Biosynthetic studies on tenellin and aminoisobutyrate metabolism in streptomyces sp

Moore, M. Caragh

BIOSYNTHETIC STUDIES ON TENELLIN AND AMINOISOBUTYRATE METABOLISM IN STREPTOMYCES SP.

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Ph.D. Thesis

Department of Chemistry

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DECLARATION

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ABSTRACT

Biosynthetic Studies on Tenellin and Aminoisobutyrate Metabolism in Streptomyces sp.

M. Caragh Moore, B.Sc.

This thesis is divided into two parts. Part 1 covers the biosynthesis of the fungal metabolite tenellin, and Part 2 the metabolism of β-aminoisobutyrate in Streptomyces sp.

Tenellin is a yellow pigment of the fungus *Beauvaria bassiana*. It is of mixed biosynthetic origin, being derived from a polyketide moiety and the amino acid L-phenylalanine. The timing of the C-methylations of the polyketide chain is discussed in Chapter 2, which describes attempts to incorporate deuterium labelled partially assembled putative intermediates into the polyketide. The biosynthesis of the pyridone ring of tenellin requires the condensation of the polyketide moiety with a rearranged phenylpropanoid unit derived from phenylalanine. The nature of this intriguing intramolecular rearrangement is discussed in Chapters 3 and 4. A phenylalanine derived tetramic acid, proposed as an intermediate in the biosynthesis, has been synthesised, and used in biosynthetic investigations. The results of these investigations and the subsequent identification of tyrosine as a closer precursor to tenellin argue against its intermediacy. The failure of [2-$^{13}C^{2}H^{15}N$]-phenylalanine to become incorporated intact suggests a transamination process for phenylalanine / tyrosine prior to incorporation. Preliminary investigations suggest *para*-hydroxy phenyllactate may be the substrate for the rearranging enzyme and a more direct precursor to tenellin.

β-Aminoisobutyrate, the end product of reductive thymine catabolism, contributes to both the propionate and butyrate pools in Streptomyces sp. The pathway of incorporation into the isobutyrate / butyrate pool has been investigated, and confirmed to be the reverse of that known to occur in L-valine metabolism. A mutant strain of *Streptomyces avermitilis*, unable to produce isobutyrate, was used due to low level incorporations into the branched-chain fatty acids. This work was carried out in collaboration with Dr. Hamish McArthur, Pfizer Central Research Division, Groton, USA, and Dr. Kevin Reynolds, Department of Pharmaceutical Science, University of Maryland.

To Dad,

who got me into all this,

and to Jonathan,

who got me out of it.

"... for Thou hast created all things

and for Thy pleasure

they are and were created."

(Revelation Chapter 4, verse 11)

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The research described would be impossible without the support and expertise of the technical staff, particularly Julia and Barry Say, Ian McKeag, and Alan Kenright for NMR analysis, and for many hours on the ^{2}H NMR spectrometer. The stores, Jimmy and Joe, the glass blowers, Gordon and Ray, and Brenda (who tirelessly scrubbed lab 8C) are all thanked for their contributions. I also thank Michael Prodigalidad for assisting me in printing the final draft, and Mr J. D. Heenan for kindly lending his laser printer to me.

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Had it not been for the encouragement and advice of Mum and Dad this much enjoyed period of research would not have been begun. Thank you for many journeys made to Durham with an overflowing car boot, for stimulating my interest in scientific research and for home-made cakes. Thank you also Brian and Brenda for friendship, fellowship and loving oversight, and for your care of Jonathan, the ghost lodger, on his many and lengthy visits from Cambridge. Lastly, thank you Jonathan, at last my husband, for your wonderful support in our marathon effort to complete and to submit our theses. Oh and I must also acknowledge Baby Moore, without whose imminent arrival I might still not have submitted!

CONTENTS

PART 1
BIOSYNTHETIC STUDIES ON TENELLIN

1. Introduction

1.1 Primary and Secondary Metabolism

Study of the vital metabolic processes of living things has revealed the detail of the complex web of cycles of enzyme catalysed reactions. Beginning with the substrates of photosynthesis, CO_2 and H_2O, many diverse classes of essential molecules are built up and used to construct the primary metabolites. Of central importance is the co-enzyme adenosine triphosphate, (ATP) which is the cell's energy source, working in co-operation with particular enzymes in the reactions they catalyse. This web of biochemical reactions referred to as primary metabolism produces metabolites including amino acids, sugars, nucleotides, acetyl-coenzyme A, mevalonic acid, all of which are essential for metabolism in all cells.[1] Secondary metabolites can be distinguished from their primary counterparts in that they are not essential for the primary functions of life, and are not found in all life forms, having a restricted distribution characteristic of individual strains or species. They are formed as products of specialised pathways originating from the metabolites of primary metabolism. Although they are not essential for life they have important and useful functions for the survival and fitness of the organism including acting as competitive weapons (antibiotics), sexual hormones and differentiation effectors between and within cells.[2]

Throughout history mankind has exploited crude plant extracts for medicines, purfumes, stimulants, narcotics, hallucinogens and poisons. Many secondary metabolites (otherwise known as natural products) have been isolated and characterised from natural sources for use in medicinal chemistry, such as the penicillin and tetracycline antibiotics, morphine and ephedrine. Sometimes these compounds appear to serve no useful purpose in the producing organism, but have been of great benefit in the service of man, motivating chemists from the early nineteenth century to seek to purify and characterise these natural products. Attempted total syntheses of these products followed, often bringing with them breakthoughs in understanding the 'rules' of organic chemistry. It was their studies on the total synthesis of vitamin B_{12}, and an examination of its thermal and photochemical reactions, that led Woodward and Hoffman to enunciate the principals of conservation of orbital theory in 1969.[3] Although traditionally primary metabolism has

been the realm of biochemists, and secondary metabolism that of organic chemists, it is an interdisciplinary approach which is now enabling exciting advances in understanding the biosynthesis and enzymology of many of these complex metabolites, the polyketides and the polypeptides in particular.[4]

Biosynthesis and accumulation of most secondary metabolites is limited to distinct developmental stages of the producer organism. Microbial cultures pass from a phase of rapid cell growth (trophophase) to a phase of cell specialization (idiophase), and it is in the latter stage that the enzymes of secondary metabolites are expressed.[5] Thus these metabolites rely on the catabolic products of primary metabolism for their biosynthesis.

Secondary metabolites, though diverse in structure, can be classified into groups on the basis of the units from which they are constructed. In fact, many metabolites have only one basic building block which is used to create the whole structure. The group of interest discussed here are the polyketide metabolites, which are assembled in a similar fashion to the fatty acids (a group of primary polyketide metabolites) by linear combination of acetate units, with reduction of the keto groups to various degrees.

1.2 Techniques in Biosynthesis

1.2.1 The use of isotopically labelled studies

Extensive information has been obtained about primary and secondary metabolism by use of feeding experiments utilising isotopically labelled precursors. This involves administration of a likely precursor to the organism and analysis of the metabolite produced to detect incorporation.

Two general types of feeding experiment have been used:

- Radioactively labelled precursors with long half lives, e.g. ^{14}C and ^{3}H and ^{32}P which can be sensitively assayed by scintillation counting.

- Stable isotopic labelling, ^{13}C, ^{15}N, ^{18}O, or ^{2}H and detection by NMR or MS studies. NMR studies provide information about regiochemistry of incorporation and have proved of greatest importance.[6]

The advantage of using radioactively labelled substrates is that a very low level of activity can be recorded. However metabolic products must be rigorously purified to eliminate errors from contamination. Site specific information requires chemical degradation of the metabolites with painstaking purification of the degradation products. High Performance Liquid Chromatography may also be used with scintillation counting to separate and detect the radioactive products.

Although methods of analysis using stable isotopes are much less sensitive than those used to assay radioactivity, they are non-destructive and reveal more information about the regiochemistry of incorporation of the precursor into the metabolite. Nuclear magnetic resonance is the preferred method of analysis, but mass spectroscopy often combined with GC or HPLC is sometimes chosen as much smaller amounts may be analysed. The number of isotopes may be assessed, but detection is not site specific. ^{13}C NMR with proton decoupling is most commonly used to follow the fate of carbon atoms. The natural abundance of carbon-13 is 1.1%, allowing detection of reasonably small

incorporations above natural abundance. Studies with double labelled precursors such as [1,2-$^{13}C_2$]-acetate have enabled very detailed analyses of bonds broken and retained during biosynthetic processes.[7] ^{2}H NMR has also been widely used with relatively inexpensively prepared deuterium labelled precursors and has similar chemical shift values to proton NMR. ^{2}H NMR however, has associated problems: low sensitivity (due to low magnetogyric ratio), poor dispersion of chemical shifts, and broad signals due to the ^{2}H nuclear quadruple (I=1). Despite this, its low natural abundance (0.015% of ^{1}H) allows incorporations to be measured at very low levels. In this context ^{2}H-NMR is effectively sixty times more sensitive than ^{13}C NMR when applied to biosynthetic incorporation studies.[8] Less frequently used is ^{3}H NMR, due to the radioactivity of tritium. However the nuclear properties of tritium (highest known magnetogyric ratio and nuclear spin of ½) combined with its negligible natural abundance make it the most sensitive isotope for NMR analysis.[9] ^{17}O NMR studies have also been successfully used, despite the difficulty of aquiring good quality ^{17}O NMR spectra. The signals are very broad due to the quadrupolar nucleus which relaxes very badly. However, the attraction again is the low natural abundance of oxygen-17 (0.04%), allowing small enrichments to be observed.[10] ^{15}N NMR may be useful, although its use as a label in α-amino acids does not always give reliable results because of rapid transamination.

Isotopes may also be detected indirectly, usually by monitoring their interaction with an adjacent ^{13}C atom by ^{13}C NMR. This approach has been frequently used with deuterium.[11] Each deuterium atom shifts the adjacent resonance of the attached carbon-13, 0.3 - 0.6 ppm to higher frequency. This is known as an α-shift. However, as the signal to noise ratio of carbon-13 atoms attached to deuterium atoms is reduced due to poor relaxation, signal multiplicity and loss of n.O.e, it can be difficult to obtain reliable levels of enrichment. Joint proton and deuterium decoupling may be used to reduce the signal to a singlet thus increasing the sensitivity. β-Shifts (which are smaller, 0.04 ppm to lower field) are also additive and observable, and do not have the disadvantages associated with α-shifts. They also provide information on the incorporation of intact units, ^{13}C-C-D.[12] The fate of oxygen-18 may similarly be followed by monitoring the induced chemical shift of an adjacent carbon-13, a technique which was used for example to establish the origin of oxygen atoms in the polyether antibiotic monensin[13] (discussed in Chapter 5). Similarly nitrogen-15 incorporation may be monitored by the observed

coupling to an adjacent carbon-13, and was a valuable aid in elucidating the structure of the fungal metabolite tenellin.[14]

Studies using stable isotopes, however, are not without disadvantages. The labelled precursor is only a small fraction of the total pool of unlabelled precursor, and therefore will only become incorporated into a fraction of the molecules of the metabolite produced. Furthermore the administered precursor is not always able to penetrate the site of biosynthesis, or may be degraded *en route*. Because of these obstacles it is necessary to administer higher concentrations of the precursor, and there is a danger of the normal metabolism being perturbed. Fungi in particular are prone to lose or change their metabolic capabilities under such conditions.[15]

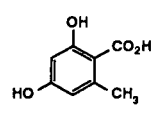
1.2.2 Enzymes, inhibitors and mutants

A complementary approach to the administration of isotopically labelled intermediates on a putative biosynthetic pathway is to seek to isolate intermediates directly from the organisms. Mutation of the wild type organism (induced by UV or chemically, or by genetic engineering) results in organisms, which if deficient in an enzyme on the pathway may accumulate biosynthetic intermediates, but are unable to synthesize the natural metabolic product. These intermediates may be isolated and analysed. Supplementation with the subsequent intermediate on the pathway should restore the production of the natural products. Applied to polyketide biosynthesis such techniques have enabled the identification of post assembly modifications (e.g. hydroxylations[16], methylations[17]), as well as identifying the biosynthetic origin of the precursors, particularly the starter units.[18] Polyketide chain elongation intermediates have even been isolated from mutant strains of the mycinamycin producing organism *Micromonospora griseorubida* sp.[19]

Production and isolation of mutants deficient in a specific enzyme activity can be a laborious process. An alternative approach has been to block potential enzymes on the pathway by use of inhibitors.[20] An extension of this technique involves the administration of isotopically labelled putative intermediates after the 'block'.[21] Specific inhibitors have also been used in parallel with feeding studies to impede degradation of the putative intermediate prior to its utilization on the pathway of interest.[22]

1.3 Biosynthesis of the Polyketide Metabolites

The polyketides are a large and very diverse group of metabolites, extending from primary into secondary metabolism. Their wide distribution makes their study a fascinating domain, involving techniques from a range of disciplines. Stuctures range from the very simple aromatics such as the fungal metabolite orsellinic acid (1.1) discussed below, to the massive marine metabolite maitotoxin (1.2), the largest non-polymeric secondary metabolite known. Incredibly, only 8.1 mgs of maitotoxin was used in the structure elucidation![23]

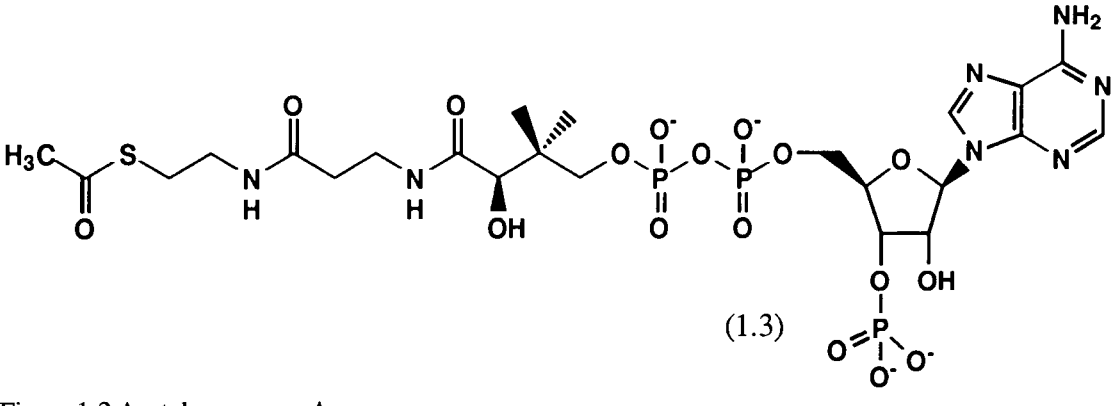


(1.1)

(1.2)

Figure 1-1 Orsellinic acid and Maitotoxin

The secondary metabolites known as the polyketides are assembled in a manner closely related to that of the primary metabolites, the fatty acids. These long hydrocarbon chain fatty acids are usually derived from a linear combination of acetate units, although like the polyketide metabolites propionate may also be incorporated. Fatty acids are activated in the cytosol of the cell by reaction with coenzyme-A and ATP yielding fatty acyl-CoA, as shown below for acetyl-CoA (1.3).



(1.3)

Figure 1-2 Acetyl coenzyme-A

For the condensation of the acetate units, acetyl-CoA is first activated by carboxylation to malonyl-CoA and then transesterified onto an acyl carrier protein (ACP) attached to a fatty acid synthase (FAS). Acyl carrier protein, like CoA, contains a phosphopantetheine group to which acyl groups are esterified.

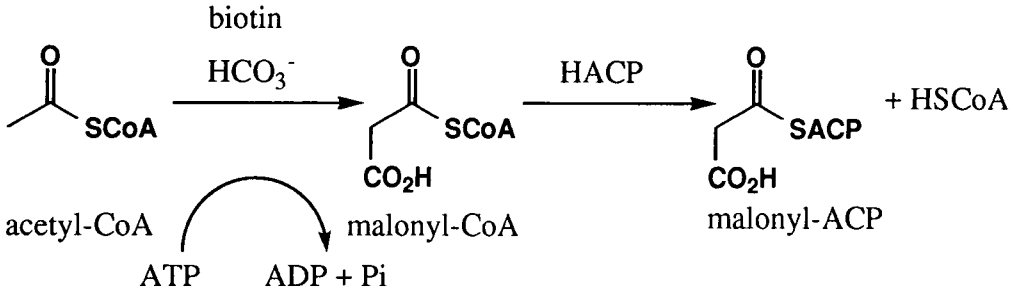


Figure 1-3 Activation of acetate units for fatty acid biosynthesis

Malonyl-CoA is transesterified onto the fatty acid synthase (FAS) condensing enzyme ready for condensation with the starter unit, usually acetyl-CoA, also held on a free thiol residue of the condensing enzyme. Condensations of the growing fatty acid chain are driven by loss of CO_2, to form β-ketoacyl-ACP. In Type II FASs a specific β-ketoacyl synthase (condensing enzyme III) usually mediates this first condensation with acetyl-CoA directly, which may account for the observed incorporation of a number of alternative starter units.[24] After each chain extension the acyl carrier protein transports the β-ketoacyl around a series of enzymes which catalyse the reduction of each β-keto group by NADPH to a saturated methylene group. The extended chain is then transferred to the keto-synthase thiol, releasing the acyl carrier protein to accept another malonyl-CoA, and to serve as the substrate for the next condensation. This cycle continues until the complete fatty acid has been synthesised, which is then released from the enzyme complex.[25]

