadensolide from D-glucose was reported establishing the absolute stereochemistry of canadensolide as (2S,3R,4R) (2)[92]. The previously discussed result from C.D. studies or from the Horeau technique is thus substantiated and the X-ray study became unnecessary and was discontinued.

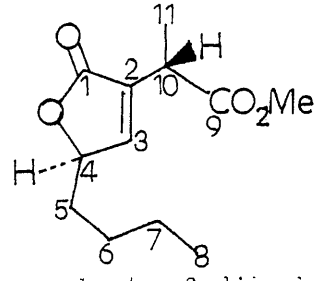
(b) Canadensic Acid

Canadensic acid (60), a metabolite of P.canadense has been shown to have trans stereochemistry relative to C-2 and C-4 as follows (Scheme 13)[93].

(60)

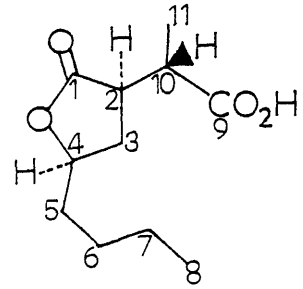


Catalytic reduction of canadensic acid gave a mixture of C-10 epimers of dihydrocanadensic acid (in which, as shown in Scheme 13(i), H-2 and H-4 are trans). This, after esterification, showed a single peak on glc. Evidently the configuration at C-10 does not affect the retention time. Canadensic acid is readily epimerised at C-2 to give an equilibrium mixture with its H-2/H-4 cis isomer. Reduction of this mixture gave a mixture of four dihydrocanadensic acid diastereoisomers (Scheme 13(ii)) which, after esterification, showed two peaks on glc, the one of longer retention time being the same as that observed previously. The new peak was the same in retention time as that of the single diastereoisomer obtained by catalytic reduction of the butenolide (31) (Scheme 13(iii))



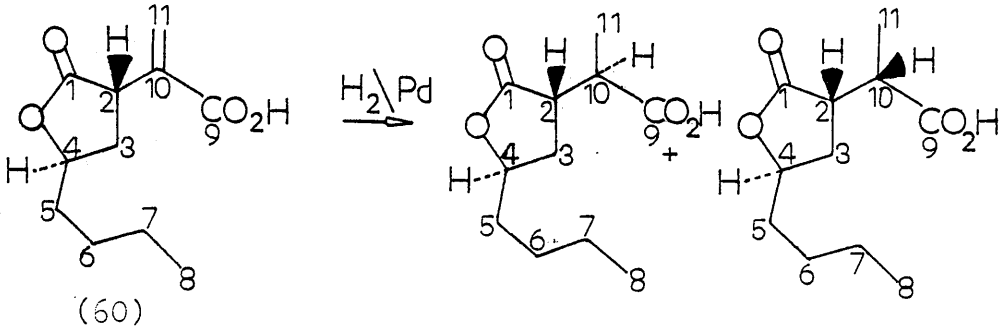
(31)

(a transformation product of dihydrocanadensolide (3)). This reduction would be expected to occur from the least hindered face to give the isomer (38) with H-2 and H-4 cis.



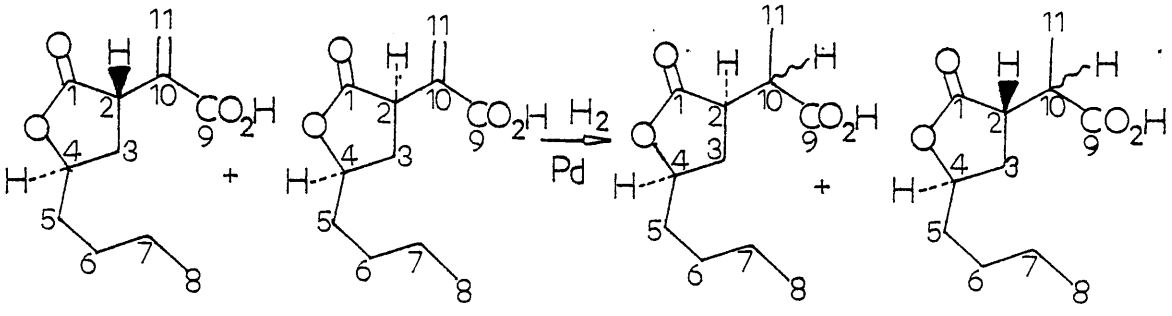
(38)

SCHEME 13

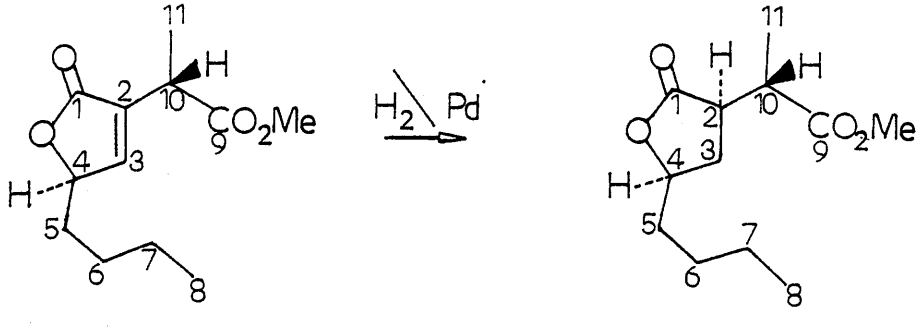


(60)

(i)



(ii)

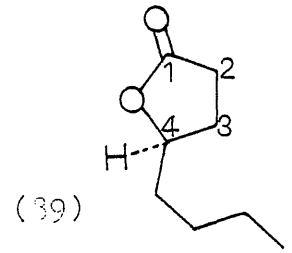


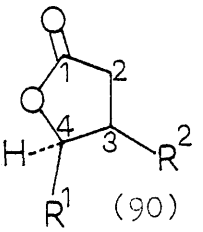
(81)

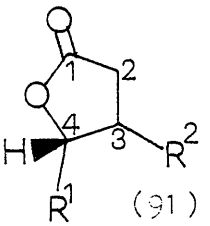
(iii)

It follows that the methyl dihydrocanadensate giving the longer retention time have H-2 and H-4 *trans*, as has canadensic acid (60)[93].

The absolute configuration of canadensic acid was originally assigned on the basis of the negative circular dichroism shown by the sample of γ-caprylolactone (89) obtained by degradation[94]. It has been shown by Klyne *et.al.*[95], that


(89)

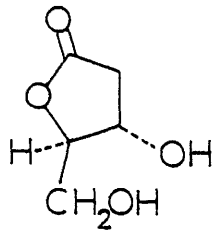
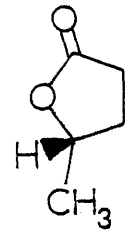
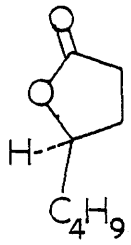

(90)


(91)

lactones of type (90) and (91) give positive and negative lactone Cotton effects respectively, and on these grounds the caprylolactone (89) was considered to have a C-4(S) configuration[94]. This interpretation conflicts with the results of biosynthetic studies which are described later. However the lactones studied by Klyne are inappropriate models for the present case in that they are all conformationally rigid, with the lactone rings fused in the 3- and 4- positions to 5- or 6- membered rings. More recently[96] the C.D. of some simple, conformationally mobile, lactones have been examined providing evidence as follows that the γ-caprylolactone (89) has a 4-(R) configuration as shown.

The (R)-2-deoxysugar lactone (92) shows negative C.D. (Table 4) although it is a type(90) lactone. The first completely valid model for γ-caprylolactone (89) is provided by (S)-γ-valerolactone (93) which gives a weakly positive C.D. curve[97] which is almost the mirror image of that observed for the γ-caprylolactone (Table 5). The latter may therefore be assigned an R-configuration (89) and canadensic acid is therefore (2S,4R) (60).

TABLE 5

COMPOUND	λ n.m./ΔE
(92) 	208/-0.71
(93) 	214/+0.08; 217/+0.04
(89) 	216/-0.17

Chapter 7

Discussion:

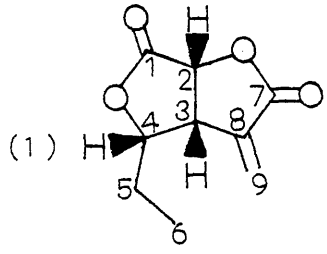
6. Incorporation Studies using [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate

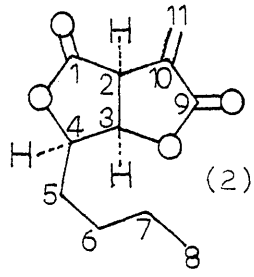
   (i) Fatty Acid Biosynthesis in P.canadense: The Stereochemistry of Double Bond Reduction

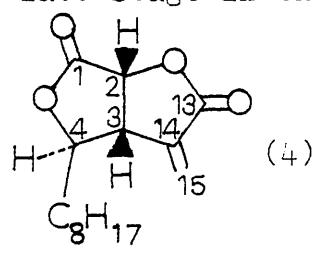
   (ii) Previous Studies on Ethisolide and Dihydrocanadensolide

   (iii) Dihydrocanadensolide, Canadensolide, Canadensic Acid and Avenaciolide

The retention or loss of acetate-derived hydrogen atoms in the biosynthesis of ethisolide (1), canadensolide (2) and avenaciolide (4) reflects a number of features of the biosynthetic sequences involved and it will be seen how this affords useful information about the stereochemistry of the hydroxylation at C-4 which occurs at a late stage in the







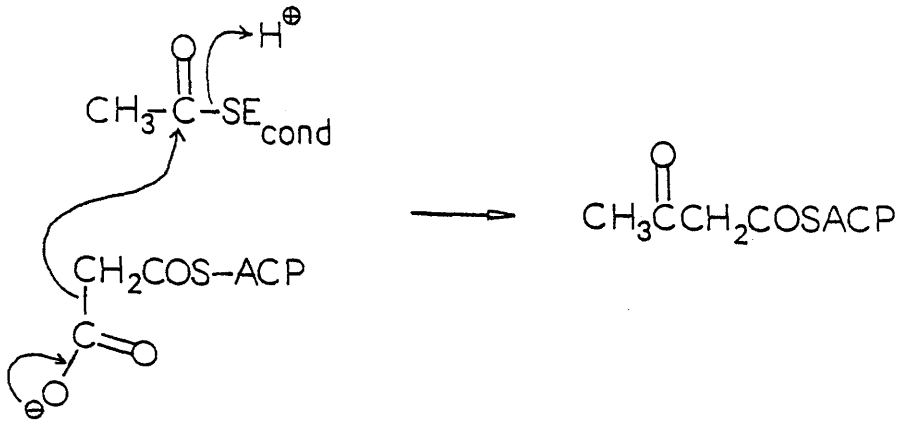
biosynthesis.

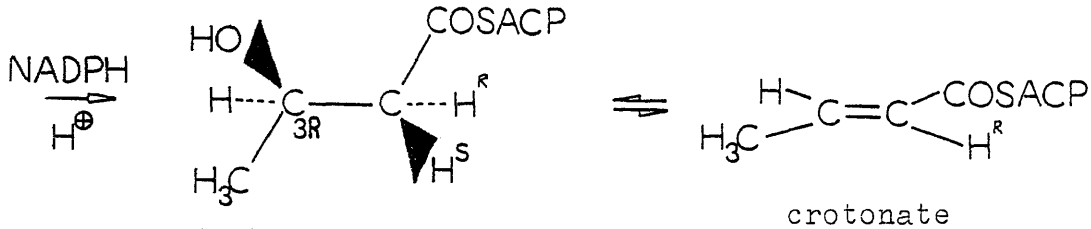
(i) <u>Fatty Acid Biosynthesis in P.canadense: The Stereochemistry of Double Bond Reduction.</u>

The accepted pathway of fatty acid biosynthesis from acetate involves a cycle of reactions including dehydration of a (3R)-hydroxy-fattyacyl derivative followed by reduction of the resulting <u>trans</u>-2-enoyl compound[98,99]. The initially formed compound is known to be the D-(-)-(3R)-hydroxybutyric acid derivative[98,99] and dehydration of this has recently been shown to be a <u>syn</u> process involving elimination of the pro-2S hydrogen atom (H^s)[100]. Hence the pro-2R hydrogen (H^f) is retained in the crotonate (Scheme 14).

This predicts that only one deuterium atom will be retained at the even numbered carbons in a fatty acid chain derived from deuterio-acetate ($^2H_3{}^{13}CCO_2Na$) . Assuming that the steps subsequent to butyrate formation and leading to stearate are repetitions of the first steps, all even numbered methylene carbons in fatty acids such as stearic acid would retain one hydrogen atom derived from acetate in the same configuration (eg. pro-R as in Scheme 14).

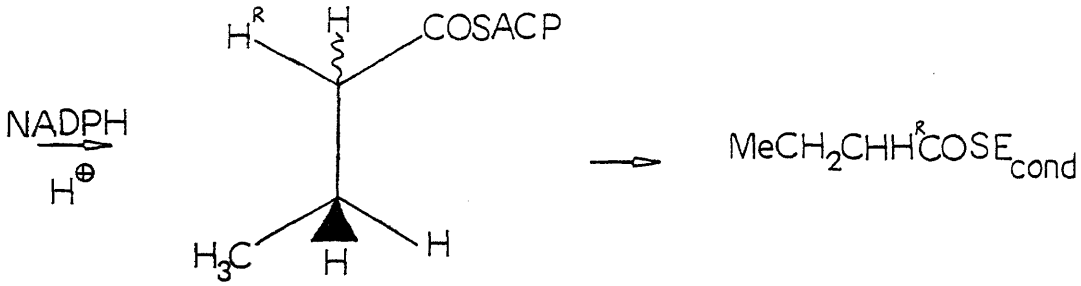
SCHEME 14



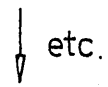


D-(-)-(3R)-hydroxybutyrate

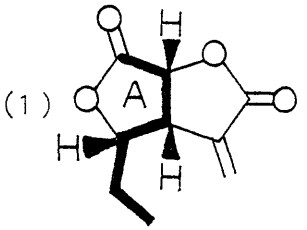
crotonate

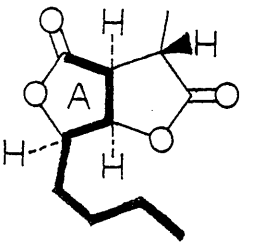


butyrate



The anti-biotics ethisolide (1) and dihydrocanadensolide (3) both contain polyacetate derived moieties (ring A and the alkyl substituent) presumed to arise by standard fatty acid type biosynthesis following studies using ^{14}C, ^{13}C, ^{3}H and ^{2}H singly and multiply labelled acetates[101]. Each is

(1) 

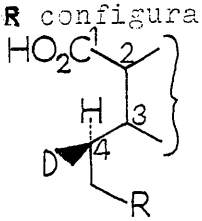
 (3)

oxygenated at C-4 of the fatty acid chain. An interesting result was obtained using $^{13}CD_3CO_2Na$ whereby deuterium was retained at C-4 in ethisolide (1) and lost from C-4 in dihydrocanadensolide (3)[102]. The absolute configuration of these anti-biotics was at that time uncertain† but the above result was correctly deduced to reflect a difference in absolute configuration at C-4

It has been established that in several systems (C.diptheriae, chlorella and chicken liver) desaturation of stearic acid to give oleic acid involves loss of the 9-pro-R and 10-pro-R hydrogens[104]. This was first illustrated using 9R and 9S and 10R and 10S tritiostearic acids as substrates with C.diptheriae[105] and similar results were obtained with erythro 9R, 10R and 9S, 10S dideuteriostearic acids and threo 9R, 10S and 9S, 10R dideuteriostearic acids as substrates for Chlorella vulgaris[106] (Scheme 14a).

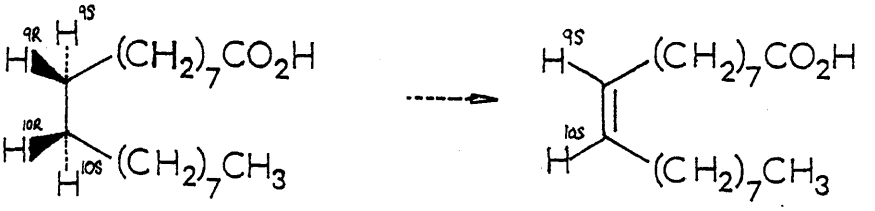
If the same process is assumed to occur in P.canadense, loss or retention of deuterium at C-10 in oleic acid derived

†These absolute configurations are now known to be as shown (86, 103) so that the retained H atom from deuterioacetate is deduced to be in an **R** configuration as in structure (94).

 (94)

This assumes that hydroxylation at C-4 and lactonisation do not affect the configuration at C-4.

SCHEME 14a



Stearic acid

Oleic acid

from deuterioacetate will indicate whether the C-10 deuterium atom in the stearate precursor was in the pro-R or pro-S configuration respectively. The mycelial extract of P.canadense afforded a substantial quantity of lipid consisting mainly of a mixture of triglycerides. It was hoped to derive evidence for the configuration of the acetate-derived hydrogen atoms in the fatty acid chains of the lipids, and apply the result to the biosynthesis of the fatty acid part of the lactone metabolites being discussed.

A method had previously been developed[107] for converting the triglyceride mixture, obtained by chromatography of the mycelial extract, into the corresponding mixture of fatty esters by successive treatments with 0.5M NaOH in MeOH and with BF_3 in MeOH[108]. ^{13}C-N.M.R. spectra of the ester mixtures obtained by feeding various labelled acetates had also been examined[107]. In the present work, methyl oleate was isolated, using $AgNO_3$-silica gel chromatography[109], from the mixture of esters obtained by culturing the P.canadense in the presence of $^{13}CD_3CO_2Na$ (95%).

The presence of a deuterium atom at a particular even numbered carbon atom will result in a 1:1:1 triplet of characteristic magnitude eg. ca.19Hz.† in the ^{13}C-N.M.R. spectrum, owing to coupling of ^{13}C with ^{2}H which has a spin number of 1. This triplet will be slightly upfield of the natural abundance signal (owing to the isotope effect) and will be relatively weak in intensity (owing to the greatly increased spin-lattice relaxation time and greatly reduced Nuclear Overhauser Effect associated with a ^{13}C-^{2}H signal as compared to ^{13}C-H [110])

† Since $J(HX)/J(DX) = \gamma_H/\gamma_D$ = ca.6.51 (110) and typical values of J(C-13/H) for saturated CH2 group and for an olefinic CH group are 128Hz. and 158Hz. respectively (110), corresponding values can be calculated for J(C-13/D) of 19.7Hz. and 24.3Hz. respectively.

It may be noted that even 20% incorporation for example of CD_3CO_2Na would not be detectable from the ^{13}C-N.M.R. spectrum since only ca. 1% of the incorporated acetate molecules will have ^{13}C on C-2. The incorporation of $^{13}CD_3CO_2Na$ would in fact be 0.2%. In view of the above mentioned signal suppression and the ^{13}C-^{2}H splittings, no multiplet would be detectable corresponding to the deuterated carbons. By feeding $^{13}CD_3CO_2Na$ it is ensured that a high proportion of incorporated deuterium atoms are attached to carbon-13 atoms so that, with a suitable level of incorporation, the typical ^{13}C-^{2}H multiplets can be seen.

The biosynthetic pathway to saturated fatty acids from $^{13}CD_3CO_2Na$ (fully deuterated) would predict that every even numbered carbon atom would retain at least one deuterium atom. In practice, enhancement of the natural abundance signals may be observed (Table 6). This must correspond to the incorporation of ^{13}C atoms bearing hydrogen rather than deuterium atoms. This might arise because of some loss of deuterium by exchange during biosynthesis. Also, if a small amount (eg.1-2%) of the fed [2-^{13}C] acetate was not fully deuterated, any isotope effect would tend to this being preferentially carboxylated and incorporated into the fatty acid chain. In a recent study in which $^{13}CD_3CO_2Na$ was incorporated into a number of polyketides, extensive loss of deuterium was also noted[111].

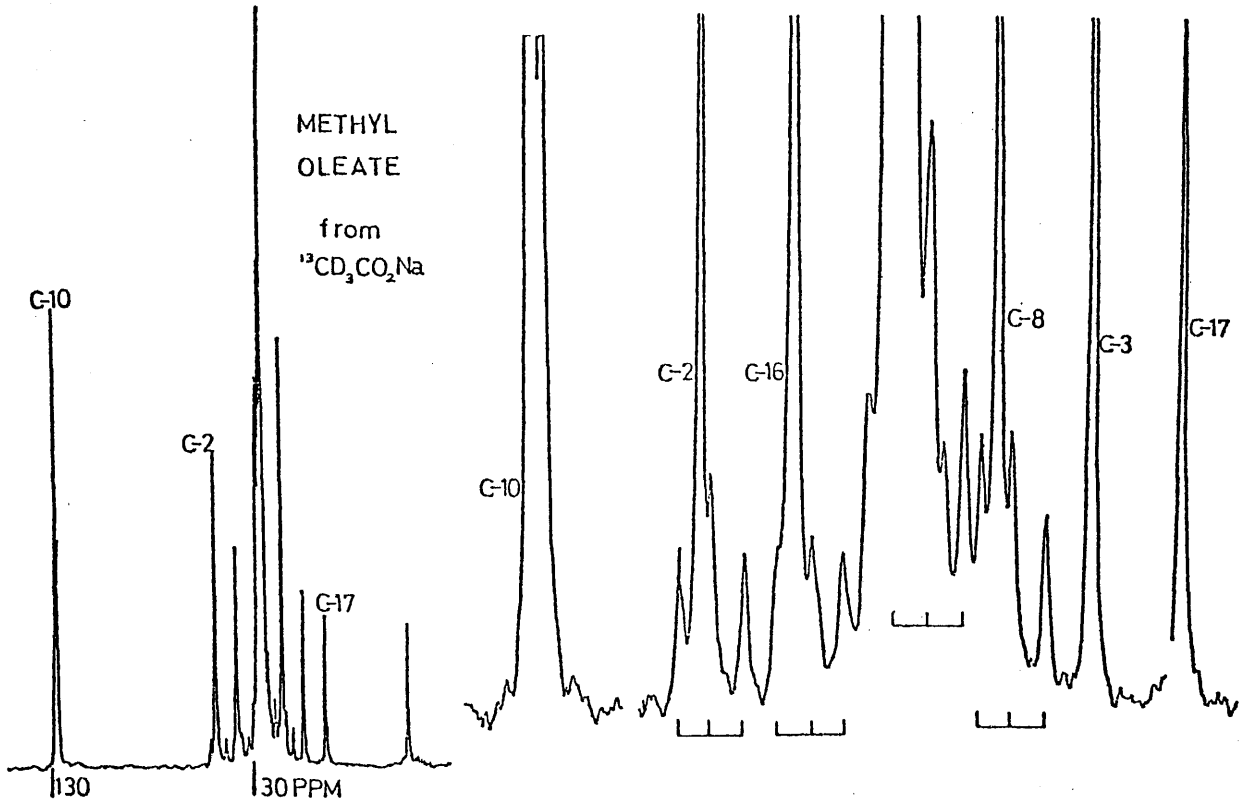
The ^{13}C-N.M.R. spectrum of the sample of methyl oleate derived after feeding $^{13}CD_3CO_2Na$ (fig.33) shows triplets (J=ca.19.3Hz., isotope shift=ca.0.35ppm) associated with C-2, C-16 and C-8 most clearly, indicating the presence of one deuterium atom at these centres[110]. The signal for C-10 is clearly enhanced relative to C-9 (more than doubled in intensity) and enhanced considerably more than any of the other

TABLE 6

Methyl Oleate from $^{13}CD_3CO_2Na$

Chemical Shift( )	Carbon Atom	Enhancement Factor
14.12	C-18	-0.14
22.71	C-17	-0.10
24.97	C-3	-0.36
27.20	C-8,C-11	1.22
29.14		0.67
29.36	$-(CH_2)_{\bar{n}}$	0.35
29.54		0.47
29.75		-0.24
31.93	C-16	0.44
34.11	C-2	0.0
51.41	OMe	-0.3
129.77	C-9	0.42
130.01	C-10	1.66
174.29	C-1	0.07

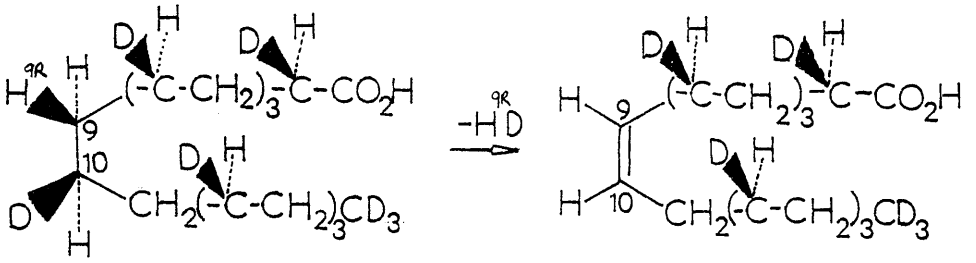
Enhancement Factor is calculated as described in the Experimental section.


METHYL
OLEATE
from
$^{13}CD_3CO_2Na$
C-10
C-2
C-17
130
30 PPM
C-10
C-2
C-16
C-8
C-3
C-17

(fig.33)

even numbered carbon atoms. Also, there is no sign of a triplet (J=ca.24.3Hz.†,isotope shift=ca.0.35ppm) corresponding to $-^{13}C^{2}H=CH-$.

