The biosynthesis of some bacterial and fungal polyketide metabolites

Sarah Victoria Rogers

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The Biosynthesis of Some Bacterial and Fungal Polyketide Metabolites

Ph.D. 1994

Abstract:-

Methylmalonyl-CoA is a key building block in the biosynthesis of propionate derived polyketide metabolites. There are several known metabolic sources of methylmalonyl-CoA, e.g. succinyl-CoA (citric acid cycle), valine and isoleucine. An objective of this research was to investigate the role of the DNA base, thymine, as a source of methylmalonyl-CoA in *Streptomyces* and hence probe the link between primary and secondary metabolism. Feeding key intermediates of the thymine and valine catabolic pathways, i.e. [$^{13}C^{2}H_3$-*methyl*]-thymine, [^{13}C-*methyl*]- and [1-^{13}C]-β-*aminoisobutyric acid, sodium* [3-^{13}C]-*isobutyrate, sodium* [^{13}C-*methyl*]-methacrylate and sodium [1-^{13}C]-methacrylate, to the monensin A producer, *Streptomyces cinnamonensis,* provided evidence of the reductive catabolism of thymine occurring in *Streptomyces,* analogous to mammals. The results also provided evidence which supports the existence of a novel deaminase enzyme mediating the transformation of β-aminoisobutyric acid and methacrylyl-CoA.

Cubensic acid, isolated from *Xylaria cubensis,* is a long chain fungal metabolite possessing eight pendant methyl groups. Its biosynthesis from acetate and L-methionine units was demonstrated with the aid of feeding experiments, proving a classical fungal mode of assembly. Attempts to incorporate an advanced methylated precursor into cubensic acid were unsuccessful. Biological intramolecular Diels-Alder reactions are implicated in the biosynthesis of a wide range of polyketide metabolites, e.g. nargenicin, solanapyrones. Attempts to demonstrate, by feeding an isotopically labelled precursor, an intramolecular Diels-Alder mechanism for the formation of the six membered ring in cytochalasin D, proved inconclusive. In the event, the precursor was degraded to acetate. This degradation was suppressed in the second attempt by the addition of a β-oxidation inhibitor, but still no incorporation of labelled precursor was evident.

THE BIOSYNTHESIS OF SOME BACTERIAL AND FUNGAL POLYKETIDE METABOLITES

Sarah Victoria Rogers
University of Durham
1991 - 1994

To Jennifer and Edward Rogers

'The only way is up'

'We are indeed in much the same position as an observer trying to gain an idea of the life of a household by a careful scrutiny of the persons or materials arriving at, or leaving the house: We keep an accurate record of the foods and commodities left at the door and patiently examine the contents of the dustbin and endeavour to deduce from such data the events occurring within the closed doors.'

M. Stephenson, *Bacterial Metabolism*, 1930, Longmans, London.

ACKNOWLEDGEMENTS

I would like to thank SERC and Sterling Winthrop for financial support and Dr. J.A. Robinson, University of Zurich, for providing a strain of *Streptomyces cinnamonensis*, Professor T. Beppu, University of Tokyo, for providing a strain of *Streptomyces sp. ATS 1287* and Dr. R.L. Edwards, University of Bradford, for providing a strain of *Xylaria cubensis*. Many thanks to Dr. K.A. Reynolds, University of Maryland at Baltimore, for analysis of the lipids of *S. cinnamonensis* and for a β-aminoisobutyric acid feeding experiment, and to Dr. J. Parkinson, University of Edinburgh, for $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. analysis.

A big thank you to all the technical staff at Durham University particularly the n.m.r. team, Mrs J. Say, Dr. A. Kenwright and Mr. I. McKeag, Miss L. Turner and Dr. M. Jones for mass spectra analysis, Mrs J. Dostal for elemental analysis and Mr. D. Hunter for high pressure work. Also thanks to the technicians, Mr. G. Metcalfe and Mr. B. Eddy for the invaluable loan of apparatus and chemicals. Thanks to Mr. R. Hart and Mr. G. Haswell for amazing glassware repairs and Mr. J. Lincoln for keeping a plentiful supply of essential disposable gloves, cotton wool and tin foil.

An enormous thank you to David O'Hagan for being a wonderful supervisor and to everybody in the research group past and present particularly Dr. R.J. Cox and Dr. M. Meyer who helped me considerably during my first year but also Dr. L. Dasharadi, Dr. J. Sanvoisin, Dr. N. Zaidi and the 'Dr.'s pending' Nikki, Dave, Jeff, Colin and Ruhul. Also thank you to all the staff at Sterling-Winthrop research centre particularly Dr. G. Duffin, my CASE award supervisor, and Karen, Glynis, Alain, Dave, John and George.

A very very big thank you to my family, mum, dad, Sean, James, Edward and William, for all their support during the three years. Last but by no means least, thank you Mark X.

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PART A : The biosynthesis of cubensic acid

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CHAPTER 1
GENERAL INTRODUCTION

1. INTRODUCTION

1.1 Metabolism

Metabolism is the collective word for all the chemical reactions which take place in living systems. Organic compounds (metabolites) are interconverted in living cells to produce both the complex molecules of life and the waste products of metabolism. Essentially the same metabolic sequences are utilised by a wide range of organisms, e.g. bacteria, plants, mammals.

Metabolic pathways can be classified into two main categories:-

i) catabolic pathways

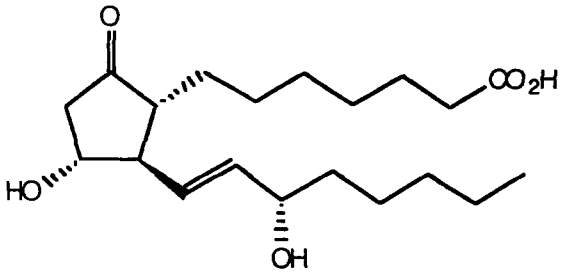
ii) anabolic pathways

The functions of the catabolic pathways are to degrade primary carbon sources, which are taken in as food, in order to produce precursors for biosynthesis and also to generate chemical energy in a form which is suitable for powering the energy requiring operations of the cell or organism. A further function is the conversion of waste material to a chemical form which is suitable for excretion from the cell or organism. The function of the anabolic pathways, on the other hand, is to synthesise the complex molecules needed to build, maintain and reproduce the living cells from smaller precursors. These two types of metabolic pathways are closely integrated, i.e. catabolic pathways service the anabolic pathways by providing the starting materials and the necessary chemical energy for the operation of the biosynthetic pathways.[1]

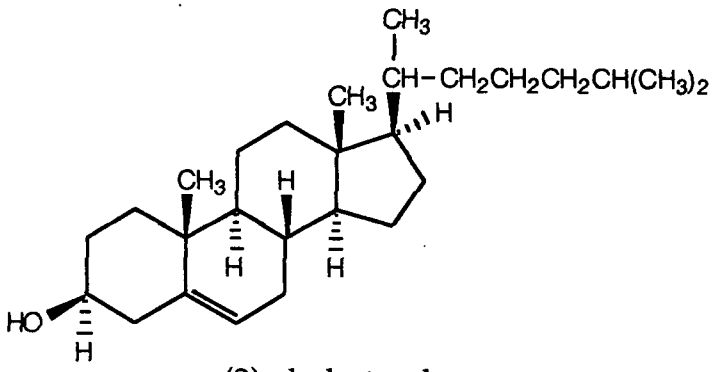
1.2 Primary and secondary metabolism

Primary metabolism has a broad distribution among all living organisms and is essential to cellular metabolism. The products of these metabolic processes are for example, amino acids, sugars and nucleotides e.t.c., and they can be contrasted with secondary metabolites, which are generally non-essential to the survival of individual cells but are important to the survival and function of the producing organism as a whole. Secondary metabolites are generally produced by the products of primary metabolism. They can have very elaborate and diverse structures, examples of which are

prostaglandins [(1) PGE_1], steroids [(2) cholesterol], terpenoids and polyketides.



(1) PGE_1



(2) cholesterol

1.3 Secondary metabolites

Generally secondary metabolites are not necessary for the growth of the producing organism, i.e. elimination of the production of secondary metabolites by mutation will not necessarily stop or slow down the growth of the organism.[2] So what is the function of secondary metabolites in nature?

This is a controversial area and attempts to define an underlying benefit common to the production of all secondary metabolites has not been forthcoming. A likely function could clearly be defence. For example, secondary metabolites are most abundant in micro-organisms which live in crowded habitats where nutrients may be limited, and it could be of benefit to the micro-organism to use chemical defence and attack to aid survival. Whatever the biological role, the secondary metabolites must have been continuously beneficial to the producing organism for the spread and survival of the genes encoding them to be selected and reinforced with each generation.[3]

A great deal of interest has been stimulated, particularly in the pharmaceutical industry after screening programmes revealed that a

number of secondary metabolites display antibiotic activity. The Actinomycetes (sporulating, soil-borne organisms), particularly, produce a wide range of secondary metabolites some of which have useful applications in clinical and veterinary medicine and agriculture. Such extensive screening programmes have led to the isolation and structure elucidation of many secondary metabolites but there still remains an extensive untapped microbial resource potentially available for drug screening.[4]

2.POLYKETIDES

2.1 History of discovery

Polyketides are one of the largest families of secondary metabolites. They are especially abundant among Actinomycete bacteria, eucaryotic fungi and higher plants, but are also present in many other groups, eg. higher animals, marine organisms.

In 1907, J. N. Collie was the first to acknowledge that many natural products contained within them a $[CH_2\text{-}CO]_n$ unit.[5] He hypothesised that a diverse range of aromatics, including some naturally occurring species, could be generated from condensations of acetic acid, acetoacetate and higher homologues of acetate, and some experimental evidence was also provided in support of this theory.[6,7] D. Rittenberg and K. Bloch, 40 years later, established the involvement of acetate in fatty acid biosynthesis by successfully incorporating labelled acetate into fatty acids.[8,9] This encouraged Sir. R. Robinson to elaborate on Collie's ideas and develop his hypothesis in a book in 1955 entitled *The structural relationships of natural products.* In this text, he predicted the origin of tetracycline antibiotics and other polyketides.[10] Independently, A. J. Birch outlined his 'acetate hypothesis', i.e. that acetate was the building block of many aromatic plant metabolites.[11,12] The discovery led by F. Lynen, that acetyl coenzyme-A acts as 'active acetate' in fatty acid and steroid construction[13,14] encouraged Birch to analyse and confirm the structures of many aromatic polyketide metabolites by demonstrating that they were compatible with the folding of extended acetate derived β-keto chains.[15,16] Birch[17] was the first to perform biosynthetic experiments on fungal aromatic metabolites and therefore proving the biosynthetic role of acetate. Another of his contributions was the demonstration that the pendant methyl groups of fungal polyketides are

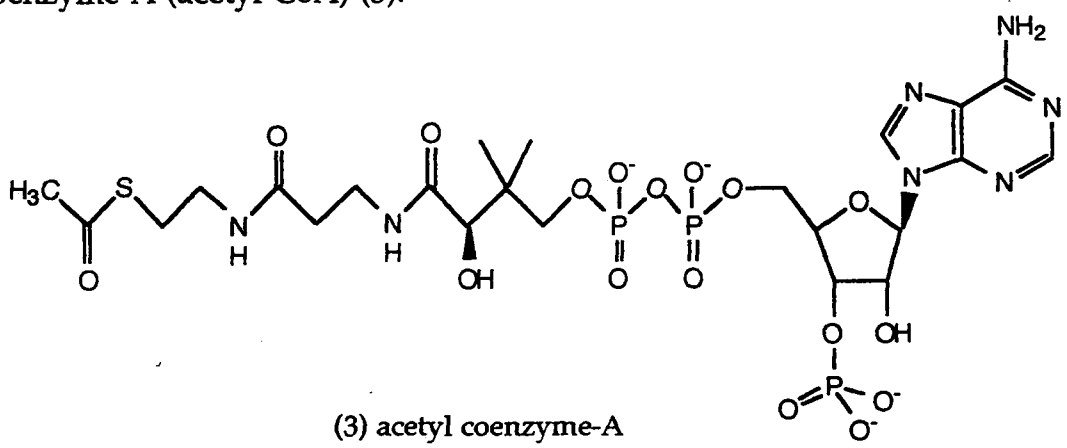
derived from the methyl group of L-methionine.[18] The basic origin of the polyketides was firmly established which opened the doors to a flood of reports of novel polyketide metabolites, their structure and biogenesis.

2.2 Fatty acids

Fatty acids are primary metabolites which are common to all living cells (with the exception of archaebacteria). They are characterised by a long hydrocarbon chain with a carboxylic acid terminus. As constituents of phospholipids, fatty acids play a critical structural role in the assembly of cell membranes. They are also found in cells as triglycerides where they are stored as a food reserve. In Actinomycete batch cultures, generally the fatty acids are produced during the first stage of growth (*tropophase*) and are then degraded by β-oxidation to acetate which can be utilised in secondary metabolism, e.g. polyketide synthesis, which takes place during the second stage of growth (*idiophase*) of the culture. It is useful to discuss the mode of assembly of fatty acids in the first instance as the process by which the saturated fatty acids are assembled offers a blueprint for the construction of polyketide metabolites.[19]

2.2.1 Fatty acid biosynthesis

There are variations in the way different organisms organize their cellular machinery to biosynthesise fatty acids, but a pivotal compound is acetyl coenzyme-A (acetyl-CoA) (3).



(3) acetyl coenzyme-A

The biosynthesis of long chain fatty acids occurs essentially in two distinct steps;[20]

i) generation of malonyl-CoA by the action of acetyl-CoA carboxylase on acetyl-CoA

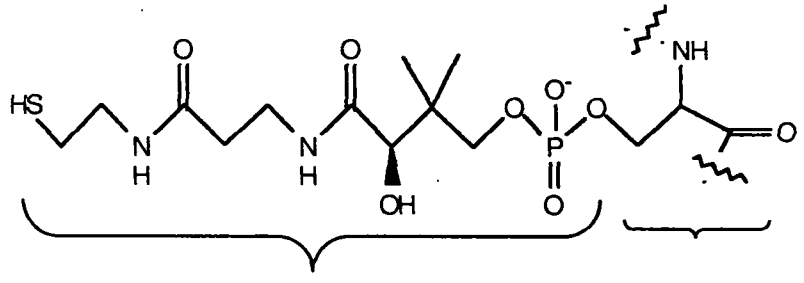
$$CH_3COSCoA + CO_2 + ATP \xrightleftharpoons{[biotin]} HO_2CCH_2COSCoA + ADP + P_i$$

ii) conversion of acetyl-CoA and malonyl-CoA into the fatty acid (palmitate) by the fatty acid synthase in the presence of NADPH as co-factor

$$CH_3COSCoA + 7HO_2CCH_2COSCoA + 14NADPH + 14H^+ \longrightarrow$$

$$CH_3CH_2(CH_2CH_2)_6CH_2CO_2H + 14NADP^+ + 8CoA\text{-}SH + 6H_2O$$

The acyl and malonyl thioesters are processed by the fatty acid synthase (FAS) and the fatty acid is fully assembled without the release of partially constructed intermediates.[20] There is a wide diversity in the structure of the FAS's utilised by different organisms but they can generally be classified as Type I or Type II FAS's.[19] Type I FAS's are found in most of the higher organisms, eg. insects, birds and mammals. These are either tightly bound complexes of individual enzymes which do not dissociate easily, or one or two multifunctional proteins upon which all the necessary activities exist. Type II FAS's consist of discrete monofunctional enzymes and an acyl carrier protein, all of which are separable and express their individual activities in isolation from each other. Examples of organisms possessing a Type II FAS are most bacteria, plants and photosynthetic eukaryotes. Yeast and fungal FAS's seem to have characteristics of both Type I and Type II complexes.[21] A few representatives of the Actinomycetes, have FAS's which appear to be clusters of multifunctional proteins with similar characteristics to yeast and fungal systems and so are classified as Type I (discussed in chapter 4, part B).[19]

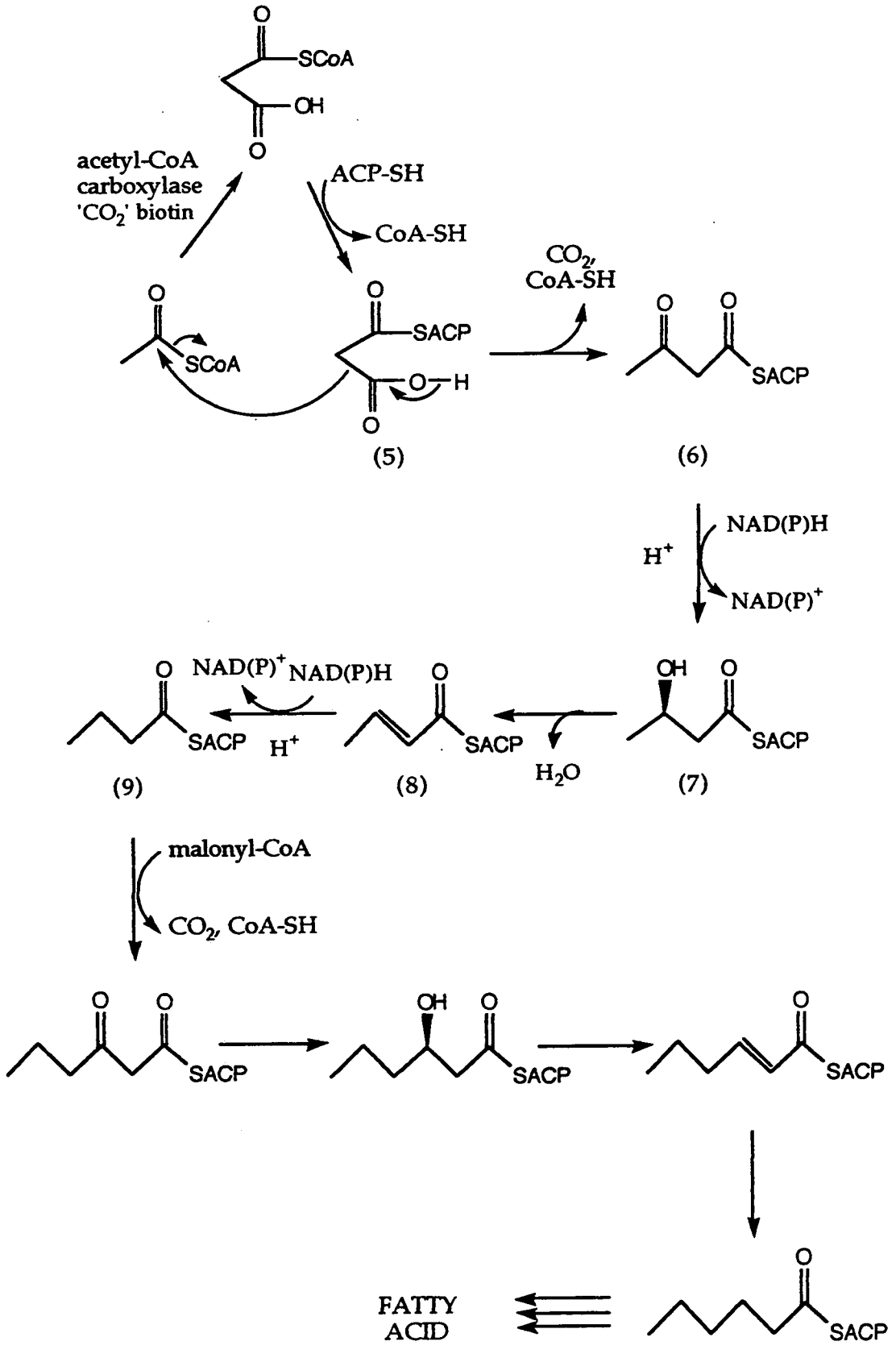


(4) acyl carrier protein

The acyl carrier protein (ACP) (4) has the role of co-ordinating fatty acid biosynthesis, by chaperoning the intermediate acyl groups as thioesters around the catalytic sites of the FAS. In Type II FAS's, the ACP is a single defined protein but in Type I FAS's, the acyl carrier protein is a domain consisting of a serine residue which covalently links the 4'-phosphopantetheine group to the synthase.[19]

A summary of the mode of assembly of fatty acids is outlined in scheme 1.[19] Acetyl-CoA becomes activated to malonyl-CoA by the action of the biotin requiring enzyme acetyl-CoA carboxylase. Malonyl-CoA is then transacylated with the 4'-phosphopantetheine moiety of ACP and the resultant malonyl-ACP (5) is located at the appropriate site of the condensing enzyme, β-ketoacyl-ACP synthase. A decarboxylative condensation takes place with a molecule of acetyl-CoA and the resultant β-ketoacyl-ACP (6) is then transported to the next enzyme, β-ketoacyl-ACP reductase, which mediates reduction to the D-alcohol in the presence of NAD(P)H to afford D-β-hydroxyacyl-ACP (7). The acyl carrier protein then guides the intermediates through the further stages of the cycle, i.e. dehydration to yield (8) mediated by β-hydroxyacyl-ACP dehydratase and then reduction to give a fully saturated C_4-ACP unit (9) mediated by enoyl-ACP reductase in the presence of NAD(P)H. In order to begin the next cycle, the C_4 unit is transesterified onto a free thiol residue at the active site of the condensing enzyme such that the thiol of ACP is now released and available to transesterify with another malonyl-CoA, and the second cycle can begin. These cycles repeat themselves until the fatty acid of optimum length is generated and is released from the fatty acid synthase to give a free carboxylic acid.

Until recently it had been generally accepted that acetyl-CoA underwent a transacylation with a free thiol residue on the condensing enzyme prior to the first condensation with malonyl-ACP. However a specific β-ketoacyl synthase (condensing enzyme III) has been isolated from *E. coli*, which mediates the first condensation between acetyl-CoA and malonyl-ACP.[22] The resultant β-ketoacyl-ACP (6) is then processed as outlined in scheme 1. This is emerging as a general feature of the dissociated Type II FAS's of bacteria and plants[23] and may explain the occurrence of unusual starter units, eg. isobutyrate, cyclohexylcarboxylate and others, in certain fatty acids. This condensing enzyme could be less selective in the condensation of CoA-esters with malonyl-ACP as no transesterification is required.


SCoA
OH
acetyl-CoA
carboxylase
'CO_2' biotin
ACP-SH
CoA-SH
CO_2,
CoA-SH
SACP
SCoA
O—H
(5)
(6)
H^+
NAD(P)H
$NAD(P)^+$
OH
$NAD(P)^+$ NAD(P)H
H^+
H_2O
(9)
(8)
(7)
malonyl-CoA
CO_2, CoA-SH
FATTY
ACID

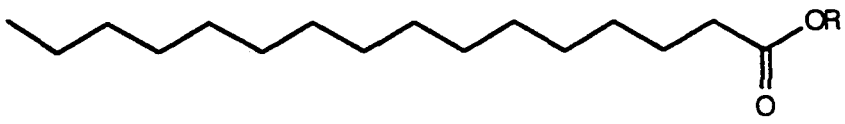
Scheme 1

In Type II FAS's, the condensing enzyme I is involved in the assembly of the fatty acid up to C_{16} chain length and condensing enzyme II takes the fatty acid from C_{16} to C_{18} and beyond.[19]

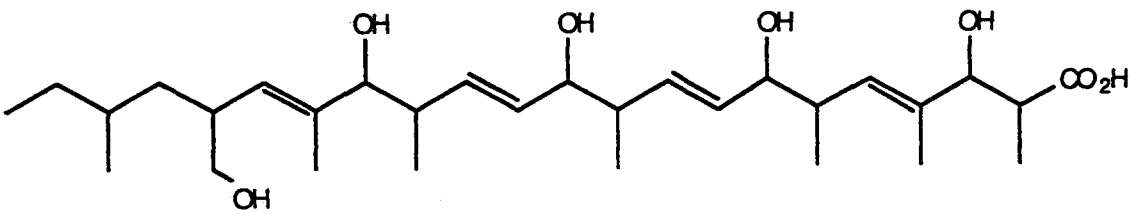
2.3 Polyketide biosynthesis

2.3.1 Fungal polyketides

The majority of polyketide secondary metabolites identified from fungal and plant sources are aromatic, the non-aromatic compounds are more rare. The process of polyketide chain assembly is analogous to fatty acid biosynthesis, i.e. acetyl-CoA is activated to malonyl-CoA. Malonyl-CoA then becomes transesterified onto the acyl carrier protein of the polyketide synthase (PKS) and a series of decarboxylative condensation, reductions etc. occur. The polyketide synthase has the full complement of reducing enzymes that the FAS possesses, but a difference lies in the ability of the PKS to stop the reduction / elimination procedure at any stage prior to the next condensation. In fatty acid biosynthesis a fully saturated chain is formed, eg. palmitate (10). In polyketide biosynthesis, however, the polyketide backbones often consist of partially reduced ketones or double bonds, leading to more complex structures, eg. cubensic acid (11).



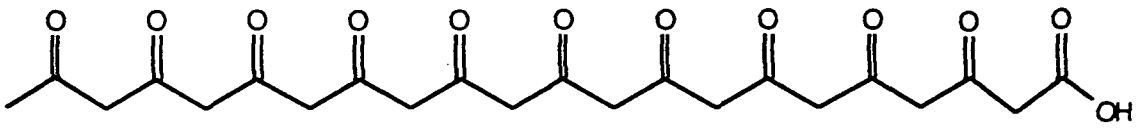
(10) palmitate



(11) cubensic acid

The process by which the oxidation level of the developing polyketide chain is adjusted to its final status, prior to the next condensation has been termed processive assembly.[24] The alternative is a non-processive assembly where a 'polyketo' chain is formed in its entirety, for example (12)

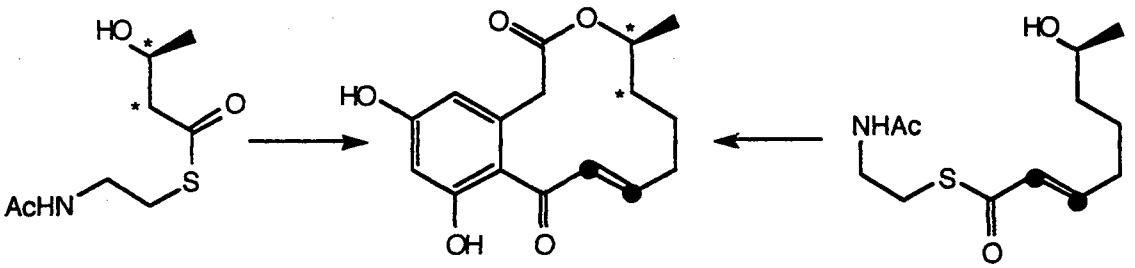
in the case of cubensic acid, and then a series of enzyme activities perform all the relevant modifications to yield the polyketide metabolite.



(12)

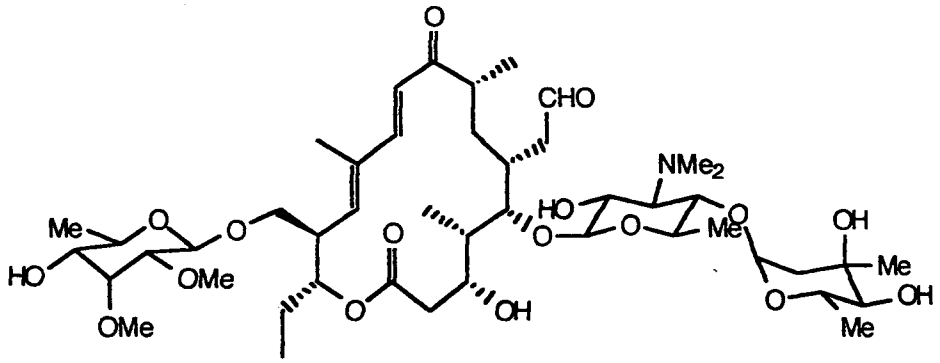
(11) cubensic acid

Experimental evidence supporting a processive assembly of fungal and bacterial polyketide metabolites has been provided in the form of incorporations of partially reduced di-, tri- and tetra- ketides into several polyketide metabolites. Dehydrocurvularin (13), a fungal metabolite produced by wild type *Alternaria cinerariae ATCCC 11784* [25] is an example. The isotopically labelled substrates (14) and (15) have been shown to be regiospecifically incorporated into dehydrocurvularin.[25]

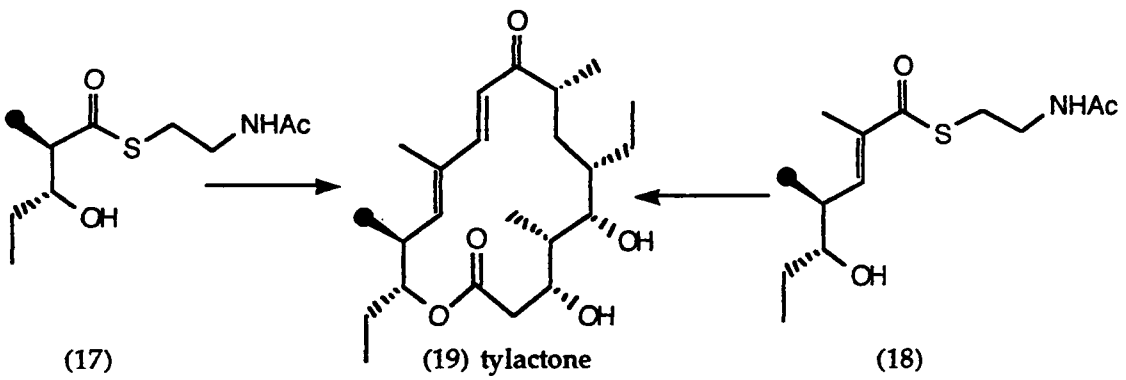


(14) (13) dehydrocurvularin (15)

Further examples are the incorporations of partially assembled fragments into the Actinomycete metabolites, nargenicin from *Nocardia argentinensis*[26] (discussed in chapter 5) and tylosin (16) produced by mutant strains of *Streptomyces fradiae*.[27] The isotopically labelled substrates (17) and (18) were shown[24] to be regiospecifically incorporated into the tylosin aglycone, tylactone (19).

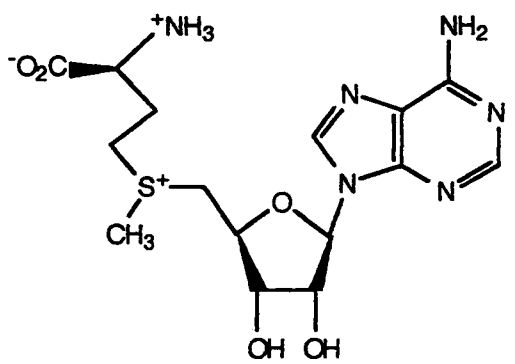


(16) tylosin



(17) (19) tylactone (18)

Often polyketides possess branched chain methyl groups, e.g. cubensic acid (11), and these pendant methyl groups, in fungal polyketides, are generally derived from the methyl group of L-methionine activated as S-adenosyl-L-methionine (SAM) (20). However in Actinomycete polyketides, the branched chain methyl groups are derived from the condensation of propionate units, e.g. tylosin (16), discussed in section 2.3.2.

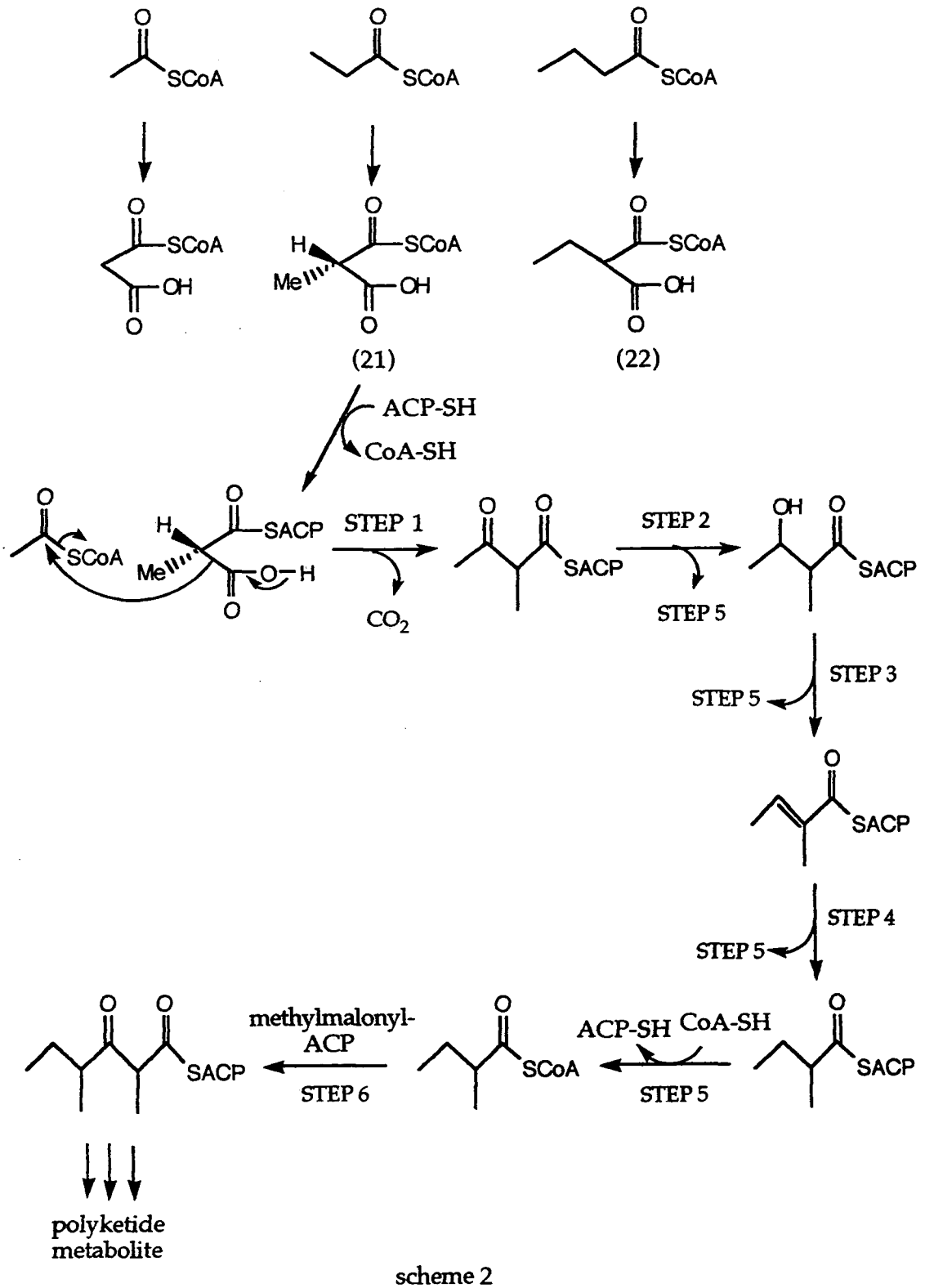


(20) S-adenosyl-L-methionine (SAM)

The exact timing of these C-methylations in fungal polyketides is unknown. There could exist a methyl transferase enzyme present on the PKS which mediates the alkylation of the methyl group from SAM to the polyketide during a processive assembly process, i.e. before the next condensation with a C_2 unit takes place (discussed in chapter 4). Alternatively the C-methylations could be post polyketide assembly modifications, like the O- and N- methylations. From a mechanistic perspective it seems reasonable to suggest that the methyl group of SAM will be attacked by either a double bond or an activated methylene prior to ketone reduction. This is more probable than nucleophilic attack by a saturated polyketide backbone. Remarkably experimental evidence to support a processive methylation mechanism is lacking, as no advanced methylated precursors have been incorporated into fungal polyketides.

2.3.2 Bacterial polyketides

The polyketide synthases of bacterial polyketides utilise not only acetyl-CoA but also, propionyl-CoA and butyryl-CoA during polyketide assembly. These intermediates become activated by carboxylase enzymes to methylmalonyl-CoA (21) and ethylmalonyl-CoA (22) respectively. Propionyl-CoA carboxylase has been isolated and purified from the erythromycin A producer *Saccharopolyspora erythraea* and it mediates the conversion of propionyl-CoA to (2S)-methylmalonyl-CoA.[28] (2S)-Methylmalonyl-CoA is then processed[29] by the PKS after transesterification onto an ACP, scheme 2. The PKS possesses all the reducing enzymes utilised in fatty acid biosynthesis and so the cycle of condensations and reductions can begin. The cycle can stop at any point and the next condensation with a C_2 - C_4 unit takes place (see section 2.3.1). The polyketide chain can therefore become very complex, comprising of pendant methyl and ethyl groups which can have either D or L configurations with respect to the direction of chain assembly, and also ketone, alcohol and double bond functionality or fully saturated methylene units.


SCoA
OH
Me
H
O
(21)
(22)
ACP-SH
CoA-SH
SACP
STEP 1
CO_2
STEP 2
STEP 5
STEP 3
STEP 4
methylmalonyl-
ACP
STEP 6
polyketide
metabolite

scheme 2

3. BIOSYNTHESIS TECHNIQUES

3.1 Radioactive isotopes

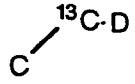
The radioactive isotopes, ^{14}C and ^{3}H (both β-emitters), were commonly used in biosynthetic studies between the 1950 - 1970's. They are used to assess accurately the level of incorporation of a substrate into a metabolite, by the sensitive detection technique of scintillation counting. Unfortunately in order to determine the regiospecific sites of incorporation, the metabolite has to be chemically degraded, which can be a long and laborious task. Also to ensure an accurate result the sample requires recrystallisation to constant radioactivity which can be difficult with small amounts of sample. With the advances in n.m.r. and analysis techniques of stable isotopes, in recent years radioactive isotopes are usually employed in exploration experiments prior to the deployment of stable isotopes.

3.2 Stable isotopes

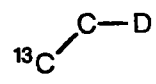
The experimental study of biosynthesis has developed largely due to the use of radioactive isotopes such as ^{14}C and ^{3}H and also the introduction of Fourier transform nuclear magnetic resonance (n.m.r.)[30] which provides the ability to detect regiospecifically, the location of stable isotopes in a molecule quickly, and without recourse to degradation of the molecule under study. The most common stable isotopes used include ^{13}C (nuclear spin = 0.5), ^{2}H (nuclear spin = 1) and ^{15}N (nuclear spin = 0.5). The use of ^{13}C isotopes is a popular tool. Enrichment of the ^{13}C signals in a ^{13}C n.m.r. spectrum of a metabolite gives information on where the label is located. However it is often difficult to accurately calculate the percentage incorporation because there are several influencing factors, eg. sample concentration and relaxation effects. A lower practical limit for the accurate estimation of enrichment, is a signal enriched to the extent of 20% of the natural abundance signal.[31]

Deployment of dual labels offers a powerful technique in biosynthesis. If two enriched carbons (^{13}C) are adjacent to each other, coupling satellites are apparent as a doublet, flanking the natural abundance signal. Incorporations can be measured at very low enrichments since the natural abundance of ^{13}C-^{13}C coupled signals is low (0.0123%). This approach is useful as it highlights the incorporation of intact C-C bonds into

the metabolite from labelled precursors. Extension of this strategy involves the use of ^{13}C-^{2}H (23) and ^{13}C-C-^{2}H (24) labelling in a precursor.



(23)



(24)

In the first case, the deuterium in a ^{13}C-^{2}H labelled precursor shifts the carbon-13 resonance of the carbon to which it is attached, to a lower frequency by 0.3 - 0.6ppm (α-shift). Hence the maximum shift for three deuteriums attached to a ^{13}C, i.e. ^{13}C-$^{2}H_3$, would be 1.8ppm. Also the nuclear spin on ^{2}H (= 1) increases the multiplicity of the carbon-13 signal in the ^{13}C n.m.r. spectrum. In the second case, the deuterium in a ^{13}C-C-^{2}H labelled precursor induces a lower frequency shift in the ^{13}C n.m.r. spectrum of the β-carbon by 0.01 - 0.1 ppm per ^{2}H (β-shift). This labelling combination can also cause a higher frequency or zero shift particularly, if the carbon-13 is a carbonyl group.[32]

The potential of ^{2}H n.m.r. was first demonstrated[33] in 1964 and deuterium is now widely used as a tracer isotope for biosynthetic studies.[34] There are several advantages of using deuterium. The chemical shift values obtained in ^{2}H n.m.r. spectra are similar to those in the corresponding ^{1}H n.m.r. spectra and hence assignments are straight forward. It is a less expensive isotope and the low natural abundance of deuterium (0.016%) compared with carbon-13 (1.1%), renders it more sensitive, and hence very low incorporations into a metabolite are detectable above natural abundance. A clear disadvantage, however, is that there is extensive line broadening in the ^{2}H n.m.r. and poor resolution.

Oxygen-18 does not give an n.m.r. signal but its presence can be detected by ^{13}C n.m.r.,[35] as ^{13}C-^{18}O causes a shift of the carbon-13 resonance to lower frequency compared with that of ^{13}C-^{16}O. The shift range extends from 0.01 - 0.035ppm for singly bonded oxygens to 0.03 - 0.55ppm for doubly bonded oxygens.[31] Oxygen-17 can be detected by n.m.r., it has a lower natural abundance than oxygen-18, but unfortunately there is line broadening in the n.m.r. and it is an expensive isotope.[31]

4. Précis

This thesis has been divided into parts 1 and 2 in order to highlight the two distinct categories of Actinomycete polyketides (part 1) and fungal polyketides (part 2). Each polyketide metabolite is discussed in an individual chapter, as there are unique aims and experimental targets.

Monensin A produced by *Streptomyces cinnamonensis* is an Actinomycete metabolite and is outlined in chapter 2. Bacterial polyketide metabolites which incorporate propionate require a metabolic source of methylmalonyl-CoA and this chapter discusses the possible sources of this key building block, from L-valine, the citric acid cycle and from the DNA base thymine. The link between primary and secondary metabolism is probed with the aid of appropriate feeding experiments. Chapter 3 discusses how the stereochemistry of leptomycin B, an Actinomycete metabolite from *Streptomyces sp. ATS 1287*, may have been established by feeding sodium [2-^{13}C,$^{2}H_2$]-propionate.

Part 2 and chapter 4 enters the arena of fungal polyketides and in particular cubensic acid, a straight chain fungal metabolite isolated from *Xylaria cubensis*. This chapter is split into parts A and B. The biosynthesis and biogenesis of cubensic acid are discussed in parts A and B respectively. A hypothetical modular polyketide assembly is proposed based upon the established gene sequence and assembly process involved in erythromycin A biosynthesis. The mode of assembly of the six membered ring in cytochalasin D isolated from *Xylaria cubensis* is discussed in chapter 5 and attempts to prove a biological intramolecular Diels-Alder reaction are outlined.

Chapter 6 includes full experimental details of the experiments covered in each chapter.

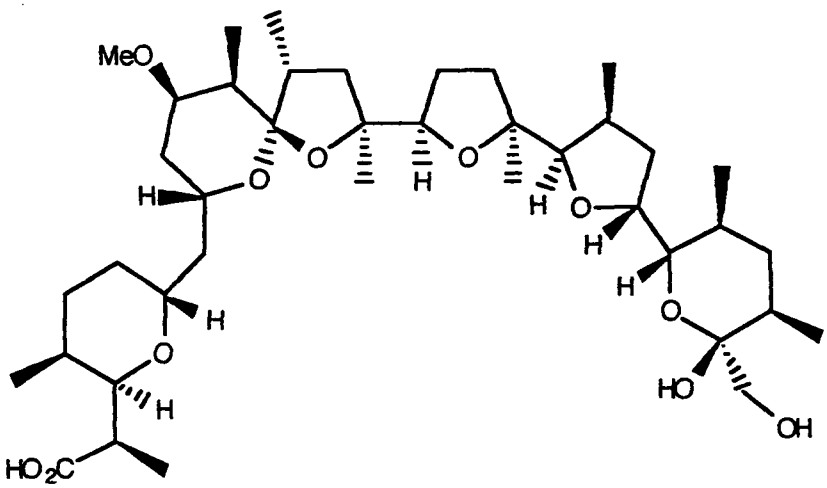
PART 1

CHAPTER 2
MONENSIN A

1. INTRODUCTION

1.1 Polyethers

The polyethers are a large class of naturally occurring ionophores which have attracted a great deal of interest due to their chemical and biochemical properties.[36] The first polyether to be isolated was nigericin (1)[37] from *Streptomyces hygroscopicus* over 30 years ago.

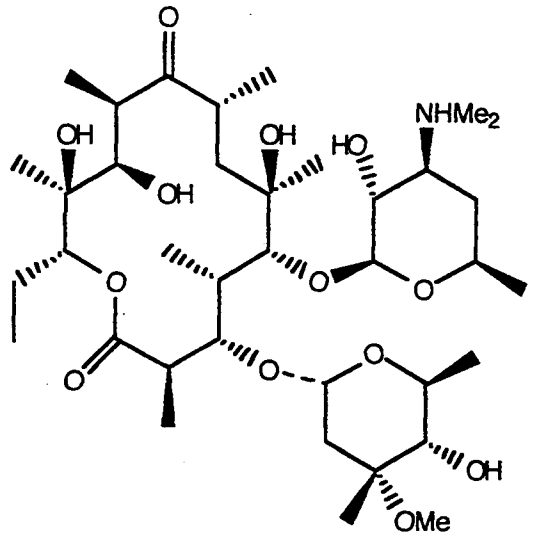


(1) nigericin

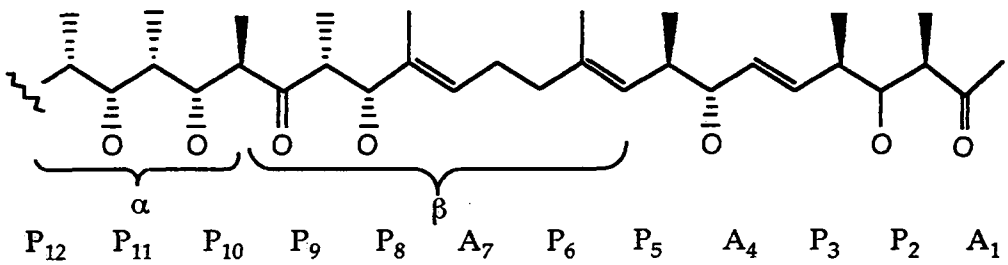
The polyethers as a class have a characteristic structural framework consisiting of tetrahydofuranyl and tetrahydropyranyl rings, and branched methyl and ethyl units. These metabolites are arranged in a conformation such that the oxygen atoms are able to complex alkali metal ions, analogous to crown ethers. The macrolide antibiotics, also products of Streptomycetes, are a second major class of polyoxygenated metabolites which bear a resemblance to the polyethers.[38] An example is erythromycin A (2) produced by *Saccharopolyspora erythraea*, a key clinical antibiotic of this class.

There are[39] structural similarities between these two important groups of natural products, both of which have a common biosynthetic origin from acetate, propionate and butyrate precursors. Celmer realised in 1965, that there exists stereochemical homologies within the macrolides and he proposed a single stereochemical model which summarised the entire

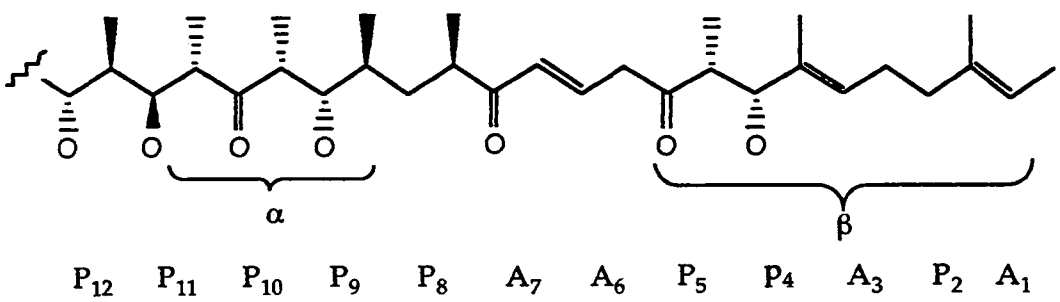
class.[40] This empirical model has served as a guide to the structural and stereochemical assignment of new macrolide antibiotics and only a few exceptions to this model have been found.[41]



(2) erythromycin A



(3a) APPA polyene prototype

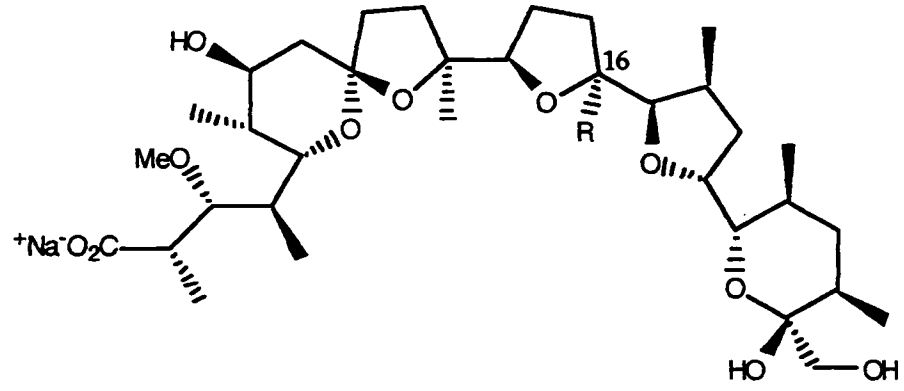


(3b) PAPA polyene prototype

A similar study of the polyether group of antibiotics has been reported.[39] Putative[42] partially reduced polyene intermediates in the biosynthesis of polyethers are microbially epoxidised to their corresponding polyepoxides. These polyepoxides may then undergo enzymatically controlled cascade cyclisations generating the polyether frameworks (discussed in section 1.2). Cane, Celmer and Westley[4] derived, from empirical observations, the two polyene prototypes APPA (3a) and PAPA (3b) which summarise the stereochemical patterns of more than 30 different polyether antibiotics. α And β highlight the recurring stereochemical units in each of the polyene prototypes indicating structural homologies. Such prototypes raise intriguing questions about the biogenetic origin of these structurally and stereochemically similar metabolites.

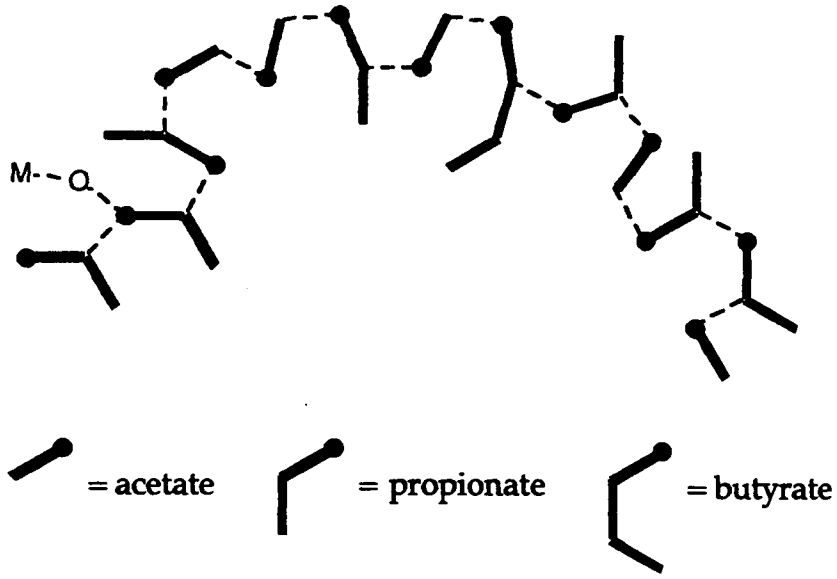
1.2 Monensin A

The polyether antibiotic monensin A (4)[43] is an important agent in the control of coccidiosis in poultry.[44] Both monensin A (4) and monensin B (5) are isolated as crystalline sodium salts from *Streptomyces cinnamonensis.* Monensin A differs from monensin B at C16 (R = Me or Et) with the incorporation of a butyrate over a propionate unit during biosynthesis.[45] The structure of monensin A was determined in 1967[46] and its ^{13}C n.m.r. spectrum was fully assigned by two groups in 1982.[47]



(4) R = CH_2CH_3
(5) R = CH_3

Monensin A was one of the first polyether antibiotics to be subjected to biosynthetic investigations,[48] and feeding experiments revealed that the carbon skeleton originates from 5 acetate, 7 propionate and 1 butyrate unit with the methoxyl carbon atom being derived from L-methionine (6).[49]



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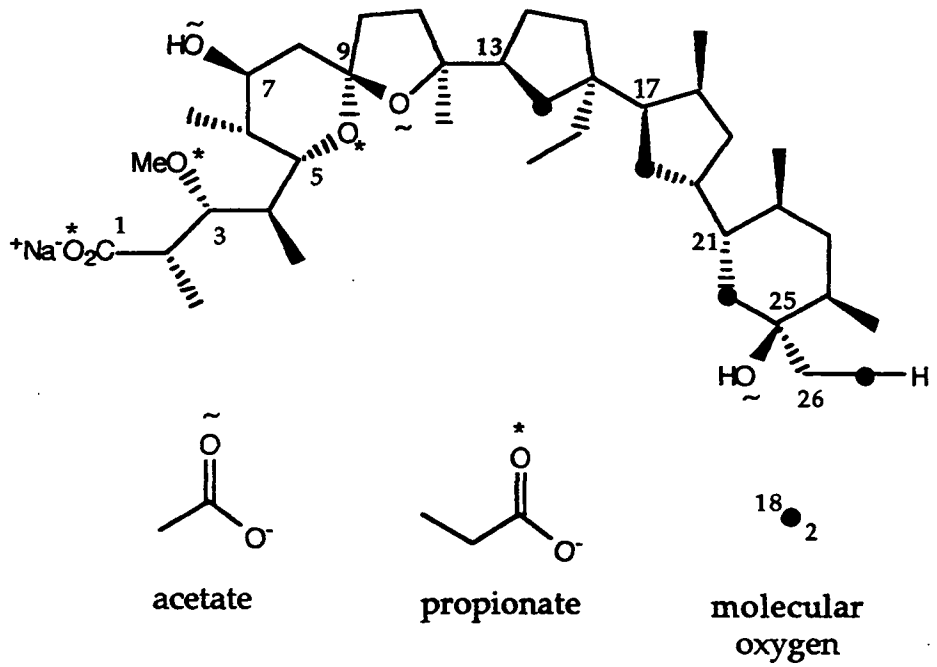
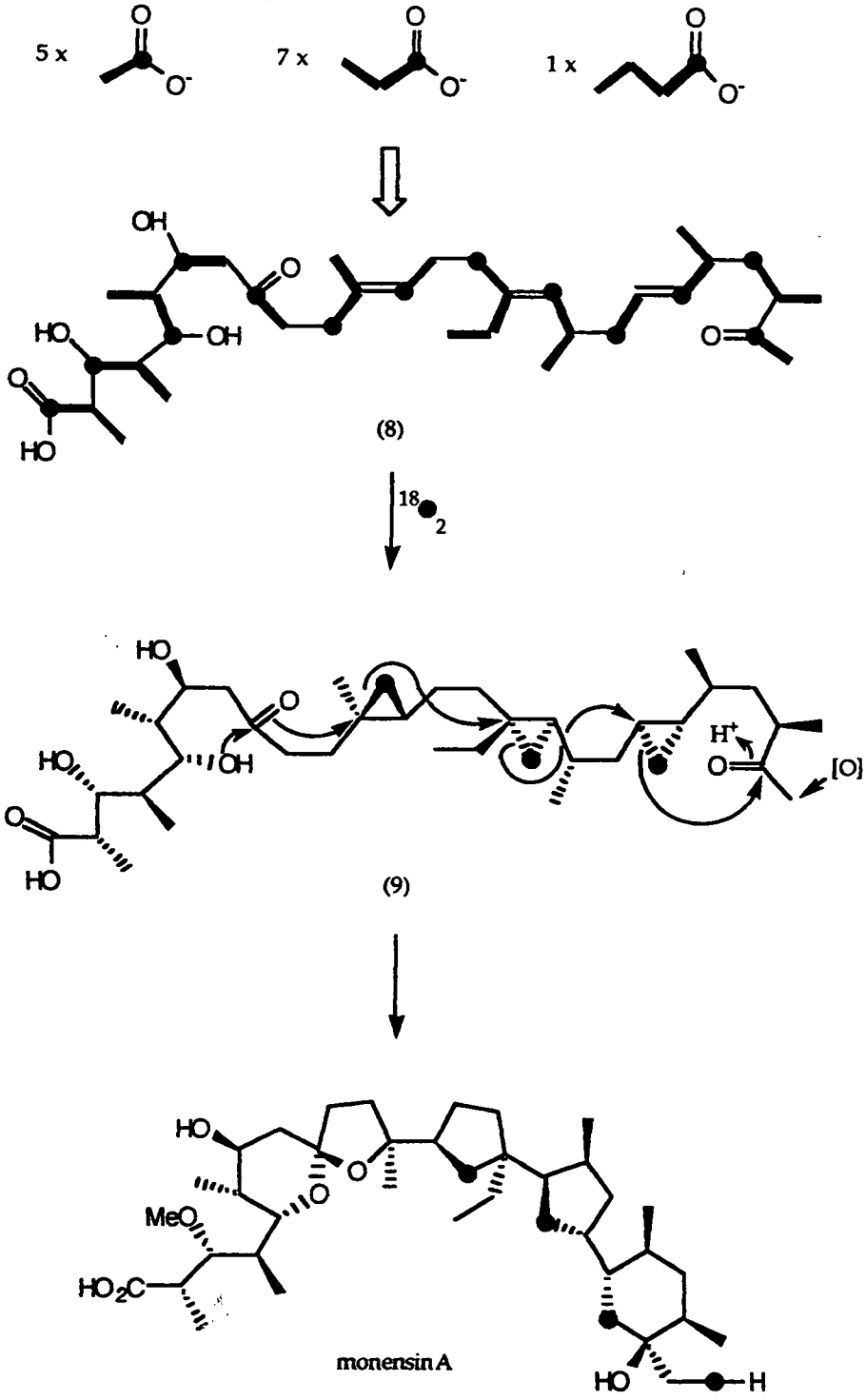


(7)

Extensive isotopic labelling experiments on monensin A, utilising sodium [1-^{18}O,1-^{13}C]-acetate and -propionate,[12(b),49] and molecular $^{18}O_2$[50] have established the origin of all the oxygen atoms in monensin A (7). The oxygen's at C1, C3, C5 were shown to derive from propionate, and those at C7, C9, C25 from acetate. The oxygen's at C13, C17, C21, C26 originate from molecular oxygen. These results led Cane *et al.*[47(b)] to speculate that

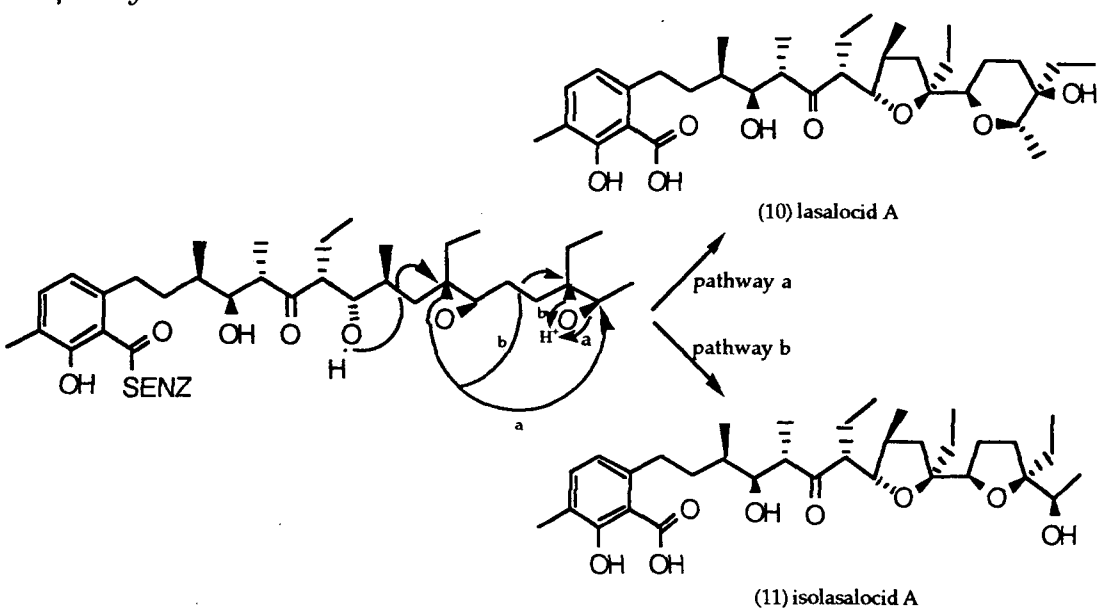
monensin A is biosynthesised from an all (E)-triene intermediate (8) which could undergo microbial epoxidation to the triepoxide (9). Such a triepoxide (9) could then be subjected to an enzymatically controlled cascade cyclisation, to generate the heterocyclic ring systems of monensin A with the correct stereochemistry.