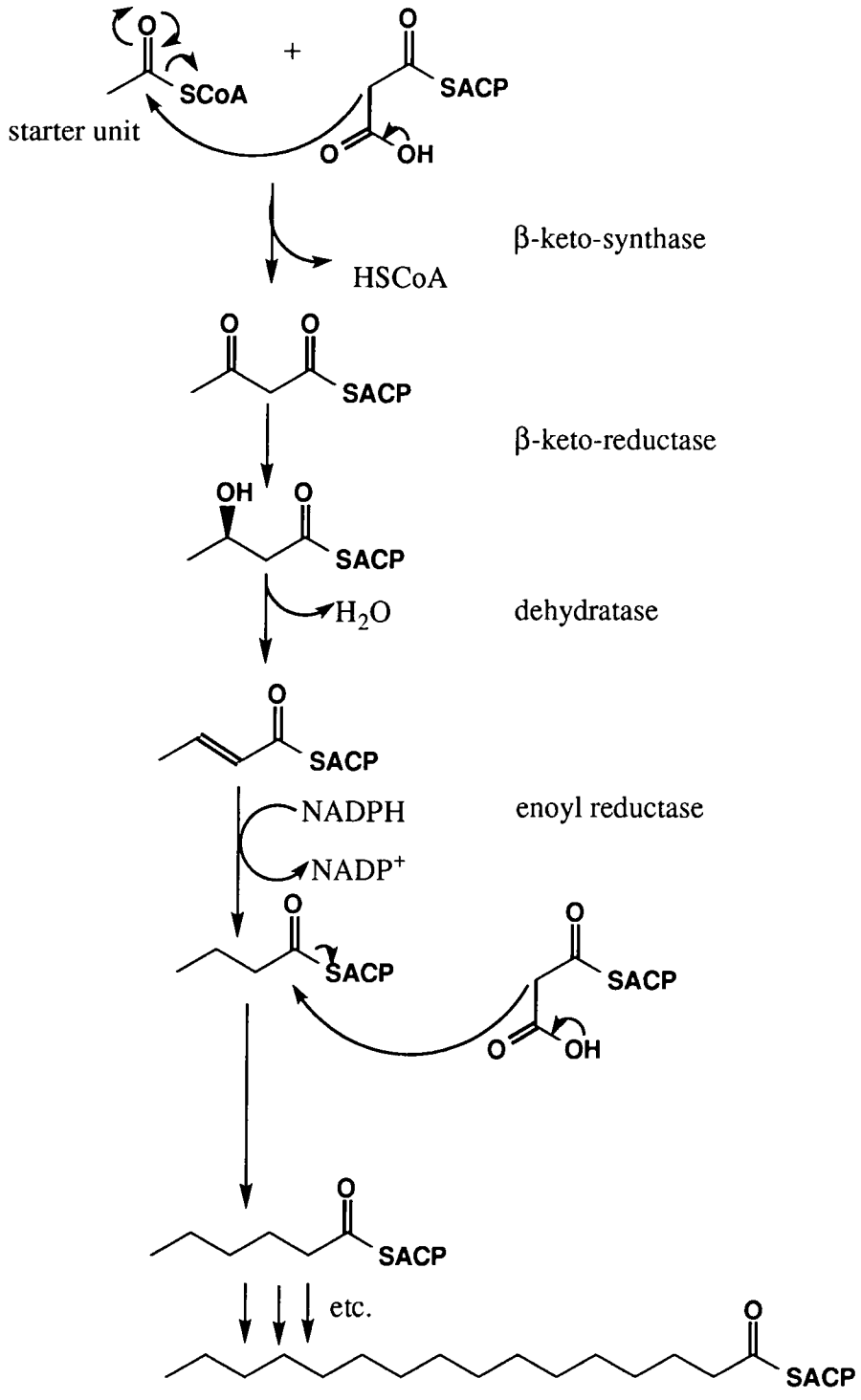


Figure 1-4 The reactions of fatty acid biosynthesis

The stereochemical course of the transformations of fatty acid biosynthesis have been established by Sedgwick and Cornforth[26] using chiral methyl methodology, and all activities apart from the final reducing enzyme, enoyl reductase appear to operate with the same stereochemistry in different organisms. Retention of configuration for the carboxylation step is followed by inversion of configuration during condensing malonyl-

ACP with acetyl-ACP. The β-ketoacyl reductase mediates a *si*-face reduction[27] and β-ketoacyl-ACP dehydratase executes the *syn*-elimination of water.[28]

Fatty acid synthases (FAS), are composed of at least six enzymatic activities, making them among the most sophisticated of known enzymes. Fungal and animal synthases are multifunctional enzymes (M_r > 200,000) consisting of single or clusters of multifunctional polypeptides, contiguous stretches of which are folded into autonomous domains of polypeptide subunits each having a specific catalytic activity. These are known as Type I synthases. For mammalian FASs two almost identical multifunctional peptides dimerize *in vivo* and it is suggested that such an arrangement orientates the thiol residues of the condensing enzymes so that full activity can be expressed. Fungal and yeast FASs often have complex molecular architecture requiring the association of a number of multifunctional subunits.[29] Bacterial and plant synthases differ from these in that they are readily dissociable into their component enzymes, and ACP, and are known as Type II synthases.[30] Each enzyme is monofunctional and may be active in isolation from the other enzymes. In either case it is the acyl carrier protein which sequentially escorts the intermediate thioesters to the catalytic sites of the FAS. In Type I FASs this acyl carrier protein is covalently linked to a serine residue on the synthase. Some bacteria (especially the Actinomycetes) possess Type II FASs similar to the yeast and fungal systems.[31] This is significant as the complex polyketides also produced by these organisms require complex multifunctional polyketide synthases.

Some fatty acids incorporate unusual starter units, instead of acetyl-CoA, resulting in fatty acids with structural variation at the terminal end. Starter units include isobutyryl-CoA, 2-methylbutyryl-CoA, 3-methylbutyryl-CoA (the catabolic products of the amino acids L-valine, L-isoleucine and L-leucine). Other unusual starter units are propionyl-CoA, cyclohexylcarboxyl-CoA, and even fluoroacetyl-CoA.[32]

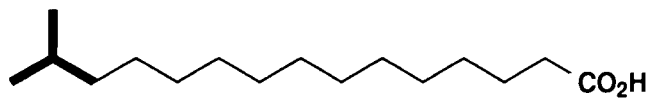


Figure 1-5 Iso-palmitate derived from starter unit isobutyryl-CoA

Although non-acetate starter units are relatively common, alternatives to acetate within the chain are rare. However, in a similar manner to bacterial polyketide synthases certain fatty acid synthases incorporate propionate units, activated first as methylmalonyl-CoA. Further diversity may also be introduced into the hydrocarbon chain, by desaturases (resulting in unsaturated fatty acids), methylations with S-adenosyl methionine, or by incomplete reduction of the polyketide.

1.3.1 Fungal polyketide biosynthesis

In a similar manner to the classical fatty acid biosynthesis the polyketides are assembled from the condensation of acetyl-CoA and malonyl-CoA units.[32]

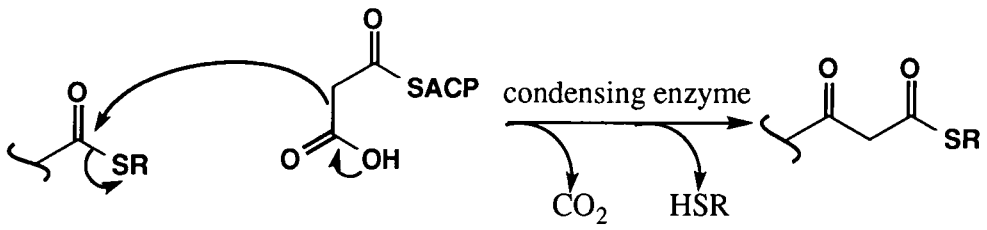


Figure 1-6 Polyketide biosynthesis

However unlike fatty acid biosynthesis the polyketide is not completely reduced to generate a fully saturated carbon chain. Each polyketide synthase (PKS) is programmed to effect a different degree of reduction leading to a range of partially reduced chains. These then fold and cyclise or cascade *via* intramolecular condensations and substitutions to generate a specific metabolite.

The majority of polyketide metabolites so far identified are products of the fungi, the fungi imperfecti being particularly prolific producers. The phenolic polyketides are a ubiquitous class, which arise from intramolecular condensations of extended, inherently unstable, unreduced polyketide chains. It has been suggested that a poly-β-keto intermediate is stabilised on the synthase, possibly by metal ion chelation (as shown in Figure 1-7).[32] Orsellinic acid (1.1) produced by both plants and fungi is assembled from four acetate units. Cyclisation, *via* aldol condensation, of the β-keto intermediate followed by dehydration and two enolisations gives the structure shown below.

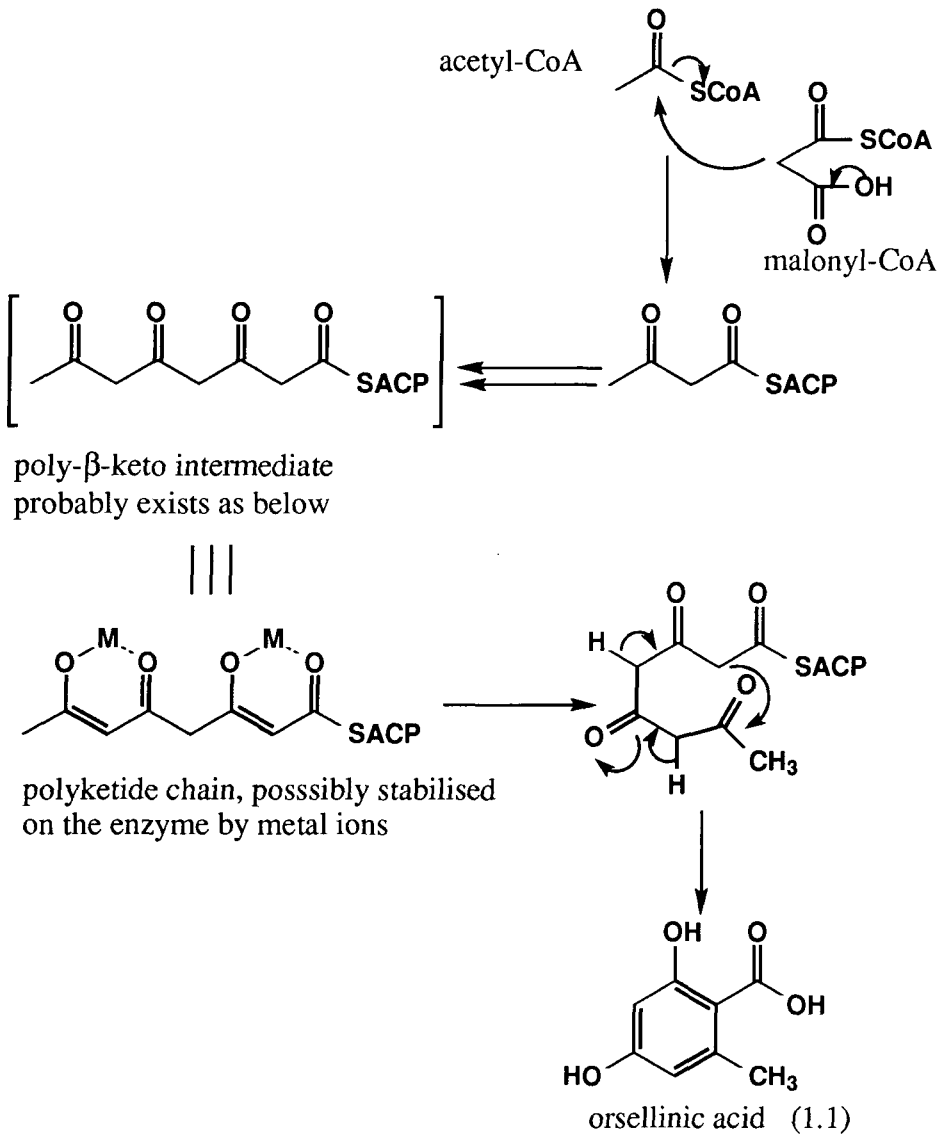
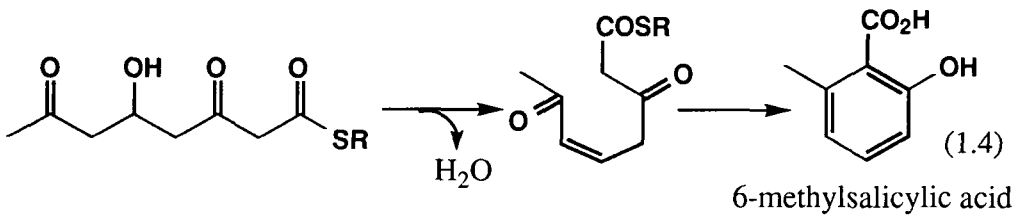


Figure 1-7 Biosynthesis of orsellinic acid

6-Methylsalicylic acid (1.4), (from *Penicillium patulum*) requires an NADPH-dependent β-keto reductase as part of the PKS, due to the absence of a hydroxyl group at carbon-4. Dehydration of the partially reduced β-keto intermediate is followed by cyclisation and aromatisation. An enzyme extract from *Penicillium patulum* has been purified which in the presence of NADPH converts acetyl-CoA and malonyl-CoA into 6-methylsalicylic acid. This enzyme was shown to be a single multifunctional protein similar to the Type 1 fatty acid synthases.[33] The reduction step appears to occur at the triacetic acid level, implying a processive mechanism of polyketide assembly.

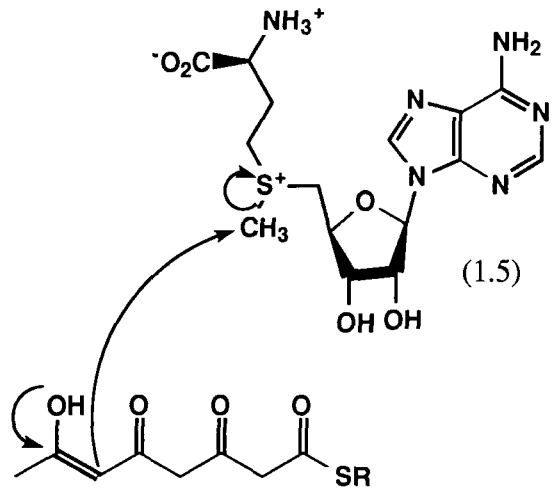


(1.4)

6-methylsalicylic acid

Figure 1-8 Biosynthesis of 6-methylsalicylic acid

Many polyketide metabolites of fungi and plants possess extra methyl groups attached to the acetogenic carbon skeleton. These are usually donated from L-methionine activated as S-adenosyl L-methionine (SAM) (1.5) by nucleophilic substitution (S_N2) of the S-methyl group. O- and N-Methylations are also well known as a feature of polyketide biosynthesis, usually occuring as post-assembly modifications. The timing of C-methylations is not well established. Presumably C-methylations occur prior to reduction of the poly-β-keto chain *via* nucleophilic attack of the methylene, or possibly at the alkene level, following the dehydration step. This implies a C-methylation enzyme operating closely with the polyketide synthase enzyme, if the mechanism of polyketide assembly is a processive one.[32] The timing of the C-methylations in tenellin biosynthesis is the subject of Chapter 2 of this thesis.

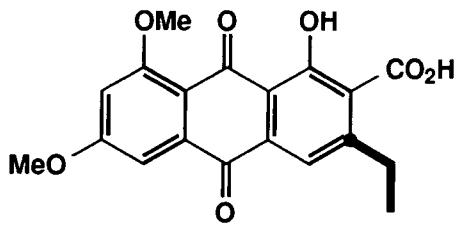


(1.5)

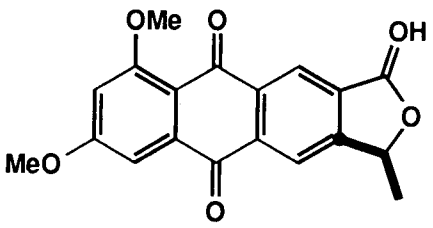
Figure 1-9 C-Methylation of a fungal polyketide by SAM

Extra methyl groups at the beginning of the polyketide chain may also originate from the incorporation of unusual starter units, as is observed in *iso*- and *anteiso*-fatty acid biosynthesis. Propionate is used as a starter unit in the biosynthesis of the anthroquinones, austrocorticinic acid (1.6) and (+)-austrocorticin (1.7), produced by the

Australian toadstool *Cortinarius dermocybe*,[34] but there are no known examples in fungi, of incorporation of propionate at any other position in the polyketide chain.



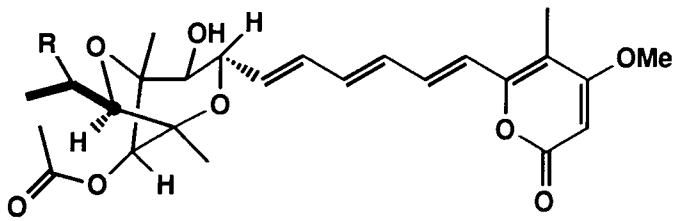
austrocorticinic acid (1.6)



(+) austrocorticin (1.7)

Figure 1-10 Fungal metabolites using propionate starter units

Intriguingly, two biosynthetic pathways appear to be operating during the biosynthesis of the aurovertins (1.8). Propionate has been shown to become incorporated as the starter unit, but acetate and methionine may also become incorporated at this site.[35]



aurovertin B R = H
aurovertin D R = OH (1.8)

Figure 1-11 Aurovertins B and D

Although the majority of fungal polyketides appear to be aromatic, a number of fungal metabolites have been isolated which have a reduced polyketide framework. Convincing evidence for a processive mechanism of assembly operating in fungal polyketide assembly has come from a number of studies involving the successful incorporation of partially assembled fragments into certain metabolites. Isotopically labelled N-acetylcysteamine thioesters of partially assembled reduced intermediates have been successfully incorported into dehydrocurvularin (1.9), in a mutant strain of *Alternaria cinerariae*. Such thioesters appear to be able to intercept the polyketide synthase, and are then further chain extended. As dual ^{13}C labels were used, and intact incorporation observed, degradation of these intermediates prior to incorporation may be discounted.[36]

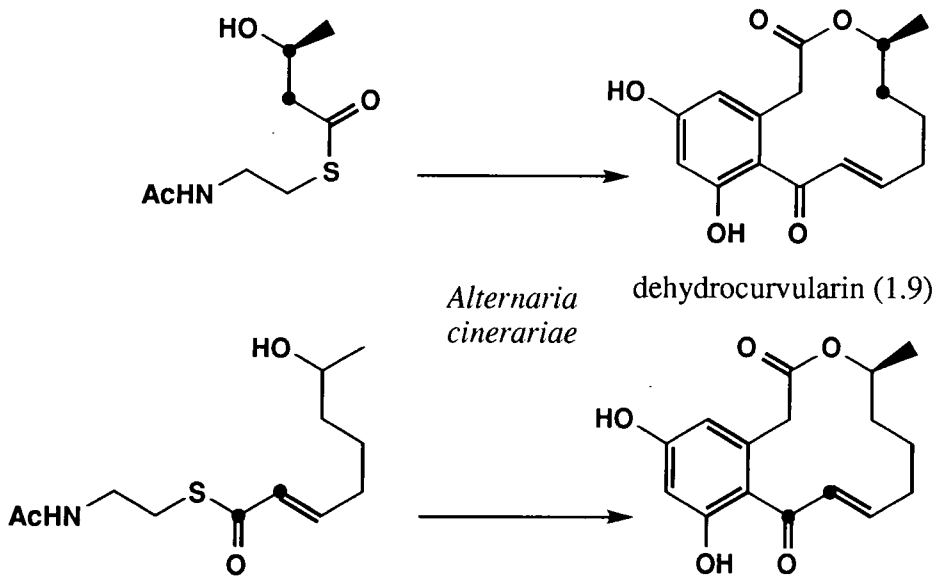


Figure 1-12 Intact incorporation of putative intermediates into dehydrocurvularin

Polyketide metabolites of mixed biosynthetic origin are not uncommon in plants and fungi. Tenellin (1.10), a fungal polyketide metabolite, which is the subject of Part 1 of this thesis, is one such metabolite, biosynthesised from a polyketide moiety and the amino acid phenylalanine or tyrosine.