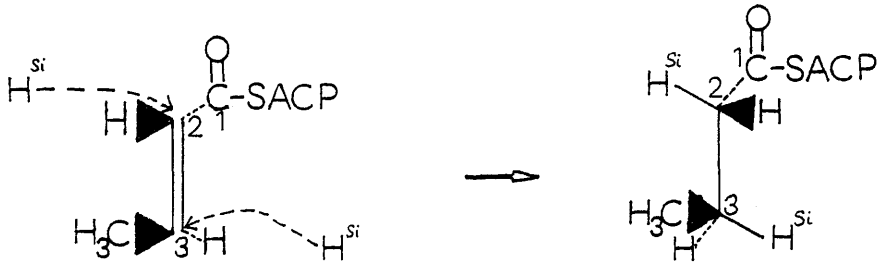
This shows almost certainly that there is no deuterium at C-10 in the methyl oleate, so that the deuterium atom at C-10 and other even numbered methylene carbons in the precursor stearic acid can be deduced to be **R** as indicated in (fig. 34).



Stearic acid Oleic acid

(fig.34)

It is now possible to deduce that the stereochemistry of reduction of the crotonate to butyrate (Scheme 14) involves delivery of hydrogen at C-2 from the <u>si</u> face ie. the hydrogen will adopt the pro-S configuration. Assuming <u>trans</u> reduction as is observed in the biosynthesis of sterols[112], in the biosynthesis of griseofulvin[113] and in the enzymic reduction of cinnamoyl alcohol using Baker's Yeast[114], the delivery of hydrogen to C-3 will also be from the <u>si</u> face with respect to C-3 ie. the hydrogen will adopt the pro-S configuration (fig.34a).



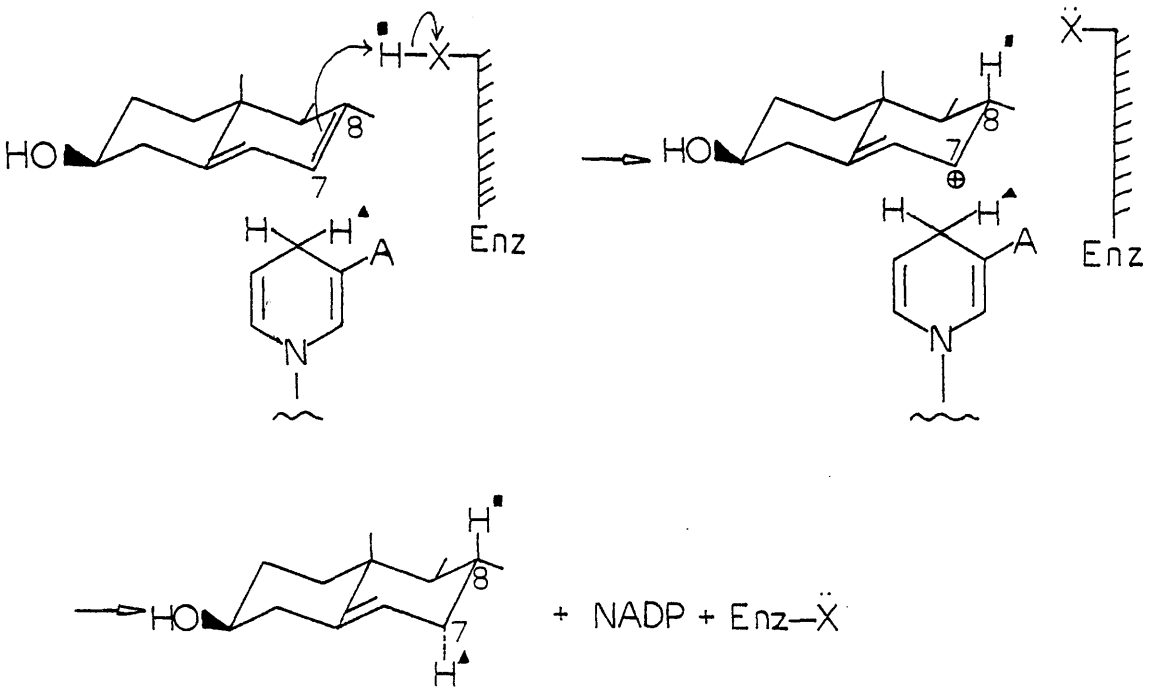
(fig.34a)

The above-mentioned naturally found <u>trans</u> reductions are illustrated overleaf (Scheme 15).

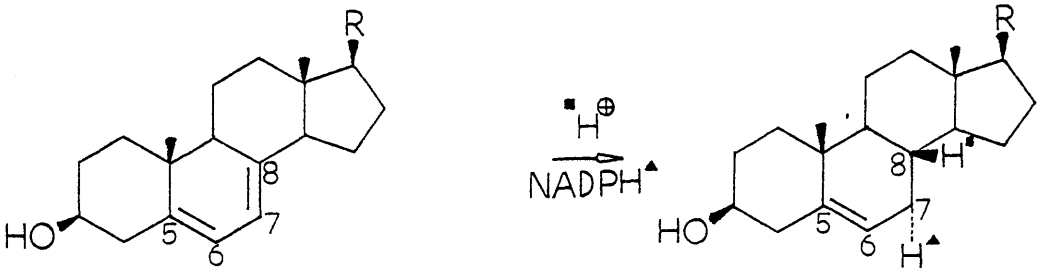
SCHEME 15

1. Reduction of Double Bonds in Sterols

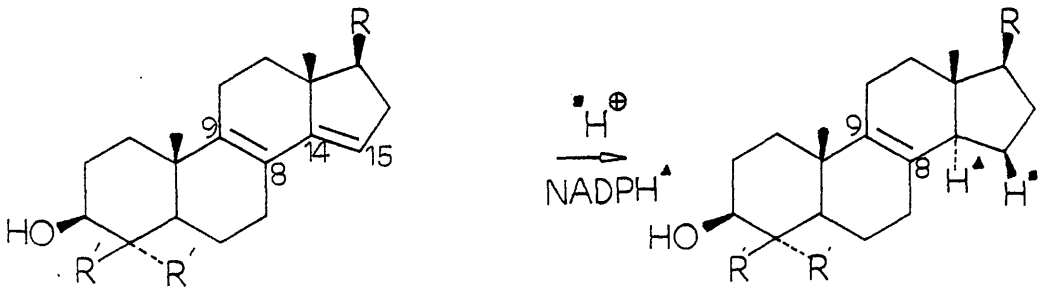
(i) General Mechanism.



(ii) Reduction of the 7,8 Double Bond of 7-Dehydro-Cholesterol to give Cholesterol.



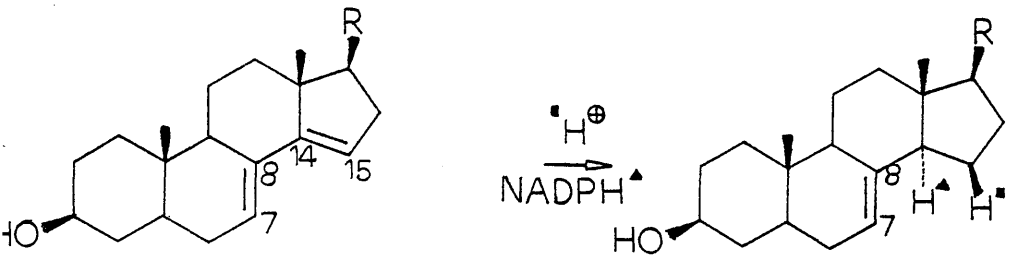
(iii) Reduction of the 14,15 Double Bond of Steroid 8,14 and 7,14 Dienes.



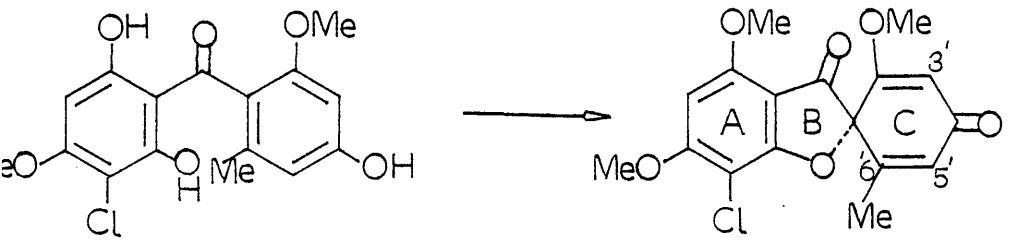
R = H, $-CH_3$

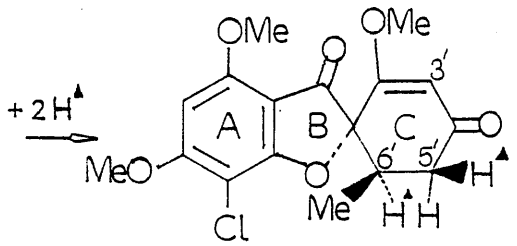
SCHEME 15 (cont.)

1. (iii) (cont.)



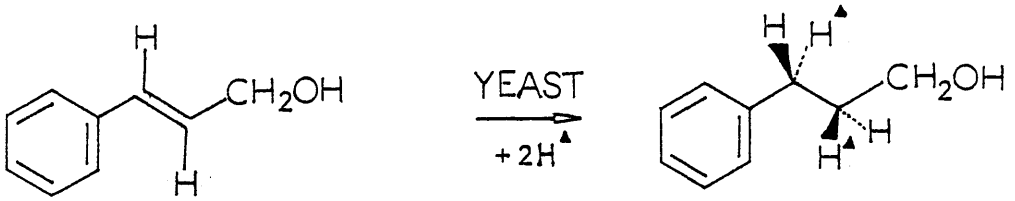
2. Trans Diaxial-type Reduction of Ring C in the Biosynthesis of Griseofulvin.





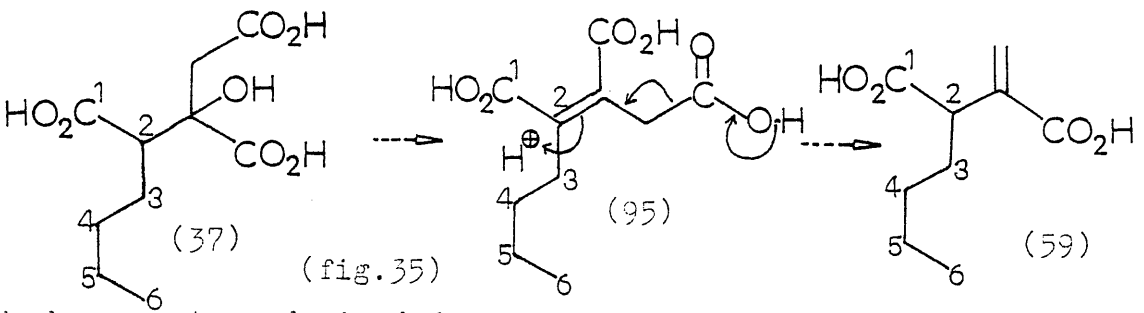
Griseofulvin

3. Reduction of Cinnamoyl Alcohol.



(ii) Previous Studies on Ethisolide (1) and Dihydrocanadensolide (3).

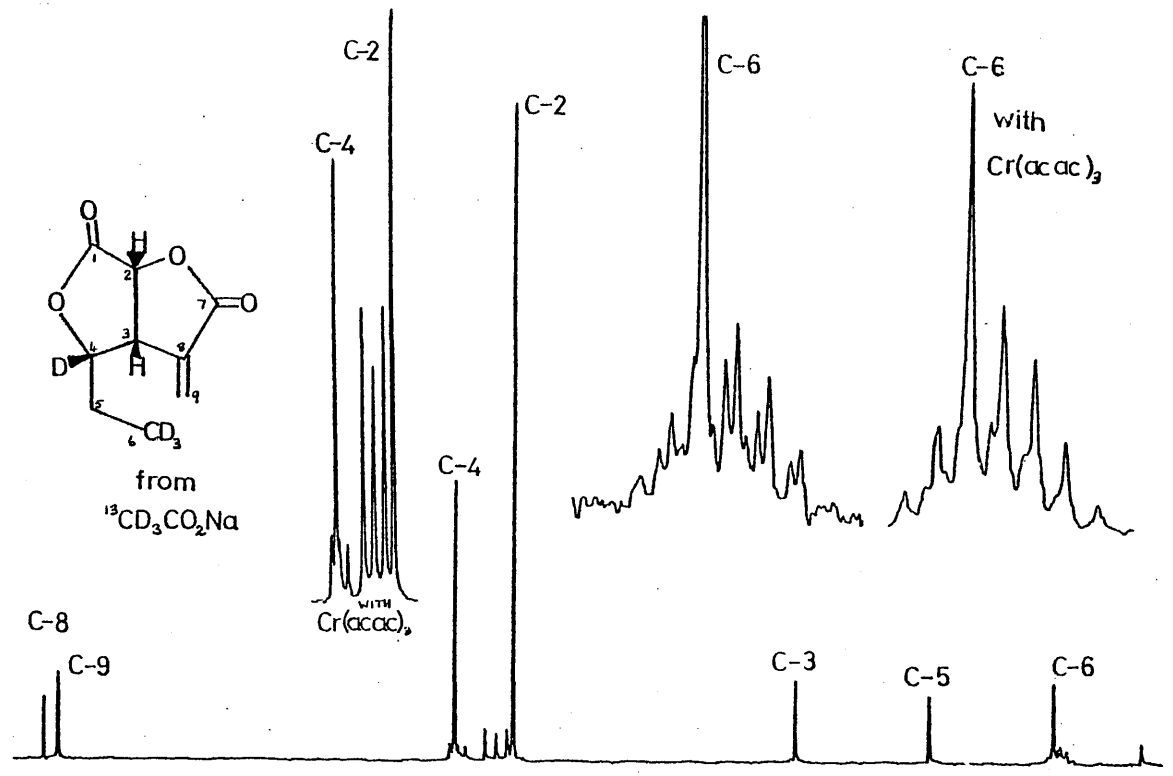
As discussed earlier (p.27, Scheme 7), ethisolide (1) has been shown in the present work to be biosynthesised via α-n-butylitaconic acid (59). This is probably produced itself from n-butylcitric acid (37) and n-butyl-cis-aconitic acid (95) (fig. 35) and hence would be expected to retain only four



(fig.35)

hydrogen atoms derived from acetate, three in the C-6 methyl group and one at C-4.

The biosynthetic route to ethisolide from α-n-butylitaconic acid (59) involves substitution at C-4 by an oxygen function but this was shown in previous work to occur without loss of the acetate-derived hydrogen atom. Careful experiments involving feeding [2-^{14}C,2-^{3}H] acetate[115] gave ethisolide (1) with a tritium to carbon ratio corresponding to the incorporation of 3.5 tritium atoms. The location of the tritium was not however established by degradations. In the present work, the ^{13}C-N.M.R. spectrum of a sample of ethisolide (1) biosynthesised from $^{13}CD_3CO_2Na$ has been obtained (fig. 36), which shows clearly that four deuterium atoms are incorporated including one at C-4 (allowing for some incorporation of ^{13}C atoms bearing no deuterium atoms due to loss of deuterium or other factors as discussed above for methyl oleate biosynthesis). Thus whereas the C-2 signal shows strong enhancement (enrichment factor 6.1) compared to natural abundance, the C-4 signal shows a much smaller enhancement (enrichment factor 2.7) and is associated with a C-D triplet

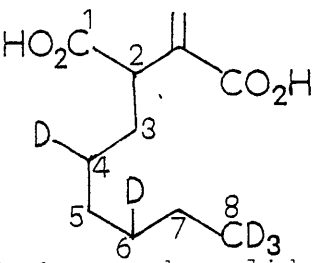

C-2
C-4
C-2
C-6
C-6
with
Cr(acac)3
from
13CD3CO2Na
C-4
with
Cr(acac)3
C-8
C-9
C-3
C-5
C-6

(fig.36)

(J=23.3Hz., Δ=0.39ppm.). The signal for the methyl carbon, C-6, shows no enhancement but is associated with a triplet, quintet and septet (J=19.3Hz., Δ=0.27ppm. per deuterium atom) corresponding to d_1, d_2 and d_3 species respectively. On addition of the relaxation reagent $Cr(acac)_3$, the septet undergoes approximately 3-fold enhancement relative to the other multiplets owing to removal of the Nuclear Overhauser Effects of the protonated species $-CD_2H$ and $-CDH_2$. This feeding experiment in fact duplicated one carried out previously[115] which gave essentially the same result. In the present experiment sterilisation was carried out using millipore filters as opposed to autoclaving and less deuterium appeared to have been lost by exchange as indicated by the relative abundance of the d_3, d_2 and d_1 species.

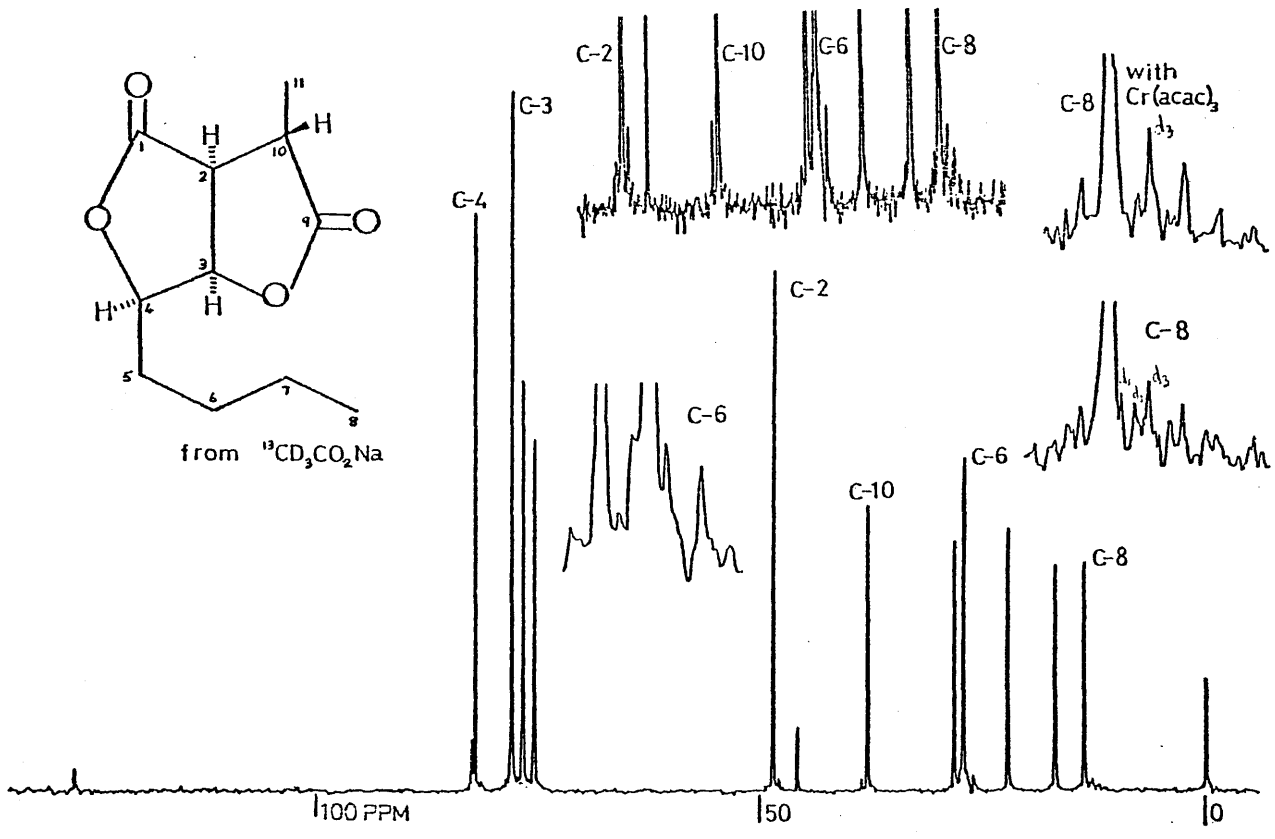
It has been shown in the present work that dihydrocanadensolide (3) is biosynthesised via ∝-n-hexylitaconic acid (65) (p.32 and Scheme 8). This would be formed via the correspondeing cis-aconitic acid and hence the expected labelling pattern from $^{13}CD_3CO_2Na$ would be as in (fig.37).

(65)



(fig.37)

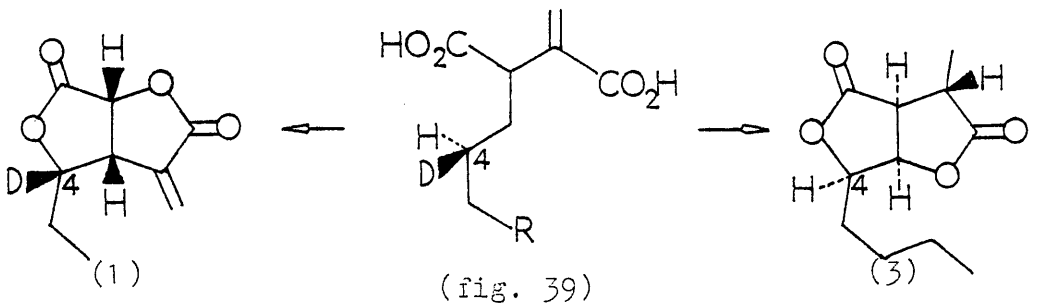
In previous work, dihydrocanadensolide (3) was obtained after feeding P.canadense with $^{13}CD_3CO_2Na$[116]. This provided evidence suggesting that the acetate-derived hydrogen atom at C-4 in (fig.37) is lost in the hydroxylation at this position en route to dihydrocanadensolide (3). In this case the signals for C-2 and C-4 were enhanced to the same extent (fig.38) and no ^{13}C-D triplet was associated with the C-4 signal. The C-8 signal was not enhanced but was associated with a triplet, quintet and septet indicating the presence of d_1, d_2 and


from $^{13}CD_3CO_2Na$
C-4
C-3
C-2
C-10
C-6
C-8
with Cr(acac)$_3$
C-8
C-2
C-6
C-8
C-10
C-6
C-8
100 PPM
50
0

(fig. 38)

d_3 species.

Since the absolute configurations of ethisolide (1) and dihydrocanadensolide (3) at C-4 are respectively S and R, this result is consistent with the acetate-derived hydrogen atom in the respective itaconic acid precursors (59 and 65) being in the pro-R configuration and being retained or lost during a subsequent hydroxylation with retention of configuration (fig. 39).



(1) (fig. 39) (3)

Unfortunately it is evident from impurity signals (fig.38) that the sample of dihydrocanadensolide (3) contained a small proportion (<10%) of canadensolide (2). This important result was however confirmed in the present work as described below.

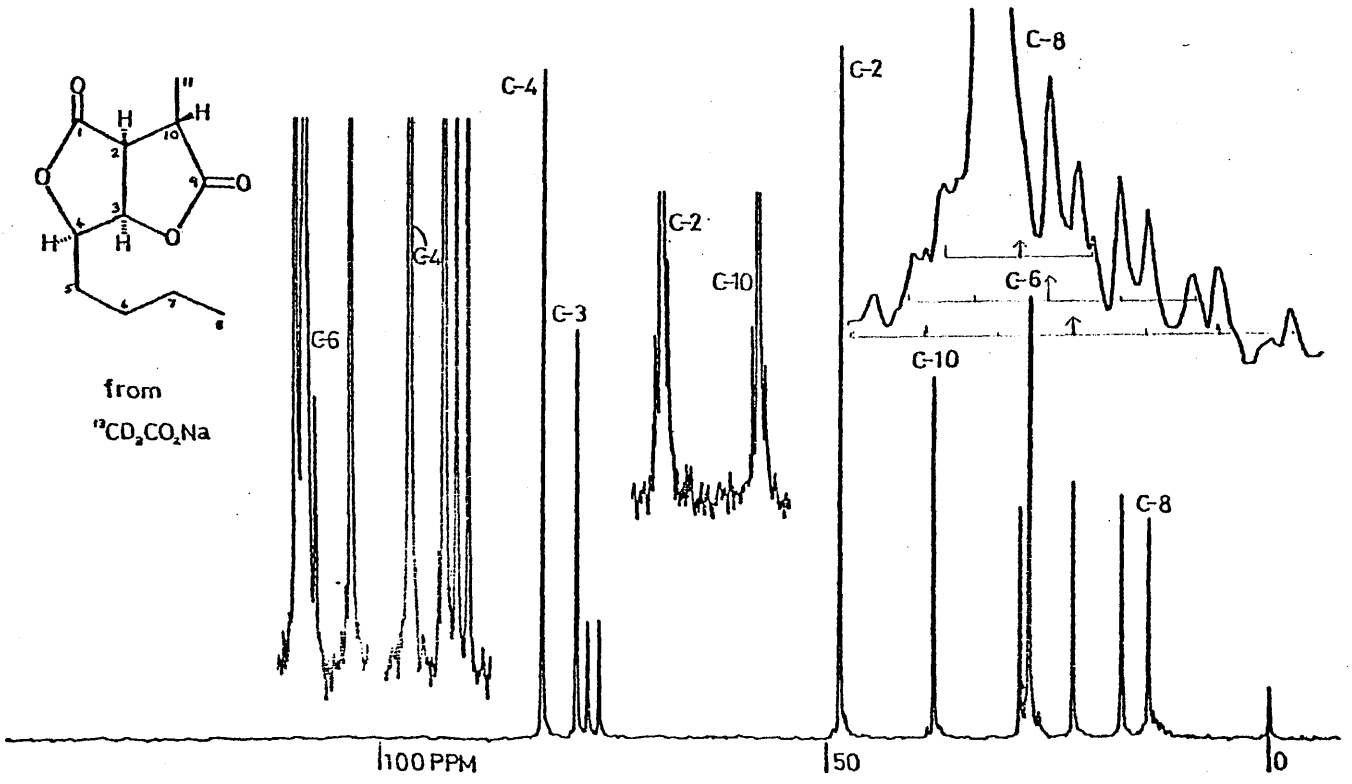
(iii) Dihydrocanadensolide, Canadensolide, Canadensic Acid and Avenaciolide.

In order to confirm the previous dihydrocanadensolide (3) result and extend this approach to further metabolites, sufficient culture of P.canadense was grown in the presence of $^{13}CD_3CO_2Na$ so that pure samples, not only of dihydrocanadensolide (3) but also of canadensolide (2) and canadensic acid (60) could be obtained in suitable quantity.