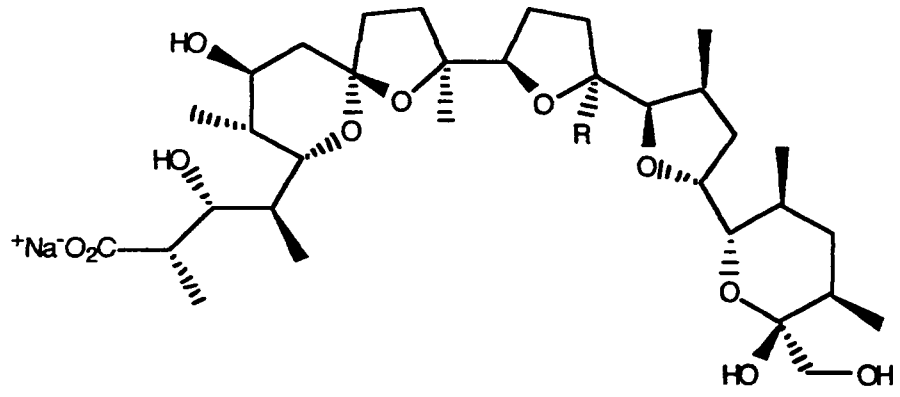
Scheme1

This hypothesis is compelling as it is also consistent with the origin of the oxygen's at C13, C17, C21 from molecular oxygen (scheme 1).

An earlier speculation by Westley[42] that lasalocid A (10) and isolasalocid A (11) may arise by differential cyclisation of a common diepoxide intermediate also reinforces this general view for polyether assembly. Westley's speculation was experimentally[51] supported by the results of ^{13}C and ^{18}O labelled precursor feeding experiments on *Streptomyces lasaliensis.*

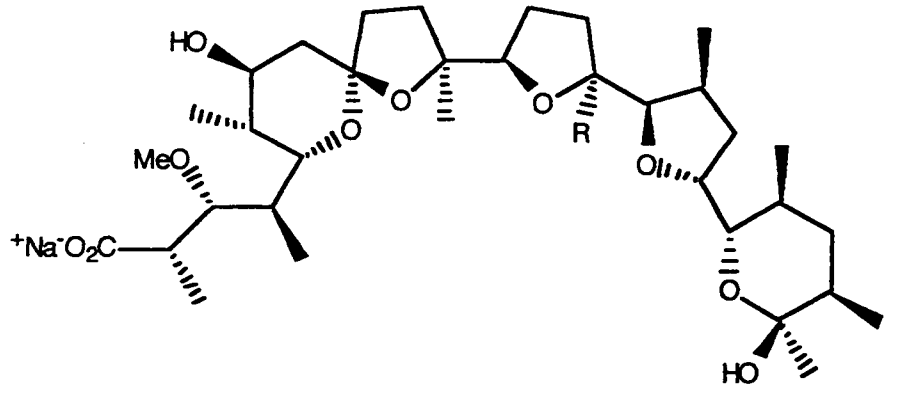


Related investigations on narasin,[52] lenoremycin,[53] ICI139603[54] and maduramicin[55] further strengthen this concept and therefore it is widely held that the polyene - polyepoxide hypothesis provides a rational explanation for the origin of these antibiotics. However there has been no definitive experimental evidence to support the hypothesis. Attempts[56] have been made to isolate biosynthetic intermediates in the monensin pathway, particularly the putative triene intermediate (8), but without success. 3-O-Demethylmonensins A and B (12)[57] (differing from the monensins by the presence of -OH over -OMe at C3) have been recovered as minor components from *S. cinnamonensis* fermentation broths.



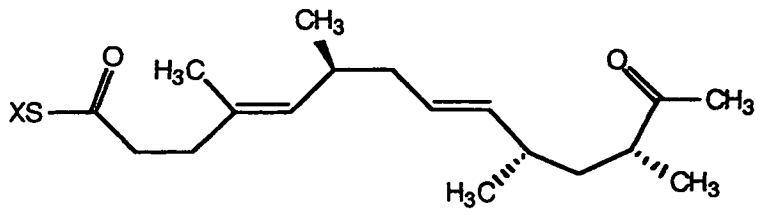
(12) 3-O-demethylmonensin A R = CH_2CH_3
3-O-demethylmonensin B R = CH_3

Also from a mutant strain of *S. cinnamonensis,* blocked in monensin production, 26-deoxymonensins A and B (13) have been isolated.[58] These intermediates were prepared in labelled form and fed to the cultures of *S. cinnamonensis,* but unfortunately there was no evidence of incorporation into the monensins.[58]

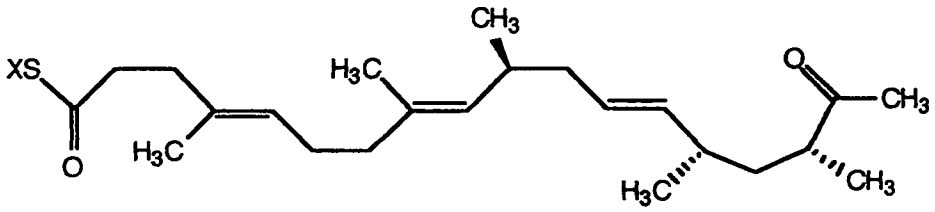


(13) 26-deoxymonensin A R = CH_2CH_3
26-deoxymonensin B R = CH_3

Robinson *et al.*[56] have pursued the synthesis of the partially assembled putative diene (14) and triene (15) intermediates in the biosynthesis of the monensins, utilising ^3H as the isotopic label and activated as a thioester (discussed in chapter 4), but upon feeding to *S. cinnamonensis* there was no evidence of an intact incorporation into the monensins. Their interpretation was that the triene (15) was probably unable to cross the cell membrane, however the diene (14) could cross the cell membrane but was efficiently degraded, presumably by β-oxidation processes (discussed in chapter 5).



(14)

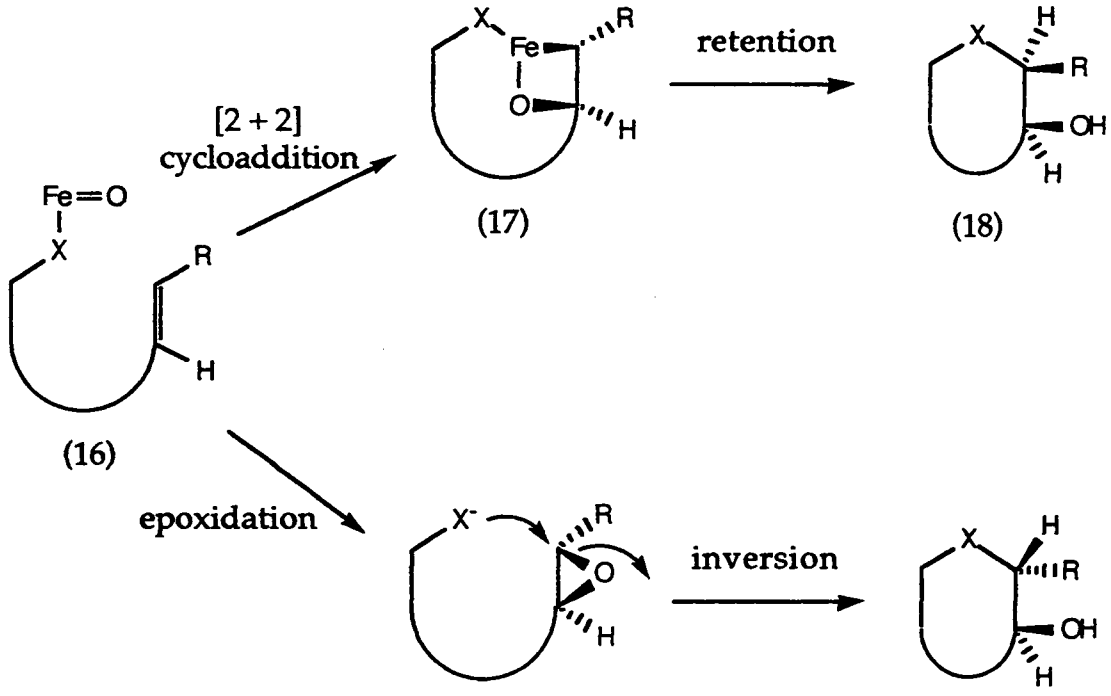


(15)

$$X = -CH_2CH_2NHCOC_7H_{15}$$

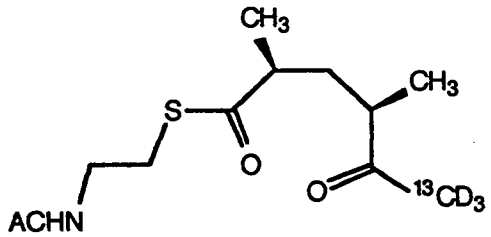
Another possibility for the lack of incorporation of (14) and (15) could be due to the presentation of the incorrect structure to the system. It has been assumed that all of the double bonds involved in the cyclisation process have (E)- geometry, as this would lead to the correct stereochemistry for the monensins *via* the triepoxide hypothesis. Townsend[59] has however speculated that the triene intermediate (8) could have all (Z)- and not (E)- double bond geometry. This hypothesis extends from the oxidative cyclisation processes involving a heteroatom - ferryl complex and particularly that mediated by clavaminate synthase (X = O) and isopenicillin-N synthase (X = S). If this system is applied to polyether biosynthesis (scheme 2), then a pathway involving [2 + 2] cycloaddition of the ferryl species (16), with an olefin, would yield an oxametallocyclobutane derivative (17) which would break down with retention of configuration to give an oxygen ether (18), i.e. the C-X bond has replaced the C-Fe bond. The alternative pathway involving the epoxide ring opening would proceed with inversion of configuration. Hence, if the [2 + 2] cycloaddition pathway is operating, then the double bond geometry of the putative polyene intermediate would have to be (Z)- and not (E)-. This suggestion remains to

be tested, but adds a new perspective to the study of the biosynthesis of these polyethers.

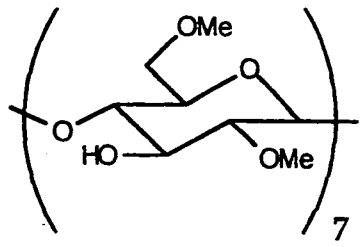


Scheme 2

Success has been obtained by Robinson *et al.*[60] recently in the incorporation into monensin A of substrate (19), labelled with [$^{13}C^{2}H_3$]- and activated as an N-acetylcysteamine thioester. This incorporation was assisted by the addition of a β-oxidation inhibitor (to prevent substrate degradation) and 2,6-O-dimethyl-β-cyclodextrin (20) which improves the production of monensin A[61] and helps to stimulate the uptake and incorporation of the substrate (19). The intact incorporation of this partially assembled intermediate (19) provides the first evidence of a processive assembly (discussed in chapter 1) during polyether biosynthesis.



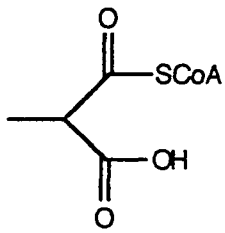
(19)



(20)

2. SOURCES OF METHYLMALONYL-CoA

2.1 Introduction



(21)

Methylmalonyl-CoA (21) is the key building block in the biosynthesis of propionate derived polyketide metabolites. There are several well established metabolic routes to this intermediate.

i) *The carboxylation of propionyl-CoA.* Propionyl-CoA carboxylase from *Saccharopolyspora erythraea* generates (2S)-methylmalonyl-CoA from propionyl-CoA.[28] (2S)-Methylmalonyl-CoA then participates in polyketide biosynthesis by undergoing a decarboxylative condensation with inversion of configuration onto the developing polyketide chain.[62]

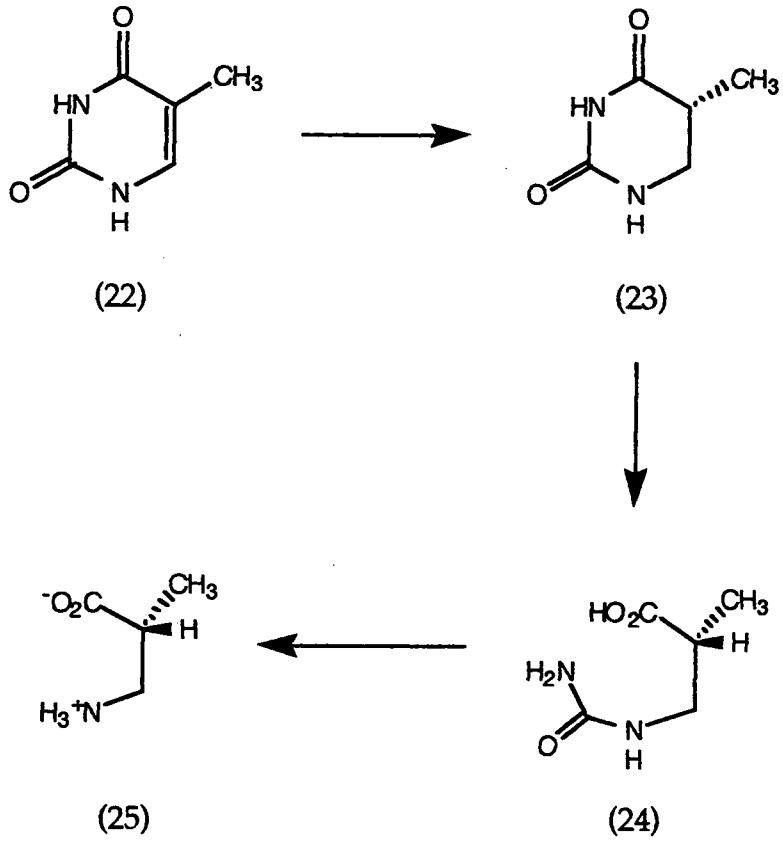
ii) *The citric acid cycle.* Succinyl-CoA can undergo a rearrangement, mediated by methylmalonyl-CoA mutase, to (2R)-methylmalonyl-CoA.[63-65]

iii) *Amino acid catabolism.* Catabolic pathways particularly that of valine and isoleucine (discussed in section 2.3.3) afford methylmalonyl-CoA as a metabolic product.

iv) *Degradation of long chain fatty acids.* This affords C_4 fatty acids (i.e. butyryl-CoA) which can be converted to isobutryl-CoA mediated by isobutyryl-CoA mutase[66] and then processed *via* the series of enzymes utilised in the catabolism of valine.[62]

It is well established[67,68] in mammalian systems, that the catabolism of thymine (22) affords (2R)-β-aminoisobutyric acid (25) *via* the

intermediates (5R)-dihydrothymine (23) and β-ureidoisobutyric acid (24) (scheme 3). So the possibility of thymine (a DNA base) degrading *via* a similar pathway in *Streptomyces* to afford methylmalonyl-CoA units is investigated. This probes further the links between primary and secondary metabolism and the possibility of nucleic acids providing a source of propionate units available for antibiotic biosynthesis.



Scheme 3

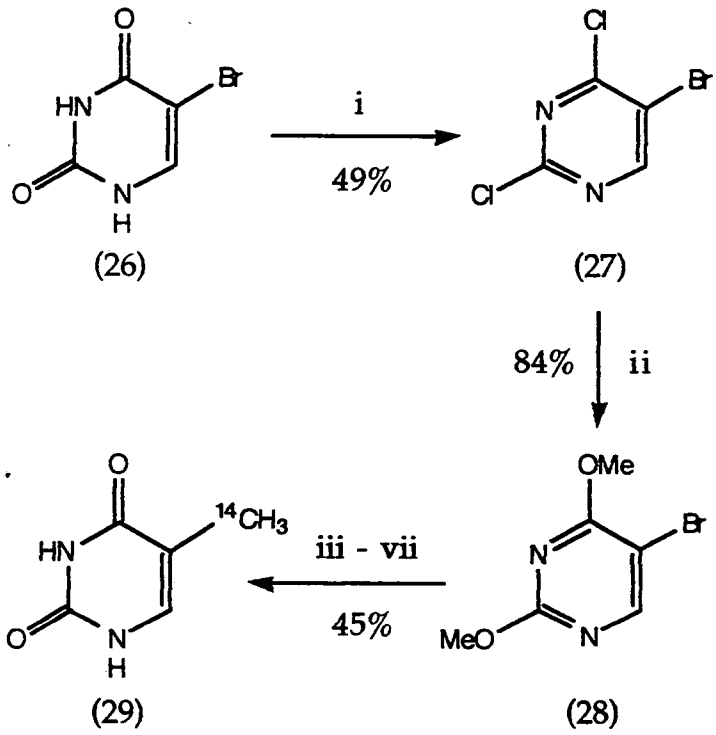
There are a small number of micro-organisms which are known to degrade pyrimidines *via* this reductive pathway,[69] e.g. various yeasts,[70] *Pseudomonas facilis*,[71,72] *Clostridium sporogenes*,[73] *Clostridium sticklandii*[74] and *Nocardia rubra*.[75] However, *C. sporogenes* and *C. sticklandii* are unable to completely metabolise thymine. Dihydrothymine and β-ureidoisobutyrate were observed as intermediates but there was no evidence of further degradation to β-aminoisobutyrate. Also the evidence for the reductive pathway in *Nocardia rubra* is based only on indirect evidence.[69] *Pseudomonas facilis*[72] and *Candida utilis*[76] are the only micro-organisms known to utilise the carbon atoms of the pyrimidines, and this is

attributed to their ability to degrade β-alanine, an end product of the catabolism of uracil.

A key objective of this research is to investigate the role of thymine as a source of methylmalonyl-CoA in *Streptomyces*. The investigation takes the form of feeding the key intermediates of the thymine and valine catabolic pathways, i.e. thymine, β-aminoisobutyric acid, sodium isobutyrate and sodium methacrylate, to the monensin A producer *Streptomyces cinnamonensis* followed by analyses of the relative incorporations.

2.2 Synthesis

2.2.1 Synthesis of [^{14}C-*methyl*]-thymine and [$^{13}C^{2}H_3$-*methyl*]-thymine



Scheme 4. Reagents and conditions: i) $POCl_3$, reflux 6d, ii) Na, MeOH, rt 0.5h, iii) ^{n}BuLi, THF, -78°C 0.25h, iv) $^{14}CH_3I$ and CH_3I, -78 to -50°C 1.5h, v) $CO_{2(g)}$, -50°C, vi) 15%HCl, reflux 24h, vii) H^+ Dowex.

[^{14}C-*methyl*]-Thymine (29) was synthesised as outlined in scheme 4.[77,78] Treatment of 5-bromouracil (26) with phosphorus oxychloride yielded 2,4-dichloro-5-bromopyrimidine (27). Reaction of (27) with sodium methoxide afforded a white precipitate of 2,4-dimethoxy-5-bromopyrimidine (28) which

was then treated with ^{n}BuLi. The reaction mixture was cooled to -198°C and $^{14}CH_3I$ (1mCi) was transferred by vacuum into the reaction flask. Unlabelled CH_3I was added to a final specific activity of $^{14}CH_3I$ of 0.475mCi/mmol and the reaction quenched by the addition of CO_2. Acid hydrolysis and purification by ion exchange (H^+ form) chromatography afforded [^{14}C-*methyl*]-thymine (29) (926μCi) with a radiochemical purity of 91.2% (determined by radiochemical t.l.c. analysis [^{n}BuOH : H_2O, (86 : 14) on silica]). Autoradio-ography also verified that the site of radioactivity on the t.l.c. plate had the same R_f value (0.69) as cold thymine, Fig. 1.

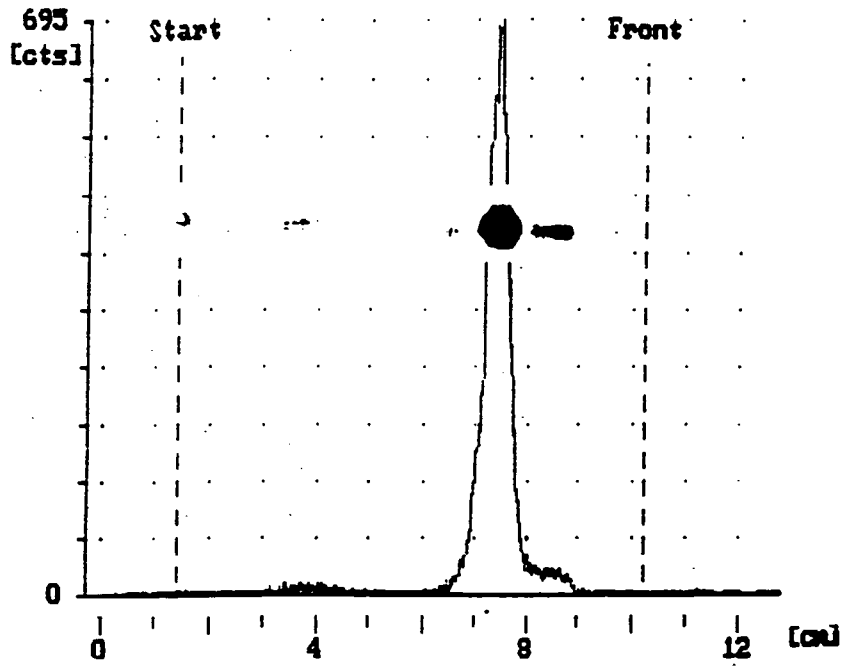
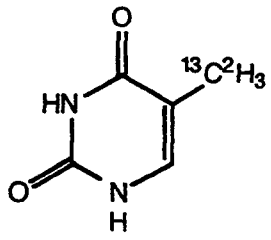


Fig. 1, thin layer chromatography radioactivity scan with the autoradioography result superimposed.

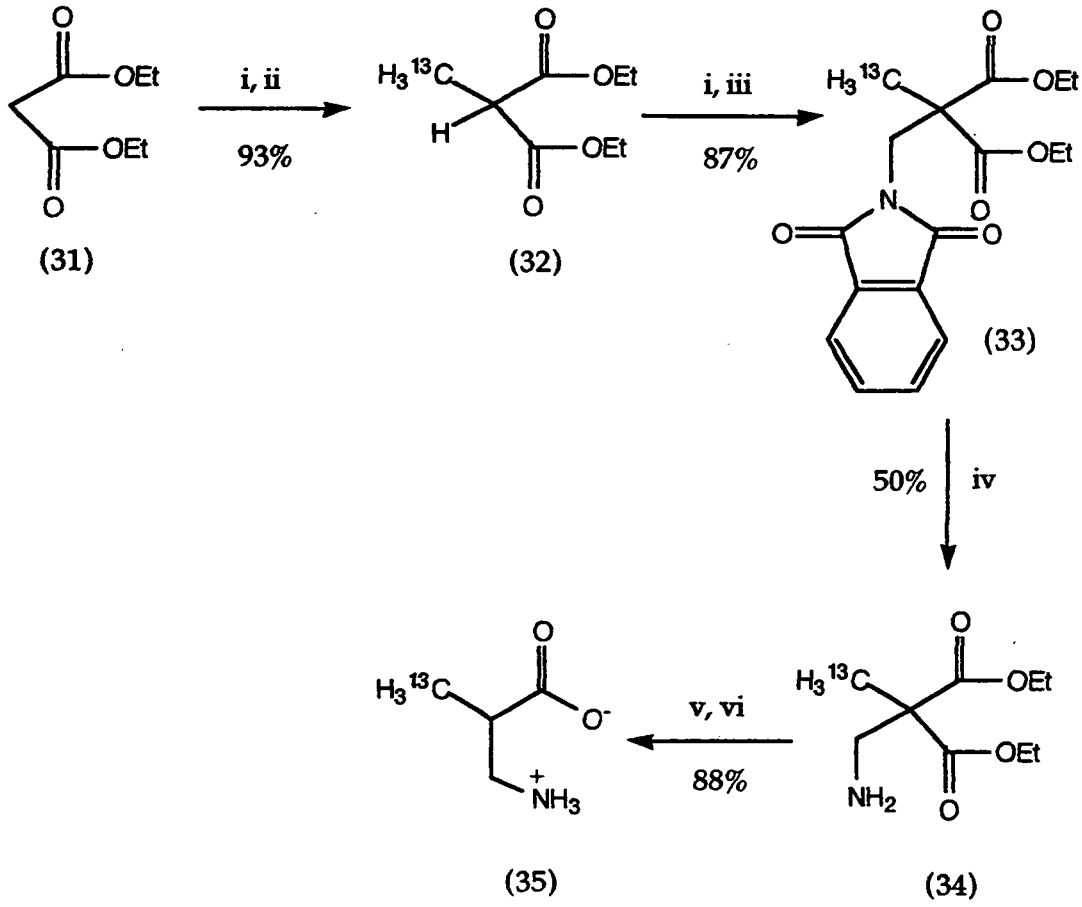
The ^{1}H n.m.r. of the prepared [^{14}C-*methyl*]-thymine indicated a small amount of uracil present (thymine : uracil, 9 : 1). The uracil is non-radioactive and therefore should have no significant effect on the results.

[$^{13}C^2H_3$-*methyl*]-Thymine (30) was prepared in a similar manner by replacing the $^{14}CH_3I$ with $^{13}C^2H_3I$. [$^{13}C^2H_3$-*methyl*]-Thymine was purified by sublimation. It sublimed as a white solid at 157°C and 0.023mmHg in a yield of 45% based upon $^{13}C^2H_3I$.



(30)

2.2.2 Synthesis of (DL)-[^{13}C-*methyl*]-β-aminoisobutyric acid



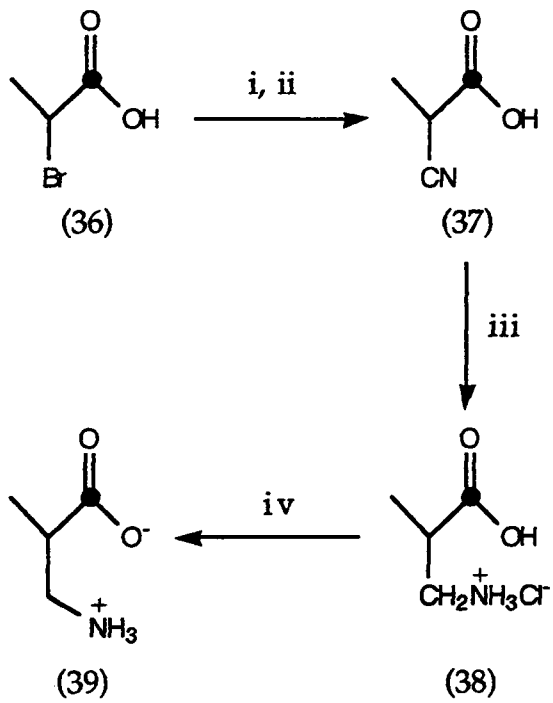
(31) (32) (33) (34) (35)

Scheme 5. Reagents and conditions: i) NaH, THF, 0°C, ii) $^{13}CH_3I$, reflux 2h, iii) N-(bromomethyl)phthalimide, 0°C to r.t. 1.5h, iv) H_2NNH_2, MeOH, r.t. 12h, v) 10% HCl, reflux 12h, vi) H^+ Dowex.

The synthesis of (DL)-[^{13}C-*methyl*]-β-aminoisobutyric acid was executed by a modification of the method of Bohme *et al.*[79] as outlined in scheme 5. The route starts with sodium hydride treatment of diethyl malonate (31) followed by quenching of the resultant anion with $^{13}CH_3I$. Further

alkylation of diethyl [^{13}C-*methyl*]-methylmalonate (32) under the same conditions, but quenching with N-(bromomethyl)-phthalimide yielded (33). Generation of the primary amine was achieved after treatment of (33) with a methanolic solution of anhydrous hydrazine to create (34), which was hydrolysed under acidic conditions and decarboxylated to yield [^{13}C-*methyl*]-β-aminoisobutyric acid hydrochloride in an overall yield of 37% from $^{13}CH_3I$. This material was used directly for feeding experiments, however, a small amount was purified, for characterisation purposes, by ion exchange (H^+ form) chromatography, to afford (DL)-[^{13}C-*methyl*]-β-aminoisobutyric acid (35) as a white solid.

2.2.3 Synthesis of (DL)-[1-^{13}C]-β-aminoisobutyric acid and (DL)-[3-$^{2}H_2$]-β-aminoisobutyric acid

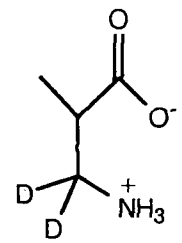


Scheme 6. Reagents and conditions: i) Na_2CO_3, CH_3CN, r.t. 2h, ii) NaCN, NaOH, H_2O, 65 - 80°C 2.5h, $HCl_{(aq)}$, iii) H_2, PtO_2 in EtOH - $CHCl_3$ (10 : 1), 2.5atm, r.t. 18h, iv) H^+ Dowex.

The synthetic route to (DL)-[1-^{13}C]-β-aminoisobutyric acid (39) is outlined in scheme 6. The synthesis of [1-^{13}C]-α-cyanopropionic acid (37) followed a previous preparation by Redwine and Whaley.[80] [1-^{13}C]-α-Bromopropionic

acid (36) was converted into its sodium salt and then treated with sodium cyanide and sodium hydroxide, to yield (37) as a colourless oil. This proved to be problematic and a poor yielding reaction in our hands. It was reported[80] that the reaction be heated to 50°C for 2.5 hours, but in the event the reaction proved temperamental and heat dependent, and was only successful if the temperature reached 80°C. Successive experiments also gave inconsistent results. [1-^{13}C]-α-Cyanopropionic acid (37) was taken through to the next step without complete characterisation due to its volatility. Hydrogenation using PtO_2 as a catalyst, in ethanol : chloroform (10 : 1), generated the amine hydrochloride (38) *in situ*.[81] Purification of (38) was executed by ion exchange (H^+ form) chromatography to afford (DL)-[1-^{13}C]-β-aminoisobutyric acid (39) in an overall yield of 8% from [1-^{13}C]-α-bromopropionic acid.

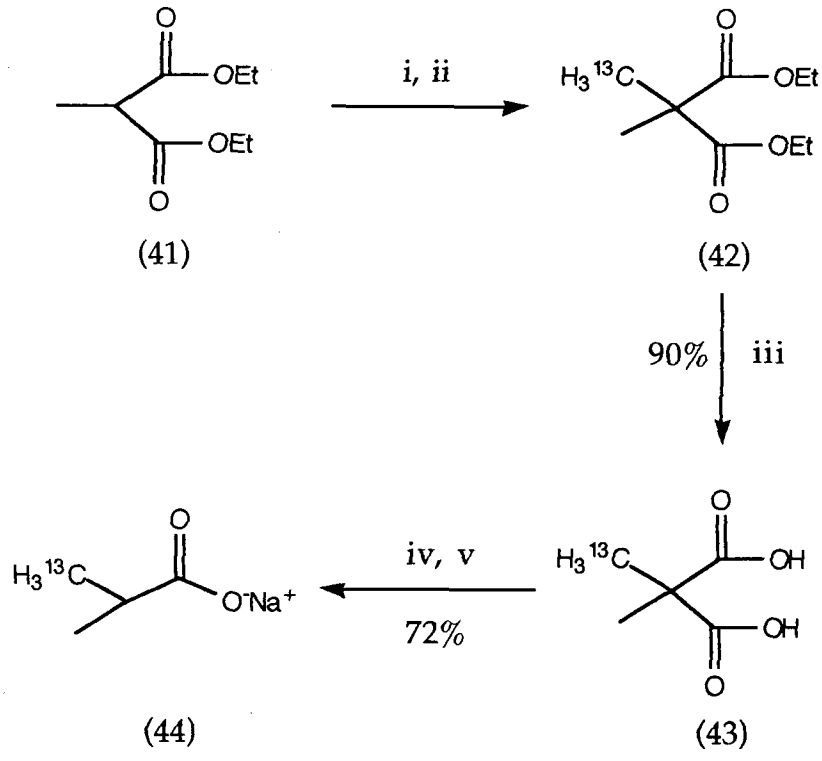
(DL)-[3-$^{2}H_2$]-β-Aminoisobutyric acid (40) was prepared in a similar manner but starting with unlabelled α-bromopropionic acid and performing a deuteration with D_2-PtO_2 in EtOH : $CHCl_3$. This generated (40) in an overall yield of 10% from α-bromopropionic acid. These materials were used directly for feeding experiments.



(40)

2.2.4 Synthesis of sodium [3-^{13}C]-isobutyrate

Sodium [3-^{13}C]-isobutyrate (44) was synthesised as outlined in scheme 7. The first step afforded (42) efficiently in 90% yield. A base mediated hydrolysis then gave [^{13}C-*methyl*]-dimethylmalonic acid (43) which after acidification and decarboxylation at 180°C generated [3-^{13}C]-isobutyric acid. This was neutralised to pH 7 to afford the sodium salt (44) in an overall yield of 69% from $^{13}CH_3I$.

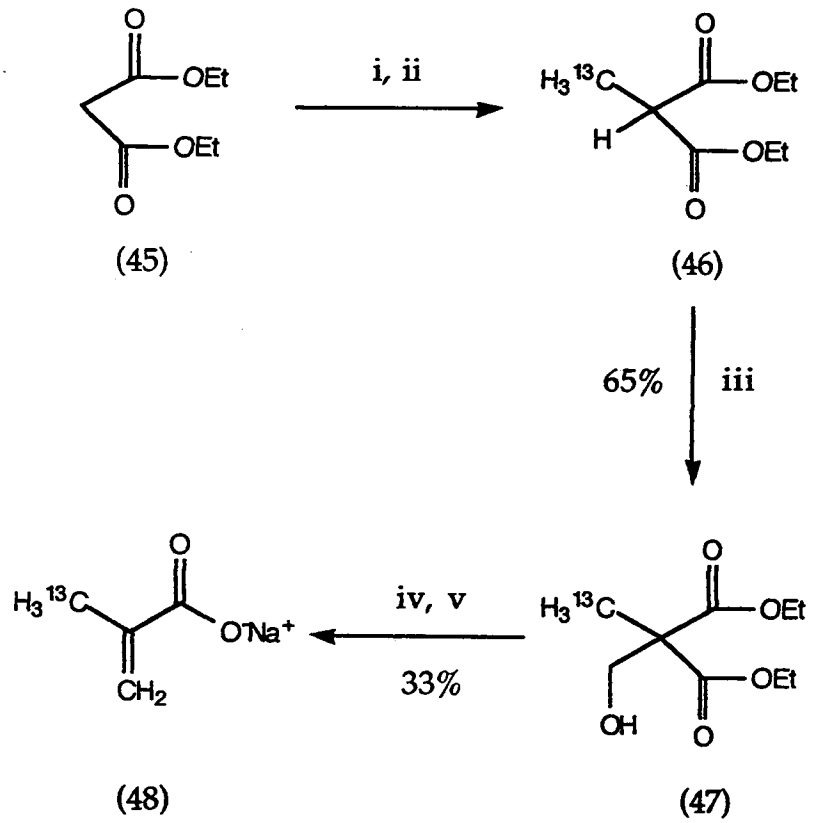


(41) (42) (43) (44)

Scheme 7. Reagents and conditions: i) NaH, THF, 0°C, ii) $^{13}CH_3I$, reflux 2h, iii) 5M KOH, reflux 24h, $HCl_{(aq)}$, iv) H_2O, 180°C 3h, $HCl_{(aq)}$, v) pH 7 with $NaOH_{(aq)}$.

2.2.5 Synthesis of sodium [^{13}C-*methyl*]-methacrylate

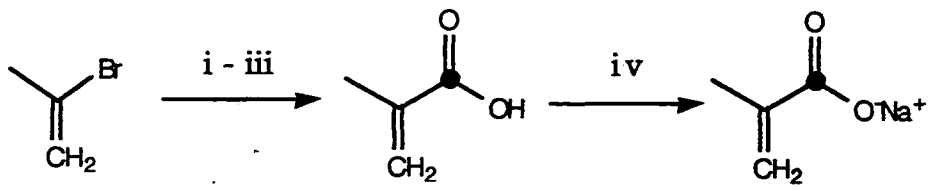
Sodium [^{13}C-*methyl*]-methacrylate (48) was synthesised as outlined in *scheme 8. Alkylation of diethyl malonate* (45) *with* $^{13}CH_3I$ *proceeded* as before (see section 2.2.2) to generate (46). This material was treated with an aqueous solution of formaldehyde and potassium bicarbonate to introduce the hydroxymethyl group. Acid hydrolysis of (47) followed by purification over silica gel afforded methacrylic acid. Neutralisation with dilute NaOH yielded the labelled compound (48) in a yield of 33% from (47). Care had to be exercised on handling methacrylic acid due to its volatility and ease of polymerisation and so the material was stored at -20°C in the dark.



(45) (46) (47) (48)

Scheme 8. Reagents and conditions: i) NaH, THF, 0°C, ii) $^{13}CH_3I$, reflux 2h, iii) $CH_2O_{(aq)}$, $KHCO_3$, 70°C 2h, r.t. 12h, iv) 7% $HCl_{(aq)}$, reflux 3d, v) pH 7 with $NaOH_{(aq)}$.

2.2.6 Synthesis of sodium [1-^{13}C]-methacrylate



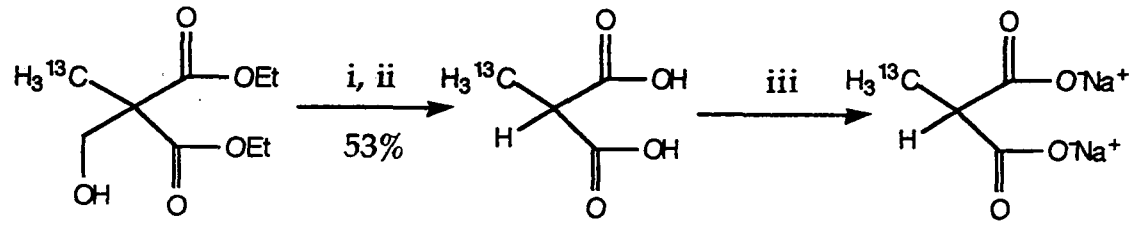
(49) (50) (51)

Scheme 9. Reagents and conditions: i) Mg, ether, r.t. 1h, reflux 1h, ii) $^{13}CO_2$, iii) pH 3 with $H_2SO_{4(aq)}$, iv) pH 7 with $NaOH_{(aq)}$

The synthesis of sodium [1-^{13}C]-methacrylate (51) (scheme 9) was executed using a Grignard reaction.[82] The Grignard reagent was generated by the cautious addition of 2-bromopropene (49) to activated magnesium turnings in ether followed by cannular transfer into a clean flask. Isotopically

enriched $^{13}CO_2$ (99%), generated *in situ* by the careful addition of $Ba^{13}CO_3$ onto H_2SO_4, was vacuum transferred into the Grignard reagent. After work up, the methacrylic acid was purified over silica gel and neutralised to pH 7 to afford sodium [1-^{13}C]-methacrylate (51) in a 22.5% yield based upon the amount of $Ba^{13}CO_3$ used.

2.2.7 Synthesis of sodium [^{13}C-*methyl*]-methylmalonate



(47) (52) (53)

Scheme 10. Reagents and conditions: i) 10M KOH, reflux 24h, ii) 0°C, $HCl_{(aq)}$, iii) pH 7 with $NaOH_{(aq)}$.

[^{13}C-*methyl*]-Methylmalonic acid (52) was synthesised by the utilisation of diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (47) (see section 2.2.5). This route was chosen over the more conventional methylation of diethyl malonate because this procedure obviates any dialkylated product, a common problem when trying to monoalkylate diethyl malonate. The presence of any [$^{13}C_2$-*methyl*]-dimethylmalonic acid is problematic and would potentially affect the feeding experiment results, because it would be decarboxylated *in vivo* to generate [$^{13}C_2$-*methyl*]-isobutyric acid. In this chosen route the hydroxymethyl group is acting as a blocking group, to prevent dimethylation, which can easily be removed again. The resultant (47) was purified over silica gel and hydrolysis under basic conditions followed by acidification, neatly generates [^{13}C-*methyl*]-methylmalonic acid (52) (free from any [$^{13}C_2$-*methyl*]-dimethylmalonic acid) in a yield of 53%. [^{13}C-*methyl*]-Methylmalonic acid (52) was neutralised to generate the sodium salt (53) prior to the feeding experiment.

2.3 Results

2.3.1 Introduction

The labelled compounds prepared above were administered to *Streptomyces cinnamonensis* and the details of incorporation into monensin A are outlined in section 2.3.2, A to H. These labelled substrates were generally administered batchwise, i.e day 3, 3.5 and 4 of a 7.5 day growth cycle, as solutions *via* a sterilised micropore filter, in order to increase the efficiency of incorporation into monensin A. It is advantageous to supplement the cultures at the stage of maximum secondary metabolite production. There are two phases of growth in batch cultures of bacteria.[83] The first is the *trophophase* during which primary metabolism is predominant and the second is the *idiophase* during which period secondary metabolism occurs. The distinction between the two phases is not always clear and there may be an overlap, therefore timing of feeding experiments is an important factor. If the substrate is fed too early in the growth cycle then it may be utilised in primary metabolism and become incorporated into the secondary metabolite *via* an indirect route resulting in excessive scrambling and low incorporation of the isotopic label. Similarly feeding the substrate too late to the cultures, when secondary metabolism has slowed down, will also give low incorporation results.

The ^{13}C n.m.r. of monensin A has previously been unambiguously assigned.[12] Table A1 (Appendix 1, pg. i) details the chemical shifts of the carbon atoms of monensin A isolated from *S. cinnamonensis*, recorded as solutions in $CDCl_3$, compared with the literature values.[47] The Incorporations of the following substrates [^{13}C-*methyl*]-β-aminoisobutyric acid, [1-^{13}C]-β-aminoisobutyric acid, sodium [^{13}C-*methyl*]-methacrylate, sodium [1-^{13}C]-methacrylate and sodium [3-^{13}C]-isobutyrate into monensin A are tabulated in Fig. 10, in order to aid direct comparison of these experiments. The values were calculated by normalising the peak heights in the ^{13}C n.m.r. spectrum relative to an unenriched carbon and then further normalised relative to a standard natural abundance spectrum. These unenriched carbons were either C10 (CH_2) or C28 (OCH_3). In Fig. 10 there also includes figures labelled with an asterisk (*) (i.e. C6 (CH), C9 (C), C17 (CH-O), C19 (CH_2)). These signals are internal references which have undergone the same calculations as the enriched carbons in order to give a further comparison of the Incorporation values of enriched to unenriched

carbons. Detailed discussion of all of these results are included in section 3, however they can be summarised as follows.

2.3.2 Feeding experiments

A

[^{14}C-*methyl*]-Thymine (29) (32.5μCi) and 14mg of unlabelled thymine (as carrier) were administered to cultures (6 x 100cm^3) of *S. cinnamonensis* before sterilisation. The solubility of thymine precluded batchwise addition by a sterilised micropore filter in the normal manner. After 7.5 days incubation, the monensins were isolated (monensin A : B, 7 : 3) with a radiochemical Incorporation (I) of 2%.

$$I = \frac{0.66\mu Ci}{32.5\mu Ci} \times 100 = 2\%$$

This experiment demonstrates that some ^{14}C isotope from [^{14}C-*methyl*]-thymine has been incorporated into the monensins, but it gave no information concerning the specific sites of enrichment. However the Incorporation was sufficient to proceed with a stable isotope study using [$^{13}C^2H_3$-*methyl*]-thymine (30) and n.m.r. analysis.

B

[^{14}C-*methyl*]-Thymine was added prior to sterilisation of the medium. This was judged to be a limitation as substrates should be fed ideally to the cultures at the point of maximum secondary metabolite production (see section 2.3.1). This limitation was also encountered with [$^{13}C^2H_3$-*methyl*]-thymine. It was overcome by keeping aside about 20cm^3 of production medium when the culture flasks were prepared. [$^{13}C^2H_3$-*methyl*]-Thymine was added to this media and the whole was sterilised and then administered on day 3 to cultures (5 x 100cm^3) *via* a sterilised glass pipette such that the final concentration was 4.2mM. Such a strategy ensured that the [$^{13}C^2H_3$-*methyl*]-thymine was integral with the medium and that there was minimum loss of substrate during the feeding procedure. Monensin A was isolated on day 7.5. A preliminary ^{13}C n.m.r. (100MHz) confirmed the presence of monensin A but for full broad - band proton and deuterium decoupling ($^{13}C\{^1H, ^2H\}$-n.m.r. (150MHz)) analysis, the sample was sent to Edinburgh University. The resulting spectra are shown in Fig. 2 and Fig. 3.

Fig. 2, $^{13}C\{^{1}H,^{2}H\}$-n.m.r. of monensin A isolated from *S. cinnamonensis* fed with [$^{13}C^{2}H_3$-*methyl*]-thymine.

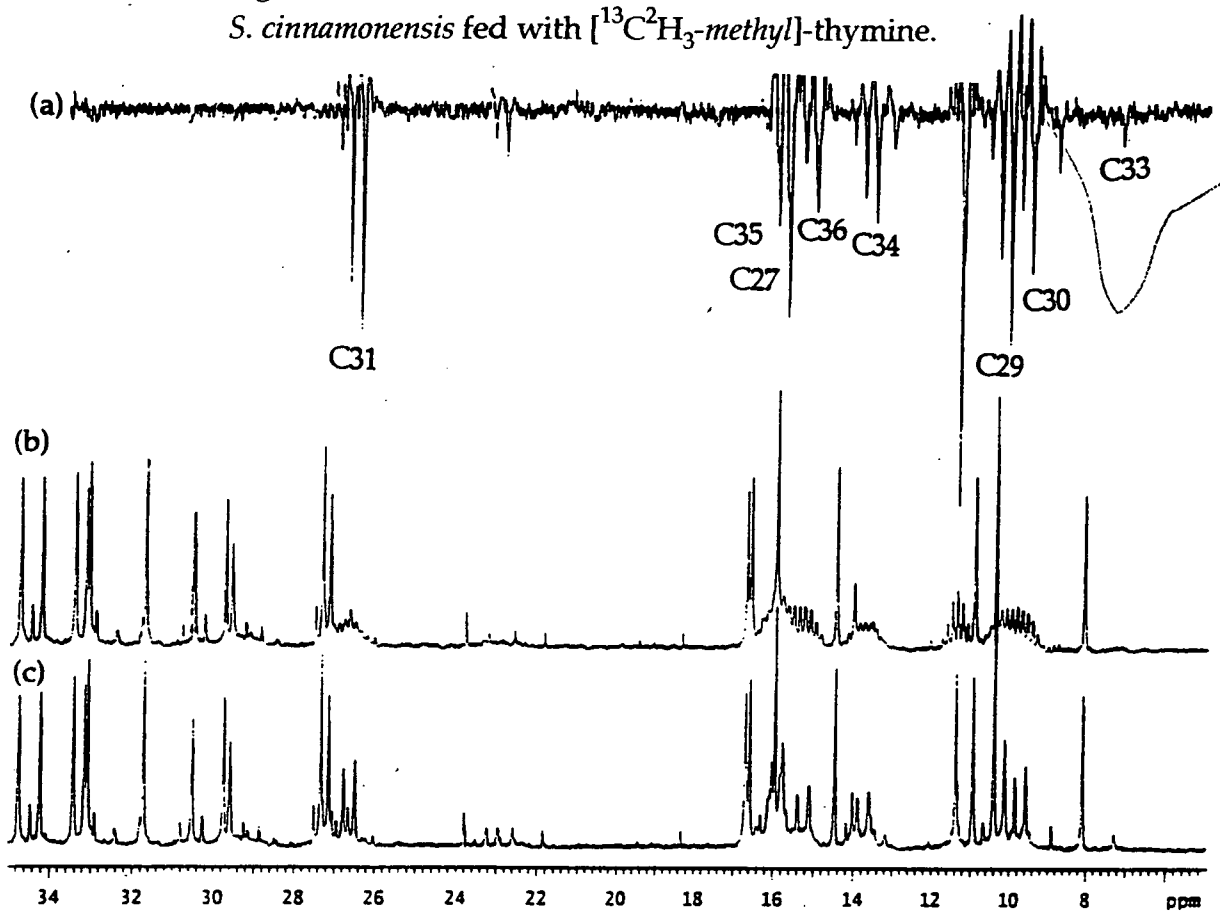
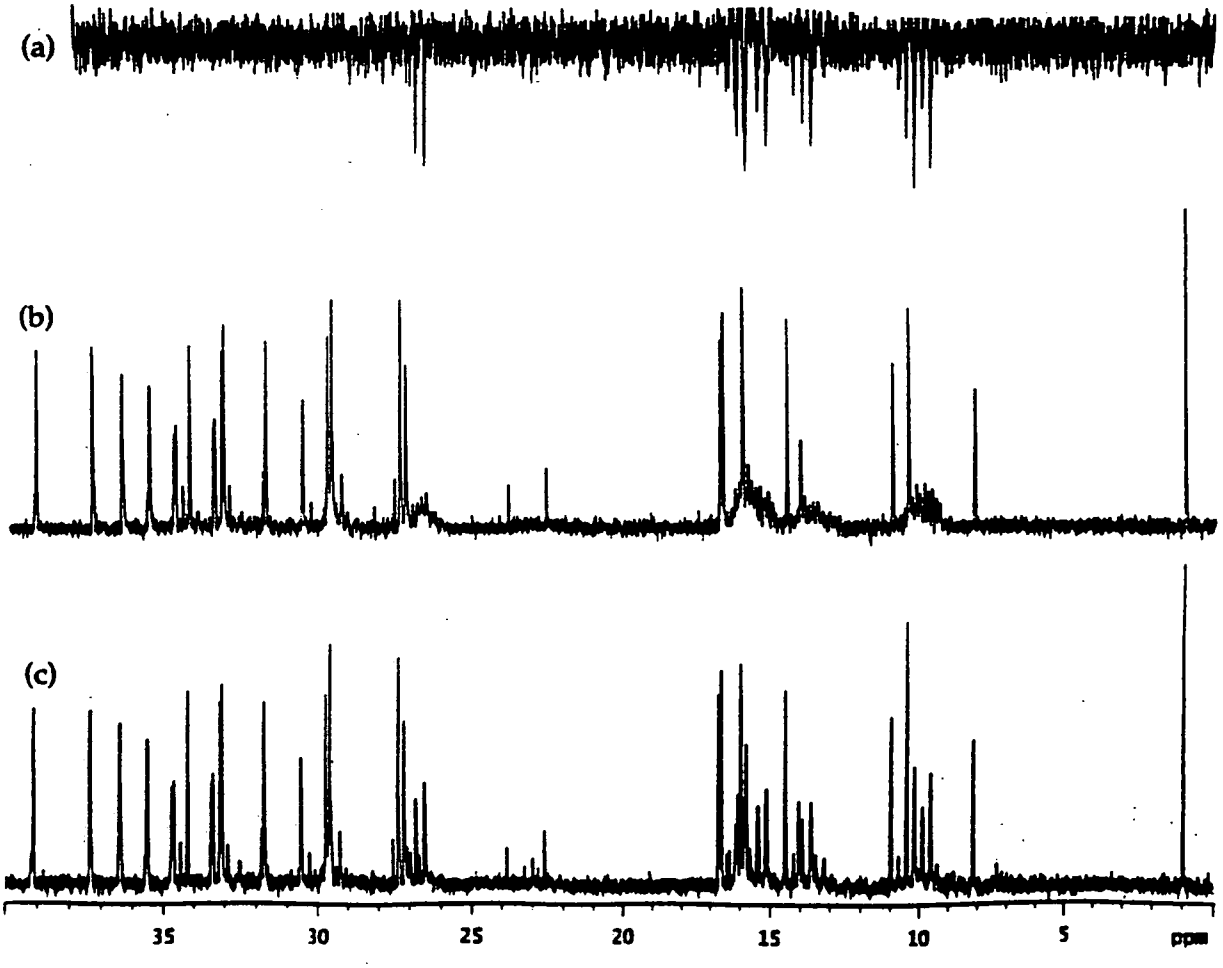
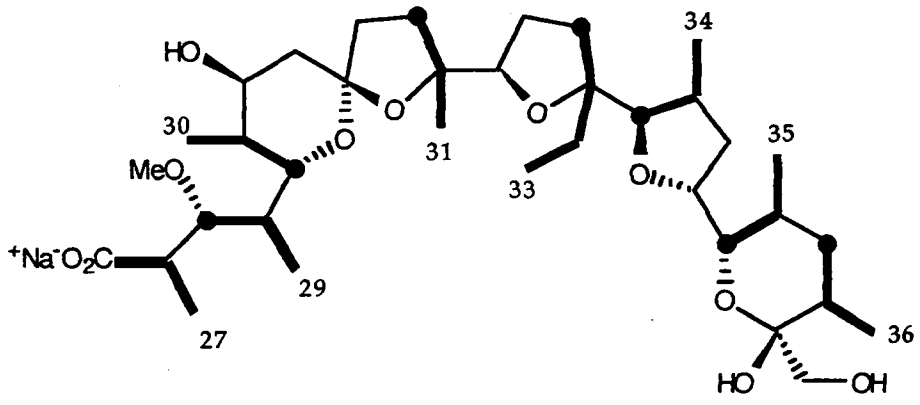


Fig. 3, $^{13}C\{^{1}H,^{2}H\}$-n.m.r. of monensin A (purified by C_{18} preparative t.l.c) isolated from *S. cinnamonensis* fed with [$^{13}C^{2}H_3$-*methyl*]-thymine.



The sample giving rise to the spectrum in Fig. 2 is rather impure and contains a labelled impurity showing at 11.4ppm. This sample was subsequently purified by C_{18} reverse phase preparative chromatography, and the $^{13}C\{^1H, ^2H\}$-n.m.r. spectrum of the purified sample is shown in Fig. 3. The Incorporation value of [$^{13}C^2H_3$-*methyl*]-thymine into monensin A is based upon Fig. 2, as this is a more concentrated sample and the spectrum is clearer. In the $^{13}C\{^1H\}$-spectrum (b), a $^{13}CD_3$- signal appears as a septet, shifted from the natural abundance signal to a lower frequency. In the $^{13}C\{^1H, ^2H\}$-spectrum (c), this broad septet collapses to a singlet, but remains shifted to a lower frequency by the same magnitude. Subtraction of these two spectra [(b) - (c)] generates the difference spectrum (a).

From the analysis of the spectra it is deduced that [$^{13}C^2H_3$-*methyl*]-thymine has been incorporated into carbon-3 of the seven propionate units of monensin A (54), i.e. C27, C29, C30, C31, C34, C35 and C36, at a level of 1 - 2 fold enrichment. For each of these carbons there exists three components, $^{13}CD_3$, $^{13}CD_2H$ and $^{13}CDH_2$, indicating that at some point there has been partial loss of deuterium on the methyl group. This can probably be accounted for by the interconversion of the methylmalonyl-CoA pool with succinyl-CoA, mediated by methylmalonyl-CoA mutase.



(54)

An Incorporation (0.2 fold) into carbon-4 of the butyrate unit (C33) is also evident in the $^{13}C\{^1H, ^2H\}$-n.m.r. spectrum (Fig.2 (c)) in the form of a small signal shifted to a lower frequency, by a magnitude of 0.84ppm from the natural abundance signal. The magnitude of one deuterium induced α-shift (^{13}C-D) is 0.28ppm, i.e. this shifted signal corresponds to a three deuterium induced shift, a $^{13}CD_3$ component. Interestingly there is no

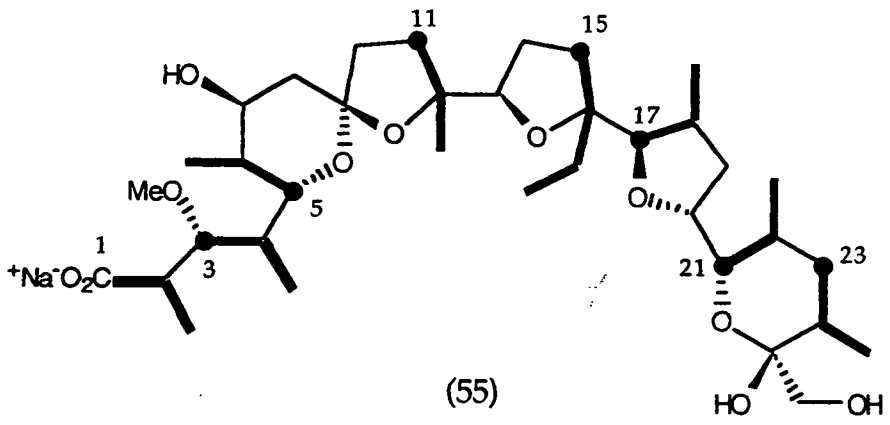
apparent $^{13}CD_2H$ or $^{13}CDH_2$ component indicating limited exchange of deuterium at this site (discussed further in section 3.3).

C

[^{13}C-*methyl*]-β-Aminoisobutyric acid hydrochloride (35) was neutralised to pH 7 with dilute sodium hydroxide solution and administered in three batches (day 3, 3.5 and 4) to cultures (6 x 100cm^3) of *S. cinnamonensis*, such that the final concentration of [^{13}C-*methyl*]-β-aminoisobutyric acid was 5.9mM. Monensin A was isolated on day 7.5 and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 4. A high level of enrichment (6 - 7 fold) at the sites corresponding to carbon-3 of the seven propionate units of monensin A (54), i.e. C27, C29, C30, C31, C34, C35 and C36, is clearly evident. There is a small but noticable enrichment (1.5 fold) at C33, carbon-4 of the butyrate unit. Therefore the ratio of Incorporation of [^{13}C-*methyl*]-β-aminoisobutyric acid into the propionate and butyrate derived units of monensin A is approximately 4 : 1 (propionate : butyrate).

D

[1-^{13}C]-β-Aminoisobutyric acid (39) was administered to cultures (2 x 100cm^3) of *S. cinnamonensis*, such that the final concentration of (39) was 2.6mM. Monensin A was isolated on day 7.5 and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 5. From this spectrum it is clearly evident that C15, derived from carbon-1 of the butyrate unit of monensin A (55), is enriched 2.3 fold, compared with natural abundance.