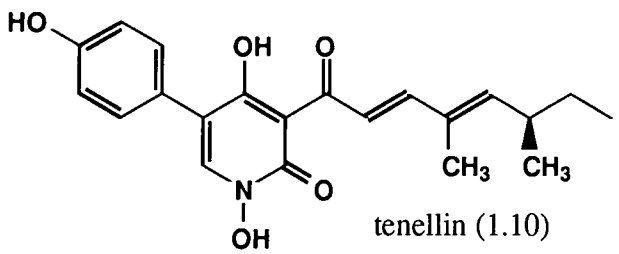
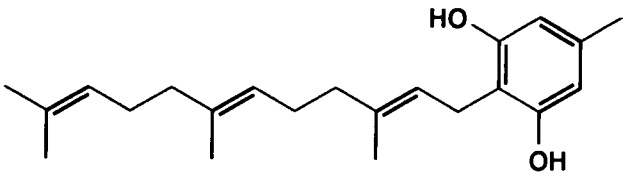


Figure 1-13 Tenellin

Other related polyketide metabolites, such as the tetramic acids, are discussed in Chapter 3. The polyketide moiety is presumably biosynthesised independently, then condensed with the amino acid moiety. This is also assumed to be the pathway of biosynthesis for the group of terpene-polyketide metabolites, such as grifolin (1.11), which have pendent isoprenoid groups, generally attached to polyketide-derived phenol ring.



grifolin (1.11)

Figure 1-14 Grifolin derived from farnesyl pyrophosphate and orcinol

However for the flavanoid metabolites originating from phenylalanine, the amino acid moiety is incorporated into the polyketide itself, as the starter unit, after conversion *via trans* cinnamic acid to 4-coumaric acid (1.12). Interestingly, the first three condensations with malonyl-CoA are controlled by chalcone synthase (or resveratrol synthase) at the CoA level. These synthases are unique amongst polyketide synthase enzymes for this reason, that they are devoid of an ACP.

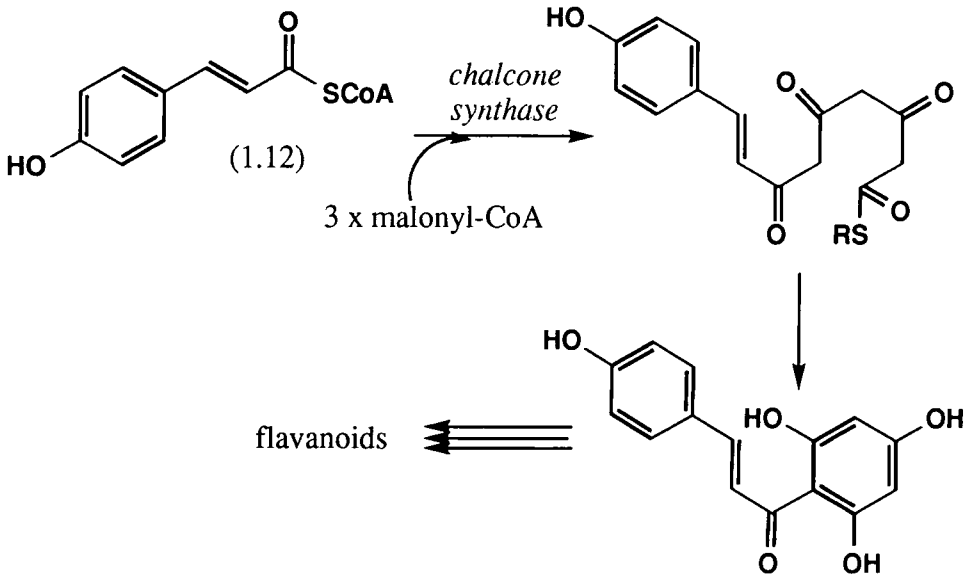


Figure 1-15 Biosynthesis of the flavanoids

1.3.2 Bacterial polyketide biosynthesis

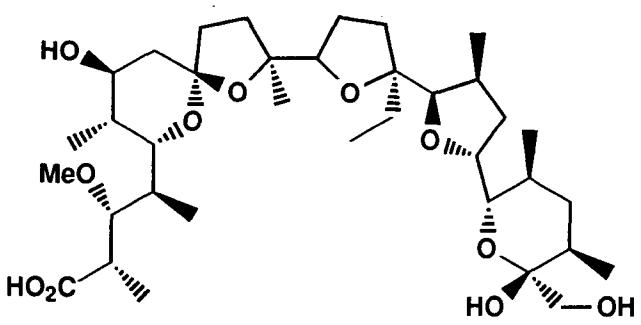
In comparison to the fungi, bacteria do not produce a great variety of polyketide metabolites, except the actinomycetes. In particular the subclass Streptomyces (soil bacteria) are able to produce polyketides ranging from simple acetogenic aromatic compounds to complex macrolide structures. As many of these compounds are antibiotics they have attracted much attention. Bacterial polyketides, are generally

structurally more complex than the fungal metabolites due to the utilisation of propionate units, and occasionally butyrate units, in addition to acetate units during biosynthesis. Incorporation of propionate and butyrate results in pendent methyl and ethyl groups respectively attached to the polyketide backbone. This is in contrast to the orgin of pendant methyl groups in fungal polyketides, which arise from C-methylations contributed by S-adenosyl methionine to the polyketide chain.

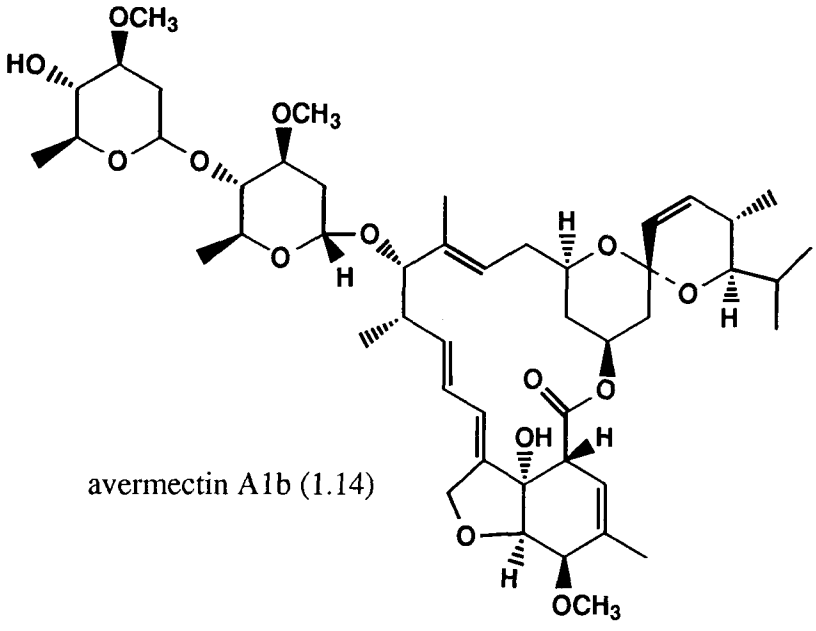
In an analogous process to fatty acid biosynthesis acetate, propionate, and butyrate units are activated by acetyl-CoA carboxylases to give malonyl-CoA, propionyl-CoA, and butyryl-CoA respectively. The synthase enzymes are programmed to select each substrate in the correct order, and on reduction the methyl and ethyl branches adopt either the D or L configuration as the chain is progressively built up.

The polyketide synthase enzymes also contain a whole complement of reducing activities which can selectively reduce or retain the β-keto functionality as the chain is assembled. As a consequence the whole range of functional groups CH_2, C=C, C-OH, C=O, may be displayed in the resulting polyketide.

The malonyl-CoA precursors to the acetate, propionate and butyrate precursors from which the polyketide chain is assembled are derived from the catabolism of various primary metabolites. Amino acids, fatty acids and the products of the citric acid cycle are known to contribute carbon atoms to the propionate and butyrate pools. Once cells enter the stationary stage of cell growth, primary metabolites are broken down to these essential precursors, and secondary metabolism can begin. Part 2 (Chapter 5) of this thesis investigates one such link between primary metabolism and the malonyl-CoA precursors of polyketide biosynthesis using the Streptomyces polyketides monensin A (1.13) and avermectin (1.14) as case studies.



monensin (1.13)



avermectin A1b (1.14)

Figure 1-16 Monensin A and avermectin A1b

The evidence strongly supports a processive mechanism of polyketide biosynthesis for these higher order polyketides. In particular the macrolides erythromycin A and tylosin have been studied due to their commercial significance as antibiotics.

It has already been dicussed that fragments of mycinolide IV have been identified in mutant strains of the mycinamycin producing *Micromonospora griseorubida*.[19] Similar fragments have been identified in mutant strains of *Streptomyces fradiae* which secretes the tylosin aglycone tylactone (1.15).[37] Furthermore isotopically labelled N-acetylthioesters of partially assembled tylactone were successfully recognised and converted into the aglycone in the same mutant strain.[38] Dual carbon-13 labelled intermediates have also been incorporated into erythromycin A (1.16)[39] and nargenicin A (1.17),[40] as their thioesters. The use of a deuterium label in diketide (1.18) demonstrated

that oxidation to the β-keto thioester was not occuring prior to incorportion into erythromycin.[41] Similar studies on these metabolites and others continue to emerge.[42]

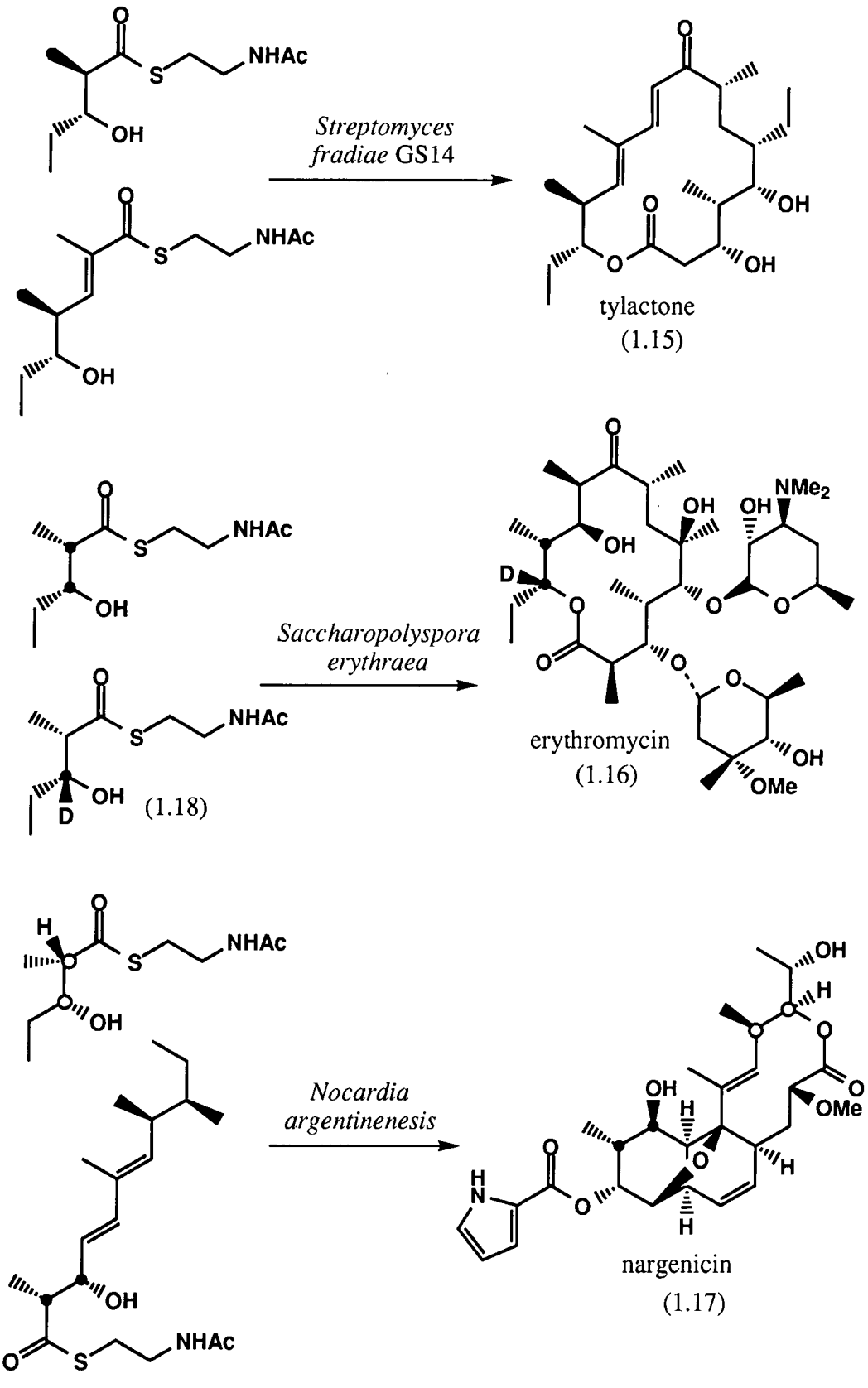


Figure 1-17 Incorporation of partially assembled intermediates into reduced bacterial polyketides

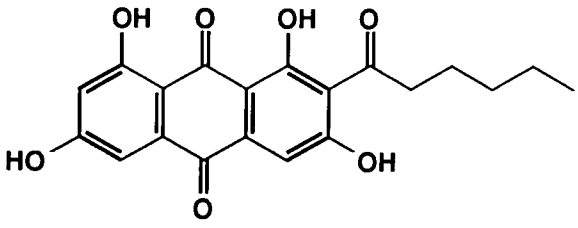
Despite the structural diversity of bacterial polyketides identified, striking stereochemical homologies may be observed between them, particularly between the macrolide antibiotics. In 1965 Celmer[43] outlined a configurational model which summarised the absolute stereochemistry of all the known 12 and 14-membered macrolides. This model may be extended to include the 16-membered macrolides with only one asymmetric centre (C-14) showing an inconsistency. The polyethers and the ansamycins also show considerable stereochemical homologies,[44] and indeed correlations between the three classes are also found.[45]

1.4 Genetic Studies on Polyketide Synthases

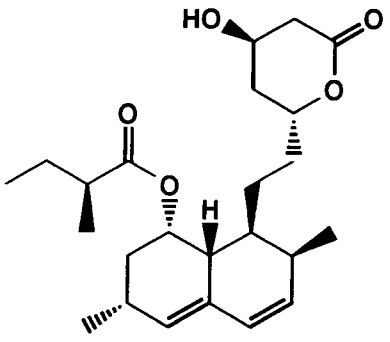
Important advances in our understanding of the polyketide synthases have been made using chemical and biochemical approaches. However a key problem is understanding the origin of programming. How do the PKSs control the construction of such diverse and elaborate metabolites? It is genetic techniques that have for the last ten years led the exceptional advances in unravelling this mystery. Cloning and sequencing of a variety of PKSs, have established that these PKSs resemble both the Type 1 and Type 2 fatty acid synthases.