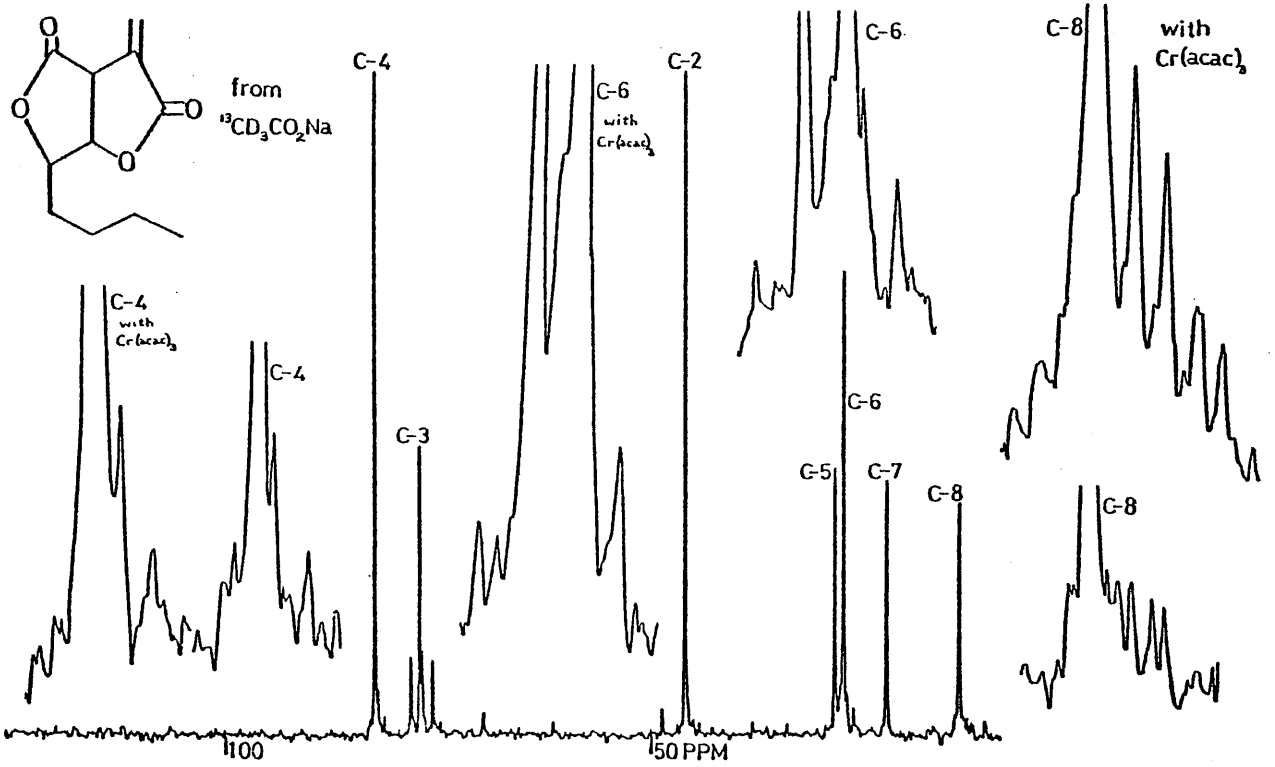
The ^{13}C-N.M.R. spectrum of the resulting dihydrocanadensolide shows it to be free from contamination by canadensolide (fig. 40). Again, the spectrum shows evidence for the presence of deuterium in the methyl group, C-8 (multiplets, J=19.2Hz., Δ=0.30ppm.) and at C-6 (triplet, J=18.9Hz., Δ=0.36ppm.). No triplets associated with the C-2 or C-4 peaks could be detected and these were enhanced to the same extent due to ^{13}C enrichment. Upon addition of $Cr(acac)_3$ selective enhancement was observed of the $-CD_3$ septet relative to the multiplets corresponding to the protonated species ($-CD_2H$ and $-CDH_2$ for C-8 and CDH for C-6).

With the good signal to noise ratio achieved, weak doublets could be detected in this spectrum associated with the signals for C-2 and C-10. These are evidently due to ^{13}C-^{13}C coupling, J=35.1Hz., and reflect a degree of ^{13}C incorporation into the C-3 unit via the Krebs cycle as discussed earlier (p. 22, Scheme 4 for ethisolide (1)).

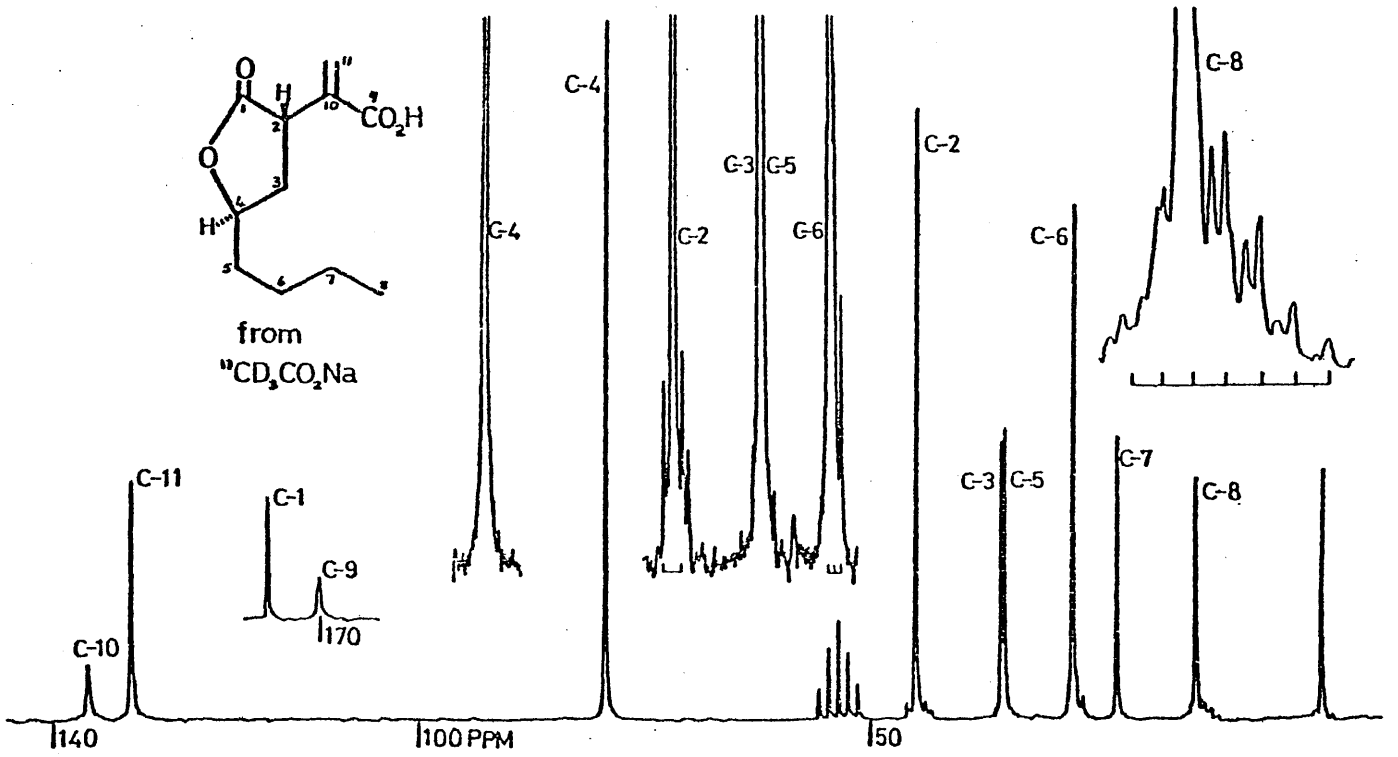
The spectra of the canadensolide (2) and canadensic acid (60) samples showed excellent evidence for exactly the same pattern of deuterium incorporation (figs. 41 and 42 respect.), ie. septets ($-CD_3$) associated with the C-8 signals, triplets (-CHD) associated with the C-6 signal and no triplets associated with the C-2 or C-4 signals. The canadensic acid spectrum which was run in CD_2Cl_2 to avoid overlap of the C-4 signal



(fig. 4O)


from
$^{13}CD_3CO_2Na$
C-4
C-3
C-2
C-6
with
Cr(acac)$_3$
C-6
C-5
C-7
C-8
C-8
with
Cr(acac)$_3$
C-8
C-4
with
Cr(acac)$_3$
C-4
100
50 PPM

(Fig. 41)


from
$^{13}CD_3CO_2Na$
C-4
C-2
C-3 C-5
C-6
C-2
C-6
C-8
C-4
C-11
C-1
C-9
170
C-10
C-3 C-5
C-7
C-8
140
100 PPM
50

(Fig. 42)

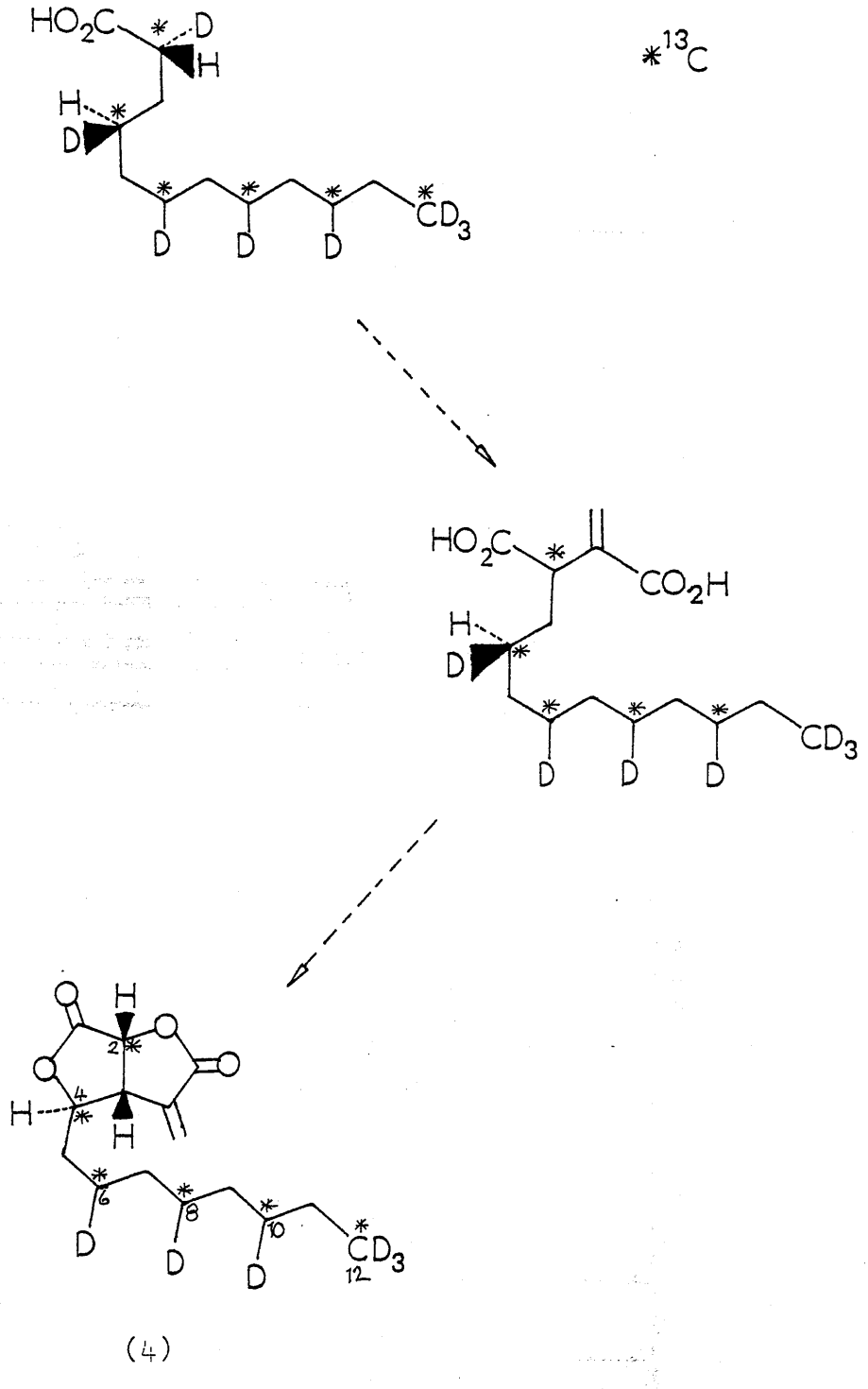
with those for $CDCl_3$, again showed a weak doublet, J=49.7Hz., associated with C-2, probably due to coupling between C-2 and C-10.

At the time of these experiments, the stereochemistry of these metabolites relative to one another was still uncertain. Since in each case no deuterium is retained at the C-4 position, it was deduced that these three metabolites all have the same absolute configuration (R) at this centre. This in fact prompted a careful scrutiny of all of the accumulated evidence for the stereochemistry and re-interpretation as previously discussed.

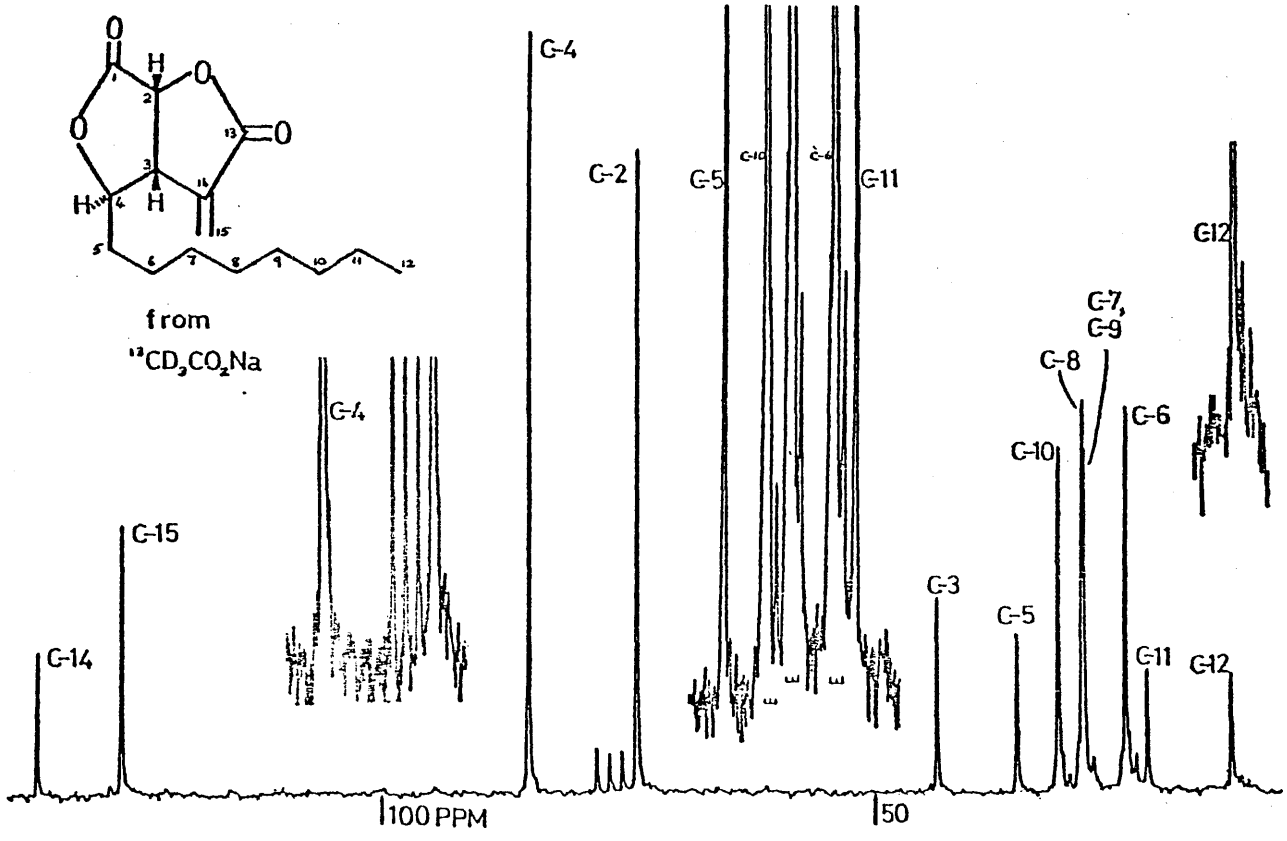
Following the results from the methyl oleate, the metabolites of P.canadense and ethisolide, the biosynthesis of avenaciolide (4) from $^{13}CD_3CO_2Na$ can be predicted with some confidence to follow the path indicated in Scheme 16. A sample of avenaciolide (4) obtained after feeding A. avenaceus with $^{13}CD_3CO_2Na$ gave a spectrum completly in accord with these predictions (fig. 43). Typical -CHD triplets associated with the signals for C-6, C-8 and C-10 are evident (although the central line of the C-8 triplet is overlapped by the signal for C-7 and C-9). The presence of deuterium in the methyl group C-12 is reflected in the absence of enhancement and the presence of typical multiplets. The C-2 and C-4 signals are strongly enhanced and no triplets are associated with them.

Iso-avenaciolide (77), which differs from avenaciolide (4) only in having the S configuration at C-4, would be expected to retain an acetate-derived hydrogen atom at this position, but this metabolite is evidently produced by A.avenaceus in only very small amounts and could not be detected in an experiment on the scale used here.

SCHEME 16



(4)


from
$^{13}CD_3CO_2Na$
C-4
C-2
C-5
C-10
C-6
C-11
C-12
C-7,
C-9
C-8
C-6
C-10
C-15
C-14
C-3
C-5
C-11
C-12
100 PPM
50

(fig.43)

APPENDICES

Appendix 1:

Natural Products derived from Tricarboxylic Acid Metabolites.

Appendix 2:

Syntheses of Bis-lactone Antibiotics.

(i) Avenaciolide and Iso-avenaciolide

(ii) Canadensolide

Appendix 3:

Biosynthetic Studies designed to follow the fate of Hydrogen Atoms in Biosynthesis.

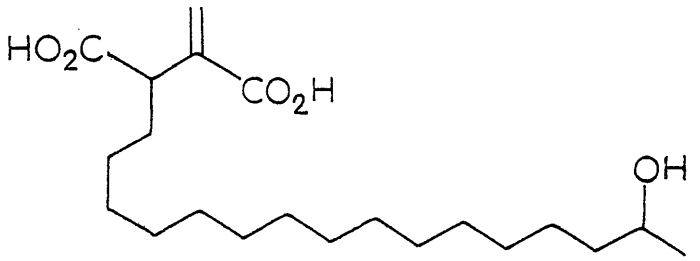
Appendix 4:

Deuterium Exchange during Biosynthesis.

APPENDIX 1

Natural Products derived from Tricarboxylic Acid Metabolites.

The recent isolation of the metabolite α-(15-OH-hexadecyl)-itaconic acid (96)[119] gave the first example of

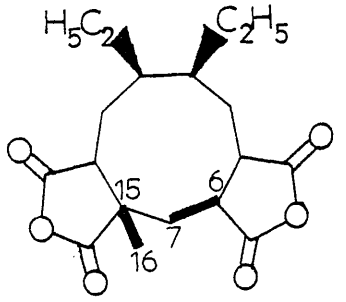


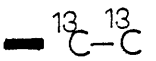
(96)

a naturally-occurring alkyl itaconic acid. The authors comment that 'the only other natural product of related structure is itaconic acid ex. A.terreus'.

The previously proposed biosynthesis of the nonadride glauconic acid (28) (discussed on pp. 12-14) has been further supported by incorporation studies using [2,3-$^{13}C_2$] succinic acid with P.purpurogenum[120] . The ^{13}C-N.M.R. spectrum of the isolated glauconic acid (28) showed intact incorporation of the [2,3-$^{13}C_2$] succinic acid which clearly illustrated the

(28)





$J_{C(15)-C(16)}$ = 33Hz.

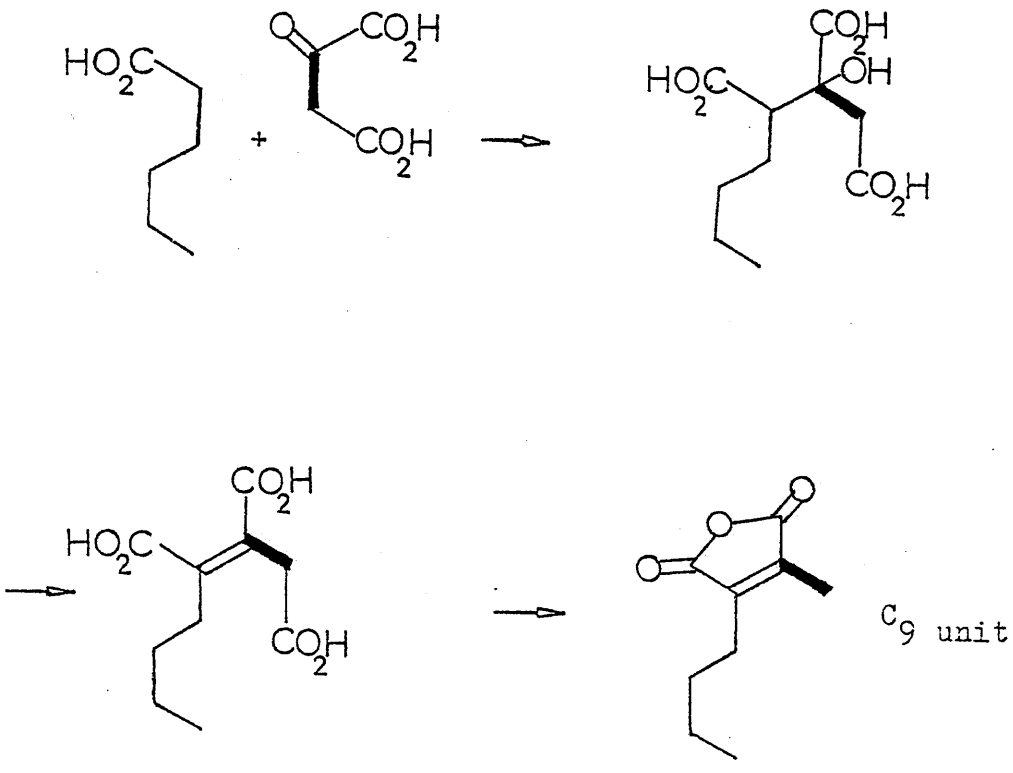
$J_{C(6)-C(7)}$ = 48Hz.

biosynthesis involving dimerisation of C_9 units derived from acetate and a TCA cycle metabolite (Scheme 17).

N.B.

It is claimed here that no randomisation of the ^{13}C label occurs and this is attributed to the particular conditions used in the inoculation.

SCHEME 17



→ Glauconic acid (28)

▬ ^{13}C-^{13}C

APPENDIX 2

Syntheses of Bis-Lactone Antibiotics.

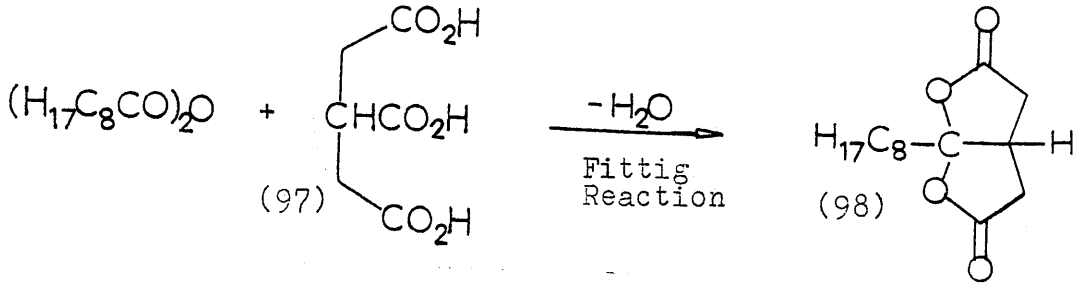
(i) Avenaciolide and Iso-avenaciolide

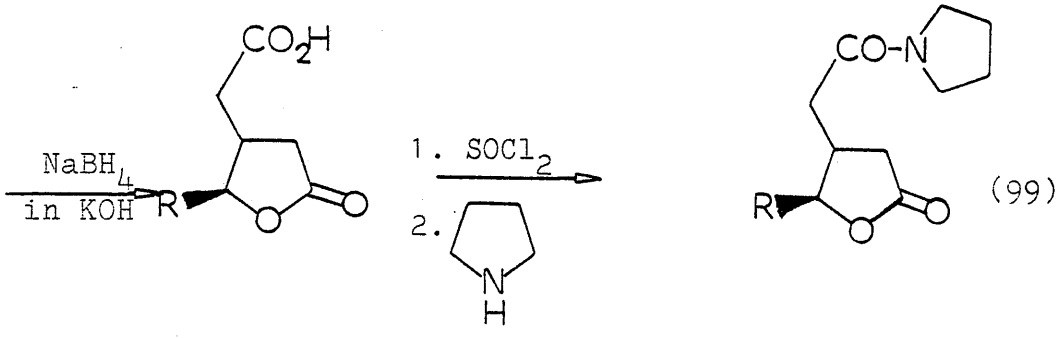
The total synthesis of (±)-avenaciolide (4) was first reported in 1969[53]. The synthetic route (Scheme 18) involved a Fittig condensation of n-nonanoic anhydride and tricarballylic acid (97) to give the bis-lactone (98) after loss of the elements of water. This was transformed into (99) by reductive cleavage of one ring and formation of the amide using pyrrolidine.