(55)

The sites corresponding to carbon-1 of the propionate units are not observably enriched. This experiment contrasts directly with the

Fig. 4, ^{13}C n.m.r. (125MHz) of monensin A isolated from *S. cinnamonensis* fed with [^{13}C-*methyl*]-β-aminoisobutyric acid

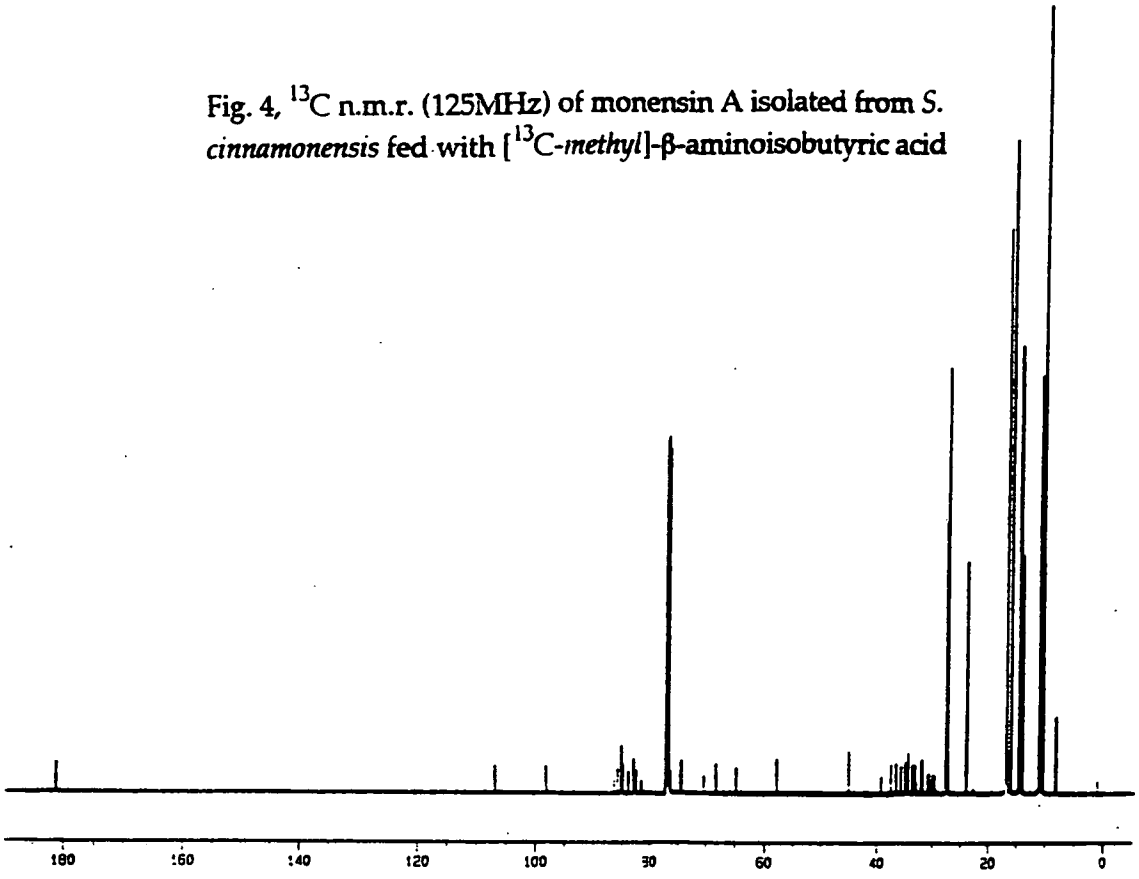
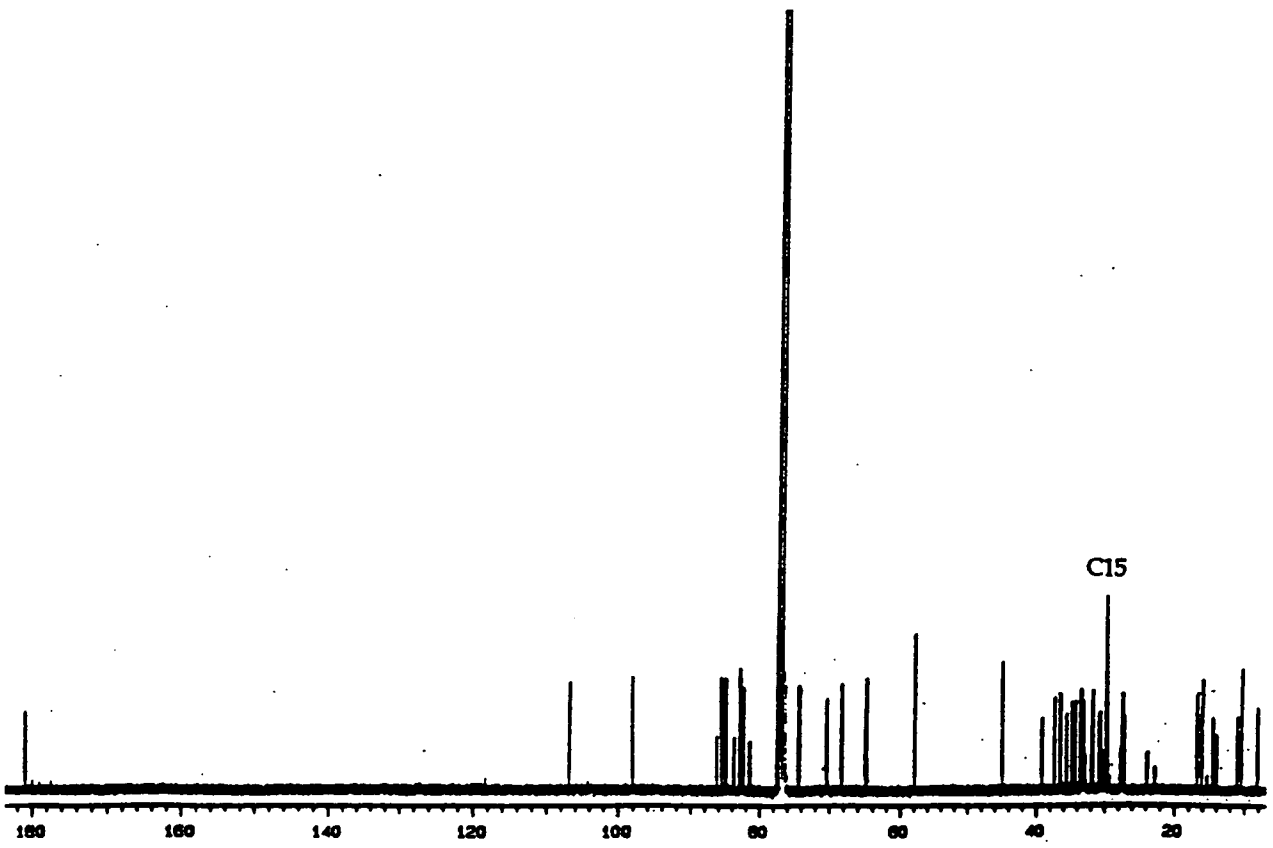


Fig. 5, ^{13}C n.m.r. (100MHz) of monensin A isolated from *S. cinnamonensis* fed with [1-^{13}C]-β-aminoisobutyric acid



[^{13}C-*methyl*]-β-aminoisobutyric acid feeding experiment, in that the same precursor is studied but the label is in a different position. In that experiment the sites corresponding to carbon-3 of the propionate units were enriched 6 - 7 fold. The implications of this are discussed in context with the other feeding experiments in section 3.2.

E

Sodium [^{13}C-*methyl*]-methacrylate (48) was administered to cultures (6 x 100cm^3) of *S. cinnamonensis*, such that the final concentration of (48) was 5.3mM. Monensin A was isolated on day 7.5 and the ^{13}C n.m.r. spectrum is shown in Fig. 6. Carbon-4 of the butyrate unit, C33, is enriched by 4.3 fold. Evidence of Incorporations into those carbons derived from carbon-3 of the propionate units, however, is not so clear. The values calculated for these carbons, i.e. C27, C29, C30, C31, C34, C35 and C36, are only slightly higher than the unenriched carbons and at best the calculated enrichment averages 1.5 fold. Accepting this value, the ratio of Incorporation of sodium [^{13}C-*methyl*]-methacrylate into the propionate and butyrate derived units of monensin A is approximately 1: 3 (propionate : butyrate).

F

Sodium [1-^{13}C]-methacrylate (51) was administered to cultures (6 x 100cm^3) of *S. cinnamonensis*, such that the final concentration was 5.2mM. Monensin A was isolated on day 7.5 and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 7. The site derived from carbon-1 of the butyrate unit, C15, is clearly enriched by a magnitude of 4.3 fold. This enrichment is identical to that at C33 (carbon-4 of the butyrate unit) after the sodium [^{13}C-*methyl*]-methacrylate feeding experiment. Similarly, carbons corresponding to carbon-1 of the propionate units of monensin A (55), i.e. C1, C3, C5, C11, C17, C21 and C23, seem to be very slightly enriched. Again the ratio (1:3) of Incorporation into the propionate and butyrate derived units of monensin A is the same as for the sodium [^{13}C-*methyl*]-methacrylate feeding experiment.

G

Sodium [3-^{13}C]-isobutyrate (44) was administered to cultures (6 x 100cm^3) of *S. cinnamonensis* to a final concentration of 5.6mM. Monensin A was isolated on day 7.5 and the ^{13}C n.m.r. spectrum is shown in Fig. 8.

Fig. 6, ^{13}C n.m.r. (125MHz) of monensin A isolated from *S. cinnamonensis* fed with sodium [^{13}C-*methyl*]-methacrylate

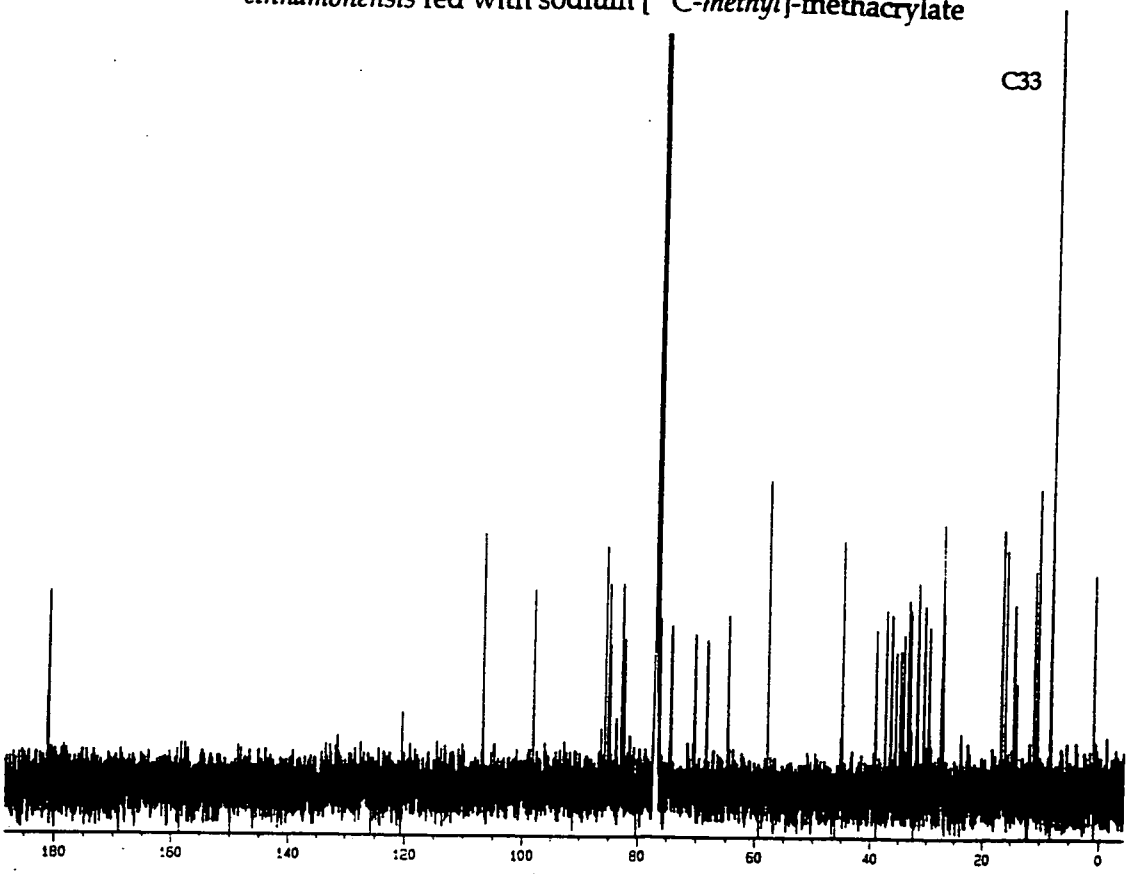
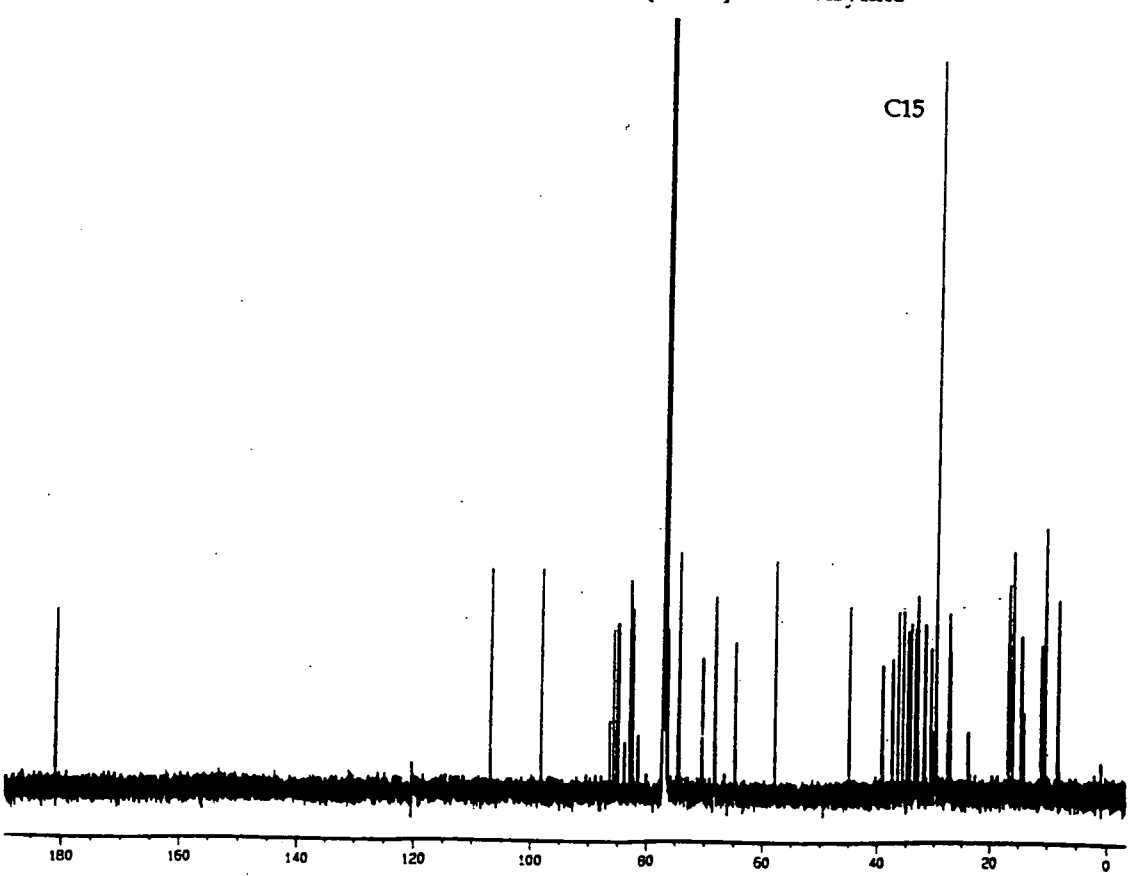
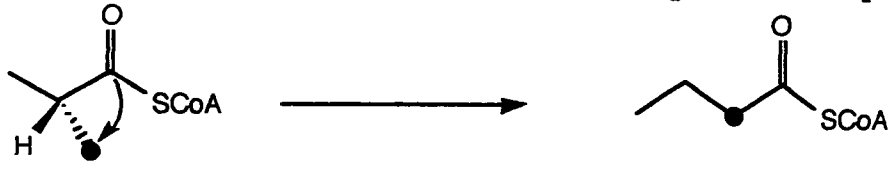


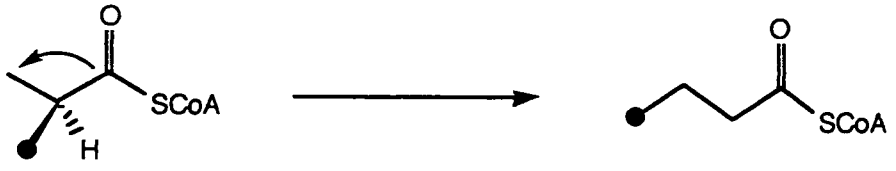
Fig. 7, ^{13}C n.m.r. (125MHz) of monensin A isolated from *S. cinnamonensis* fed with sodium [1-^{13}C]-methacrylate



An enzyme activity has been identified from *S. cinnamonensis* which mediates the stereospecific interconversion of isobutyryl-CoA and butyryl-CoA. This activity has been termed isobutyryl-CoA mutase.[66] The rearrangement involves migration of the carboxyl carbon of isobutyryl-CoA to the *pro*-S methyl group. The resultant butyryl-CoA then becomes incorporated into the butyrate unit of monensin A, presumably *via* activation to ethylmalonyl-CoA. Sodium [3-^{13}C]-isobutyrate was prepared in racemic form, i.e. half of the molecules had the *pro*-S methyl group labelled with ^{13}C (56) and half had the *pro*-R methyl group labelled (57), such that each is processed to butyrate labelling the C2 and C4 positions, respectively.



(56)



(57)

As expected the carbons derived from carbon-2 (C16) and carbon-4 (C33) of the butyrate unit are enriched by a magnitude of 9 fold, by ^{13}C n.m.r. analysis of monensin A (Fig. 8). Also the carbons derived from carbon-3 of the propionate units of monensin A (54) are enriched 2.5 fold. Therefore the ratio of propionate to butyrate Incorporation of sodium [3-^{13}C]-isobutyrate into monensin A is 1 : 3.5 (propionate : butyrate) which mirrors the ratio of Incorporation of the isotopically labelled methacrylate substrates into monensin A.

H

Sodium [^{13}C-*methyl*]-methylmalonate (53) was administered to cultures (4 x 100cm^3) of *S. cinnamonensis* to a final concentration of 4.6mM. Monensin A was isolated on day 7.5 and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 9. Very little information can be obtained from this experiment as the labelled methylmalonate (53) has been highly metabolised, presumably, *via*

Fig. 8, ^{13}C n.m.r. (125MHz) of monensin A isolated from *S. cinnamonensis* fed with sodium [3-^{13}C]-isobutyrate

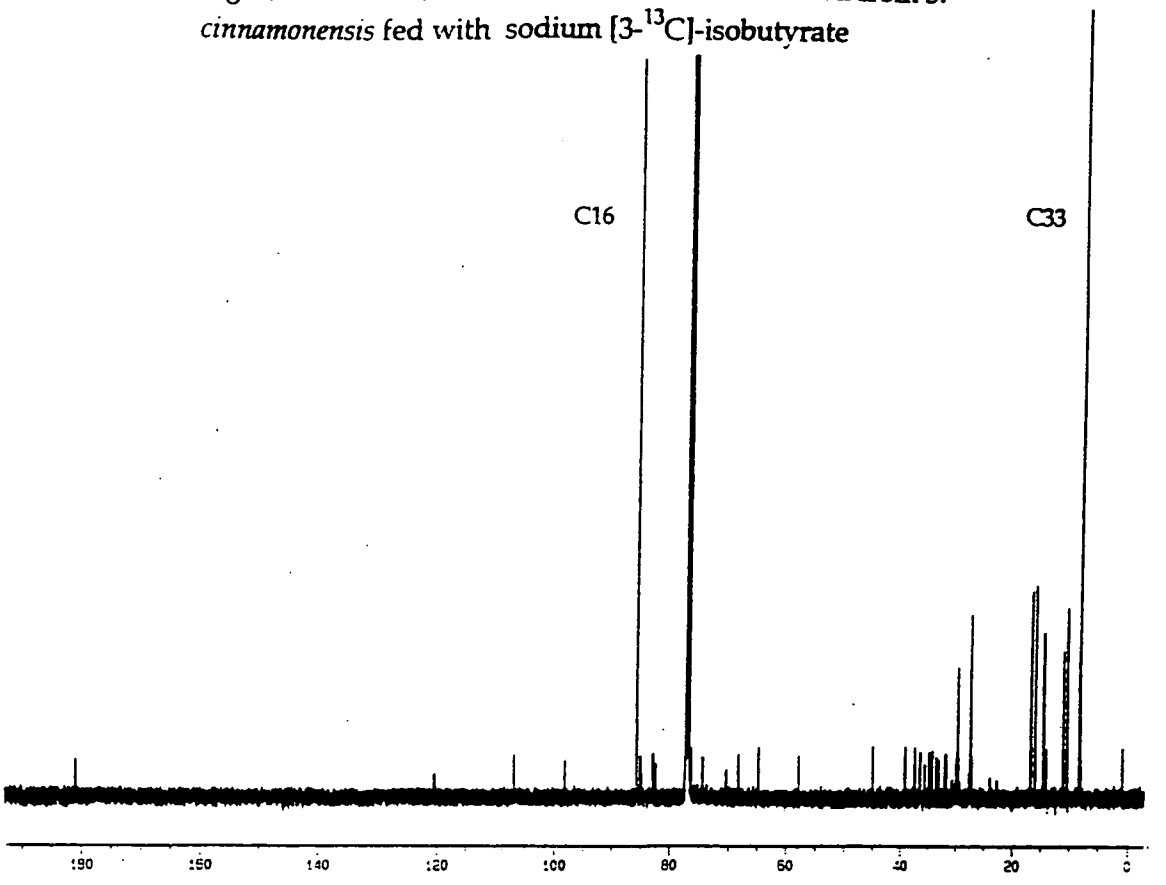
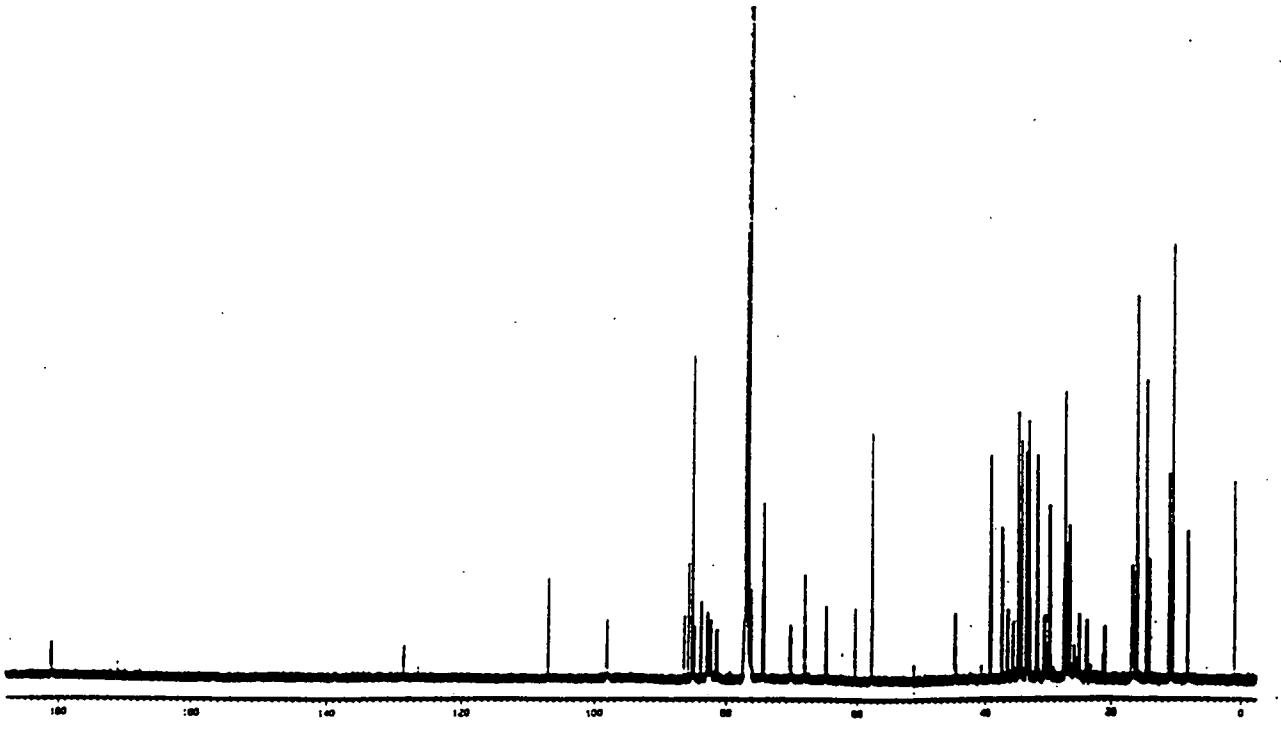


Fig. 9, ^{13}C n.m.r. (125MHz) of monensin A isolated from *S. cinnamonensis* fed with sodium [^{13}C-*methyl*]-malonate



succinyl-CoA and the citric acid cycle causing scrambling of the isotopic label and incorporation into monensin A in an indiscriminate manner. Also the signals in the ^{13}C n.m.r. spectrum are unlike a 'normal' natural abundance spectrum. Some are distinctively smaller than expected and broader and this makes it harder to assess accurately, if and where ^{13}C label has been incorporated. To gain more information in the future on the possible Incorporation of methylmalonate into monensin A, a double labelling experiment is advisable and then the Incorporation of intact units can be identified unambiguously.

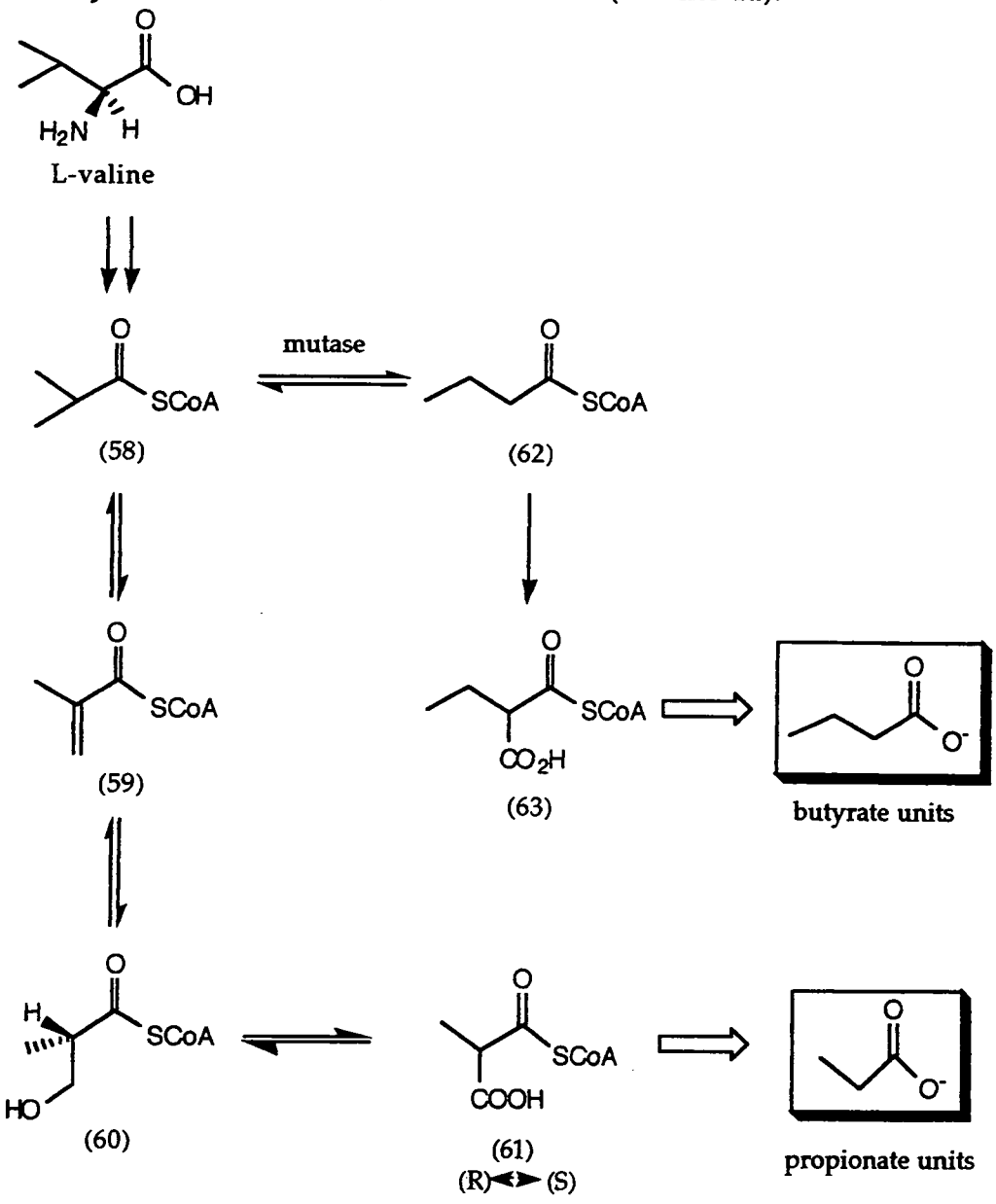
	^{13}Cnmr/ ppm	[^{13}C-*methyl*]-β-AIBA (a, c)	[1-^{13}C]-β-AIBA (a, d)	[^{13}C-*methyl*]-methacr. (b, c)	[1-^{13}C]-methac-rylate (a, c)	[3-^{13}C]-IBA (a, c)
C1	181.26	-	0.94	-	1.14	-
C3	82.93	-	0.99	-	0.93	-
C5	68.32	-	1.04	-	1.02	-
C6	34.83	0.93*	0.87*	0.91*	0.88*	0.70*
C9	106.94	0.71*	0.87*	1.20*	0.93*	0.54*
C11	33.16	-	1.08	-	1.26	-
C15	29.81	-	2.33	-	4.3	-
C16	85.79	-	-	-	-	9.09
C17	84.97	0.84*	1.03	0.9*	0.88	0.38*
C19	33.24	1.07*	1.03*	1.32*	1.11*	0.49*
C21	74.46	-	0.96	-	1.16	-
C23	35.60	-	1.10	-	1.35	-
C27	16.69	6.55	-	1.4	-	2.31
C29	11.07	7.11	-	1.74	-	2.67
C30	10.50	7	-	1.24	-	2.41
C31	27.44	5.58	-	1.57	-	2.66
C33	8.23	1.58	-	4.36	-	9.86
C34	14.57	7.08	-	1.43	-	2.78
C35	16.82	6.14	-	1.21	-	2.45
C36	16.07	6.85	-	1.19	-	2.71

Fig. 10: ^{13}C enrichments of the carbons of monensin A,
a normalised relative to C10 = 1, b normalised relative to C28 = 1,
c 125MHz, d 100MHz, * unenriched C as standard.

3. DISCUSSION OF RESULTS

3.1 Sodium [3-^{13}C]-isobutyrate, sodium [^{13}C-*methyl*]-methacrylate and sodium [1-^{13}C]-methacrylate feeding experiments

The purpose of these experiments was to investigate the link between primary and secondary metabolism in *Streptomyces*, utilising the monensin A producing strain *Streptomyces cinnamonensis* (ATCC 15413). One of the best established metabolic routes linking primary and secondary metabolism in such systems is the catabolism of L-valine (scheme 11).[84]



Scheme 11

The degradative pathway of valine metabolism has been studied extensively both in bacteria and in mammals and where relevant the stereochemical course has been defined. It was shown originally in *Pseudomonas putida*, that isobutyryl-CoA (58) undergoes α,β-dehydrogenation with loss of H from the 2-*pro*-S-methyl group, followed by hydration by hydrogen addition to the re- face of the α-C of methacrylate (59) to produce (S)-β-hydoxyisobutyryl-CoA (60).[85] The intermediacy of methacrylyl-CoA has only been implied because the feeding experiments are consistent with the existence of such an unsaturated intermediate.[85] The stereochemical course of isobutyrate dehydrogenation / hydration is the same in mammals[68] as in bacteria.

L-Valine was soon recognised as a source of not only the propionate units in secondary metabolism but also the butyrate units.[45,86] The existence of a reversible rearrangement, in whole cells of *S. cinnamonensis*, of isobutyryl-CoA to butyryl-CoA was demonstrated in 1988.[66] This rearrangement is catalysed by a coenzyme-B_{12}-dependent mutase and involves the intramolecular migration of the carboxyl carbon of isobutyrate to the 2-*pro*-S methyl, with a concomitant back migration of a H atom from the methyl group. The discovery of this rearrangement revealed an important link between straight chain and branched chain fatty acid metabolism in these antibiotic producing organisms. The incorporation of sodium isobutyrate into monensin A was first demonstrated by Vanek and co-workers[45] who showed that monensin A was efficiently and similarly enriched by either [1-^{13}C]-butyrate or [1-^{13}C]-isobutyrate. This was a neccessary reference experiment, in our case, for comparison with the other feeding experiments. As expected, sodium [3-^{13}C]-isobutyrate efficiently enriched C16 and C33 of monensin A.

As discussed, the intermediacy of methacrylyl-CoA in valine catabolism has only been implied. Therefore it was deemed appropriate to carry out the definitive experiment and feed, in the first instance, sodium [^{13}C-*methyl*]-methacrylate to *S. cinnamonensis*. This proved to be a good substrate and resulted in efficient incorporation into monensin A at C33 (carbon-4 of the butyrate unit) by a magnitude of 4.3 fold. This can be rationalised by following valine catabolism as shown in scheme 11. The [^{13}C-*methyl*]-methacrylate will afford isobutyryl-CoA (58) after a stereospecific reduction resulting in ^{13}C label exclusively on the *pro*-R methyl group (57). Stereospecific migration, mediated by isobutyryl-CoA

mutase, of the carboxyl group to the *pro*-S methyl group of isobutyryl-CoA would generate butyryl-CoA now labelled exclusively at the carbon-4 position. If methacrylyl-CoA is a true intermediate between isobutyryl-CoA and (2S)-methylmalonyl-CoA, sodium [^{13}C-*methyl*]-methacrylate should be incorporated into the seven sites derived from carbon-3 of propionate. As discussed these Incorporations were low (1.5 fold) but significant.

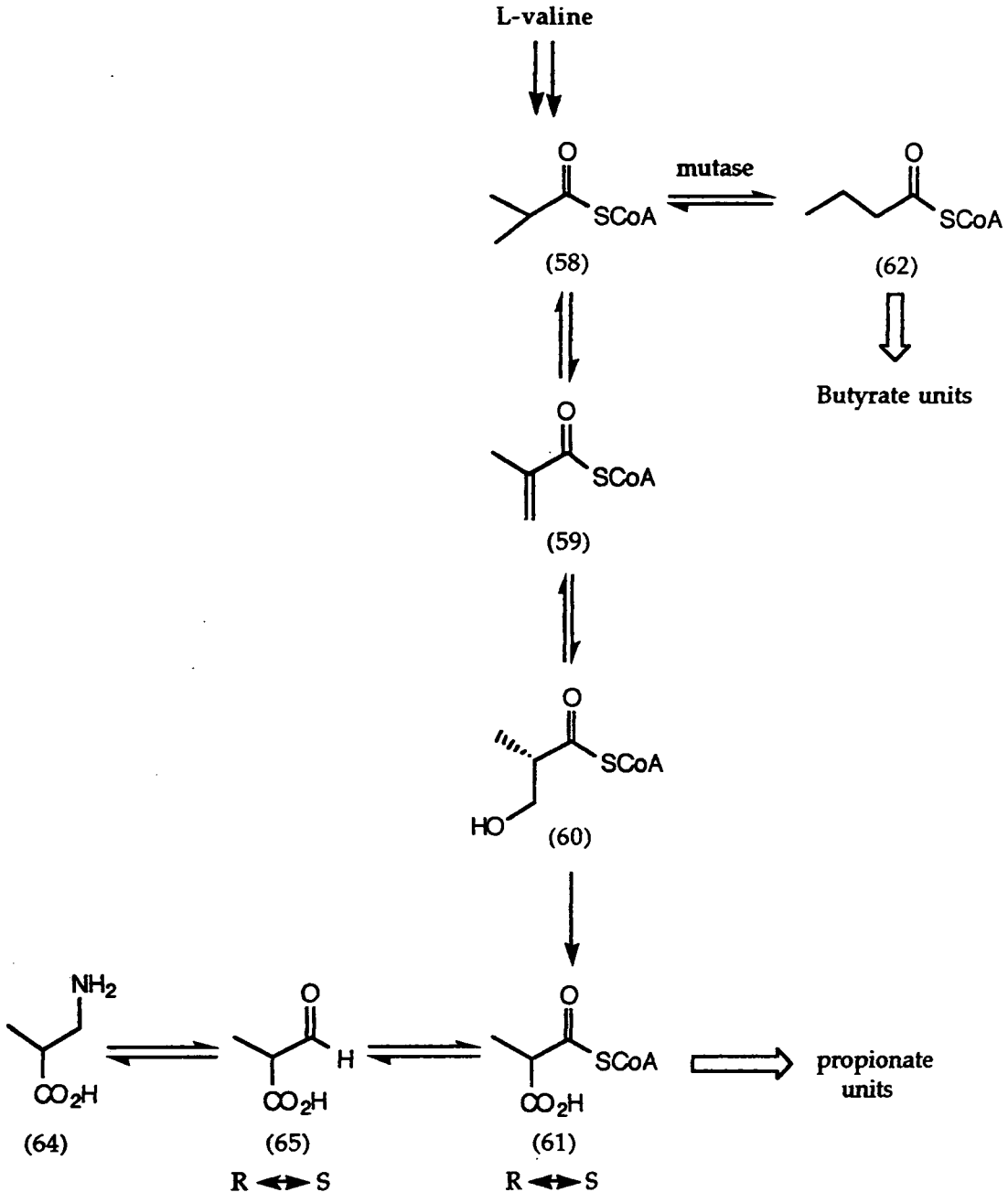
To support the hypothesis for the intermediacy of methacrylyl-CoA in this pathway, the Incorporation of sodium [1-^{13}C]-methacrylate into monensin A was studied. Incorporation of the substrate into C15, carbon-1 of the butyrate derived unit of monensin A, was evident as a 4.3 fold enrichment by ^{13}C n.m.r. Significantly this is exactly the same level of enrichment into the butyrate unit as was recorded in the sodium [^{13}C-*methyl*]-methacrylate feeding experiment. Similarly, the carbons derived from carbon-1 of the propionate units are enriched slightly (1.2 fold). Therefore the [1-^{13}C]-methacrylate and the [^{13}C-*methyl*]-methacrylate feeding experiments complement each other.

These results give an insight into the relative flux of the conversion of methacrylyl-CoA to isobutyryl-CoA and methylmalonyl-CoA. It would appear that methacrylyl-CoA (and isobutyryl-CoA) furnishes the butyrate pathway to a greater extent than the propionate pathway (an approximate ratio of 3 : 1 respectively).The potential reversibility of these steps in *Streptomyces* could be further investigated by feeding experiments utilising isotopically labelled (S)-β-hydroxyisobutyrate.

3.2 (DL)-[^{13}C-*methyl*]-β-Aminoisobutyric acid, (DL)-[1-^{13}C]-β-aminoisobutyric acid and (DL)-[3-$^{2}H_2$]-β-aminoisobutyric acid feeding experiments.

A further objective was to investigate the potential of thymine and it's degradation product, β-aminoisobutyric acid, to act as a source of propionate units *via* (2S)-methylmalonyl-CoA and hence providing evidence of the reductive catabolism of thymine occurring in *Streptomyces*, similar to that known to occur in mammals (scheme 3).[67,68,87]

The first experiment involved feeding (DL)-[^{13}C-*methyl*]-β-aminoisobutyric acid to *S. cinnamonensis*. The results were very interesting as the sites derived from carbon-3 of the propionate units were enriched by a magnitude of 6 - 7 fold and the site derived from carbon-4 of the butyrate unit was also enriched 1.5 fold. So β-aminoisobutyric acid is readily incorporated into the propionate units.



Scheme 12

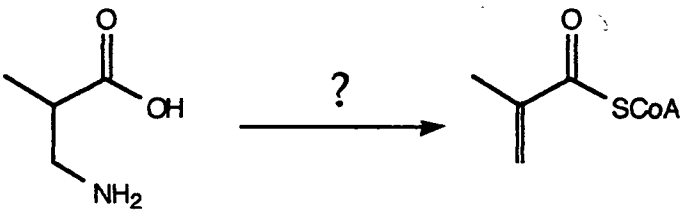
This can be explained by the transamination of β-aminoisobutyric acid (64) to methylmalonic acid semialdehyde (65) as an initial event. The stereochemistry of the process has not been established in bacteria, but in mammals (S)-β-aminoisobutyric acid is converted to (S)-methylmalonic acid semialdehyde, mediated by S-β-aminoisobutyrate-α-ketoglutarate transaminase.[88] Methylmalonic acid semialdehyde would then be converted to methylmalonyl-CoA (61), by an aldehyde dehydrogenase

activity, which would in turn furnish the propionate units of the antibiotic. Scheme 12 highlights this link between valine catabolism and β-aminoisobutyric acid. It is of interest to note that it is (S)-β-aminoisobutyric acid which is an end product of valine metabolism in mammals,[89,90] but (R)-β-aminoisobutyric acid which is the end product of thymine catabolism. So further work is required to establish if it is the (R)- enantiomer of β-aminoisobutyric acid which is produced by the thymine degradation in *Streptomyces*, as in mammals, and to establish if the enantiomers of β-aminoisobutyric acid are processed differently.

The carbon signal corresponding to carbon-4 of the butyrate unit (C33) of monensin A, was also enhanced after feeding of [^{13}C-*methyl*]-β-aminoisobutyric acid, by a magnitude of 1.5 fold. This can be explained conventionally by going through the following intermediates in scheme 12:-

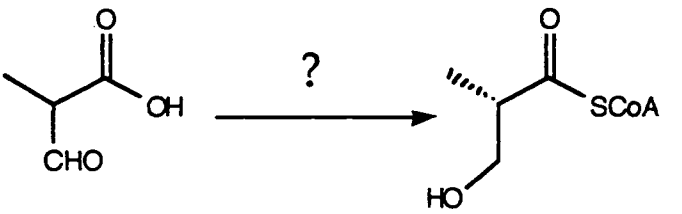
(65) ⟶ (61) ⟶ (60) ⟶ (59) ⟶ (58) ⟶ (62) ⟶ butyrate

However this is a long reductive metabolic route, and perhaps there exists a short cut which bypasses the intermediacy of methylmalonyl-CoA (61). In particular we have been testing the hypothesis of a deaminase enzyme which could act on β-aminoisobutyric acid (64) to afford, after activation, methacrylyl-CoA (59). Alternatively there could be an activity mediating the reduction of methylmalonic acid semialdehyde (65) to β-hydroxyisobutyryl-CoA (60).



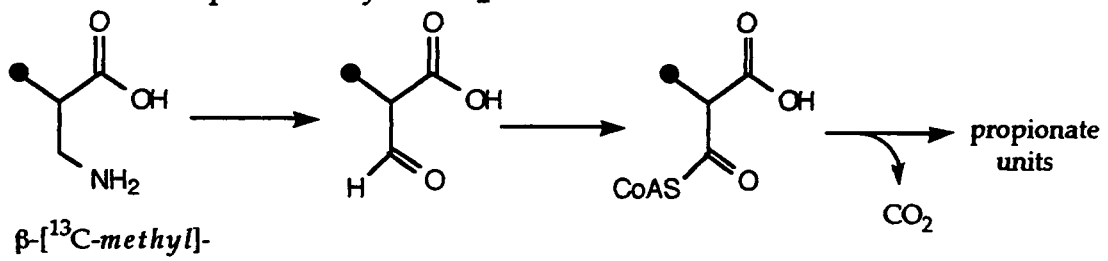
(64) (59)

or



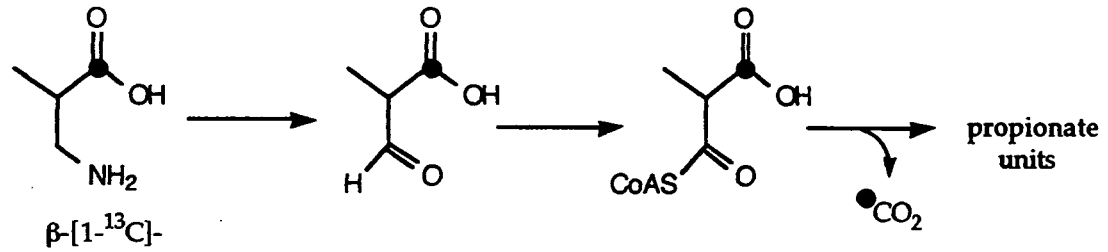
(65) (60)

To assess further these alternatives, β-aminoisobutyric acid was prepared with the isotopic label in a different position, i.e. [1-^{13}C]-β-aminoisobutyric acid. The results of this experiment show an Incorporation into C15 (carbon-1 of the butyrate unit of monensin A) by a magnitude of 2.3 fold, but no observable Incorporation into the propionate units of monensin A. In the first experiment, [^{13}C-*methyl*]-β-aminoisobutyric acid was readily incorporated into monensin A and the ^{13}C label was evident in those carbons derived from carbon-3 of the propionate units (scheme 13), whereas in this [1-^{13}C]-β-aminoisobutyric acid experiment (scheme 14) the ^{13}C label is lost presumably as CO_2.



β-[^{13}C-*methyl*]-aminoisobutyric acid

Scheme 13

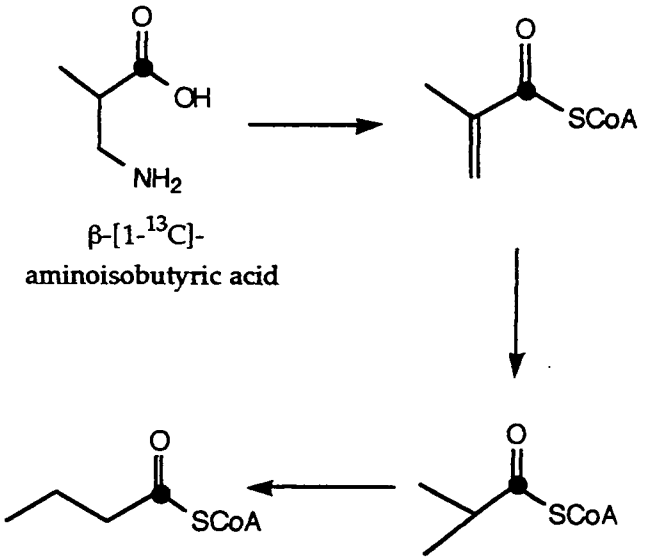


β-[1-^{13}C]-aminoisobutyric acid

Scheme 14

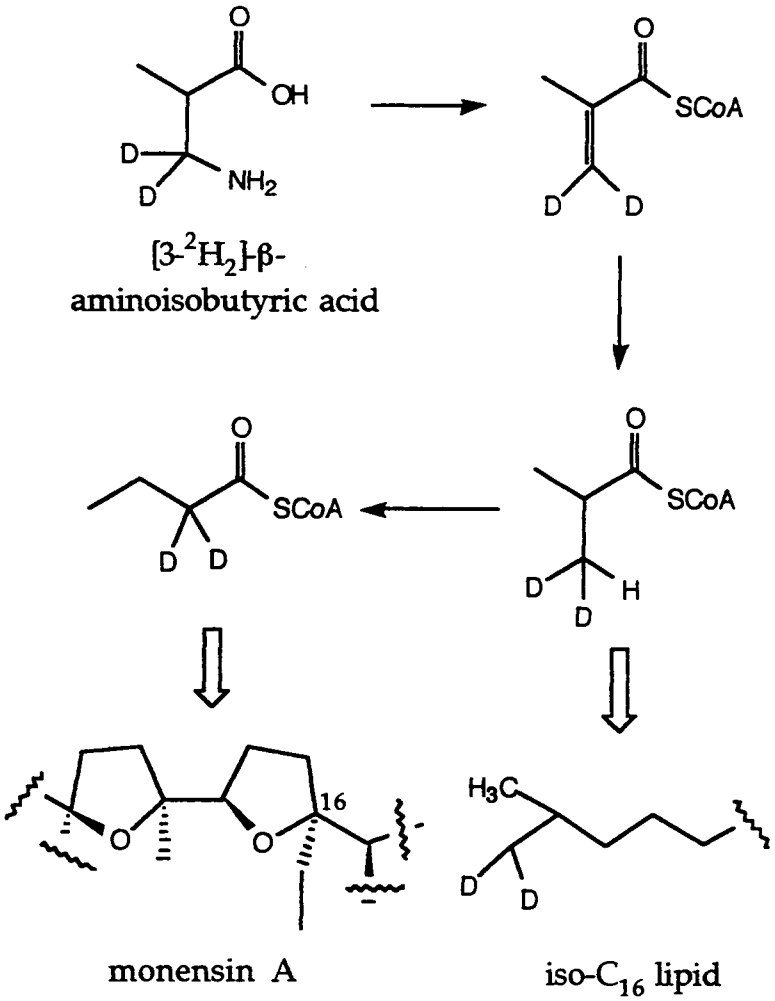
So the isotope from [1-^{13}C]-β-aminoisobutyric acid is not incorporated into the propionate units as it is lost due to decarboxylation of (2S)-methylmalonyl-CoA during polyketide biosynthesis (scheme 14). If this isotopically labelled methylmalonyl-CoA (labelled at the carboxylate carbon, scheme 14), generated from feeding [1-^{13}C]-β-aminoisobutyric acid, proceeds through the intermediates (S)-β-hydroxyisobutyryl-CoA, methacrylyl-CoA and isobutyryl-CoA to furnish the butyrate unit (outlined in scheme 12) then one may expect to see an enrichment of C16 (corresponding to carbon-2 of the butyrate unit) in the ^{13}C n.m.r. spectrum of monensin A. On close analysis there is no enrichment of C16, but there is an enrichment of C15 (carbon-1 of the butyrate unit) by a magnitude of 2.3 fold. This Incorporation

into carbon-1 of the butyrate unit of monensin A is consistent with the hypothesis of a deaminase enzyme mediating the conversion between β-aminoisobutyric acid and methacrylyl-CoA (scheme 15), i.e. no involvement of methylmalonyl-CoA.



Scheme 15

In an attempt to investigate the existence of a deaminase enzyme further, a different approach was taken. Incorporation of deuterium from (DL)-[3-$^2H_2$]-β-aminoisobutyric acid was analysed in the lipids rather than monensin A. The carbon carrying these deuteriums becomes quaternary in monensin A and so all the deuterium are of course lost. However, the incorporation of deuterium could be investigated by analysing the lipids produced by *S. cinnamonensis*. The mutase rearrangement of isobutyryl-CoA to butyryl-CoA is an important link between straight chain and branched chain fatty acid metabolism in *S. cinnamonensis* and one of the major lipids produced by this strain is *iso*-C_{16} fatty acid which has an isobutyryl-CoA starter unit. Therefore analysis of the lipids produced by *S. cinnamonensis* after administration of [3-$^2H_2$]-β-aminoisobutyric acid would hopefully reveal incorporation of deuterium in the *pro*-S methyl group of the starter unit of the branched chain lipid (scheme 16).



Scheme 16

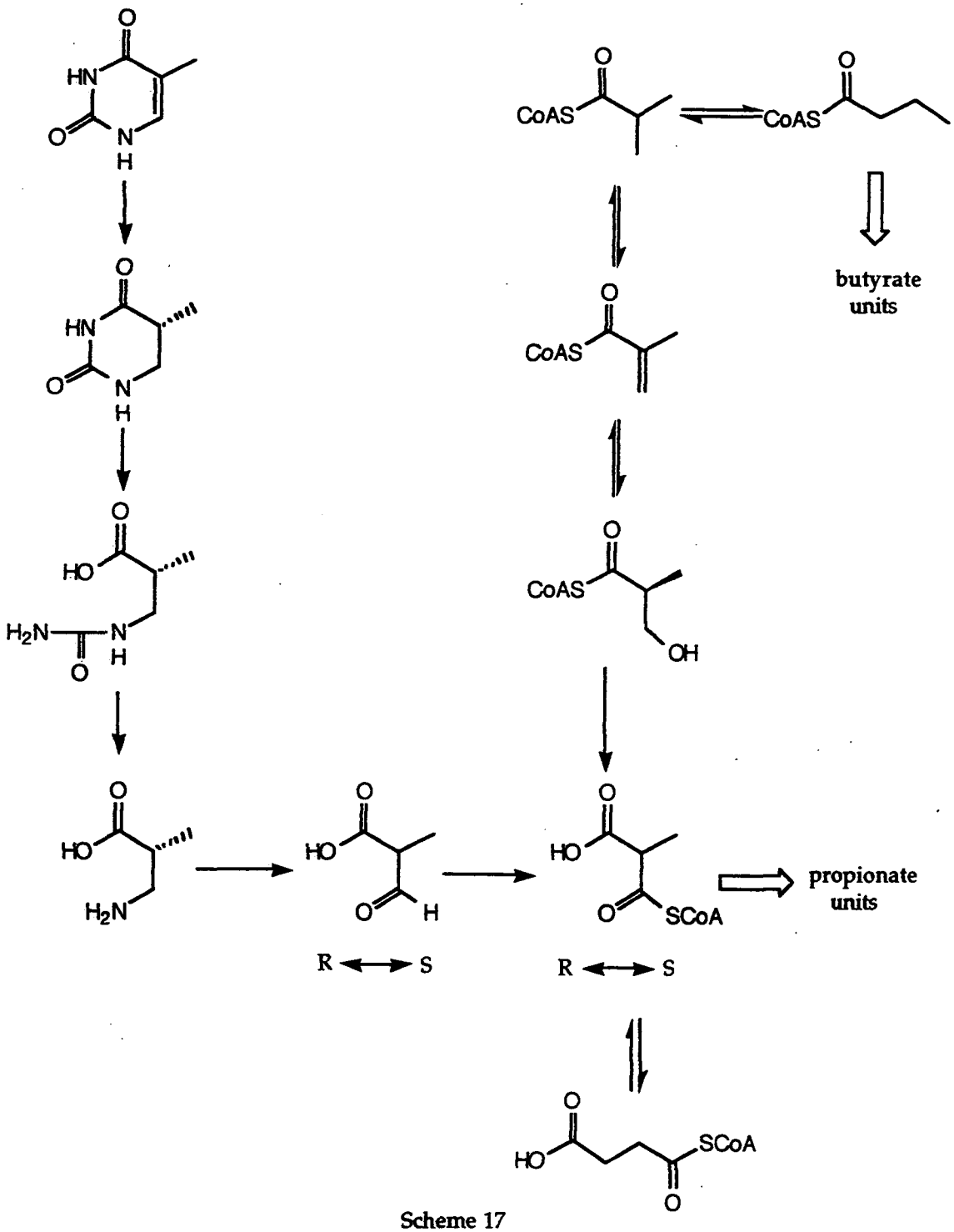
For this experiment a different *S. cinnamonensis* production media had to be employed as the media used to date contained lipids which interfered with the analysis procedure. A sample of cells was sent to Dr. K. Reynolds for lipid analysis. This procedure involved base mediated hydrolysis of the cells to release the lipids, followed by methylation, and then gas chromatography / mass spectra (G.C. / M.S.) analysis. In the G.C. / M.S. spectrum, an Incorporation of [3-$^2H_2$]-β-aminoisobutyric acid into the starter unit of the branched chain lipids would be evident by an increase of the M + 2 signal. However, an Incorporation of only about 2% was expected (based upon incorporation values of previous β-aminoisobutyric acid feeding experiments) and in the event this proved to be below the threshold of detection by G.C. / M.S. In the future, the experiment could be repeated

on a larger scale and the lipids analysed by deuterium n.m.r. to see if deuterium has been incorporated into the methyl group.

3.3 [^{14}C-*methyl*]-Thymine and [$^{13}C^{2}H_3$-*methyl*]-thymine feeding experiments.

After successfully demonstrating the Incorporation of β-aminoisobutyric acid into the polyketide metabolite, monensin A, the scene was set to investigate whether the catabolism of thymine occurs in *Streptomyces* and generates β-aminoisobutyric acid, as in mammals. This β-aminoisobutyric acid could access the methylmalonyl-CoA pool and become available for secondary metabolite biosynthesis.

[^{14}C-*methyl*]-Thymine was administered to *S. cinnamonensis* and, in the event, a 2% Incorporation of radioactivity into the isolated monensins was evaluated. In order to establish the regiospecific incorporation of thymine into monensin A, a stable isotope experiment was designed. Accordingly [$^{13}C^{2}H_3$-*methyl*]-thymine was synthesised and administered to the cultures of *S. cinnamonensis* and the resultant monensin A analysed by $^{13}C\{^{1}H, ^{2}H\}$ n.m.r. The results clearly show a 1 - 2 fold enrichment of the methyl groups derived from propionate units. Scheme 17 shows the postulated link between thymine and valine metabolism in *Streptomyces*. Incorporation of [$^{13}C^{2}H_3$-*methyl*]-thymine into the propionate units can be rationalised by degradation to β-aminoisobutyric acid and then as detailed previously, through methylmalonic acid semialdehyde and (2S)-methylmalonyl-CoA. This entry into the methylmalonyl-CoA pool would account for the exchange of deuterium on the methyl groups, discussed in section 2.3.2 B, i.e. an interconversion of (2R)-methylmalonyl-CoA and succinyl-CoA mediated by methylmalonyl-CoA mutase.

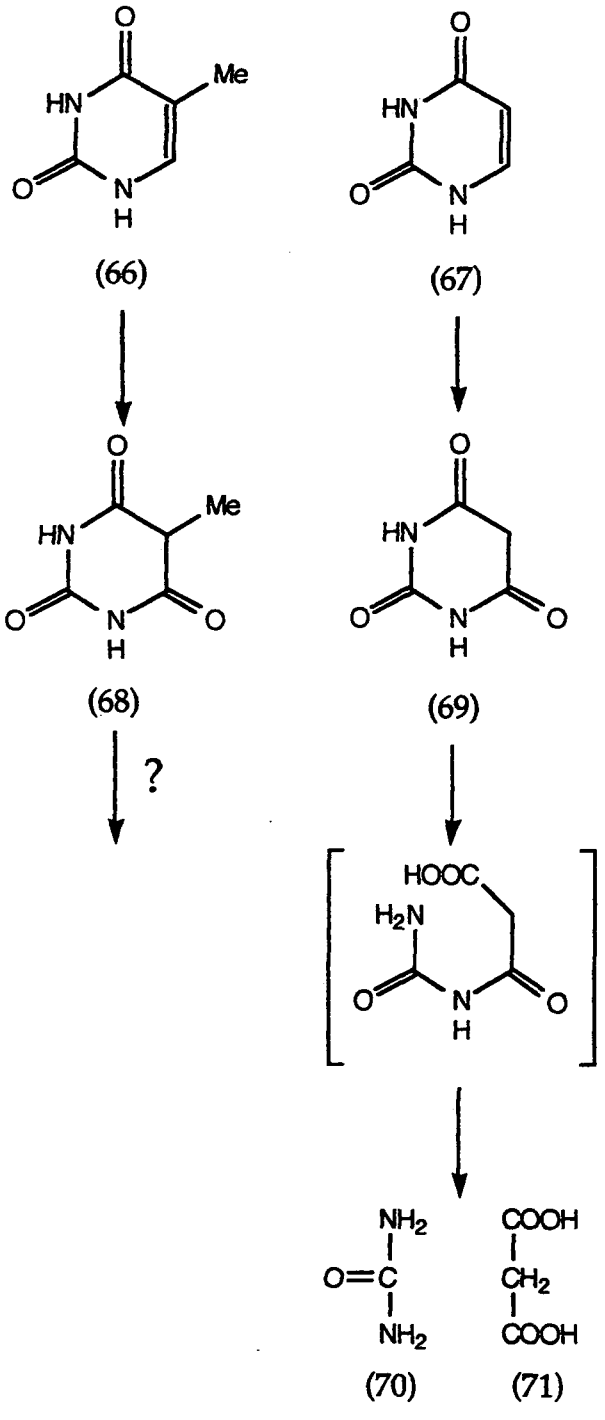


Scheme 17

[$^{13}C^{2}H_3$-*methyl*]-Thymine is also incorporated to a small extent (0.2 fold enrichment) into carbon-4 of the butyrate unit, C33. This observation illustrates the power of ^{13}C-^{2}H dual labelling followed by $^{13}C\{^{1}H, ^{2}H\}$ n.m.r. analysis. Incorporation of [^{13}C-*methyl*]-thymine into the butyrate unit would have been impossible to detect at this level by ^{13}C n.m.r. analysis,

however by utilising [$^{13}C^2H_3$-*methyl*]-thymine, the $^{13}CD_3$ incorporation into carbon-4 of the butyrate unit (C33) is detectable in the difference spectrum, Fig. 2(a). It is distinct and shifted to a lower frequency by a magnitude of 0.84ppm from the natural abundance signal (i.e. three deuterium induced α-shifts). The existence of only a $^{13}CD_3$ component for carbon-4 of the butyrate unit, lends further support for the existence of a deaminase enzyme mediating the transformation between β-aminoisobutyric acid and methacrylyl-CoA because there has been no significant deuterium exchange prior to incorporation into the butyrate unit, i.e. methylmalonyl-CoA is unlikely to have been an intermediate in the pathway. Thus thymine does contribute to the methylmalonyl-CoA pool available for secondary metabolite biosynthesis. The incorporation of β-aminoisobutyric acid into the propionate and butyrate units of monensin A, which mirrors the thymine incorporation into these units, lends support for the reductive catabolism of thymine *via* β-aminoisobutyric acid, analogous to mammalian systems.

There is, however, another pathway that can not be excluded from this discussion. This is the oxidative catabolism of thymine.[69] This pathway degrades thymine (66) to 5-methylbarbituric acid (68) mediated by uracil dehydrogenase[91,92] but the fate of this intermediate is unknown. Analogously, uracil (67) is degraded by a similar mechanism to barbituric acid (69) which is transformed, by the action of barbiturase, to urea (70) and malonic acid(71) [91,93,94] (scheme 18). Some aerobically grown bacteria have been shown[91,92,94-96] to grow in media containing uracil and thymine as a sole source of nitrogen and carbon and in these bacteria an oxidative pathway has been found.[69] The results from our feeding experiments indicate a reductive catabolism of thymine occurring in *Streptomyces*, however, feeding experiments with [$^{13}C^2H_3$-*methyl*]-dihydrothymine and [$^{13}C^2H_3$-*methyl*]-β-aminoisobutyric acid to assess if these incorporation patterns are similar to [$^{13}C^2H_3$-*methyl*]-thymine would unambiguously confirm or otherwise the degradation of thymine *via* a reductive pathway.



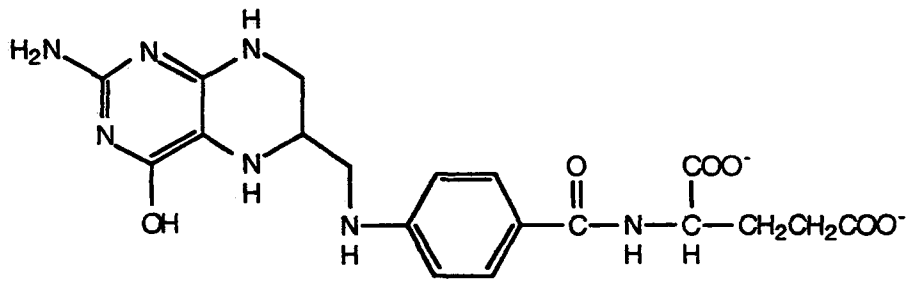
Scheme 18

3.4 L-[3-^{14}C]-Serine feeding experiments

All of these results which provide evidence of a reductive catabolism of thymine occurring in *Streptomyces*, fuels an interesting discussion about the possible links between DNA synthesis / degradation and secondary metabolite production. Several studies have been performed to determine

the timing of growth and production of secondary metabolites in certain micro-organisms. Studies on *Streptomyces griseus* (production of candicidin)[97] and *Streptomyces coelicolor A3(2)* (production of methylenomycin)[98] have both demonstrated that the maximum concentration of the antibiotic in the culture medium is reached when DNA synthesis ceases. Therefore the products of DNA degradation could in principle be channelled into secondary metabolite production. Such a study has not been performed on *S. cinnamonensis*.