1.4.1 Fungal polyketide synthases

Although the multifunctional, Type 1 PKS for 6-methylsalicylic acid biosynthesis from *Penicillium patulum* was the first functional PKS protein isolated, the genetics of fungal polyketide synthases have not yet been widely explored. The gene coding for this PKS has since been cloned and sequenced.[46] Subsequently PKS genes from various filamentous fungi have been sequenced, and all show a Type 1 structure. Most lack the three reductive functions, and a thioesterase, and code for aromatic polyketides such as norsolorinic acid (1.19), an intermediate of aflatoxin biosynthesis in *Aspergillus parasiticus*.[47] The mevinolin (1.20) PKS from *Aspergillus terreus* has also been sequenced and is of particular interest as it is reduced, and also requires a methyltransferase domain for the C-6 methylation.[48]



norsolorinic acid (1.19)

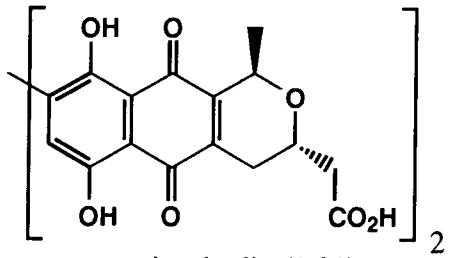


mevinolin (1.20)

Figure 1-18 Norsolorinic acid and mevinolin

1.4.2 Bacterial polyketide synthases

Most of the genetic work with both the Type I and Type II polyketide synthases has concerned the Streptomycetes and their near relatives among the Actinomycetes. Important generalisations emerged from early attempts to isolate genes; that all of the biosynthetic genes needed to make a particular antibiotic from primary metabolites occur together in a single cluster, and that the genes for self resistance are also to be found there. An early demonstration of this was the cloning of the entire cluster of genes (*act* genes) responsible for the biosynthesis of the benzoisochromanequinone actinorhodin (1.21) from *Streptomyces coelicolor* A3(2). This has led to the development of two main routes to the isolation of complete sets of genes for other polyketides. Furthermore cloned DNA carrying the gene cluster for the polyketide synthases were found to be sufficiently alike to be used as probes to isolate other polyketide synthases.[49] However it gradually became apparent that not all of the genes isolated actually encoded the PKS sought. For example when genes isolated from the monensin producer *Streptomyces cinnamonenesis* were disrupted, monensin production was undisturbed.[50] It is now clear that the *act* probes are only useful for isolating aromatic polyketide synthases, but the complex reduced polyketides do not show sufficient homologies.



actinorhodin (1.21)

Figure 1-19 Actinorhodin

As for the bacterial fatty acid synthases, Type II PKSs, encoded for by adjacent genes on the chromosome are found for bacterial aromatic polyketides, each open reading frame (ORF) encoding an individual protein with one enzymatic activity and for a discrete acyl carrier protein. The expressed proteins presumably cluster togther into a complex. Thus for the actinorhodin PKS there are six genes, the minimal PKS genes (*act* 1 composed of ketosynthase, chain length factor, acyl carrier protein), together with ketoreductase (*act* III), aromatase (*act* VII), and cyclase (*act* IV).[51]

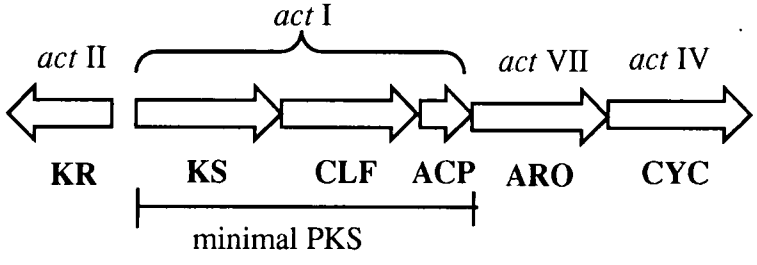


Figure 1-20 Arrangement of actinorhodin genes

The programming mechanism for complex or reduced bacterial macrolides is quite different from that for the aromatic polyketides. Each ORF encodes a single multifunctional protein with activities for the reduction or partial reduction of the β-keto acyl group to the appropriate degree, and with the required stereochemistry i.e. a Type I PKS. Several of these ORFs are involved in the complete synthesis of the metabolite.

Erythromycin (1.16) is the most thoroughly investigated aliphatic polyketide at the genetic level.[52] The polyketide synthase, responsible for the sequential condensation and reduction cycles of propionyl Co-A with six units of methylmalonyl Co-A provides the first enzyme free intermediate, 6-deoxyerythronolide B, which is further tailored to the active antibiotics erythromycin A-D. The evidence for a processive mechanism of assembly was presented above. Following the pioneering work of Hopwood and

coworkers (who located and sequenced the genes responsible for the actinorhodin PKS),[53] the region in the vicinity of the gene encoding erythromycin resistance was sequenced revealing three large open readng frames (*eryAI, eryAII,* and *eryAIII*), coding for giant multifunctional proteins (DEBS1, DEBS2, and DEBS3), the eythromycin PKS.[54] Sequence homology with the equivalent domains in fatty acid synthases, allowed the catalytic centres (*domains*) to be assigned. There are in fact two *modules* per protein (*cassette*), each of which is able to perform a condensation step followed by appropriate reduction steps. Clearly then these are multifunctional Type I PKSs. The cassettes co-operate to form a modular assembly line in which the intermediates remain enzyme bound *via* thioester links. This modular arrangement is also seen in other PKSs, such as rapamycin, tylosin, avemectin, but the number of modules per cassette, and their order along the chromosome, can vary. The rapamycin PKS includes the largest PKS known (RAP 3) containing six modules![55] The modular arrangement of the erythromycin genes, and their corresponding activities are shown below.

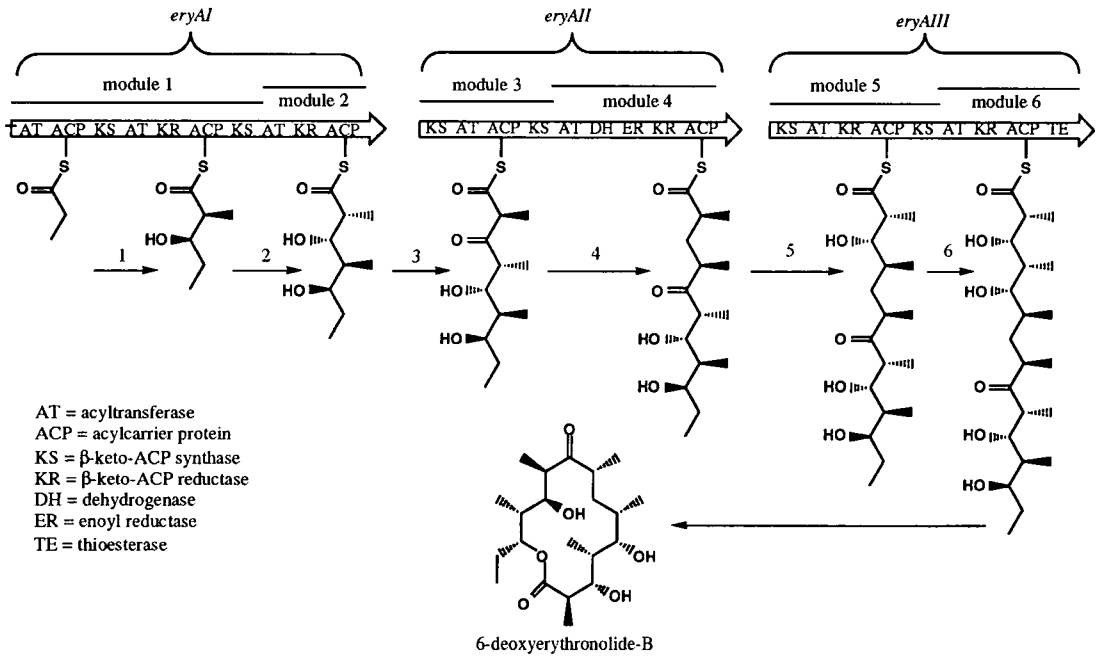


Figure 1-21 Modular arrangement of the erythromycin genes

Evidence for the correlation between the structure of the growing polyketide chain and the sequential activity of the domains in the module came from gene disruption experiments (Katz *et al.*[56]). Disruption of the β-keto reductase (KR) of module 5 led to an erythromycin analogue (1.22) with an unreduced keto functionality in the expected

position. If the enoyl reductase (ER) in module 4 is disrupted, analogue (1.23) is produced.[57]

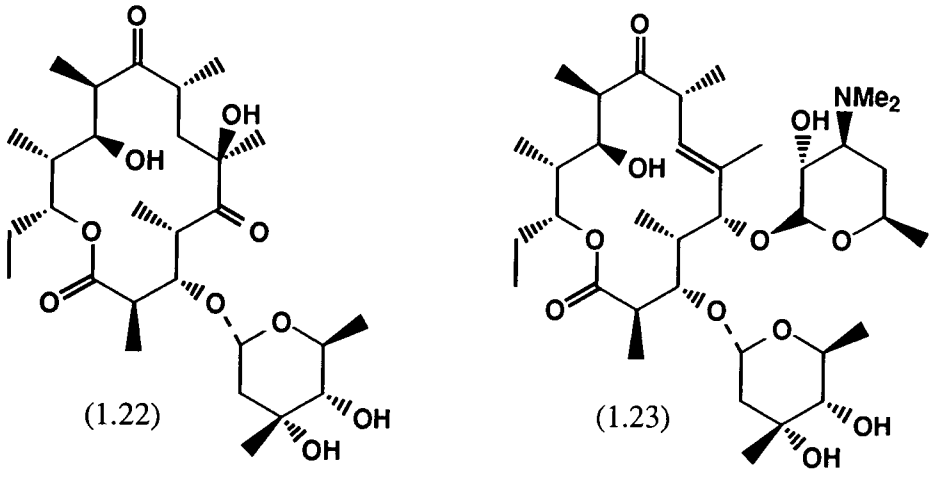


Figure 1-22 Novel erythromycin analogues from gene disruption experiments

All three DEBS proteins have been overexpressed and purified to homogeneity, indicating that the DEBS multifunctional proteins like the animal FAS, are homodimeric.[58] There are two models suggested, one based on the linear head to tail model of the animal FAS.[59] The evidence (proteolysis studies[60]) however, seems to point to the alternative dimeric double helical structure with close interchain cooperation between KS, ACP and AT domains, a presumed requirement of the condensation steps. The DH, ER and KR form loops which protrude outwards from the central core. This new model also adequately explains the data relating to FASs, and may be a more accurate model.

The erythromycin PKS has been genetically engineered in many ways. The gene disruption stategy of Katz has been mentioned. Since the development by Hopwood and Khosla[61] of the expression plasmid pRM5, allowing engineered contructs to be expressed in *Streptomyces coelicolor,* domains have been relocated along the gene, resulting in the isolation of novel compounds. The first example of such was the repositioning of the thioesterase (thought to catalyse lactonisation of the polyketide) from the terminus of DEBS3 to the terminus of DEBS1[62]. Erythromycin production was blocked after module 2 and lactone (1.24) was isolated. A purified DEBS1-TE contruct protein has also been used to show unambiguously that, despite the opposite stereochemistries of the methyl groups in the product lactone, only the *S* isomer of

methyl malonate is the substrate for the enzyme.[63] Attempts to elucidate the molecular basis for Celmer's model have shown that condensation of (2S)-methylmalonyl-CoA in module 2 proceeds with decarboxylative inversion without cleavage of the C-H bond adjacent to the methyl group whereas in module 1 the chain extension process involves loss of the hydrogen attached to C-2 of the methylmalonyl-CoA precursor. The production of the D-methyl center in module 2 the asymmetric center of the (2S)-methylmalonyl-CoA establishes that condensation takes place with inversion of configuration. The loss of hydrogen to produce the L-methyl center generated in module 1 implies that an additional obligatory epimerization step takes place in that module. The nature and timing of the epimerization remain to be established.[64]

Even more ambitiously, a complete loading module has also been transplanted from the avermectin PKS into a DEBS1-TE contruct.[65] The impressive result was the production of two novel lactones with starter acyl units characteristic of the avermectins (1.25, 1.26).

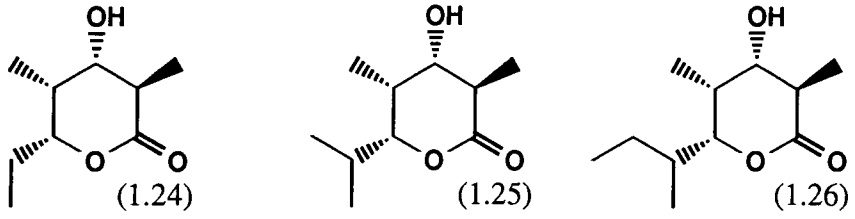
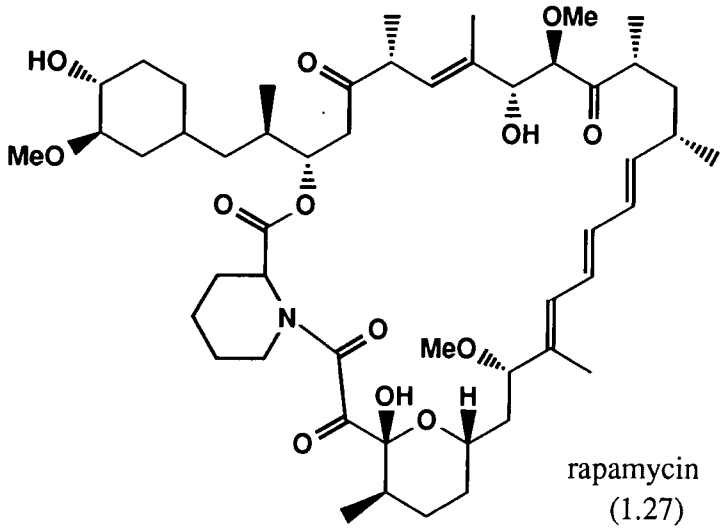


Figure 1-23 Novel lactones from genetic engineering of the erythromycin PKS

The construction of hybrids for both aromatic and reduced polyketides continues to be a challenging field, which has enticing potential for the production of novel antibiotics, and other active compounds.[66]

The gene cluster for the biosynthesis of rapamycin (1.27) has been sequenced,[67] and is worth noting as the structure of this polyketide incorporates a pipecolate unit, probably derived from the amino acid lysine. The thioesterase is replaced by a specialised monomeric multidomain protein (pipecolate incorporating enzyme) believed to catalyse formation of the ester and amide bonds to pipecolic acid.[68] It is probable that other reduced polyketides of mixed biosynthetic origin, such as the tetramic acids, and indeed tenellin have similar biosynthetic machinery.



rapamycin
(1.27)

Figure 1-24 Rapamycin derived from a polyketide and a pipecolate unit

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PART 1

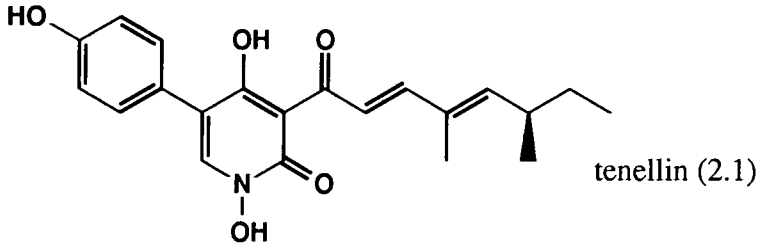
BIOSYNTHETIC STUDIES ON TENELLIN

2. The Timing of C-Methyltransferases in Tenellin Biosynthesis: An unresolved problem.

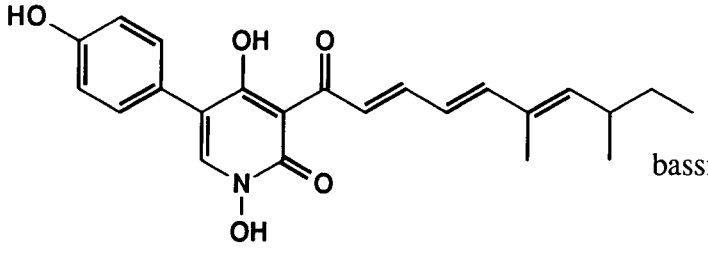
2.1 Introduction and Background

2.1.1 The biosynthesis of tenellin

Tenellin (2.1) is a yellow metabolite of the insect pathogenic fungus *Beauvaria bassiana.* It was first isolated and purified along with a related metabolite, bassianin (2.2), and a dibenzoquinone pigment oosporein (2.3) in 1967.[1]



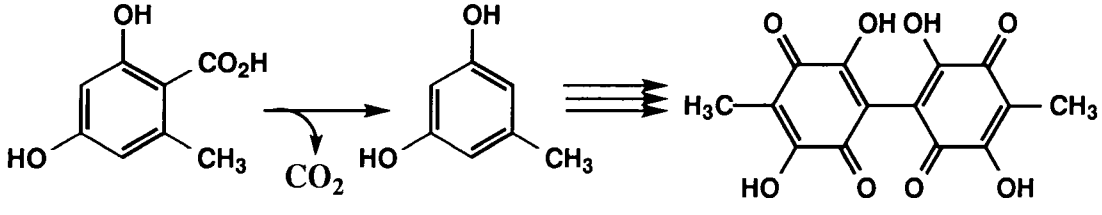
tenellin (2.1)



bassianin (2.2)

Figure 2-1 Structures of tenellin and bassianin

Oosporein (a red pigment) was shown to be of polyketide origin, being enriched by carbon-14 labelled acetate and malonate, as well as by orsellinic acid and orcinol.[2]



orsellinic acid orcinol oosporein (2.3)

Figure 2-2 Biosynthesis of oosporein

Tenellin and bassianin were shown to derive from the amino acid phenylalanine and a polyketide moiety. The polyketide chains are partially reduced and are elaborated by pendent methyl groups derived from L-methionine.

The biosyntheses and structures were elucidated by feeding ^{15}N- and ^{13}C- doubly labelled compounds and analysis of the metabolite with ^{13}C NMR spectroscopy (Vining *et al.*, 1974).[3] The ^{1}H and ^{13}C-NMR spectra and fragmentation ions from the mass spectrum have since been fully assigned.[4] Carbon atoms in and adjoining the hydroxamic group were identified by ^{15}N-^{13}C coupling observed in tenellin enriched with ^{15}N from potassium nitrate-^{15}N. This illustrates the usefulness of feeding experiments, not merely as a tool for unravelling the biosynthesis, but as an aid to structural analysis.

The polyketide moiety of tenellin was labelled as expected from intact incorporation of singly and doubly labelled ^{13}C-acetates, and L-[$^{13}CH_3$]-methionine enriched the methyl groups, presumably after activation to S-adenosyl-L-methionine (SAM). The timing of such C-methylations in fungal polyketide biosynthesis remains unknown at present. Tenellin is an ideal metabolite in which to explore this issue. The nitrogen of the pyridone ring of tenellin was proposed to derive from the intact incorporation of L-phenylalanine as enrichments from ^{15}N-phenylalanine were comparable to those from ^{13}C-labelled phenylalanines. The labelling patterns from DL-[1-^{13}C]- and DL-[2-^{13}C]-phenylalanines indicated an intriguing rearrangement of the propanoid component occuring, involving migration of the original carboxyl carbon of L-phenylalanine to become C4 of the pyridone ring.[5] This was shown to be intramolecular in a definitive feeding experiment with [1,3-$^{13}C_2$]-phenylalanine, in which the labelled carbons came together and became C-4 and C-5 in the pyridone ring of tenellin (Figure 2-3). The ^{13}C-NMR clearly showed two satellite peaks due to spin-spin coupling symmetrically located about the C-4 and C-5 singlet peaks.[6] The mechanism and proposed intermediates of this intriguing rearrangement are the subject of Chapters 3 and 4 of this thesis.