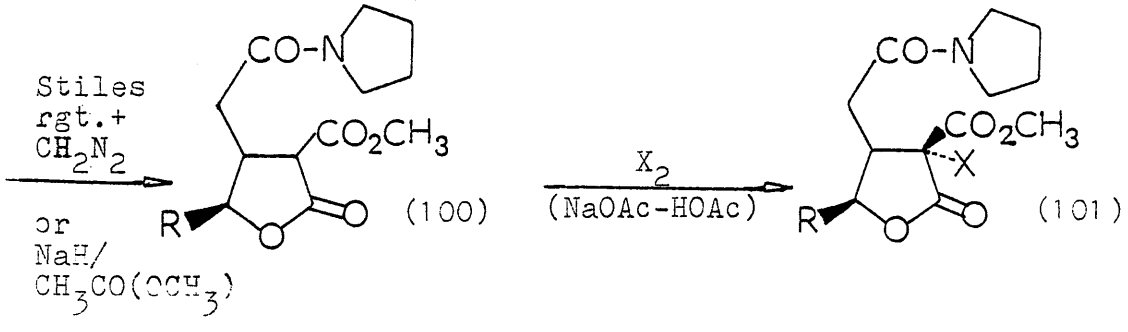
Carbomethoxylation of (99) to (100) was achieved either by carboxylation using Stiles reagent and treatment with diazomethane or directly using sodium hydride and dimethyl carbonate in the presence of trace quantities of methanol. (100) reacted rapidly with halogens in basic media (NaOAc-HOAc) to give a mixture of (101) and (102) although (102) could be obtained directly by brief treatment of (100) with aqueous NaOCl.

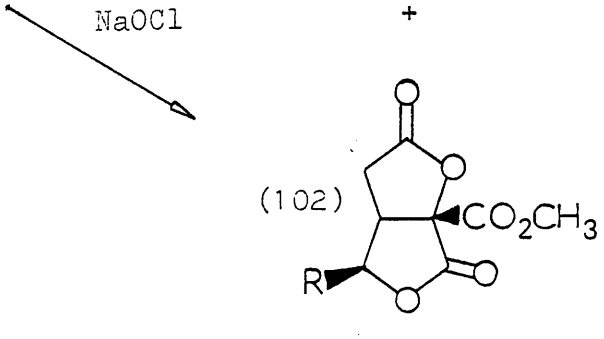
After decarbomethoxylation to give (103) the neutral compound was carboxylated using Stiles reagent to (104) and the exocyclic methylene group was introduced by a Mannich reaction of formaldehyde and dimethylamine in weakly basic media. This gave (±)-avenaciolide (4) which was identical spectroscopically to the natural material.

SCHEME 18



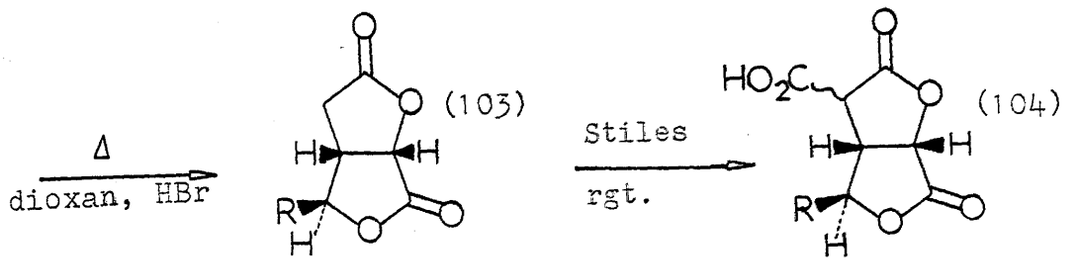


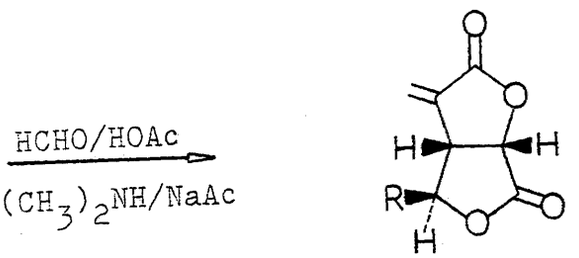




R= C_8H_{17}

SCHEME 18 (contd.)



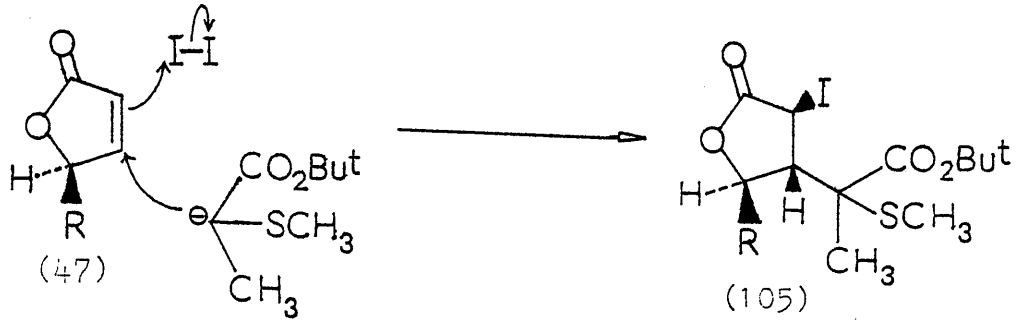


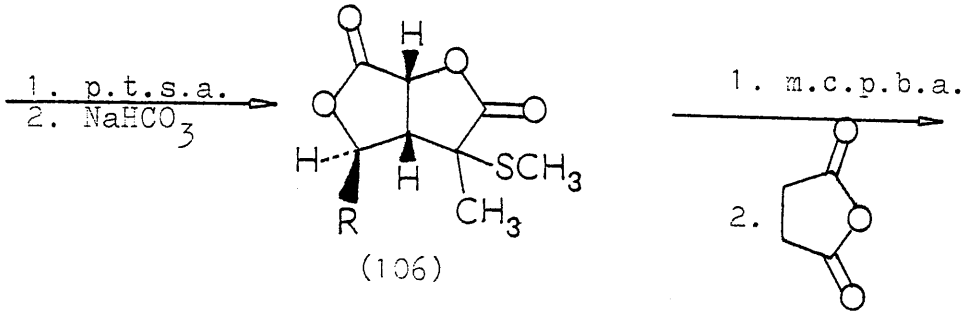
(4)

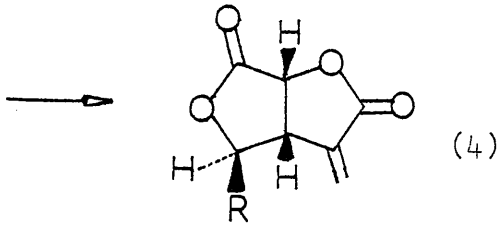
R= C_8H_{17}

A simple stereospecific synthesis of (±)-avenaciolide (4) was reported in 1973[48] (a full report has recently been published[121]). This synthesis (Scheme 19) was used as the basis of the attempted synthesis of ethisic acid (38) described earlier. The synthesis involved substitution of both the β- and α- positions of a Michael receptor (47) using a conjugate addition-halogenation sequence. The acid hydrolysis of the ^{t}Butyl ester of the resulting iodo-lactone (105) gave the bis-lactone (106) where the grouping >C(Me)-SMe represents a potential terminal methylene group >C=CH_2. Oxidation of the sulphide to sulphoxide and subsequent cyclic elimination of methane thiol gave (±)-avenaciolide (4).

SCHEME 19







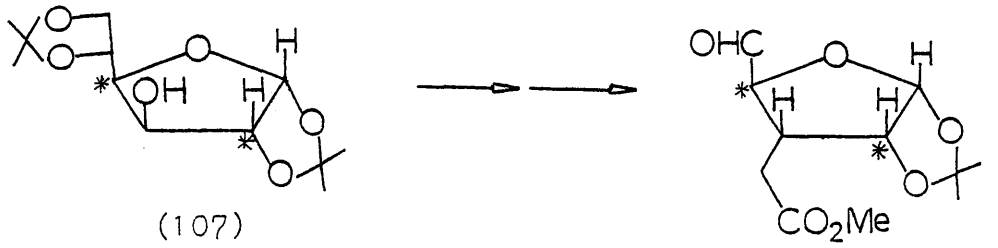
(4)

R= C_8H_{17}

The first synthesis of natural avenaciolide reported in the literature (1975)[82] used as starting material 'diacetone glucose' (107) (Scheme 20). This molecule was converted to the aldehyde (108) by the method of Rosenthal and Nguyen[122]. The Wittig reaction was used to introduce the alkyl group and catalytic hydrogenation of the resulting alkene gave (109). After acid treatment which caused both the removal of the acetonide and lactonisation to the hemiacetal (110), Jones oxidation afforded the bis-lactone (103) with the indicated configuration (3R, 4R) (this compound had previously been obtained[53] as a racemic mixture). The exo-cyclic methylene function was introduced using Stile's reagent (methyl magnesium carbonate) followed by reaction of the resulting carboxyl group with formaldehyde and dimethylamine giving avenaciolide (4) identical spectroscopically and optically to natural material. The absolute stereochemistry of avenaciolide is therefore as shown (2R, 3R, 4R) (4).

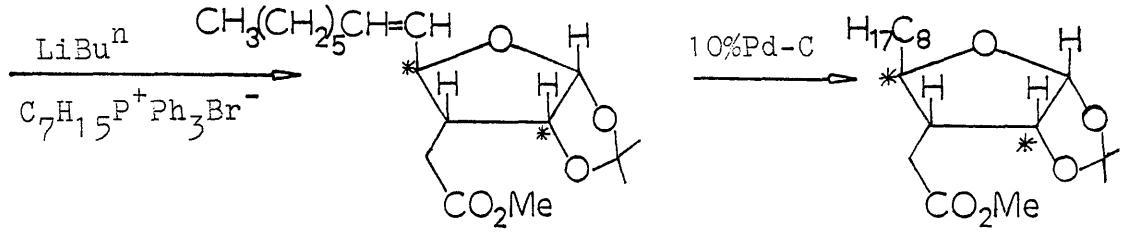
This finding was confirmed independantly by Ohrui and Emoto[81] who used a very similar synthetic route with Wittig introduction of the alkyl chain and Jones oxidation of hemiacetal (110)

SCHEME 20

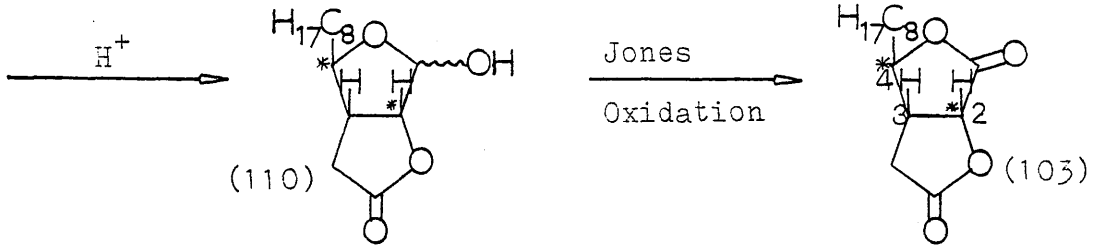


(107)

(108)



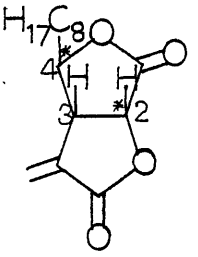
(109)



(110)

(103)

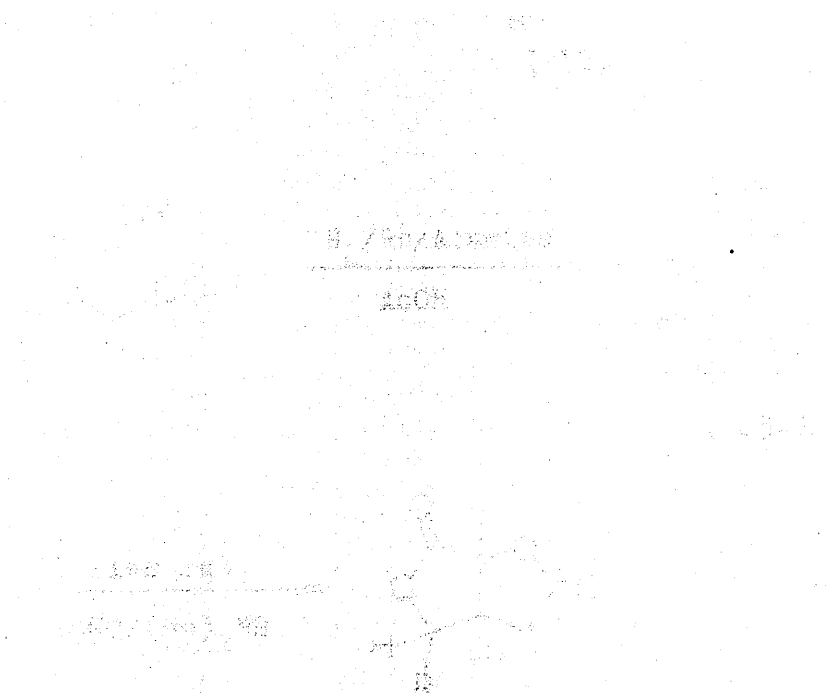
1. Stile's rgt.
2. HCHO/HOAc; $(Me)_2NH$/NaOAc



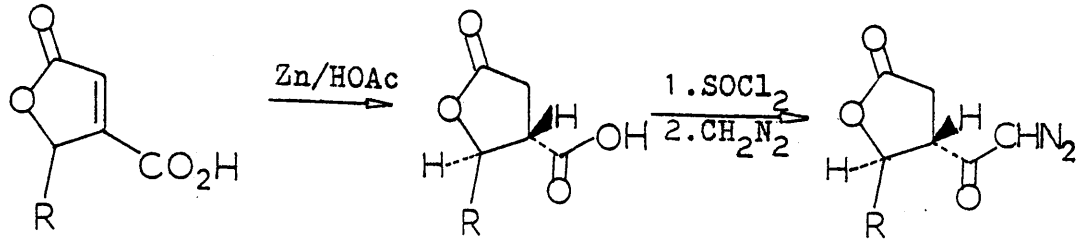
(4)

* Configuration is retained at these carbon atoms

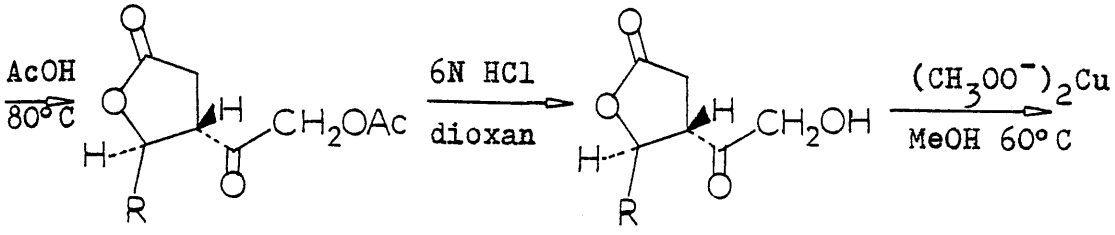
The total synthesis of (±)-4-isoavenaciolide (77) was reported in 1973[54]. The compound was synthesised from the aconic acid (111) (Scheme 21). The process involves a series of simple reactions and the α-keto ester (112) was converted under acidic conditions (2N-HCl-dioxan 1:1 v/v) at 45°C into the α-hydroxy-α,β-unsaturated-γ-lactone (113). Catalytic hydrogenation of (113), expected to occur at the least hindered face, gave the bis-lactone (103A) and the exo-cyclic methylene group was introduced using a similar Mannich reaction to that already described for avenaciolide (4). The product (±)-4-isoavenaciolide (77) was identical spectroscopically to a sample of natural 4-isoavenaciolide.

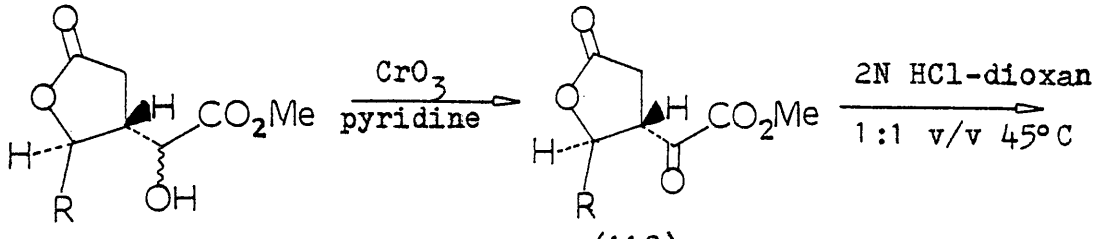


SCHEME 21

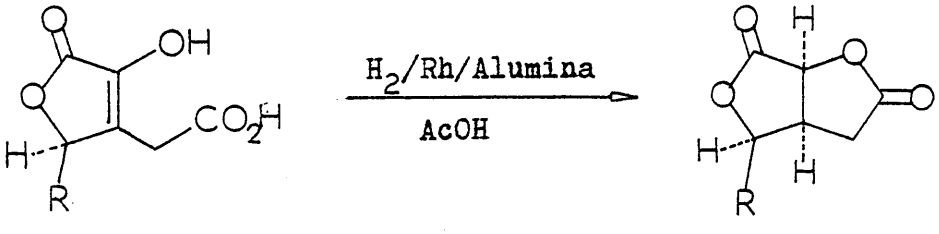


(111)





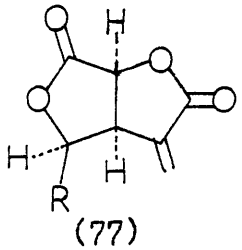
(112)



(113)

(103A)

1. Stiles rgt.
2. $HCHO/(Me)_2NH$

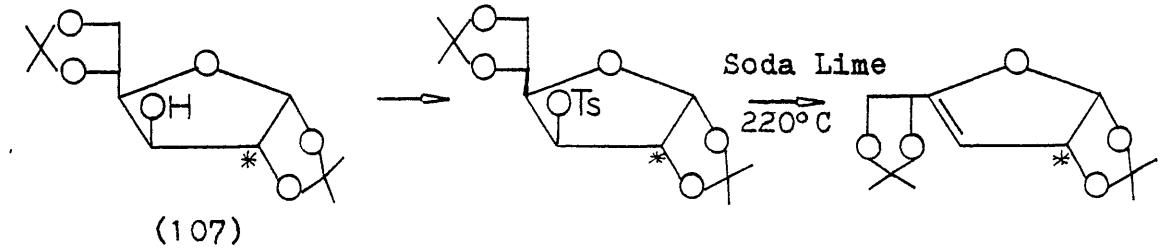


(77)

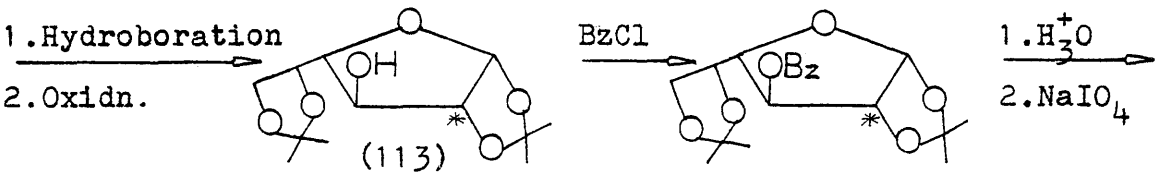
$R = C_8H_{17}$

The synthesis of naturally occurring (-)-4-isoavenaciolide (77) was reported in 1977[85] (Scheme 22). This synthesis is closely related to that of natural avenaciolide described earlier (p. 62). The starting material was once more 'diacetone glucose' (107). The key differences are the isomerisation of (107) to (113) via pyrolysis of the tosylate of (107) in the presence of soda lime followed by hydroboration and oxidation of the resulting olefin. This isomerisation allows the correct configuration (S) at C-4 of isoavenaciolide to be obtained. The alkyl group and ester function are introduced as before via Wittig reactions and the exocyclic methylene function is introduced as previously described. This process yields 4-isoavenaciolide (77) which was identical spectroscopically and optically to the natural material. Tha absolute configuration of isoavenaciolide is therefore (2R,3R,4S) (77).

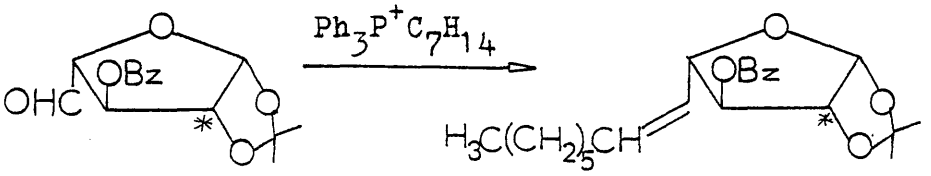
SCHEME 22

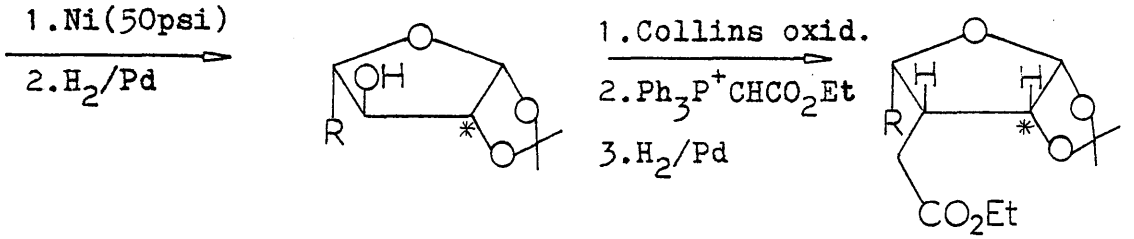


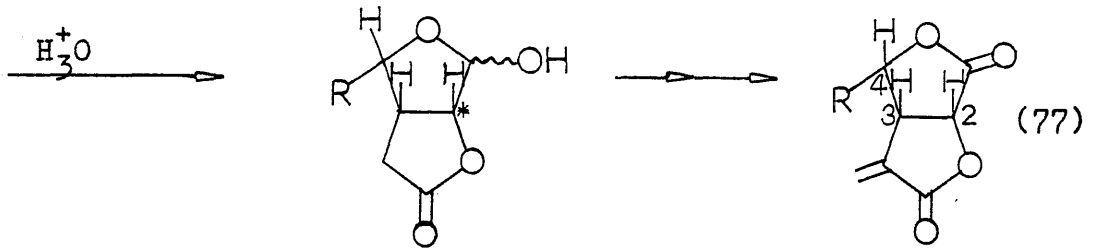
(107)



(113)







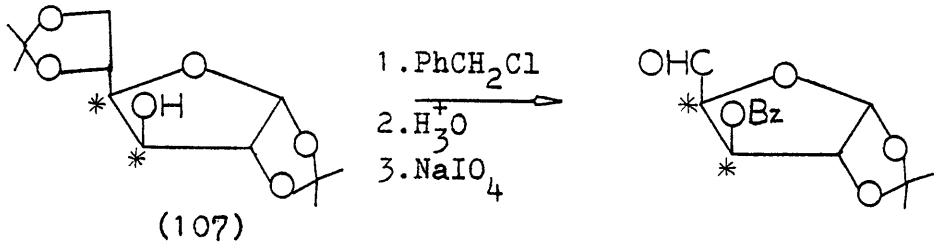
(77)

* Configuration retained here

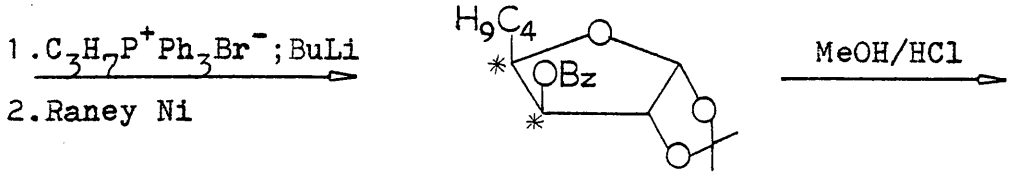
(ii) Canadensolide

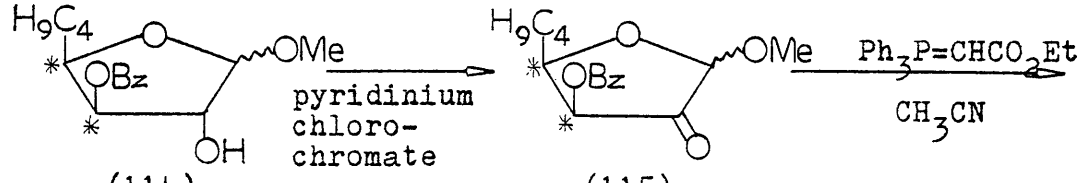
The synthesis of (±)-canadensolide (2)[66] has already been discussed in some detail (p. 24). The synthesis of naturally occurring canadensolide (2) was reported in 1978[92] and is similar in concept to the previously described syntheses of avenaciolide (4) (p. 62) and 4-isoavenaciolide (77) (p. 64) from the same laboratory and is shown in (Scheme 23). This synthesis used 'diacetone glucose' (107), as before, as the starting material. The alcohol (114) was not easily oxidisable and pyridinium chlorochromate was found to be the most suitable reagent - the resulting ketone (115) had to be reacted immediately to minimise decomposition and the final products of Wittig condensation and reduction were the diastereoisomers (116a+b) and (117a+b). Only (116a+b) cis isomers gave the bis-lactone (118) on hydrogenolysis of the benzyl group. Gentle acid catalysis (pyridinium p-toluene sulphonate (PPTS)) protected the alcohol group in (119) and ethoxycarbonylation using ethyl chloroformate followed by deprotection of the alcohol and Jones oxidation gave (120). The ethoxycarbonyl group was gently hydrolysed and the exocyclic methylene group introduced via a Mannich reaction as previously described. The canadensolide (2) isolated thus had absolute stereochemistry (2S,3R,4R) and was identical in all respects to natural material.