We made an attempt to probe the link between DNA synthesis / degradation and monensin A production in *S. cinnamonensis*. Carbon-3 of L-serine enters the C1 pool of the growing micro-organism and thymine is formed from uracil by utilising carbons from the C1 pool, aided by tetrahydrofolate (72) (which is a highly versatile carrier of activated one carbon units).



(72)

Therefore an experiment was designed whereby L-[3-^{14}C]-serine (22.2μCi) and 10mg of cold serine as carrier were administered to cultures (6 x 100cm^3) of *S. cinnamonensis* before innoculation such that the specific activity was 0.233mCi/mmol. The monensins were isolated on day 7.5 and the Incorporation of radioactive serine into the monensins was shown to be 2.5%. A parallel experiment was also performed, where L-[3-^{14}C]-serine (23.3μCi) and 10mg of cold serine were administered at day 3.5 to cultures (6 x 100cm^3) of *S. cinnamonensis*. The specific activity of the L-[3-^{14}C]-Serine added was 0.244mCi/mmol and the monensins which were isolated at day 7.5 showed an Incorporation of radioactivity of 3.2%. Feeding L-[3-^{14}C]-serine at day 0 to the cultures of *S. cinnamonensis* was anticipated to generate *in vivo* [^{14}C-*methyl*]-thymine (from uracil and carbon-3 of serine)

which would then be utilised in DNA synthesis. The degradation products of DNA could then contribute towards secondary metabolite production, i.e. monensin A production, and hence this monensin A may be more radioactive than the monensin A isolated from a feeding experiment whereby L-[3-^{14}C]-serine was administered at day 3.5, a period when DNA and primary metabolism in general should have ceased or at least slowed down.

However, this experiment was complicated by the fact that monensin A possesses a methoxyl carbon, which is known to derive also from the C1 pool, so radioactivity incorporated in both experiments is quite probably due to this methoxyl carbon. Nevertheless, it was judged an interesting experiment to assess any large differences in the amount of radioactivity incorporated into monensin A during the *trophophase* and *idiophase.*

4. CONCLUSION

The links between primary and secondary metabolism in *Streptomyces* have been extensively probed with the aid of feeding experiments. It has been demonstrated that thymine can contribute to the methylmalonyl-CoA pool and be available for secondary metabolite biosynthesis. The evidence suggests that its entry is *via* a reductive pathway and β-aminoisobutyric acid, however an oxidative pathway of thymine degradation *via* 5-methylbarbiturate can not be ruled out. The relative importance of thymine as a source of methylmalonyl-CoA is unknown. Relatively large amounts of thymine was added to the cultures of *S. cinnamonensis* therefore the biological system has the capacity to utilise thymine in the manner discussed, but the experiment gives no information about the true flux in batch fermentation.

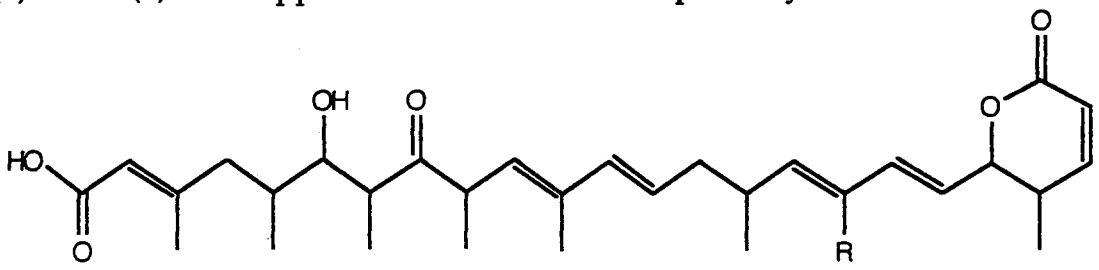
During this chapter, the existence of a novel deaminase enzyme, mediating the transformation of β-aminoisobutyric acid to methacrylyl-CoA has been implied, and some supporting evidence has been highlighted. An enzyme has been identified in *Clostridium propionicum* which catalyses the amination of acrylyl-CoA to β-alanyl-CoA, however the reversibility of this process was not demonstrated.[99]

CHAPTER 3
LEPTOMYCIN B

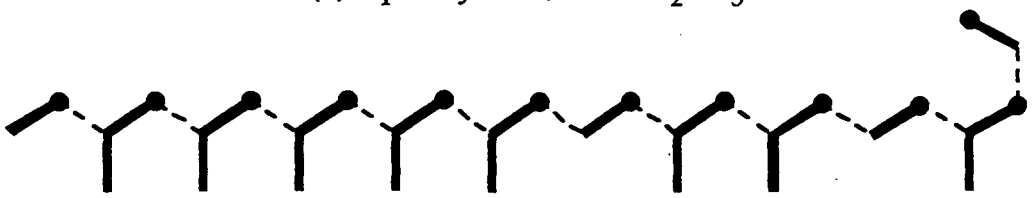
1. INTRODUCTION

1.1 Leptomycins

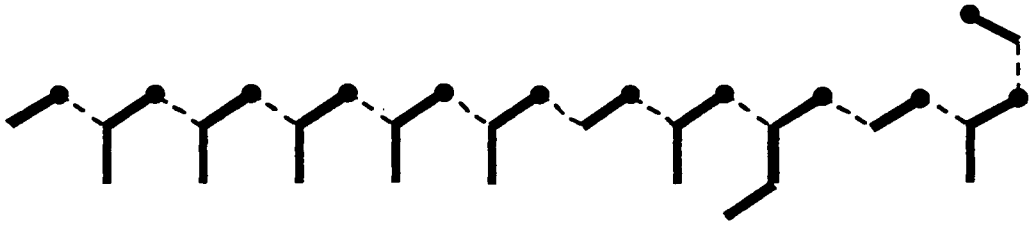
The leptomycins are antifungal antibiotics found in the course of a screening program for substances which cause abnormal morphology on the growth of various fungi.[100] Elucidation of the chemical structures of these compounds have shown that they are long unsaturated branched chain dicarboxylic acids in which one of the carboxyl groups has formed a δ-lactone ring.[101,102] *Streptomyces sp. ATS1287* produces both leptomycin A (1) and B (2) in an approximate ratio of 1 : 6 respectively.



(1) leptomycin A, R = CH_3
(2) leptomycin B, R = CH_2CH_3



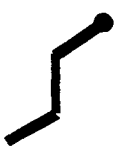
leptomycin A



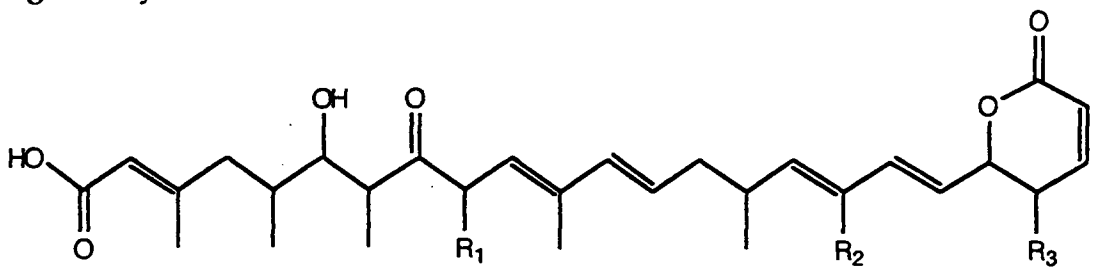
leptomycin B

 = acetate

 = propionate

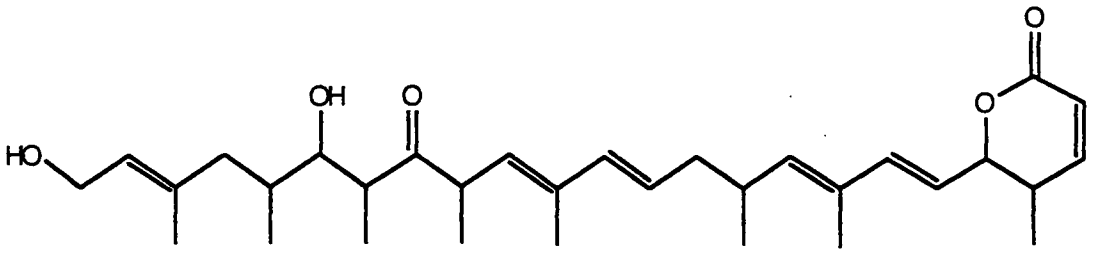
 = butyrate

Leptomycin A is biosynthesised from 4 acetate and 8 propionate units and leptomycin B from 4 acetate, 7 propionate and 1 butyrate unit.[103] Several antibiotics have been isolated from other strains of *Streptomyces* with very similar chemical structures, e.g. kazusamycins A and B[104] and anguinomycins A and B.[105]



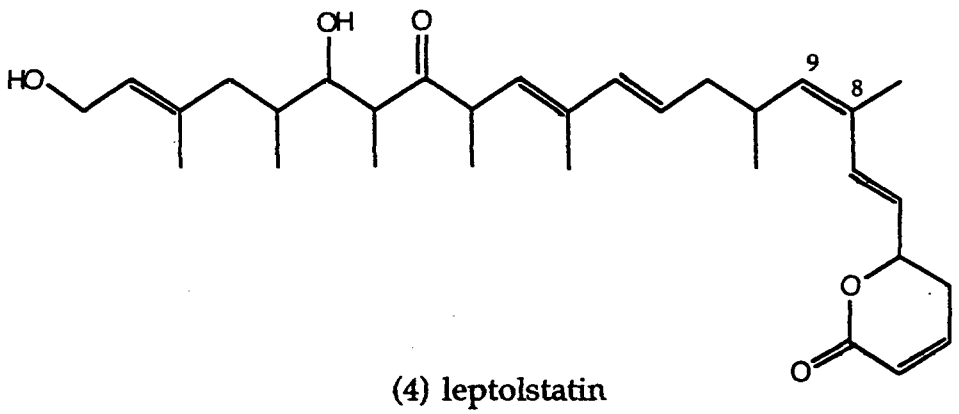
leptomycin A, $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$
leptomycin B, $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = CH_3$
kazusamycin A, $R_1 = CH_2OH$, $R_2 = CH_2CH_3$, $R_3 = CH_3$
kazusamycin B, $R_1 = CH_2OH$, $R_2 = CH_3$, $R_3 = CH_3$
anguinomycin A, $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$
anguinomycin B, $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = H$

Recently there has been reports of the isolation of two further antibiotics which have very similar structures to the leptomycins, with the exception that the terminal carboxyl group has been replaced with a hydroxymethyl group, i.e. reductoleptomycin A (3)[106] isolated from *Streptomyces sp. MJ132NF5* and leptolstatin (4)[107] isolated from *Streptomyces sp. SAM1595.*



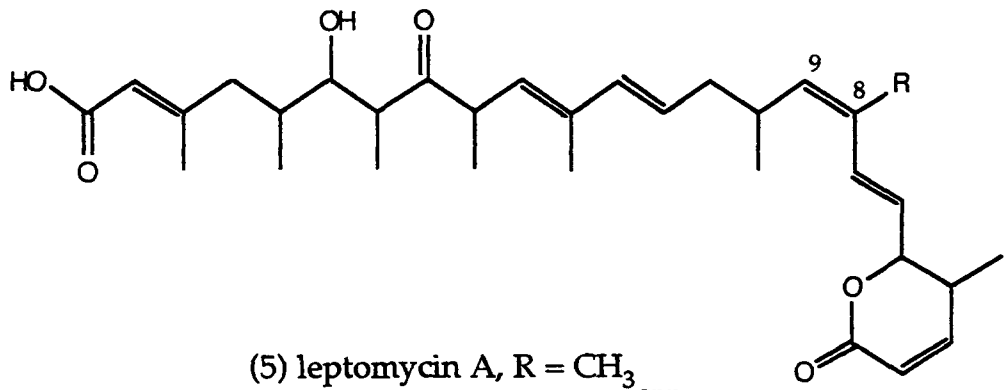
(3) reductoleptomycin A

The N.O.E. study of leptolstatin (4) demonstrated that the double bond between C8 - C9 was the Z- geometric isomer.[107] When the structure of the leptomycins was elucidated,[102] the configuration of this C8 - C9 bond was ambiguous and left for further investigation.



(4) leptolstatin

So after the N.O.E. study of leptolstatin, a similar study was performed on leptomycin B and this C8 - C9 double bond was also found to have Z-geometry.[107] It seems likely, therefore, that all the members of this group possess a similar skeleton and hence a Z- double bond at this site. Therefore the leptomycins (5) and (6) should be redrawn with the correct double bond geometry.

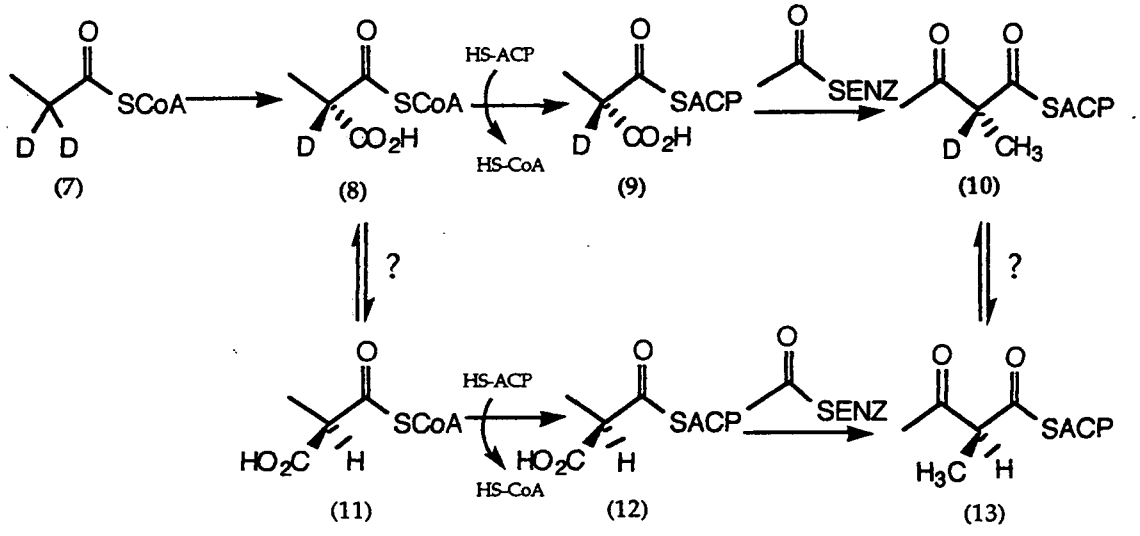


(5) leptomycin A, R = CH_3
(6) leptomycin B, R = CH_2CH_3

1.2 Stereochemical analysis of leptomycin B

It is established that propionyl-CoA is activated by carboxylation to (2S)-methylmalonyl-CoA, a biosynthetic precursor to the propionate subunits. The enzyme mediating this transformation, propionyl-CoA carboxylase, has been purified to near homogeneity from the erythromycin producing organism *Saccharopolyspora erythraea*.[28] This isomer of methylmalonyl-CoA is then utilised in polyketide biosynthesis by undergoing a decarboxylative condensation with inversion of configuration.[108] This has been demonstrated with the aid of feeding experiments to several *Streptomyces* metabolites, e.g. lasalocid A,[109] monensin A,[110] erythromycin

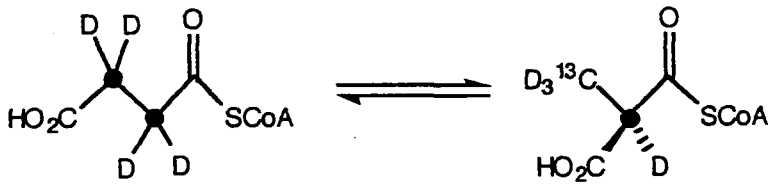
A.[63] Sodium [2-^{13}C,$^{2}H_2$]-propionate was administered to the fermentations and the isolated polyketide metabolites were analysed to establish if any deuterium had been retained at the methine centres of the polyketide backbone. Deuterium is only expected to be retained at sites where the functionality of the condensation unit is either a keto group or an alcohol. If the functionality has been reduced further to a double bond or a fully saturated methylene group then all the deuteriums are washed out. From these experiments it was established that deuterium was retained only at the D-methine sites, the L-methine sites were devoid of deuterium enrichment. This can be rationalised as outlined in scheme 1. [2-$^{2}H_2$]-Propionyl-CoA (7) undergoes carboxylation giving specifically (2S)-methylmalonyl-CoA (8) with one deuterium retained. Transesterification onto the polyketide synthase affords (9), which undergoes a decarboxylative condensation proceeding with inversion of configuration to give D-α-methyl-β-keto-thioester (10) with retention of deuterium. However, deuterium is not retained at the L-methine site, i.e. L-α-methyl-β-keto-thioester (13), and therefore an epimerisation must be occurring causing loss of the deuterium.



Scheme1

This epimerisation could occur at one of two possible stages, i.e. the polyketide synthase utilises only (2S)-methylmalonyl-CoA and an enzyme mediates the epimerisation between D-α-methyl-β-keto-thioester (10) and L-α-methyl-β-keto-thioester (13), or there could be an epimerisation between (2S)-methylmalonyl-CoA (8) and (2R)-methylmalonyl-CoA (11) and the

polyketide synthase is capable of processing each diastereoisomer, the (S) affording D-alkylated centres and (R) the L-alkylated centres. Both options would afford L-methine sites devoid of deuterium. Attempts to delineate between these processes have been directed at accessing (2R)-methylmalonyl-CoA specifically labelled *in vivo*. The experiments performed on erythromycin A[63] and lasalocid A[109,64] utilised isotopically labelled succinyl-CoA (14) which would generate (2R)-methylmalonyl-CoA (15) (with the respective isotopic labels) by the action of methylmalonyl-CoA mutase.[111] However in these studies, there was extensive wash out of deuterium probably due to the direct entry of succinate into the citric acid cycle and no conclusions could be drawn.



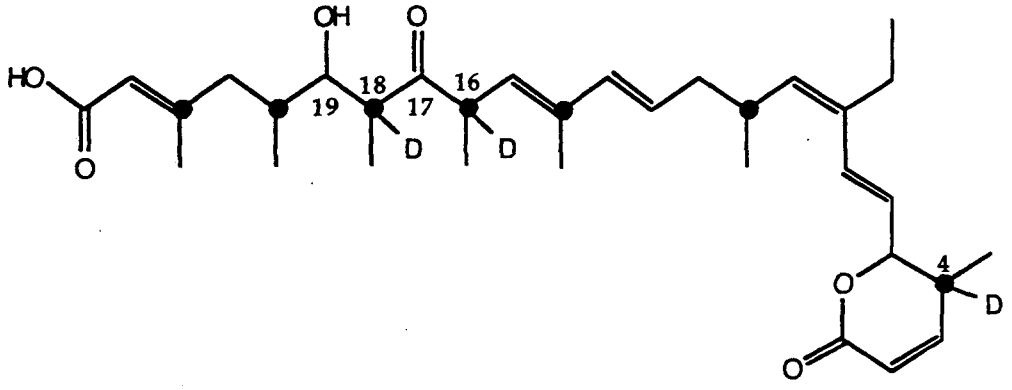
(14) (15)

The timing of epimerisation, which will lead to both D and L alkylated centres during polyketide growth, has now been established by Leadlay and co-workers.[29] Detailed work with the three DEBS multi-enzymes, which catalyse the formation of the macrolide core of erythromycin A, produced by *Saccharopolyspora erythraea* (discussed in detail in chapter 4B), has shown that all the units used in the construction of erythromycin A come from (2S)-methylmalonyl-CoA, i.e. there must be additional epimerisation steps catalysed by the synthase in order to generate the L-alkylated centres. Actinomycete metabolites are constructed in a similar manner, i.e. a processive assembly from similar biosynthetic precursors, and so it seems likely that chain assembly in all of these related polyketide synthases requires only this one stereoisomer of methylmalonyl-CoA.[29]

So studies with Actinomycete metabolites, e.g. lasalocid A,[109] monensin A[110] and erythromycin A,[63] have shown that the administration of sodium [2-^{13}C,$^{2}H_2$]-propionate to the antibiotic followed by analysis of deuterium incorporation is potentially a good biosynthetic tool for stereochemical assignment of polyketide metabolites. Deuterium incorporation at methine sites allows assignment of the D-configuration, whereas loss of deuterium provides evidence for the L-configuration. As

the stereochemistry of leptomycin B (and related metabolites) has not been reported, it was of interest to attempt this strategy for the first time with leptomycin B to *assign* stereochemistry.

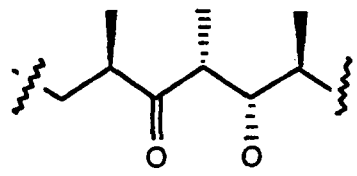
There are only three sites in leptomycin B (16) where deuterium could realistically be retained by employing this strategy of feeding sodium [2-^{13}C,$^{2}H_2$]-propionate to cultures of the producing strain, i.e. C4, C16, C18.



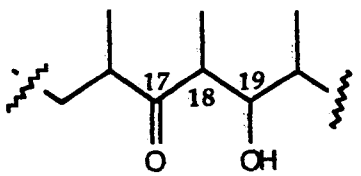
(16)

If any of these three sites show deuterium enrichment upon isolation of the leptomycin B then this will indicate a D-configuration and lack of deuterium may indicate an L-configuration (due to an epimerisation).

The macrolide antibiotics are a major class of polyoxygenated metabolites produced by *Streptomyces*,[38] e.g. erythromycin A. Celmer, in 1965, proposed a single stereochemical model to represent the entire class of macrolides.[40] It is of interest to highlight that a section of the Celmer model (17) is structurally analogous to a section of leptomycin B (18). Therefore it would be interesting to determine the stereochemistry of leptomycin B to assess or otherwise a stereochemical correlation to the Celmer model, and thus to macrolide antibiotics more generally.



(17) Celmer model



(18) fragment of leptomcin B

2. RESULTS AND DISCUSSION

2.1 Synthesis of sodium [2-^{13}C,$^{2}H_2$]-propionate

The synthetic route[47(b)] to sodium [2-^{13}C,$^{2}H_2$]-propionate is outlined in scheme 2. The synthesis started with a relatively large amount of sodium [1-^{13}C]-acetate (6g) which was converted into (20), its p-phenylphenacyl derivative. [1-^{13}C,$^{2}H_2$]-Ethanol (21) was carefully generated by treating (20) with lithium aluminium deuteride. The reaction was quenched by the addition of phenoxyethanol and the [1-^{13}C,$^{2}H_2$]-ethanol was carefully distilled from the high boiling solvent and collected in a liquid nitrogen cooled trap. The resultant [1-^{13}C,$^{2}H_2$]-ethanol was then directly derivatised with p-toluenesulphonyl chloride. The crystals of [1-^{13}C,$^{2}H_2$]-ethyl tosylate (22) were collected by filtration and stored at 0°C in a dessicator. This material was treated with KCN in MeOD : D_2O and the reaction product [2-^{13}C,$^{2}H_2$]-propionitrile (23) distilled into a clean flask. Utilisation of KOD in D_2O for the base hydrolysis of (23) afforded [2-^{13}C,$^{2}H_2$]-propionic acid after acidification and lyophilisation. The lyophilisate was neutralised with dilute NaOH and a further lyophilisation afforded sodium [2-^{13}C,$^{2}H_2$]-propionate (24) in an overall yield of 16.5% from sodium [1-^{13}C]-acetate.

The ^{13}C n.m.r. spectrum of sodium [2-^{13}C,$^{2}H_2$]-propionate is shown in Fig. 1(a). Fig. 1(b) shows an expansion of the $^{13}CD_2$ signal where a mixture of $^{13}CD_2$ (pentet) and ^{13}CDH (triplet) is obvious, in an approximate ratio of 88% : 12% respectively, indicating a small amount of deuterium exchange during the synthesis. Fig. 1 (c) is an expansion of the $^{13}CD_2$ signal from the first attempted synthesis of sodium [2-^{13}C,$^{2}H_2$]-propionate. A triplet clearly predominates over the pentet, indicative of a high proportion of ^{13}CDH. There is also a singlet present which corresponds to complete loss of deuterium. This extensive exchange of deuterium in the first synthesis was presumably due to utilising KOH and D_2O for the base hydrolysis.

Fig. 1(a), ^{13}C nmr (50MHz) of sodium [2-^{13}C,$^{2}H_2$]-propionate in a solution of D_2O.

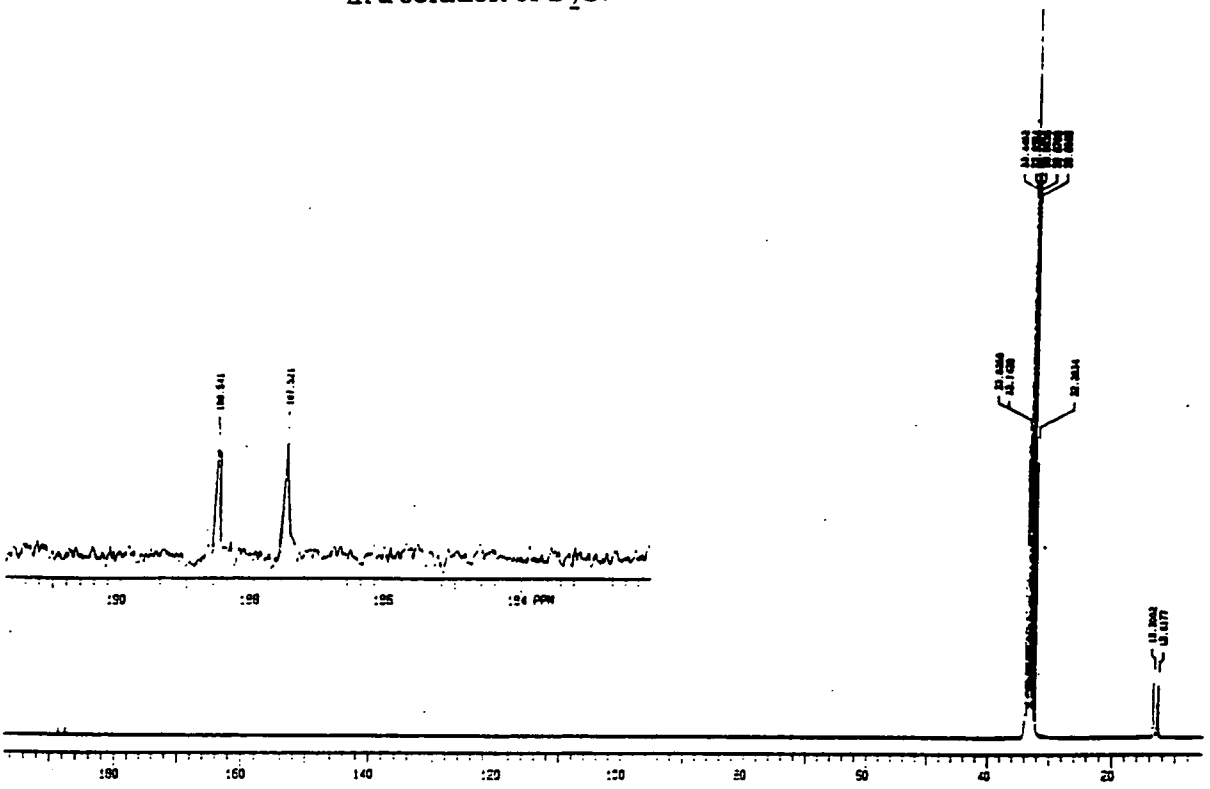


Fig. 1(b)

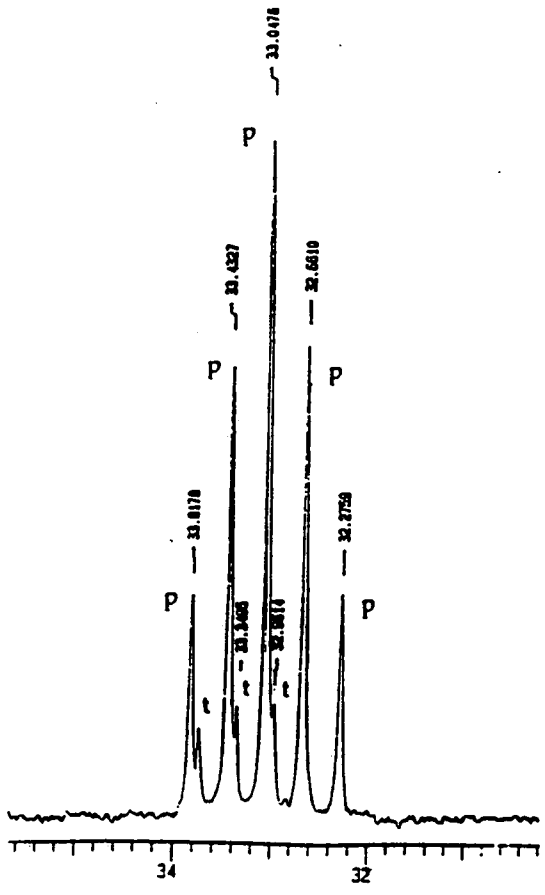
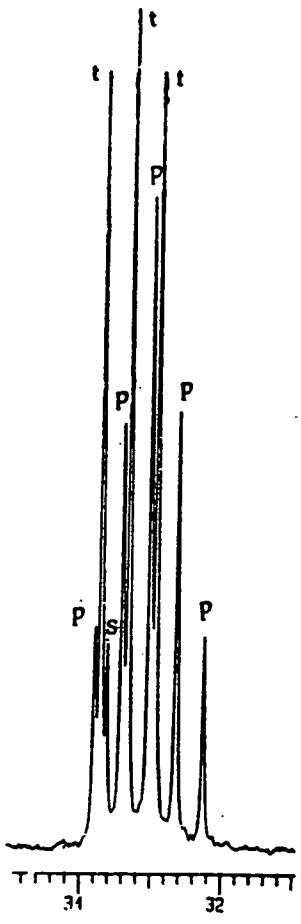
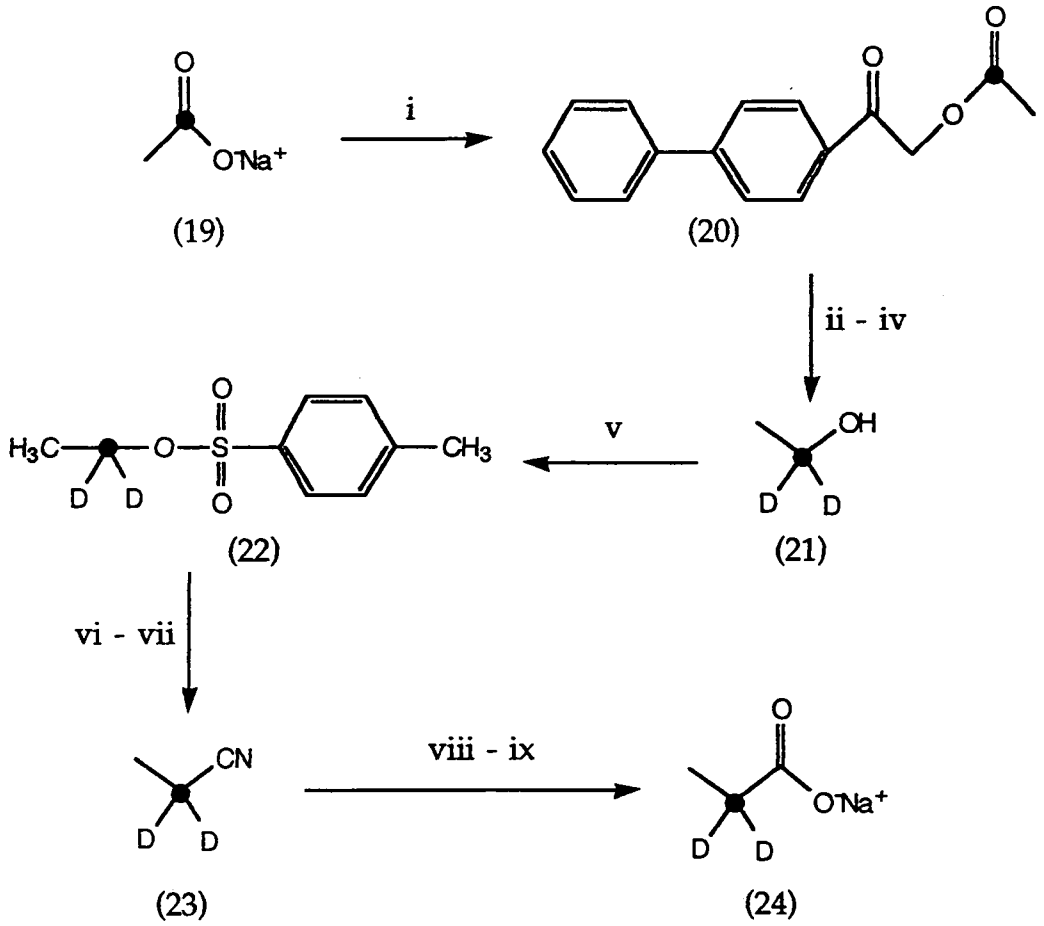


Fig. 1(c)





Scheme 2. Reagents and conditions:- i) p-phenylphenacyl bromide, 18-crown-6, C_6H_6 : CH_3CN (1 : 1), reflux 16h, ii) diglyme, -23°C, $LiAlD_4$, r.t. 4h, iii) 0°C, phenoxyethanol, iv) 50°C 8h distillation, v) p-toluenesulphonyl chloride, pyridine, -23°C 2h, vi) KCN, MeOD : D_2O (3 : 1), reflux 2h, vii) distillation, viii) KOD, D_2O, reflux 3d, ix) $HCl_{(aq)}$, lyophilise, pH 7 $NaOH_{(aq)}$.

2.2 Feeding experiments

2.2.1 Feeding of sodium [1-^{13}C]-propionate to *Streptomyces sp. ATS1287*

In the first instance, sodium [1-^{13}C]-propionate was fed to *Streptomyces sp.ATS1287* to determine the level of incorporation before sodium [2-^{13}C,$^{2}H_2$]-propionate was synthesised and fed. The sodium [1-^{13}C]-propionate was administered batchwise at day 3, 3.5 and 4 to *Streptomyces sp. ATS1287*. Leptomycin B was isolated on day 9 and converted to its methyl ester (about 10mg/800cm^3) and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 2. The sample is not very pure as there are related co-metabolites which could not

be separated, however the signals corresponding to leptomycin B methyl ester can be clearly identified (marked with a black spot in Fig. 2). The full ^{13}C n.m.r. assignment of leptomycin B methyl ester has been reported by Seto *et al.*[102] and Table A2 (Appendix 1, pg. ii) lists the chemical shifts for each carbon of a standard sample of leptomycin B methyl ester, compared with the literature value.

The Incorporation of sodium [1-^{13}C]-propionate into leptomycin B was evident by the enrichment in the ^{13}C n.m.r. spectrum of the carbons corresponding to carbon-1 of the propionate units, i.e. C3, C9, C13, C15, C17, C19 and C21, by a magnitude of 3 - 4 fold. The enrichments are detailed in Fig. 3. These values were calculated by normalising the height of each signal relative to C29 (CH_3), and then a further normalisation relative to a natural abundance spectrum. The figure corresponding to the enrichment at C21 is much higher than the others. The origin of this discrepancy is not clear but it could be due to n.m.r. relaxation effects or perhaps another coincident signal (corresponding to an impurity) at the same chemical shift and therefore effecting the calculation.

	^{13}C/ppm	sodium [1-^{13}C]-propionate
C3	151.57	3.54
C9	136.96	3.25
C13	135.17	3.13
C15	128.17	2.70
C17	215.25	2.91
C19	74.31	4.56
C21	45.28	6.10
C23*	116.95	0.99
C5*	81.50	0.90

Fig. 3, * unenriched C as standard

2.2.2 Feeding sodium [2-^{13}C,$^{2}H_2$]-propionate to *Streptomyces sp. ATS1287*

The synthetic scheme and corresponding figures outlined in section 2.1 corresponds to the second preparation of sodium [2-^{13}C,$^{2}H_2$]-propionate. In the first attempt at the synthesis, precautions were not taken to prevent

Fig. 2, ^{13}C nmr (100MHz) of leptomycin B methyl ester isolated from *Streptomyces sp. ATS1287* fed with sodium [1-^{13}C]-propionate

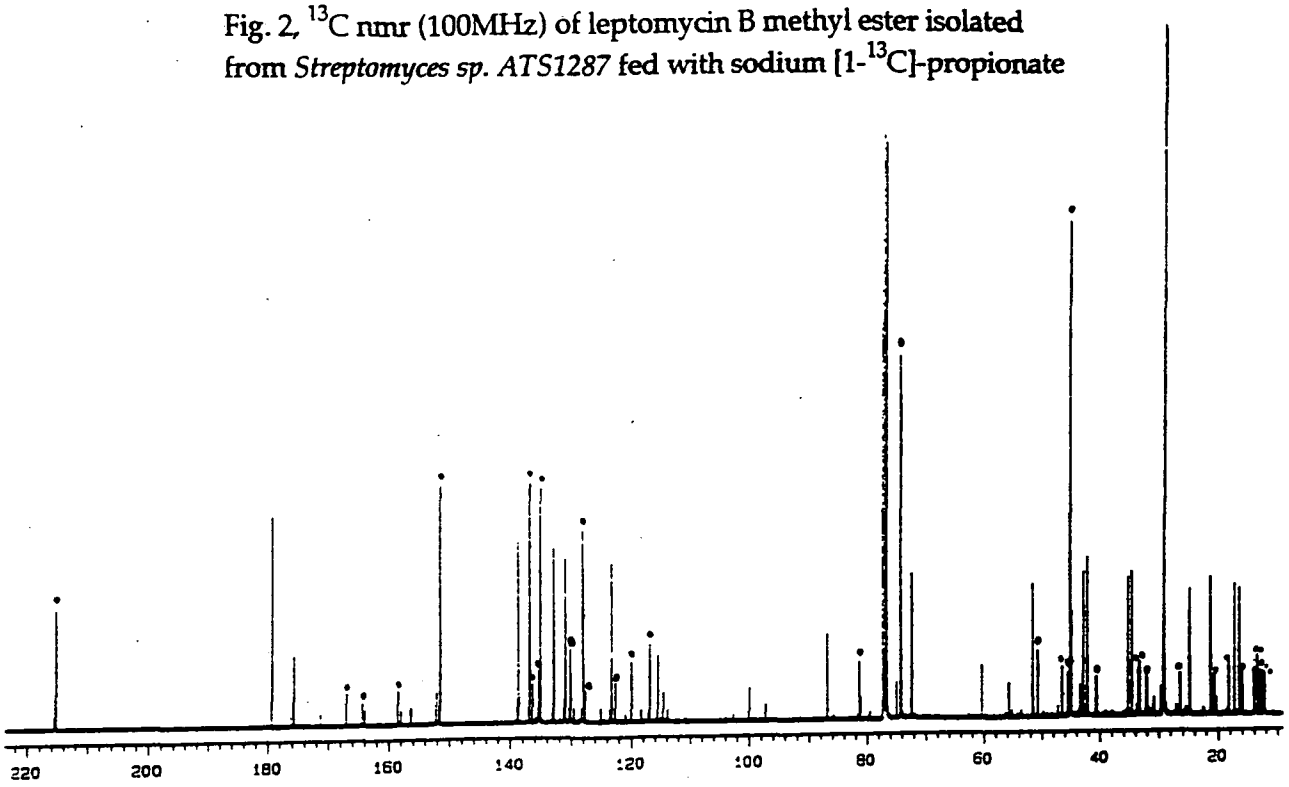
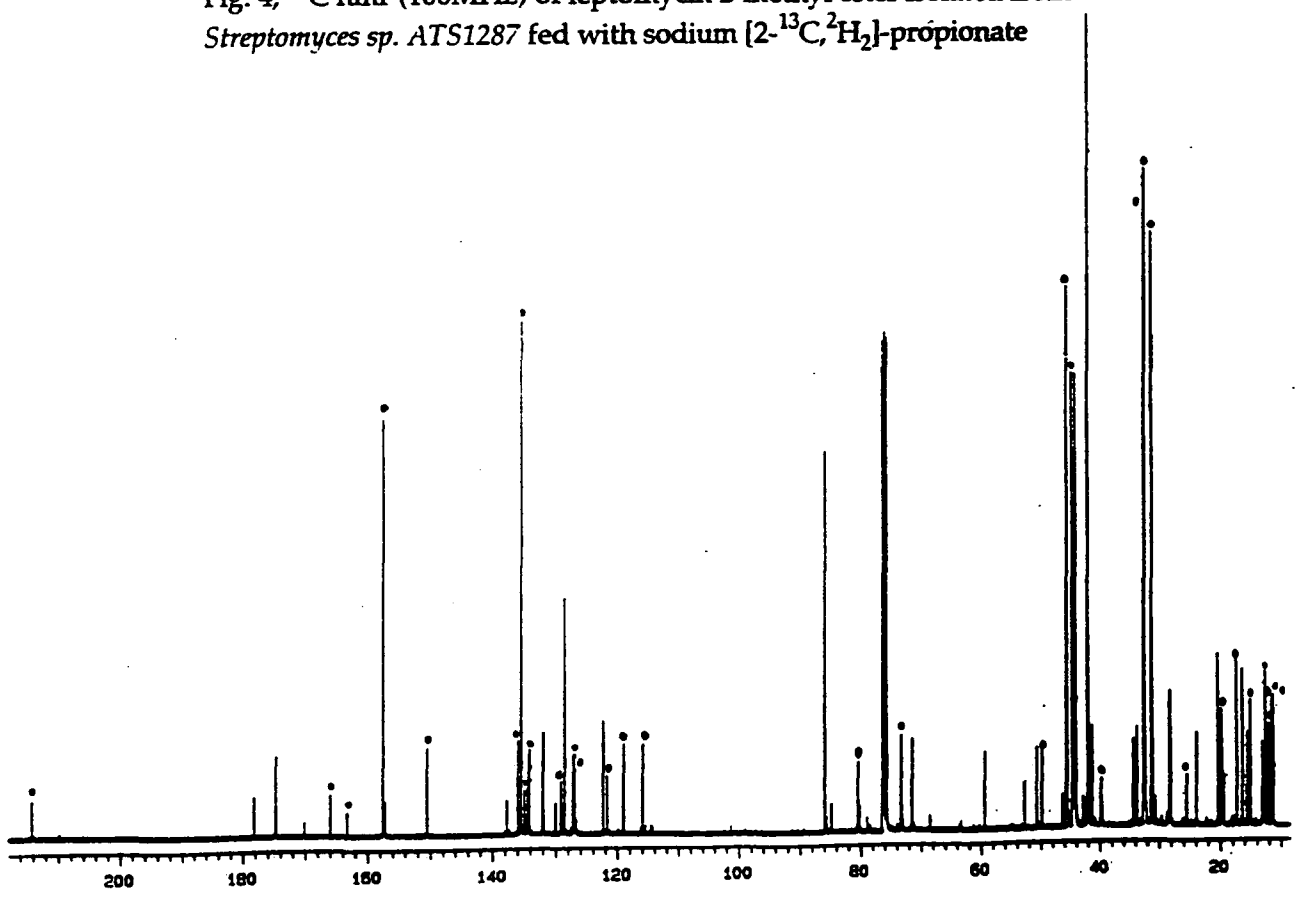


Fig. 4, ^{13}C nmr (100MHz) of leptomycin B methyl ester isolated from *Streptomyces sp. ATS1287* fed with sodium [2-^{13}C,$^{2}H_2$]-propionate



exchange of the deuterium and this lead to half of the material with only one deuterium, and a small amount carrying no deuteriums. The feeding experiment was however attempted, with the anticipation that the primary kinetic isotope effect would help the situation, i.e. loss of H favoured over loss of D.

Sodium [2-^{13}C,$^{2}H_2$]-propionate was fed as outlined in section 2.2.1 to *Streptomyces sp.ATS1287* and leptomycin B methyl ester was isolated on day 9. The corresponding ^{13}C n.m.r. spectrum is shown in Fig. 4. Again the sample is lacking in purity, but leptomycin B methyl ester is identifiable (marked with black spots). The Incorporation of sodium [2-^{13}C,$^{2}H_2$]-propionate into leptomycin B methyl ester is clearly evident by the enrichment in the ^{13}C n.m.r. spectrum of the carbons derived from carbon-2 of the propionate units, i.e. C4, C10, C14, C16, C18, C20 and C22 by a magnitude of 7 - 9 fold. The enrichments are detailed in Fig. 5, these values were calculated by normalising relative to C11 (CH_2).

	^{13}C/ppm	Sodium [2-^{13}C,$^{2}H_2$]-propionate
C4	32.43	9.05
C10	31.14	7.98
C14	135.43	9.33
C16	44.60	6.10
C18	45.58	7.95
C20	32.37	9.47
C22	157.49	8.37
C23*	115.93	1.2
C5*	80.45	1.10

Fig. 5, * unenriched C as standard

An Incorporation of a ^{13}C-D unit into leptomycin B methyl ester would appear as a triplet shifted to a lower frequency by a magnitude of about 0.3ppm from the natural abundance signal in the ^{13}C n.m.r. spectrum. However there was no evidence of this in the ^{13}C n.m.r. spectrum and so the sample was analysed by $^{13}C\{^{1}H, ^{2}H\}$ n.m.r. This analysis is very clear and low level incorporations will be identifiable (e.g. chapter 2, a 0.2 fold enrichment of the butyrate unit of monensin A when fed with [$^{13}C^{2}H_3$-

methyl]-thymine). The resultant spectrum however showed no evidence of deuterium incorporation. This result could be explained in several ways :-
i) the methine sites at C4, C16 and C18 all possess L-configuration, i.e. the deuterium has been lost due to epimerisation.
ii) there wasn't sufficient deuterium content in the sodium [2-^{13}C,$^{2}H_2$]-propionate.
To have confidence in the results, it was necessary to prepare again sodium [2-^{13}C,$^{2}H_2$]-propionate, taking care to avoid deuterium loss (section 2.1) such that the feeding experiment could be repeated.

In the interim, leptomycin B production ceased. Many attempts were made to induce production and the *Streptomyces* strain did appear to produce leptomycin B again. After several sub-cultures the second sodium [2-^{13}C,$^{2}H_2$]-propionate feeding experiment was performed but in the event no leptomycin B could be isolated.

3. CONCLUSION

Little conclusive information can be gleaned from these experiments due to the diminishing leptomycin B production. Stereochemical information could be gained by other means. For example an X-ray crystal structure but this would require sufficient leptomycin B. In the future if the stereochemistry of the leptomycins or the structurally related metabolites (leptolstatin, kazusamycins e.t.c.) can be assigned then it will be of interest to see if the configurations correlate with the Celmer macrolide model.[40]

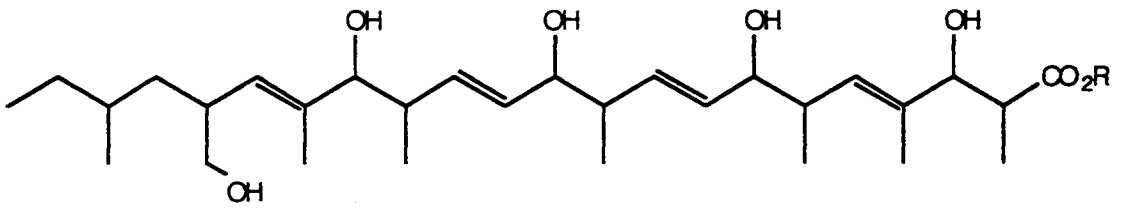
PART 2

CHAPTER 4
PART A
THE BIOSYNTHESIS OF CUBENSIC ACID

1. INTRODUCTION

1.1 Cubensic acid

Cubensic acid (1) is a straight chain fungal metabolite secreted by *Xylaria cubensis* (Mont) Fr. collected in the Peruvian Amazon rain forest. The genus *Xylaria* contains a large and undetermined number of species, most of them inhabiting tropical and sub-tropical regions. Their exact physiological role is unknown but most of them produce a white rot in wood. The isolation and structure elucidation of cubensic acid (1) has recently been reported by Edwards *et al.*[112]



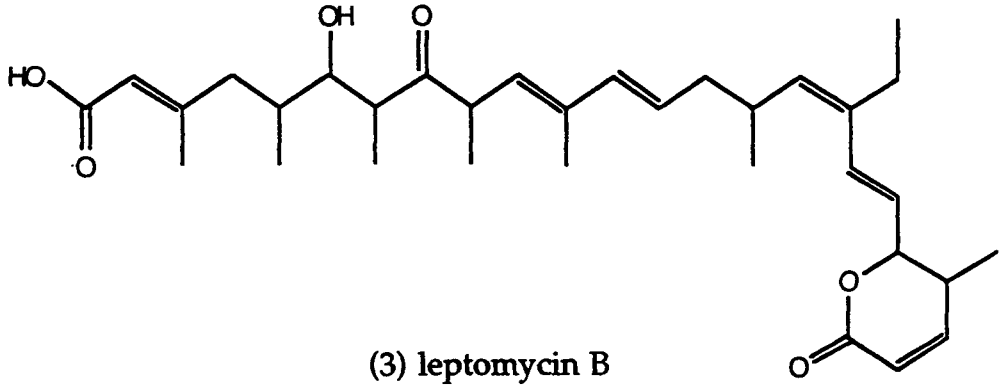
(1) R = H
(2) R = Me

The acid is isolated from the static surface grown mycelium after eight weeks growth on 3% malt extract medium. Conversion to its methyl ester (2) by treatment with diazomethane aids purification of this metabolite. Cytochalasin D is isolated from the supernatant of these cultures, and studies on the biosynthesis of cytochalasin D are discussed in chapter 5.

1.2 Biosynthesis of cubensic acid

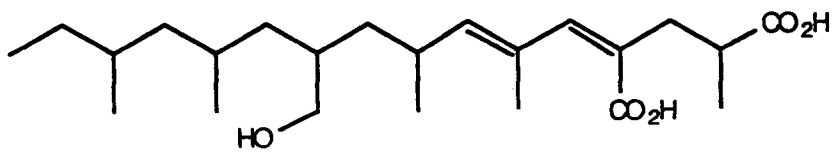
The long extended polyketide chain structure of cubensic acid (1) with its eight pendant methyl groups looks at first glance reminiscent of many of the polyketide metabolites of Actinomycete bacteria. An example of such a metabolite is leptomycin B (3)[101] isolated from *Streptomyces sp.* ATS 1287 (discussed in chapter 3). It too has an extended polyketide chain with pendant methyl groups and is biosynthesised from the condensation of acetate C_2- and propionate C_3- units.[103] Hence by extending this analogy it was hypothesised, by Edwards *et al.*,[112] that cubensic acid (1) could also

derive from the condensation of acetate and propionate units. There is a flaw in this hypothesis however, as leptomycin B is an Actinomycete metabolite whereas cubensic acid is of fungal origin, and fungal metabolites are not known to incorporate propionate, mid-chain, into their metabolites.[19] There are however several fungal metabolites where propionate is utilised as a starter unit, e.g. aurovertins B and D, and asteltoxin which are metabolites of *Emericella variecolor*.[113,114]

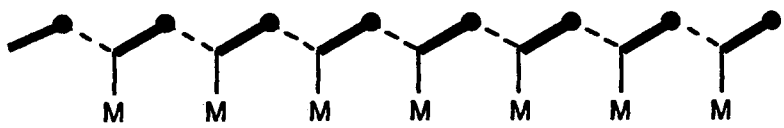


(3) leptomycin B

A more attractive proposal for cubensic acid is that it is constructed from acetate and the pendant methyls from L-methionine, cf. radiclonic acid (4)[115] a fungal metabolite isolated from *Penicillium sp.* Radiclonic acid (4) and cubensic acid are unusual metabolites as they are highly reduced long chain systems, whereas the majority of fungal metabolites are aromatic in character. The biosynthesis of radiclonic acid from 8 acetate and 7 L-methionine units has been established.[116] It appeared appropriate in view of the errant proposal in the literature and the extensive degree of methylation, to assess the biosynthetic make up of cubensic acid.



(4) radiclonic acid



= acetate M = L-methionine

2. RESULTS AND DISCUSSION

2.1 Feeding of L-[^{13}C-*methyl*]-methionine, sodium [1-^{13}C, 2-$^{2}H_3$]-acetate and sodium [$^{13}C_2$]-acetate to *Xylaria cubensis*

L-[^{13}C-*methyl*]-Methionine was administered to the *Xylaria cubensis* cultures in one batch on day 4 (i.e. when the mycelium appeared to have healthy growth) at a final concentration of 3.7mM. Cubensic acid was isolated from the mycelium on week 8. Methylation with diazomethane yielded methyl cubensate which gives a distinctive blue colouration on a silica t.l.c. plate, after spraying with an anisaldehyde : glacial acetic acid : sulphuric acid solution.

^{13}C Nuclear magnetic resonance spectrum of crude methyl cubensate clearly identified that the L-[^{13}C-*methyl*]-methionine had been incorporated into the methyl groups of methyl cubensate, i.e. C23, C24, C25, C26, C27, C28, C29 and C30 were enriched by a magnitude of 10 - 17 fold. The specific values given in Fig. 1, were calculated by normalising relative to the methoxyl carbon (OMe). The sample isolated was rather impure but purification with preparative C_{18} reverse phase chromatography afforded clean material and the resultant ^{13}C n.m.r. spectrum is shown in Fig. 2. There was little sample after the purification and so only the enriched methyl signals are evident in the ^{13}C n.m.r. spectrum. A full ^{13}C n.m.r. assignment of cubensic acid has been reported by Edwards *et al.*[112] and Table A3 (Appendix 1, pg. iii) contains all of the chemical shift values of methyl cubensate in comparison with the literature values.

	^{13}C/ppm	[^{13}C-*methyl*]-methionine
C23	14.89	15.53
C24	11.11	15.71
C25	17.22	13.91
C26	17.44	17.63
C27	18.04	17.01
C28	12.08	10.50
C29	66.82	17.31
C30	19.12	17.50
OMe*	51.46	-
C14*	40.70	1.09

Fig. 1, * unenriched C as standard

Fig. 2, ^{13}C nmr (100MHz) of methyl cubensate isolated from *Xylaria cubensis* fed with [^{13}C-*methyl*]-methionine.

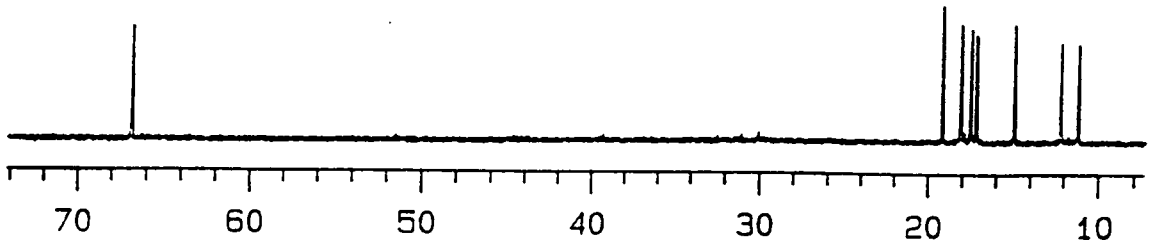
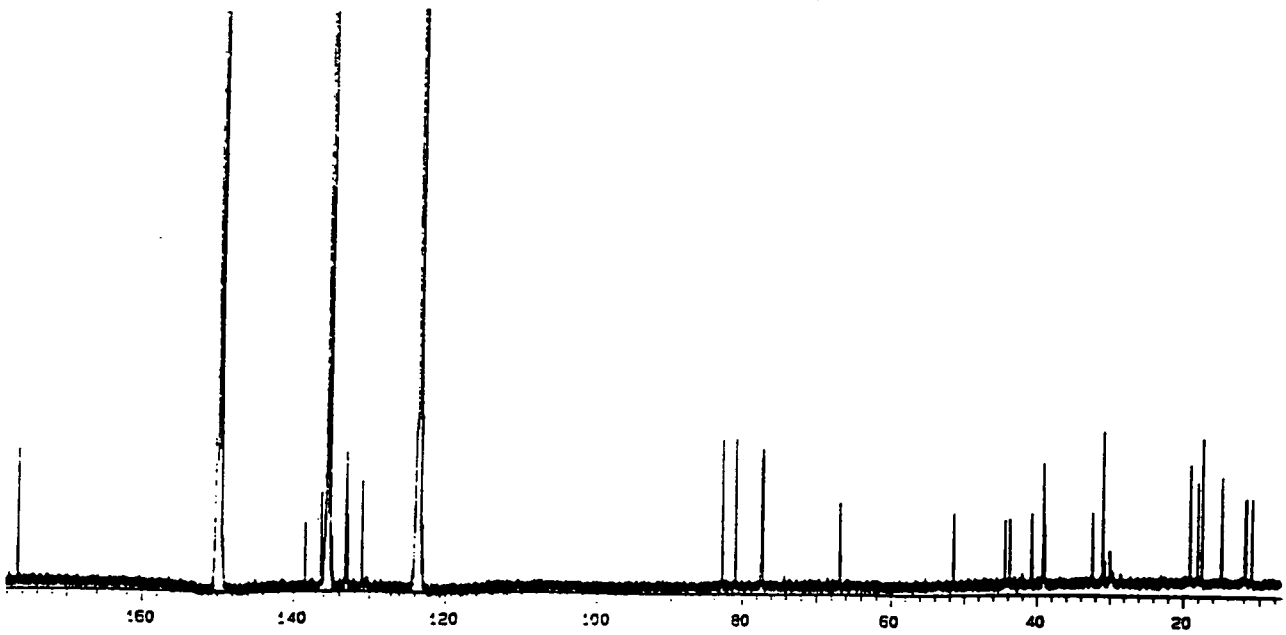


Fig. 3, ^{13}C nmr (100MHz) of methyl cubensate isolated from *Xylaria cubensis* fed with sodium [1-^{13}C, 2-$^{2}H_3$]-acetate.

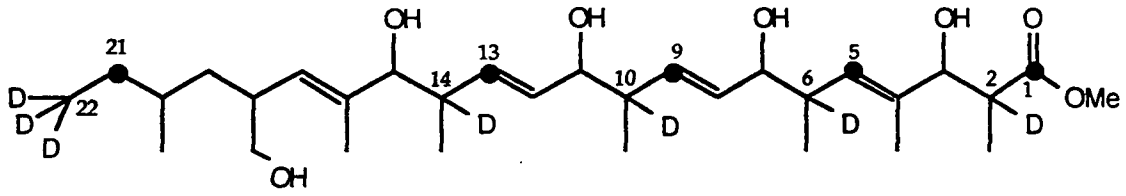


After successfully demonstrating that the eight methyl groups of cubensic acid are heavily enriched by L-[^{13}C-*methyl*]-methionine, the next experiment was designed to confirm that the backbone of cubensic acid is constructed from acetate units. Accordingly sodium [1-^{13}C,2-^{2}H$_3$]-acetate was administered to cultures of *Xylaria cubensis* at day 0 such that the final concentration was 16.15mM. The resultant cubensic acid was isolated on week 8 and the ^{13}C n.m.r. spectrum of the purified methyl cubensate is shown in Fig. 3. The Incorporation of sodium [1-^{13}C,2-^{2}H$_3$]-acetate into methyl cubensate is evident by the enhancement of the ^{13}C signals corresponding to carbon-1 of the acetate units, i.e. C1, C3, C5, C7, C9, C11, C13, C15, C17, C19 and C21, in the ^{13}C n.m.r. spectrum. The Incorporation is quite low at a magnitude of about 1.5 - 2 fold. The specific values are given in Fig. 4, calculated by normalising relative to C14 (CH).