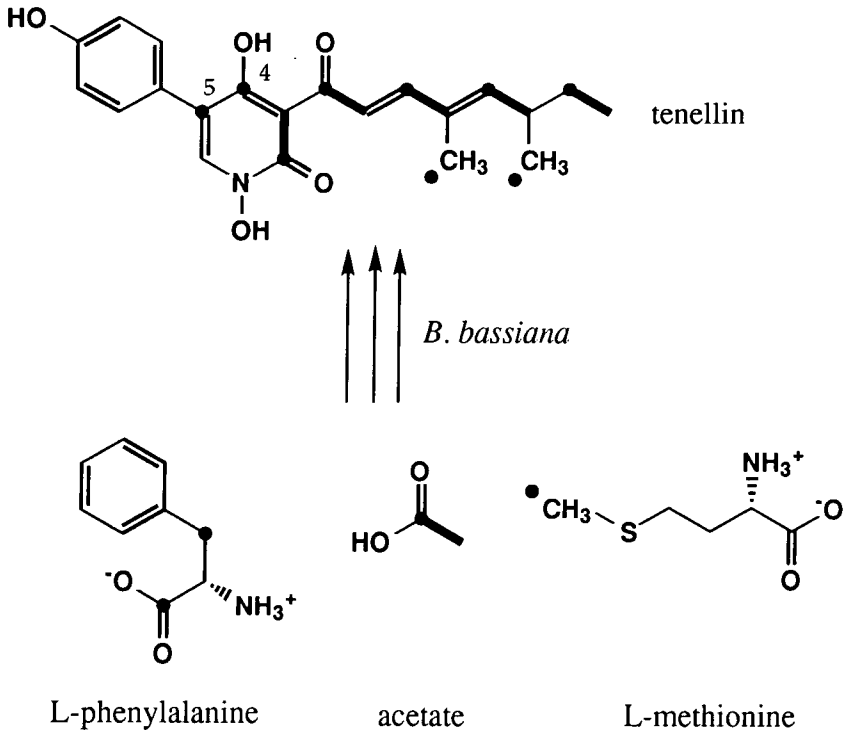


Figure 2-3 Origin of the skeletal carbon atoms in tenellin

The absolute configuration of tenellin was established by Cox by oxidative degradation of crude tenellin releasing the 2-methylbutyric acid fragment. Complexation with Parker's chiral solvating agent (1R, 2R)-(-)-1,2-diphenylethane-1,2-diamine[7] followed by ^{1}H NMR analysis, and comparison against complexes with (RS)-2-methylbutyric acid and (S)-(+)-2-methylbutyric acid resulted in the (R)-assignment of the chiral centre. Interestingly this is consistent with the configurations found for the 2-pyridones funiculosin and ilicicolin-H, although fungal polyketide synthases generally possess the (S)-configuration at such methylated centres.[8]

2.1.2 Timing of the C-methylations in fungal polyketide assembly

A number of investigations have addressed the issue of the timing and mode of the C-methylation process during fungal polyketide biosynthesis. An early investigation into citrinin (2.4) biosynthesis established that methylation inhibitors, such as ethionine, suppressed the formation of any aromatic intermediates. This is consistent with direct methylation of the poly-β-ketone.[9]

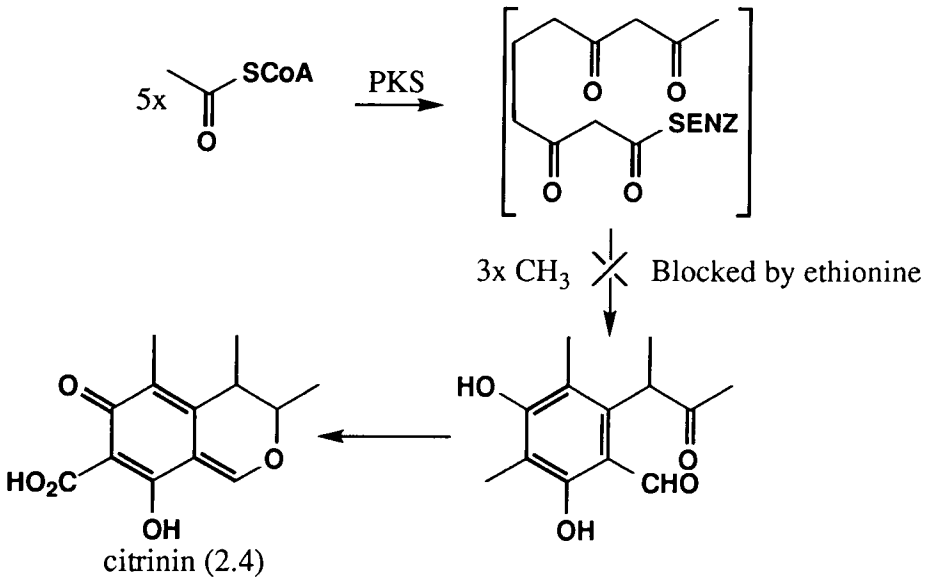


Figure 2-4 Ethionine inhibition of citrinin biosynthesis

It has been shown by Lederer *et al.*[10] that transfer of the CH_3^+ from the sulphonium ion of methionine to a weakly nucleophilic double bond, results in the loss of one of the three original hydrogen atoms as a result of rearrangement to the most stable intermediate carbocation. In contrast all hydrogens are retained if the CH_3^+ attacks a strongly nucleophilic double bond such as an enolate.

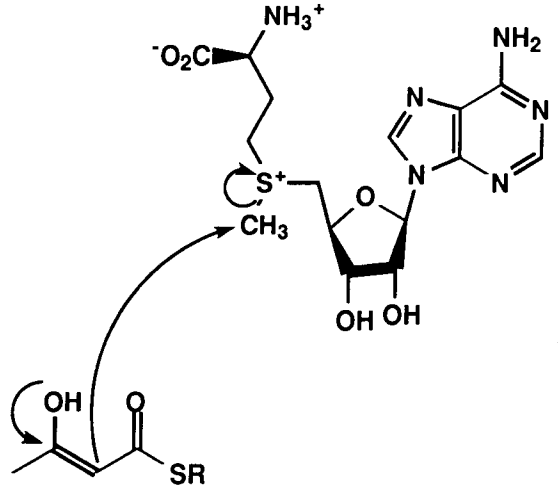


Figure 2-5 Methylation of enolate by S-adenosyl-L-methionine

Poignantly, a number of investigations with [$^{13}C^2H_3$-*methyl*]-L-methionine have established that all the deuteriums are retained in the resultant methylated polyketides.[11] An investigation with tenellin gave similar results. All three deuterium atoms from[$^{13}C^2H_3$-*methyl*]-L-methionine were incorporated intact into the pendent methyl

groups of tenellin.[8] On the basis of a similar investigation, it had been suggested by Wyss and Tamm[12] that methylation may occur before reduction of a poly-β-keto chain during the biosynthesis of the reduced fungal polyketides, the cytochalasins (2.5)

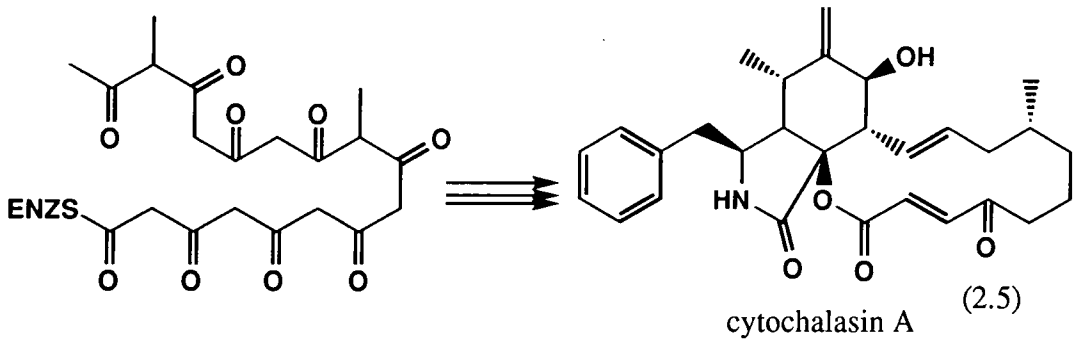


Figure 2-6 Proposed mechanism of cychalasin assembly, involving early methylation

However, as discussed in Chapter 1, there is mounting evidence (i.e. the incorporation of intact, reduced intermediates) for a processive mechanism of assembly for the reduced fungal polyketides, as well as for the reduced bacterial polyketides. This involves a reduction cycle following each chain extension, rather than the formation of a complete β-keto chain prior to reduction. The above results with [$^{13}C^{2}H_3$-*methyl*]-L-methionine suggest that, for a processive mechanism, the methyl groups must be incorporated into the polyketide chain during the assembly process at the β-keto stage, rather than after the polyketide chain is assembled and reduced. The β-keto acyl group formed by chain extension with malonyl-CoA could readily be attacked by the electrophilic methyl group of S-adenosyl methionine, followed by the usual cycle of β-keto reduction. Thus a processive mechanism would implicate the involvement of a C-methyl transferase enzyme operating closely with, or possibly expressed as a part of, a Type I polyketide synthase enzyme (PKS).

A biosynthetic investigation on the fungal polyketide cubensic acid (Figure 2-7) (produced by *Xylaria cubensis*) which has eight pendent methyl groups, demonstrated that each of those is derived from SAM.[13] It is also interesting to note that the structure of cubensic acid can be divided into five biosynthetic regions, (three different modules, two of which are repeated) each requiring a series of enzymatic activities to achieve the correct functionality along the backbone. These structural units are observed in other reduced fungal polyketides.[14] It was therefore suggested that a processive modular

mechanism of assembly, similar to the Type I PKS modular assembly of bacterial macrolides (discussed in Chapter 1) could be operative in reduced fungal polyketide biosynthesis. For cubensic acid each module could code for a multifunctional protein including a methyl transferase activity.

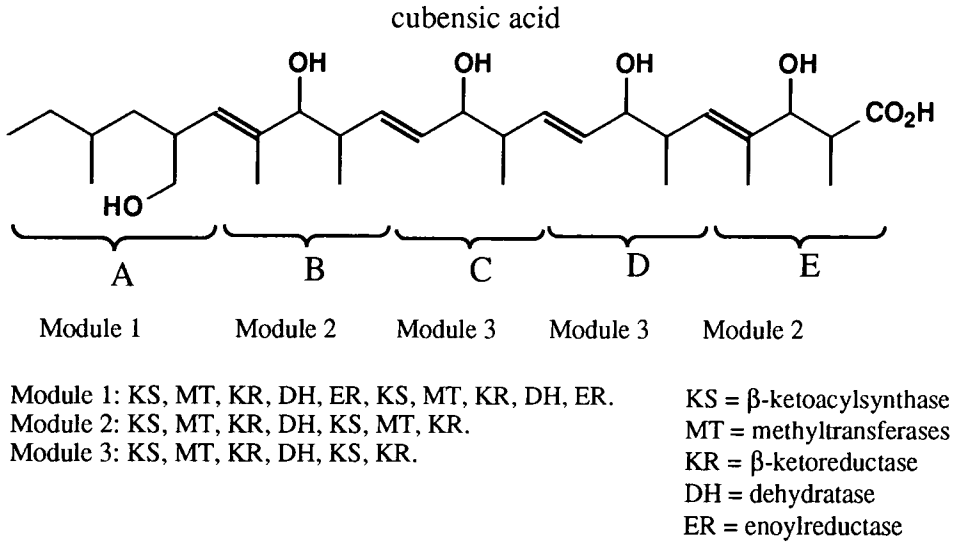


Figure 2-7 Modular assembly of cubensic acid

Similar speculations can be made concerning the mechanism of polyketide assembly in tenellin biosynthesis. A modular PKS is plausible, and structural similarities can be highlighted with other fungal pyridones e.g. bassianin (2.2) and funiculosin (2.6). It is noteworthy however that the R-configuration for the 2-methylbutyrate unit of tenellin is not a general feature of fungal PKSs.[8]

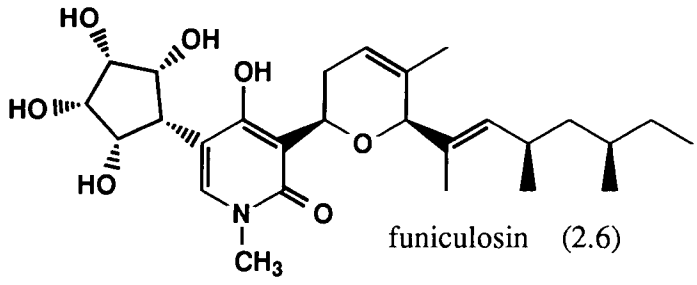


Figure 2-8 Structure of funiculosin

2.1.3 Incorporation studies with partially assembled fungal polyketides

There have been a number of successful experiments reported in which isotopically labelled, partially assembled polyketide intermediates have been incorporated into

polyketides, lending strong support for a processive mechanism of assembly. As already stated, Vederas *et al.* have reported successful incorporations of such intermediates into dehydrocurvularin (2.10), a macrolide phytotoxin from *Alternaria cineriae.* Early studies with wild-type *A. cinerariae* resulted in complete degradation to acetate prior to incorporation. However an unprecedented level of intact incorporation of the di- (2.7) and tetra- ketides (2.9) (>12% of incorporated precursor) was achieved with a UV mutant deficient in the ability to grow on fatty acids, high glucose replacement media and and the addition of β-oxidation inhibitors.[15] Subsequent investigations with a range of potential β-oxidation inhibitors, using high glucose replacement medium, and carefully controlled multiple pulse feeding enabled low level intact incorporation of the diketide into the wild-type organism. Attempts to incorporate the triketide (2.8) were unsuccessful, and it was suggested that the polyketide synthase may only permit loading of external precursors at certain chain lengths or oxidation states. However, impressive incorporations of the intact tetraketide (70% of incorporated precursor) were achieved using the β-oxidation inhibitor 3-tetradecylthiopropanoic acid. It is noteworthy that some (ca. 7%) intact incorporation of the tetraketide was observed under the same experimental conditions even without the use of β-oxidation inhibitors.[16] In the successful studies, the observed absolute incorporation of the precursor used was 1-2%.

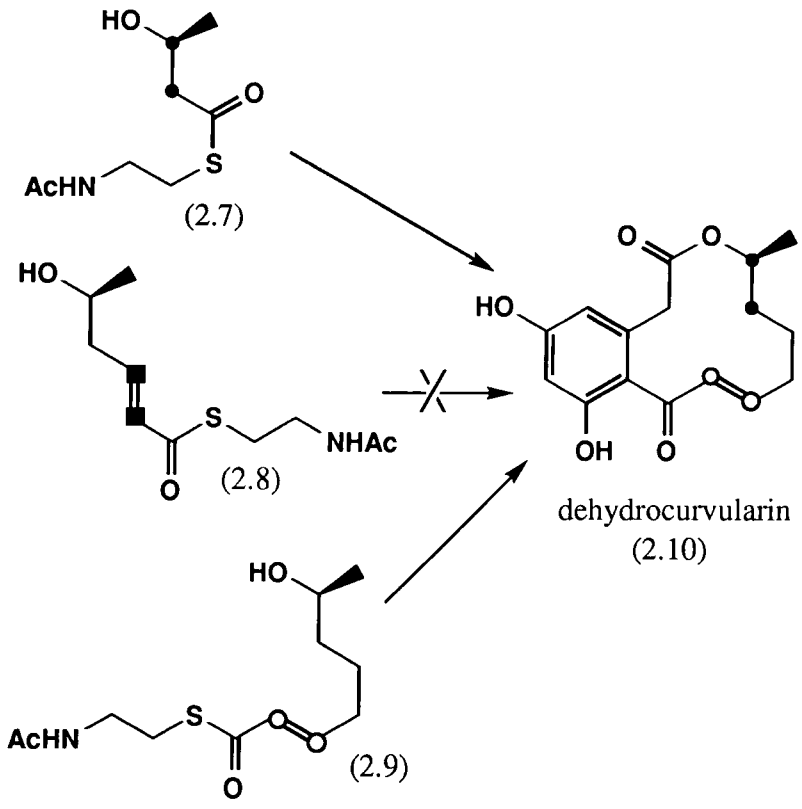


Figure 2-9 Incorporation of NAC-thioesters into dehydrocurvularin

However it is widely accepted that the failure of such incorporation experiments far exceeds the number of successful reports. When incorporations are observed they are generally very low, due to difficulties in cell transport across membranes and the need to intercept the polyketide synthase. Futhermore these experiments can be plagued by β-oxidation of the precursors prior to incorporation. However Staunton *et al.* reported a successful series of experiments on aspyrone (2.17), a fungal polyketide produced by *Aspergillus melleus*. Not only were the reduced di- (2.12), tri- (2.14), and tetra-ketides (2.16) incorporated,[17] but this study reported the first incorporation of mid-chain-extension cycle intermediates (2.11), (2.13) and (2.15).[18] This allowed the investigation of the stereochemistry of the hydroxyacyl intermediates, and the stereochemistry of the dehydration of the diketide (2.12). The success of these experiments was due in part to the choice of deuterium as a very sensitive label, allowing the detection of low level enrichments, ^{2}H having a natural abundance of only 0.015%.