SCHEME 23

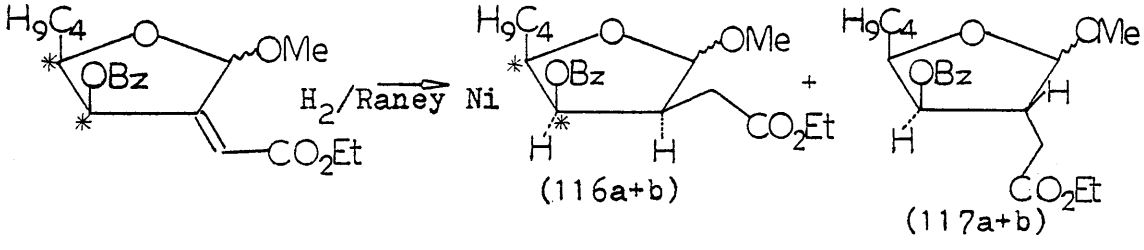


(107)

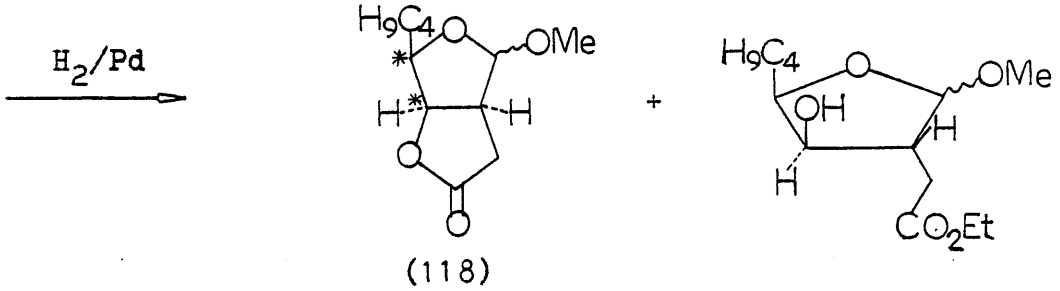




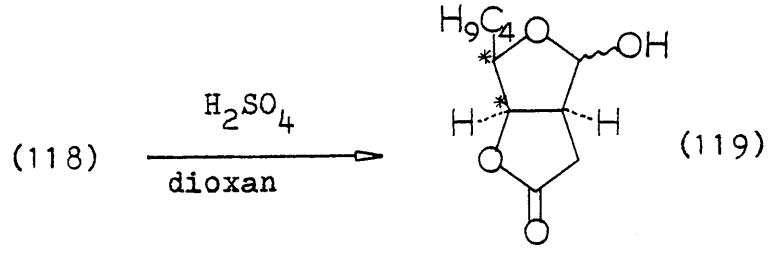
(114) (115)



(116a+b) (117a+b)

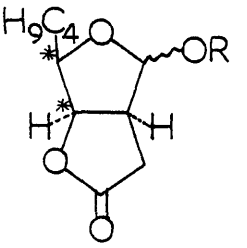


(118)



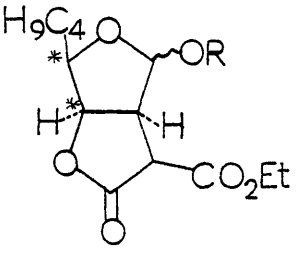
SCHEME 23(contd.)

(119) —ethyl vinyl ether / PPTS→

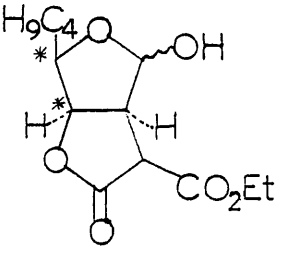


R = α-ethoxyethyl

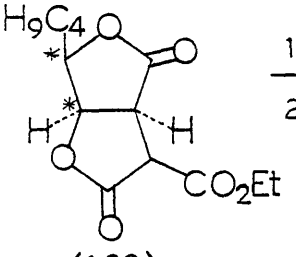
—$ClCO_2Et$ / $Li\ N(Pr^i)_2$→



—EtOH / PPTS 50°C→

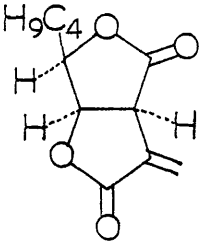


—Jones oxidn.→



(120)

—1.HCl/dioxan / 2.$HCHO/(CH_3)_2NH$→



(2)

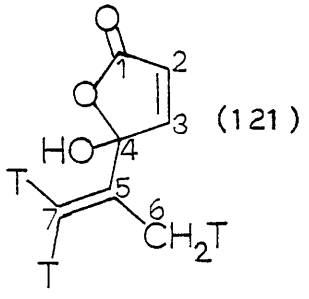
✳ configuration retained here

APPENDIX 3

Biosynthetic Studies designed to follow the Fate of Hydrogen Atoms in Biosynthesis.

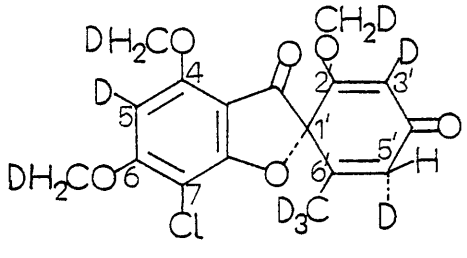
At the time that this work was started, there were several literature examples of the use of N.M.R. spectroscopy to follow labelled hydrogen atoms in the biosynthesis of natural products.

^{3}H-N.M.R. spectroscopy has been used to study the biosynthesis of penicillinic acid (121) in P.cyclopium[123], [2-C^{3}H$_{3}$] -acetate giving (121) with tritium at C-2, C-6 and C-7.



(121)

A study of the biosynthesis of griseofulvin (122) by P.urticae using [2-^{2}H$_{3}$] -acetate has been reported[124]. The location of the deuterium atoms in the isolated griseofulvin was determined using F.T. ^{2}H-N.M.R. The pattern of labelling (122) was determined by comparison of the biosynthetically labelled metabolite with several synthetically labelled models.

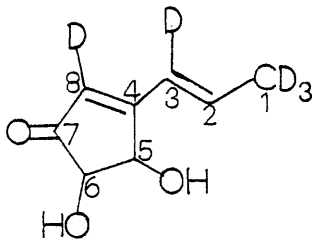


(122)

The advantage of the method using ^{13}C-D couplings to determine the position of deuterium in the metabolites is that it enables conclusive identification of the labelling pattern without the need for labelled standards as above.

The intact incorporation of seven methyl groups into the macrocycle of vitamin B_{12} was proved using deuterium noise-decoupled ^{13}C-F.T. N.M.R.[125,126] and also using [^{1}H, ^{13}C] -N.M.R. difference spectroscopy[127], of [methyl-$^{13}C^{2}H_3$]-methionine enriched dicyanocobalamin.

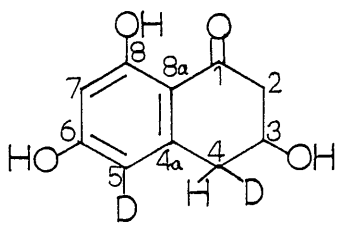
In the course of our studies it was reported[128] that [2-$^{13}CD_3$]-acetate is incorporated into the polyketide terrein (123) at C-1, C-3 and C-8 as indicated. The presence



(123)

of deuterium was shown using ^{13}C-N.M.R. and deuterium-decoupled ^{13}C-N.M.R. However, the resolution, particularly of the ^{13}C-D triplets is very much less good than has been found in our studies. This is due to excessive exchange in the biosynthesis.

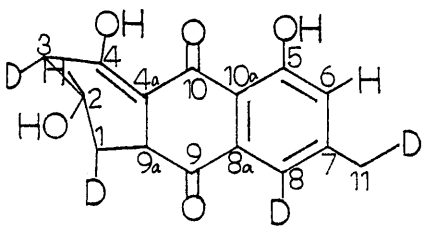
The method has also been reported[148] in a study of the biosynthesis of the polyketide scytalone (124). Enriched



(124)

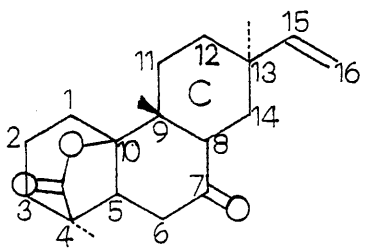
^{13}C-N.M.R. signals due to C-2, C-4, C-5, C-7 and C-8a were noticed, however, ^{13}C-D coupling was noticed only at C-4 - in this case a very clear triplet (1:1:1, J=20Hz.). A later study[149] using deuterium - decoupled ^{13}C-N.M.R. showed also the presence ofdeuterium at C-5 but none was observed at C-2 or C-7.

Deuterium noise decoupling ^{13}C-N.M.R. of the [2-$^{13}CD_3$]-acetate derived polyketide (+)-rugulosin (125)[149] showed clearly the incorporation of deuterium at C-1, C-3, C-8 and C-11.



(125)

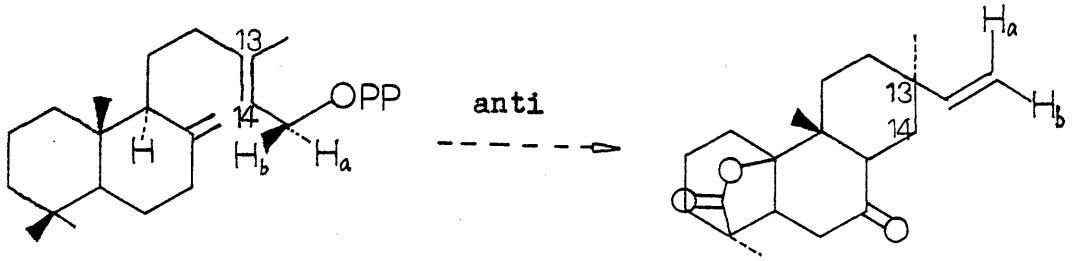
^{2}H-N.M.R. spectroscopy has been used[150] to study the formation of ring C in the biosynthesis of the fungal diterpene rosenonolactone (126) by T.roseum.



(126)

Samples of [5-$^{2}H_2$]-mevalonate, (5R)-[5-^{2}H]-mevalonate and (5S)-[5-^{2}H]-mevalonate were administered to cultures of T. roseum and the isolated rosenonolactone analysed using ^{2}H-N.M.R.

It was established that the 5-pro-R hydrogen of mevalonate becomes the 16Z hydrogen of (126) and conversely that the 16E hydrogen of (126) is derived from the 5-pro-S hydrogen of mevalonate. These results (taken with the known direction of attack at the 13,14 double bond of precursor (127)) established that the allylic displacement (fig. 44) leading to ring C of rosenonolactone occurs with overall anti stereochemistry.

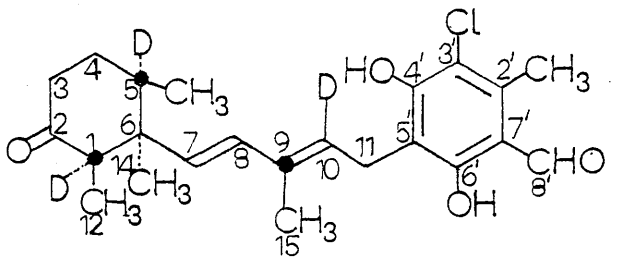


(127) (126)

(fig.44)

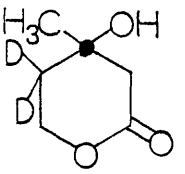
The mechanism of carbocyclic ring formation during the biosynthesis of asochlorin (128) in Nectria coccinea has

(128)



• ^{13}C

been studied[151] by administration of [3-^{13}C, 4-$^{2}H_2$] -mevalonic acid (129).



(129)

• ^{13}C

In the ^{13}C-N.M.R. spectrum of $^{13}C/^{2}H$ enriched asochlorin (128) the C-9 signal appeared as two lines, separated by 2Hz., and showed a 1.2 fold enhancement relative to natural abundance, which is consistent with its being derived from C-3 of mevalonic acid. The line at higher field was due to $^{2}H/^{13}C$ enriched molecules of (128) containing ^{13}C at C-9 and ^{2}H at C-10. The line at lower field was due to natural

abundance ^{13}C at C-9 in unenriched molecules of (128). This is in agreement with the previously observed[152] secondary isotopic substitution effect (^{13}C-C-^{2}H vs. ^{13}C-C-^{1}H).

The observed collapse of the ^{13}C signals due to C-1 and C-5 relative to C-9, in $^{2}H/^{13}C$ enriched asochlorin (128), is due to the presence of deuterium at these positions. Indeed, in the case of C-5, two lines of the ^{13}C-D triplet (Δ= 0.36ppm.) were clearly visible (J = 23.9Hz.) but were of weak intensity. The remaining line of the triplet was obscured by the natural abundance C-5 signal.

No triplet was observed for C-1, perhaps due to the longer relaxation time of this carbon atom. This study provided strong evidence for a series of 1,2 hydrogen shifts in the biosynthesis of asochlorin and illustrates the high degree of sensitivity obtainable using this method of detection of deuterium in a natural product.

APPENDIX 4

TABLE 7

		d_1	d_2	d_3
Canadensolide	(fig.41)	21.1%	25.8%	53.1%
	$Cr(acac)_3$	24%	22.9%	53.1%
Ethisolide	(fig.36)	9.4%	25.4%	65.2%
	$Cr(acac)_3$	11.8%	26%	62.2%
Avenaciolide	(fig.43)	12.8%	25.0%	61.2%
Dihydro-canadensolide	(fig.38)	19.4%	16.4%	64.2%
	$Cr(acac)_3$	12.2%	24.5%	63.3%
Dihydro-canadensolide	(fig.40)	21.4%	26.7%	51.9%
	$Cr(acac)_3$	19.4%	33.6%	47.0%
Canadensic Acid	(fig.42)	19.0%	19.0%	61.0%

In all of these cases d_3 is the main contributor, usually greater than 50%. Hence approximately one half of the deuterium remains unexchanged.

Chapter 8

Experimental Section

I. General.

II. Isolation of Decumbic Acid from *P.decumbens*.

III. Ethisolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies using *P.decumbens*.

1. Attempted Syntheses of Ethisic Acid
   - (i) Via Ethyl (trans) hex-2-enoate
   - (ii) Via Ethyl 3-carbethoxy-2-oxo-hexanoate
2. Attempted Synthesis of α-Alkylitaconic Acids from Itaconic acid
3. Synthesis of α-*n*-Butylitaconic Acid via 1,1,2-Tricarbomethoxyethene
4. Incorporation of [1-^{14}C]2,3-Dicarboxyhept-1-ene into Ethisolide by cultures of *P.decumbens*
5. Distribution of the Label in the [^{14}C] Ethisolide

IV. Canadensolide and Dihydrocanadensolide: Preparation of Potential Biosynthetic Intermediates and Incorporation Studies using *P.canadense*.

1. Attempted Preparations of 1,1,2-Tricarbomethoxyoctane
2. Synthesis of α-*n*-Hexylitaconic acid
3. Administration of [1-^{14}C]2,3-Dicarboxynon-1-ene to Cultures of *P.canadense*
4. Administration of [^{14}C-methylidine] Canadensic Acid to cultures of *P.canadense*
5. Administration of [^{14}C-methylidine] Canadensolide to Cultures of *P.canadense*

V. Avenaciolide: Attempted Synthesis of a Potential Biosynthetic Intermediate and Incorporation Studies using A.avenaceus.

1. Attempted Synthesis of [1-^{14}C]2,3-Dicarboxytridec-1-ene
2. Incorporation Studies using A.avenaceus

VI. Stereochemistry.

1. Studies Related to the Absolute Stereochemistry of Canadensolide and Ethisolide
2. Relative Stereochemistry of Canadensolide and Dihydrocanadensolide
3. Feeding Experiments with [2-^{13}C, 2-$^{2}H_3$]Sodium Acetate

I. General.

The three cultures studied in this thesis are Penicillium decumbens (3903 OOT), Penicillium canadense (Commonwealth Mycological Insitute No.95493) and Aspergillus avenaceus (AGG 4558). The fungi were subcultured on to 2% malt agar slants and thence to agar seed bottles (15×9 cms.). A spore suspension, prepared from twelve such bottles and distilled water (2l.) was used to innoculate 100 Roux surface culture bottles containing culture medium (200ml.) which had previously been sterilised (0.5hrs. with steam at 117°C and 12p.s.i.).

P.decumbens and A.avenaceus were grown on a culture medium (Czapek-Dox + 0.1% Yeast extract) which contained glucose (50g.), $NaNO_3$ (2g.), KCl (1g.), $MgSO_4.7H_2O$ (1g.), K_2HPO_4 (0.5g.), $FeSO_4.7H_2O$ (0.01g.) and yeast extract (0.1g.) per litre of distilled water, while P.canadense was grown on a culture medium containing glucose (50g.), ammonium tartrate (2.8g.), K_2HPO_4 (5g.), $MgSO_4.7H_2O$ (1.0g.), NaCl (1.0g.), yeast extract (0.5g.), $FeSO_4.7H_2O$ (0.1g.), $CuSO_4.5H_2O$ (0.015g.), $ZnSO_4.7H_2O$ (0.05g.), $MnSO_4$ (0.01g.) and Na_2MoO_4 (0.01g.) per litre of distilled water.

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

Thanks and recognition are due to the staff of the Glasgow University Mycology Unit who prepared all of the cultures used in this work.

The samples used in the feeding experiments were sterilised in the way described above for the Roux culture bottles, except in the case of the $[2\text{-}^{13}C, 2\text{-}^{2}H_3]$ sodium acetate samples which were sterilised using a millipore filter.

Radio-active assays were carried out using a Phillips Liquid Scintillation Counter. Samples were weighed on metal foil, transferred to Packard Scintillation Vials and dissolved in toluene scintillation solution (15ml.). The scintillation solution contained 2,5-diphenyloxazole (4g.) and 1,4-bis-2(4-methyl-5-phenyloxazalyl)-benzene (0.1g.) per litre of toluene. The samples were crystallised until constant activity was achieved and counted sufficiently long to achieve an error of less than 3%.

^{13}C-N.M.R. spectra were recorded using a Varian XL-100 spectrometer operating in the Fourier Transform mode with a probe temperature of ca. 35°C. Unless otherwise stated, the spectra were determined in $CDCl_3$ solutions containing tetramethylsilane as an internal reference. Proton noise decoupling was carried out using a wide-band noise decoupler.

^{1}H-N.M.R. spectra were recorded using Varian T-60, H.A.-100 and Perkin-Elmer R32 spectrometers using $CDCl_3$ solutions with tetramethylsilane as internal standard.

I.R. spectra were recorded with a Perkin-Elmer 227 spectrophotometer. Mass Spectra were recorded on a G.E.C.-A.E.I. M.S. 12 spectrometer (low resolution) and a G.E.C.-A.E.I. M.S. 902 spectrometer (high resolution). G.C.-M.S. results were obtained using an L.K.B.-9000 instrument.

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

Unless otherwise stated, all organic extracts were washed with satd. aqueous NaCl and dried over anhyd. Na_2SC_4 prior to evaporation. Light petroleum refers to the fraction boiling between 60-80°C unless otherwise stated.

Abbreviations:

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

Experimental Section

II. Isolation of Decumbic Acid from *P.decumbens*.

Isolation of Decumbic acid (40) from P.decumbens.

The aqueous broth from 25 bottles of a 14-day culture of P.decumbens was continuously extracted with ethyl acetate for 40hrs. The extract was washed with satd. aqueous $NaHCO_3$, dried and evaporated to give ethisolide (1),(3.25g.),m.p. 121-123°C from ethanol. (Lit.[44] m.p. 122-123°C).

The alkaline extract was acidified with conc. HCl, saturated with solid NaCl, and extracted with ethyl acetate. The ethyl acetate was dried and evaporated to yield a brown oily solid (0.52g.). Preparative TLC on silica gel GF_{254} (using ether:hexane:acetic acid (50:50:1) as eluent) gave decumbic acid (40),Rf.0.6,(0.35g.), m.p. 98-99°C from ethyl acetate - light petroleum.

I.R. (KBr disc)

3500-2300 cm^{-1} ; 1780 cm^{-1} ; 1745 cm^{-1} ; 1710 cm^{-1}.

^{1}H-N.M.R. (100 MHz.)

5.15δ (1H, m, H-3); 1.42δ (4H, m, H-4,H-5); 2.14δ (3H, d, J=2Hz., H-9); 0.92δ (3H, t, J=7Hz., H-6).

Mass Spectrum m/e (rel. abundance)

M^+. 184(8), 155(80), 142(100; $M-C_3H_6^{\cdot}$), 124(40), 96(70).

Analysis

Found: C, 58.44 ; H, 6.93

$C_9H_{12}O_4$ requires C, 58.69; H, 6.52%.

Circular Dichroism. (methanol) λ n.m/ΔE

256n.m./-0.79 , 223n.m./+2.76.

Experimental Section

III. Ethisolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies using *P.decumbens*

1. Attempted Syntheses of Ethisic Acid
   - (i) Via Ethyl (trans) hex-2-enoate
   - (ii) Via Ethyl 3-carbethoxy-2-oxo-hexanoate
2. Attempted Synthesis of α-Alkylitaconic Acids from Itaconic Acid
3. Synthesis of α-*n*-Butylitaconic Acid via 1,1,2-Tricarbomethoxyethene
4. Incorporation of [1-^{14}C]2,3-Dicarboxyhept-1-ene into Ethisolide by cultures of *P.decumbens*
5. Distribution of the Label in the [^{14}C] Ethisolide

Ethisolide: Synthesis of Potential Biosynthetic Intermediates and Incorporation Studies with P.decumbens.

1. Attempted syntheses of Ethisic acid (38).

(i) Via ethyl (trans) hex-2-enoate (45).

Preparation of Ethyl (trans) hex-2-enoate.[129]

Re-distilled n-butyraldehyde (88g.) was added over 1hr. to a cooled (4°C) mixture of malonic acid (100g.), pyridine (84ml.) and dry ethanol (133ml.). The mixture was then gently warmed to dissolve the malonic acid and was stirred at room temperature for 2hrs. The reaction was then refluxed for 3hrs. and allowed to cool. It was acidified using 6N-aqueous HCl and extracted thoroughly with ether. The resulting crude product was esterified by refluxing with dry ethanol (58ml.) and conc. H_2SO_4 (5ml.) for 5hrs. After dilution with ether, the product was washed with satd. aqueous Na_2CO_3 and the ether was dried and evaporated to give the ester, b.p. 60-64°C/10mm, (45.9g.,26.4%). (Lit.[130] b.p. 73-74°C/18mm).

I.R. (CCl_4)

1725 cm^{-1}; 1660 cm^{-1} ; 980 cm^{-1} .

^{1}H-N.M.R. (60Mhz.)

6.97δ (1H, d.t , J=15Hz., 7Hz., H-3); 5.81δ (1H, d, J=15Hz., H-2); 4.20δ (2H, q, J=7Hz., ester $-CH_2-$); 2.16δ (2H, b.q, J=7Hz., H-4); 1.27δ (3H, t, J=7Hz., ester $-CH_3$); 1.27δ (2H, m, H-5); 0.92δ (3H, t, J=7Hz., H-6).

Preparation of 2-bromopropionic acid[131]

Ethyl 2-bromopropionate (51g.) was shaken with a solution of potassium hydroxide (32g.) in distilled water (250ml.) until it dissolved. The solution was washed with ether, acidified with 6N-aqueous H_2SO_4, saturated with NaCl and thoroughly extracted with ether. The ether was dried and evaporated to give the bromo-acid, b.p. 90-95°C/10mm,

(28.2g., 65.4%). (Lit.[131] b.p. 102-104°C/15-16mm).

I.R. (CCl_4)

1730 cm^{-1}; 1180 cm^{-1} .

^{1}H-N.M.R. (60MHz.)

4.41δ (1H, q, J=7Hz., H-2); 1.85δ (3H, d, J=7Hz., H-3). These spectra were identical to those of an authentic sample of 2-bromopropionic acid.

Preparation of ^{t}Butyl 2-bromopropionate[132]

To a stirred solution of 2-bromopropionic acid (22.4g.) in sodium-dried benzene (45ml.) was added gently oxalyl chloride (37.2g.). The solution was gently refluxed for 2hrs. and excess oxalyl chloride was evaporated at atmospheric pressure (<90°C) to give the crude acid chloride, (26.15g., 95.5%).

A mixture of anhydrous tert. butanol (20.3g.) and redistilled N,N-dimethylaniline (35.9g., b.p. 190-192°C) in dry ether (30ml.) was stirred and refluxed gently on a water bath. The crude acid chloride was added dropwise so that refluxing continued in the absence of heating and the reaction was refluxed gently for a further hour and was then cooled to room temperature. Water (30 ml.) was added and the reaction stirred at room temperature. The organic layer was separated and washed thoroughly with portions of cold 10%-aqueous H_2SO_4, followed by saturated aqueous $NaHCO_3$. The organic solution was dried and evaporated to give the bromo-ester, b.p. 54-58°C/10mm, (13.8g., 47.1%). (Lit.[133] b.p. 62.2°C/15 mm).

I.R. (CCl_4)

1740 cm^{-1}; 1370 cm^{-1}; 1240 cm^{-1}; 1145 cm^{-1}.

^{1}H-N.M.R. (60 MHz.)

4.26δ (1H, q, J=7Hz., H-2); 1.75δ (3H, d, J=7Hz., H-3); 1.48δ (9H, s, $-C(CH_3)_3$).

Preparation of Sodium Methyl Mercaptide[134]

Methanethiol (100g.) was cooled to -78°C (acetone/dry-ice) and the liquified thiol was poured into a sealed system fitted with an acidic potassium permanganate vapour trap, and containing a solution of metallic sodium (47.9g.) in anhydrous ethanol (400ml.). The reaction mixture was brought to room temperature and was then refluxed for one hour. Anhydrous benzene (100ml.) was added at room temperature and the solvent evaporated to give a pale yellow solid which was washed with anhydrous ether, filtered and dried to give sodium methyl mercaptide, (116g., 79%).

^{1}H-N.M.R. (60MHz.) in CD_3OD

1.98δ (3H, s, CH_3-S^-).