	^{13}C/ppm	Na [1-^{13}C,2-^{2}H$_3$]-acetate		^{13}C/ppm	Na [1-^{13}C,2-^{2}H$_3$]-acetate
C1	176.67	1.50	C15	82.86	2.03
C3	80.96	1.91	C17	130.96	1.63
C5	133.14	1.46	C19	-	-
C7	77.37	1.89	C21	30.97	1.52
C9	-	-	OMe*	51.48	0.81
C11	77.26	1.97	C10*	43.74	0.95
C13	136.41	1.68			

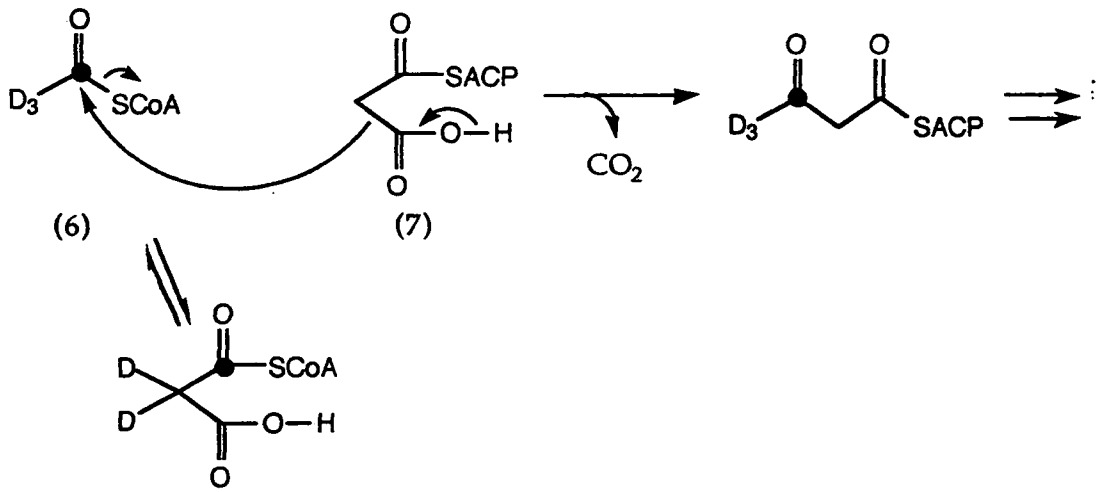
Fig.4, *unenriched C as standard

The ^{13}C signal corresponding to C9 is hidden by the C_5D_5N solvent signal and the signals corresponding to C18 and C19 coalesce, so calculation of these peak heights is difficult. The location of any residual deuterium isotope in cubensic acid was investigated by assessing the presence of β-shifted signals. Deuterium can only be retained at the following sites in methyl cubensate (5), i.e. C2, C6, C10, C14 and C22. This conclusion is drawn as the functionality along the backbone after the condensation has to be a ketone or reduced to an alcohol. If an elimination to a double bond has occurred or reduction to a fully saturated methylene, then deuterium will be lost.



(5)

The ^{13}C n.m.r. spectrum was analysed for deuterium induced β-shifts (^{13}C-C-D) of the signals corresponding to C1, C5, C9, C13, C21. This shift is normally to a lower frequency by a magnitude of between 0.01 - 0.1ppm per deuterium.[31] Due to the low incorporation of acetate into methyl cubensate, the β-shifted signal will be of low intensity. There was no apparent β-shift of the ^{13}C resonances of the carbons C1, C5, C9 and C13, but expansion of the ^{13}C n.m.r. spectrum, Fig. 5, shows the signal corresponding to C21 and to a lower frequency there are three small signals corresponding to a (a) ^{13}C-C-D, (b) ^{13}C-C-D_2 and (c) ^{13}C-C-D_3 component. The resonance corresponding to a β-shift induced by three deuterium atoms is shifted by 0.26ppm from the ^{13}C natural abundance signal of C21. There is a higher incorporation of sodium [1-^{13}C,2-$^{2}H_3$]-acetate into this C21 - C22 unit as it is the starter unit in the construction of the polyketide, i.e. malonyl-ACP (7) undergoes a decarboxylative condensation with acetyl-CoA (6) directly. Exchange of the deuterium is probably due to the conversion of acetyl-CoA to malonyl-CoA and then decarboxylation back to acetyl-CoA again.



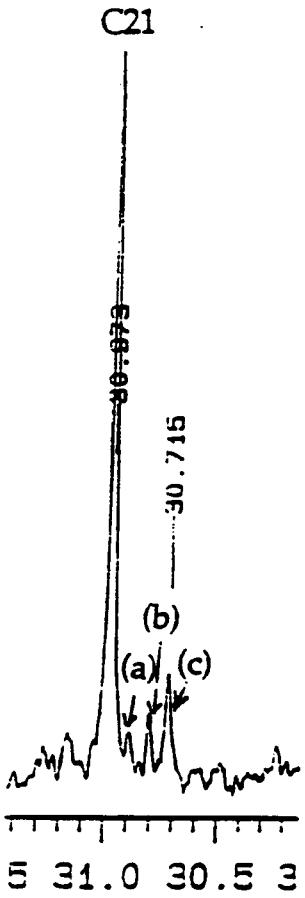


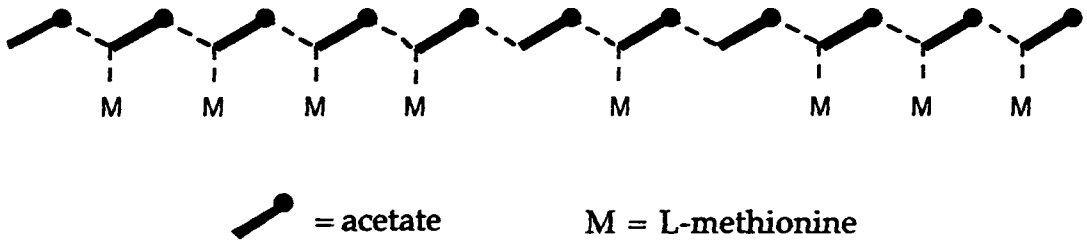
Fig. 5, expansion of Fig. 3 to show C21

The lack of evidence for incorporation of deuterium at the other sites, i.e. C1, C5, C9 and C13 may be due to the lack of sensitivity of the ^{13}C n.m.r. technique or in some cases the β-shift can be to a higher frequency or zero, particularly when the enriched carbon is a carbonyl,[32] e.g. C1, and therefore the presence of deuterium would not be observable in the ^{13}C n.m.r. In order to confirm or otherwise the presence of deuterium at the methine sites the sample was submitted for ^{2}H n.m.r. Unfortunately this result was inconclusive due to insufficient sample.

To sustantiate further the derivation of the polyketide backbone of cubensic acid from acetate units, sodium [$^{13}C_2$]-acetate was also administered to the cultures of *Xylaria cubensis* at day 0 and at a final concentration of 16.5mM. Cubensic acid was isolated at week eight, however the cubensic acid production was very low. The ^{13}C n.m.r. spectrum of the resultant methyl cubensate is shown in Fig. 6 (a). An expansion of this spectrum is

shown in Fig. 6 (b) which shows more clearly the ^{13}C-^{13}C coupling satellites flanking the natural abundance signals. Each acetate derived carbon, i.e. C1 to C22, possess the ^{13}C-^{13}C coupling satellites which indicate incorporation of intact acetate units along the backbone of cubensic acid. The incorporation of sodium [$^{13}C_2$]-acetate is approximately 0.5 - 1 fold.

These results demonstrate the incorporation of acetate into the backbone of cubensic acid and show that the methyl groups are enriched by L-[^{13}C-*methyl*]-methionine, demonstrating unambiguously that cubensic acid (8) is assembled in a classical fungal mode from eleven acetate and eight L-methionine units.



(8)

2.2 Feeding of N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate and L-[$^{13}C^2H_3$-*methyl*]-methionine to *Xylaria cubensis*

It is a challenge in polyketide biosynthetic studies to try and determine intermediates and possible advanced starter units in a biosynthetic pathway. There have been several reports of successful incorporations of isotopically labelled advanced biosynthetic intermediates into such metabolites, e.g. nargenicin from *Nocardia argentinensis*[26] (discussed in chapter 5) and dehydrocurvularin from wild type *Alternaria cinerariae ATCCC 11784*[25] (discussed in detail in chapter 1). These results reinforce the existence of a processive mode of assembly for polyketide metabolites. In fungal systems, however, there still remains a certain amount of ambiguity over the timing of the methylations by S-adenosyl-L-methionine (discussed in chapter 1). It seems likely that there are methyltransferase enzymes which are integral with the polyketide synthase and which mediate the methylations processively (scheme 1), i.e. as the polyketide chain is constructed, rather than a series of methylations occurring at the end of polyketide construction (scheme 2). There is no definitive experimental evidence to support either

Fig. 6 (a), ^{13}C nmr (125MHz) of methyl cubensate isolated from *Xylaria cubensis* fed with sodium [$^{13}C_2$]-acetate.

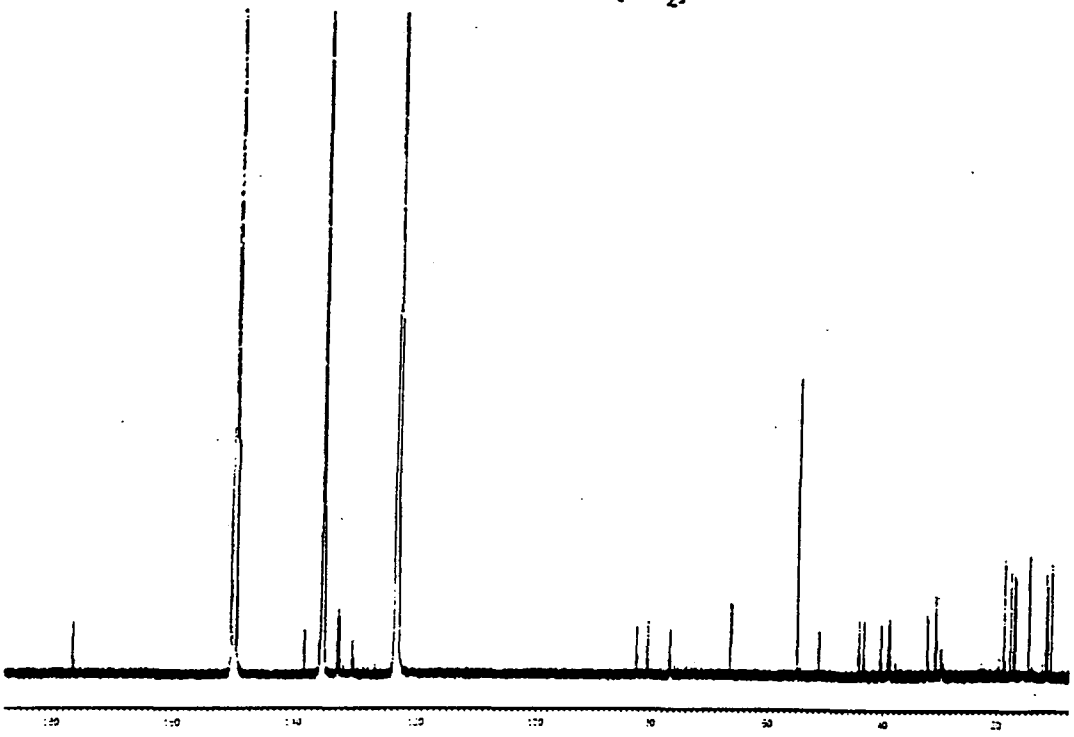
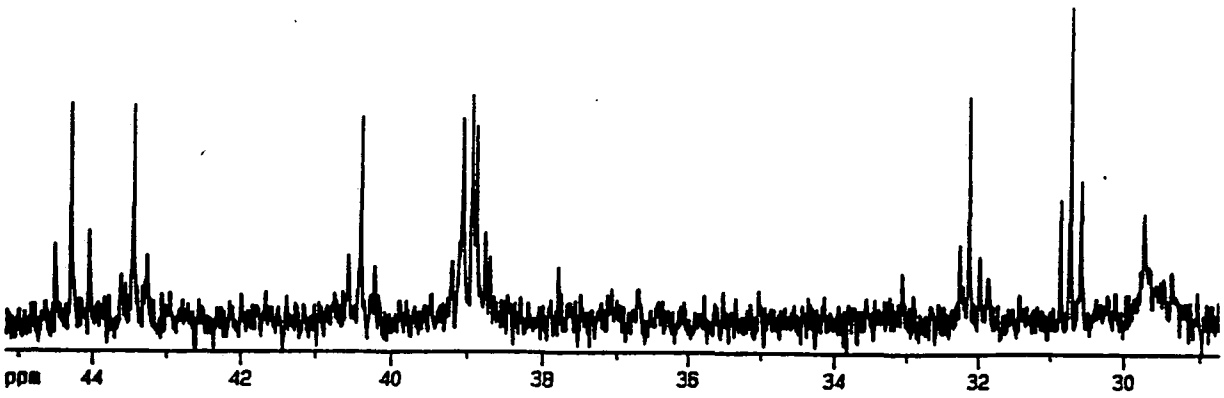
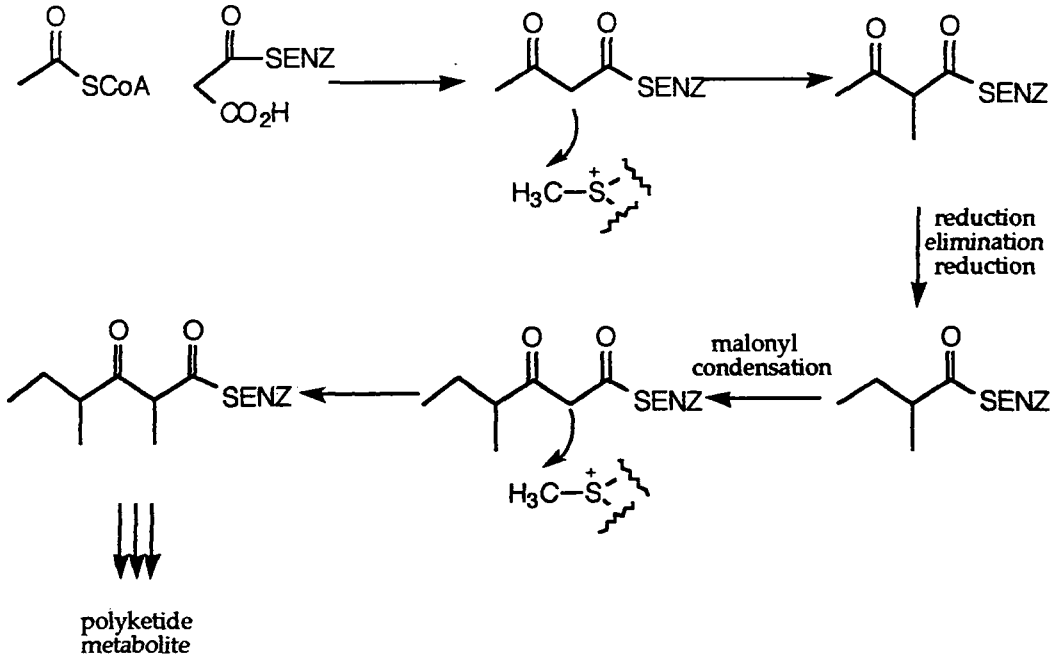


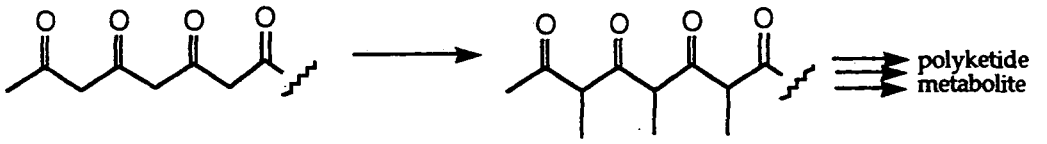
Fig. 6 (b), expansion of part of Fig. 6 (a).



methylation pathway as there has been no reported incorporations of partially assembled fragments which contain a methyl group derived from SAM into fungal polyketides.

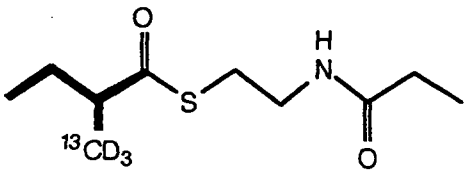


Scheme1

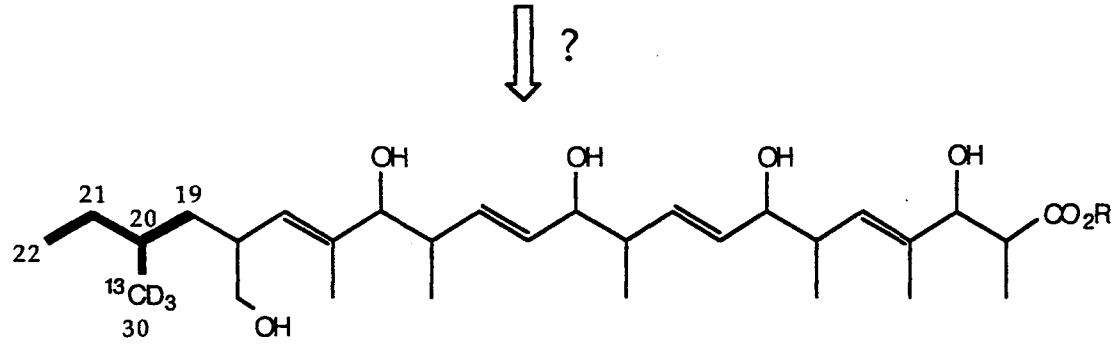


Scheme2

Cubensic acid, with its eight pendant methyl groups, is an attractive metabolite to try to test this processive methylation hypothesis. Our approach was to feed N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (9), a partially assembled methylated precursor, to *Xylaria cubensis*. Assessment of the resultant cubensic acid may determine if the substrate was utilised by the polyketide synthase and regiospecifically incorporated into the polyketide metabolite cubensic acid (10) at the positions C19, C20, C21, C22 and C30. [$^{13}C^2H_3$-*methyl*]-2-Methylbutyrate was administered as its N-acetylcysteamine thioester (NAC) as this unit has been used as a coenzyme A mimic (discussed in chapter 1) and aids transfer of the labelled acyl residue onto the appropriate thiol residues of the polyketide synthase.[24,117]



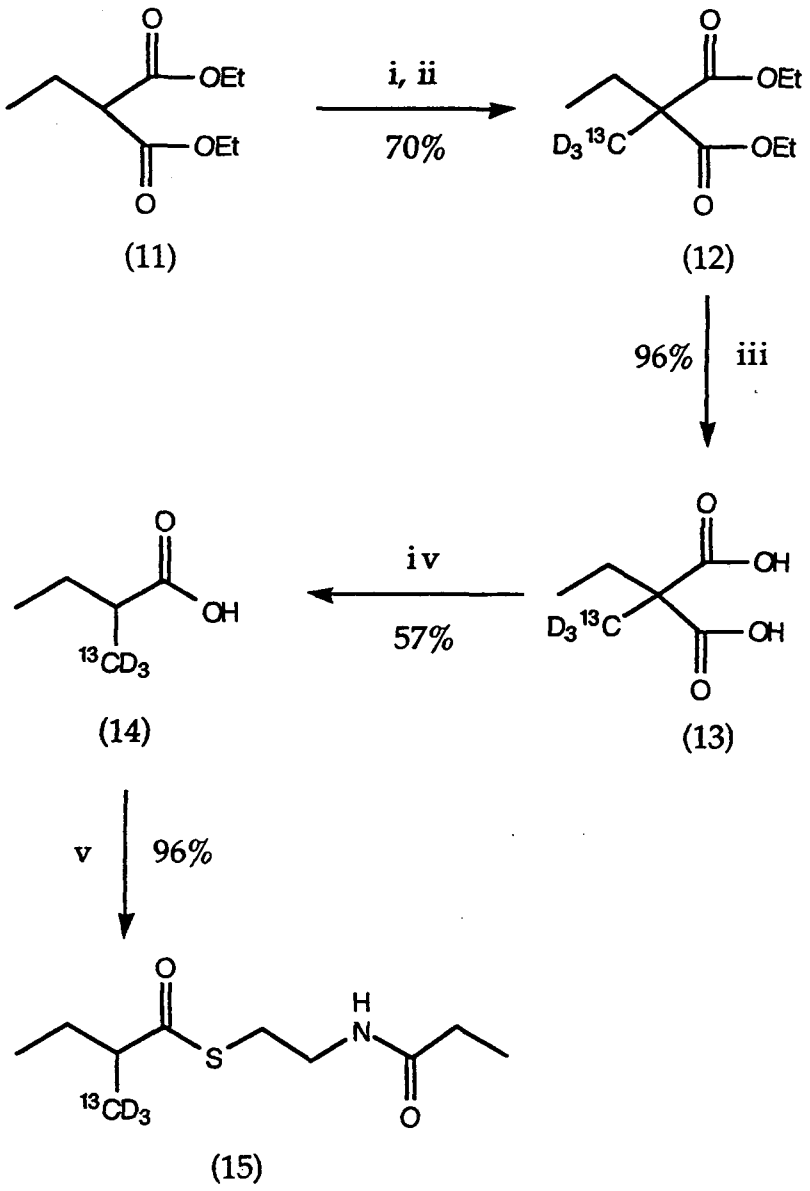
(9)



(10)

2.2.1 Synthesis of N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate

The synthesis of N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (15) is outlined in scheme 3. Diethyl ethylmalonate (11) was treated with sodium hydride to generate the sodium enolate, which was quenched with $^{13}C^2H_3I$. The resultant alkylated product (12) was hydrolysed to afford [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonic acid (13). Decarboxylation was mediated in a steel bomb to yield (14). Coupling[118] of (14) with N-propionylcysteamine[119] was performed in the presence of 1,3-dicyclohexylcarbodiimide and 4-dimethylaminopyridine to afford N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (15) in an overall yield of 38% from $^{13}C^2H_3I$.



Scheme 3. Reagents and conditions: i) NaH, THF, 0°C, ii) $^{13}CD_3I$, reflux 2h, iii) 5M KOH, reflux 24h, $HCl_{(aq)}$, iv) H_2O, 180°C 3h, $HCl_{(aq)}$, v) N-propionylcysteamine, DCC, DMAP, ether, r.t. 12h.

2.2.2 Feeding experiment

N-Acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate was administered on day 3 to *Xylaria cubensis* such that the final concentration was 5mM. Cubensic acid was isolated after week 8 and the ^{13}C n.m.r. spectrum of the purified sample is shown in Fig. 7. Particular attention was focused on assessing whether there was a regiospecific Incorporation of

Fig.7, ^{13}C nmr (125MHz) of methyl cubensate isolated from *Xylaria cubensis* fed with N-acetylcysteamine-[$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate.

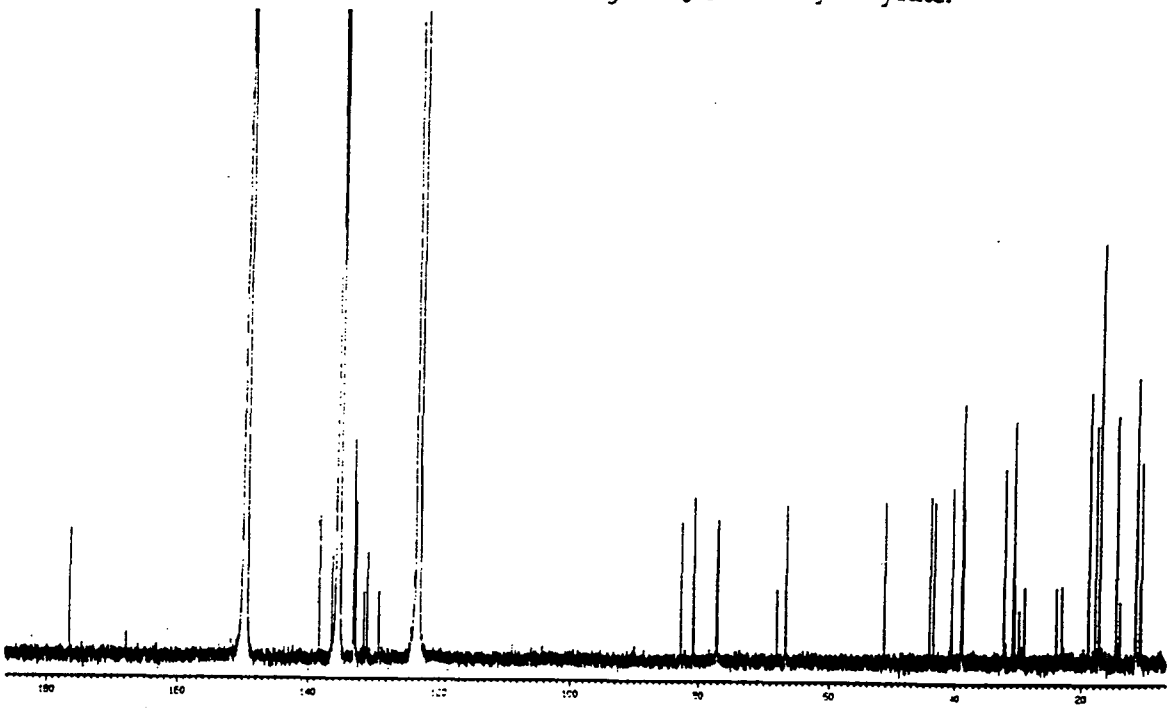
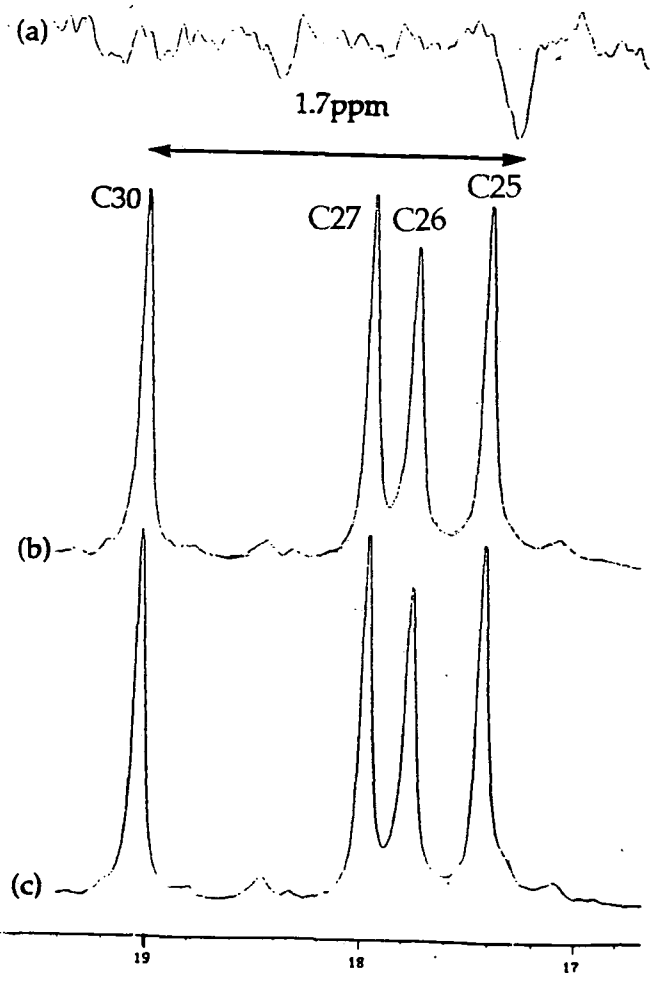


Fig. 8



[$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate into cubensic acid (10) at C19, C20, C21, C22 and C30. No Incorporation was evident in the $^{13}C\{^{1}H\}$-n.m.r. spectrum therefore the sample was sent to Edinburgh University for $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. analysis. The resultant spectrum is shown in Fig. 8. Spectrum (b) is a $^{13}C\{^{1}H\}$-n.m.r. spectrum and in this spectrum the $^{13}CD_3$ appears as a septet but in spectrum (c), the $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. spectrum, the septet collapses to a singlet and subtraction of the two spectra gives the difference spectrum, (a). Upon close examination of Fig. 8 (a), there is a signal which is shifted to a lower frequency by 1.7ppm from the C30 natural abundance signal. Typically deuterium induced α-shifts (^{13}C-D) can shift resonances between the range of 0.3 to 0.6ppm per deuterium.[31] So theoretically the maximum α-shift for three deuteriums could be 1.8ppm. Therefore the peak in the difference spectrum, Fig. 8 (a), could be due to an incorporation of $^{13}CD_3$ into the C30 methyl group. In order to confirm this deduction it was judged necessary to establish the typical magnitude of an α-shift corresponding to three deuteriums in cubensic acid at this carbon. So following from the L-[^{13}C-*methyl*]-methionine experiment, it was appropriate to feed L-[$^{13}C^{2}H_3$-*methyl*]-methionine and assess the magnitude of the α-shift on the methyl carbons.

The production of cubensic acid was low and when L-[$^{13}C^{2}H_3$-*methyl*]-methionine was fed to seven *Xylaria cubensis* cultures, no cubensic acid could be isolated. The feeding experiment was repeated on a larger scale, i.e. L-[$^{13}C^{2}H_3$-*methyl*]-methionine was administered on day 4 at a concentration of 2.42mM and the cultures were worked up on week 8. An impure sample was isolated and sent for $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. analysis. The resultant difference spectrum ($^{13}C\{^{1}H\}$-n.m.r. subtracted by $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. spectrum) showed the presence of eight signals corresponding to the eight methyl groups of cubensic acid which are derived from L-methionine, Fig. 9. Because of the impurities it was difficult to assign with confidence the natural abundance signals of methyl cubensate which therefore hampered the calculation of the α-shifts. So a sample of reference methyl cubensate was added to the n.m.r. tube to enhance the natural abundance signals.

From this experiment it could be ascertained that the α-shift corresponding to three deuteriums (i.e. $^{13}CD_3$) at C30 is about 1ppm. Therefore the signal evident in Fig. 8 (a), 1.7 ppm to lower frequency from the C30 natural abundance signal (in the [$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate feeding experiment) is too large a shift to be due to three deuteriums, and

therefore it must correspond to an impurity which is not associated with cubensic acid. This result forces the conclusion that [$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate was not incorporated into cubensic acid in an intact manner.

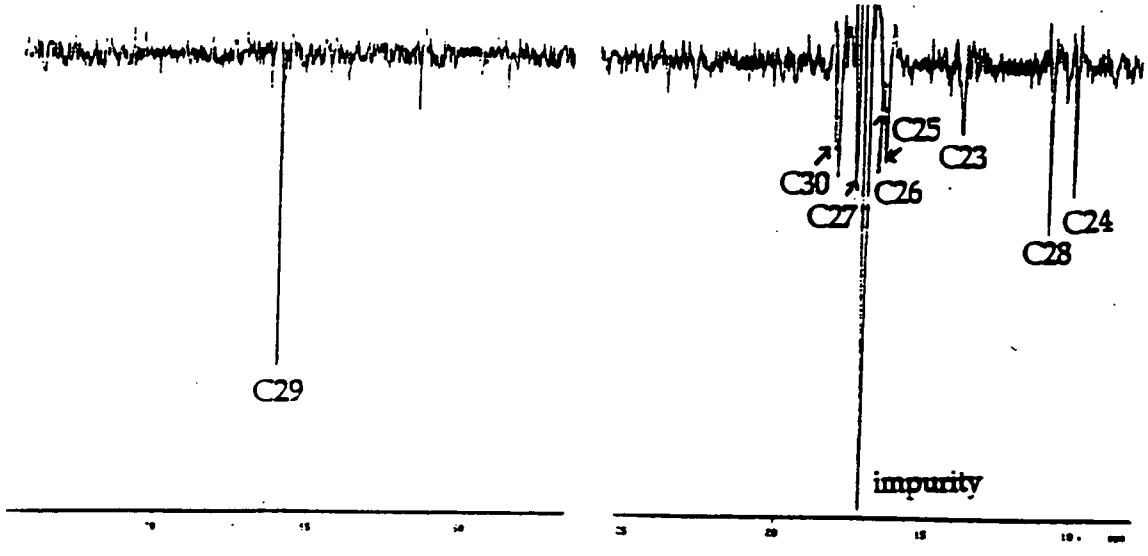
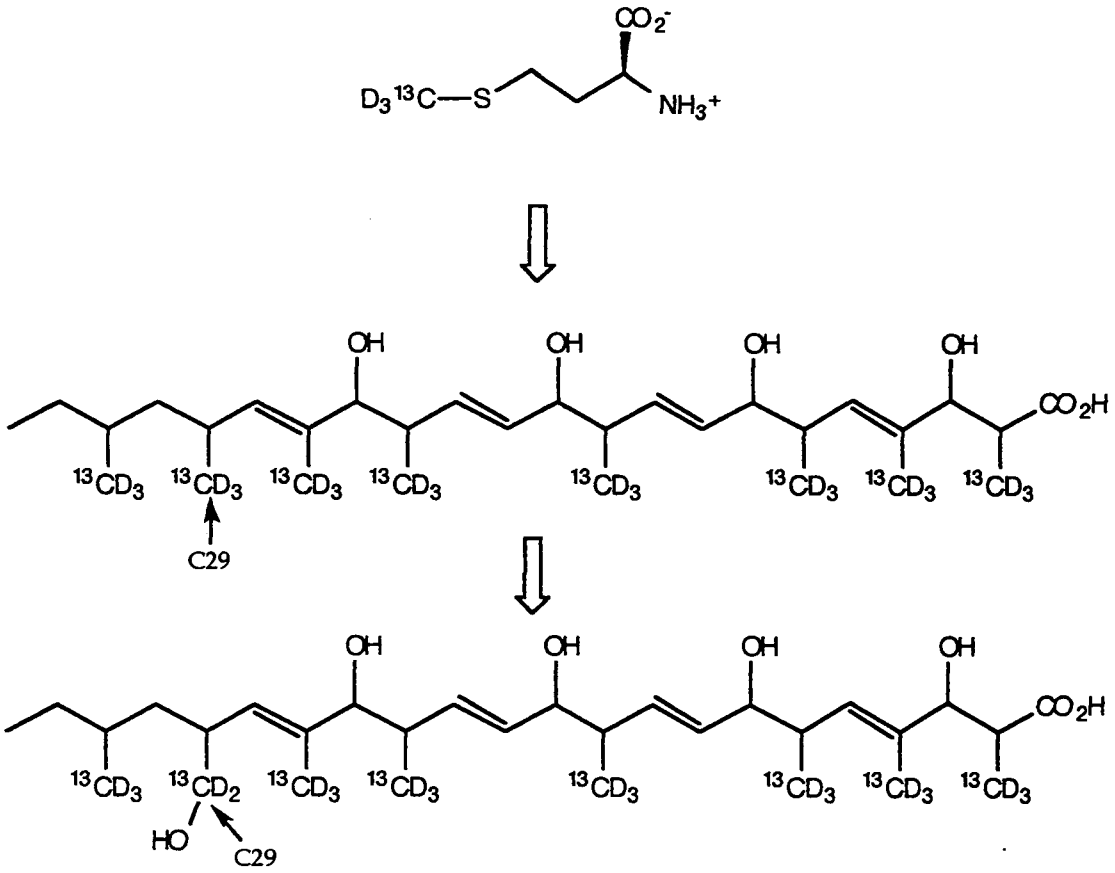


Fig.9, $^{13}C\{^{1}H, ^{2}H\}$-n.m.r. difference spectrum of methyl cubensate isolated from *Xylaria cubensis* when fed with L-[$^{13}C^{2}H_3$-*methyl*]-methionine.

Further information can be ascertained from the L-[$^{13}C^{2}H_3$-*methyl*]-methionine feeding experiment, because the difference spectrum in Fig. 9 shows the deuterium induced α-shifted signals of all the methyl carbons and that each methyl carbon possesses three deuteriums, except C29 which carries two deuteriums and an alcohol moiety. This proves that C29 is derived from L-methionine and the retention of the two deuteriums demonstrates that the hydroxylation is a post assembly modification, i.e. C29 hasn't been oxidised to an aldehyde moiety and then reduced to an alcohol during the construction of the polyketide backbone, scheme 4.



Scheme 4

3. FAST ATOM BOMBARDMENT (FAB) MASS SPECTRA ANALYSIS OF METHYL CUBENSATE

Methyl cubensate is a long chain fatty acid ester with five -OH groups pertruding from the backbone, and potentially it may have ionophoric properties, i.e. the stereochemistry of methyl cubensate is unknown but it's backbone may curve around and present the -OH groups in the right conformation to complex to metal cations, analogous to the polyether antibiotics and macrocyclic crown ethers more generally.

Fast atom bombardment (FAB) is a technique which is commonly used to obtain mass spectra from high molecular weight compounds (eg. peptides) and relatively unstable molecules. It can also be used for examining complex formation between metal cations and ionophoric ligands in solution.[120] A simple explanation of the technique is that there is a beam of fast moving neutral atoms (in this case xenon atoms) which is directed at the tip of a probe upon which is placed the sample (held in a glycerol matrix). The energy of the beam is dissipated in the surface layers,

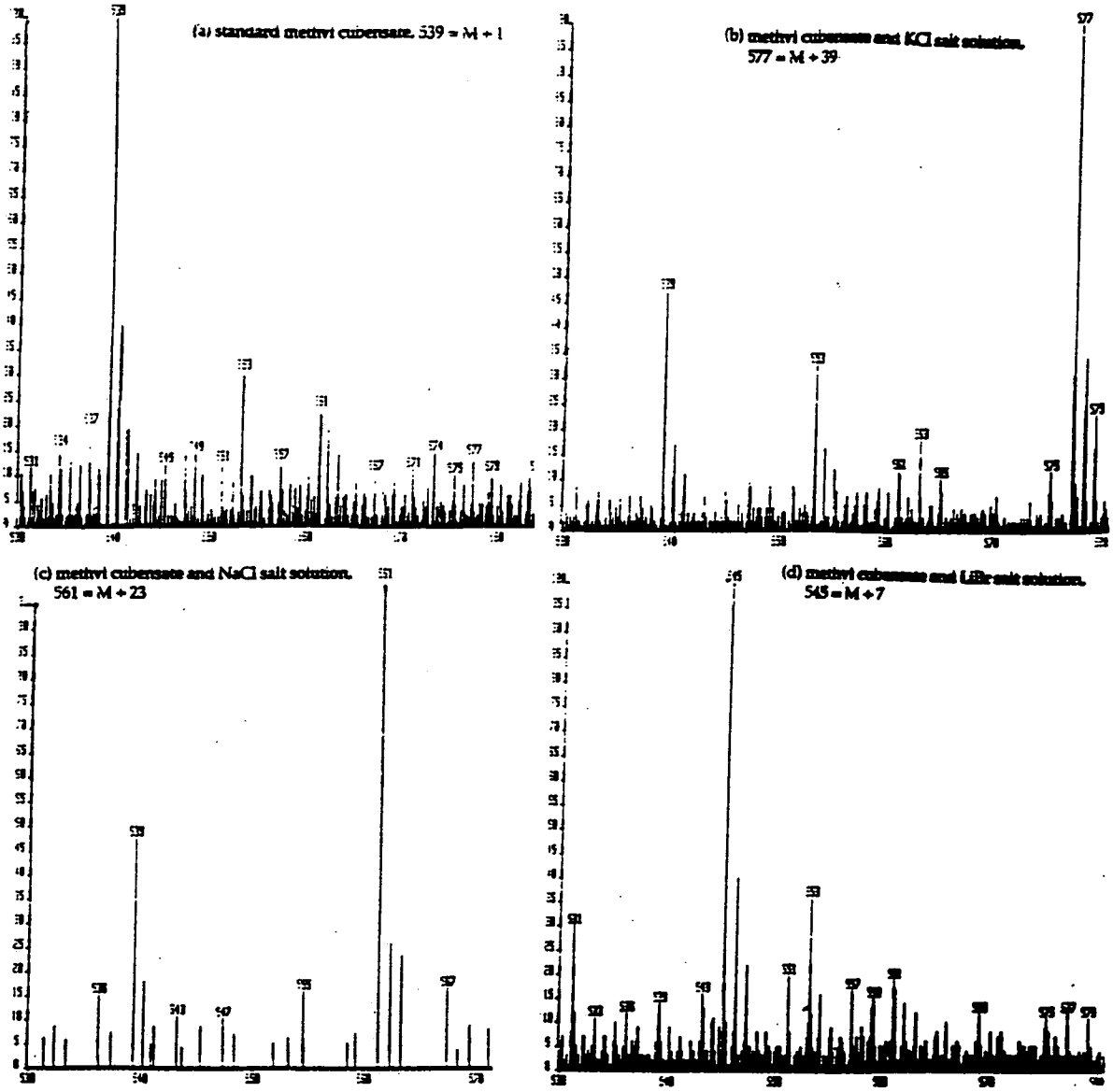
causing molecules to be released which are either positive, negative or neutral in charge. It is possible to detect either the positive or negative ions that are emitted (in this case positive ions were detected) by reversing the potential difference of the metal target to the source slits.

The aim of this study was to determine if methyl cubensate would complex to any of the following mono- and di-valent cations K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+} and Zn^{2+}. The following aqueous salt solutions of equal concentrations were prepared, i.e. KCl, NaCl, LiBr, $CaCl_2$, $MgSO_4$ and $ZnSO_4$. Methyl cubensate was dissolved in ethanol. The concentration of the salt solutions was higher than the methyl cubensate concentration in order to maximise the possibility of complexation. Six individual FAB mass spectra were recorded (run on a VG analytical 7070E mass spectrometer) by placing one drop of methyl cubensate solution on the tip of the probe and one drop of KCl solution and then a FAB mass spectra was recorded, the probe was wiped clean and the process was repeated with a different salt solution.

The resulting mass spectra are shown in Fig. 10. Fig. 10 (a) is the FAB mass spectra of a standard sample of methyl cubensate, which clearly shows the M + 1 peak of 539. Spectrum (b) results from a solution of methyl cubensate and KCl with a peak at 577, evident, corresponding to M + 39. Spectrum (c) is the mass spectrum of methyl cubensate and NaCl with a peak at 561 (M + 23) and finally (d) is of methyl cubensate with LiBr, and a peak at 545 (M + 7) is evident. The same treatment with the salt solutions of divalent cations gave only the methyl cubensate signal, i.e. M + 1 = 539.

This suggests that methyl cubensate can complex with the monovalent cations K^+, Na^+ and Li^+ but not with the divalent cations Mg^{2+}, Ca^{2+} and Zn^{2+}. These results are interesting and may suggest a mode of action for cubensic acid in promoting transportation of monovalent cations across cell membranes, c.f. transport antibiotics. This type of antibiotic makes membranes permeable to ions in two different ways. One group of antibiotics known as channel formers (e.g. gramicidin A, an open chain polypeptide) form channels that traverse the membrane and ions can enter through this channel at one side and diffuse through to the other side. The second group of antibiotics (e.g. valinomycin, a cyclic molecule made up of amino acid residues) are called carriers and they function by carrying ions through the hydrocarbon region of the membrane.[121]

Fig. 10. FAB mass spectra



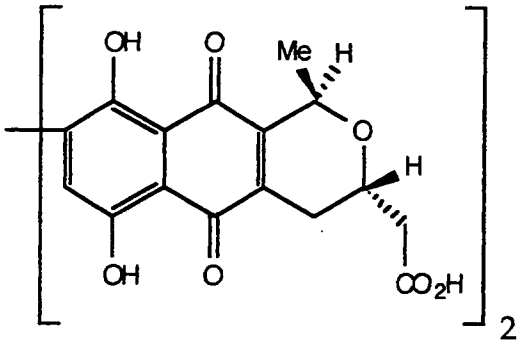
4. CONCLUSION

The biosynthetic origin of cubensic acid from acetate and L-methionine units has been firmly established. N-Acetylcysteamine-[$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate showed no incorporation into cubensic acid. This was shown by determining that the α-shift due to three deuteriums was about 1ppm, by feeding L-[$^{13}C^{2}H_3$-*methyl*]-methionine. Therefore the signal in the difference spectrum, Fig. 8 (a), of the N-acetylcysteamine-[$^{13}C^{2}H_3$-*methyl*]-2-methylbutyrate feeding experiment, at 1.7ppm to lower frequency from C30, must be due to an impurity, i.e. it is not associated with C30 of cubensic acid.

Cubensic acid is an interesting fungal metabolite because it possesses so many pendant methyl groups. It would clearly be interesting to know the absolute stereochemistry of cubensic acid and to assess further its potential ionophoric capabilities.

PART B
THE BIOGENESIS OF CUBENSIC ACID

In recent years genetic studies have dominated polyketide chemistry. The lead has been taken with research into the biosynthesis of the bacterial aromatic actinorhodin (16).[122] Actinorhodin is a dimeric isochromanequinone antibiotic isolated from *Streptomyces coelicolor* and is the only polyketide to have its gene cluster fully elucidated.

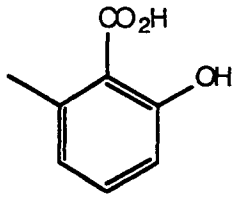


(16) actinorhodin

The six *act* genes which have been identified in the assembly of actinorhodin are located adjacent to each other in a cluster and they are believed to encode for the individual enzymes which are involved in polyketide assembly.[19] The individual genes are discrete and encode enzyme activities which are not covalently linked. This resembles the fatty acid synthase (FAS) systems of most bacteria and is known as a Type II polyketide synthase (PKS), i.e. an acyl carrier protein chaperones the assembling polyketide around a series of individual enzymes. PKS gene clusters associated with the generation of several other *Streptomyces* originating polyacetate aromatic compounds are emerging with a similar organisation.[122]

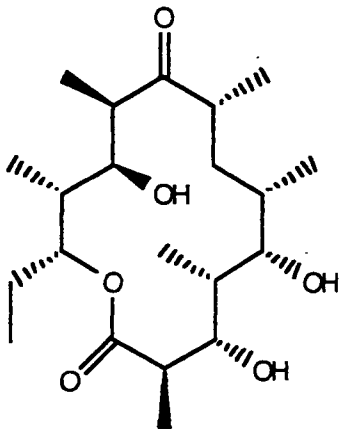
The breakthrough in locating the actinorhodin PKS genes stimulated the search for PKS genes in other organisms and there is currently a large amount of information available on the structure of different polyketide synthases. For example, in contrast to the PKS for actinorhodin biosynthesis, the PKS 6-methylsalicyclic acid (17) has been isolated and purified from *Penicillium patulum*. It is composed of a set of covalently linked enzyme activities which together form a large multifunctional protein.[123] This is a

classic Type I PKS (analogous to the Type I FAS of yeast and mammals, which consists of large multifunctional proteins with individual activities, including an acyl carrier protein, located in catalytic domains of the protein).[124]



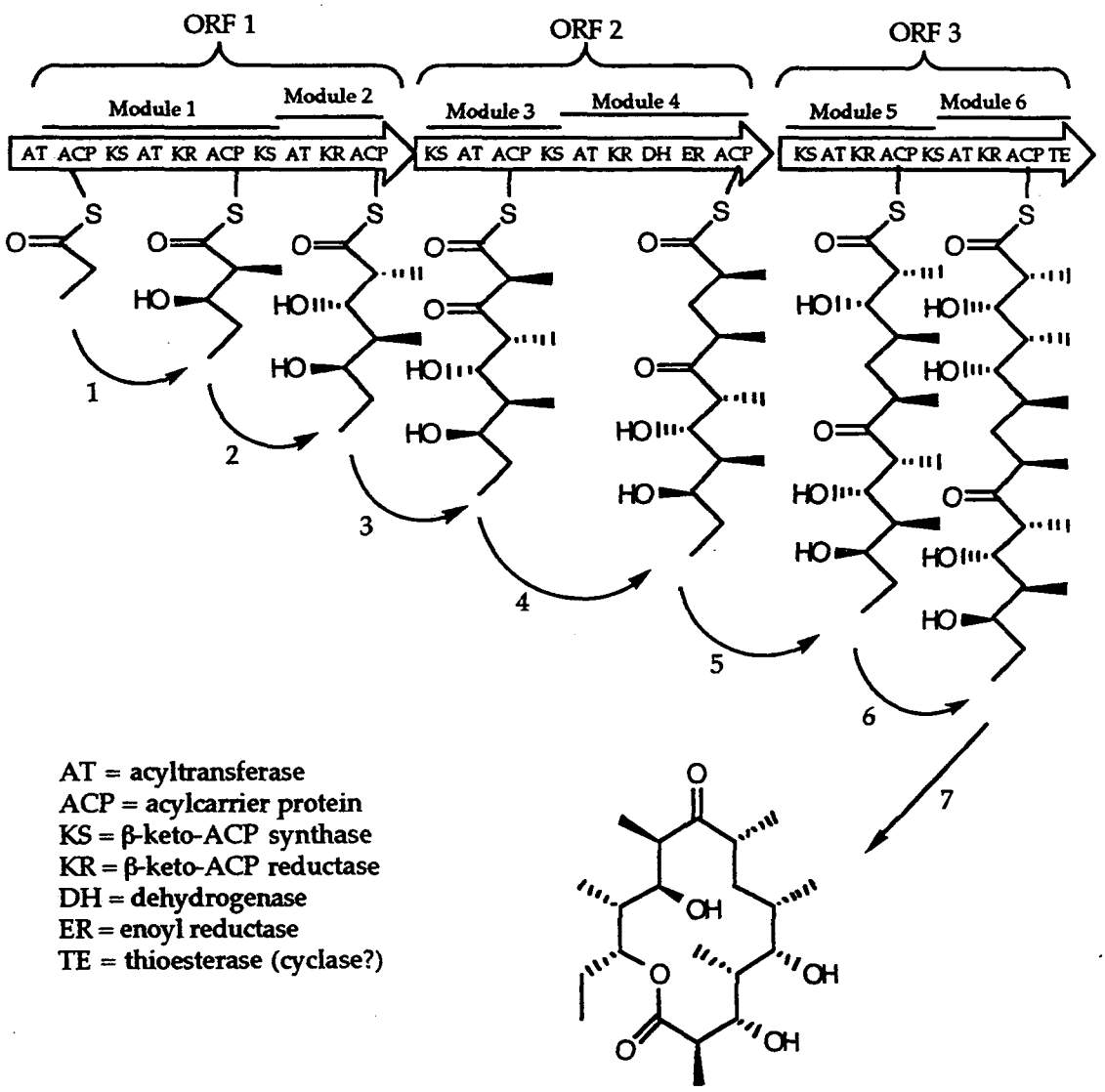
(17) 6-methylsalicyclic acid

The PKS genes of *Saccharopolyspora erythraea* which are responsible for the production of the erythromycin aglycone, 6-deoxyerythronolide B (18), have been identified. 6-Deoxyerythronolide B is biosynthesised from a propionyl-CoA starter unit and six methylmalonyl-CoA condensations and it is the first enzyme free intermediate to be secreted in the erythromycin biosynthetic pathway.



(18) 6-deoxyerythronolide B

Two research groups have cloned the entire PKS of *S. erythraea* and demonstrated that there are three open reading frames (ORF's) involved in the expression of 6-deoxyerythronolide B synthase.[125,126] A topographical illustration of the three ORF's activities is outlined in scheme 5.[124]

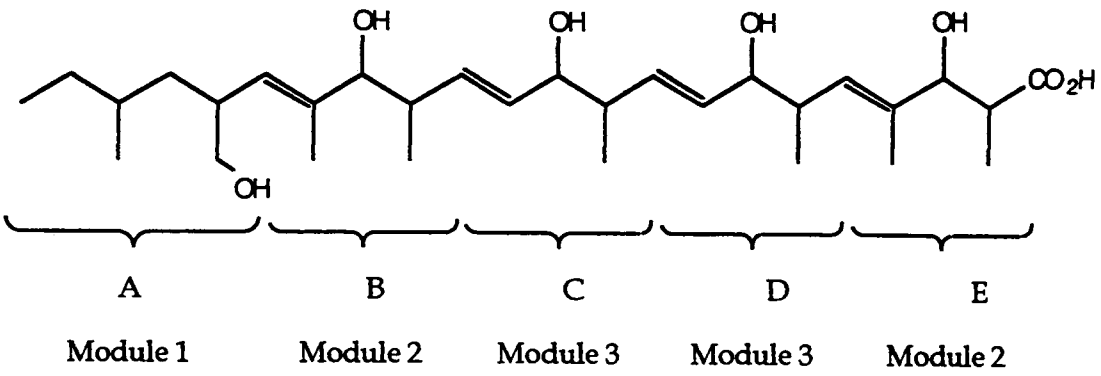


(17) 6-deoxyerythronolide B

Scheme 5

Each open reading frame encodes for a large multifunctional protein (i.e. DEBS 1, DEBS 2 and DEBS 3), each of which is thought to contain all the activities necessary for two of the six chain extension cycles, i.e. there exists an individual activity for each step carried out by the erythromycin PKS. The activities are organised into six modules which may be linked further into pairs,[127] and one pair of modules is expressed by one DEBS polypeptide. This type of PKS is classified as a Type I PKS due to the multifunctional nature of the proteins. Progress in this field has been rapid and the three DEBS multifunctional enzymes have been isolated and purified from *S. erythraea*[128] and the molecular basis of their actions is now under study.

Attention is currently focused on the genetic analysis of PKS genes from bacterial systems, particularly the commercially important Actinomycete antibiotic producers,[122,129] whereas the many fungal systems are relatively unexplored. The bacterial Type I PKS and the fungal Type I PKS may be very similar in structure and function. So applying a Type I PKS gene organisation to cubensic acid, similar to that of erythromycin A, would lead to five discernible biosynthetic regions, A - E, which could be further summarised as three enzymatic modules, 1 - 3, with modules 2 and 3 repeating themselves (as outlined in scheme 6).

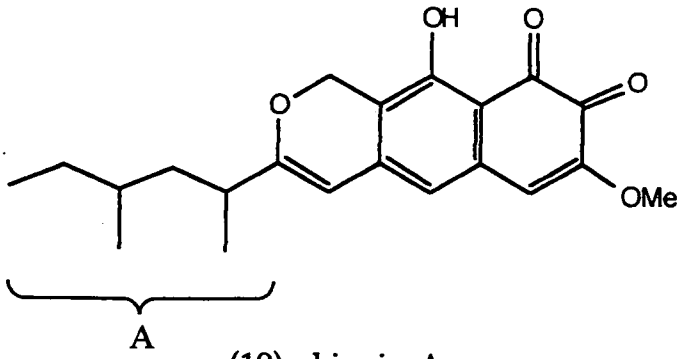


Module 1 KS, MT, KR, DH, ER, KS, MT, KR, DH, ER
Module 2 KS, MT, KR, DH, KS, MT, KR
Module 3 KS, KR, DH, KS, MT, KR

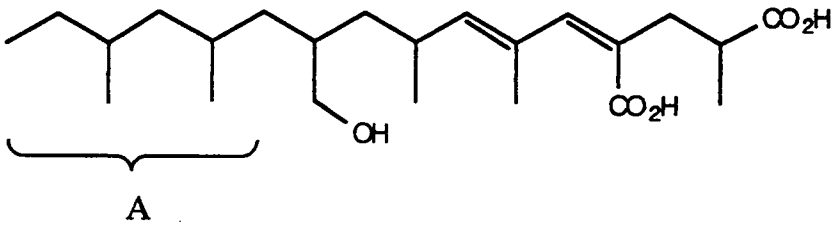
KS = β-ketoacyl synthase, MT = methyl transferase
KR = β-keto reductase, DH = dehydratase, ER = enoyl reductase

Scheme 6

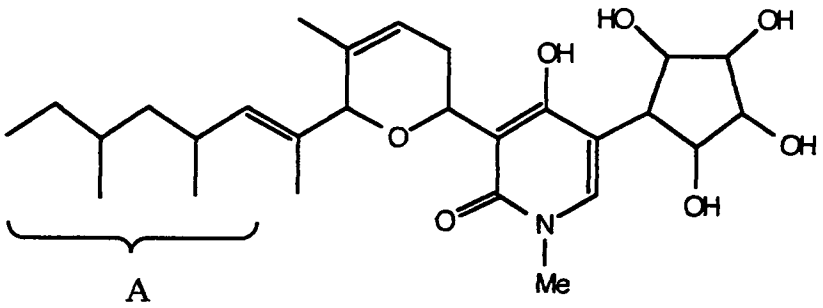
This hypothesis assumes that there exists a methyltransferase activity which is integral with the polyketide synthase and hence the methylations occur processively during polyketide construction. If this is the case then the cubensic acid PKS may be even larger and of greater complexity than the erythromycin PKS. However this remains to be tested. Even so it must be noted that the structure of region A (Module 1 in scheme 6) is a common 'starter unit' found in several fungal metabolites (the hydroxylation is a post assembly modification), e.g. obionin A (19) isolated from a marine fungus *Leptosphaeria obiones*,[130] radiclonic acid (3) isolated from *Penicillium sp.*[115] and funiculosin (20) isolated from *Penicillium funiculosum*.[131]



(19) obionin A

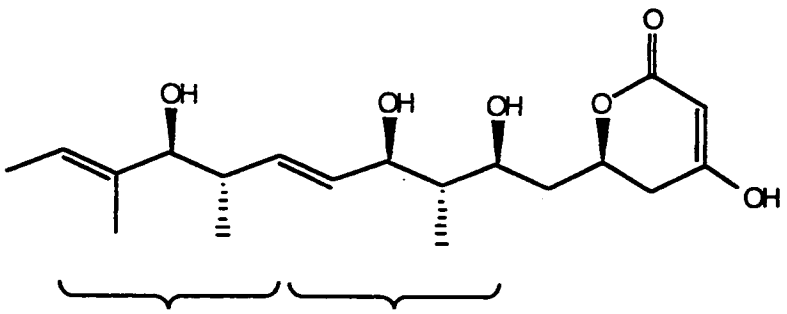


(3) radiclonic acid



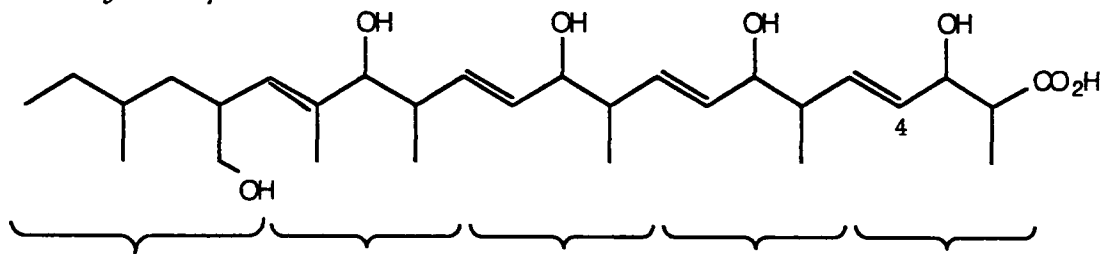
(20) funiculosin

The Modules 2 and 3 are also identifiable in ACRL Toxin I (21) isolated from *Alternaria citri*.[132]



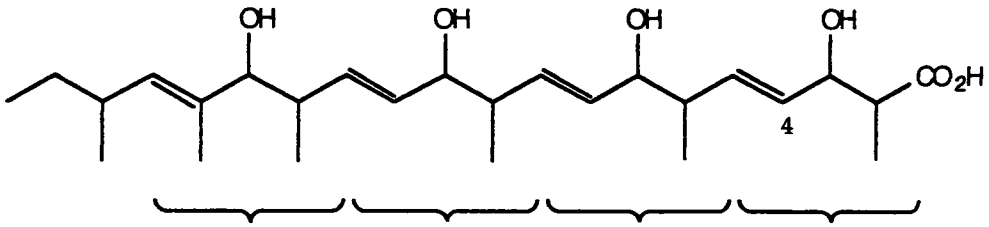
(21) ACRL toxin I

Similarly malaysionic acid (22) and camaronic acid (23) have been isolated from *Xylaria sp.*[133]



Module 1 Module 2 Module 3 Module 3 Module 3

(22) malaysionic acid



Module 2 Module 3 Module 3 Module 3

(23) camaronic acid

Both structures are very similar to cubensic acid, and by extending the module hypothesis, it can be interpreted that malaysionic acid (22) has the same modular sequence as cubensic acid but replaces the enzymatic Module 2 of cubensic acid at C4 with Module 3. The same is apparent for camaronic acid (23), but the starter unit is smaller in this metabolite than in cubensic acid.

From this analysis it can be suggested that there exists a gene cluster for Module 1 which has been replicated and selected through the evolutionary process such that region A is evident in several fungal metabolites. It would also be of considerable interest to determine the absolute stereochemistry of cubensic acid to establish, or otherwise, if the stereochemistry of the regions corresponding to Modules 2 and 3 (which are very similar and differ only by degree of methylation) is the same as that found in ACRL Toxin I from *Alternaria citri*, thereby giving evidence of modules evolving from common ancestral proteins.[127]

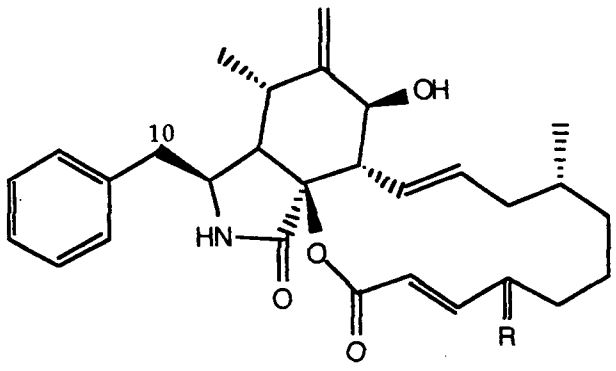
CHAPTER 5
CYTOCHALASIN D

1. INTRODUCTION

1.1 Cytochalasans

The cytochalasans are a class of biologically active fungal metabolites which were first isolated in 1966. They exert unique biological effects on mammalian cells in tissue culture, e.g. they inhibit cell movement and cytoplasmic cleavage. The isolation of these compounds resulted from the observation that culture filtrates of certain micro-organisms had a cytostatic action on cell cultures *in vitro*.[134]

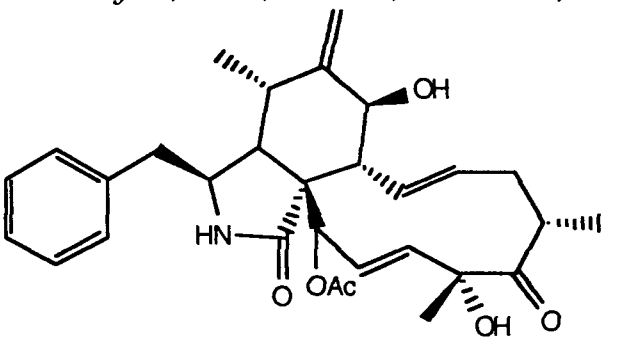
The isolation and characterisation of the first two substances of this new class of metabolites was achieved independently by Aldridge and Turner[135] at ICI and by Tamm[136] in Zurich. In the ICI laboratories, the metabolites named cytochalasin A (1) and B (2) were isolated from cultures of *Helminthosporium dematioideum* and in Zurich, Tamm isolated the metabolites named phomin (2) and dehydrophomin (1) from the micro-organism *Phoma*, strain S298. A direct comparison of these two discoveries showed that phomin was identical with cytochalasin B and dehydrophomin with cytochalasin A.