As all intermediates remain covalently bound to the enzyme system until the completed chain is released, intercepting the polyketide synthase (PKS) and introducing partially assembled fragments is difficult. For this reason N-acylcysteamine thioesters of the

putative intermediates were administered to cultures. It is proposed that this residue can mimic the thiol terminus of the pantetheine group present in coenzyme-A and in the ACP of the PKS system.[19] Such thioester precursors undergo transesterification onto the enzyme more readily than oxygen-esters, which reduces the opportunity for the catabolism of the precursors prior to incorporation. The majority of the successful feeding experiments with advanced precursors to polyketides have used N-acylcysteamine thioesters.[20]

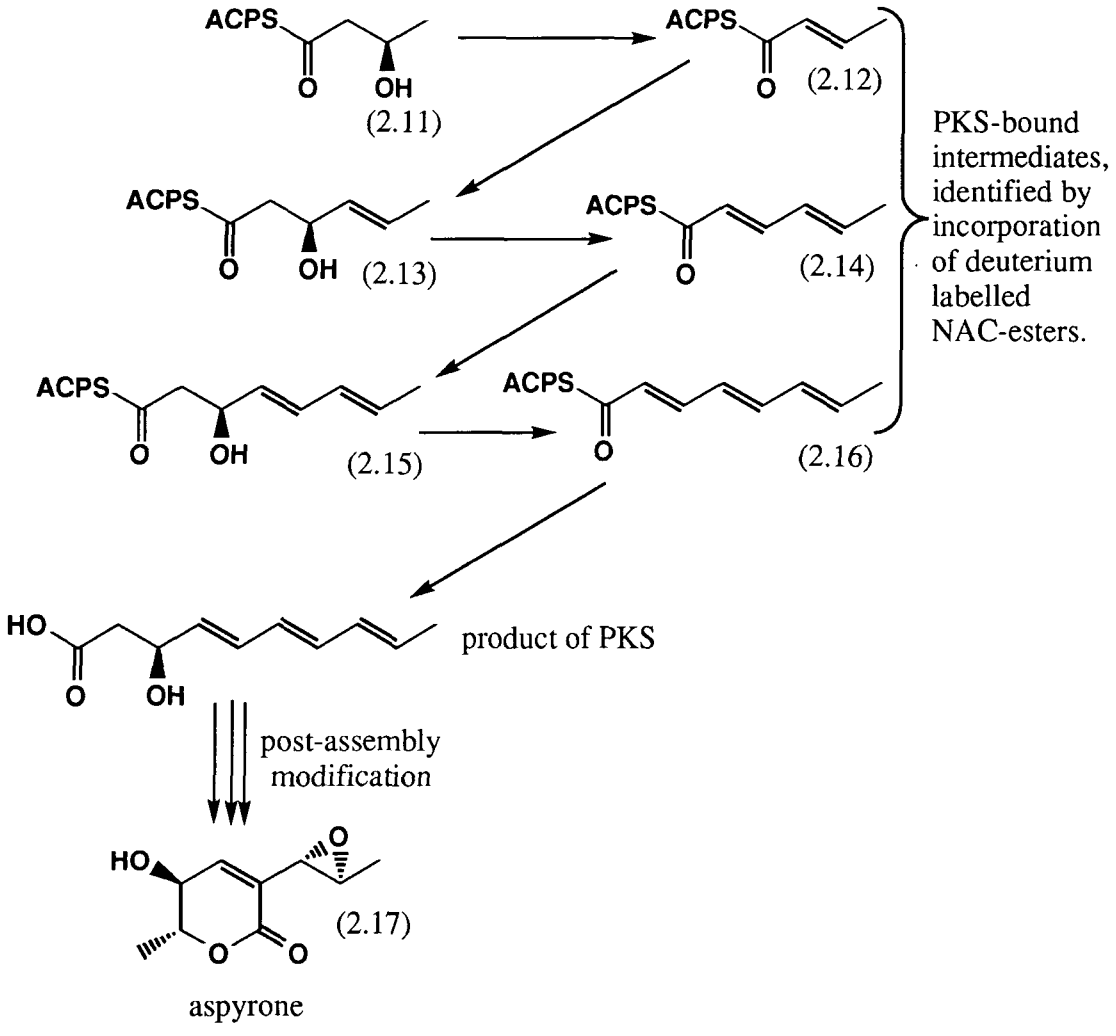
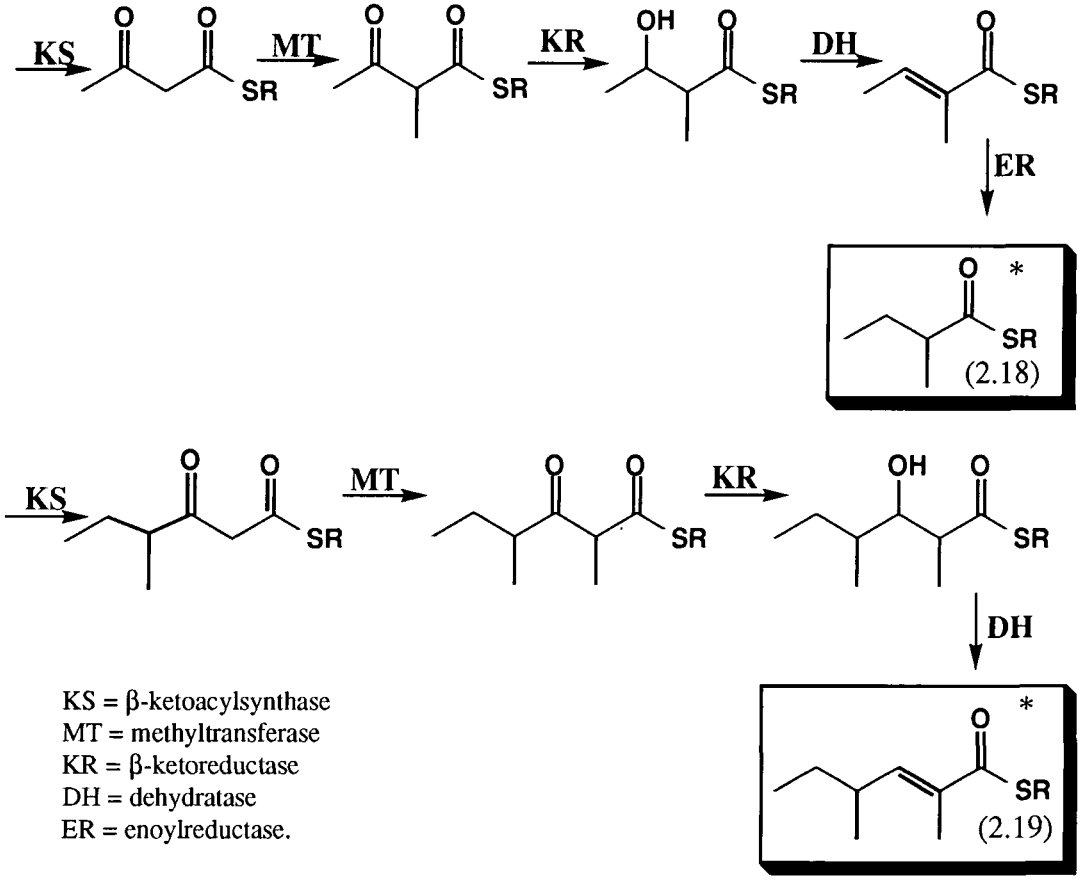


Figure 2-10 Intermediates in aspyrone biosynthesis

A processive mechanism of polyketide assembly is presumably occuring in tenellin biosynthesis. This would require a methyltransferase activity integral with the polyketide synthase. No incorporations of partially assembled intermediates into fungal polyketides, which require a C-methylation step by S-adenosylmethionine have been reported to date.

However if fragments such as 2-methylbutyrate (2.18) and (E)-2,4-dimethyl-2-hexenoate (2.19) can be shown to become incorporated intact into tenellin, this would demonstrate that they were early intermediates is the assembly process, confirming a processive model for methylation.



* = deuterium labelled intermediates fed to *B. bassiana*

Figure 2-11 Proposed intermediates in tenellin biosynthesis

It was anticipated that appropriate intermediates would be administered as N-acylcysteamine thioesters to promote their incorporation onto the ACP thiol group of the PKS, ready to be carried through subsequent chain extension steps. [2-^{13}C-*Methyl*]-2-methylbutyrate had previously been prepared, and fed to *B. bassiana* both as its sodium salt and as its N-propionylcysteamine thioester.[8] However no incorporation into tenellin was observed in these experiments. The sensitivity of such experiments is very low. Carbon-13 has a natural abundance 1.1% relative to carbon-12. To accurately detect enrichment (a 20% increase in peak height) greater than 0.32 % incorporation of the labelled precursor into the metabolite is required. Given the inherent insensitivity of this

experiment a negative result does not necessarily negate the intermediacy of 2-methylbutyrate. However, as demonstrated by the Staunton group, the sensitivity is substantially increased using deuterium labelled studies. With the low natural abundance of deuterium (0.015%), only 0.003% incorporation is required to observe a 20% increase in peak height in the resultant ^2H NMR. Accordingly N-propionylcysteamine thioesters of [2-*methyl*-$^2H_3$]-2-methylbutyrate and [2-*methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoate have been prepared and administered to production cultures of *Beauvaria bassiana*. The results of these experiments are discussed below.

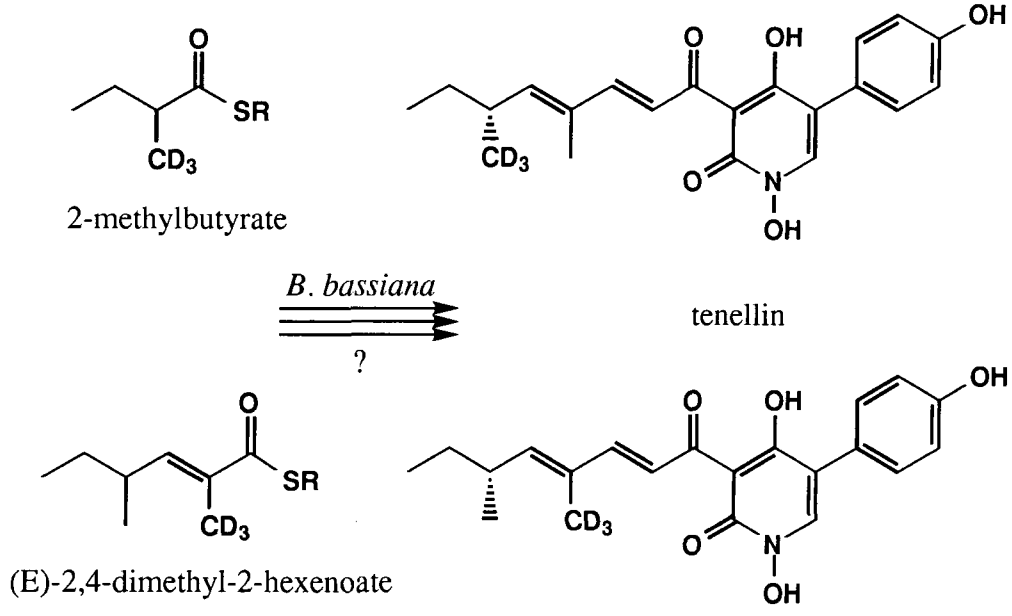
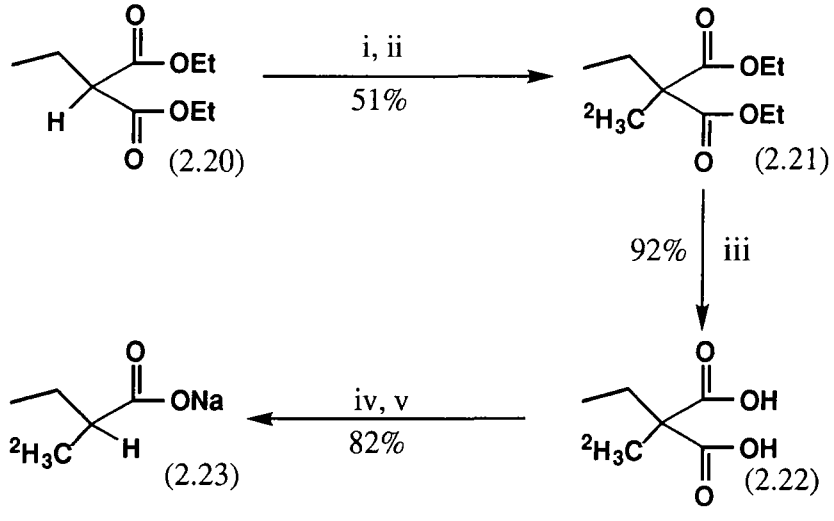


Figure 2-12 Proposed feeding experiments to *B. bassiana*

2.2 Synthesis of Proposed Intermediates

2.2.1 Synthesis of sodium [2-*methyl*-$^2H_3$]-2-methylbutyrate

Sodium [2-*methyl*-$^2H_3$]-2-methylbutyrate was synthesised by alkylation of diethyl 2-ethylmalonate (2.20) with [$^2H_3$]-methyliodide. Base mediated ester hydrolysis followed by acidic work-up afforded [2-*methyl*-$^2H_3$]-2-methyl-2-ethylmalonic acid (2.22). Decarboxylation of this was accomplished by heating in a carius tube at 180°C yeilding [2-*methyl*-$^2H_3$]-2-methylbutyric acid. The acid was separated from involatile impurities by lyophilisation, followed by neutralisation to generated an aqueous solution of the salt sodium [2-*methyl*-$^2H_3$]-2-methylbutyrate (2.23). Involatile impurities and water were removed by further lysophilisation. Prior to preparation of the N-propionyl cysteamine thioester the acid salt was reacidified, extracted into diethyl ether, and the solvent removed *in vacuo*, yielding [2-*methyl*-$^2H_3$]-2-methylbutyric acid.

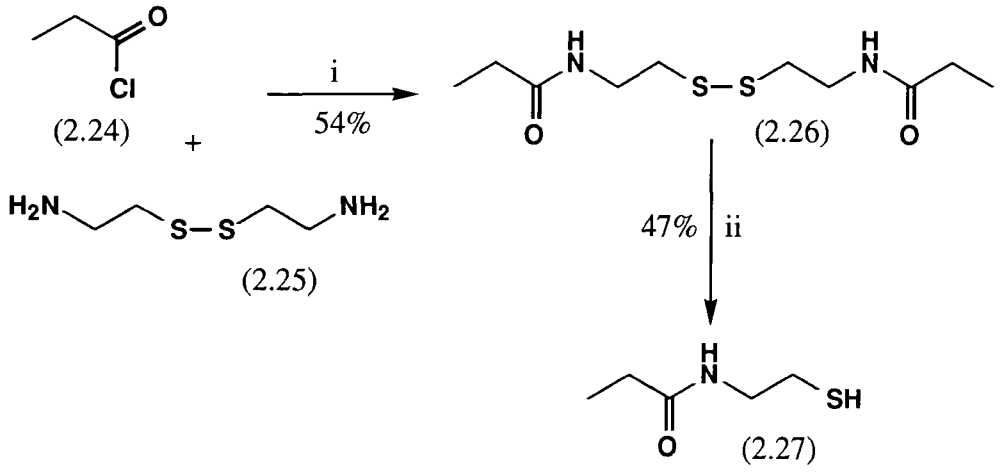


(i) NaH, THF, 0°C, (ii)C^2H_3I, THF, reflux 2hr,
(iii) KOH(aq), reflux 24hr, HCl(aq), (iv) H_2O, 180°C, 3hr, HCl(aq),
(v) pH 8 with NaOH(aq).

Figure 2-13 Synthesis of [2-*methyl*-$^2H_3$]-2-methylbutyrate

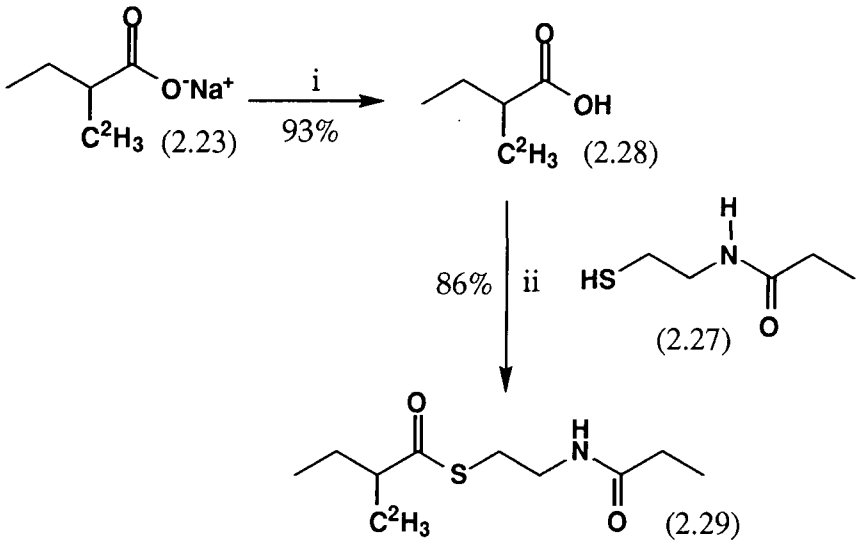
Cysteamine dihydrochloride (2.25) was neutralised and acylated with propionyl chloride (2.24) to give N, N'-dipropionyl cystamine (2.26). Reduction with sodium amalgam in dry methanol afforded N-propionyl cystamine (2.27), which was coupled with [2-*methyl*-$^2H_3$]-2-methylbutyric acid using 1,3-dicyclohexylcarbodiimide and

4-dimethylaminopyridine in ether. After purification over silica the N-propionyl cysteamine thioester (2.29) was could be stored under nitrogen at 4°C.



(i) 0°C, pH > 8.2, (ii) NaHg amalgam, MeOH.

Figure 2-14 Synthesis of N,N'-dipropionylcystamine



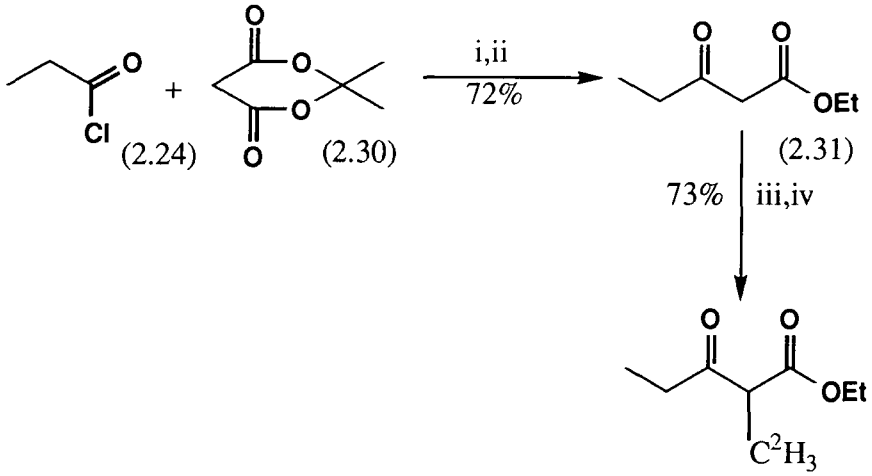
(i) HCl(aq), H_2O, (ii) DCC, DMAP, ether, 20°C, 12hr.