Preparation of ^{t}Butyl 2-(thiomethyl)-propionate (44).[134]

^{t}Butyl 2-bromopropionate (21.3g.) in anhydrous ether (20ml.) was added dropwise to a suspension of sodium methyl-mercaptide (11.2g.) in anhydrous ether (80ml.). The resulting cloudy suspension was stirred at room temperature for 6hrs. and filtered, and the ethereal filtrate evaporated to give the thio-ester (44), b.p. 66°C/10mm, (14.6g., 85.3%).

I.R. (CCl_4)

1735 cm^{-1} (d) ; 1370 cm^{-1}; 1235 cm^{-1}; 1145cm^{-1} ; 1067 cm^{-1} .

^{1}H-N.M.R. (60Mhz.)

3.27δ (1H, q, J=7Hz., H-2); 2.19δ (3H, s, S-CH_3); 1.48δ (9H, s, -$C(CH_3)_3$); 1.39δ (3H, d, J=7Hz., H-3).

Mass Spectrum m/e (rel. abundance)

M^+. 176(12), 75(35, H_3C-$\overset{+}{C}H$-SCH_3), 57(100, $\overset{+}{C}(CH_3)_3$), 47(32, $\overset{+}{S}CH_3$).

Mass Measurement

Found: 176.08702.

$C_8H_{16}SO_2$ requires 176.08708 a.m.u.

Preparation of Sodium Thiophenoxide[134]

Thiophenol (3.2ml.) was added dropwise to a stirred solution of metallic sodium (2.1g.) in anhydrous ethanol (80ml.). The experimental method used was the same as for sodium methyl mercaptide above, and sodium thiophenoxide was isolated as a white solid,(11.1g., 93.4%).

^{1}H-N.M.R. (60MHz.) in CD_3OD

7.26δ (2H, d.d, J=1Hz., 8Hz., H-1,H-5); 6.71 (3H, m, H-2,H-3,H-4).

Preparation of ^{t}Butyl 2-(thiophenyl)-propionate.[134]

^{t}Butyl 2-bromopropionate (15g.) in anhydrous ether (20ml.) was added to a suspension of sodium thiophenoxide (10g.) in anhydrous ether. Using the same method as for the preparation of ^{t}butyl 2-(thiomethyl)-propionate (44), the reaction gave ^{t}butyl 2-(thiophenyl)-propionate b.p. 86-88°C/8mm, (20.4g., 75.6%).

I.R. (CCl_4)

1735 cm^{-1}; 1585 cm^{-1}; 1145 cm^{-1}; 845 cm^{-1}; 685 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

7.35δ (5H, m, aromatic H); 3.72δ (1H,q, J=7Hz., H-2); 1.41δ (3H, d, J=7Hz., H-3); 1.35δ (9H, s, $-C(CH_3)_3$).

Mass Spectrum m/e (rel. abundance)

M^+. 238(30), 137(100,$H_3C-\overset{+}{C}H-SC_6H_5$), 109(90, $\overset{+}{S}C_6H_5$).

Analysis

Found: C, 65.72; H, 7.14; S, 13.12.

$C_{13}H_{18}SO_2$ requires C, 65.55; H, 7.56; S, 13.44%.

Attempted addition of ^{t}Butyl 2-(thiomethyl)-propionate(44) and Ethyl (trans) Hex-2-enoate (45).[48]

(a) Dry di-isopropylamine (2.87g.) was added dropwise under nitrogen to a cooled (4°C) solution of n-butyl lithium (20ml. of 2.4M soln. in hexane) in anhydrous tetrahydrofuran (28ml.), and the mixture stirred at room temperature for 20mins.

The resulting pale solution of lithium di-isopropylamide[135] was cooled to -78°C and to this was added dropwise ^{t}butyl 2-(thiomethyl)-propionate (44) (5g.). The mixture was then stirred at -78°C for 30mins. Ethyl (trans) hex-2-enoate (45) (4.03g.) in anhydrous tetrahydrofuran (28mls.) was added during 20mins. and the stirring at -78°C continued for 2hrs. Iodine (4.33g.) in anhydrous tetrahydrofuran (34mls.) was added dropwise (still at -78°C). After stirring for a further 30mins., the reaction was allowed to come to 0°C and ether (30mls.) was added. The organic solution was washed successively with 6N-aqueous HCl and saturated aqueous Na HCO_3, dried and evaporated to give a red, oily mixture (8.4g.) which consisted chiefly of starting materials eg. ^{1}H-N.M.R. (60MHz.) [6.81δ(1H, m); 5.72δ(1H, d, J=15Hz.)] .

Gas Chromatography-Mass Spectroscopy ,(SE30 at 85°C), rel. retention time/integral/M^{+}.

1, 1.5, m/e 142	ethyl(trans)hex-2-enoate
1.6, 1.5, m/e 142	ethyl(cis)hex-2-enoate
1.95, 1.0, m/e 176	^{t}butyl 2-(thiomethyl)-propionate
4.0, 0.7, m/e 132	unidentified component

(b) Reaction as above, followed by treatment with Acid.

The above reaction was repeated using ^{t}butyl 2-(thiomethyl)-propionate (3g.), lithium di-isopropylamide (prepared from di-isopropylamine (2.43g.) and n-butyl lithium solution (10ml.)) and ethyl (trans) hex-2-enoate (2.41g.). The product isolated was a pale yellow oily mixture (5.82g.) in which no unsaturated esters were present and which had an I.R. max. at 1730 cm^{-1}.

The above oily mixture (3g.) in anhydrous benzene (6ml.) was refluxed for three hours with p-toluenesulphonic acid (1.2g.). The reaction was then stirred for 30mins. at room temperature with solid $NaHCO_3$. The resulting solution was

filtered, washed with aqueous $Na_2S_2O_3$, diluted with ether (20ml.) and dried and evaporated to give a pale yellow oil (0.54g.) which showed I.R. maxima at 1785 cm^{-1} (w) and 1740 cm^{-1}.

Preparative TLC of a sample (100mgs.) on kieselgel GF_{254} (using $CHCl_3$:light petroleum: HOAc (50:50:1) as eluent) gave a band, Rf. 0.2, (20mgs.) which still appeared to be a mixture, possibly containing some lactonic material.

^{1}H-N.M.R. (60MHz.)

4.20 δ (m); 3.39 (m); 2.36 δ (s); 2.11 (s); 1.30 δ (m); 0.95 δ (t, J=7Hz.).

Also obtained were ethyl (trans) hex-2-enoate (45), Rf. 0.5, (23mgs.), [^{1}H-N.M.R. (60MHz.) eg. 6.81 δ (m) and 5.72 δ (d, J=15Hz.)] and tert.butanol, Rf. 0.9, (12mgs.) [^{1}H-N.M.R. (60MHz.) eg. 1.25 δ (s)]. The reaction was not investigated further.

(ii) Via Ethyl 3-carbethoxy-2-oxo-Hexanoate (50).

Preparation of Ethyl 3-carbethoxy-2-oxo-Hexanoate (50).[136]

A mixture of diethyl oxalate (58.4g.) and ethyl valerate (52g.) was added dropwise under nitrogen to a stirred suspension of sodium hydride (12.0g.) in sodium-dried ether (200ml.). Once the initial hydrogen evolution had ceased, stirring was continued under gentle reflux. TLC (with 10% ether in light-petroleum as eluent) was used to determine when the reaction was complete, (20hrs.). After addition of dry ethanol (5ml.), the mixture was stirred for 15mins. at room temperature, and then poured on to ice (ca. 100g.) to which conc. H_2SO_4 (ca. 5ml.) had been added. Extraction with ether gave the desired keto-ester (50), b.p. 90-92°C/ 1.0mm, (61.2g., 66.5%). (Lit.[136] b.p. 120°C/1.5-2.0mm).

^{1}H-N.M.R. (100MHz.)

4.21 δ (5H, m, ester $-CH_2-$, H-3); 1.87 δ (2H, m, H-4); 1.32 δ (8H, m, ester $-CH_3$, H-5); 0.95 δ (3H, t, J=7Hz., H-6).

I.R. (CCl_4)

3440 cm^{-1} (w); 1785 cm^{-1}; 1755 cm^{-1}; 1730 cm^{-1}; 1655 cm^{-1}.

Mass Spectrum m/e (rel. abundance)

M^+. 230(1), 229(10), 201(20, M-29), 183(15), 182(16), 157(100, M-73).

Analysis

Found: C, 57.39; H, 7.79.

Calcd. for $C_{11}H_{18}O_5$: C, 57.39; H, 7.82%.

Preparation of Ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-Hexanoate (51).[137]

Ethyl 3-carbethoxy-2-oxo-hexanoate (10g.) in sodium dried benzene (30ml.) was added under nitrogen to a stirred suspension of sodium hydride (1.25g.) in sodium-dried benzene (30ml.). When the initial evolution of hydrogen had ceased, the mixture was heated to gentle reflux and ethyl bromoacetate (3.7g.) in sodium-dried benzene (10ml.) was added dropwise over 2hrs. After stirring and refluxing for a further 45mins., the mixture was allowed to cool, and was poured on to an ice-water mixture. The organic layer was separated and the aqueous layer thoroughly extracted with ether. The combined organic layers were washed successively with 6N-aqueous HCl and brine before drying and evaporating to give the tricarboxylic ester (51), b.p. 104°C/0.2mm, (3.5g., 25.4%). (Lit.[137] b.p. 142-147°C/1.0-1.2mm).

^{1}H-N.M.R. (100MHz)

4.23 δ (6H, m, ester $-CH_2-$); 2.84 δ (2H, m, H-7); 1.90 δ (2H, m, H-4); 1.35 δ (11H, m, ester $-CH_3$, H-5); 0.95 δ (3H, t, J=7Hz., H-6).

I.R. (CCl_4)

1740 cm^{-1} (br.)

Mass Spectrum m/e (rel. abundance)

M^+. 316(0.2), 243(30, M-73), 230(10, M-86), 215(10), 202(25), 156(100, M-73-87).

Analysis

Found: C, 57.58; H, 8.16.

Calcd. for $C_{15}H_{24}O_7$: C, 56.96; H, 7.59%.

Reactions of Ethyl 3-Carbethoxy-3-Carbethoxymethyl-2-oxo-Hexanoate (51) with Potassium Hydroxide.

(a) Formation of n-Propyl Succinic acid (55).

A suspension of ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51), (2.5g.) in 3M-aqueous KOH (30ml.) was stirred overnight at room temperature. The resulting clear solution was acidified at 0°C with 6N-aqueous H_2SO_4, saturated with solid NaCl and thoroughly extracted with ether. The ether solution was dried and evaporated to give an oily solid (1.54g.), identified as n-propyl succinic acid (55). (Lit.[138] m.p. 95°C).

^{1}H-N.M.R. (60MHz.)

2.72 δ (1H, m, H-3); 2.29 δ (2H, t, J=7Hz., H-2); 1.41 δ (4H, m, H-4,H-5); 0.91 δ (3H, t, J=7Hz., H-6).

^{13}C-N.M.R.

Chemical Shift (δ)	Carbon	Multiplicity	$J_{res.}$ (Hz)
181.61	C-1		
178.65	C-7		
40.75	C-3	d.	24.4
35.50	C-2	t.	23.8
33.77	C-4	t.	13.3
20.09	C-5	t.	10.3
13.79	C-6	q.	7.5

A sample (0.235g.), of the crude n-propyl succinic acid (55) was dissolved in anhydrous methanol (10ml.) and added dropwise at 0°C to a stirred solution prepared from thionyl chloride (10ml.) and anhydrous methanol (20ml.). After stirring overnight, the solvent was evaporated, the residue taken up in ethyl acetate, washed successively with satd. aqueous Na_2CO_3 and brine and then dried and evaporated giving a pale yellow oil (182mg.). Preparative TLC on silica gel GF_{254} (using 50:50 ether:light petroleum as eluent) gave dimethyl n-propyl succinic acid (55), Rf 0.6, (63mg.). (Lit.[139] b.p. 110°C/14mm).

^{1}H-N.M.R. (60MHz.)

3.64 δ (3H, s, ester $-CH_3$); 3.62 δ (3H, s, ester $-CH_3$); 2.48 δ (3H, m, H-2,H-3); 1.39 δ (4H, m, H-4, H-5); 0.92 δ (3H, t, J=7Hz., H-6).

I.R. (CCl_4)

1745 cm^{-1}; 1160 cm^{-1}.

Mass Spectrum m/e (rel. abundance)

M^+-31. 157(30), 146(20, M-42), 115(95, M-73), 114(100, M-74), 87(60, M-42-59).

$C_9H_{16}O_4$ requires M^+ m/e 188

(b) Formation of n-Propyl Succinic Acid (55) and the Rearranged Tri-acid (56).

Ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51) (0.96g.) was treated as in (a) above with 1M-aqueous KOH (15ml.). The product was obtained as a pale yellow gum (0.76g.) which was esterified using excess ethereal diazomethane (prepared from nitrosan (5g.)) at room temperature. Evaporation of the ether gave a mixture (0.57g.). Preparative TLC on silica gel GF_{254} (using 20% ethyl acetate in hexane as eluent) of a sample of the mixture (140mg.) gave the

methoxy-triester (56), Rf 0.2, as an oil (47mg.).

^{1}H-N.M.R. (60MHz.)

4.55 δ (3H, s, methoxyl $-CH_3$); 3.84 δ (3H, s, ester $-CH_3$); 3.81 δ (3H, s, ester $-CH_3$); 3.78 δ (3H, s, ester $-CH_3$); 2.49 δ (1H, t, J=7Hz., H-4); 1.42 δ (4H, m, H-5, H-6); 0.95 δ (3H, t, J=7Hz., H-7).

I.R. (CCl_4)

1770 cm^{-1}; 1740 cm^{-1}; 1640 cm^{-1}.

Mass Spectrum m/e (rel. abundance)

M^+. 288(5), 243(100, M-45), 242(90, M-31-15), 229(10, M-59), 217(80), 215(90), 201(60).

$C_{13}H_{20}O_7$ requires M^+ m/e 288

Also obtained was dimethyl n-propyl succinic acid (55), Rf 0.4, (25mg.), which was identical (Rf, IR, ^{1}H-NMR) to the sample produced in (a).

Reactions of Ethyl 3-Carbethoxy-3-Carbethoxymethyl-2-oxo-Hexanoate (51) with 2N-aqueous HCl.

(a) A mixture of ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51) (2.2g.) and 2N-aqueous HCl (17.5ml.) was stirred at reflux overnight. The cooled solution was saturated with solid NaCl and extracted with ethyl acetate. The organic solution was dried and evaporated to yield a pale yellow oil (1.63g.).

A portion of this (220mg.), was treated with an excess of ethereal diazomethane (prepared from nitrosan (1g.)) at room temperature overnight and the solution evaporated to give an oil (71mg.). Preparative TLC on silica gel GF_{254} (using 80% $CHCl_3$ in light petroleum as eluent) gave the butenolide (58) as a colourless oil, Rf 0.3, (19mg.).

^{1}H-N.M.R. (60MHz.)

6.08 δ (1H, s(br.), H-2); 3.78 δ (3H, s, ester $-CH_3$); 2.21 δ (3H, s, H-9); 1.39 δ (4H, m, H-4, H-5); 0.90 δ (3H, t,

J=7Hz., H-6).

I.R. (CCl_4)

1740 cm^{-1}; 1705 cm^{-1}; 1620 cm^{-1}.

Mass Spectrum m/e (rel. abundance)

M^+-29. 169(20), 167(10), 125(30), 123(25), 111(35), 110(10), 109(30), 97(100,M-59-42), 95(80, M-59-44).

$C_{10}H_{14}O_4$ requires M^+. m/e 198

(b) The crude oily mixture (250mg.), prepared as in (a) by treatment of ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51) (0.75g.) with 2N-aqueous HCl (24ml.), was dissolved in anhydrous methanol (10ml.) and added dropwise at 0°C to a stirred solution prepared from thionyl chloride (0.35g.) and anhydrous methanol (15ml.). After stirring overnight at room temperature, the solvent was evaporated and the residue taken up in ethyl acetate, washed successively with saturated aqueous Na_2CO_3 and brine, dried and evaporated, giving a yellow oil (160mg.). Preparative TLC on silica gel GF_{254} (using 3:2 $CHCl_3$: hexane as eluent) gave an oil, Rf 0.3, (41mg.). This was found to be a mixture (^{1}H-N.M.R. (60MHz.) eg. 3.86 (s), 3.84 (s), 3.82 (s) and I.R. (CCl_4) 1820 cm^{-1}, 1790 cm^{-1}, 1740 cm^{-1}, 1640 cm^{-1}.) and was not investigated further.

(c) Ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51) (163mg.) was stirred at reflux with 6N-aqueous HCl (30ml.) for 20hrs. The cooled aqueous solution was saturated with solid NaCl and thoroughly extracted with ethyl acetate which was dried and evaporated to give an oil (65mg.). The oil was taken up in anhydrous methanol (5ml.) and esterified as in (b) above to give an oily mixture (33mg.). Only traces of lactonic material were removed after preparative TLC, Rf 0.2, (8mg.),(I.R.,(CCl_4),1775 cm^{-1} ; 1760 cm^{-1} ; 1740 cm^{-1}.) and the

reaction was not investigated further.

Attempted De-carbethoxylation of Ethyl 3-Carbethoxy-3-Carbethoxymethyl-2-oxo-Hexanoate (51) in Dimethyl Sulphoxide[57,58]

A mixture of ethyl 3-carbethoxy-3-carbethoxymethyl-2-oxo-hexanoate (51) (200mg.), sodium chloride (37mg.), dimethyl sulphoxide (10ml.) and distilled water (0.7ml.) was stirred at reflux for 4hrs. and allowed to cool. Distilled water (25ml.) was added and the aqueous solution thoroughly extracted with ether to yield an oil (114mg.).

Preparative TLC on silica gel GF_{254} (using 30% ether in hexane as eluent) gave a colourless oil Rf 0.4, (50mg.) which, from the observed multiplicity of signals in the ^{1}H-N.M.R. spectrum eg. 4.22 δ (s); 4.20 δ (s), ester $-CH_3$ + superimposed (m), ester $-CH_2-$, was evidently a mixture, which was only partially resolved by distillation in a sublimation tube. This reaction was not investigated further.

2. Attempted Synthesis of α-Alkylitaconic Acids from Itaconic Acid.

(i) Preparation of 4-Methoxybenzyl Alcohol.[140]

A mixture of 4-methoxybenzaldehyde (136g.) and 37%-aqueous formaldehyde (100ml.) in methanol (100ml.) was added dropwise to a stirred solution of potassium hydroxide (167g.) in methanol (250ml.). The reaction mixture was stirred at 70°C for 3hrs., and excess methanol removed by distillation at room pressure. The residue was diluted with distilled water (300ml.) and extracted thoroughly with benzene.

The benzene was dried and evaporated to give 4-methoxybenzyl alcohol b.p. 82-84°C/2mm, (81g., 58.6%). (Lit.[141] b.p. 134-135°C/12mm).

^{1}H-N.M.R. (60MHz.)

7.10 δ (4H, q, J=16Hz., 7Hz., aromatic H); 4.52 δ (2H, s, $-CH_2OH$); 3.75 δ (3H, s, $-OCH_3$); 3.20 δ (1H, s(br.), -OH).

I.R. (CCl_4)

3450 cm^{-1}; 1610 cm^{-1} .

(ii) Preparation of Itaconic Anhydride.[64]

A mixture of itaconic acid (100g.) and acetyl chloride (200ml.) was stirred at reflux for 1hr. The solution was evaporated giving a yellow acidic solid which, after washing with anhydrous benzene and drying, gave itaconic anhydride m.p. 68-69°C, (44.7g., 45%). (Lit.[64] m.p. 68-69°C).

^{1}H-N.M.R. (60MHz.)

6.55 δ (1H, t, J=3Hz., H-5); 5.95 δ (1H, t, J=3Hz., H-5); 3.62 δ (2H, t, J=3Hz., H-2).

I.R. (CCl_4)

1860 cm^{-1}; 1850 cm^{-1}; 1805 cm^{-1}; 1785 cm^{-1}; 1770 cm^{-1}; 1660 cm^{-1}.

(iii) Preparation of 4-Methoxybenzylitaconate (60).[64]

A mixture of itaconic anhydride (15g.) and

4-methoxybenzyl alcohol (28g.) was stirred at 55-60°C for 30hrs. On cooling, the reaction mixture solidified and was recrystallised from benzene-hexane to give 4-methoxybenzyl-itaconate (60) m.p. 87°C, (20g., 60%). (Lit.[64] m.p. 86.8-87.2°C).

^{1}H-N.M.R. (60MHz.)

6.95 δ(4H, q, J=24Hz., 8Hz., aromatic H); 6.30 δ(1H, s, H-5); 5.74 δ(1H, s, H-5); 5.00 (2H, s, CH_2-O); 3.75 δ(3H, s, $-OCH_3$); 3.34 δ(2H, s, H-2).

I.R. (CCl_4)

3540 cm^{-1}; 3400-2400 cm^{-1}; 1740 cm^{-1}; 1700 cm^{-1}; 1635 cm^{-1} 1610 cm^{-1}.

(iv) Reactions of 4-Methoxybenzylitaconate (60) with 1-Bromobutane.[63]

Dry diisopropylamine (12.1g.) was added dropwise under nitrogen to a cooled (4°C) solution of n-butyl lithium (50ml. of 2.4M soln. in hexane) in anhydrous tetrahydrofuran (60ml.) and the mixture stirred at room temperature for 20 mins. The resulting pale yellow solution of lithium diisopropylamide[135] was cooled to -78°C and 4-methoxybenzylitaconic acid (10g.) in anhydrous tetrahydrofuran (20ml.) added dropwise.

The mixture was stirred at -78°C for 2hrs. and a solution of 1-bromobutane (6.9g.) in anhydrous tetrahydrofuran (40ml.) added over 30mins. The reaction was stirred at -78°C for 7hrs. and glacial acetic acid (ca. 5ml.) added. The solution was brought to 0°C, poured on to ice, acidified with 6N-aqueous H_2SO_4 and thoroughly extracted with ether. The ether was dried and evaporated to give crystals (5g.), m.p. 87°C from benzene-hexane which were identified as unreacted 4-methoxybenzylitaconic acid (60) by comparison of I.R. and ^{1}H-N.M.R. spectra.

In a similar experiment, hexamethylphosphortriamide (ca.

0.5ml.) was added to solubilise the anion prepared from 4-methoxybenzylitaconic acid (60) (5g.) before addition of the 1-bromobutane. The mixture was then stirred at 0°C for 2hrs.[65] before working up as before. Preparative TLC of the resulting product on silica gel GF_{254} (using 50:50 ethyl acetate/light petroleum as eluent) gave 4-methoxybenzyl alcohol (4.0g.), identified by comparison of Rf (0.7) and ^{1}H-N.M.R. spectra. This reaction was not investigated further.

3. Synthesis of α-n-Butylitaconic acid (59) via 1,1,2-Tricarbomethoxyethene (61).

(i) Preparation of 1,1,2-Tricarbomethoxyethane[67]

To a stirred solution of metallic sodium (3.8g.) in anhydrous methanol (150ml.), was added dropwise dimethyl malonate (43g.). The mixture was refluxed for 30mins. and methyl bromoacetate (25g.) added gently to the refluxing solution. TLC (with 25% chloroform in light petroleum as eluent) was used to determine when the reaction was complete, (8hrs.). The reaction mixture was allowed to cool, poured on to ice (ca. 100g.) and was thoroughly extracted with ether. The ether was dried and evaporated to give 1,1,2-tricarbomethoxyethane b.p. 92-94°C/1mm, (15g., 45%). (Lit.[67] b.p. 86-95°C/0.2-0.4mm).

I.R. (CCl_4)

1760 cm^{-1}; 1745 cm^{-1}; 1165 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

3.85 δ (1H, t, J=7Hz., H-1); 3.76 δ (6H, s, ester $-CH_3$); 3.70 δ (3H, s, ester $-CH_3$); 2.92 δ (2H, d, J=7Hz., H-2).

Mass Spectrum m/e (rel. abundance)

M^+-31. 173(30), 145(100, M-59), 113(95, M-59-32), 101(20).

Analysis

Found: C, 46.86; H, 5.87.

Calcd. for $C_8H_{12}O_6$: C, 47.05; H, 5.88%.

(ii) Preparation of 1,1,2-Tricarbomethoxyethene (61)[67]

A solution of bromine (5ml.) in methylene chloride (20ml.) was added dropwise to a cooled (0°C) stirred solution of 1,1,2-tricarbomethoxyethane (10g.) in methylene chloride (50ml.). The reaction mixture was stirred at reflux for 30mins. and allowed to cool. The solution was washed thoroughly with satd. aqueous Na_2CO_3, dried and evaporated to give 1-bromo-1,1,2-tricarbomethoxyethane

as a colourless oil (13.9g., 96.2%).

I.R. (CCl_4)

1785 cm^{-1} (w); 1750 cm^{-1} (s).

^{1}H-N.M.R. (60MHz.)

3.89 δ (6H, s, ester $-CH_3$); 3.74 δ (3H, s, ester $-CH_3$); 3.49 δ (2H, s, H-2).

Mass Spectrum m/e (rel. abundance)

M^+. 284,282(5), 253,251(30, M-31), 240,238(50, M-44), 225,223(30, M-59), 181,179(100, M-73-30).

This product (13.7g.) was dissolved in pyridine (70ml.) and the solution stirred at 90°C for 1hr. The reaction mixture was allowed to cool and was poured on to crushed ice (ca. 100g.) and extracted thoroughly with ether. The organic extract was washed successively with water, 6N-aqueous HCl, satd. aqueous Na_2CO_3, and was dried and evaporated to give 1,1,2-tricarbomethoxyethene (61) b.p. 85-90°C/0.5mm, m.p. 35-36°C, (6.06g., 62%). (Lit.[142] m.p. 40-41°C).