(1) R = O
(2) R = H, OH

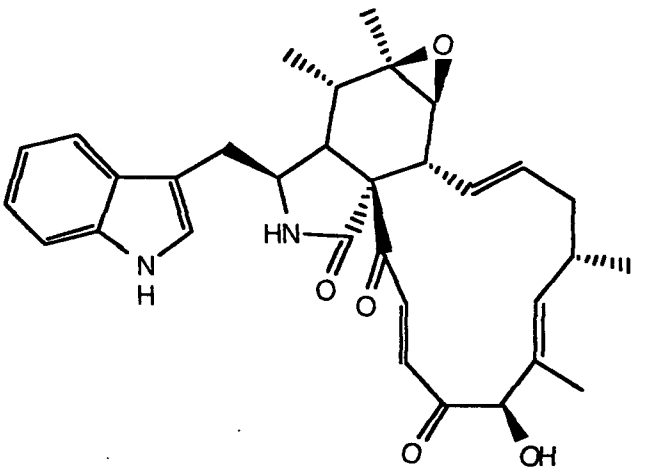
Since this initial discovery a great range of cytochalasans have been isolated and the class consists of five groups of compounds all with similar chemical structures, i.e.

[1] cytochalasins (Greek: *cytos*, a cell; *chalasis*, relaxation)



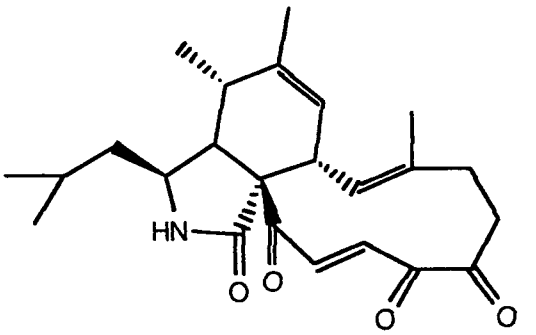
(3) cytochalasin D [137,138]

[2] chaetoglobosins



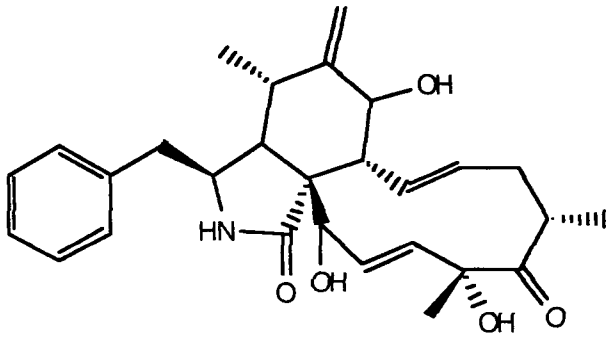
(4) chaetoglobosin A [139]

[3] aspochalasins



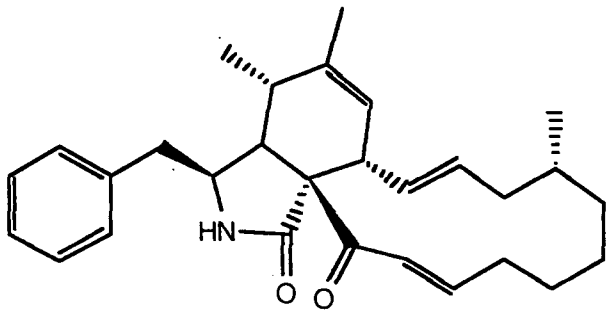
(5) aspochalasin A [140]

[4] zygosporins



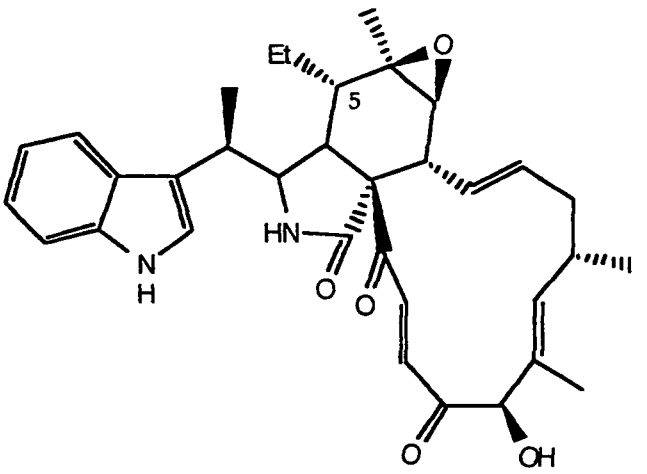
(6) zygosporin D [141]

[5] phomins



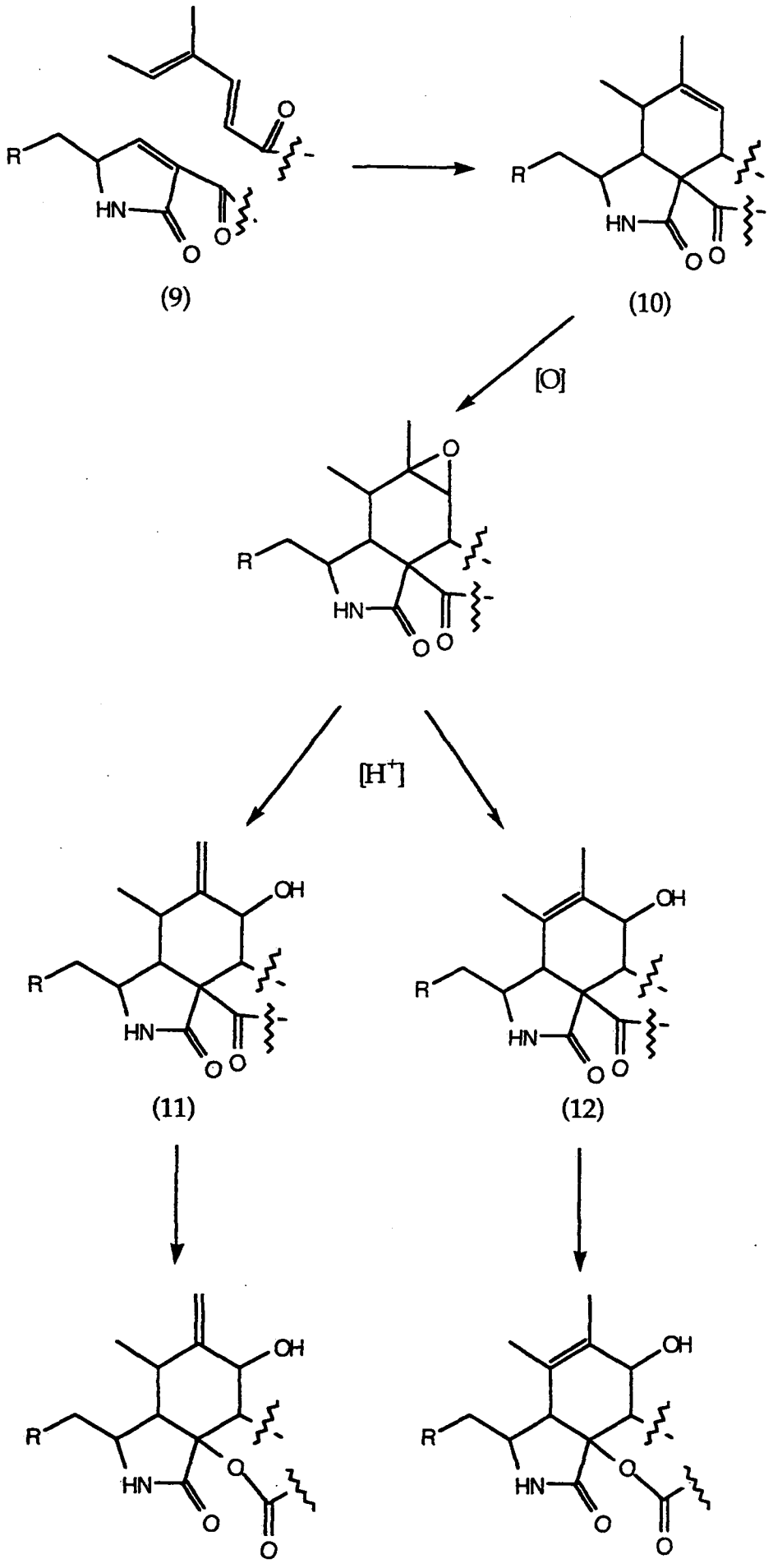
(7) proxiphomin [142]

All are characterised by a highly substituted isoindolinone ring system to which is fused a macrocyclic ring. The latter is either carbocyclic, a lactone or a cyclic carbonate. The structural differences occur at the 10 position where there exists a phenyl group in cytochalasins, indoyl group in chaetoglobosins and an isopropyl group in aspochalasins. Chaetoglobosin K (8) from *Diplodia macrospora*,[143] is an unusual member of the cytochalasans. It consists of a methyltryptophan residue and also possesses an ethyl group at C5 which may possibly originate from a propionate starter unit, c.f. aurovertins B and D and asteltoxin, metabolites produced by *Emericella variecolor*.[113,114]



(8) chaetoglobosin K

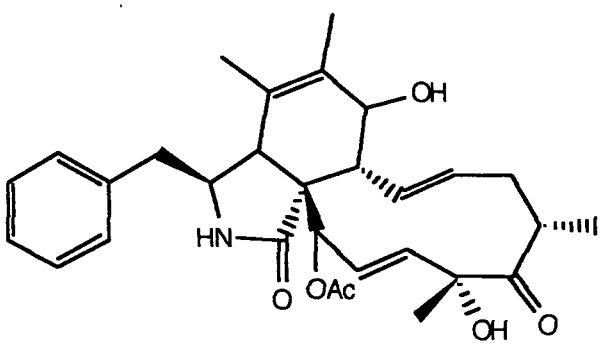
The closely related structures of the cytochalasan metabolites is suggestive of a similar biogenesis. A mechanism can be speculated originating with a polyketide derived chain which is linked to a phenylalanine (leucine or tryptophan) residue through an amide linkage to give the intermediate (9). This could then undergo a cycloaddition reaction to (10) which has the ring system present in some of the cytochalasans, e.g. aspochalasin A (5). Epoxidation of the double bond would yield another type of ring system which exists among the cytochalasans, e.g. chaetoglobosin A (4). The epoxides could then be transformed *in vitro* to a mixture of isomeric allylic alcohols (11) and (12), another cytochalasan ring system. The lactone system that exists in cytochalasin B (2), for example, arises by a Baeyer-Villiger type oxidation of the carbocyclic system[144] and likewise the cyclic carbonate system of cytochalasin E arises after a second oxidation.[145] The mechanism[146] (scheme 1) for the formation of the cyclohexane ring is speculative and there have been alternatives proposed.[147]



Scheme 1 :- A general hypothesis for the biosynthesis of cytochalasans

1.2 Cytochalasin D :- isolation

Both cytochalasin C (13) and D (3) were first isolated and characterised from the cultures of *Metarrhizium anisopliae* by Aldridge and Turner[138] and independently Minato et al.[137] described a new antibiotic isolated from the cultures of *Zygosporium masonii*. This metabolite was given the name zygosporin A. Aldridge and Turner[148] deduced that zygosporin A was in fact identical to cytochalasin D and this was established by an independent elucidation of the structure.[149]

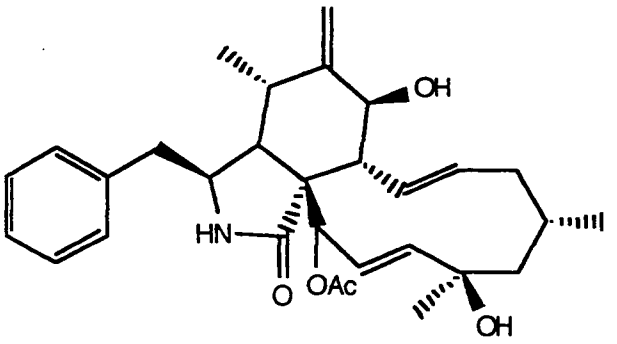


(13) cytochalasin C

More recently there has been the first report of the occurrence of a cytochalasan metabolite from a *Xylaria* species. Cytochalasin D (3) was isolated from the culture medium of *Xylaria cubensis*.[112] It was expected that the *Xylaria cubensis* mycelium would also yield cytochalasin D because large quantities of cytochalasins constitute the mycelium of *Hypoxylon terricola*.[150] This was however not the case and another unrelated metabolite, cubensic acid, predominated.

1.3 Cytochalasin D :- synthesis

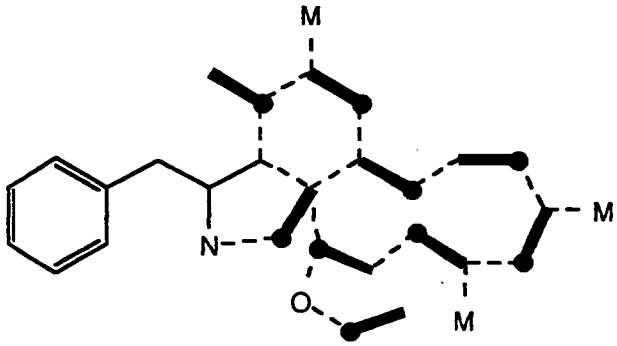
The combination of the challenging structural complexity and the unusual cytological properties of the cytochalasans has created an interest in their syntheses. The total synthesis of cytochalasin D (3) has been achieved[151] using a strategy that had been successful for other naturally occurring cytochalasans including cytochalasin H (14).[152,153] This approach involved the use of an intramolecular Diels-Alder reaction to form the reduced isoindolone, and large ring fragments, simultaneously.



(14) cytochalasin H

1.4 Cytochalasin D:- biosynthesis

Extensive biosynthetic studies[154-159] utilising both radioactive and stable isotopes have been performed on cytochalasin D from *Zygosporium masonni* and the origin of each carbon atom is now well established. Cytochalasin D is generated from nine acetate units, eight of which are coupled in a head to tail fashion to form the C_{16} polyketide moiety, three L-methionine derived methyl groups and one L-phenylalanine unit.

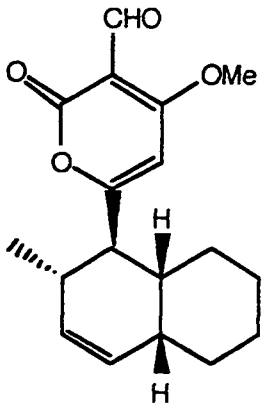


= acetate M = L-methionine

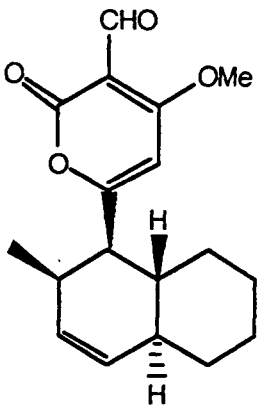
An interesting and controversial feature of the cytochalasans is the mechanism of formation of the six membered ring . An attractive theory involves a biological intramolecular Diels-Alder reaction, a process which has chemical precedence, and would create the correct relative stereochemistry around the ring. The involvement of a biological Diels-Alder reaction has been proposed in the biosynthesis of a variety of secondary metabolites, particularly decalin-based compounds such as betaenones,[160] and mevinolin[161] and in dimeric indole alkaloids such as

the presecamines.[162] A further example is ikarugamycin, an acyltetramic acid antibiotic isolated from *Streptomyces phaeochromogenes var. ikaruganensis*[163] and lachnanthocarpone, a major pigment found in the seed pods of *Lachnanthes tinctoria*.[164]

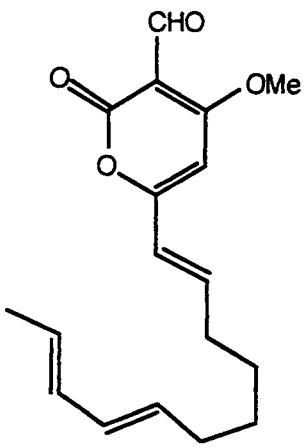
It has however proved difficult to obtain conclusive experimental evidence for the participation of a biological intramolecular Diels-Alder. All other alternative mechanisms have to be eliminated and proof that the reaction actually takes place in a living organism is not straightforward. There has however been some very promising results obtained from work on several metabolites in which a Diels-Alder reaction can be implicated. One example is solanapyrone A (15), a phytoxin isolated from *Alternaria solani.*



(15)
solanapyrone A



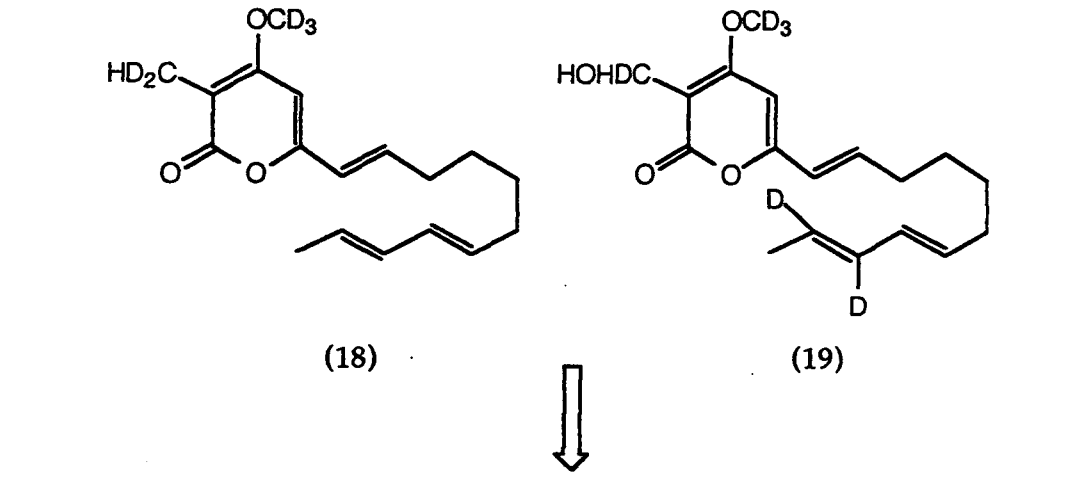
(16)
solanapyrone D



(17)

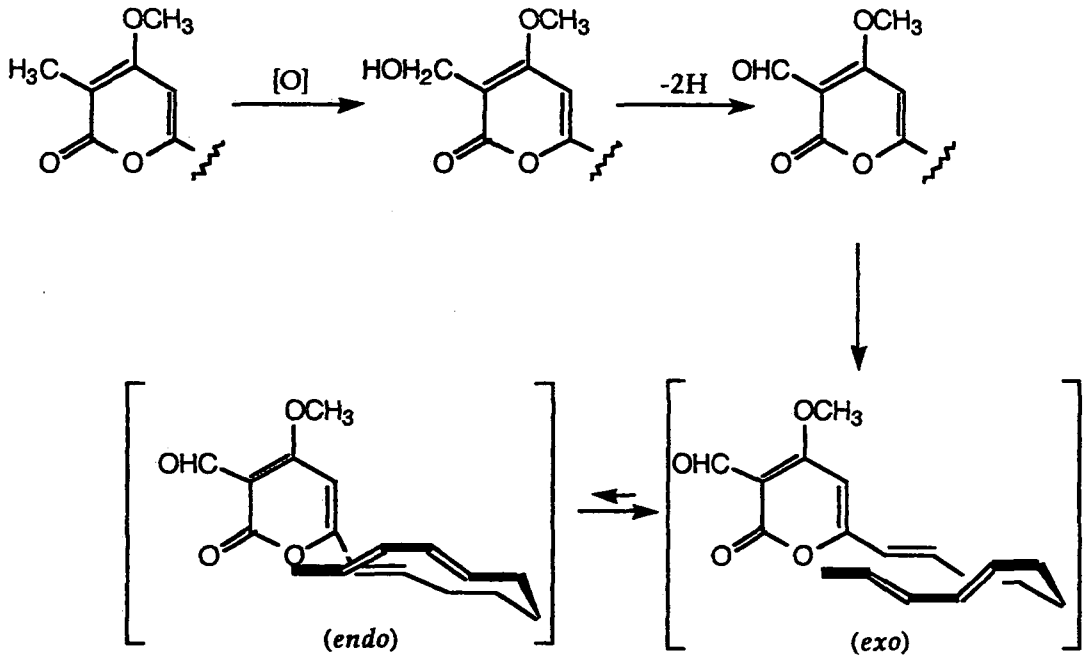
Extensive feeding experiments have been performed[165] and show that solanapyrone A (15) is derived from eight acetate units and two L-methionine derived methyl groups. After feeding sodium [2-^{13}C,$^{2}H_3$]-acetate, the sites of retained deuterium are consistent with an intramolecular Diels-Alder reaction. Additional evidence[166] came from the isolation of an optically active diastereoisomer, solanapyrone D (16). This co-exists with solanapyrone A as a minor component in the cultures of *Alternaria solani,* and could clearly arise from an *endo* cycloaddition of the postulated triene intermediate (17) in a biological Diels-Alder reaction. In more than 30 decalin polyketides, solanapyrones represent the only example of a putative triene intermediate which would not possess any chiral centres and this may be the reason for the biological production of two diastereoisomers, the *endo* and *exo* cycloaddition products. Most recently

the solanapyrone story has gone one step futher and the deuterium labelled precursors (18) and (19) have been regiospecifically incorporated[167] into solanapyrone A providing strong experimental support for the operation of a biosynthetic Diels-Alder reaction. The pathway of incorporation into the solanapyrones is outlined in scheme 2.[167]



(18) (19)

(15) solanapyrone A

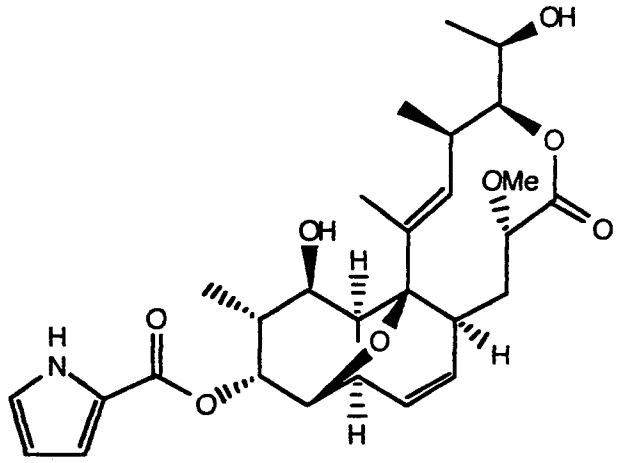


intramolecular Diels-Alder reaction

(16) solanapyrone D (15) solanapyrone A

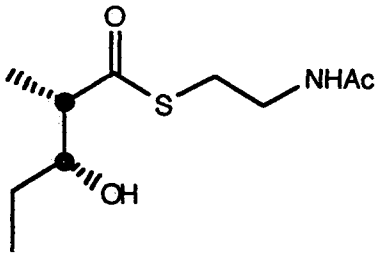
Scheme 2

Further evidence of an intramolecular biological Diels-Alder reaction comes from nargenicin (20), an antibiotic isolated from *Nocardia argentinensis.*

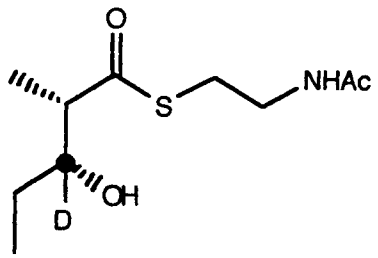


(20) nargenicin

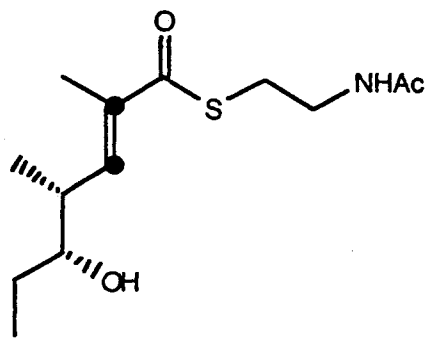
Cane *et al.*[26] have been successful in incorporating the following ^{13}C and deuterium labelled substrates (21a, b, c, d) into nargenicin, in a regiospecific manner.



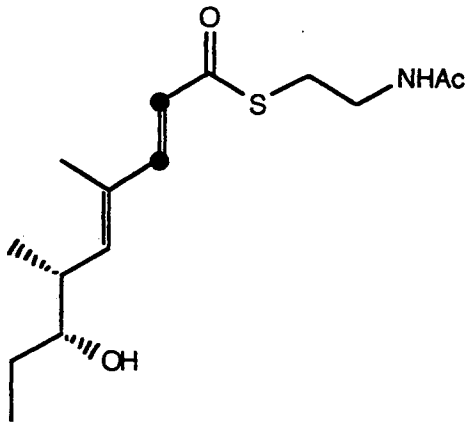
(21a)



(21b)

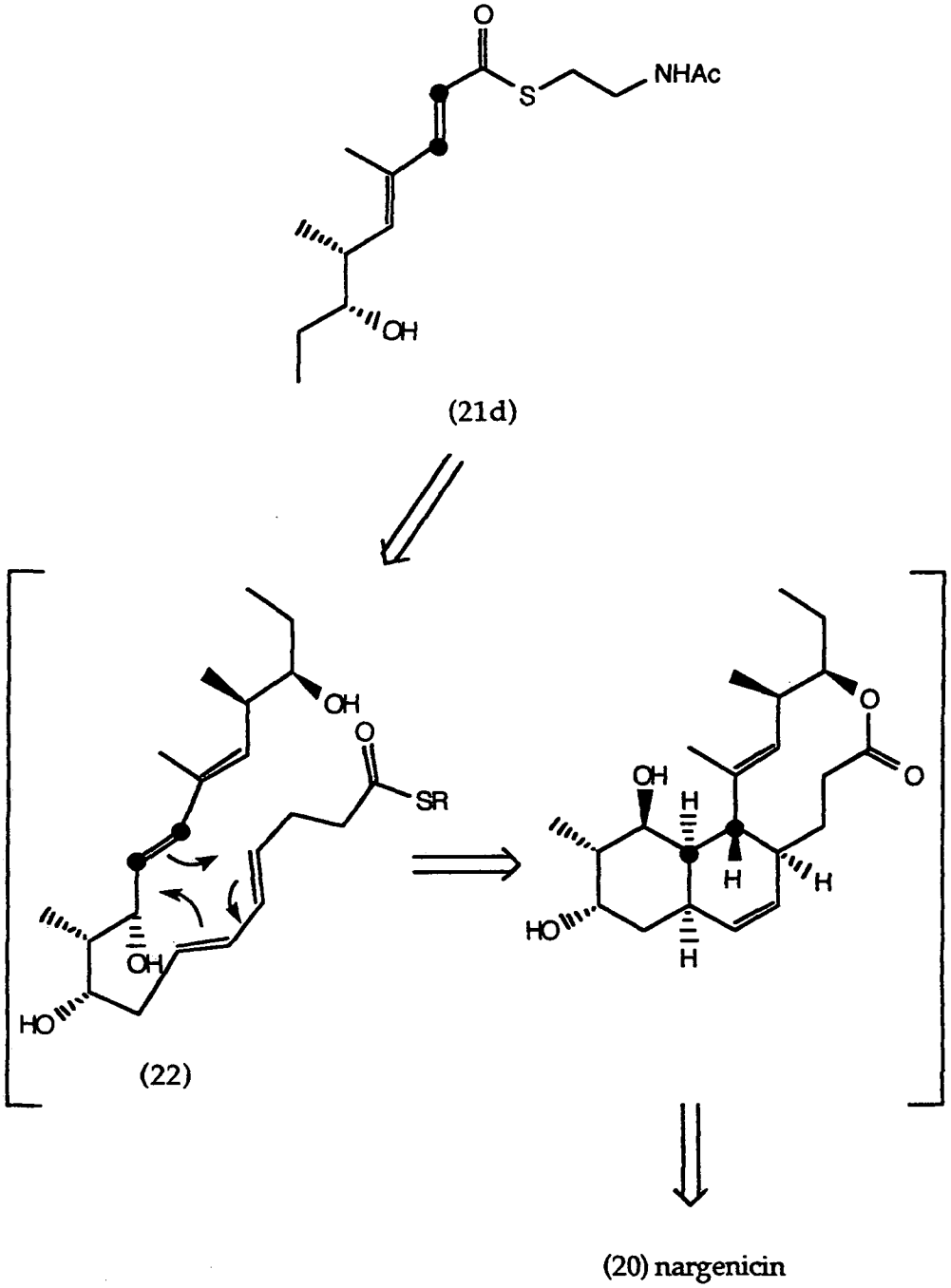


(21c)



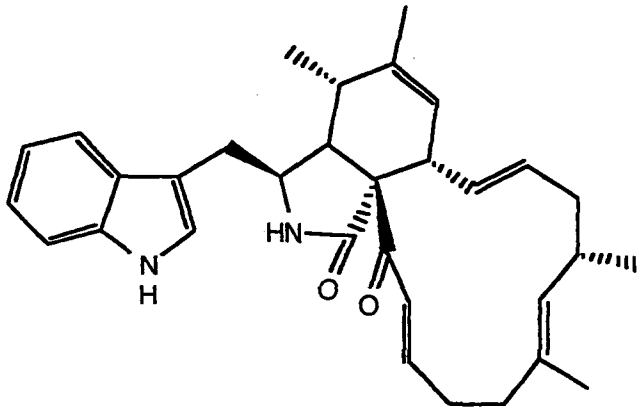
(21d)

These incorporations provide evidence for a processive model of polyketide assembly and chain elongation in which the stereochemistry and oxidation level are adjusted prior to each successive condensation. Also the incorporation of the tetraketide precursor (21d) is consistent with an intermediate which undergoes an intramolecular Diels-Alder reaction to form the octalin ring system in the biosynthesis of nargenicin (scheme 3).



Scheme 3

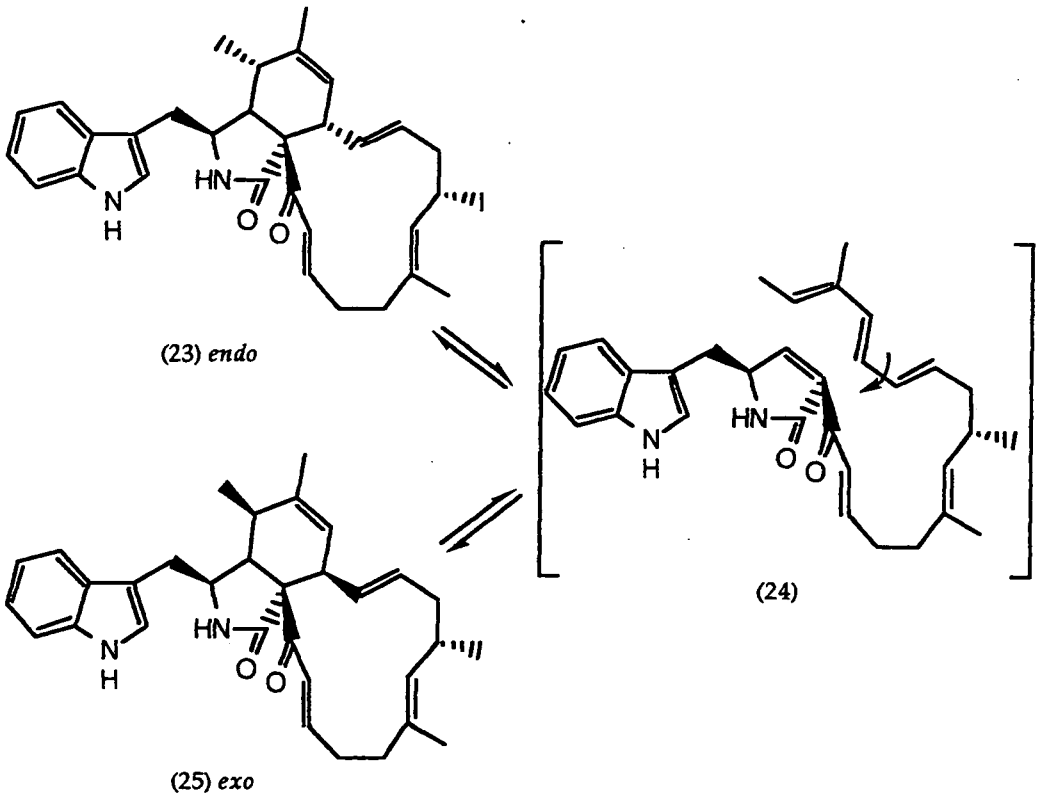
Biosynthetic studies on chaetoglobosin A (4)[168,169] isolated from *Chaetomium subaffine* have revealed indirect evidence of a biological Diels-Alder reaction to form the cyclohexane ring. This was obtained by isolating a non-oxidised derivative called prochaetoglobosin I (23) by adding a cytochrome P-450 inhibitor to the cultures of *Chaetomium subaffine.*



(23) prochaetoglobosin I

It is believed that this compound is one of the first intermediates in post-cyclisation and that a variety of modifications produce a number of analogues.[170,171] The occurrence of non-oxidised derivatives, eg. proxiphomin, have also been reported from cytochalasins.[172]

In order to provide unambiguous evidence for the existence of an intramolecular Diels-Alder reaction, studies involving a retro-Diels-Alder reaction on prochaetoglobosin I were performed in order to obtain the putative hexaene (24)[169] (scheme 4). In the event the reaction produced not the hexaene intermediate (24), as expected, but a new compound (25) which was shown to be the diastereoisomer of prochaetoglobosin I. With this result it was suggested that the *endo* and *exo* transition states do not possess a sufficient energy difference and therefore both adducts (23) and (25) result. In a biological system however, the enzyme responsible for the cycloadditions may stabilise the *endo* transition state and therefore only prochaetoglobosin I (23) is generated.

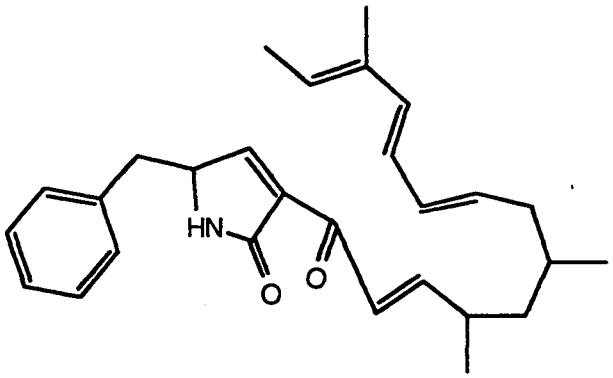


Scheme4

2. RESULTS AND DISCUSSION

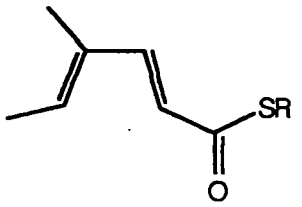
2.1 Aim

If an intramolecular Diels-Alder reaction is assumed in the biosynthesis of cytochalasin D then a possible intermediate could be (26).



(26)

Cyclisation and then post assembly modifications such as oxidation would then generate cytochalasin D. It is envisaged that incorporation of the early putative intermediate (27) into cytochalasin D would support an intramolecular Diels-Alder cycloaddition during the biosynthesis of cytochalasans. It would also provide evidence of a processive mechanism of methylation as the polyketide chain is constructed, i.e. the existence of a methyltransferase enzyme integral with the polyketide synthase, mediating the methylations at some point before the next condensation.



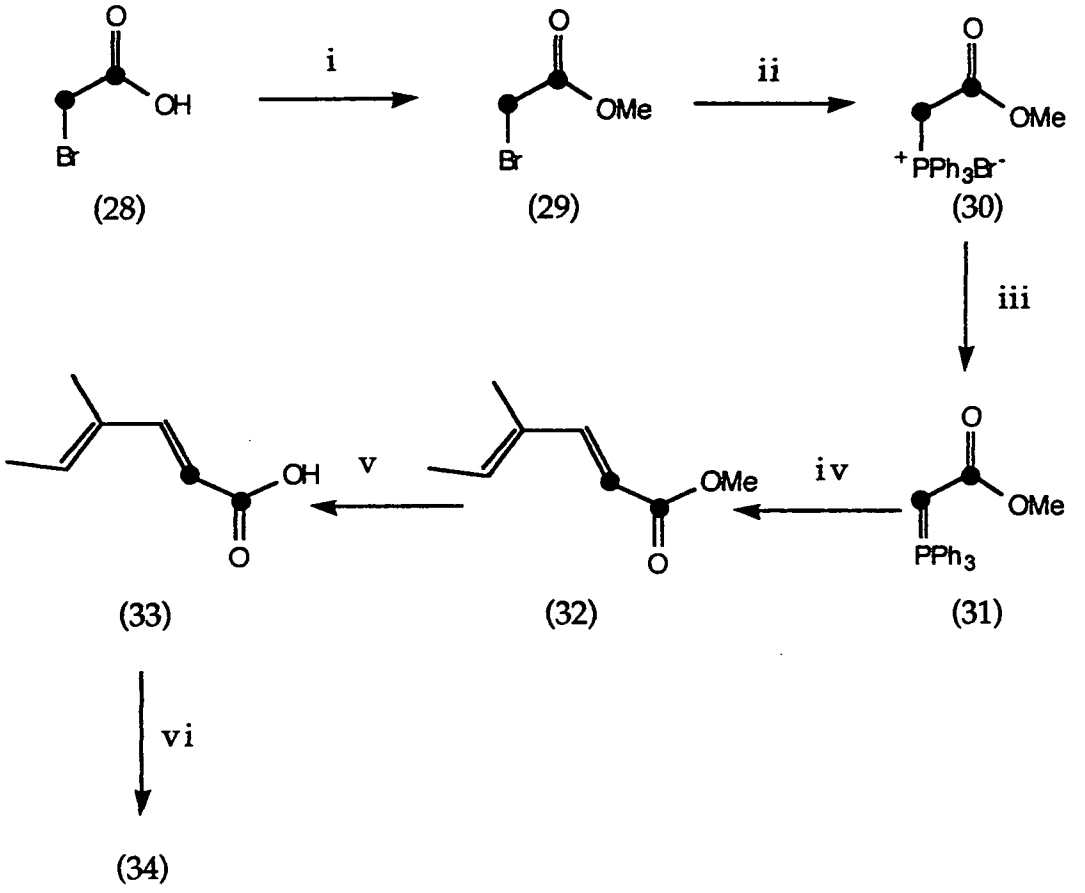
(27)

Therefore it was judged appropriate to prepare the N-acetylcysteamine thioester (NAC) of (27) with a ^{13}C-^{13}C label, to incubate to cultures of *Xylaria cubensis* in order to assess Incorporation into cytochalasin D. Substrates are generally fed[24,117] as their NAC thioesters as this unit mimics coenzyme-A (discussed in chapter 1) and aids transfer of the labelled acyl residue onto the appropriate thiol binding site on the polyketide synthase. The use of a double ^{13}C-^{13}C label was employed to provide greater sensitivity in the detection of low levels of enrichment, and to help identify the presence of an intact unit, observable by the ^{13}C-^{13}C coupling satellites around the natural abundance signal in the ^{13}C n.m.r. spectrum.

2.2 Synthesis of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate

The approach to the synthesis of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate is outlined in scheme 5. [$^{13}C_2$]-Bromoacetic acid (1g) was methylated to afford (29) which was carried on directly to the next reaction by treatment with triphenylphosphine in acetonitrile. Collection by filtration of the precipitated (30) and treatment with aqueous sodium hydroxide solution afforded (31). A Wittig reaction with tiglic aldehyde generated methyl (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate which was hydrolysed to the corresponding carboxylic acid (33). The final reaction involved the coupling[118] of (33) with N-acetylcysteamine[119] in the presence

of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to afford N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (34) in an overall yield from [$^{13}C_2$]-bromoacetic acid of 8.7%. A small amount, about 4%, of (Z,E)-material was evident in the ^{13}C n.m.r. spectrum. N-Acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate was unstable and decomposed over several months in storage at 4°C.



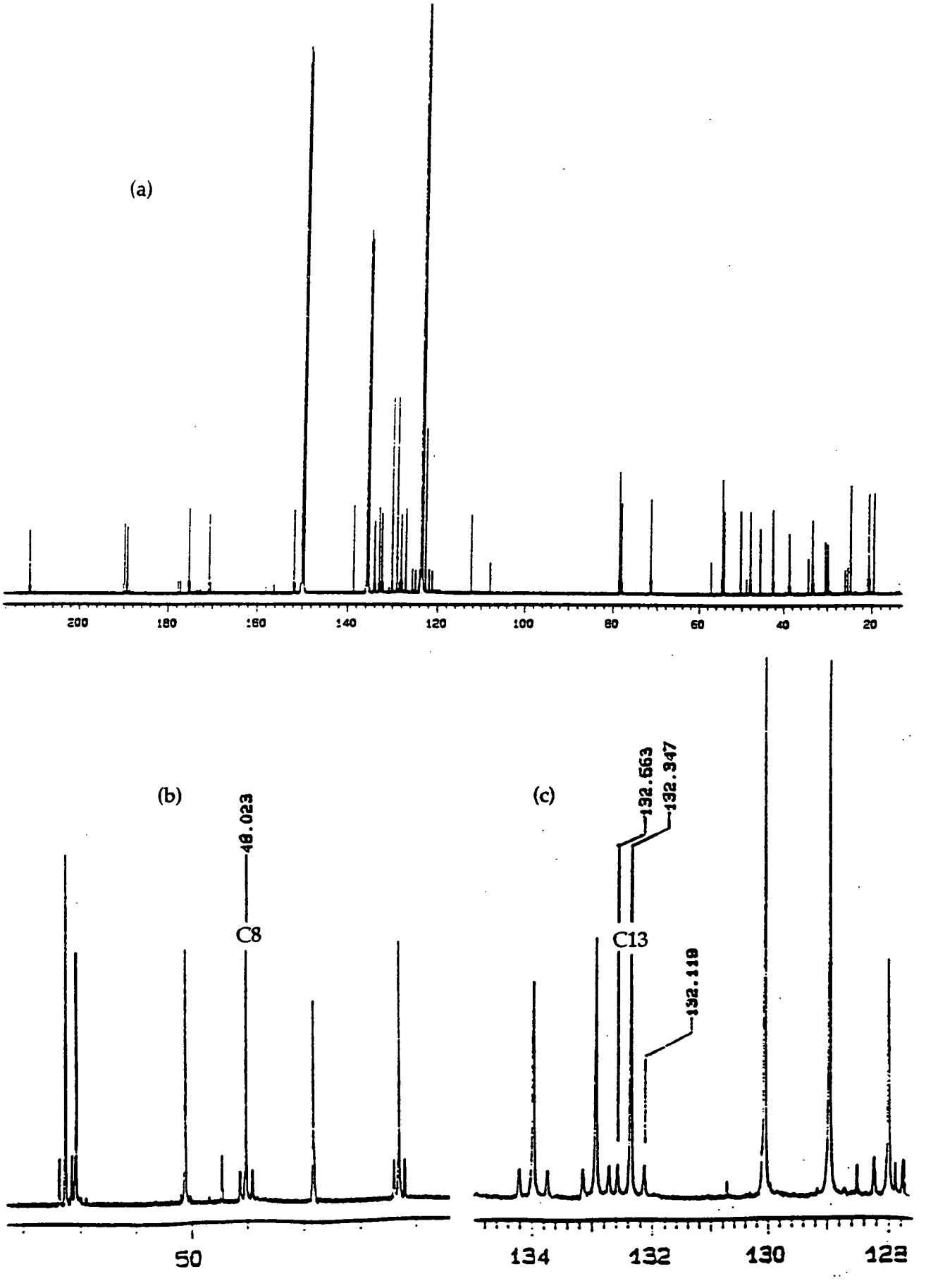
(28) (29) (30) (31) (32) (33) (34)

Scheme 5 :- Reagents and conditions: i) CH_2N_2, ii) Ph_3P, acetonitrile, rt 3d, iii) aq. NaOH, iv) *trans*-2-methyl-2-butenal, ethanol, reflux 24h, v) 1M NaOH, methanol, rt 16h, vi) DCC, DMAP, N-propionylcysteamine, ether, rt 12h.

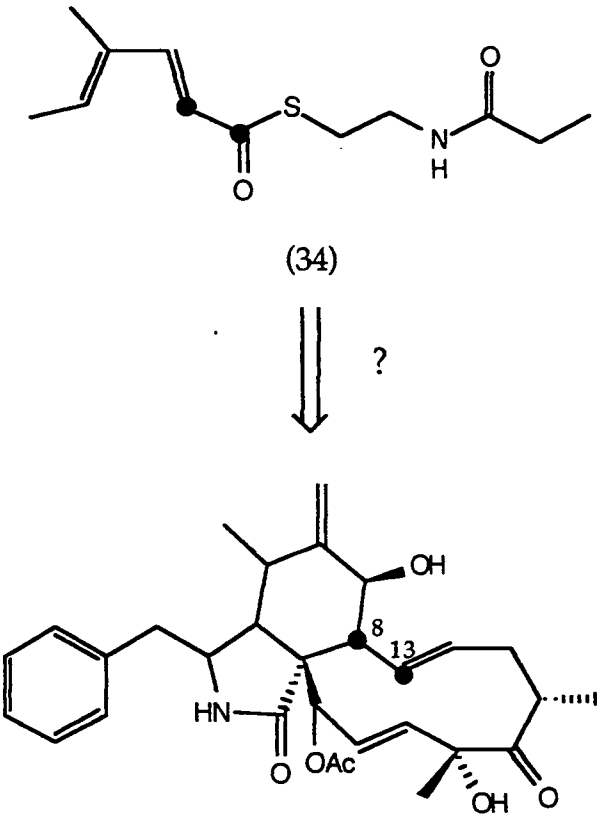
2.3 Feeding of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate to *Xylaria cubensis*

N-Acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate was fed in one batch to growing cultures of *Xylaria cubensis* on day 4 (i.e. when the mycelium showed a degree of healthy growth) at a final concentration of 1.3mM. Cytochalasin D was isolated after three weeks and the resultant

Fig. 1, ^{13}C nmr spectrum [C_5D_5N, 100MHz], (a) cytochalasin D fed with N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate, (b) C8 of cytochalasin D, (c) C13 of cytochalasin D

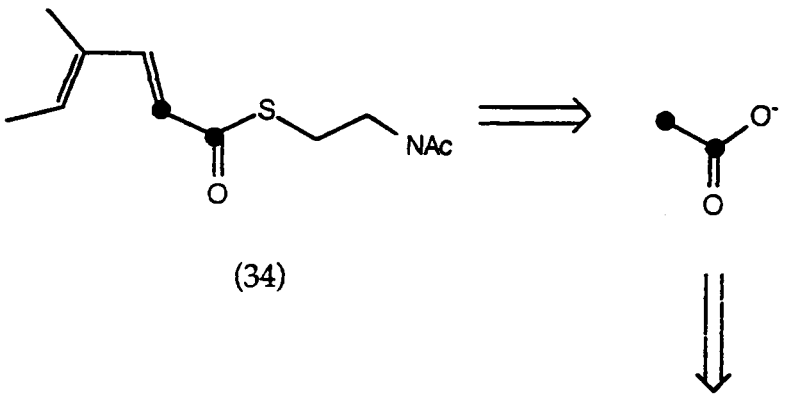


^{13}C n.m.r. spectrum is shown in Fig. 1. The ^{13}C n.m.r. spectrum of cytochalasin D has previously been fully assigned[156] (Table A4, Appendix 1, pg. iv). The two carbons of particular interest in assessing a regiochemical incorporation of the labelled substrate (34) into cytochalasin D are C8 and C13 (scheme 6).



Scheme 6

^{13}C-^{13}C Coupling satellites, in the ^{13}C n.m.r. spectrum, at each of the signals corresponding to C8 and C13 would be indicative of an intact incorporation of substrate (34) into cytochalasin D. Inspection of the ^{13}C n.m.r. spectrum Fig. 1 (a), reveals that each of the signals corresponding to the acetate derived carbon atoms possess ^{13}C-^{13}C coupling satellites. Expansion of the spectrum to reveal C8 Fig. 1(b), and C13 Fig. 1(c), demonstrates that the C8-C13 coupling satellites are of equal intensity to the satellites of the other acetate derived carbons. This observation indicates that (34) has been degraded by β-oxidation[173] to [$^{13}C_2$]-acetate and that it is this that has been utilised by the polyketide synthase enzymes and incorporated into cytochalasin D (scheme 7).



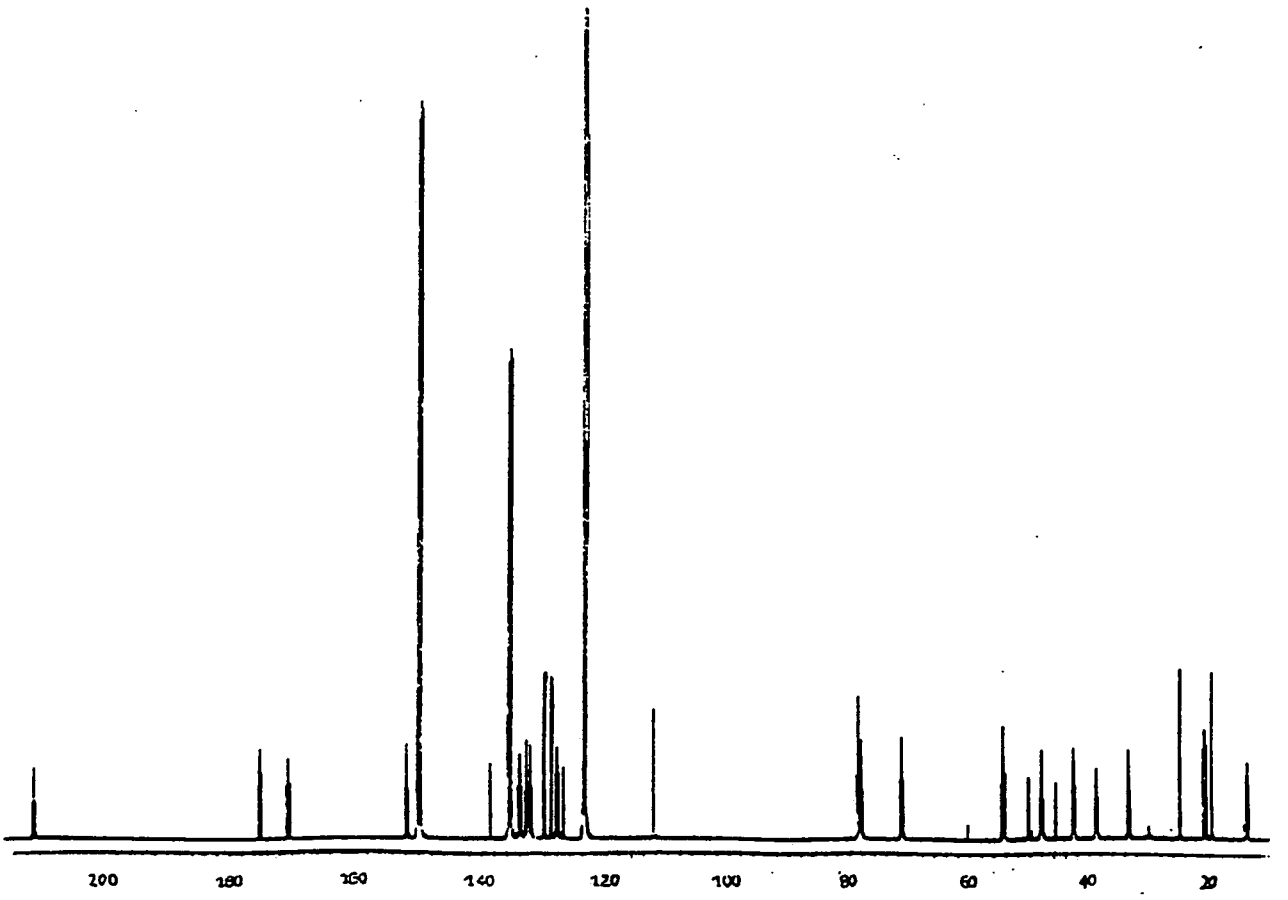
(34)

Cytochalasin D

Scheme 7

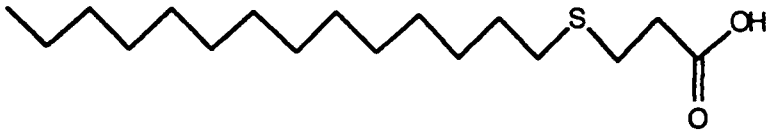
So we have obtained a ^{13}C n.m.r. spectrum of cytochalasin D which has a similar enrichment pattern to that of cytochalasin D isolated from cultures fed with sodium [$^{13}C_2$]-acetate, Fig. 2.

Fig. 2, ^{13}C nmr spectrum of cytochalasin D fed with sodium [$^{13}C_2$]-acetate [C_5D_5N, 100MHz]



2.4 Feeding of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate with a β-oxidation inhibitor to *Xylaria cubensis*

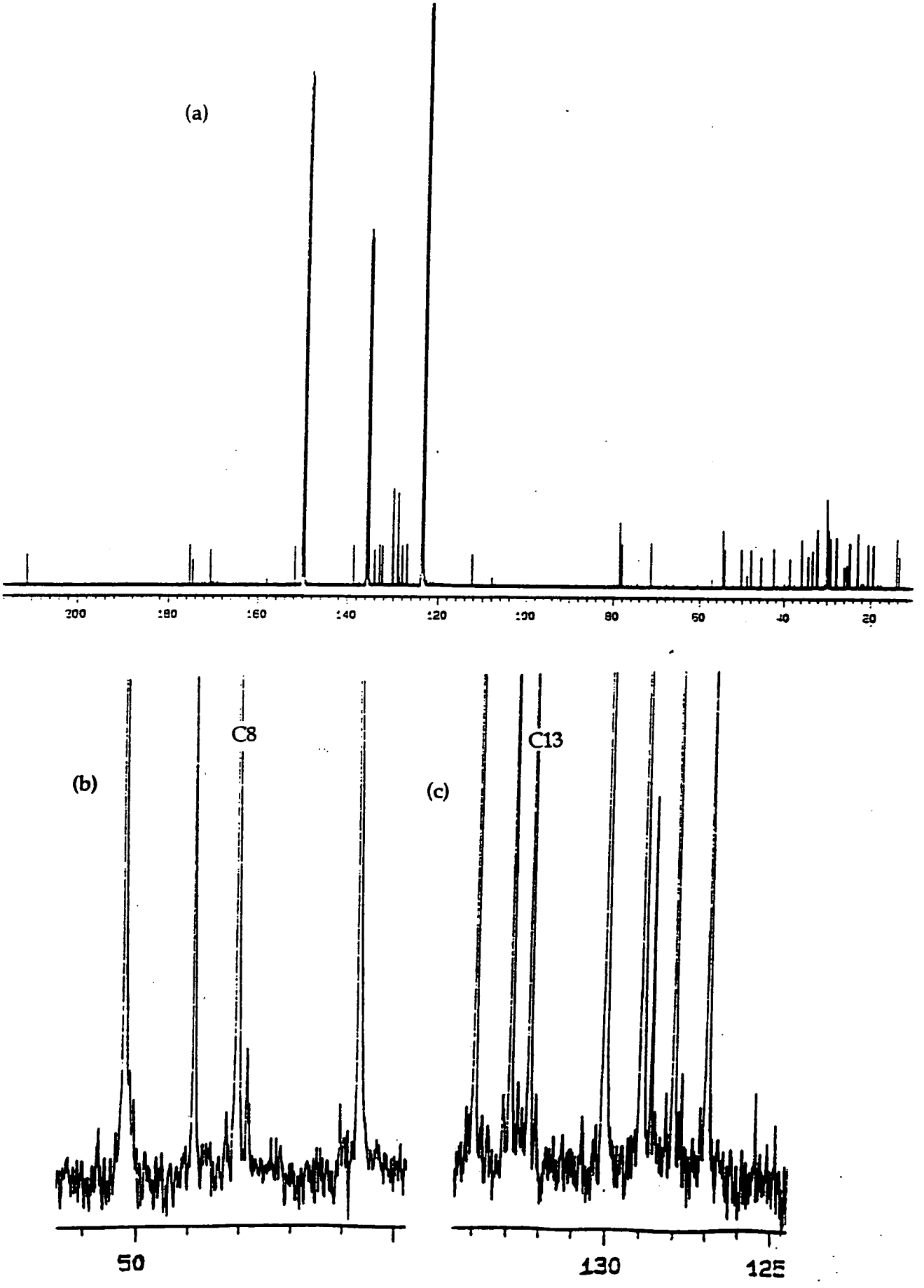
Experiments aimed at the intact incorporation of functionalised di- and tri-ketides into metabolites are generally plagued by rapid degradation of precursors by β-oxidation.[173,56] Vederas *et al.*[25] examined the effect of several β-oxidation inhibitors to supress the breakdown of the ^{13}C labelled precursors into dehydrocurvularin by wild-type *Alternaria cinerariae* ATCC 11784. When 3-(tetradecylthio)propanoic acid (35) was used as a β-oxidation inhibitor little breakdown, if any, of the tetraketide substrate by β-oxidation was observed. In an effort to omit β-oxidation in our system this inhibitor was employed. Thus 3-(tetradecylthio)propanoic acid (35)[174] was synthesised in a straightforward manner by coupling 3-mercaptopropanoic acid and 1-bromotetradecane in a methanol solution of potassium hydroxide.



(35)

The feeding experiment with *Xylaria cubensis* was repeated as before with the addition of 2 batches (day 0 and day 4) of 3-(tetradecythio)propanoic acid (it had to be dissolved in ethanol with heating and then administered to the cultures rapidly before it cooled and precipitated out) to a final concentration of 3.7mM. N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate was also administered on day 4 at a final concentration of 1.3mM. Cytochalasin D was isolated after three weeks and the ^{13}C n.m.r. spectrum is shown in Fig. 3. A small amount of residual 3-(tetradecylthio)propanoic acid is evident from the ^{13}C n.m.r. analysis. It can clearly be seen, however, that the degradation of the substrate has been substantially suppressed with only a very small amount of [$^{13}C_2$]-acetate incorporated into cytochalasin D. Expansion of the spectrum to see C8, Fig. 3 (b), and C13, Fig. 3 (c), signals more clearly reveals small ^{13}C-^{13}C satellites indicative of acetate incorporation (also evident at the same level in all of the acetate derived carbon atoms). There is no increased intensity of the satellites at C8 and C13 which would indicate regiospecific incorporation of substrate (34) into cytochalasin D.

Fig. 3, ^{13}C nmr spectrum [C_5D_5N, 100MHz], (a) cytochalasin D fed with N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate and β-oxidation inhibitor, (b) C8 of cytochalasin D, (c) C13 of cytochalasin D



3. CONCLUSION

The feeding experiments to investigate the mode of ring formation in cytochalasin D, isolated from *Xylaria cubensis*, have proved inconclusive. In the first experiment, the [$^{13}C_2$]-substrate (34) was degraded to [$^{13}C_2$]-acetate and this incorporation was evident in the ^{13}C n.m.r. spectrum of cytochalasin D. In the second experiment, a β-oxidation inhibitor was employed which was successful in suppressing β-oxidation, however there was no evidence for regiospecific incorporation of (34) into cytochalasin D.

The lack of regiospecific incorporation of (34) into cytochalasin D does not rule out the possibility that the acyl moiety is an intermediate on the polyketide synthase. There could be several factors governing the incorporation of such a substrate.[56] For example, it may be unable to access the polyketide biosynthesis enzymes due to its inability to cross the cell membrane, possibly as a result of the natural mechanism for excreting metabolites from the cell. Alternatively compartmentalisation within the cell may present a problem. Another possibility is that the substrate is not suitably primed for transesterification onto the polyketide synthase. Of course the structure of the substrate may be wrong, e.g. the true intermediate may be the alternative geometric isomers.

Finally the ^{13}C-^{13}C double labelling technique may not be sensitive enough to detect a very low level of incorporation. Labelling of (34) with deuterium and analysis by ^{2}H n.m.r.[34] may provide both increased sensitivity (due to the low natural abundance of deuterium) and economy. Such an approach has been successful in the recent work of Staunton *et al.*[175] investigating the incoporation of partially assembled intermediates in the biosynthesis of tetronasin, an antibiotic produced by *Streptomyces longisporoflavus*.