Figure 2-15 Synthesis of N-propionylcystamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate

2.2.2 Synthesis of (E)-[2-*methyl*-$^2H_3$]-2,4-dimethyl-2-hexenoic acid

Meldrum's acid (2.30) was selected as an appropriate malonate for acylation with propionyl chloride (2.24). Heating under reflux in ethanol resulted afforded the ester ethyl 3-oxo-pentanoate (2.31). This was alkylated with [$^2H_3$]-methyl iodide using

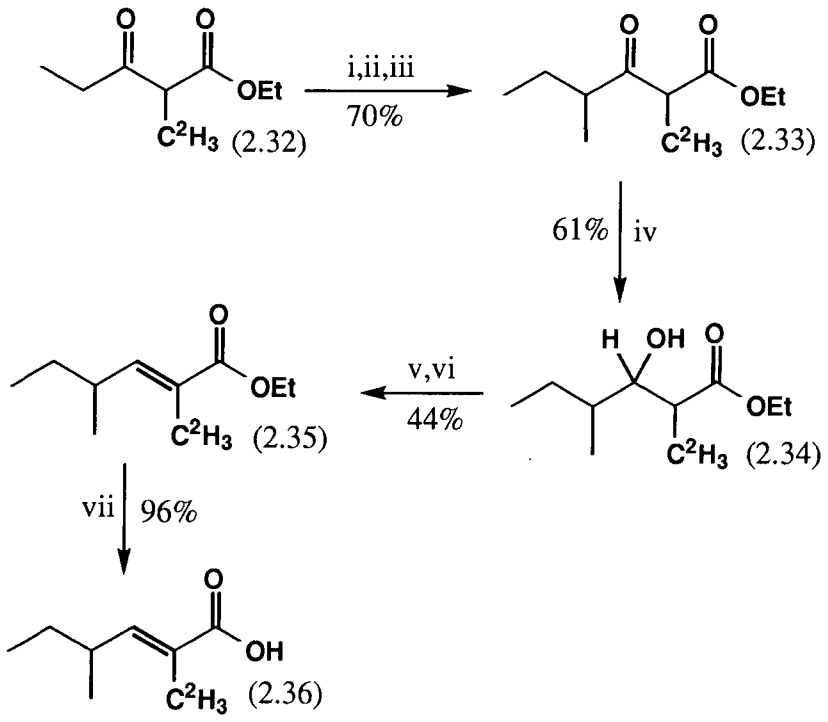
sodium ethoxide in ethanol, yielding ethyl [2-*methyl*-$^2H_3$]-3-oxo-2-methylpentanoate (2.32), which was purified by distillation.



(i) pyridine, DCM, 20°C, 16hr, (ii) ethanol reflux, 5hr, (2.32)
(iii) NaOEt, ethanol, 13°C, (iv) C^2H_3I, ethanol, reflux, 24hr.

Figure 2-16 Synthesis of [2-*methyl*-$^2H_3$]-ethyl 3-oxo-2-methylpentanoate

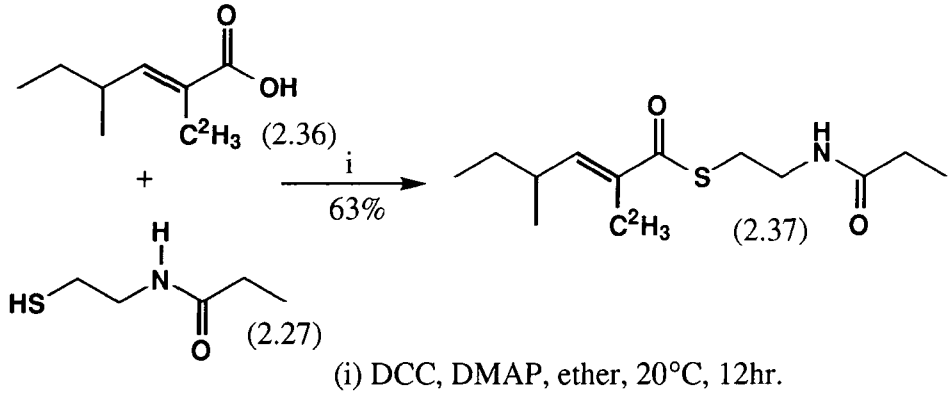
(E)-[2-*Methyl*-$^2H_3$]-2,4-dimethyl-2-hexenoic acid was prepared from ethyl [2-*methyl*-$^2H_3$]-3-oxo-2-methylpentanoate (2.32), according to the procedure described by Katzenellenbogen and Utawanit.[21] The reduction and dehydration steps in the method mirror transformations of the putative biosynthetic pathway, and it was envisaged that intermediates (2.33) and (2.34) could potentially be used in feeding experiments. Ethylation of the dianion of ethyl [2-*methyl*-$^2H_3$]-3-oxo-2-methylpentanoate (2.32) with ethyl bromide, followed by distillation afforded the β-ketoester (2.33). Subsequent reduction with sodium borohydride gave the β-hydroxyester, ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-hydroxyhexanoate (2.34) Purification of this mixture of diasteriomers required both silica and alumina chromatography. The β-hydroxyester was dehydrated using lithium diisopropylamide with diethoxyaluminium chloride to give ethyl (E)-[2-*methyl*-$^2H_3$]-2,4-dimethyl-2-hexenoate (2.35). Hydrolysis under basic conditions, followed by acidic work up, afforded the desired carboxylic acid (2.36) which was used directly for thioester preparation. Intermediates (2.33) and (2.34) were complex mixtures of diastereoisomers and were not fully characterised.



(i) NaH, THF, 0°C, (ii) BuLi, hexane, 0°C, (iii) EtBr, THF, 20°C, 30min, (iv) $NaBH_4$, ethanol, O°C, 30min, (v) $Al(OEt)_3$, $AlCl_3$, THF, 20°C, 30min, (vi) LDA, THF, 65°C, 1hr, (vii) NaOH, methanol, reflux, 5hr.

Figure 2-17 Synthesis of (E)-[2-*methyl*-$^2H_3$]-2,4-dimethyl-2-hexenoic acid

[2-C^2H_3-*Methyl*]-N-propionyl cysteamine-(E)-2,4-dimethyl-2-hexenoate (2.37) was prepared by coupling N-propionyl cystamine (2.26) with (E)-[2-*methyl*-$^2H_3$]-2,4-dimethyl-2-hexenoic acid (2.36) using 1,3-dicyclohexylcarbodiimide and 4-dimethylaminopyridine in dry ether. This could be stored under nitrogen at 4°C, after purification over silica.



(i) DCC, DMAP, ether, 20°C, 12hr.

Figure 2-18 Synthesis of [2-C^2H_3-*methyl*]-N-propionyl cysteamine-(E)-2,4-dimethyl-2-hexenoate

The key step in this synthesis is the treatment of the β-hydroxyester (2.38) with diethoxyaluminium chloride followed by warming with diisopropylamide to produce the corresponding α,β-unsaturated ester (2.40). The process is completely regiospecific, and has high stereospecificity with only the E isomer formed. This was confirmed by an n.O.e. study which showed no interaction between the CH-3 and CH_3-7. The reaction is thought to proceed through a β-alanoxyenolate intermediate (2.41), the stereoselectivity resulting from the minimization of 1,4-interactions in the boat like transition state.

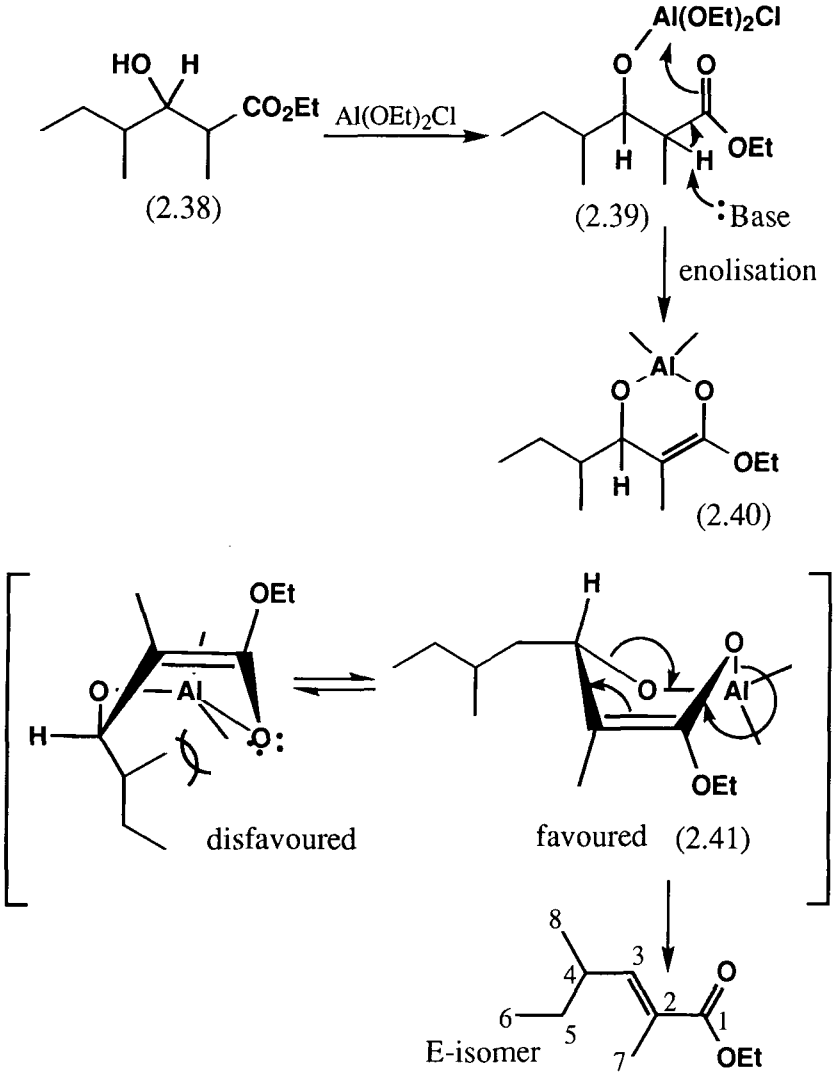


Figure 2-19 Dehydration of ethyl 2,4-dimethyl-3-hydroxyhexanoate

2.3 Biosynthetic Investigations

2.3.1 Growth of *Beauvaria bassiana*

Frozen samples of *B. bassiana* production media were used to inoculate fresh production media flasks. Tenellin production was good, with an average of 50mg of tenellin from 100ml of production medium. The yellow colouration characteristic of tenellin production usually appeared on day 4 after innoculation.

2.3.2 Isolation and analysis of tenellin from *Beauvaria bassiana*

Tenellin shows only limited solubility in aprotic solvents such as dichloromethane and carbon tetrachloride, and therefore ^{2}H NMR spectra were recorded in dimethylsulphoxide (DMSO). This was a disadvantage due to a large solvent signal observed at 2.49 ppm, however it did not mask the methyl signals of tenellin, and any incorporations into these sites should be directly observable. A natural abundance ^{2}H NMR spectrum was obtained. However the signal to noise was too low to obtain accurate integrals, but the signals for the methyl, methylene, and methine site of the polyketide moiety were clearly observed. The chemical shift values for the ^{2}H NMR signals correlate closely to those of the ^{1}H NMR (Figure 2-20). The assignments of the ^{1}H NMR resonances of tenellin are tabulated below The hydroxyl protons, which resonate above 9.0 ppm are not listed.

		chemical shift (ppm)
aromatic	3', 5'	6.78
	6', 2'	7.28
pyridone	6	8.18
aliphatic	8	7.54
	9	7.99
	11	5.98
	12	2.40 (under DMSO)
	13	1.36
	14	0.81
	15	0.97
	16	1.83

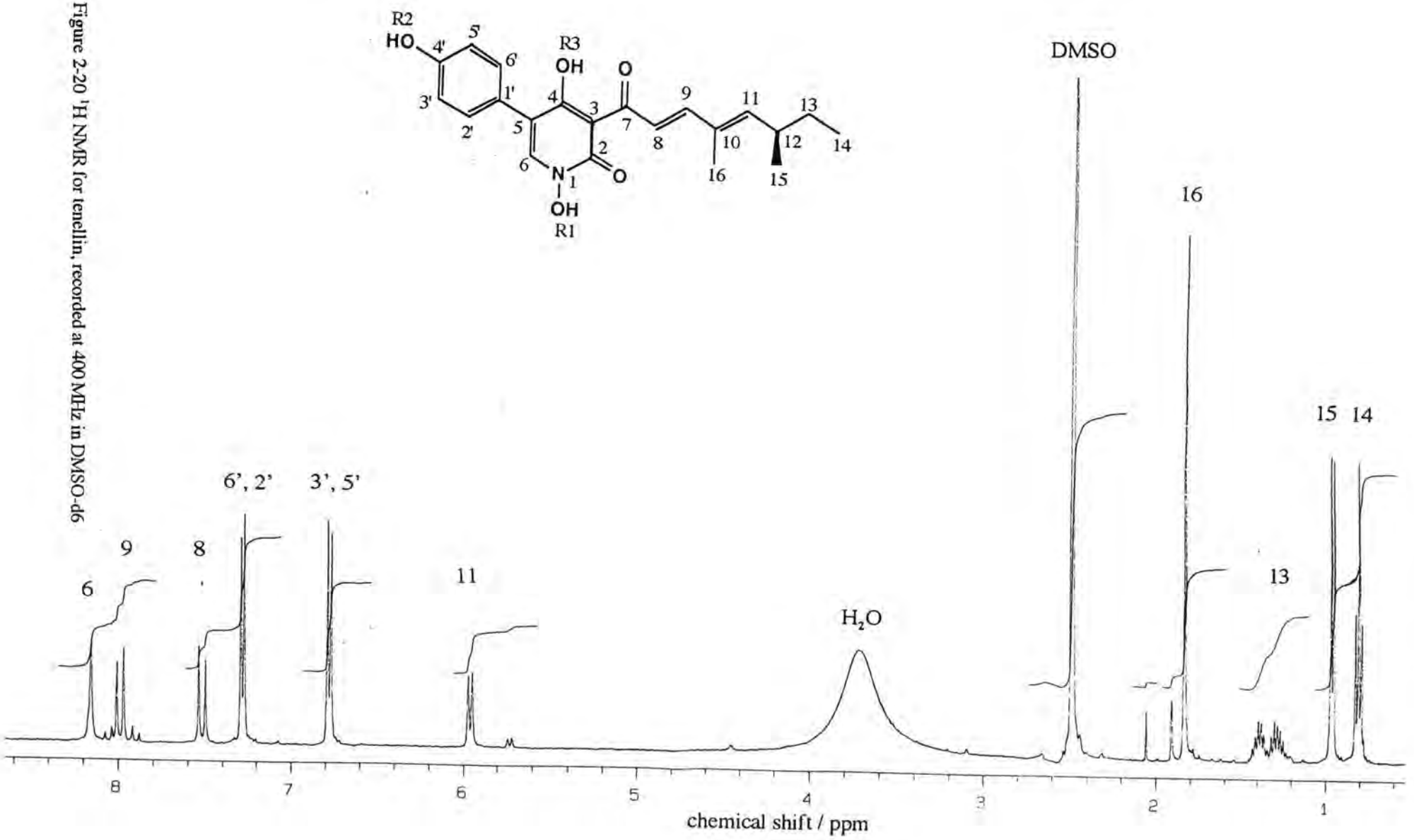


Figure 2-20 ^{1}H NMR for tenellin, recorded at 400 MHz in DMSO-d6

2.3.3 Growth of *Beauvaria bassiana* in 6.6% D_2O

Two flasks of production media (50ml) prepared with 6.6% D_2O in H_2O were innoculated with 5ml of 7 day old *Beauvaria bassiana* production media cultures, and incubated for 8 days. The resultant tenellin was isolated, and purified by HPLC as discussed below. Clear deuterium enrichments were observed in the aliphatic region corresponding to isotope enrichments at CH_3-14, CH_3-15, CH_2-13 and CH_3-16 at 0.77 ppm, 0.92 ppm, 1.23 ppm and 1.79 ppm respectively. The aromatic region was clearly enriched *ortho* to hydroxyl at 6.76 ppm, with a minor enrichment at 7.31 ppm *meta* to the hydroxyl. CH_3-15 and CH_3-16 are derived from methionine. In this case the deuterium may become incorporated *via* NADPD reduction of a C_1-tetrahydrofolate cofactor to N^5-methyl-THF, required for methylation of homocysteine to methionine. The enrichment at CH_3-14 must derive from a deuterium labelled acetate unit, possibly *via* direct exchange with the medium. It is probable that acetate is in rapid equilibrium with malonyl-CoA. However the high enrichment at CH_3-14 (directly derived form acetate) is not so readily explained, as negligible incorporation is observed at CH-8 (derived from malonate). Incorporation of deuterium from the NADPD is as expected at CH_2-13, as a result of reduction of the C-12 to C-13 double bond during polyketide biosynthesis. Deuterium enrichment of the aromatic protons *ortho* and *meta* to the phenolic hydroxy may be rationalised by deuterium exchange with the medium, in the shikimic acid precursors to phenylalanine / tyrosine.