I.R. (CCl_4)

1750 cm^{-1}; 1735 cm^{-1}; 1650 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

6.83 δ (1H, s, H-2); 3.87 δ (6H, d, J=2Hz., ester $-CH_3$); 3.80 δ (3H, s, ester $-CH_3$).

Mass Spectrum m/e (rel. abundance)

M^+. 202(3), 172(35), 171(100, M-31), 143(15), 140(30), 113(45).

Analysis

Found: C, 47.68; H, 5.10.

Calcd. for $C_8H_{10}O_6$: C, 47.52; H, 4.95%.

(iii) Formation of 1,1,2-Tricarbomethoxybutane (63) during the Attempted Preparation of 1,1,2-Tricarbomethoxyhex-3-yne.[66]

To a stirred solution of ethyl bromide (0.44g.) in anhydrous tetrahydrofuran (10ml.), was added activated

magnesium (0.12g.). The mixture was stirred at room temperature until all the magnesium had dissolved (1hr.) and was cooled to 0°C. A solution of but-1-yne (ca. 0.2g.) in anhydrous tetrahydrofuran (10ml.) was added dropwise and the reaction stirred at room temperature for 10hrs. The solution was cooled to 0°C and anhydrous cuprous chloride (10mgs.) added followed by 1,1,2-tricarbomethoxyethene (61) (0.5g.) in anhydrous tetrahydrofuran (20ml.). After stirring at room temperature overnight, the reaction mixture was poured on to satd. aqueous NH_4Cl, acidified with 6N-aqueous HCl and thoroughly extracted with ether. The ether extract was dried and evaporated to give a red oil (0.5g.) which contained no alkyne (I.R. (CCl_4) 1740 cm^{-1}). However, preparative TLC on silica gel GF_{254} (using 80% ether in light petroleum as eluent) afforded 1,1,2-tricarbomethoxybutane (63) Rf 0.7, (0.15g., 26.2%) as an oil.

I.R. (CCl_4)

1755 cm^{-1}; 1740 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

4.18 δ (2H, AB system, J=22Hz., 8Hz., H-1 and H-2); 3.75 δ (6H, s, ester $-CH_3$); 3.73 δ (3H, s, ester $-CH_3$); 1.60 δ (2H, m, H-3); 1.16 δ (3H, t, J=7Hz., H-4).

Mass Spectrum m/e (rel. abundance)

M^+. 232(1), 205(20), 204(15, $[(MeO_2C)_2CH=C(OH)OMe]^+$), 190(65), 173(25), 172(100, M-60).

$C_{10}H_{16}O_6$ requires M^+ at m/e 232

(iv) Preparation of 1,1,2-Tricarbomethoxyhexane (64).[66]

Activated magnesium (0.36g.) was added (under N_2) to a stirred solution of 1-bromobutane (2.33g.) in anhydrous tetrahydrofuran (50ml.). The mixture was stirred at room temperature until all the metal dissolved, anhydrous cuprous chloride (10mgs.) was added and the mixture stirred at 0°C

for 30mins.

1,1,2-Tricarbomethoxyethene (61) (2g.) in anhydrous tetrahydrofuran (50ml.) was added dropwise at 0°C, and the reaction stirred at room temperature for 20hrs. The reaction mixture was quenched at 0°C in satd. aqueous NH_4Cl, acidified with 6N-aqueous HCl and thoroughly extracted with ether which was dried and evaporated. Preparative TLC on silica gel GF_{254} (using 80% ether in light petroleum as eluent) gave 1,1,2-tricarbomethoxyhexane (64) Rf 0.9, (1.65g., 65%) as an oil.

I.R. (CCl_4)

1755 cm^{-1}; 1740 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

3.85 δ (3H, s, ester $-CH_3$); 3.75 δ (6H, s, ester $-CH_3$); 3.33 δ (2H, m, H-1,H-2); 1.55 (2H, m, H-3); 1.30 δ (4H, m, H-4, H-5); 0.90 δ (3H, t, J=7Hz., H-6).

Mass Spectrum m/e (rel. abundance)

M^+-31. 229(15), 204(15), 201(25), 172(35), 137(20), 132(100, $[MeO_2CCH=C(OH)OMe]^+$), 129(95, $[MeO_2C\overset{+}{C}HC_4H_9]$).

Analysis

Found: C, 55.19; H, 7.69.

$C_{12}H_{20}O_6$ requires C, 55.38; H, 7.69%.

(v) Preparation of 1,1,2-Tricarboxyhexane (65a).[66]

1M-aqueous sodium hydroxide (32ml.) was added to 1,1,2-tricarbomethoxyhexane (64) (1.65g.) and the reaction mixture stirred overnight at room temperature. The solution was stirred at 70°C for three hours, 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 (65a) (0.74g., 54%), m.p. 152-154°C from light petroleum (100-120°C).

I.R. (KBr disc)

3600-2300 cm^{-1}; 1700 cm^{-1} (br.)

Analysis

Found: C, 49.30; H, 6.70

$C_9H_{14}O_6$ requires C, 49.50; H, 6.42%.

(vi) Preparation of α-n-Butylitaconic acid (2,3-Dicarboxyhept-1-ene) (59).[66]

A solution of 1,1,2-tricarboxyhexane (65a) (0.68g.) in methanol (2ml.) was treated with 40%-aqueous dimethylamine, stirred at -20°C for five mins., and 37%-aqueous formaldehyde (5ml.) in methanol (10ml.) was added gently.

The reaction mixture was stirred overnight at room temperature, refluxed for 1hr. and evaporated at reduced pressure. The residue was taken up in distilled water (10ml.), acidified at 0°C with 6N-aqueous HCl, saturated with solid NaCl and thoroughly extracted with ether. The ether was dried and evaporated to give α-n-butylitaconic acid (2,3-dicarboxyhept-1-ene) (59) (0.29g., 40%), m.p. 97-99°C from heptane.

I.R. (KBr disc)

3500-2300 cm^{-1}; 1690 cm^{-1}; 1630 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

6.55 δ (1H, s, H-9); 5.90 δ (1H, s, H-9); 3.47 δ (1H, t(br.), J=7Hz., H-2); 1.85 δ (2H, m, H-3); 1.40 δ (4H, m, H-4, H-5); 0.90 δ ((3H, t, J=7Hz., H-6).

Mass Spectrum m/e (rel. abundance)

M^+. 186(5), 143(10), 141(70, M-45), 129(100, $[HO_2C\overset{+}{C}HC(CO_2H)=CH_2]$).

Analysis

Found: C, 58.10; H, 7.27.

$C_9H_{14}O_4$ requires C, 58.06; H, 7.53%.

(vii) Synthesis of [1-^{14}C] 2,3-Dicarboxyhept-1-ene (59).

To a stirred solution containing 1,1,2-tricarboxyhexane (65a) (50mgs.), methanol (1ml.) and 40%-aqueous dimethylamine (0.2ml.) at -20°C, was added [^{14}C] -formaldehyde (250μCi) in distilled water (1ml.). The reaction mixture was stirred at -20°C for 10 mins., 37%-aqueous formaldehyde (0.5ml.) was added and the solution stirred at room temperature overnight. After stirring at reflux for 1hr., the solution was evaporated and the residue taken up in water (5ml.), acidified with 6N-aqueous HCl and saturated with solid NaCl. The acid mixture was thoroughly extracted with ethyl acetate, which was dried and evaporated to give [1-^{14}C] 2,3-dicarboxyhept-1-ene (59) (18mgs., 42.2%, 3.63×10^5 dpm/mg.), m.p. 97-99°C from light petroleum.

4. Incorporation of [1-^{14}C]2,3-Dicarboxyhept-1-ene (59) into Ethisolide (1) by cultures of P.decumbens.

A solution of [1-^{14}C]2,3-dicarboxy hept-1-ene (59) (10mg.) in 0.005M-aqueous $NaHCO_3$ (3ml.) and distilled water (4ml.) was sterilised and fed, in three 24hr. pulses, namely on the 7th, 8th and 9th days after inoculation, to two Roux bottles containing cultures of P.decumbens. On the 14th day after inoculation, the aqueous broth was separated by decantation and continuously extracted with ethyl acetate for 48hrs., after which, the ethyl acetate was extracted with satd. aqueous $NaHCO_3$, dried and evaporated. Preparative TLC on silica gel GF_{254} (using chloroform as eluent) gave ethisolide (1) Rf 0.3 (79.8mg., 7855 dpm/mg.), m.p. 122-123°C from ethanol. (Lit.[44] m.p. 122-123°C). The total activity isolated as [^{14}C] ethisolide was 6.94×10^5 dpm., giving an incorporation of 10.4% from [1-^{14}C]2,3-dicarboxyhept-1-ene.

The aqueous broth was evaporated, the residue taken up in anhydrous methanol (30ml.) and added at 0°C, to a stirred solution prepared from thionyl chloride (23ml.) and anhydrous methanol (30ml.). The reaction was stirred at room temperature overnight and evaporated to dryness. The residue was dissolved at room temperature in ethyl acetate, washed with satd. aqueous $NaHCO_3$, dried and evaporated to give an oil (0.313g.). Preparative TLC (using 80% ether in light petroleum as eluent) gave trimethyl citrate Rf 0.3, (54mg., 7dpm/mg.), m.p. 75-77°C from diisopropyl ether. (Lit.[143] m.p. 75-76°C).

5. Distribution of label in the [^{14}C] Ethisolide.[144]

[^{14}C] ethisolide (18.8mg., 7855dpm/mg.) was diluted with inactive ethisolide (25.1mg.), and the combined sample (43.9mg.) dissolved in glacial acetic acid (10ml.). A stream of ozonised oxygen was passed through the solution for 1.5 hrs. at room temperature, 2N-aqueous HCl (6ml.) was added, and the solution left for 2hrs. After the addition of glacial acetic acid (6ml.), the solution was left overnight and steam distilled under N_2 into a solution of dimedone (67mg.) and piperidine (0.5ml.) in ethanol (10ml.). Once ca. 50ml of distillate had been collected, the dimedone solution was boiled for 15mins. and allowed to cool to give methylene-bis-dimedone (24.1mg., 34.2%, 2127dpm/mg.), m.p. 190-191°C from ethanol-water. (Lit.[145] m.p. 191-191.5°C).

The [^{14}C] ethisolide sample used had activity of 6.094x 10^5dpm/m.mol. This showed that 102% of the activity of the ^{14}C ethisolide was located in the terminal methylene carbon (C-9).

Experimental Section

IV. Canadensolide and Dihydrocanadensolide: Preparation of Potential Biosynthetic Intermediates and Incorporation Studies using *P.canadense.*

1. Attempted Preparations of 1,1,2-Tricarbomethoxy-octane
2. Synthesis of α-*n*-Hexylitaconic Acid
3. Administration of [1-^{14}C]2,3-Dicarboxynon-1-ene to Cultures of *P.canadense*
4. Administration of [^{14}C-methylidine]Canadensic Acid to Cultures of *P.canadense*
5. Administration of [^{14}C-methylidine]Canadensolide to Cultures of *P.canadense*

Canadensolide and Dihydrocanadensolide: Preparation of Potential Biosynthetic Intermediates and Incorporation Studies with P.canadense.

1. Attempted Preparations of 1,1,2-Tricarbomethoxyoctane (66).

To a stirred solution of 1-bromohexane (0.165g.) in anhydrous tetrahydrofuran (10ml.) was added activated magnesium (0.28g.). Once the magnesium had dissolved (1hr. at reflux), anhydrous cuprous chloride (10mg.) was added and the mixture stirred at 0°C for 30mins.

1,1,2-tricarbomethoxyethene (61) (0.116g.) in anhydrous tetrahydrofuran (10ml.) was added dropwise at 0°C and the reaction stirred at room temperature for 20hrs. The reaction mixture was quenched at 0°C in satd. aqueous NH_4Cl, acidified with 6N-aqueous HCl and thoroughly extracted with ether which was dried and evaporated. Preparative TLC on silica gel GF_{254} (using 60% ether in light petroleum as eluent) gave an ester Rf 0.1 (0.1g.) which, from its ^{1}H-N.M.R. spectrum, did not appear to contain an alkyl substituent (I.R. (CCl_4) 1760 cm^{-1}; 1740 cm^{-1}). The product was not investigated further.

In another reaction carried out under similar conditions, the crude product was found to be an oil, Rf 0.2 which was evidently a mixture from its ^{1}H-N.M.R. spectrum (3.75 δ, s; 3.70 δ, s; 1.85 δ, m(br.).). This reaction was not investigated further.

2. Synthesis of α-n-Hexylitaconic Acid (2,3-Dicarboxynon-1-ene) (65).

(i) Preparation of 1,1,2-Tricarbomethoxyoctane (66).[66]

Activated magnesium turnings (0.12g.) were added to a stirred solution of 1-bromohexane (1.32g.) in anhydrous ether (30ml.) and the mixture stirred at room temperature until the magnesium dissolved (1hr.). Anhydrous cuprous chloride (15mg.) was added and the reaction stirred at 0°C for 30mins.

1,1,2-Tricarbomethoxyethene (61) (1.35g.) in anhydrous ether (30ml.) was added dropwise at 0°C and the reaction stirred at room temperature for 20hrs. The reaction mixture was quenched at 0°C in satd. aqueous NH_4Cl, acidified with 6N-aqueous HCl and thoroughly extracted with ether which was dried and evaporated. Preparative TLC on silica gel GF_{254} (using 60% ether in light petroleum as eluent) gave 1,1,2-tricarbomethoxyoctane (66) Rf 0.7, (1.08g., 56.4%).

I.R. (CCl_4)

1760 cm^{-1}; 1740 cm^{-1}; 1165 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

3.37 δ (1H, d, J=7Hz., H-1); 3.75 δ (3H, s, ester $-CH_3$); 3.73 δ (6H, s, ester $-CH_3$); 3.18 δ (1H, m, H-2); 1.27 δ (10H, m, H-3 to H-7); 0.89 δ (3H, t(br.), J=7Hz., H-8).

Mass Spectrum m/e (rel. abundance)

M^++1. 289(10), M^+. 288(5), 257(60), 229(70), 204(100, M-84, McLafferty product)

Analysis

Found: C, 58.59; H, 8.59

$C_{14}H_{22}O_6$ requires C, 58.33; H, 8.33%.

(ii) Preparation of 1,1,2-Tricarboxyoctane (67).

1M-aqueous sodium hydroxide (40ml.) was added to

1,1,2-tricarbomethoxyoctane (1g.) and the reaction mixture stirred overnight at room temperature. The solution was stirred at 70°C for 3hrs., 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-tricarboxyoctane (67) (0.596g., 69.7%), m.p. 140-141°C from light petroleum (b.p. 100-120°C).

I.R. (KBr disc)

3600 cm^{-1} - 2400 cm^{-1}; 1720 cm^{-1}; 1695 cm^{-1}.

Analysis

Found: C, 53.91; H, 7.10

$C_{11}H_{18}O_6$ requires C, 53.66; H, 7.32%.

(iii) Preparation of α-n-Hexylitaconic Acid (2,3-Dicarboxy-non-1-ene) (65).

A solution of 1,1,2-tricarboxyoctane (0.5g.) in methaol (2ml.) was treated with 40%-aqueous dimethylamine (2ml.), stirred at -20°C for 5mins., and 37%-aqueous formaldehyde (5ml.) in methanol was added dropwise. The reaction mixture was stirred overnight at room temperature, refluxed for 1hr. and evaporated at reduced pressure. The residue was taken up in distilled water (10ml.), acidified at 0°C with 6N-aqueous HCl, saturated with solid NaCl and thoroughly extracted with ether. The ether was dried and evaporated to give the α-n-hexylitaconic acid (2,3-dicarboxy-non-1-ene) (65) (0.14g., 32.2%), m.p. 76-77°C from light petroleum 100-120°C.

I.R. (CCl_4)

3300 cm^{-1} - 2500 cm^{-1}; 1710 cm^{-1}; 1630 cm^{-1}.

^{1}H-N.M.R. (90MHz.)

11.00 δ (2H, s(br.), acid H); 6.41 δ (1H, s, H-11); 5.72 δ (1H, s, H-11); 3.20 δ (1H, t, J=7Hz., H-2); 1.83 δ (2H, m, H-3); 1.30 δ (8H, m, H-4 to H-7); 0.89 δ (3H, t, J=7Hz., H-8).

Mass Spectrum m/e (rel. abundance)

M^+. 214(5), 170(10), 169(100, M-45), 168(15), 143(25), 129(90,M-85).

Analysis

Found: C, 61.46; H, 8.52

$C_{11}H_{18}O_4$ requires C, 61.68; H, 8.41%.

(iv) Synthesis of [1-^{14}C] -2,3-Dicarboxynon-1-ene (65).

To a stirred solution containing 1,1,2-tricarboxyoctane (67) (50mg.), methanol (1ml.) and 40%-aqueous dimethylamine (0.2ml.) at -20°C, was added [^{14}C]-formaldehyde (250μCi) in distilled water (1ml.).

The reaction mixture was stirred at -20°C for 10mins., 37%-aqueous formaldehyde (0.5ml.) was added, and the solution stirred at room temperature overnight. After stirring at reflux for 1hr., the solution was evaporated and the residue taken up in water (5ml.), acidified with 6N-aqueous HCl and saturated with solid NaCl. The acidic mixture was thoroughly extracted with ethyl acetate which was dried and evaporated to give [1-^{14}C] 2,3-dicarboxynon-1-ene (65) (17.4mg., 40%, 7.16×10^5 dpm/mg.), m.p. 76-77°C from light petroleum (b.p. 100-120°C).

3. Administration of [1-^{14}C] 2,3-Dicarboxynon-1-ene (65) to cultures of P.canadense.

(i) Production and Isolation of the Metabolites.

A solution of [1-^{14}C] 2,3-dicarboxynon-1-ene (65) was dissolved in 0.005M-aqueous $NaHCO_3$ (2ml.) and distilled water (7ml.). The solution was sterilised and fed, in three 24hr. pulses, namely on the 7th, 8th and 9th days after inoculation to three Roux bottles containing surface cultures of P.canadense. On the 11th day after inoculation, the aqueous broth was separated by decantation, acidified to pH 2 with 6N-aqueous HCl and continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated.

Preparative TLC on silica gel GF_{254} (eluting four times with 2:1 chloroform/light petroleum) gave dihydrocanadensolide (3) Rf 0.5, (65.7mg., 2257dpm/mg.), m.p. 94-95°C from ether-light petroleum. (Lit.[72] m.p. 94-94.5°C)

Preparative TLC on silica gel GF_{254} (eluting four times with chloroform-light petroleum) of an oily fraction Rf 0.4, (51mg.) gave canadensolide (2) Rf 0.2, (43.6mg., 5889dpm/mg.), m.p. 48-49°C from ether-light petroleum. (Lit.[72] m.p. 46-47.5°C).

Preparative TLC on silica gel GF_{254} (using 2% acetic acid in chloroform as eluent) of an oily fraction Rf 0.1, (292mg.), gave canadensic acid (60) Rf 0.4, (65mg., 6432 dpm/mg.), m.p. 114-115°C from ether-light petroleum. (Lit.[72] m.p. 113-114°C).

The total activity isolated as [^{14}C] dihydrocanadensolide (3) was 1.48×10^5 dpm; as [^{14}C] canadensolide (2) was 2.57×10^5 dpm and as [^{14}C] canadensic acid (60) was 4.18×10^5 dpm; giving incorporations of 11.9%, 20.6% and 33.6% respectively from [1-^{14}C] 2,3-dicarboxynon-1-ene (65).

(ii) Ozonolysis of [^{14}C]Canadensolide (2).[144]

[^{14}C]Canadensolide (2) from the above feeding experiments (8.1mg., 5889dpm/mg.) was diluted with inactive canadensolide (25.2mg.) and the combined sample (33.3mg.) dissolved in acetic acid (10ml.). A stream of ozonised oxygen was passed through the solution for 1.5hrs. at room temperature, 2N-aqueous HCl (6ml.) was added and the solution left for 2hrs. After the further addition of acetic acid (6ml.), the solution was left overnight and steam distilled under N_2 into a solution of dimedone (43.7mg.) and piperidine (0.5ml.) in ethanol (10ml.). Once ca. 50ml. of distillate had been collected, the dimedone solution was boiled for 15mins., and allowed to cool to give methylene-bis-dimedone (9mg., 19.4%, 949dpm/mg.), m.p. 190-191°C from ethanol-water. (Lit.[145] m.p. 191-191.5°C). The [^{14}C]canadensolide used had activity 3.01×10^5 dpm/m.mole, and the methylene-bis-dimedone recovered had activity 2.77×10^5dpm/m.mole. This indicated that 92.7% of the ^{14}C activity of the canadensolide was located in the terminal methylene carbon (C-11).

(iii) Ozonolysis of [^{14}C]Canadensic Acid (60).[144]

[^{14}C] Canadensic acid (60) also from the above feeding experiment [(i)] (8.8mg., 6432 dpm/mg.) was diluted with inactive canadensic acid (48.5mg.) and the combined sample (57.3mg.) dissolved in ethanol (10ml.) and cooled to -25°C. A stream of ozonised oxygen was passed through the solution for 1.5hrs. at -25°C, followed by nitrogen at 0°C for 30mins. Water (1ml.) was added and the solution left overnight at room temperature. The reaction mixture was steam distilled under N_2 into a solution of dimedone (33.5mg.) and piperidine (0.5ml.) in ethanol (10ml.). Once ca. 50ml. of distillate had been collected, the dimedone solution was boiled for 15mins. and evaporated to give methylene-bis-

dimedone (5.7mg., 7.4%, 705dpm/mg.), m.p. 191°C from ethanol-water. (Lit.[145] m.p. 191-191.5°C). The [^{14}C] canadensic acid sample used had activity 2.09×10^5 dpm/m.mole, while the methylene-bis-dimedone isolated had activity of 2.06×10^5 dpm/mg. This indicated that 98.3% of the ^{14}C activity of the canadensic acid was located in the terminal methylene carbon (C-11).

4. Administration of [^{14}C-methylidene] Canadensic Acid (60) to cultures of P. canadense.

[^{14}C-methylidene] Canadensic acid (60) (56.2mg. 6432 dpm/mg.) was dissolved in 0.005M-aqueous $NaHCO_3$ (5ml.) with the addition of dimethyl sulphoxide (1ml.) and distilled water (3ml.). The solution was sterilised and fed, in three 24hr. pulses, namely on the 7th, 8th and 9th days after inoculation to three bottles containing surface cultures of P. canadense. The aqueous broth was separated by decantation on the 11th day after inoculation, acidified to pH 2 with 6N-aqueous HCl and continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated. Preparative TLC on silica gel GF_{254} (eluting four times with 2:1 chloroform/light petroleum) gave an oil Rf 0.3, (112mg.). Preparative TLC on silica gel GF_{254} (eluting three times with 2:1 chloroform/light petroleum) of the oil gave canadensolide (2) Rf 0.3, (23.5mg., 5.2 dpm/mg.), m.p. 48-49°C from ether-light petroleum. (Lit.[72] m.p. 46-47.5°C). The total activity isolated as [^{14}C] canadensolide was 122.2 dpm; giving an incorporation of 0.034% from [^{14}C-methylidene] canadensic acid (60). Dihydrocanadensolide was not isolated from this culture of P. canadense.

5. Administration of [^{14}C-methylidene] Canadensolide (2). to P. canadense.

[^{14}C-methylidene] canadensolide (2) (19.8mg., 5889 dpm/mg.) was dissolved in distilled water (7ml.) with the addition of dimethyl sulphoxide (2ml.). The solution was sterilised and fed, in three 24hr. pulses, namely on the 8th, 9th and 10th days after inoculation, to three bottles containing surface cultures of P. canadense. The aqueous broth was separated by decantation on the 12th day after inoculation, acidified to pH 2 using 6N-aqueous HCl, and continuously extracted with ethyl acetate for 48hrs. after which the ethyl acetate was dried and evaporated. Preparative TLC on silica gel GF_{254} (eluting four times with 2:1 chloroform/light petroleum) gave dihydrocanadensolide (3) Rf 0.3, (14mg., 71.4dpm/mg.), m.p. 94-95°C from ether-light petroleum. (Lit.[72] m.p. 94-95°C). The total activity isolated as [^{14}C]dihydrocanadensolide (3) was 999.6 dpm; giving an incorporation of 0.857% from [^{14}C-methylidene] canadensolide (2).

Experimental Section

V. Avenaciolide: Attempted Synthesis of a Potential Biosynthetic Intermediate and Incorporation Studies using *A.avenaceus*.

1. Attempted Synthesis of [1-^{14}C]2,3-Dicarboxytridec-1-ene
2. Incorporation Studies using *A.avenaceus*

Avenaciolide: Attempted Synthesis of a Potential Biosynthetic Intermediate and Incorporation Studies with A. avenaceus.

1. Attempted synthesis of [1-^{14}C] 2,3-Dicarboxytridec-1-ene ([^{14}C-methylidene] -α-n-decylitaconic acid).

(i) Preparation of 1-Bromodecane[146]

48%-aqueous hydrobromic acid (160ml.) was added over 15mins. to a stirred mixture of 1-decanol (87g.) and concentrated sulphuric acid (62g.), the mixture was stirred and heated under reflux for 2hrs. and then diluted with distilled water (100ml.). The organic layer was separated and washed successively with cold conc. H_2SO_4 (10ml.), water and satd. aqueous Na_2CO_3. The combined aqueous portions were extracted with ether and the organic portions dried and evaporated to give 1-bromodecane b.p. 66-68°C/1.2mm, (92.7g., 78.9%). (Lit.[147] b.p. 104-104.4°C/8mm).

I.R. (CCl_4)

2960 cm^{-1}; 2855 cm^{-1}; 1460 cm^{-1}; 575 cm^{-1}.

^{1}H-N.M.R. (60MHz.)

3.34 δ (2H, t, J=7Hz., H-1); 1.82 δ (2H, m, H-2); 1.23 δ (14H, m, H-3 to H-9); 0.89 δ (3H, t(br.), J=7Hz., H-10).