CHAPTER 6
EXPERIMENTAL

GENERAL

^1H Nuclear magnetic resonance spectra were recorded on a Gemini (200MHz) spectrometer operating at 199.97MHz. ^{13}C Nuclear magnetic resonance spectra were recorded on a Varian VXR 400S (400MHz) spectrometer operating at 100.57 MHz. Chemical shifts are quoted in ppm relative to TMS [$(CH_3)_4Si$] in $CDCl_3$. Coupling constants are quoted in Hz. Mass spectra were recorded on a VG analytical 7070E mass spectrometer. Infrared spectra were recorded on a Perkin-Elmer F.T. 1600 spectrometer. Melting points were performed on a Gallenkamp melting point apparatus. Flash chromatography was undertaken with the use of Fluka silica gel-60 (35 - 70μm) or Sorbsil-C60-H (40 - 60μm). All sterilisations were carried out in an autoclave at 121°C/ 20 min. Organic extracts were dried with $MgSO_4$ and the solvent removed under reduced pressure unless stated otherwise. Solvents were always dried and distilled before use. Sodium hydride is a 60% dispersion in mineral oil.

PART 1

CHAPTER 2

2,4-Dichloro-5-bromopyrimidine (27)

A suspension of 5-bromouracil (7.64g, 40mmol) in phosphorus oxychloride (50 cm^3) was heated under reflux (125°C) for 6 days. Excess phosphorus oxychloride was removed by distillation (25°C, 1.5mmHg) and the residue subsequently cooled with an ice bath. Ice was very cautiously added to the flask resulting in the evolution of HCl gas. The resultant dark brown oily tar was extracted into ether. The ether extract was dried and then concentrated to yield a yellow cloudy oil (4.49g, 49.2%); ν_{max} (neat)/cm^{-1} 3080 and 3030, 1530 and 1515, 760, 680, 530; δ_H ($CDCl_3$) 8.73 (1H, s, CH); δ_C ($CDCl_3$) 161.4 (C), 158.8 (C), 118.9 (CH); m/z (EI) 230 (M^+, 44.5%), 228 (100%), 226 (61.0%), 195 (13.1%), 193 (52.75%), 191 (41.45%).

2,4-Dimethoxy-5-bromopyrimidine (28)

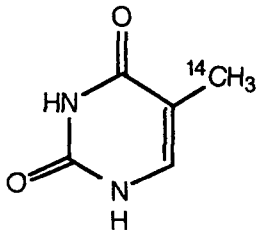
A solution of sodium (0.73g, 31.61mmol) in dried and distilled methanol ($20cm^3$) was added to a solution of 2,4-dichloro-5-bromopyrimidine (2g, 8.77mmol) in methanol ($5cm^3$) under N_2. After stirring for 30 min., the fawn coloured precipitate was removed by filtration and discarded. Concentration of the filtrate followed by the addition of H_2O ($15cm^3$) resulted in a white precipitate of 2,4-dimethoxy-5-bromopyrimidine (1.62g, 84.4%) which was collected by filtration; m.p. 63.9 - 64.9°C (lit. [176] m.p. 63 - 64°C); (Found: C, 32.73; H, 3.17; N, 12.71%. $C_6H_7N_2O_2Br$ requires C, 32.90; H, 3.22; N, 12.79%); ν_{max} (KBr)/cm^{-1} 3080 - 2900, 1580, 1470, 1400; δ_H ($CDCl_3$) 8.30 (1H, s, CH), 4.03 (3H, s, CH_3), 3.98 (3H, s, CH_3); δ_C ($CDCl_3$) 168 (C), 167.27 (C), 159.64 (C), 98.63 (CH), 55.76 (CH_3), 55.43 (CH_3); m/z (EI) 220 (97.5%), 218 (100%).

Thymine

A solution of n-butyllithium ($3.2cm^3$, 1.6M, 5.1mmol) in hexane was carefully added to a solution of 2,4-dimethoxy-5-bromopyrimidine (0.82g, 3.73mmol) in THF ($10cm^3$) whilst under an atmosphere of nitrogen and cooled with a dry ice / acetone bath. A solution of CH_3I ($0.23cm^3$, 3.73mmol) in THF ($5cm^3$) was then added. After stirring for 90mins at -65°C, the reaction was quenched by the cautious addition of solid CO_2. Upon stirring for 1h at room temperature, the solution was added to water and the organic layer extracted into ether. Concentration afforded an orange residue. 6M HCl ($3.7cm^3$ conc. HCl in $20cm^3$

H_2O) was added to this residue and heated under reflux for 2h. The subsequent solution was concentrated to dryness and the residue suspended in ether, filtered and the solid sublimed *in vacuo* to afford thymine (170°C/0.06mmHg) (0.12g, 26.4%); ν_{max} (KBr)/cm^{-1} 3540 - 3300, 3220, 3070, 2940, 1735, 1680, 1390; δ_H (DMSO-d_6) 11.03 (1H, broad s, NH), 10.60 (1H, broad s, NH), 7.24 (1H, s, CH), 1.73 (3H, s, CH_3); δ_C (DMSO-d_6) 165.30 (CO), 151.78 (CO), 138.03 (CH), 108.05 (C), 12.04 (CH_3); m/z (EI) 127 (M+1, 100%).

[^{14}C-*methyl*]-Thymine (29)



A solution of n-butyllithium (1.47cm^3, 1.6M, 2.35mmol) in hexane was carefully added to a solution of 2,4-dimethoxy-5-bromopyrimidine (0.57g, 2.61mmol) in THF (15cm^3) under N_2 and cooled with a dry ice / acetone bath. After stirring for 15 min. at -78°C, the resultant orange solution was further cooled to -198°C. Under vacuum, the solution was charged firstly with $^{14}CH_3I$ (1mCi), and then the flask was purged with nitrogen and CH_3I (0.13cm^3, 2.09mmol) was added *via* a syringe. The reaction mixture was allowed to warm to -78°C at which temperature it stirred for 1h followed by a further 30 min. between -50 and -60°C. The reaction was quenched by the addition of CO_2 [generated *in situ* by the careful addition of $BaCO_3$ (1.03g, 5.22mmol) to concentrated H_2SO_4 (2cm^3)] at -198°C and under vacuum. The flask was allowed to gradually warm from -198°C to -78°C and then to -50°C and finally to room temperature. The organics were extracted into ether and washed with saturated sodium bicarbonate solution followed by drying and concentration to afford an oil. The residue was hydrolysed by heating under reflux for 24h in 6M HCl (47.4cm^3) followed by concentration and purification over Dowex (50X2-400). Loaded onto the column in NH_4OH (2cm^3) and elution with water afforded a mixture of [^{14}C-*methyl*]-thymine and uracil (9:1, [^{14}C-*methyl*]-thymine : uracil). [^{14}C-*methyl*]-Thymine (926.5μCi, 289mg) was isolated in a radiochemical yield 92.6% and the radiochemical purity by t.l.c. (^{n}BuOH : H_2O [86 : 14 v/v] on silica) 91.2%; δ_H (DMSO-d_6) 7.23 (1H, s, CH), 1.71 (3H, s, CH_3); δ_C (DMSO-d_6) 165.49 (CO), 152.04 (CO), 138.28 (CH), 108.23 (C), 12.36 (CH_3).

[$^{13}C^2H_3$-*methyl*]-Thymine (30)

A solution of n-butyllithium (3.25cm^3, 1.6M, 5.21mmol) in hexane was carefully added to a solution of 2,4-dimethoxy-5-bromopyrimidine (1.2g, 5.48mmol) in THF (30cm^3) under an atmosphere of nitrogen and cooled with a dry ice / acetone bath. The solution was stirred for 15 min. at -78°C and then $^{13}C^2H_3I$ (0.31cm^3, 4.93mmol) was added. The solution was stirred for 1h at -78°C and then for a further 30min at a temperature between -50 to -60°C. The reaction was quenched with CO_2 gas at -78°C and the temperature was gradually allowed to rise to -50°C. The reaction was then allowed to reach room temperature and work up followed by acid hydrolysis proceeded as above to yield a yellow solid. Purification was accomplished by sublimation (157°C/ 0.023mmHg) affording [$^{13}C^2H_3$-*methyl*]-thymine (0.29g, 44.9%) as a white solid; (Found M$^+$, 130.0663. $C_4{}^{13}CH_3D_3O_2N_2$ requires M$^+$, 130.0651); ν_{max} (KBr)/cm^{-1} 3560 - 3300, 3159.8, 3044.8, 2814.4, 1734.1, 1672.9, 1448.3; δ_H (DMSO-d_6) 7.21 (1H, d, J 3.6, CH); δ_C (DMSO-d_6) 165.11 (CO), 151.60 (CO), 137.88 (CH), - (C), 11.08 (septet, $^1J_{CD}$ 19.41, $^{13}CD_3$); m/z (EI) 130 (M$^+$, 99.28%).

Dihydrothymine

Rh (~5%) on alumina (100mg) was added to a solution of thymine (0.10g, 0.83mmol) in 0.001M HCl (40cm^3) and stirred under H_2 at room temperature for 16h. Filtration of the reaction mixture through a celite pad and then concentration afforded dihydrothymine (0.10g, 96.4%) as a white solid; m.p. 261.3-263°C (lit. [177] m.p. 262.5 - 263°C); ν_{max} (KBr)/cm^{-1} 3240 and 3090, 2980, 2940, 2900 and 2840, 1740 and 1715, 1680, 1390; δ_H (D_2O) 3.35 (1H, dd, J 12.7 and 6.3, 6CH_S), 3.05 (1H, dd, J 12.6 and 10.0, 6CH_R), 2.67 (1H, m, 5-CH), 1.06 (3H, d, J 7.0, CH_3); δ_C (D_2O) 180.23 (CO), 177.0 (CO), 44.79 (CH), 36.97 (CH_2), 14.79 (CH_3); m/z (EI) 128 (M$^+$, 100%).

[^{14}C-*methyl*]-Dihydrothymine

Rh (~5%) on alumina (19.67mg) was added to a solution of [^{14}C-*methyl*]-thymine (37.77μCi, 11.78mg, 0.093mmol) in 0.001M HCl (5cm^3). Under the same hydrogenation conditions and work up as above, [^{14}C-*methyl*]-dihydrothymine (30.4μCi, 16.34mg) was isolated with a radiochemical yield 80.5% and radiochemical purity by t.l.c. (^{n}BuOH : H_2O [86 : 14 v/v] on silica) 92.06%).

Diethyl methylmalonate

A solution of diethyl malonate (2.25g, 14.09mmol) in THF (5cm^3) was added to a suspension of sodium hydride (0.55g, 13.67mmol) in THF (40cm^3) under N_2 and at 0°C. After effervescence had ceased, a solution of CH_3I (2g, 14.09mmol) in THF (5cm^3) was added and the reaction mixture was heated under reflux for 2h. Upon cooling a drop of ethanol, then water, was added and the organics extracted into ether. After drying, concentration of the ether extract yielded diethyl methylmalonate (1.95g, 79.4%) as a yellow oil; (Found: M+1, 175.0939. $C_8H_{14}O_4$ requires M+1, 175.0970); ν_{max} (neat)/cm^{-1} 2970, 2927, 1734, 1458; δ_H ($CDCl_3$) 4.19 (4H, q, J 7.1, CH_2), 3.42 (1H, q, J 8, CH), 1.40 (3H, d, J 6.84, CH_3), 1.27 (6H, t, J 7.16, CH_3); δ_C ($CDCl_3$) 170.19 (CO), 61.37 (CH_2), 46.29 (CH), 14.19 (CH_3), 13.64 (CH_3); m/z (EI) 175 (M+1, 100%), 129 (10.73%).

Diethyl [^{13}C-*methyl*]-methylmalonate (32)

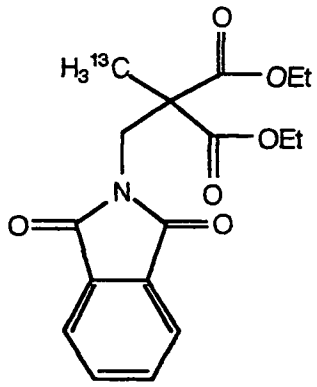
A solution of diethyl malonate (5.64g, 35.23mmol) in THF (15cm^3) was added to a suspension of sodium hydride (1.37g, 34.18mmol) in THF (100cm^3) under N_2 and at 0°C. After the effervescence had ceased, $^{13}CH_3I$ (5g, 34.97mmol) in THF (10cm^3) was added and the reaction proceeded as above to afford diethyl [^{13}C-*methyl*]-methylmalonate (5.76g, 93.5%); ν_{max} (neat)/cm^{-1} 2980, 2910, 1730, 1455; δ_H ($CDCl_3$) 4.10 (4H, q, J 7.2, CH_2), 3.32 (1H, dq, J 7.2 and $^2J_{CH}$ 4.6, CH), 1.31 (3H, dd, $^1J_{CH}$ 130.6 and 7.3, $^{13}CH_3$), 1.18 (6H, t, J 6.6, CH_3); δ_C ($CDCl_3$) 170.50 (CO), 61.64 (CH_2), 46.52 (d, J 34.3, CH), 14.39 (CH_3), 13.87 ($^{13}CH_3$); m/z (EI) 176 (M+1, 7.99%), 130 (37.11%).

Diethyl methyl(N-methylphthalimide)malonate

A solution of diethyl methylmalonate (1.94g, 11.15mmol) in THF (5cm^3) was added to a suspension of sodium hydride (0.46g, 11.71mmol) in THF (100cm^3) under N_2 and at 0°C. N-(bromomethyl)phthalimide (2.68g, 11.15mmol) was subsequently added and the mixture stirred for 1h at 0°C and then for a further 30 mins. at room temperature. A drop of ethanol, then water, was added and the product extracted into ether. Drying and concentration yielded diethyl methyl(N-methylphthalimide)malonate (2.79g, 75.2%) as a yellow solid; m.p. 93.2 - 95.8°C (lit. [79] m.p. 96°C); (Found: M+1, 334.1251. $C_{17}H_{19}O_6N$ requires M+1, 334.1291); ν_{max} (KBr)/cm^{-1} 2960 and 2920, 1775, 1720, 1610, 1440, 1420, 1390, 715; δ_H (DMSO-d_6) 7.90 (4H, m, Ph), 4.18 (4H, q, J 7.1, CH_2), 4.08 (2H, s, CH_2), 1.28 (3H, s, CH_3), 1.22 (6H, t, J 7.1, CH_3); δ_C (DMSO-d_6) 170.23 (CO), 168.08 (CO), 134.98 (CH), 131.54 (C), 123.56 (CH), 61.74 (CH_2), 53.26 (C), 41.06

(CH_2), 18.43 (CH_3), 13.99 (CH_3); m/z (EI) 334 (M+1, 26.97%), 288 (33.52%), 160 (100%).

Diethyl [^{13}C-*methyl*]-methyl(N-methylphthalimide)malonate (33)



A solution of diethyl [^{13}C-*methyl*]-methylmalonate (5.76g, 32.92mmol) in THF (10cm^3) was added to a suspension of sodium hydride (1.38g, 34.57mmol) in THF (200cm^3) under N_2 and at 0°C. N-(Bromomethyl)phthalimide (7.90g, 32.92mmol) was subsequently added and the reaction proceeded as above to afford diethyl [^{13}C-*methyl*]-methyl(N-methylphthalimide)malonate (9.59g, 87.24%); m.p. 93.5 - 94.8°C (lit. [79] m.p. 96°C); (Found: M+1, 335.1324. $C_{16}{}^{13}CH_{19}O_6N$ requires M+1, 335.1324); ν_{max} (KBr)/cm^{-1} 2980, 2930 and 2860, 1780, 1725, 1620, 1470 and 1440, 1395; δ_H (DMSO-d_6) 7.90 (4H, m, Ph), 4.18 (4H, q, J 7.0, CH_2), 4.09 (2H, d, $^3J_{CH}$ 3.8, CH_2), 1.29 (3H, d, $^1J_{CH}$ 130.9, $^{13}CH_3$), 1.22 (6H, t, J 7.2, CH_3); δ_C (DMSO-d_6) 170.25 (CO), 168.11 (CO), 135 (CH), 131.55 (C), 123.58 (CH), 61.75 (CH_2), 53.1 (d, $^1J_{CC}$ 30.2, C), 41.89 (CH_2), 18.43 ($^{13}CH_3$), 14.00 (CH_3); m/z (CI) 352 (M+NH_4^+, 1.25%), 335 (M+1, 8.35), 206 (4.96%).

Diethyl (aminomethyl)methylmalonate

Anhydrous hydrazine (2.5cm^3, 82.2mmol) was added to a solution of diethyl methyl(N-methylphthalimide)malonate (2.74g, 8.22mmol) in dry methanol (70cm^3) under N_2 and at room temperature. The reaction mixture was stirred for 12h. The resulting white precipitate was removed by filtration and discarded. The filtrate was added to water and extracted into dichloromethane. Drying and then concentration of the extract afforded diethyl (aminomethyl)methylmalonate (1.04g, 62.2%) as a yellow oil; ν_{max} (neat)/cm^{-1} 3500 - 3300, 2930 and 2860, 1735, 1465, 1380; δ_H ($CDCl_3$) 4.1 (4H, q, J 7.2, CH_2), 2.93 (2H, s, CH_2), 2.1 (broad s, NH_2), 1.31 (3H, s, CH_3), 1.16 (6H, t, J 7.0, CH_3);

δ_C ($CDCl_3$) 171.70 (CO), 61.24 (CH_2), 56.17 (C), 47.85 (CH_2), 18.55 (CH_3), 14.10 (CH_3); m/z (EI) 204 (M+1, 19.79%), 174 (71.78%), 128 (33.70%).

Diethyl [^{13}C-*methyl*]-(aminomethyl)methylmalonate (34)

Anhydrous hydrazine (9cm^3, 287.1mmol) was added to a solution of diethyl [^{13}C-*methyl*]-methyl(N-methylphthalimide)malonate (9.59g, 28.71mmol) in dry methanol (100cm^3) under N_2 and the reaction proceeded as above to afford diethyl [^{13}C-*methyl*]-(aminomethyl)methylmalonate (2.95g, 50.4%); ν_{max} (neat)/cm^{-1} 3500 - 3300, 2930 and 2850, 1730, 1470, 1380; δ_H ($CDCl_3$) 4.20 (4H, q, J 7.1, CH_2), 3.05 (2H, d, $^3J_{CH}$ 3.5, CH_2), 1.42 (3H, d, $^1J_{CH}$ 130.4, $^{13}CH_3$), 1.26 (6H, t, J 7.1, CH_3); δ_C ($CDCl_3$) 172.10 (CO), 61.61 (CH_2), 56.17 (d, $^1J_{CC}$ 26.6, C), 48.28 (CH_2), 18.97 ($^{13}CH_3$), 14.44 (CH_3); m/z (EI) 205 (M+1, 44.42%), 191 (6.17%), 176 (7.04%), 175 (7.22%).

(DL)-β-Aminoisobutyric acid

A solution of diethyl (aminomethyl)methylmalonate (1.04g, 5.12mmol) in HCl (5cm^3) and H_2O (5cm^3) was heated under reflux for 12h. Residual organics were extracted into ether and the remaining aqueous layer was concentrated to dryness to yield a pale yellow coloured solid of β-aminoisobutyric acid hydrochloride (0.55g, 76.6%) which was used directly for feeding experiments; ν_{max} (KBr)/cm^{-1} 3420, 3050, 1720, 1590, 1460; δ_H (D_2O) 3.02 (2H, m, CH_2), 2.73 (1H, m, CH), 1.07 (3H, d, J 7.2, CH_3); δ_C (D_2O) 180.16 (CO), 44.03 (CH_2), 39.68 (CH), 17.04 (CH_3); m/z (EI) 104 (M-HCl, 100%), 86 (14.64%).

A small amount was purified over Dowex (50X2-400), elution with NH_4.OH solution. After concentration to dryness of the active fractions, the residue was recrystallised with ethanol to afford white crystals of β-aminoisobutyric acid; m.p. 174.8 - 175.8°C (lit. [178] m.p. 176.5 - 177.5°C); (Found: M+1, 104.0669. $C_4H_9O_2N$ requires M+1, 104.0712); ν_{max} (KBr)/cm^{-1} 3354.5, 3043.0, 1676.2, 1628.9, 1560.1; δ_H (D_2O) 2.91 (2H, m, CH_2), 2.46 (1H, m, CH), 1.04 (3H, d, J 7.2, CH_3); δ_C (D_2O) 184.39 (CO), 45.15 (CH_2), 42.04 (CH), 17.98 (CH_3); m/z (EI) 104 (M+1, 100%), 86 (52.08%).

(DL)-[^{13}C-*methyl*]-β-Aminoisobutyric acid hydrochloride (35)

A solution of diethyl [^{13}C-*methyl*]-(aminomethyl)methylmalonate (2.95g, 14.46mmol) in HCl (15cm^3) and H_2O (15cm^3) was heated under reflux for 12h. Extraction as above yielded a pale yellow solid of [^{13}C-*methyl*]-β-aminoisobutyric acid hydrochloride (1.80g, 88.60%); δ_H (D_2O) 3.03 (2H, m,

CH_2), 2.77 (1H, m, CH), 1.14 (3H, dd, $^1J_{CH}$ 129.1 and J 6.6, $^{13}CH_3$); δ_C (D_2O) 180.32 (CO), 44.04 (CH_2), 39.76 (d, $^1J_{CC}$ 32.7, CH), 17.00 ($^{13}CH_3$).

α-Cyanopropionic acid

Sodium carbonate (1.04g, 9.81mmol) was carefully added to a solution of α-bromopropionic acid (3g, 19.61mmol) in acetonitrile (20cm^3). After stirring at room temperature for 2h, sodium α-bromopropionate was collected by filtration and dried under reduced pressure. A solution of this sodium salt (2.94g, 16.82mmol), NaOH (0.05g, 1.26mmol) and NaCN (0.82g, 16.82mmol) in H_2O (10cm^3) was heated between 65 - 80°C for 2.5h. After cooling, acidification with dilute HCl followed by extraction of the organics into ether, drying and concentration afforded α-cyanopropionic acid (1.39g, 83.5%) as a colourless oil; ν_{max} (neat)/cm^{-1} 3471.9, 2257.5, 1736.6; δ_H ($CDCl_3$) 3.64 (1H, q, J 7.4, CH), 1.60 (3H, d, J 7.4, CH_3); δ_C ($CDCl_3$) 170.73 (CO), 117.81 (CN), 32.09 (CH), 15.61 (CH_3); m/z (EI) 100 (M+1, 32.60%).

[1-^{13}C]-α-cyanopropionic acid (37)

Sodium carbonate (0.34g, 3.25mmol) was carefully added to a solution of [1-^{13}C]-α-bromopropionic acid (1g, 6.49mmol) in acetonitrile (7cm^3). Reaction proceeded as above to afford [1-^{13}C]-α-cyanopropionic acid which was carried on to the next step without characterisation.

(DL)-β-Aminoisobutyric acid

PtO_2 (100mg) was added to a solution of α-cyanopropionic acid (0.24g, 2.37mmol) in dried and distilled ethanol : chloroform (10 : 1, 27.5cm^3) and shook under 2.5atm H_2 at room temperature for 18h. Filtration of the reaction mixture through a celite pad followed by concentration of the filtrate afforded an oil which was purified with Dowex (H^+ form). Elution with NH_4OH afforded β-aminoisobutyric acid (0.018g, 7.4%) which was recrystallised with ethanol; Characterisation the same as before.

(DL)-[1-^{13}C]-β-Aminoisobutyric acid (39)

[1-^{13}C]-α-Cyanopropionic acid was hydrogenated utilising the same conditions as above to afford, after Dowex (H^+ form) chromatography, [1-^{13}C]-β-aminoisobutyric acid (0.054g, 8%); δ_H (D_2O) 2.96 (2H, m, CH_2), 2.54 (1H, m, CH), 1.06 (3H, dd, $^3J_{CH}$ 4.6 and J 7.2, CH_3); δ_C (D_2O) 183.41 (^{13}CO), 44.90 (CH_2), 41.48 (d, $^1J_{CC}$ 51.74, CH), 17.05 (CH_3).

(DL)-[3-$^2H_2$]-β-Aminoisobutyric acid (40)

α-Cyanopropionic acid was deuterated utilising the conditions of PtO_2 (200mg), MeOD : $CDCl_3$ (10 : 1, 33cm^3), 2.5atm D_2, room temperature for 18h. Dowex chromatography afforded [3-$^2H_2$]-β-aminoisobutyric acid (0.07g, 10%); δ_H (D_2O) 2.52 (1H, q, CH), 1.05 (3H, d, J 7.2, CH_3); δ_C (D_2O) 183.62 (CO), - (CD_2), 41.49 (CH), 17.82 (CH_3); δ_D (H_2O) 3.21 (s, CD), 3.11 (s, CD).

Diethyl dimethylmalonate

A solution of diethyl methylmalonate (3.61g, 20.71mmol) in THF (5cm^3) was carefully added to a stirred suspension of sodium hydride (0.845g, 21.14mmol) in THF (50cm^3) at 0°C and under N_2. A solution of CH_3I (3g, 21.14mmol) in THF (5cm^3) was added and the mixture was heated under reflux for 2h. Water was added to the reaction mixture and the whole was extracted with ether. The organic extracts were dried and concentrated to afford diethyl dimethylmalonate (4.01g, 102.9%) as a yellow oil; (Found: M^+, 188.1050. $C_9H_{16}O_4$ requires M, 188.1049); ν_{max} (neat)/cm^{-1} 2984.2, 2938.6, 1732.1, 1469.9, 1447.6, 1386.1, 1366.2; δ_H ($CDCl_3$) 4.05 (4H, q, J 6.4, CH_2), 1.29 (6H, s, CH_3), 1.13 (6H, t, J 6.36, CH_3); δ_C ($CDCl_3$) 172.34 (CO), 60.88 (CH_2), 49.59 (C), 22.54 (CH_3), 13.89 (CH_3); m/z (EI) 189 (M+1, 100%), 143 (29.67%).

Diethyl [^{13}C-*methyl*]-dimethylmalonate (42)

A solution of diethyl methylmalonate (3.61g, 20.71mmol) in THF (5cm^3) was carefully added to a stirred suspension of sodium hydride (0.845g, 21.14mmol) in THF (50cm^3) at 0°C and under N_2. A solution of $^{13}CH_3I$ (3g, 21.14mmol) in THF (5cm^3) was added and the reaction proceeded as above to yield diethyl [^{13}C-*methyl*]-dimethylmalonate (4.17g, 104.2%); (Found: M+1, 190.1076. $C_8{}^{13}CH_{16}O_4$ requires M+1, 190.1160); ν_{max} (neat)/cm^{-1} 2983.9, 2935.9, 1734.0, 1470.8, 1448.5, 1383.8, 1365.9; δ_H ($CDCl_3$) 4.08 (4H, q, J 7.08, CH_2), 1.32 (3H, d, $^1J_{CH}$ 130.14, $^{13}CH_3$), 1.32 (3H, d, $^3J_{CH}$ 4.6, CH_3), 1.15 (6H, t, J 7.12, CH_3); δ_C ($CDCl_3$) 172.96 (CO), 61.33 (CH_2), 49.97 (d, $^1J_{CC}$ 34.92, C), 22.91 ($^{13}CH_3$), 14.23 (CH_3); m/z (EI) 190 (M+1, 100%), 144 (44.67%).

Dimethylmalonic acid

Diethyl dimethylmalonate (4.01g, 21.32mmol) was added to a solution of 5M KOH (4.2g KOH in 15cm^3 H_2O) and heated under reflux for 24h. Acidification with dilute HCl and extraction of the whole into ether afforded a white solid of dimethylmalonic acid (2.18g, 79.7%) after drying and concentration; m.p. 194.0 -

195.7°C; (Found: M+1 133.0405. $C_5H_8O_4$ requires M+1, 133.0501); ν_{max} (KBr)/cm^{-1} 3005.1, 2923.5, 1700.3, 1466.0, 1403.7; δ_H (D_2O) 1.13 (6H, s, CH_3); δ_C (D_2O) 179.77 (CO), 52.60 (C), 24.84 (CH_3); m/z (EI) 133 (M+1, 100%), 115 (46.24%).

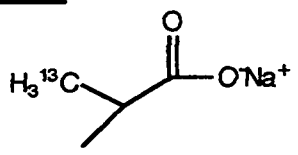
[^{13}C-*methyl*]-Dimethylmalonic acid (43)

Diethyl [^{13}C-*methyl*]-dimethylmalonate (4.17g, 22.04mmol) was added to a solution of 5M KOH. The reaction and work up as outlined above afforded [^{13}C-*methyl*]-dimethylmalonic acid (2.65g, 90.3%); m.p. 192.0 - 193.0°C; (Found: M+1 134.0476. $C_4{}^{13}CH_8O_4$ requires M+1, 134.0534); ν_{max} (KBr)/cm^{-1} 3088.3, 2922.8, 1703.0, 1460.3, 1403.5; δ_H (D_2O) 1.24 (3H, d, $^1J_{CH}$ 130.42, $^{13}CH_3$), 1.24 (3H, d, $^3J_{CH}$ 4.62, CH_3); δ_C (D_2O) 180.03 (CO), 52.75 (d, $^1J_{CC}$ 35.22, C), 24.92 ($^{13}CH_3$); m/z (EI) 134 (M+1, 100%), 116 (69.69%).

Sodium isobutyrate

A solution of dimethylmalonic acid (2g, 15.14mmol) in H_2O (10cm^3) was heated to 180°C for 3h. Careful acidification with conc. HCl and then extraction of the whole into ether afforded isobutyric acid after drying and concentration. Neutralisation to pH 7 with dilute NaOH solution, followed by washing with ether to remove unwanted organics, and removal of the water yielded sodium isobutyrate (1.13g, 67.9%) as a white solid; m.p. 249.8 - 251.4°C; ν_{max} (KBr)/cm^{-1} 2968.2, 2932.7, 1547.1, 1477.2, 1420.8; δ_H (D_2O) 2.27 (1H, septet, J 7.04, CH), 0.94 (6H, d, J 7.00, CH_3); δ_C (D_2O) 190.75 (CO), 39.82 (CH), 22.27 (CH_3).

Sodium [^{13}C-*methyl*]-isobutyrate (44)

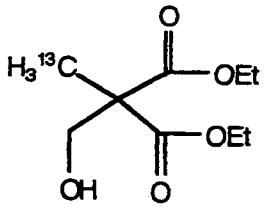


A solution of [^{13}C-*methyl*]-dimethylmalonic acid (2.65g, 19.92mmol) in H_2O (10cm^3) was heated to 180°C for 3h. After careful acidification with conc. HCl the work up was as outlined above to afford sodium [^{13}C-*methyl*]-isobutyrate (1.60g, 68.65%); m.p. 244.4 - 245.7°C; ν_{max} (KBr)/cm^{-1} 2967.1, 2931.5, 1548.1, 1477.3, 1420.7; δ_H (D_2O) 2.26 (1H, ds, J 6.96 and $^2J_{CH}$ 4.05, CH), 0.93 (3H, dd, $^1J_{CH}$ 126.6 and J 6.9, $^{13}CH_3$), 0.93 (3H, dd, J 6.92 and $^3J_{CH}$ 5.48, CH_3); δ_C (D_2O) 190.73 (CO), 39.80 (d, $^1J_{CC}$ 33.80, CH), 22.28 ($^{13}CH_3$).

Diethyl (hydroxymethyl)methylmalonate

A solution of diethyl methylmalonate (2.69g, 15.42mmol), formaldehyde (37% in H_2O, 1.25g, 15.42mmol) and potassium bicarbonate (0.2g, 2mmol) was heated to 70°C for 2h and stirred for a following 12h at room temperature. The reaction mixture was washed with saturated ammonium sulphate solution and the organics were extracted into ether. Drying and concentration yielded an oil which was purified by silica gel column chromatography. The active fractions were eluted with a solvent system starting with 100% dichloromethane but gradually increasing the ethyl acetate concentration until 100%, afforded diethyl (hydroxymethyl)methylmalonate (2.36g, 74.8%) as a colourless oil; ν_{max} (neat)/cm^{-1} 3530, 2980, 2935, 1730; δ_H ($CDCl_3$) 4.21 (4H, q, J 7.1, CH_2), 3.86 (2H, d, J 6.64, CH_2), 3.36 (1H, t, J 6.64, OH), 1.45 (3H, s, CH_3), 1.27 (6H, t, J 7.1, CH_3); δ_C ($CDCl_3$) 171.70 (CO), 66.55 (CH_2), 61.71 (CH_2), 56.13 (C), 17.83 (CH_3), 14.20 (CH_3); m/z (EI) 205 (M+1, 47.63%), 175 (67%), 129 (79.21%).

Diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (47)



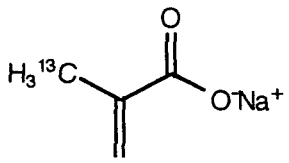
A solution of diethyl [^{13}C-*methyl*]-methylmalonate (3.72g, 21.23mmol), formaldehyde (37% in H_2O, 1.72g, 21.23mmol) and potassium bicarbonate (0.3g, 3mmol) was heated to 70°C for 2h and stirred for a following 12h at room temperature. Work up and purification was as outlined above to yield diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (0.93g, 64.8%); (Found: M+1, 206.1081. $C_8{}^{13}CH_{16}O_4$ requires M+1, 206.1110); ν_{max} (neat)/cm^{-1} 3400, 2983, 2930, 1730, 1465; δ_H ($CDCl_3$) 4.21 (4H, q, J 7.14, CH_2), 3.86 (2H, dd, J 6.77 and $^3J_{CH}$ 3.16, CH_2), 3.27 (1H, t, J 6.86, OH), 1.44 (3H, d, $^1J_{CH}$ 130.76, $^{13}CH_3$), 1.27 (6H, t, J 7.1, CH_3); δ_C ($CDCl_3$) 171.21 (CO), 66.09 (CH_2), 61.17 (CH_2), 55.51 (d, $^1J_{CC}$ 34.69, C), 17.22 ($^{13}CH_3$), 13.64 (CH_3); m/z (EI) 206 (M+1, 36.28%), 176 (83.25%), 130 (96.92%).

Sodium methacrylate

A solution of diethyl (hydroxymethyl)methylmalonate (1.10g, 5.39mmol) and dilute hydrochloric acid (1.5cm^3 conc. HCl and 20cm^3 H_2O) was gently heated

under reflux for 3 days. The organics were extracted into ether. Drying and concentration afforded a yellow oil which was purified by silica gel column chromatography. The active fractions were eluted with a solvent system starting with 100% dichloromethane and gradually increasing the ethyl acetate concentration until 100%. Addition of water to the concentrated active fractions and neutralisation to pH7 with dilute NaOH solution afforded, after concentration to dryness, sodium methacrylate (0.34g, 58.37%) as a white solid; m.p. >360°C; δ_H (D_2O) 5.51 (1H, m, CH), 5.21 (1H, m, CH), 1.72 (3H, m, CH_3); δ_C (D_2O) 180.28 (CO), 145.13 (C), 123.55 (CH_2), 21.90 (CH_3).

Sodium [^{13}C-*methyl*]-methacrylate (48)



A solution of diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (0.93g, 4.53mmol) and dilute hydrochloric acid (1.5cm^3 conc. HCl and 20cm^3 H_2O) was gently heated under reflux for 3 days. The isolation and purification was as outlined above to afford [^{13}C-*methyl*]-methacrylic acid as a colourless oil; ν_{max} (neat)/cm^{-1} 3000, 2926.4, 1696.8, 1635.8; δ_H ($CDCl_3$) 6.25 (1H, dm, $^3J_{CH}$ 10.1, CH), 5.70 (1H, dm, $^3J_{CH}$ 5.90, CH), 1.96 (3H, dm, $^1J_{CH}$ 128.2, $^{13}CH_3$); m/z (CI) 87 (M^+, 1.8%).

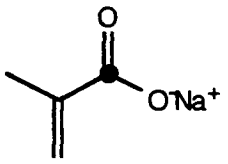
[^{13}C-*methyl*]-Methacrylic acid was neutralised to pH7 with dilute NaOH solution to afford sodium [^{13}C-*methyl*]-methacrylate (0.16g, 32.38%); ν_{max} (KBr)/cm^{-1} 3100, 2958, 2924, 1555, 1458, 1419; δ_H (D_2O) 5.51 (1H, dm, $^3J_{CH}$ 9.56, CH), 5.20 (1H, dm, $^3J_{CH}$ 6.14, CH), 1.72 (3H, dm, $^1J_{CH}$ 127.14, $^{13}CH_3$); δ_C (D_2O) 179.60 (d, $^2J_{CC}$ 3.32, CO), 144.43 (d, $^1J_{CC}$ 43.25, C), 122.58 (CH_2), 21.10 ($^{13}CH_3$).

Sodium methacrylate

A solution of 2-bromopropene (2.05cm^3, 23.03mmol) in dry ether (6cm^3) was carefully added to a stirred suspension of activated Mg turnings (6.16g, 253.35mmol) in dry ether (6cm^3) over 1h. The reaction mixture was cautiously heated under reflux for a further 1h using a water bath at 45°C. After cannular transfer, the Grignard reagent was quenched with CO_2 [generated *in situ* by the careful addition of $BaCO_3$ (5g, 25.34mmol) onto sulphuric acid (15cm^3)]. After quenching, the reaction mixture was concentrated to dryness. Water was added

and the solution was altered to pH3 with dilute sulphuric acid. The organics were extracted into ether, dried and concentrated to yield a yellow oil which was chromatographed over silica. The activity was eluted with a solvent system starting with 100% dichloromethane and gradually increasing to 100% ethyl acetate. Concentration of active fractions yielded methacrylic acid as a colourless oil. Neutralisation to pH7 with dilute NaOH solution afforded, after concentration, sodium methacrylate (0.50g, 18.10%) as a white solid; δ_H (D_2O) 5.51 (1H, m, CH), 5.20 (1H, m, CH), 1.72 (3H, m, CH_3); δ_C (D_2O) 180.50 (CO), 145.21 (C), 123.35 (CH_2), 21.86 (CH_3).

Sodium [1-^{13}C]-methacrylate (51)



A solution of 2-bromopropene (2.05cm^3, 23.03mmol) in dry ether (7cm^3) was carefully added to a stirred suspension of activated Mg turnings (6.16g, 253.35mmol) in dry ether (7cm^3) over 1h. The reaction was as outlined above with the exception that the Grignard reagent was quenched with $^{13}CO_2$ [generated *in situ* by the addition of $Ba^{13}CO_3$ (5g, 25.21mmol) onto sulphuric acid (20cm^3)]. Work up and purification afforded [1-^{13}C]-methacrylic acid which was neutralised to pH7 with dilute NaOH solution. Concentration of this aqueous extract afforded sodium [1-^{13}C]-methacrylate (0.62g, 22.51%); δ_H (D_2O) 5.53 (1H, dm, $^3J_{CH}$ 4.16, CH), 5.21 (1H, dm, $^3J_{CH}$ 11.72, CH), 1.72 (3H, dm, $^3J_{CH}$ 2.72, CH_3); δ_C (D_2O) 180.21 (^{13}CO), 145.06 (d, $^1J_{CC}$ 61.60, C), 123.67 (d, $^2J_{CC}$ 2.16, CH_2), 21.97 (d, $^2J_{CC}$ 3.22, CH_3).

[^{13}C-*methyl*]-Methylmalonic acid (52)

Diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (0.71g, 3.47mmol) was added to a solution of 10M KOH (4.2g KOH in 7.5cm^3 H_2O) and heated under reflux for 24h. Acidification with dilute HCl and extraction of the whole into ether afforded a white solid of [^{13}C-*methyl*]-methylmalonic acid (0.22g, 53.2%) after drying and concentration. This material was stored as the dicarboxylic acid and converted to the sodium salt before the feeding experiment; m.p. 128.7 - 130.1°C; (Found: C, 40.51; H, 5.09; N, 0%. $C_3{}^{13}CH_6O_4$ requires C, 40.68; H,

5.11: N, 0%); ν_{max} (KBr)/cm^{-1} 3838.6, 2726.2, 1542.1; δ_H (D_2O) 3.37 (1H, dq, J 7.2 and $^2J_{CH}$ 4.8, CH), 1.16 (3H, dd, $^1J_{CH}$ 131 and J 7.3, $^{13}CH_3$); δ_C (D_2O) 177.19 (CO), 48.79 (d, $^1J_{CC}$ 33.72, CH), 15.80 ($^{13}CH_3$); m/z (CI) 137 ($M+NH_4^+$, 100%).

Growth of *Streptomyces cinnamonensis* ATCC 15413

Streptomyces cinnamonensis was grown on yeast - malt agar plates (yeast extract 2g, malt extract 5g, glucose 2g, agar 7.5g, distilled water 500cm^3, pH adjusted to 7.2 with dilute KOH solution) for 14 days at 30°C.

The spores from the producing strain were used to innoculate two Erlenmeyer flasks (250cm^3), each containing 70 cm^3 of seed medium (glucose 3.36g, soybean flour 2.1g, $CaCO_3$ 0.42g, $FeSO_4.7H_2O$ 0.77g, MgCl 0.004g, distilled H_2O 140cm^3). These flasks were incubated on a orbital shaker (32°C, 150rpm) for 10 days.

9 Erlenmeyer flasks (500cm^3) each containing 100cm^3 of production medium (glucose 45g, soybean flour 13.5g, $CaCO_3$ 2.7g, $FeSO_4.7H_2O$ 4.94g, MgCl 0.027g, distilled H_2O 900cm^3) were innoculated with 3cm^3 of seed culture. The fermentation was carried out at 32°C and 200rpm for 7.5 days.

Isolation and purification of monensin A

Methanol (400cm^3) was added to the medium and the broth was homogenised and filtered. The pellet was resuspended in methanol (50cm^3), homogenised and re-filtered. The supernatant was extracted into dichloromethane, dried and concentrated to dryness to yield a brown coloured oil. This residue was chromatographed over silica. Activity was eluted with a solvent system starting with 100% dichloromethane and gradually increasing the ethyl acetate concentration until 100%. Activity was detected by the appearance of orange colouration on thin layer chromatography (CH_2Cl_2 : EtOAc [50 : 50 v/v]) sprayed with anisaldehyde : glacial acetic acid : sulphuric acid (0.5cm^3 : 50cm^3 : 1cm^3 v/v) reagent and heated. Combination and concentration of active fractions yielded an off-white coloured solid which could be further purified with C_{18} reverse phase preparative chromatography, elution with MeOH : H_2O (9 : 1), to afford a mixture of the monensins A and B [8 : 2 v/v] (10mg). A full ^{13}C n.m.r. assignment is given in Table A1 (Appendix 1, pg. i).

Feeding of sodium methacrylate to *Streptomyces cinnamonensis*

Sodium methacrylate (0.25g, 2.31mmol) was dissolved in water (18cm^3) and pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of sodium methacrylate was 3.86mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of sodium [^{13}C-*methyl*]-methacrylate to *Streptomyces cinnamonensis*

Sodium [^{13}C-*methyl*]-methacrylate (0.35g, 3.16mmol) was dissolved in water (15cm^3) and pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of sodium [^{13}C-*methyl*]-methacrylate was 5.27mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of (DL)-[^{13}C-*methyl*]-β-aminoisobutyric acid to *Streptomyces cinnamonensis*

(DL)-[^{13}C-*methyl*]-β-Aminoisobutyric acid hydrochloride salt (0.5g, 3.56mmol) was dissolved in water (18cm^3) and neutralised to pH7 with dilute NaOH. It was pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of [^{13}C-*methyl*]-β-aminoisobutyric acid was 5.93mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of [^{14}C-*methyl*]-thymine to *Streptomyces cinnamonensis*

[^{14}C-*methyl*]-Thymine (32.46μCi) and thymine (14mg) were added to 6 x 100cm^3 flasks of production medium before sterilisation. After innoculation, monensin A (0.66μCi, 2% incorporation) was subsequently isolated on day 7.5 as outlined above.

Feeding of [^{13}C^{2}H$_3$-*methyl*]-thymine to *Streptomyces cinnamonensis*

[^{13}C^{2}H$_3$-*methyl*]-Thymine (0.27g, 2.10mmol) was dissolved in water (18cm^3) and sterilised. It was administered to the cultures (5 x 100cm^3) of *Streptomyces cinnamonensis* via a sterilised pipette on day 3, such that the final concentration of [^{13}C^{2}H$_3$-*methyl*]-thymine was 4.21mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of sodium [3-^{13}C]-isobutyrate to *Streptomyces cinnamonensis*

Sodium [3-^{13}C]-isobutyrate (0.40g, 3.60mmol) was dissolved in water (18cm^3) and pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of sodium [3-^{13}C]-isobutyrate was 5.59mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of sodium [3-^{13}C]-isobutyrate and sodium methacrylate to *Streptomyces cinnamonensis*

Sodium [3-^{13}C]-isobutyrate (0.40g, 3.60mmol) and sodium methacrylate (0.39g, 3.60mmol) were dissolved together in water (18cm^3) and pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of sodium [3-^{13}C]-isobutyrate was 5.59mM and sodium methacrylate was 6mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of sodium [1-^{13}C]-methacrylate to *Streptomyces cinnamonensis*

Sodium [1-^{13}C]-methacrylate (0.34g, 3.11mmol) was dissolved in water (18cm^3) and pulse fed at 3, 3.5 and 4 days to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of sodium [1-^{13}C]-methacrylate was 5.18mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of L-[3-^{14}C]-serine to *Streptomyces cinnamonensis* at day 0

L-[3-^{14}C]-Serine (22.18μCi) and L-serine (10mg) were dissolved in H_2O (6cm^3) and fed before innoculation to 6 x 100cm^3 sterilised flasks of production medium via a micropore filter such that the specific activity was 0.24mCi/mmol. After innoculation, monensin A (0.33μCi, specific activity 0.00584mCi/mmol, 2.51% incorporation) was isolated on day 7.5 as outlined above.

Feeding of L-[3-^{14}C]-serine to *Streptomyces cinnamonensis* at day 3.5

L-[3-^{14}C]-Serine (23.25μCi) and L-serine (10mg) were dissolved in H_2O (6cm^3) and administered at day 3.5 to 6 x 100cm^3 cultures of *Streptomyces cinnamonensis* via a micropore filter such that the specific activity was 0.24mCi/mmol. Monensin A (0.35μCi, specific activity 0.007884mCi/mmol, 3.3% incorporation) was isolated on day 7.5 as outlined above.

Feeding of (DL)-[1-^{13}C]-β-aminoisobutyric acid to *Streptomyces cinnamonensis*

(DL)-[1-^{13}C]-β-Aminoisobutyric acid (0.054g, 0.52mmol) was dissolved in water (6cm^3) and pulse fed at 3, 3.5 and 4 days to 2 x 100cm^3 cultures of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of [1-^{13}C]-β-aminoisobutyric acid was 2.6mM. Monensin A was subsequently

isolated on day 7.5 as outlined above.

Feeding of sodium [^{13}C-*methyl*]-methylmalonate to *Streptomyces cinnamonensis*

[^{13}C-*methyl*]-Methylmalonic acid (0.22g, 1.85mmol) was dissolved in water ($12cm^3$) and neutralised to pH7 with dilute NaOH solution. The resulting sodium salt was pulse fed at 3, 3.5 and 4 days to 4 x $100cm^3$ cultures of *Streptomyces cinnamonensis* *via* a micropore filter, such that the final concentration of [^{13}C-*methyl*]-methylmalonic acid was 4.6mM. Monensin A was subsequently isolated on day 7.5 as outlined above.

Feeding of (DL)-[3-$^2H_2$]-β-aminoisobutyric acid to *Streptomyces cinnamonensis*

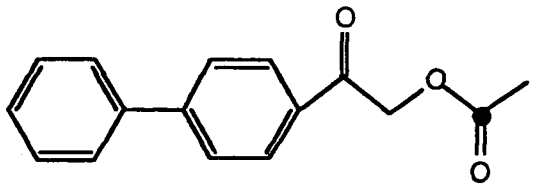
Streptomyces cinnamonensis was grown on a different medium (yeast extract 0.3g, peptone 0.5g, malt extract 0.3g, glucose 1g, distilled H_2O $100cm^3$, pH adjusted to 7.3). [3-$^2H_2$]-β-Aminoisobutyric acid (0.07g, 0.67mmol) was dissolved in water ($3cm^3$) and administered at day 0 to 1 x $100cm^3$ culture of *Streptomyces cinnamonensis* using a micropore filter, such that the final concentration of [3-$^2H_2$]-β-aminoisobutyric acid was 6.67mM. Samples were taken at day 1, 2 and 3 and filtered to separate the cells, which were sent to Dr. K.A. Reynolds, University of Maryland at Baltimore, for lipid analysis.

CHAPTER 3

p-Phenylphenacyl acetate

A solution of sodium acetate (3g, 36.56mmol), p-phenylphenacyl bromide (9.33g, 36.56mmol) and 18-crown-6 (732mg) in a mixture of benzene : acetonitrile (1 : 1, 100cm^3) was heated under reflux for 16h. The solvent was concentrated to afford a pale brown solid. After the addition of dichloromethane, filtration removed undissolved impure solid and the filtrate was concentrated to dryness. The solid residue was applied to the top of a silica gel column and eluted with dichloromethane. The active fractions were combined and concentrated to yield a white crystalline solid of p-phenylphenacyl acetate (5.25g, 56.5%); ν_{max} (KBr)/cm^{-1} 3600 - 3300, 3100 - 3020, 2940, 1720, 1703, 1608, 1585; δ_H ($CDCl_3$) 8.1- 7.4 (9H, m, Ph), 5.36 (2H, s, CH_2), 2.24 (3H, s, CH_3).

p-Phenylphenacyl [1-^{13}C]-acetate (20)



A solution of sodium [1-^{13}C]-acetate (6g, 72.26mmol), p-phenylphenacyl bromide (19.88g, 72.26mmol) and 18-crown-6 (1.44g) in benzene : acetonitrile (1 : 1, 140cm^3) was heated under reflux for 16h. The solvent was evaporated to yield a cream coloured solid. Addition of dichloromethane followed by filtration and concentration of the filtrate afforded p-phenylphenacyl [1-^{13}C]-acetate (21.43g, 116%), which was used without further purification; m.p. 106.7 - 109°C (lit. [179] m.p. 111°C; ν_{max} (KBr)/cm^{-1} :- same as above; δ_H ($CDCl_3$) 8.1-7.4 (9H, m, Ph), 5.37 (2H, d, $^3J_{CH}$ 4.4, CH_2), 2.25 (3H, d, $^2J_{CH}$ 6.9, CH_3); δ_C ($CDCl_3$) 170.95 (^{13}CO), 169.24 (CO), 147.2 (C), 140.03 (C), 133.32 (C), 129.50 (CH), 128.85 (CH), 127.93 (CH), 127.75 (CH), 69.92 (CH_2), 21.15 (d, $^1J_{CC}$ 55.30, CH_3); m/z (EI) 256 (M+1, 24.25%), 181 (100%).

Ethanol

A solution of p-phenylphenacyl acetate (5.25g, 20.67mmol) in anhydrous diglyme (50cm^3) under nitrogen was cooled with a dry ice / CCl_4 bath. Lithium

aluminium hydride (1.14g, 30.07mmol) was carefully added over a period of 20 min and the contents of the flask were stirred for 4h under a constant flow of nitrogen at room temperature. The flask was cooled with an ice bath, the nitrogen flow removed, and phenoxyethanol (25cm^3) was added dropwise to quench the reaction. The flask was heated to 50°C, and over a period of 8h, the ethanol passed over a glass bridge into a trap. The ethanol was carried onto the next step without characterisation.

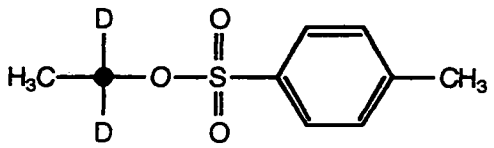
[1-^{13}C,1-$^2H_2$]-Ethanol (21)

Lithium aluminium deuteride (5.13g, 122.25mmol) was added to a solution of p-phenylphenacyl [1-^{13}C]-acetate (21.43g, 84.05mmol) in anhydrous diglyme (90cm^3) as described above, resulting in the formation of [1-^{13}C,1-$^2H_2$]-ethanol.

Ethyl tosylate

A solution of ethanol in dry pyridine (5cm^3) was cooled with a dry ice / CCl_4 bath (-23°C) whilst under N_2. *p*-Toluenesulphonyl chloride (3.11g, 16.34mmol) was carefully added and the solution stirred for 2h. After warming to 0°C with an ice bath, the reaction was quenched with ice cold concentrated HCl (15cm^3). Ethyl tosylate precipitated upon standing at 0°C. The solid was collected by filtration, dried under vacuum and then finally dried in a dessicator at 0°C to give white crystals of ethyl tosylate (0.6g, 14.6%); δ_H ($CDCl_3$) 7.8 (2H, d, J 7.92, CH), 7.35 (2H, d, J 8.08, CH), 4.12 (2H, q, J 7.16, CH_2), 2.45 (3H, s, CH_3), 1.3 (3H, t, J 7.06, CH_3).

[1-^{13}C,1-$^2H_2$]-Ethyl tosylate (22)



p-Toluenesulphonyl chloride (12.67g, 64.44mmol) was carefully added to a solution of [1-^{13}C,1-$^2H_2$]-ethanol in dry pyridine (16cm^3) whilst under N_2 and at -23°C. Following the procedure described above, crystals of [1-^{13}C,1-$^2H_2$]-ethyl tosylate (3.99g, 27.3%) were isolated; m.p. 29.4- 30.6°C (lit. [47(b)] m.p. 32-34°C); (Found: M^+, 203.0536. $C_8{}^{13}C^2H_2H_{10}O_3S$ requires M^+, 203.0664); ν_{max} (KBr)/cm^{-1} 3120 - 3020, 3000 - 2930, 1600, 1500, 1360, 1190; δ_H ($CDCl_3$) 7.79 (2H, d, J 8, CH), 7.34 (2H, d, J 8, CH), 2.45 (3H, s, tos-CH_3), 1.28 (3H, dp, $^2J_{CH}$ 4.4 and

$^{3}J_{DH}$ 0.9, CH_3); δ_C ($CDCl_3$) 145.18, 133.76, 130.33 and 128.33 (Ph), 66.74 (p, $^{1}J_{CD}$ 22.9, $^{13}CD_2$), 22.14 (CH_3), 14.97 (d, $^{1}J_{CC}$ 38.1, CH_3); m/z (EI) 203 (M^+, 21.21%), 172 (13.37%), 155 (38.73%), 108 (14.9%), 107 (13.34%), 91 (100%).

Propionitrile

Potassium cyanide (0.19g, 2.97mmol) was added to a solution of ethyl tosylate (0.6g, 3.02mmol) in methanol : water (2 : 1, 9cm^3). After heating under reflux for 2h, t.l.c. (petroleum ether : dichloromethane [70 : 30 v/v]) indicated that all the ethyl tosylate had been consumed. The reaction mixture was distilled and used directly for the next reaction.

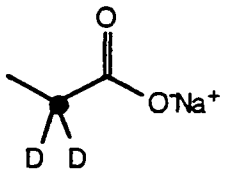
[2-^{13}C,2-$^{2}H_2$]-Propionitrile (23)

Potassium cyanide (1.26g, 19.37mmol) was added to a solution of [1-^{13}C,1-$^{2}H_2$]-ethyl tosylate (3.99g, 19.68mmol) in MeOD : D_2O (3 : 1, 16cm^3) under an atmosphere of nitrogen. Reaction and isolation conditions as above afforded [2-^{13}C,2-$^{2}H_2$]-propionitrile.

Sodium propionate

A solution of KOH (2g, 35.65mmol) in H_2O (2cm^3) was added to the propionitrile distillate prepared above and the resulting mixture was heated under reflux for 3 days. The lyophilisate collected after acidification was neutralised with dilute NaOH. Final lyophilisation afforded sodium propionate (0.27g, 93.3%) as a white amorphous solid; ν_{max} (KBr)/cm^{-1} 3620 - 3380, 2900 - 2700, 1570, 1470; δ_H (D_2O) 2.04 (2H, q, J 7.6, CH_2), 0.9 (3H, t, J 7.6, CH_3); m/z (CI) 96 (M^+, 15%), 72 (15%).

Sodium [2-^{13}C,2-$^{2}H_2$]-propionate (24)



A solution of KOD (3g, 52.53mmol) in D_2O (3cm^3) was added to the [2-^{13}C,2-$^{2}H_2$]-propionitrile distillate prepared above and the resulting mixture was heated under reflux for 3 days under an atmosphere of nitrogen. The lyophilisate collected after acidification was neutralised with dilute NaOH. A

final lyophilisation yielded sodium [2-^{13}C,2-$^{2}H_2$]-propionate (1.18g, 60.6%); m.p. 281.2 - 283°C (lit. [180] 285 - 286°C); ν_{max} (KBr)/cm^{-1} 3600 - 3200, 2980 - 2840, 1630, 1560, 1420; δ_H (D_2O) 0.93 (3H, dp, $^{2}J_{CH}$ 4.4 and $^{3}J_{DH}$ 1.1, CH_3); δ_C (D_2O/TMS salt) 188.03 (d, $^{1}J_{CC}$ 51.3, CO), 33.35 (t, $^{1}J_{CD}$ 19.5, ^{13}CD), 33.05 (p, $^{1}J_{CD}$ 19.4, $^{13}CD_2$), 12.96 (d, $^{1}J_{CC}$ 34.3, CH_3).

Diazomethane

A solution of N-methyl-N-nitrosotoluene-p-sulphonamide (Dizald) (2.14g, 10mmol) in ether (60cm^3) was placed in an Erlenmeyer flask (250cm^3). A second Erlenmeyer flask was charged with a solution of the sample to be methylated in ether (70cm^3). A smooth glass bridge connected the two flasks which were cooled to 0°C and a solution of potassium hydroxide (0.8g, 14.26mmol) in ethanol (20cm^3) was added to the Dizald flask. After 5 min the solution of Dizald was heated and an ethereal solution of diazomethane (explosive, carcinogenic) distilled across into the reaction flask. Reaction was complete when the yellow colour persisted in this reaction flask. Excess diazomethane was removed by purging with nitrogen. The ethereal solution was concentrated to dryness to afford the methylated sample.

Growth of *Streptomyces sp. ATS 1287*

Streptomyces sp. ATS 1287 was grown on yeast - malt agar plates (yeast extract 2g, malt extract 5g, glucose 2g, agar 7.5g, distilled water 500cm^3, pH adjusted to 7.2 with dilute KOH solution) for 16 days at 26.5°C.

The spores from the producing strain were used to innoculate six Erlenmeyer flasks (250cm^3), each containing 70 cm^3 of seed medium (glucose 2.1g, malt extract 2.1g, yeast extract 0.84g, KCl 1.68g, distilled water 420cm^3, pH adjusted to 7.3 with dilute KOH solution). These flasks were incubated on a orbital shaker (26.5°C, 200rpm) for 4 days.

10 Erlenmeyer flasks (500cm^3) each containing 100cm^3 of production medium (soybean flour 50g, soybean oil 30g, dried yeast 3g, KCl 3g, K_2HPO_4 0.2g, $CaCO_3$ 3g, distilled water 1000cm^3, pH adjusted to 7.2 with dilute KOH solution) were innoculated with 3cm^3 of seed culture. The fermentation was carried out at 26.5°C and 200rpm for 9 days.

Isolation and purification of leptomycin B

The cells were harvested by centrifugation (14000rpm, 4°C, 45min) and the mycellium exhaustively extracted into acetone using a Soxhlet apparatus. The acetone extract was dried and concentrated to dryness to yield a green/brown residue. This was washed with ethyl acetate (200cm^3) and filtered to remove unwanted solid. After concentration, the oil was extracted into methanol (2 x 20cm^3) to remove lipids. Concentration of the methanol layer afforded a residue containing leptomycin B. The supernatant, from the centrifugation, was extracted into ethyl acetate. The extract was dried and concentrated to afford an oil which was combined with the oil obtained from the mycellium. Methylation with an etheral solution of diazomethane afforded an orange residue which was chromatographed over silica, activity eluted with chloroform and ethyl acetate (70 : 30 v/v). Using a reference sample, the active fractions were combined and concentrated to dryness and then rechromatographed over silica, activity eluted with chloroform and ethyl acetate (85 : 15 v/v). This yielded a residue containing leptomycin A/B methyl ester (10mg). A full ^{13}C n.m.r. assignment is given in Table A2 (Appendix 1, pg. ii).

Feeding of sodium [1-^{13}C]-propionate to *Streptomyces sp. ATS 1287*

Sodium [1-^{13}C]-propionate (1.29g, 13.30mmol) was dissolved in water (30cm^3) and pulse fed at 3, 3.5 and 4 days to 10 x 100cm^3 cultures of *Streptomyces sp. ATS 1287* using a micropore filter, such that the final concentration of sodium

[1-^{13}C]-propionate was 13.3mM. Leptomycin A/B was subsequently isolated on day 9 as outlined above.

Feeding of sodium [2-^{13}C,2-$^{2}H_2$]-propionate to *Streptomyces sp. ATS 1287*

Sodium [2-^{13}C,2-$^{2}H_2$]-propionate (1.09g, 11.01mmol) was dissolved in water (30cm^3). It was pulse fed at 3, 3.5 and 4 days to 8 x 100cm^3 cultures of *Streptomyces sp. ATS 1287* using a micropore filter, such that the final concentration of sodium [2-^{13}C,2-$^{2}H_2$]-propionate was 13.76mM. Leptomycin A/B was subsequently isolated on day 9 as outlined above.

PART 2

CHAPTER 4

Diethyl ethylmethylmalonate

Diethyl ethylmalonate (3.76g, 20mmol) was added carefully under N_2 to a suspension of NaH (0.8g, 20mmol) in THF (20cm^3) cooled to 0°C. After initial effervescence, CH_3I (2.84g, 20mmol) was added and the reaction mixture heated under reflux for 2h. A drop of ethanol was added to ensure that all the sodium hydride had been consumed. After the addition of water (10cm^3), the product was extracted into dichloromethane, dried and concentrated to afford diethyl ethylmethylmalonate (2.75g, 68%) as a colourless oil; ν_{max} (neat)/cm^{-1} 2980, 2930, 1735, 1470, 1390 - 1370; δ_H ($CDCl_3$) 4.05 (4H, q, J 7, CH_2), 1.78 (2H, q, J 7.5, CH_2), 1.25 (3H, s, CH_3), 1.13 (6H, t, J 7.1, CH_3), 0.75 (3H, t, J 7.5, CH_3); δ_C ($CDCl_3$) 172.02 (CO), 60.87 (CH_2), 53.98 (C), 28.58 (CH_2), 19.23 (CH_3), 14.04 (CH_3), 8.61 (CH_3); m/z (EI) 203 (M+1, 100%), 157 (45.83%), 129 (13.74%), 73 (15%).

Diethyl [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonate (12)

Diethyl ethylmalonate (2.58g, 13.7mmol) was added to a suspension of NaH (0.55g, 13.7mmol) in THF (20cm^3) cooled to 0°C and under N_2. $^{13}C^2H_3I$ (2g, 13.7mmol) was added and the reaction proceeded as above to afford diethyl [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonate (1.98g, 70.2%); ν_{max} (neat)/cm^{-1} 2980, 2940, 2230, 1740, 1465, 1390, 1370; δ_H ($CDCl_3$) 4.17 (4H, q, J 7.0, CH_2), 1.89 (2H, m, CH_2), 1.25 (6H, t, J 7.1, CH_3), 0.87 (3H, t, J 7.5, CH_3); δ_C ($CDCl_3$) 172.23 (CO), 60.99 (CH_2), 53.91 (d, $^1J_{CC}$ 34.5, C), 28.58 (CH_2), 18.55 (septet, $^1J_{CD}$ 19.7, $^{13}CD_3$), 14.13 (CH_3), 8.73 (CH_3); m/z (EI) 207 (M+1,100%), 161 (75.24%), 133 (28.06%), 73 (34.02%).

Ethylmethylmalonic acid

A solution of diethyl ethylmethylmalonate (2.75g, 13.61mmol) in 5M KOH (4.2g KOH in 15cm^3 H_2O) was heated under reflux for 12h. Upon cooling, the reaction mixture was acidified with dilute HCl and the organics extracted into ether. The ether extract was dried and concentrated to afford a white solid of ethylmethylmalonic acid (1.56g, 78.5%); ν_{max} (KBr)/cm^{-1} 3600 - 2500, 1705, 1460; δ_H (D_2O) 1.57 (2H, q, J 7.6, CH_2), 1.08 (3H, s, CH_3), 0.58 (3H, t, J 7.5, CH_3); δ_C (D_2O) 179.28 (CO), 56.98 (C), 31.13 (CH_2), 21.61 (CH_3), 10.83 (CH_3).

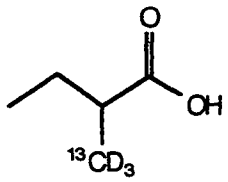
[$^{13}C^2H_3$-*methyl*]-Ethylmethylmalonic acid (13)

A solution of diethyl [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonate (1.98g, 9.61mmol) in 5M KOH was heated under reflux for 12h. The reaction was worked up as above to afford [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonic acid (1.39g, 96.5%); ν_{max} (KBr)/cm^{-1} 3560 - 3300, 3100 - 2900, 2220, 1700, 1400; δ_H (D_2O) 1.71 (2H, dq, J 7.5 and $^3J_{CH}$ 3.2, CH_2), 0.72 (3H, t, J 7.5, CH_3); δ_C (D_2O) 179.83 (CO), 57.00 (d, $^1J_{CC}$ 34.6, C), 31.29 (CH_2), 21.09 (septet, $^1J_{CD}$ 19.5, $^{13}CD_3$), 10.97 (CH_3); m/z (CI) 168 (M+ NH_4^+, 2.35%), 106, 89.

2-Methylbutyric acid

A solution of ethylmethylmalonic acid (1.56g, 10.68mmol) in H_2O ($15cm^3$) was carefully sealed under vacuum in a Carius tube, and heated to 180°C for 3h. After cooling, the tube was opened and the contents were extracted into ether. The ether extracts were dried and concentrated to give 2-methylbutyric acid (0.4g, 36.4%); ν_{max} (neat)/cm^{-1} 3500 - 3100, 2980, 2940, 2880, 1710, 1460, 1410 and 1390; δ_H ($CDCl_3$) 12.05 (1H, broad s, OH), 2.4 (1H, sextet, J 7.5, CH), 1.61 (2H, dq, J 7.4, CH_2), 1.23 (3H, d, J 7.3, CH_3), 0.95 (3H, t, J 7.5, CH_3); δ_C ($CDCl_3$) 183.97 (CO), 40.94 (CH), 26.85 (CH_2), 16.59 (CH_3), 11.83 (CH_3); m/z (EI) 104 (M+1, 89.63%), 86 (100%), 75 (54.78%), 58 (66.81%).

[$^{13}C^2H_3$-*methyl*]-2-Methylbutyric acid (14)



A solution of [$^{13}C^2H_3$-*methyl*]-ethylmethylmalonic acid (1.39g, 9.27mmol) in H_2O ($10cm^3$) was carefully sealed under vacuum in a steel bomb, and heated to 180°C for 3h. Upon cooling, the contents of the steel bomb were worked up as previous to afford [$^{13}C^2H_3$-*methyl*]-2-methylbutyric acid (0.56g, 57%); ν_{max} (neat)/cm^{-1} 3600 - 2800, 2960 - 2930, 2220, 1710, 1420; δ_H ($CDCl_3$) 12.05 (1H, broad s, OH), 2.37 (1H, m, CH), 1.61 (2H, ddq, CH_2), 0.95 (3H, t, J 7.2, CH_3); δ_C ($CDCl_3$) 184.09 (CO), 41.12 (d, $^1J_{CC}$ 33.95, CH), 26.82 (CH_2), 15.82 (septet, $^1J_{CD}$ 19.5, $^{13}CD_3$), 11.85 (CH_3); m/z (EI) 107 (M+1, 31.08%), 78 (100%), 61 (72.04%).

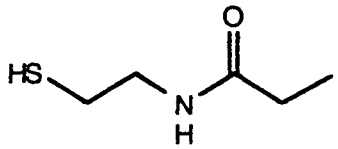
N,N'-Dipropionyl cystamine

Propionyl chloride (20g, 216mmol) was very carefully added dropwise to an ice cooled solution of cystamine dihydrochloride (22.5g, 100mmol) in H_2O (20cm^3). The pH of the reaction mixture was kept above pH 8.2 throughout the addition of the propionyl chloride by the addition of 12.5M KOH solution (35g of KOH and 50cm^3 H_2O). The resulting white precipitate was collected by filtration and washed thoroughly with cold water. Upon drying, the white solid of N,N'-dipropionyl cystamine (6.7g, 25.4%) was stored in a dessicator; m.p. 92-94.2°C; ν_{max} (Nujol)/cm^{-1} 3260, 3060, 1625, 1545; δ_H ($CDCl_3$) 6.59 (2H, broad s, NH), 3.57 (4H, dt, J 6.3 and 5.8, CH_2), 2.84 (4H, t, J 6.5, CH_2), 2.27 (4H, q, J 7.6, CH_2), 1.16 (6H, t, J 7.4, CH_3); δ_C ($CDCl_3$) 175.09 (CO), 38.90 (CH_2), 38.32 (CH_2), 30.03 (CH_2), 10.31 (CH_3); m/z (CI) 265 (M+1, 4.18%), 209 (15.94%), 134 (40.88%).

3% Sodium amalgam

Distilled mercury (430g, 2.14mol) was carefully dropped onto small pieces of sodium (13g, 0.56mol) under an atmosphere of N_2. The reaction was kept hot with the aid of a bunsen burner to maintain continuous reaction. The hot liquid was subsequently poured into a glass dish and cut up into small pieces before the sodium amalgam cooled and solidified as a solid block.

N-Propionyl cysteamine

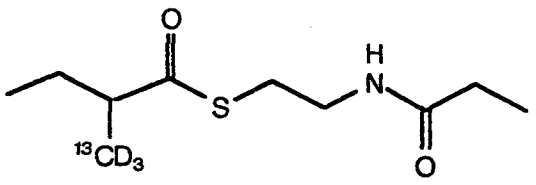


3% Sodium amalgam (26g) was added to a solution of N,N'-dipropionyl cystamine (2.64g, 10mmol) in dry methanol (50cm^3) under N_2 and the reaction mixture stirred for 90 min. at room temperature. The remaining mercury was removed by filtration, and the resulting cloudy liquid was added to dilute HCl and extracted into dichloromethane. Drying and concentration of the dichloromethane extract afforded N-propionyl cysteamine (1.88g, 70.7%) as a colourless oil; ν_{max} (neat)/cm^{-1} 3300, 3080, 2980 - 2940, 2580 - 2480, 1650, 1545, 1465, 1380; δ_H ($CDCl_3$) 7.73 (1H, broad s, NH), 3.43 (2H, dt, J 7.9 and 6.2, CH_2), 2.68 (2H, dt, J 8.1 and 5.5, CH_2), 2.29 (2H, q, J 7.6, CH_2), 1.64 (1H, t, J 9.4, SH), 1.17 (3H, t, J 7.5, CH_3); δ_C ($CDCl_3$) 175.12 (CO), 42.97 (CH_2), 29.58 (CH_2), 24.42 (CH_2), 10.30 (CH_3); m/z (EI) 134 (M+1, 100%), 74 (40.59%), 57 (38.47%).

N-Acetylcysteamine-2-methylbutyrate

1,3-Dicyclohexylcarbodiimide (1.01g, 3.8mmol), 4-dimethylaminopyridine (0.46g, 3.8mmol) and N-propionyl cysteamine (1.24g, 9.32mmol) were added to a solution of 2-methylbutyric acid (0.4g, 3.8mmol) in dry ether (25cm^3) under N_2. The reaction mixture was stirred for 12h at room temperature. A white precipitate formed which was removed by filtration and discarded. The filtrate was concentrated and then purified over silica gel (chloroform : ethyl acetate [70 : 30 v/v]). The active fractions were combined and concentrated to afford N-acetylcysteamine-2-methylbutyrate (0.59g, 71%) as a colourless oil, which was stored at 4°C; ν_{max} (neat)/cm^{-1} 3300, 3080, 2980, 2940, 2880, 1690, 1655, 1550, 1460, 1380; δ_H ($CDCl_3$) 7.08 (1H, broad s, NH), 3.43 (2H, dt, J 8.0 and 6.0, CH_2), 3.04 (2H, t, J 6.8, CH_2), 2.61 (1H, sextet, J 6.0, CH), 2.22 (2H, q, J 7.0, CH_2), 1.60 (2H, dq, J 7.2, CH_2), 1.15 (3H, d, CH_3), 1.15 (3H, t, CH_3), 0.91 (3H, t, J 7.6, CH_3); δ_C ($CDCl_3$) 204.16 (CO), 174.76 (CO), 50.04 (CH), 39.78 (CH_2), 29.75 (CH_2), 28.38 (CH_2), 27.35 (CH_2), 17.36 (CH_3), 11.83 (CH_3), 10.22 (CH_3); m/z (EI) 218 (M+1, 6.81%), 134 (9.70%), 85 (14.04%), 57 (25.50%).

N-Acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (15)



1,3-Dicyclohexylcarbodiimide (1.41g, 5.28mmol), 4-dimethylaminopyridine (0.65g, 5.28mmol) and N-propionyl cysteamine (0.9g, 6.77mmol) were added to a solution of [$^{13}C^2H_3$-*methyl*]-2-methylbutyric acid (0.56g, 5.28mmol) in dry ether (25cm^3) under N_2. The reaction and work up proceeded as above to afford N-acetylcysteamine-[$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (1.16g, 96%); (Found: M+1, 222.1407. $C_9{}^{13}C^2H_3H_{16}O_2SN$ requires M+1, 222.1437); ν_{max} (neat)/cm^{-1} 3342.1, 2972.0, 2928.6, 1688.9, 1657.2, 1555.5; δ_H ($CDCl_3$) 7.42 (1H, broad s, NH), 3.38 (2H, dt, J 6.1 and 5.9, CH_2), 3.03 (2H, t, J 8.0, CH_2), 2.55 (1H, m, CH), 2.23 (2H, q, J 7.7, CH_2), 1.59 (2H, m, CH_2), 1.14 (3H, t, J 7.4, CH_3), 0.90 (3H, t, J 7.4, CH_3); δ_C ($CDCl_3$) 203.65 (CO), 174.72 (CO), 50.09 (d, $^1J_{CC}$ 33.95, CH), 39.59 (CH_2), 29.58 (CH_2), 28.29 (CH_2), 27.25 (CH_2), 16.49 (septet, $^1J_{CD}$ 19.4, $^{13}CD_3$), 11.76 (CH_3), 10.16 (CH_3); m/z (EI) 222 (M+1, 100%), 133 (81.86%), 89 (38.12%), 61 (86.87%).

Growth of *Xylaria cubensis*

Ten Erlenmeyer flasks (500cm^3) containing 90cm^3 of medium (malt extract broth 27g, distilled water 900cm^3) were innoculated from the producing strain. The culture was incubated in the dark at 23°C for 8 weeks.

Isolation and purification of cubensic acid

The thick brittle mycellium was removed, cut up and air dried overnight. The mycellium was exhaustively extracted (Soxhlet, 2 x 10h) into chloroform and the chloroform extract dried and concentrated to yield an orange/red gum. This residue was treated with etheral diazomethane and concentrated to afford an off-white precipitate, which was purified over silica gel (100% ethyl acetate). Active fractions gave a blue colouration by t.l.c. (100% ethyl acetate) sprayed with anisaldehyde : glacial acetic acid : sulphuric acid (0.5ml : 50ml : 1ml v/v) and then heated. Combination and concentration of these active fractions followed by further purification with C_{18} reverse phase preparative chromatography, elution with MeOH : H_2O (9 : 1), afforded methyl cubensate (10mg). A full ^{13}C n.m.r. assignment is detailed in Table A3 (Appendix 1, pg. iii).

Feeding of L-[^{13}C-*methyl*]-methionine to *Xylaria cubensis*

L-[^{13}C-*methyl*]-Methionine (0.50g, 3.33mmol) was dissolved in water (20cm^3). It was fed on day 4 to cultures (10 x 90cm^3) of *Xylaria cubensis via* a micropore filter, such that the final concentration of L-[^{13}C-*methyl*]-methionine was 3.7mM. After eight weeks, cubensic acid was isolated as above.

Feeding of L-[$^{13}C^2H_3$-*methyl*]-methionine to *Xylaria cubensis*

L-[$^{13}C^2H_3$-*methyl*]-Methionine (0.50g, 3.26mmol) was dissolved in water (15cm^3) and administered *via* a micropore filter to 15 x 90cm^3 cultures of *Xylaria cubensis* on day 4 such that the final concentration of L-[$^{13}C^2H_3$-*methyl*]-methionine in each flask was 2.42mM. After eight weeks, cubensic acid was isolated as previously described.

Feeding of sodium [1-^{13}C,2-$^2H_3$]-acetate to *Xylaria cubensis*

Sodium [1-^{13}C,2-$^2H_3$]-acetate (1g, 11.63mmol) was added to the culture medium before sterilisation. Thus the final concentration of sodium [1-^{13}C,2-$^2H_3$]-acetate was 16.15mM. Cubensic acid was isolated from the cultures (8 x 90cm^3) eight weeks after innoculation as described above.

Feeding of sodium [$^{13}C_2$]-acetate to *Xylaria cubensis*

Sodium [$^{13}C_2$]-acetate (1g, 11.91mmol) was added to the culture medium before sterilisation. Thus the final concentration of sodium [$^{13}C_2$]-acetate was 16.5mM. Cubensic acid was isolated from the cultures (8 x 90cm^3) eight weeks after innoculation as described above.

Feeding of N-acetylcysteamine-[2-$^{13}C^2H_3$-*methyl*]-2-methylbutyrate to *Xylaria cubensis*

N-acetylcysteamine-[2-$^{13}C^2H_3$-*methyl*]-2-methylbutyrate (1g, 4.52mmol) was dissolved in ethanol (2.7cm^3) and administered *via* a micropore filter on day 3 to 10 x 90cm^3 cultures of *Xylaria cubensis* such that the final concentration of N-acetylcysteamine-[2-$^{13}C^2H_3$-*methyl*]-2-methylbutyrate was 5mM. After eight weeks, cubensic acid was isolated as previously described.

CHAPTER 5

Methyl bromoacetate

An ethereal solution of diazomethane was added to a solution of bromoacetic acid (1g, 7.20mmol) in ether (70cm^3). Excess diazomethane was quenched cautiously with acetic acid. After washing with saturated sodium bicarbonate solution (30cm^3), the ether was removed by distillation at atmospheric pressure. The resultant methyl bromoacetate was not characterised.

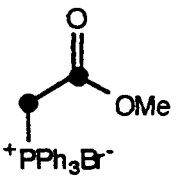
Methyl [$^{13}C_2$]-bromoacetate (29)

An ethereal solution of diazomethane was added to a solution of [$^{13}C_2$]-bromoacetic acid (1g, 7.09mmoles) in ether (70cm^3). The isolation of methyl [$^{13}C_2$]-bromoacetate was as outlined above.

Carbomethoxymethyltriphenylphosphonium bromide

Triphenylphosphine (1.98g, 7.56mmol) was added to a solution of methyl bromoacetate in acetonitrile (10cm^3). The solution was stirred at room temperature for 3 days. A precipitate was collected by filtration and washed with hexane (5cm^3). Drying under vacuum afforded carbomethoxymethyltriphenylphosphonium bromide (1.34g, 44.9%) as a white solid; m.p. 148.8 - 150.7°C (lit. [181] m.p. 163°C); (Found: C, 60.65; H, 4.81; N, 0%. $C_{21}H_{20}O_2BrP$ requires C, 60.74; H, 4.85; N, 0%); ν_{max} (KBr)/cm^{-1} 3053.4, 3006.1, 2800.4, 2731.5, 1723, 1586.6, 1485.0, 1439.5; δ_H ($CDCl_3$) 7.96 to 7.65 (15H, m, Ph), 5.61 (2H, d, $^2J_{PH}$ 13.4, CH_2), 3.60 (3H, s, CH_3); δ_C ($CDCl_3$) 165.22 (d, $^2J_{PC}$ 3.12, CO), 135.20 (d, $^4J_{PC}$ 3.1, p-CH), 134.01 (d, $^3J_{PC}$ 10.7, m-CH), 130.32 (d, $^2J_{PC}$ 13.4, o-CH), 117.93 (d, $^1J_{PC}$ 89.3, C), 53.47 (CH_3), 33.04 (d, $^1J_{PC}$ 58.0, CH_2); m/z (EI) 277 (41.98%), 262 (10.58%).

[1,2 -$^{13}C_2$]-Carbomethoxymethyltriphenylphosphonium bromide (30)



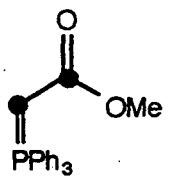
Triphenylphosphine (1.98g, 7.56mmol) was added to a solution of methyl [$^{13}C_2$]-bromoacetate in acetonitrile (8cm^3). Reaction and isolation conditions as

outlined above afforded [1,2-$^{13}C_2$]-carbomethoxymethyltriphenylphosphonium bromide (1.99g, 67.2%); m.p. 151.7 - 153.2°C; (Found: C, 60.95; H, 4.64; N, 0%. $C_{19}{}^{13}C_2H_{20}O_2BrP$ requires C, 60.93; H, 4.83; N, 0%); ν_{max} (KBr)/cm^{-1} 3053.5, 3008.1, 2791.8, 2728.8, 1681.8, 1586.7, 1485.0, 1439.6; δ_H ($CDCl_3$) 7.92 - 7.65 (15H, m, Ph), 5.54 (2H, ddd, $^1J_{CH}$ 134.4 and $^2J_{PH}$ 13.6 and $^2J_{CH}$ 7.7, $^{13}CH_2$), 3.59 (3H, d, $^3J_{CH}$ 4.06, CH_3); δ_C ($CDCl_3$) 165.51 (dd, $^1J_{CC}$ 62.9 and $^2J_{PC}$ 3.3, ^{13}CO), 135.71 (d, $^4J_{PC}$ 3.0, p-CH), 134.38 (d, $^3J_{PC}$ 10.8, m-CH), 130.79 (d, $^2J_{PC}$ 13.3, o-CH), 118.40 (d, $^1J_{PC}$ 85.5, C), 54.0 (CH_3), 33.37 (dd, $^1J_{CC}$ 59.6 and $^1J_{PC}$ 58.5, $^{13}CH_2$); m/z (EI) 303 (31.93%), 277 (100%), 262 (60.84%).

Carbomethoxymethylenetriphenylphosphine

A solution of carbomethoxymethyltriphenylphosphonium bromide (1.31g, 3.15mmol) in H_2O (15cm^3) was warmed until all the solid dissolved. Once cooled, dilute NaOH solution was added until the solution was just alkaline. The reaction was stirred for 30 min. at room temperature. H_2O was added and the product extracted into chloroform. Drying the organic extract and concentration yielded an oil which immediately precipitated a white solid upon the addition of ether. This solid was collected by filtration and dried to afford carbomethoxymethylenetriphenylphosphine (0.94g, 89.2%); m.p. 161.9 - 163.5°C (lit. [181] m.p. 162 - 163°C); (Found: C, 75.66; H, 5.81; N, 0%. $C_{21}H_{19}O_2P$ requires C, 75.44; H, 5.72: N, 0%); ν_{max} (KBr)/cm^{-1} 3057.7, 2958.8, 2842.0, 1618.3, 1482.0, 1434.5, 1347.9; δ_H ($CDCl_3$) 7.75 - 7.4 (16H, m, Ph and CH), 3.52 (3H, s, CH_3); δ_C ($CDCl_3$) 171.75 (d, $^2J_{PC}$ 4.0, CO), 133.04 (d, $^3J_{PC}$ 10.0, m-CH), 131.9 (d, $^4J_{PC}$ 2.3, p-CH), 128.73 (d, $^2J_{PC}$ 12.4, o-CH), 128.10 (d, $^1J_{PC}$ 91.9, C), 49.69 (CH_3), 29.72 (d, $^1J_{PC}$ 126.6, CH); m/z (CI) 303 (6.94%), 279 (29.05%).

[1,2-$^{13}C_2$]-Carbomethoxymethylenetriphenylphosphine (31)



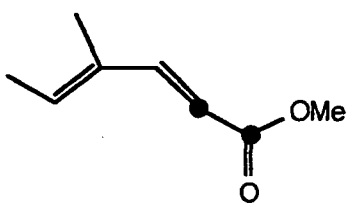
A solution of [1,2-$^{13}C_2$]-carbomethoxymethyltriphenylphosphonium bromide (1.99g, 4.77mmol) in H_2O (20cm^3) was warmed until all the solid had dissolved. Reaction and isolation proceeded as above to yield the first crop of [1,2-$^{13}C_2$]-carbomethoxymethylenetriphenylphosphine (1.26g, 3.74mmol). The ether

filtrate was concentrated to yield a second crop of [1,2-$^{13}C_2$]-carbomethoxymethylenetriphenylphosphine (0.33g, 0.99mmol). These two crops were combined (1.59g, 99.3%); ν_{max} (KBr)/cm^{-1} 3053.9, 3006.1, 2790.6, 1681.8, 1579.9, 1482.7, 1439.5; δ_H ($CDCl_3$) 7.9 - 7.4 (15H, m, Ph and CH), 3.56 (3H, d, $^3J_{CH}$ 3.9, CH_3); δ_C ($CDCl_3$) 171.90 (dd, $^1J_{CC}$ 87.2 and $^2J_{PC}$ 12.1, ^{13}CO), 133.51 (dd, $^4J_{CC}$ 26.8 and $^3J_{PC}$ 10.1, m-CH), 132.5 (d, $^4J_{PC}$ 5.2, p-CH), 129.12 (dd, $^3J_{CC}$ 33.5 and $^2J_{PC}$ 12.8, o-CH), - (d, J -, C), 50.3 (CH_3), 30.41 (dd, $^1J_{CC}$ 124.6 and $^1J_{PC}$ 87.3, ^{13}CH); m/z (CI) 337 (0.32%), 305 (1.90%), 279 (2.59%).

Methyl (E,E)-4-methyl-2,4-hexadienoate

Trans-2-methyl-2-butenal (0.29cm^3, 2.97mmol) was added to a solution of carbomethoxymethylenetriphenylphosphine (0.90g, 2.70mmol) in dried and distilled ethanol (20cm^3) under an atmosphere of N_2. The solution was heated under reflux for 24h. The ethanol was evaporated under reduced pressure and purification over silica gel (100% dichloromethane) afforded methyl (E,E)-4-methyl-2,4-hexadienoate (0.24g, 64.1%) as a colourless oil; ν_{max} (neat)/cm^{-1} 2949.8, 1718.1, 1620.1, 1432.9; δ_H ($CDCl_3$) 7.32 (1H, d, J 15.7, CH), 5.97 (1H, q, J 6.0, CH), 5.78 (1H, d, J 15.1, CH), 3.74 (3H, s, CH_3), 1.81 (3H, d, J 7.6, CH_3), 1.77 (3H, s, CH_3); δ_C ($CDCl_3$) 168.05 (CO), 149.73 (CH), 136.50 (CH), 133.78 (C), 114.85 (CH), 51.40 (OMe), 14.56 (CH_3), 11.76 (CH_3); m/z (EI) 141 (M+1, 100%), 125 (56.45%), 109 (61.32%).

Methyl (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (32)



Trans-2-methyl-2-butenal (0.50cm^3, 5.20mmol) was added to a solution of [1,2-$^{13}C_2$]-carbomethoxymethylenetriphenylphosphine (1.59g, 4.73mmol) in dried and distilled ethanol (30cm^3) under an atmosphere of N_2. Reaction and purification as above afforded methyl (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (0.53g, 80.4%); ν_{max} (neat)/cm^{-1} 2949.1, 1672.4, 1631.2, 1594.0, 1433.2; δ_H ($CDCl_3$) 7.32 (1H, ddd, $^3J_{HH}$ 15.7 and $^2J_{CH}$ 6.6 and $^3J_{CH}$ 2.6, CH), 5.98 (1H, q, J 7.0, CH), 5.77 (1H, ddd, $^1J_{CH}$ 161.97 and $^3J_{HH}$ 15.7 and $^2J_{CH}$ 3.2, CH), 3.74 (3H, d, $^3J_{CH}$ 3.8, CH_3), 1.81 (3H, d, J 7.6, CH_3), 1.77 (3H, s, CH_3); δ_C ($CDCl_3$)

168.22 (d, $^{1}J_{CC}$ 76.3, ^{13}CO), 149.75 (d, $^{1}J_{CC}$ 75.4, CH), 136.72 (d, $^{3}J_{CC}$ 7.9, CH), 134.2 (d, $^{2}J_{CC}$ 10.5, C), 115.14 (d, $^{1}J_{CC}$ 76.4, ^{13}CH), 51.8 (OMe), 14.79 (CH_3), 12.2 (CH_3).

(E,E)-4-Methyl-2,4-hexadienoic acid

A solution of methyl (E,E)-4-methyl-2,4-hexadienoate (0.24g, 1.72mmol) in methanol (12cm^3) and 1M NaOH (8cm^3) was stirred for 16h at room temperature. The methanol was removed under reduced pressure. After addition of water, the aqueous extract was acidified with dilute HCl at 0°C. The organics were extracted into ether, dried and concentrated to yield (E,E)-4-methyl-2,4-hexadienoic acid (0.19g, 85.1%) as a white solid; m.p. 91.0 - 92.7°C (lit. [182] 94 - 95°C); ν_{max} (KBr)/cm^{-1} 2995.4, 2702.2, 2593.4, 1705.9, 1674.5, 1616.1, 1417.8; δ_H ($CDCl_3$) 11.98 (1H, s, OH), 7.41 (1H, d, J 15.7, CH), 6.06 (1H, q, J 10.0, CH), 5.79 (1H, d, J 15.6, CH), 1.83 (3H, d, J 7.4, CH_3), 1.79 (3H, s, CH_3); δ_C ($CDCl_3$) 173.47 (CO), 151.90 (CH), 137.91 (CH), 133.85 (C), 114.49 (CH), 14.68 (CH_3), 11.76 (CH_3); m/z (EI) 126 (M^+, 64.52%), 111 (100%), 81 (63.39%).

(E,E)-[1,2-$^{13}C_2$]-4-Methyl-2,4-hexadienoic acid (33)

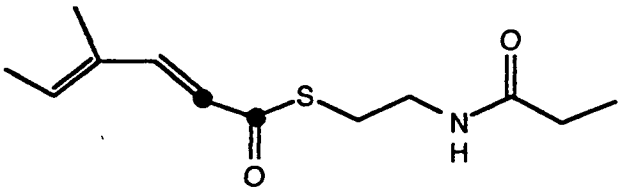
A solution of methyl (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (0.53g, 3.75mmol) in methanol (18cm^3) and 1M NaOH (12cm^3) was stirred for 16h at room temperature. Isolation as outlined above yielded (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoic acid (0.18g, 38.4%); δ_H ($CDCl_3$) 12.15 (1H, s, OH), 7.40 (1H, ddd, $^{3}J_{HH}$ 15.7 and $^{2}J_{CH}$ 6.5 and $^{3}J_{CH}$ 2.2, CH), 6.04 (1H, q, J 6.8, CH), 5.78 (1H, ddd, $^{1}J_{CH}$ 162.5 and $^{3}J_{HH}$ 15.6 and $^{2}J_{CH}$ 2.8, CH), 1.83 (3H, d, J 7.7, CH_3), 1.79 (3H, s, CH_3); δ_C ($CDCl_3$) 173.89 (d, $^{1}J_{CC}$ 69.7, ^{13}CO), 152.5 (d, $^{1}J_{CC}$ 70.4, CH), 138.6 (d, $^{3}J_{CC}$ 20.1, CH), 134.3 (d, C), 114.91 (d, $^{1}J_{CC}$ 73.8, ^{13}CH), 15.07 (CH_3), 11.01 (CH_3); m/z (EI) 128 (M^+, 69.23%), 113 (100%), 82 (71.34%).

N-Acetylcysteamine-(E,E)-4-methyl-2,4-hexadienoate

1,3-Dicyclohexylcarbodiimide (0.29g, 1.40mmol) and 4-dimethylaminopyridine (0.17g, 1.40mmol) and N-propionyl cysteamine (0.19g, 1.40mmol) was added to a solution of (E,E)-4-methyl-2,4-hexadienoic acid (0.18g, 1.40mmol) in dried and distilled ether (20cm^3) under an atmosphere of N_2. The reaction mixture was stirred at room temperature for 12h. The white precipitate was removed by filtration and discarded. The filtrate was concentrated and purified over silica gel (ethyl acetate : acetone [85 : 15 v/v]). The active fractions combined afforded N-acetylcysteamine-(E,E)-4-methyl-2,4-hexadienoate (0.22g, 66%) as a

white solid; m.p. 86.1-87.9°C; ν_{max} (KBr)/cm^{-1} 3301.7, 3080.6, 2980.6, 2931.0, 1673.0, 1641.4, 1599.8, 1550.6; δ_H ($CDCl_3$) 7.26 (1H, d, J 15.5, CH), 6.44 (1H, m, CH), 6.09 (1H, d, J 15.5, CH), 3.48 (2H, dt, J 6, CH_2), 3.13 (2H, t, J 8.5, CH_2), 2.21 (2H, q, J 7.7, CH_2), 1.84 (3H, d, J 7.0, CH_3), 1.78 (3H, s, CH_3), 1.14 (3H, t, J 7.4, CH_3); δ_C ($CDCl_3$) 190.43 (CO), 174.20 (CO), 146.20 (CH), 138.99 (CH), 133.62 (C), 122.10 (CH), 39.73 (CH_2), 29.59 (CH_2), 28.36 (CH_2), 14.88 (CH_3), 11.74 (CH_3), 9.84 (CH_3); m/z (CI) 242 (M+1, 100%), 109 (38.49%).

N-Acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (34)



1,3-Dicyclohexylcarbodiimide (0.30g, 1.44mmol) and 4-dimethylaminopyridine (0.18g, 1.44mol) and N-propionyl cysteamine (0.26g, 1.95mmol) were added to a solution of (E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoic acid (0.18g, 1.44mmol) in dried and distilled ether (25cm^3) under an atmosphere of N_2. Reaction and isolation conditions as outlined above yielded N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (0.15g, 43.5%) which had to be stored at 4°C; (Found: M+1, 244.1291. $C_{10}{}^{13}C_2H_{19}O_2NS$ requires M+1, 244.1282); δ_H ($CDCl_3$) 7.26 (1H, ddd, $^3J_{HH}$ 15.5 and $^2J_{CH}$ 7.2 and $^3J_{CH}$ 1.5, CH), 6.44 (1H, q, J 7.0, CH), 6.09 (1H, ddd, $^1J_{CH}$ 160.9 and $^3J_{HH}$ 15.3 and $^2J_{CH}$ 5.4, CH), 3.47 (2H, dt, J 6.4, CH_2), 3.13 (2H, t, J 5, CH_2), 2.21 (2H, q, J 7.6, CH_2), 1.84 (3H, d, J 7.0, CH_3), 1.77 (3H, s, CH_3), 1.14 (3H, t, J 7.7, CH_3); δ_C ($CDCl_3$) 190.85 (d, $^1J_{CC}$ 63.4, ^{13}CO), 174.65 (CO), 146.8 (d, $^1J_{CC}$ 55.3, CH), 139.5 (d, $^3J_{CC}$ 10.1, CH), 134.02 (d, C), 122.51 (d, $^1J_{CC}$ 63.5, ^{13}CH), 40.15 (CH_2), 30.02 (CH_2), 28.79 (CH_2), 15.03 (CH_3), 12.03 (CH_3), 10.27 (CH_3).

3-Tetradecylthiopropionic acid

3-Mercaptopropionic acid (0.41cm^3, 4.71mmol) was carefully added to a stirred solution of KOH (0.64g, 11.35mmol) in dried and distilled methanol (10cm^3) under N_2 and at room temperature. 1-Bromotetradecane (0.55cm^3, 1.84mmol) was then added dropwise and the reaction mixture stirred for 12h at room temperature. H_2O (20cm^3) was added and the mixture heated until a turbid solution was obtained. Acidification with conc. HCl afforded a white

precipitate which was collected by filtration. Recrystallisation with acetone : water (9 : 1, 10cm^3) yielded 3-tetradecylthiopropionic acid (0.23g, 41.4%) as a white crystalline solid; m.p. 70.0 - 70.9°C; (Found: M^+, 302.2277. $C_{17}H_{34}O_2S$ requires M^+, 302.2280); ν_{max} (KBr)/cm^{-1} 2917.7, 2846.9, 1686.0, 1462.5, 1407.5; δ_H ($CDCl_3$) 2.78 (2H, broad t, J 6.8, CH_2), 2.67 (2H, t, J 6.8, CH_2), 2.54 (2H, broad t, J 7.20, CH_2), 1.58 (2H, p, J 8.0, CH_2), 1.37 (2H, p, J 7.20, CH_2), 1.26 (20H, broad s, CH_2), 0.88 (3H, t, J 6.4, CH_3); δ_C ($CDCl_3$) 177.96 (CO), 34.64 (CH_2), 32.19 (CH_2), 31.92 (CH_2), 29.65 (6 x CH_2), 29.52 (CH_2), 29.36 (CH_2), 29.22 (CH_2), 28.86 (CH_2), 26.56 (CH_2), 22.69 (CH_2), 14.12 (CH_3); m/z (EI) 302 (M^+, 22.58%), 229 (100%).

Isolation and purification of cytochalasin D

The supernatant from the *Xylaria cubensis* cultures (10 x 90cm^3) was extracted into ethyl acetate (400cm^3) and this organic extract was dried and concentrated. Purification by silica gel column chromatography (100% ethyl acetate) afforded cytochalasin D (100mg) as a white solid. A full ^{13}C n.m.r. assignment is detailed in Table A4 (Appendix 1, pg. iv); m.p. 238.5 - 240.7°C.

Feeding of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate to *Xylaria cubensis*

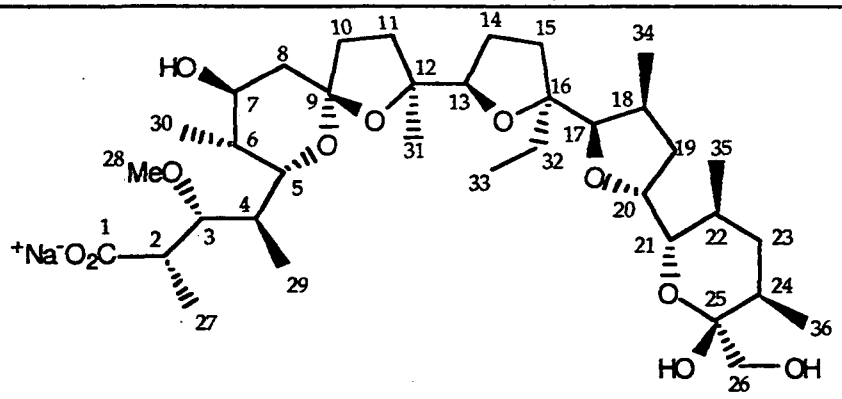
N-Acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (55.8mg, 0.23mmol) was dissolved in ethanol (4cm^3) and administered to 2 x 90cm^3 cultures of *Xylaria cubensis via* a micropore filter on day 4, such that the final concentration of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate was 1.3mM. Cytochalasin D was isolated after three weeks as outlined above.

Feeding of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate and 3-tetradecylthiopropionic acid to *Xylaria cubensis*

3-Tetradecylthiopropionic acid (50mg, 0.17mmol) was dissolved in ethanol (0.75cm^3) with heating and was administered quickly before cooling (in order to prevent it crashing out of solution) *via* a micropore filter to 1 x 90cm^3 flask of *Xylaria cubensis* before innoculation. On day 4, 3-tetradecylthiopropionic acid (50mg) was administered in a similar manner, followed by the addition of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate (30mg, 0.12mmol) which was dissolved in ethanol (1cm^3) and administered *via* a micropore filter such that the final concentration of N-acetylcysteamine-(E,E)-[1,2-$^{13}C_2$]-4-methyl-2,4-hexadienoate was 1.37mM. Cytochalasin D was isolated after three weeks as outlined above.

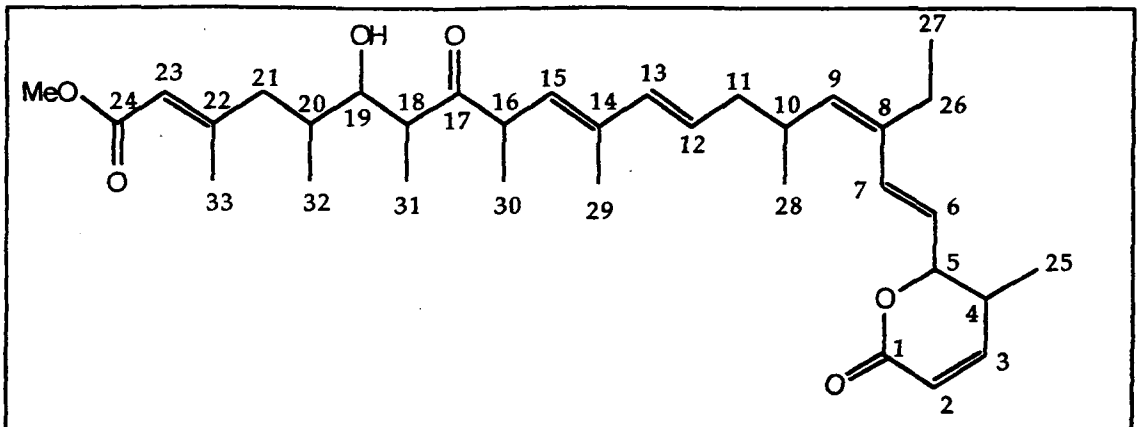
APPENDIX 1

TABLES



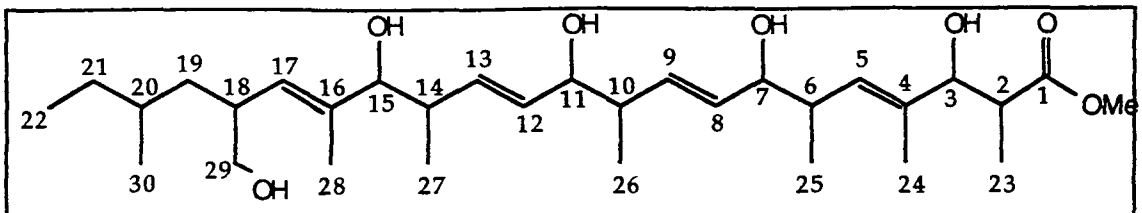
	^{13}C/ppm	lit.[47] ^{13}C/ppm		^{13}C/ppm	lit.[47] ^{13}C/ppm
C1	181.26	181.20	C19	33.24	33.33
C2	44.98	45.07	C20	76.40	76.45
C3	82.93	83.11	C21	74.46	74.51
C4	37.44	37.50	C22	31.83	31.87
C5	68.32	68.33	C23	35.60	35.75
C6	34.83	34.87	C24	36.50	36.56
C7	70.41	70.51	C25	98.26	98.31
C8	33.51	33.56	C26	64.85	64.92
C9	106.94	107.03	C27	16.69	16.72
C10	39.21	39.28	C28	57.92	57.84
C11	33.16	33.25	C29	11.07	10.97
C12	85.19	85.26	C30	10.50	10.48
C13	82.49	82.52	C31	27.44	27.47
C14	27.26	27.26	C32	30.65	30.60
C15	29.81	29.91	C33	8.23	8.14
C16	85.79	85.90	C34	14.57	14.56
C17	84.97	84.94	C35	16.82	16.80
C18	34.30	34.39	C36	16.07	16.03

Table A1: ^{13}C n.m.r. chemical shifts of monensin A recorded as a solution in $CDCl_3$ at 100MHz compared with literature values.[47]



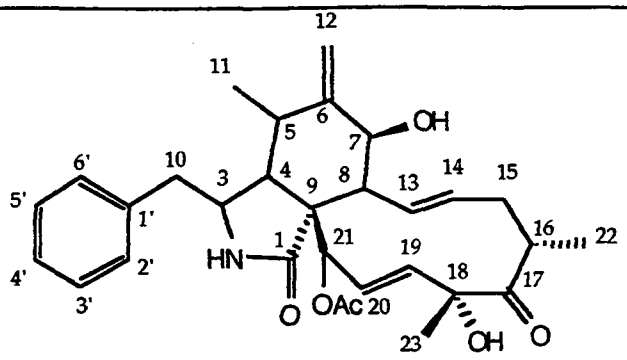
	^{13}C/ppm	lit.[102] ^{13}C/ppm		^{13}C/ppm	lit.[102] ^{13}C/ppm
C1	164.3	164.2	C18	46.5	47.0
C2	120.1	120.0	C19	74.3	74.2
C3	151.5	151.7	C20	33.4	33.6
C4	33.6	33.6	C21	45.2	45.7
C5	81.5	81.5	C22	158.5	158.4
C6	122.7	122.8	C23	117.0	117.1
C7	130.2	130.2	C24	167.0	167.0
C8	135.5	135.5	C25	12.4	12.4
C9	137.0	137.0	C26	26.6	26.6
C10	32.2	32.2	C27	13.6	13.6
C11	40.8	40.8	C28	13.1	13.1
C12	128.2	128.2	C29	18.4	18.4
C13	135.2	135.3	C30	12.5	12.4
C14	136.5	136.4	C31	20.9	20.8
C15	128.1	128.0	C32	13.7	13.7
C16	45.3	45.7	C33	16.1	16.1
C17	215.3	215.2	OMe	50.9	50.8

Table A2: ^{13}C n.m.r. chemical shifts of leptomycin B methyl ester recorded as a solution in $CDCl_3$ at 100MHz compared with literature values.[102]



	^{13}C/ppm	lit.[112] ^{13}C/ppm		^{13}C/ppm	lit.[112] ^{13}C/ppm
C1	176.59	176.61	C17	131.17	131.81
C2	44.22	43.97	C18	39.03	38.91
C3	80.98	81.27	C19	39.03	38.79
C4	135.67	135.62	C20	32.37	32.41
C5	133.19	133.58	C21	30.89	30.91
C6	38.80	38.55	C22	11.74	11.72
C7	77.48	78.01	C23	14.73	14.71
C8	133.21	133.66	C24	10.88	10.73
C9	135.92	136.64	C25	17.39	17.48
C10	43.68	43.74	C26	17.47	17.84
C11	77.35	77.81	C27	17.96	18.02
C12	132.92	133.12	C28	11.66	11.53
C13	136.60	137.32	C29	66.82	66.93
C14	40.69	40.78	C30	19.04	19.05
C15	82.93	83.3	OMe	51.43	51.50
C16	138.40	138.33			

Table A3: ^{13}C n.m.r. chemical shifts of methyl cubensate recorded as a solution in C_5D_5N at 100MHz compared with literature values.[112]



	^{13}C/ppm	lit.[156] ^{13}C/ppm		^{13}C/ppm	lit.[156] ^{13}C/ppm
C1	175.27	174.9	C16	42.60	42.45
C3	54.16	54.00	C17	211.03	210.7
C4	50.22	50.00	C18	78.56	78.32
C5	33.30	33.12	C19	128.00	127.7
C6	151.77	151.4	C20	133.97	133.7
C7	71.44	71.20	C21	78.14	77.92
C8	48.02	47.8	C1′	138.59	138.3
C9	54.52	54.37	C2′, C6′	130.06	129.9
C10	45.64	45.49	C3′, C5′	128.96	128.7
C11	13.76	13.65	C4′	126.97	126.8
C12	112.34	112.2	C22	19.52	19.44
C13	132.35	132.10	C=O (Ac)	170.64	170.3
C14	132.92	132.7	CH_3 (Ac)	20.69	20.61
C15	38.71	38.58	C23	24.77	24.64

Table A4: ^{13}C n.m.r. chemical shifts of cytochalasin D recorded as a solution in C_5D_5N at 100MHz compared with literature values[156]

APPENDIX 2

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APPENDIX 3

COLLOQUIA, LECTURES AND SEMINARS FROM INVITED SPEAKERS

Sept. 1991 - Sept. 1992

17/10/91 Dr. J.A. Salthouse, University of Manchester,
Son et Lumiere - a demonstration lecture

31/10/91 Dr. R. Keeley, Metropolitan Police Forensic science,
Modern forensic science

6/11/91 Prof. B.F.G. Johnson, Edinburgh University,
Cluster-surface analogies

7/11/91 Dr. A.R. Butler, St. Andrews University,
Traditional Chinese herbal drugs: a different way of treating disease

13/11/91 ∞ Prof. D. Gani, St. Andrews University,
The chemistry of PLP-dependent enzymes

19/11/91 ∞ At University of Newcastle,
Dr. I. Fleming, University of Cambridge
Stereocontrol in organic synthesis using silicon

20/11/91 ∞ Dr. R. More O'Ferrall, University College, Dublin,
Some acid-catalysed rearrangements in organic chemistry

28/11/91 Prof. I.M. Ward, IRC in Polymer Science, Univerity of Leeds,
The SCI lecture: the science and technology of orientated polymers

4/12/91 ∞ Prof. R. Grigg, Leeds University,
Palladium-catalysed cyclisation and ion-capture processes

5/12/91 Prof. A.L. Smith, ex Unilever,
Soap, detergents and black puddings

11/12/91 Dr. W.D. Cooper, Shell Research,
Colloid science: theory and practice

18/12/91 ∞ **Modern aspects of stereochemistry at Sheffield University**
Prof. D.A. Evans, Studies in asymmetric synthesis
Prof. S.V. Ley, Synthesis of insect antifeedants
Prof. P.B. Dervan, A chemists approach toward a general solution for the sequence specific recognition of double helical DNA

Prof. Dr. D. Bellus, Some stereochemical challenges in biologically oriented industrial research
Prof. Sir. D. Barton, The invention of chemical reactions

22/1/92 Dr. K.D.M. Harris, St. Andrews University,
Understanding the properties of solid inclusion compounds

29/1/92 ∞ Dr. A. Holmes, Cambridge University,
Cycloaddition reactions in the service of the synthesis of piperidine and indolizidine natural products

30/1/92 ∞ Dr. M. Anderson, Sittingbourne Research centre, Shell Research,
Recent advances in the safe and selective chemical control of insect pests

12/2/92 ∞ Prof. D.E. Fenton, Sheffield university,
Polynuclear complexes of molecular clefts as models for copper biosites

13/2/92 Dr. J. Saunders, Glaxo Group Research Limited,
Molecular modelling in drug discovery

19/2/92 ∞ Prof. E.J. Thomas, Manchester University,
Applications of organostannanes to organic synthesis

20/2/92 ∞ Prof. E. Vogel, University of Cologne,
The Musgrave Lecture, Porphyrins: Molecules of interdisciplinary interest

25/2/92 ∞ Prof. J.F. Nixon, University of Sussex,
The Tilden Lecture, Phosphaalkynes: new building blocks in inorganic and organometallic chemistry

26/2/92 Prof. M.L. Hitchman, Strathclyde University,
Chemical vapour deposition

5/3/92 Dr. N.C. Billingham, University of Sussex,
Degradable plastics - myth or magic?

11/3/92 ∞ Dr. S.E. Thomas, Imperial College,
Recent advances in organoiron chemistry

12/3/92 Dr. R.A. Hann, ICI Imagedata,
Electronic photography - an image of the future

18/3/92 ∞ Dr. H. Maskill, Newcastle University,
Concerted or stepwise fragmentation in a deamination-type reaction

7/4/92 Prof. D.M. Knight, Philosophy Department, University of Durham,
Interpreting experiments: the beginning of electrochemistry

13/5/92 ∞ Dr. J-C. Gehret, Ciba Geigy, Basel,
Some aspects of industrial agrochemical research

3/7/92 ∞ Dr. B. Parmar,
Biological systems in organic synthesis

Sept. 1992 - Sept. 1993

15/10/92 Dr. M. Glazer and Dr. S. Tarling, Oxford University and Birbeck college, London,
It pays to be British! - The chemist's role as an expert witness in patent litigation

20/10/92 Dr. H.E. Bryndza, Du Pont Central Research,
Synthesis, reactions and thermochemistry of metal (alkyl) cyanide complexes and their impact on olefin hydrocyanation catalysis

22/10/92 Prof. A. Davies, University College, London,
The Ingold-Albert Lecture, The behaviour of hydrogen as a pseudometal

28/10/92 Dr. J.K. Cockcroft, University of Durham,
Recent developments in powder diffraction

29/10/92 Dr. J. Emsley, Imperial College, London,
The shocking history of Phosphorus

4/11/92 Dr. T.P. Kee, University of Leeds,
Synthesis and co-ordination chemistry of silylated phosphites

5/11/92 ∞ Dr. C.J. Ludman, University of Durham,
Explosions, A demonstration lecture

11/11/92 ∞ Prof. D. Robins, Glasgow University,
Pyrrolizidine alkaloids: biological activity, biosynthesis and benefits

12/11/92 Prof. M.R. Truter, University College, London,
Luck and logic in host - guest chemistry

18/11/92 Dr. R. Nix, Queen Mary College, London,
Characterisation of heterogeneous catalysts

25/11/92 Prof. Y. Vallee, University of Caen,

Reactive thiocarbonyl compounds

25/11/92 Prof. L.D. Quin, University of Massachusetts, Amherst,
Fragmentation of phosphorus heterocycles as a route to a phosphoryl species with uncommon bonding

26/11/92 ∞ Dr. D. Humber, Glaxo, Greenford,
AIDS - The development of a novel series of inhibitors of HIV

2/12/92 ∞ Prof. A.F. Hegarty, University College, Dublin,
Highly reactive enols stabilised by steric protection

2/12/92 ∞ Dr. R.A. Aitken, University of St. Andrews,
The versatile cycloaddition chemistry of $Bu_3P.CS_2$

3/12/92 Prof. P. Edwards, Birmingham University,
The SCI lecture - What is metal?

9/12/92 ∞ Dr. A.N. Burgess, ICI Runcorn,
The structure of perfluorinated ionomer membranes

6/1/93 ∞ Dr. K.A. Reynolds, University of Maryland at Baltimore,
Comparison of two enoyl-CoA reductases in *Streptomyces collinus*

20/1/93 Dr. D.C. Clary, University of Cambridge,
Energy flow in chemical reactions

21/1/93 Prof. L. Hall, Cambridge,
NMR - Window to the human body

27/1/93 ∞ Dr. W. Kerr, University of Strathclyde,
Development of the Pauson-Khand annulation reaction : Organocobalt mediated synthesis of natural and unnatural products

28/1/93 ∞ Prof. J. Mann, University of Reading,
Murder, magic and medicine

3/2/93 ∞ Prof. S.M. Roberts, University of Exeter,
Enzymes in organic synthesis

10/2/93 Dr. D. Gillies, University of Surrey,
NMR and molecular motion in solution

11/2/93 ∞ Prof. S. Knox, Bristol University,
The Tilden Lecture, Organic chemistry at polynuclear metal centres

17/2/93 Dr. R.W. Kemmitt, University of Leicester,
Oxatrimethylenemethane metal complexes

18/2/93		Dr. I. Fraser, ICI Wilton, Relative processing of composite materials
22/2/93		Prof. D.M. Grant, University of Utah, Single crystals, molecular structure and chemical-shift anisotropy
24/2/93		Prof. C.J.M. Stirling, University of Sheffield, Chemistry on the flat-reactivity of ordered systems
10/3/93		Dr. P.K. Baker, University College of North Wales, Bangor, Chemistry of highly versatile 7-coordinate complexes
11/3/03		Dr. R.A.Y. Jones, University of East Anglia, The chemistry of wine making
17/3/93	∞	Dr. R.J.K. Taylor, University of East Anglia, Adventures in natural product synthesis
24/3/93		Prof. I.O. Sutherland, University of Liverpool, Chromogenic reagents for cations
23/4/93	∞	At Sterling Winthrop Research Centre, Alnwick, Dr. C. Gibson, University of Strathclyde, Tritium NMR
13/5/93		Prof. J.A. Pople, Carnegie-Mellon University, Pittsburgh, USA, *The Boys-Rahman Lecture*, Applications of molecular orbital theory
21/5/93		Prof. L. Weber, University of Bielefeld, Metallo-phospha alkenes as synthons in organometallic chemistry
1/6/93		Prof. J.P. Konopelski, University of California, Santa Cruz, Synthetic adventures with enantiomerically pure acetals
2/6/93		Prof. F. Ciardelli, University of Pisa, Chiral discrimination in the stereospecific polymerisation of alpha olefins
7/6/93		Prof. R.S. Stein, University of Massachusetts, Scattering studies of crystalline and liquid crystalline polymers
16/6/93		Prof. A.K. Covington, University of Newcastle, Use of ion selective electrodes as detectors in ion chromatography

17/6/93 Prof. O.F. Nielsen, H. C. Ørsted Institute, University of Copenhagen,
Low frequency IR- and Raman studies of hydrogen bonded liquids

Sept. 1993 - Sept. 1994

13/9/93 Prof. Dr. A.D. Schlüter, Freie Universität Berlin, Germany,
Synthesis and characterisation of molecular rods and ribbons

13/9/93 Dr. K.J. Wynne, Office of Naval Research, Washington, USA,
Polymer surface design for minimal adhesion

14/9/93 Prof. J.M. DeSimone, University of North Carolina, Chapel Hill, USA,
Homogeneous and heterogeneous polymerisations in environmentally responsible carbon dioxide

28/9/93 ∞ Prof. H. Ila, North Eastern Hill University, India,
Synthetic strategies for cyclopentanoids via oxoketene dithioacetals

4/10/93 Prof. F.J. Feher, University of California, Irvine, USA,
Bridging the gap between surfaces and solution with sessilquioxanes

6/10/93 ∞ At Newcastle University,
Prof. D. Seebach, ETH Zurich,
The Clemo Lecture, TADDOLS, versatile auxillarys for the preparation of enantiomerically pure compounds

14/10/93 Dr. P. Hubberstey, University of Nottingham,
Alkali metals: Alchemist's nightmare, Biochemist's puzzle and technologist's dream

20/10/93 ∞ Dr. P. Quayle, University of Manchester,
Aspects of aqueous ROMP chemistry

21/10/93 Prof. R. Adams, University of South Carolina, USA,
Chemistry of metal carbonyl cluster complexes:
development of cluster based alkyne hydrogenation catalysts

27/10/93 ∞ Dr. R.A.L. Jones, Cavendish Laboratory, Cambridge,
Perambulating polymers

10/11/93 Prof. M.N.R. Ashfold, University of Bristol,

		High resolution photofragment translational spectroscopy: A new way to watch photodissociation
17/11/93		Dr. A. Parker, Rutherford Appleton Laboratory, Didcot, Applications of time resolved resonance Raman spectroscopy to chemical and biochemical problems
18/11/93	∞	Prof. D. Parker, University of Durham, Complex chemistry with simple solutions
24/11/93	∞	Dr. P.G. Bruce, University of St. Andrews, Structure and properties of inorganic solids and polymers
25/11/93		Dr. R.P. Wayne, University of Oxford, The origin and evolution of the atmosphere
1/12/93	∞	Prof. M.A. McKervey, Queens University, Belfast, Synthesis and applications of chemically modified calixarenes
8/12/93		Prof. O. Meth-Cohn, University of Sunderland, Friedel's folly revisited - A super way to fused pyridines
16/12/93		Prof. R.F. Hudson, University of Kent, Close encounters of the second kind
26/1/94	∞	Prof. J. Evans, University of Southampton, Shining light on catalysts
2/2/94		Dr. A. Masters, University of Manchester, Modelling water without using pair potentials
9/2/94	∞	Prof. D. Young, University of Sussex, Chemical and biological studies on the coenzyme tetrahydrofolic acid
16/2/94		Prof. K.H. Theopold, University of Delaware, USA, Paramagnetic chromium alkyls : Synthesis and reactivity
23/2/94		Prof. P.M. Maitlis, University of Sheffield, Across the border : From homogeneous to heterogeneous catalysis
25/2/94	∞	Prof. Liulle, Estonian Academy of Sciences, Estonia, Studies in prostaglandins
2/3/94	∞	Dr. C. Hunter, University of Sheffield, Noncovalent interactions between aromatic molecules
9/3/94		Prof. F. Wilkinson, Loughborough University of Technology, Nanosecond and picosecond laser flash photolysis

10/3/94	∞	Prof. S.V. Ley, University of Cambridge, New methods for organic synthesis
25/3/94		Dr. J. Dilworth, University of Essex, Technetium and rhenium compounds with applications as imaging agents
28/4/94		Prof. R.J. Gillespie, McMaster University, Canada, The molecular structure of some metal fluorides and oxofluorides: apparent exceptions to the VSEPR model
12/5/94		Prof. D.A. Humphreys, McMaster University, Canada, Bringing knowledge to life

∞ indicates lectures attended by the writer

RESEARCH CONFERENCES ATTENDED

18/12/91		Modern aspects of stereochemistry at Sheffield University
13/12/93	†	One day postgraduate symposium on bio-organic chemistry, University of Exeter
16/3/94	†	Symposium on recent progress in polyketide biosynthesis, St. Johns College, University of Cambridge
1/6/94	†	1994 Graduate colloquia, University of Durham

† indicates oral presentation given by the writer

PAPERS PUBLISHED

D. O'Hagan, S.V. Rogers, G.R. Duffin and R.L. Edwards, *Tetrahedron Lett.*, 1992, 33, 5585.

D. O'Hagan, S.V. Rogers, K.A. Reynolds and G.R. Duffin, *J. Chem. Soc., Chem. Commun.*, 1994, 1577.