(ii) Attempted Syntheses of 1,1,2-Tricarbomethoxydodecane (73).

(a) With Formation of n-Decyl Magnesium Bromide in anhydrous THF using Ethylene Bromide.[80]

Activated magnesium turnings (51mg.) were added (under N_2) to a stirred solution of 1-bromodecane (219mg.) in dry tetrahydrofuran (10ml.). Since the magnesium did not react even if the mixture was refluxed for 7hrs., a solution of ethylene bromide (189mg.) in dry tetrahydrofuran (10ml.) was added dropwise to the stirred, refluxing mixture. Stirring was then continued at room temperature until the magnesium dissolved (20hrs.).

The solution was filtered under N_2, cooled to 0°C and a

catalytic quantity of cuprous chloride added (ca. 10mg.). A solution of 1,1,2-tricarbomethoxyethene (61) (186mg.) in dry tetrahydrofuran (10ml.) was added and the reaction stirred overnight at room temperature. The reaction was quenched at 0°C in satd. aqueous NH_4Cl, acidified with 6N-aqueous HCl and extracted with ether which was then dried and evaporated. TLC on silica gel GF_{254} (using 50/50 ether/light petroleum as eluent) gave a mixture containing only unsaturated products (visible using dilute aqueous $KMnO_4$) and the reaction was not investigated further.

(b) <u>With Formation of n-Decyl Magnesium Bromide in ether using Ethylene Bromide, followed by replacement of the ether by anhydrous THF.</u>

To a stirred solution of n-decyl magnesium bromide in ether (25ml.) [prepared as above from activated magnesium (48mg.), 1-bromodecane (221mg.) and ethylene bromide (188mg.)] was added anhydrous tetrahydrofuran (20ml.). The solution was stirred at room temperature, the ether blown off in a stream of nitrogen, the resulting solution filtered and a catalytic quantity of cuprous chloride (ca. 10mg.) added. 1,1,2-tricarbomethoxyethene (61) (100mg.) in dry tetrahydrofuran (20ml.) was added dropwise at 0°C and the reaction stirred at room temperature overnight. Again, only unsaturated products were obtained, preparative TLC on silica gel GF_{254} (using 50:50 ether/light petroleum as eluent) giving unreacted 1,1,2-tricarbomethoxyethene (61) Rf 0.3 (17.3mg., 17%) (identified by its ^{1}H-N.M.R. spectrum) and several more polar products which were not investigated further.

(iii) <u>Preparation of 1,1,2-Tricarbomethoxydodecane (73).</u>[66]

To activated magnesium metal (12mg.) was added a solution of 1-bromodecane (120mg.) in dry ether (15ml.) and the mixture was stirred at room temperature under nitrogen

until all the metal dissolved (3hrs.). The solution was cooled to 0°C and a catalytic quantity of anhydrous cuprous chloride (ca. 10mg.) added. 1,1,2-Tricarbomethoxyethene (61) (69mg.) in dry ether (10ml.) was added dropwise at 0°C and the reaction stirred overnight at room temperature. The solution was poured at 0°C on to satd. aqueous NH_4Cl (150ml.), acidified with 6N-aqueous HCl and extracted with ether, which was dried and evaporated. Preparative TLC on silica gel GF_{254} (using 50:50 ether/light petroleum as eluent) gave 1,1,2-tricarbomethoxydodecane (73) Rf 0.5 (8.3mg., 7%).

I.R. (CCl_4)

1750 cm^{-1} (br.)

^{1}H-N.M.R. (60MHz.)

4.20 δ (1H, d.t., J=9Hz. and 18Hz., H-1); 3.87 δ (1H, m, H-2); 3.78 δ (6H, s, ester $-CH_3$); 3.70 δ (3H, s, ester $-CH_3$) 1.55 δ (2H, m, H-3); 1.22 δ (16H, m, H-4 to H-11); 0.90 δ (3H, t(br.), J=7Hz., H-12).

2. Incorporation Studies using A.avenaceus.

(i) Administration of [1-^{14}C] Sodium Acetate to Cultures of A.avenaceus.

[1-^{14}C] sodium acetate (0.3mCi) was dissolved in distilled water (9ml.) and the solution was sterilised and fed, in three 24hour pulses, on days 7,8 and 9 after inoculation to three bottles containing surface cultures of A.avenaceus. The aqueous broth was separated by decantation on day 14 after inoculation, acidified to pH2 using conc. HCl, and continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated. Preparative TLC on silica gel GF_{254} (using chloroform as eluent) gave avenaciolide (4) Rf 0.7, (45mg., 3.65×10^5 dpm/mg.) m.p. 54-55°C from ether-light petroleum (Lit.[77] m.p. 54-56°C). The total activity isolated as [^{14}C] avenaciolide was 1.62×10^7 dpm giving an incorporation of 2.46% from [1-^{14}C] sodium acetate.

(ii) Administration of [2,3-$^{13}C_2$] Succinic acid to Cultures of A.avenaceus

[2,3-$^{13}C_2$] succinic acid (300mg., 90% 2,3-$^{13}C_2$) was dissolved in distilled water (30ml.) and the solution sterilised and fed in five 12hr. pulses, on days 7,8 and 9 after inoculation to six bottles containing surface cultures of A.avenaceus. The aqueous broth was separated by decantation on day 14 after inoculation, acidified to pH2 using conc. HCl, and continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated. Preparative TLC on silica gel GF_{254} (using $CHCl_3$ as the eluent) gave the ^{13}C enriched avenaciolide Rf 0.7 (142mg.),m.p. 54-55°C from ether-light petroleum (Lit.[77] m.p. 54-56°C).

^{13}C-N.M.R.

Chemical Shift (δ)	Carbon	$J[^{13}C-^{13}C]$(Hz.)
14.1	C-12	$J_{12,11}$ 33.9
22.6	C-11	$J_{11,12}$ 34.0
24.9	C-6	$J_{6,5}$ 33.7
29.1	C-7, C-8, C-9	
31.8	C-10	$J_{10,9}$ 34.1
35.9	C-5	$J_{5,6}$ 34.5
44.0	C-3	$J_{3,4}$ 32.6
74.5	C-2	$J_{2,1}$ 58.2
85.5	C-4	$J_{4,3}$ 32.6
126.4	C-15	$J_{15,14}$ 75.3
134.8	C-14	$J_{14,15}$ 75.2 $J_{14,13}$ 63.3
167.8	C-13	$J_{13,14}$ 65.0
170.3	C-1	

STEREOCHEMISTRY

1. Studies Related to the Absolute Stereochemistry of Canadensolide and Ethisolide

(i) Preparation of (9-Thiophenyl)-8,9-dihydroethisolide (78).[83]

To a stirred solution of ethisolide (1) (4g.) and thiophenol (2.75g.) in anhydrous tetrahydrofuran (50ml.) was added an aqueous borate buffer solution (50ml., pH 9.2). The reaction was stirred at room temperature overnight and extracted with ethyl acetate. The ethyl acetate was dried and evaporated and gave (9-thiophenyl)-8,9-dihydroethisolide (78) (1.5g., 23.4%), m.p. 87°C from ether-hexane.

I.R. (CCl_4)

1795 cm^{-1}; 1075 cm^{-1}

^{1}H-N.M.R. (60MHz.)

7.37 δ (5H, m, aromatic H); 5.10 δ (1H, d, J=8Hz., H-2); 4.51 δ (1H, d.t, J=5Hz., 7Hz., H-4); 4.16 δ (1H, m, H-3); 3.52 (1H, m, H-8); 3.32 δ (2H, d, J=7Hz., H-9); 1.53 δ (2H, m, H-5); 1.08 δ (3H, t, J=7Hz., H-6).

Mass Spectrum m/e (rel. abundance)

M^+. 292(5), 186(10), 110(100, $[C_6H_5SH]^+$), 109(25).

Analysis

Found: S, 10.62

$C_{15}H_{16}SO_4$ requires S, 10.69%.

(ii) Preparation of 9-(S-1-phenylethylamino)-8,9-dihydro-Ethisolide (79).[84]

A solution of ethisolide (1g.) and S-1-phenylethylamine (0.73g.) in dry ethanol (25ml.) was stirred (under N_2) at 95°C overnight. A solid separated on cooling, consisting of 9-(S-1-phenylethylamino)-8,9-dihydroethisolide (79) (0.24g., 14.3%), m.p. 224°C from ether-light petroleum.

I.R. (CCl_4)

3500 cm^{-1}; 3400 cm^{-1}; 1770 cm^{-1}; 1750 cm^{-1}; 1640 cm^{-1}

^{1}H-N.M.R. (60MHz.)

7.40 δ (5H, s(br.), aromatic H); 5.90 δ (1H, d.t, J=7Hz., 7Hz., N-H); 4.65 δ (1H, d.t, J=5Hz., 7Hz., H-4); 4.15 δ (1H, d, J=9Hz., H-2); 4.15 δ (1H, m, H-3); 3.25 δ (3H, m, H-8, H-9); 2.60 δ (3H, d, J=7Hz., H-10); 2.60 δ (2H, m, H-5); 2.25 δ (1H, m, H-7); 1.05 δ (3H, t(br.), J=7Hz., H-6).

Mass Spectrum m/e (rel. abundance)

M^+. 303(70), 288(5), 260(5), 120(5), 105(100, $[CH_3\overset{+}{C}HC_6H_5]$)

Analysis

Found: C, 67.55; H, 6.82; N, 4.75

$C_{17}H_{21}NO_4$ requires C, 67.32; H, 6.82; N, 4.62%.

(iii) Preparation of 11-(S-1-phenylethylamino)-10,11-Dihydrocanadensolide (37).[34]

A solution of canadensolide (2) (82mg.) and S-1-phenylethylamine (73mg.) in dry ethanol was stirred (under N_2) at 95°C overnight and allowed to cool. The solvent was evaporated to give a red oil and preparative TLC on silica gel GF_{254} (using chloroform as eluent) gave 11-(S-1-phenylethylamino)-10,11-dihydrocanadensolide Rf 0.5, (27.2mg., 21%), m.p. 137-138°C from ether-heptane.

I.R. (CCl_4)

3620 cm^{-1}; 3460 cm^{-1}; 1785 cm^{-1}; 1695 cm^{-1}; 1675 cm^{-1}.

^{1}H-N.M.R. (90MHz.)

7.30 δ (5H, s, aromatic H); 5.49 δ (1H, d.t, J=7Hz., 7Hz., N-H); 4.60 δ (1H, m; irr. at ca. 3.2 δ → s, H-3); 4.25 δ (1H, m, H-4); 3.61 δ (3H, ABX m, H-10, H-11); 3.23 δ (1H, m, H-2); 2.80 δ (1H, d(br.), J=7Hz., H-12); 1.45 δ (3H, d, J=7Hz., H-13); 1.45 δ (6H, m, H-5, H-6, H-7); 0.89 δ (3H, t(br.), J=7Hz., H-8).

Mass Spectrum m/e (rel. abundance)

M^+. 331(15), 316(10), 313(5), 287(15), 274(10), 245(95, M-42-44), 188(100, M-57-86).

Mass Measurement

Found: 331.17800

$C_{19}H_{25}NO_4$ requires M^+, 331.14330 a.m.u.

2. Relative Stereochemistry of Canadensolide (2) and Dihydrocanadensolide (3).

(i) Hydrogenations of Canadensolide

(a) Using H_2/Pd catalyst (Formation of 10-epi-dihydrocanadensolide (86)).[86]

A solution of canadensolide (60mg.) in glacial acetic acid (10ml.) was shaken with 10% palladium on charcoal for two hours under hydrogen (100 p.s.i.). The reaction mixture was filtered through celite, diluted with chloroform (100ml.) and washed with satd. aqueous $NaHCO_3$. The organic solution was dried and evaporated to give 10-epi-dihydrocanadensolide (86) (48mg., 79.2%), m.p. 70-71°C from ether-heptane. (Lit.[36] m.p. 70°C).

I.R. (CCl_4)

1800 cm^{-1}; 1790 cm^{-1}

^{1}H-N.M.R. (90MHz.)

5.00 δ (1H, d.t, J=7Hz., 1Hz.; irr. at ca. 4.5 δ → d(br.), J=7Hz.; irr. at ca. 3.0 δ → t, J=7Hz., H-3); 4.48 δ (1H, m; irr. at ca. 5.0 δ → t, J=7Hz., H-4); 3.42 δ (1H, d.d, J=7Hz., 7Hz.; irr. at ca. 5.0 δ → d, J=7Hz., H-2); 3.05 δ (1H, m; irr. at ca. 1.44 δ → d, J=7Hz., H-10); 1.84 δ (2H, m, H-5); 1.44 δ (4H, m, H-6, H-7); 1.44 δ (3H, d, J=7Hz., H-11); 0.89 δ (3H, t(br.), J=6Hz., H-8).

Rotation

$[\alpha]_D$ = 16.9° (c. 0.883, $CHCl_3$), (Lit. $[\alpha]_D^{86}$ = 17°).

Several unsuccessful attempts were made to epimerise the centre at C-10, using 2M-methanolic KOH, but these reactions yielded only polar products and were not investigated further.

(b) Using Zinc Dust, (Formation of Dihydrocanadensolide (3)).

Activated zinc dust (40mg.) was added to a solution of canadensolide (40mg.) in glacial acetic acid (2ml.) and

concentrated hydrochloric acid (2ml.). The reaction was refluxed, with the addition of excess zinc dust (10mg.) after one hour, for two hours; diluted with distilled water and extracted with ethyl acetate. The ethyl acetate was washed with satd. aqueous $NaHCO_3$, dried and evaporated and preparative TLC on silica gel GF_{254} (eluting four times with 2:1 $CHCl_3$/ hexane) gave dihydrocanadensolide (3) Rf 0.5, (10.3mg., 25.5%), m.p. 95-96°C from ether-light petroleum. (Lit.[72] m.p. 94-94.5°C). Mixed melting point with a sample of natural dihydro-canadensolide was 95°C.

I.R. (CCl_4)

1805 cm^{-1}; 1790 cm^{-1}

^{1}H-N.M.R. (60MHz.)

5.00 δ (1H, m, H-3); 4.50 δ (1H, m, H-4), 3.43 δ (1H, d(br.), J=7Hz., H-2); 3.05 δ (1H, m, H-10); 1.36 δ (2H, m, H-5); 1.45 δ (4H, m, H-6, H-7); 1.45 δ (3H, d, J=7Hz., H-11); 0.9 δ (3H, t, J=7Hz., H-8).

These spectra were completely in agreement with those obtained from a sample of natural canadensolide.

Rotation

$[\alpha]_D$ = -35.45° (c. 0.598, $CHCl_3$), (Lit. $[\alpha]_D^{72}$ = -31°).

3. Feeding Experiments with [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate.

Preparation of Sodium Deuteroxide (NaOD).

A solution of solid sodium hydroxide (5g.) in deuterium oxide (D_2O) (100ml.) was stirred at room temperature for 30 mins. and then evaporated to dryness at 40°C under reduced pressure. The residue was re-dissolved in deuterium oxide (100ml.) and filtered through celite to give a 5% solution of NaOD in D_2O.

Preparation of [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate.

A solution of [2-^{13}C]sodium acetate (150mg.) in 5% NaOD in D_2O (1ml.) and D_2O (3ml.) was sealed in a pyrex tube and heated at 180-200°C (Wood's metal) for 24hrs. The solution was evaporated to dryness (40°C) at reduced pressure, re-dissolved in D_2O (4ml.) and the above process repeated. Analysis of the samples produced showed them to be 97% [2-^{13}C, 2-$^{2}H_3$] sodium acetate (see ^{13}C-N.M.R., fig.45). The solutions were kept in sealed tubes until needed for feeding experiments.

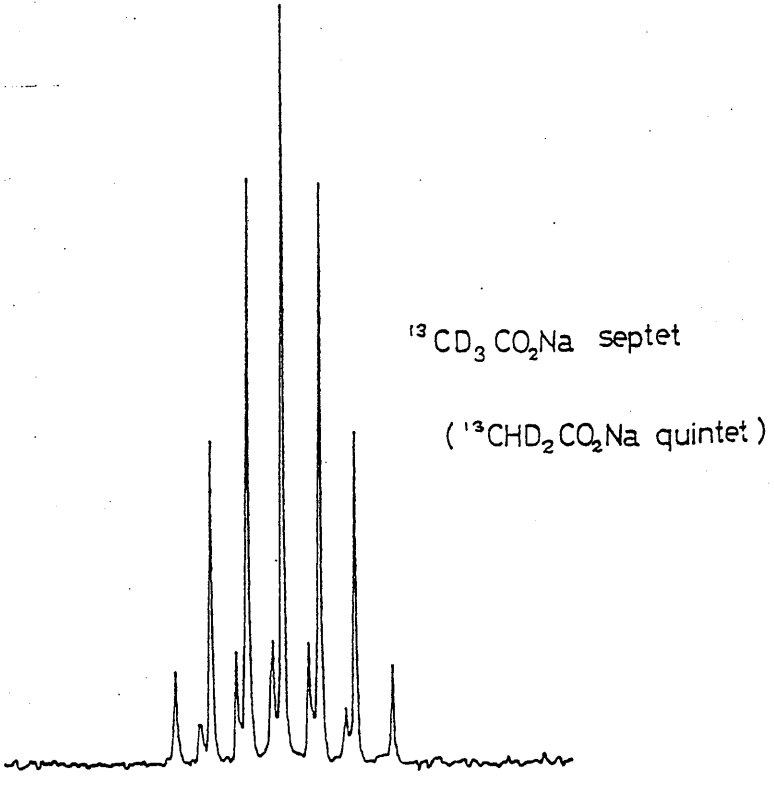


fig.45

Administration of [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate to Cultures of P.canadense.

An alkaline solution of [2-^{13}C, 2-$^{2}H_3$] sodium acetate (450mg.) in D_2O (12ml.), prepared as above, was acidified to pH 8 using a 20% solution of DCl in D_2O and the solvent was evaporated at reduced pressure (40°C). The residue was dissolved in D_2O (30ml.), filtered, sterilised using a millipore filter and fed in five 12hr. pulses, on days 7, 8 and 9 after inoculation, to six bottles containing surface cultures of P.canadense. The aqueous broth was separated by decantation on day 12 after inoculation, acidified to pH 2 using conc. HCl, and was continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated.

Preparative TLC on silica gel GF_{254} (eluting four times with 2:1 $CHCl_3$/light petroleum) gave the ^{13}C enriched dihydrocanadensolide Rf 0.5, (103mg.), m.p. 94-95°C from ether-light petroleum (Lit.[72] m.p. 94-94.5°C).

Preparative TLC on silica gel GF_{254} (eluting three times with 2:1 $CHCl_3$-light petroleum) of an oil Rf 0.4 (163mg.) gave the ^{13}C enriched canadensolide Rf 0.3,(51mg.), m.p. 48-49°C from ether-light petroleum (Lit.[72] m.p. 46-47.5°C).

Preparative TLC on silica gel GF_{254} (using 2.5% acetic acid in $CHCl_3$ as eluent) of an oil Rf 0.1, (673mg.) gave ^{13}C enriched canadensic acid Rf 0.3, (97mg.), m.p. 112-113°C from ether-light petroleum (Lit.[72] m.p. 113-114°C).

The mycelium was washed with chloroform, dried, crushed and continuously extracted for 48hrs. in a Soxhlet with chloroform, which was dried and evaporated to give an oily solid (0.65g.). This solid was refluxed with 0.5M-methanolic sodium hydroxide (25ml.) for 15mins., a 14% solution of boron trifluoride in methanol (30ml.) was added, and the reaction

stirred at reflux for a further 30mins. The solution was allowed to cool, poured on to crushed ice (ca. 50g.) and extracted with ether which was dried and evaporated. Preparative TLC on $AgNO_3$ impregnated silica gel GF_{254}^{109} [prepared from silica gel (150g.) and 5%-aqueous-$AgNO_3$ (120ml.); bands viewed by end-spraying with satd. aqueous dichlorofluorescein and irradiating with 350 n.m. u.v. light] (using 5% ether in light petroleum as eluent) gave an oil Rf 0.5, (62mg.) which was further purified by preparative TLC on silica gel GF_{254} (using light petroleum as eluent) to give pure ^{13}C enriched oleic acid methyl ester Rf 0.1, (28mg.).

^{1}H-N.M.R. (60MHz.)

5.40 δ (2H, t, J=7Hz., H-9, H-10); 3.67 δ (3H, s, ester $-CH_3$) 2.10 δ (6H, m, H-2, H-8, H-11); 1.28 δ (22H, m, H-3 to H-7 and H-12 to H-17); 0.85 δ (3H, t(br.), J=7Hz., H-18).

The oleic acid methyl ester was stored under N_2 until ^{13}C-N.M.R. spectra could be obtained.

^{13}C-N.M.R. Spectra of P.canadense Metabolites from the above Feeding Experiment.

(i) Oleic acid methyl ester from [2-^{13}C, 2-$^2H_3$] Sodium Acetate (fig. 33).

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, J $^{13}C/D$, Isotope Shift (ppm)
14.12	C-18	-0.14	
22.71	C-17	-0.10	
24.97	C-3	-0.36	
27.20	C-8, C-11	1.22	t, ca 19.3Hz., 0.35
29.14	$(CH_2)_n$	0.67	
29.36		0.35	
29.54		0.47	
29.75		-0.24	

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
31.93	C-16	0.44	t, ca 19.3Hz., 0.35
34.11	C-2	0.0	t, ca 19.3Hz., 0.35
51.41	OMe	-0.3	
129.77	C-9	0.42	
130.01	C-10	1.66	
174.29	C-1	0.07	

N.B.

The Enhancement Factor is calculated by comparing the peak intensities of the ^{13}C-N.M.R. spectra of natural abundance and ^{13}C enriched material. A conversion factor (C.F.) is calculated

$$C.F. = \frac{[2\text{-}^{13}CD_3] - \text{Natural abundance}}{\text{Natural abundance}}$$

and is used to calculate the Enhancement Factor as below.

$$\text{Enhancement Factor} = \frac{[2\text{-}^{13}CD_3] - \text{Natural abundance}(C.F.+1)}{\text{Natural abundance}(C.F.+1)}$$

This value is calculated for all the carbon atoms of the metabolite.

(ii) Dihydrocanadensolide from [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate (fig. 40).

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
13.82	C-8	-0.09	m, 19.2Hz., 0.30
16.94	C-11	-0.24	
22.40	C-7	-0.18	
27.49	C-6	0.28	t, 18.9Hz., 0.36
28.48	C-5	-0.33	
38.42	C-10	-0.12	d, [^{13}C-^{13}C], 35.1Hz.
43.93	C-2	0.96	d, [^{13}C-^{13}C], 35.1Hz.
78.65	C-3	-0.25	

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
82.34	C-4	0.83	
175.10	C-1	0.42	
177.10	C-9	0.76	

(iii) Canadensolide from [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate (fig.41)

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
13.82	C-8	0.03	m, 19.1Hz., ca 0.3/D atom
22.40	C-7	0.06	
27.49	C-6	0.76	t, 18.7Hz., 0.36
28.52	C-5	-0.19	
46.29	C-2	1.36	
77.41	C-3	-0.02	
82.76	C-4	1.42	
127.06	C-11	0.15	
130.12	C-10	0.44	
167.59	C-9	0.20	
172.31	C-1	-0.06	

(iv) Canadensic acid from [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate (fig.42).

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
14.09	C-8	0.01	m, ca.19.0Hz., ca.0.3/D
22.84	C-7	-0.05	
27.72	C-6	0.79	t, 18.7Hz., 0.34
35.33	C-5	-0.01	
35.68	C-3	0.17	
45.13	C-2	0.94	d,[^{13}C-^{13}C], 49.7Hz.
79.67	C-4	1.29	
131.62	C-11	0.03	
136.26	C-10	-0.21	
170.64	C-9	-0.50	
176.41	C-1	0.72	

Administration of [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate to Cultures of A.avenaceus.

An alkaline solution of [2-^{13}C, 2-$^{2}H_3$] sodium acetate (450mg.) in D_2O (12ml.), prepared as previously described, was acidified to pH 8 using a 20% solution of DCl in D_2O and the solvent was evaporated at reduced pressure (40°C). The residue was dissolved in D_2O (30ml.), filtered, sterilised using a millipore filter and fed in five 12hr. pulses on days 7, 8 and 9 after inoculation, to six bottles containing surface cultures of A.avenaceus. The aqueous broth was separated by decantation on day 14 after inoculation, acidified to pH 2 using conc. HCl and was continuously extracted with ethyl acetate for 48hrs., after which the ethyl acetate was dried and evaporated.

Preparative TLC on silica gel GF_{254} (using $CHCl_3$ as eluent) gave the ^{13}C enriched avenaciolide Rf 0.7, (104mg.), m.p. 54-55°C from ether/light petroleum. (Lit.[77] m.p. 54-56°C).

^{13}C-N.M.R. Spectrum of Avenaciolide from [2-^{13}C, 2-$^{2}H_3$] Sodium Acetate (fig. 43).

Chemical Shift(δ)	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
14.1	C-12	0.15	m, ca 19Hz., ca 0.3/D
22.6	C-11	0.01	
24.9	C-6	1.12	t, 19.1Hz., 0.36
29.1	C-7, C-9	0.1	
29.3	C-8	1.19	t, 18.7Hz., 0.42
31.8	C-10	1.28	t, 19.2Hz., 0.43
35.9	C-5	-0.17	
44.0	C-3	-0.01	
74.5	C-2	2.83	
85.5	C-4	2.61	
126.4	C-15	0.78	

Chemical Shift( )	Carbon Atom	Enhancement Factor	Multiplicity, $J^{13}C/D$, Isotope Shift (ppm)
134.8	C-14	0.36	
167.8	C-13	0.14	
170.3	C-1	0.04	

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