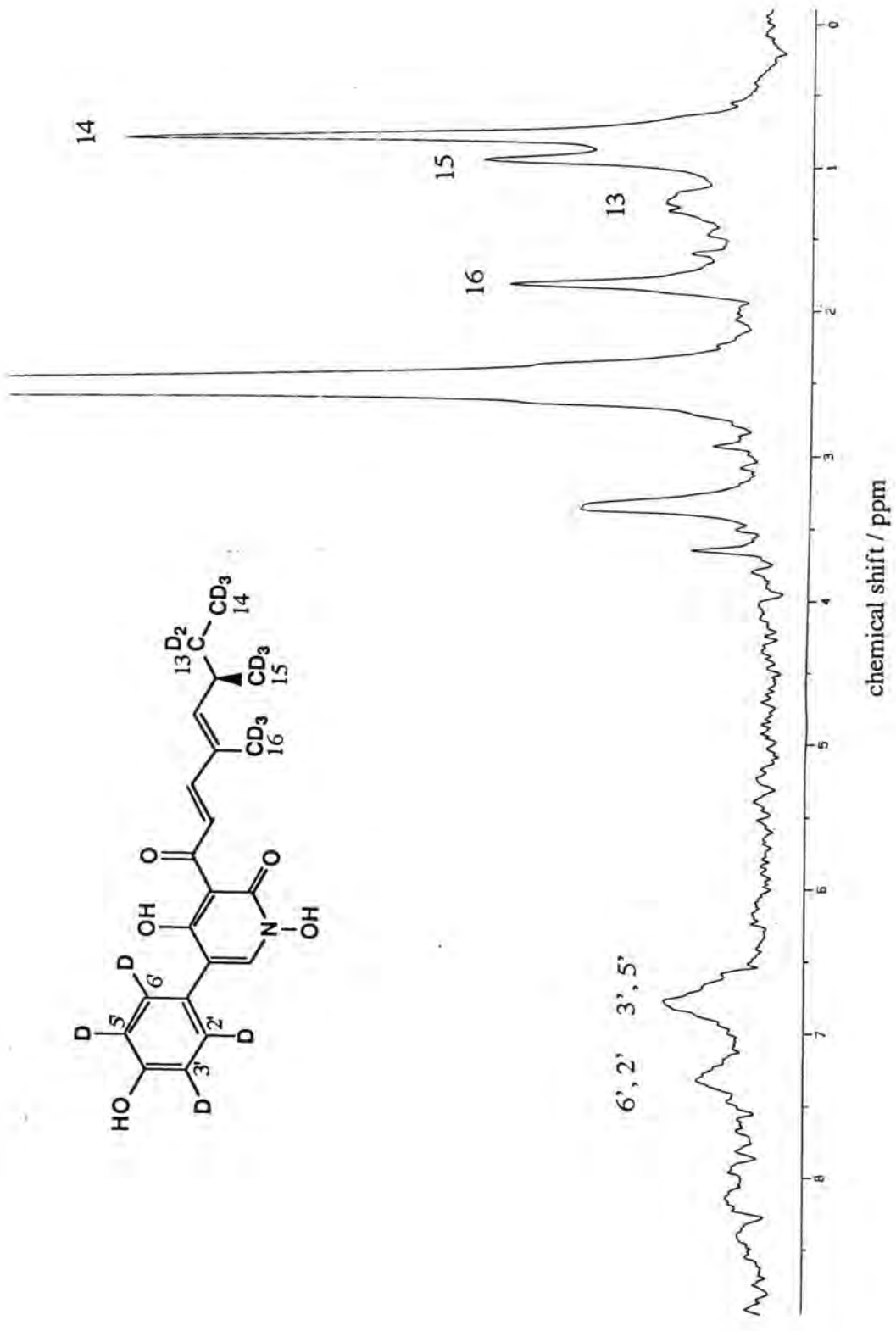


Figure 2-21 ^{2}H NMR of tenellin grown in 6.6% D_2O after purification by HPLC

2.3.4 Feeding of N-propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate to *Beauvaria bassiana*

N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate was initially pulse fed (in ethanol / water, 3 : 4) to a final concentration 6.0 mM to *Beauvaria bassiana* production medium cultures at the beginning of culture growth (trophophase). After isolation of the resultant tenellin, ^2H NMR analysis did not indicate any incorporation of deuterium. This was perhaps not unexpected as tenellin production does not commence until the stationary stage of cell growth. The added compound may have been degraded by β-oxidation prior to tenellin production, and the deuterium-labelled products used in primary metabolism.

When N-propionylcysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate was administered after tenellin production was evinced (idiophase), ^2H NMR analysis of the resultant crude tenellin sample showed signals at 0.72 ppm, 0.88 ppm and 1.77 ppm (in approximate intensity ratio 5 : 5: 1). These chemical shifts suggested incorporation into C-14, C-15, and C-16 of tenellin respectively. A signal at 5.65 ppm was also observed in one experiment which could not be assigned to a signal in tenellin and was presumably an impurity peak. Tenellin is notoriously difficult to purify, due to its highly polar nature. The purity of the tenellin isolated by the standard work-up procedure is generally adequate for analysis of incorporation experiments with carbon-13 labelled precursors. In general 'crude' tenellin shows respectably clean ^{13}C NMR and ^1H NMR spectra. However due to the sensitive nature of ^2H NMR, even minor impurities are readily detected. Thus the observed enrichments could not confidently be attributed to incorporation into tenellin at this stage.

Accordingly, an HPLC method was developed for purifying tenellin. Analytical reverse phase HPLC (methanol : water, 85 : 15, 0.1% trifluoroacetic acid) was used to check the purity of the tenellin isolated. Tailing could be minimised and sharp peaks obtained when a BDS-protected analytical column was used. The feeding experiments with N-propionylcysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate were repeated on a larger scale (10x 50ml production media flasks, to a final concentration 5.0 mM) and 300 mg of tenellin was isolated. Again enrichments were observed in the ^2H NMR at 0.70 ppm,

0.85 pm and 1.77 ppm, but the relative intensity of the signal at 0.85 ppm was now much reduced, (approximate intensity ratio 7 : 2: 1) as shown in Figure 2-22. This confirmed that labelling at 0.85 ppm is due at least in part to contamination by labelled impurities, probably the precursor [2-*methyl*-$^2H_3$]-2-methylbutyrate.

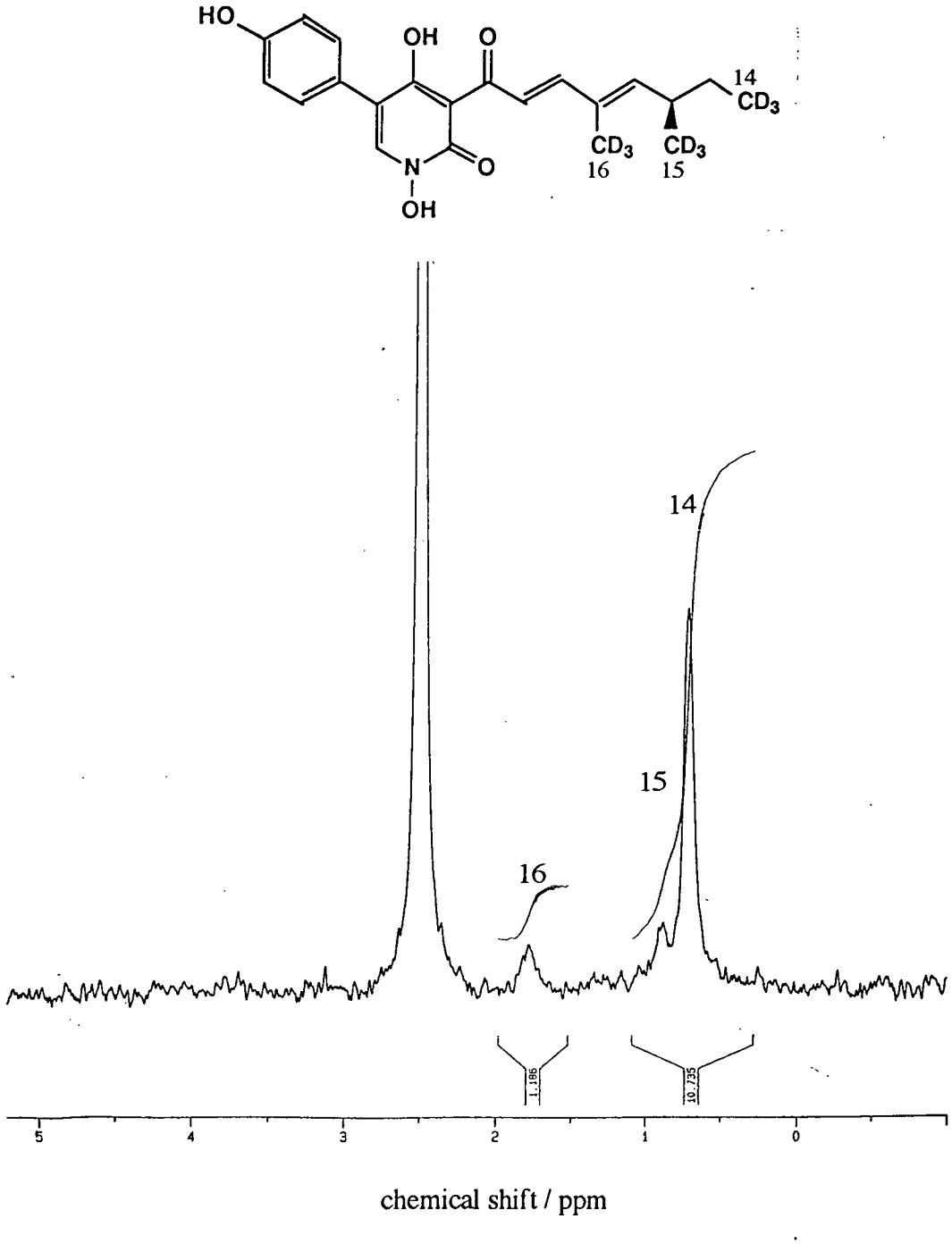


Figure 2-22 ^2H NMR of tenellin fed with N-propionylcystamine-[2-methyl-$^2H_3$]-2-methylbutyrate after purification by HPLC.

The apparent enrichment of CH_3-16 is unexpected, and requires explanation. Although only one peak was observable by HPLC, this was broad and it is conceivable that the tenellin sample still contained impurities. HPLC purification was repeated and fractions collected before and after the mid-point of the elution peak. Both these fractions gave similar ^2H NMR spectra, suggesting that the deuterium labelling was indeed in tenellin rather than a deuterated impurity. The labelling of CH_3-16 requires deuterium labelling of methionine. If this is occuring, then the labelling at CH_3-15 should also be enriched *via* the same route, with approximately the same level of enrichment, and this is indeed observed. Thus there is no evidence for the direct incorporation of deuterium into CH_3-15 from [2-*methyl*-$^2H_3$]-2-methylbutyrate. Both these methionine derived sites are also strongly enriched in tenellin when *B. bassiana* is grown in 6.6% D_2O, implying that the deuterium is exchanged with the medium, possibly after catabolism to succinate (see below). Deuterium may then be reincorporated *via* NADPD into methionine.

The significant enrichment of CH_3-14 is more readily rationalised. This site derives from the methyl group of acetate and isotope incorporation here is likely to be the result of a partial degradation of the intermediate by β-oxidation to deuterium labelled acetate. Branched chain fatty acids with a methyl group on an even numbered carbon atom are degraded to propionate, the branching appearing as C-3.[22] Propionyl-CoA is converted to succinyl-CoA *via* methylmalonyl-CoA and metabolism of succinate results in acetate. The labelled [$C^1H^2H_2$]-acetate could subsequently become incorporated into tenellin, giving rise to C-14 labelling (Figure 2-23).

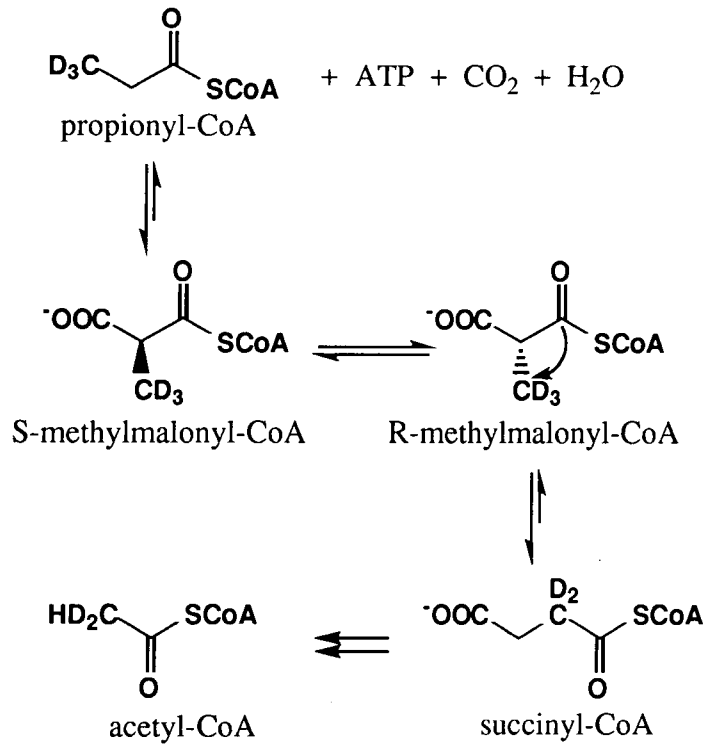


Figure 2-23 Catabolism of propionate

2.3.5 Feeding of [C^2H_3]-acetate to *Beauvaria bassiana*

In an attempt to verify the observed labelling of CH_3-14 of tenellin, [C^2H_3]-acetate was pulse fed to *B. bassiana* cultures to a final concentration of 7.4 mM. The isolated tenellin was purified by HPLC and analysed by ^2H NMR analysis. Incorporation was clearly seen into the methyl-14 peak at 0.72 ppm, as shown in Figure 2-24. This labelling pattern lends support to the above hypothesis that the enrichments observed at 0.72 ppm from tenellin fed with (R,S)-[2-*methyl*-$^2H_3$]-2-methylbutyrate, were the result of acetate incorporation after β-oxidation. Surprisingly, however, no incorporation of deuterium was observed at CH-8, the hydrogen which is also expected to be derived from the methyl group of acetate. However as this acetate must first be activated to malonyl-CoA for condensation with the growing polyketide chain, rapid exchange of the acidic deuteriums with the protic media would account for isotope washout.

Prior to HPLC purification in this experiment an enriched impurity peak at 8.2 ppm was observed which had the same intensity as the methyl-14 peak of tenellin. This was also observed in the feeding experiment below. It is likely this is due to a minor aromatic polyketide produced by the cultures.

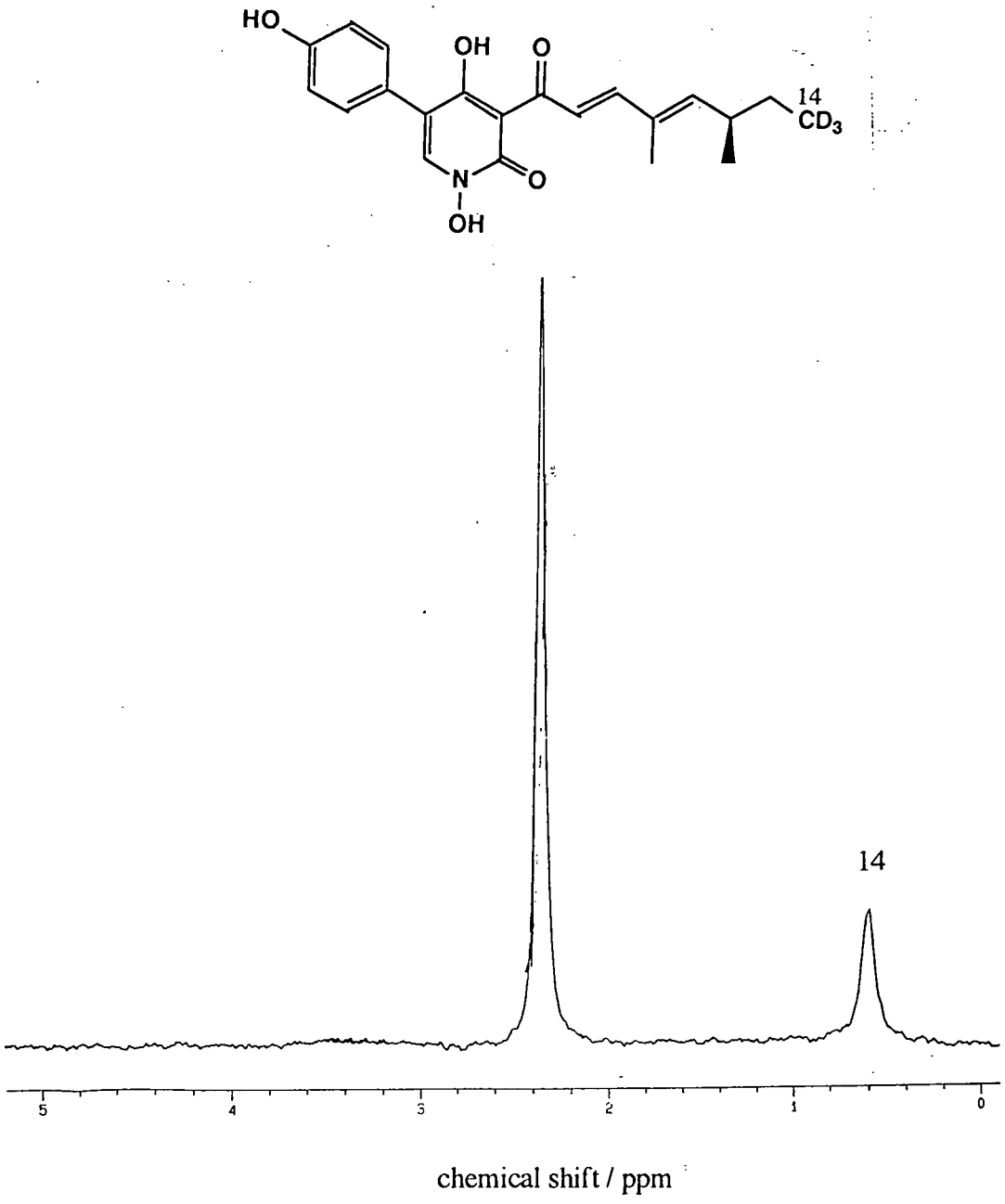


Figure 2-24 ^{2}H NMR of tenellin fed with [$C^{2}H_{3}$]-acetate after purification by HPLC.

2.3.6 Feeding of N-propionylcysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate to senescent cultures of *Beauvaria bassiana*

Incorporation experiments in the Leadlay / Staunton group, with partially assembled polyketide intermediates into tetronasin (2.45) produced by *Streptomyces longisporoflavus*, have shown that the N-acetyl cysteamine analogues of precursors are less likely to suffer degradation by β-oxidation processes late in the growth cycle. Secondary metabolism is observed to continue, albeit at a reduced rate, but the enzymes of fatty acid degradation have become less active. Thus at this stage there is greater chance of the precursor becoming incorporated intact. However incorporation is less readily detected late in the growth cycle, due to the dilution effect by previously biosynthesised metabolite. None-the-less, detectable incorporations of the tri- (2.43) and tetra- ketide (2.44) precursors of tetronasin were observed.[23]

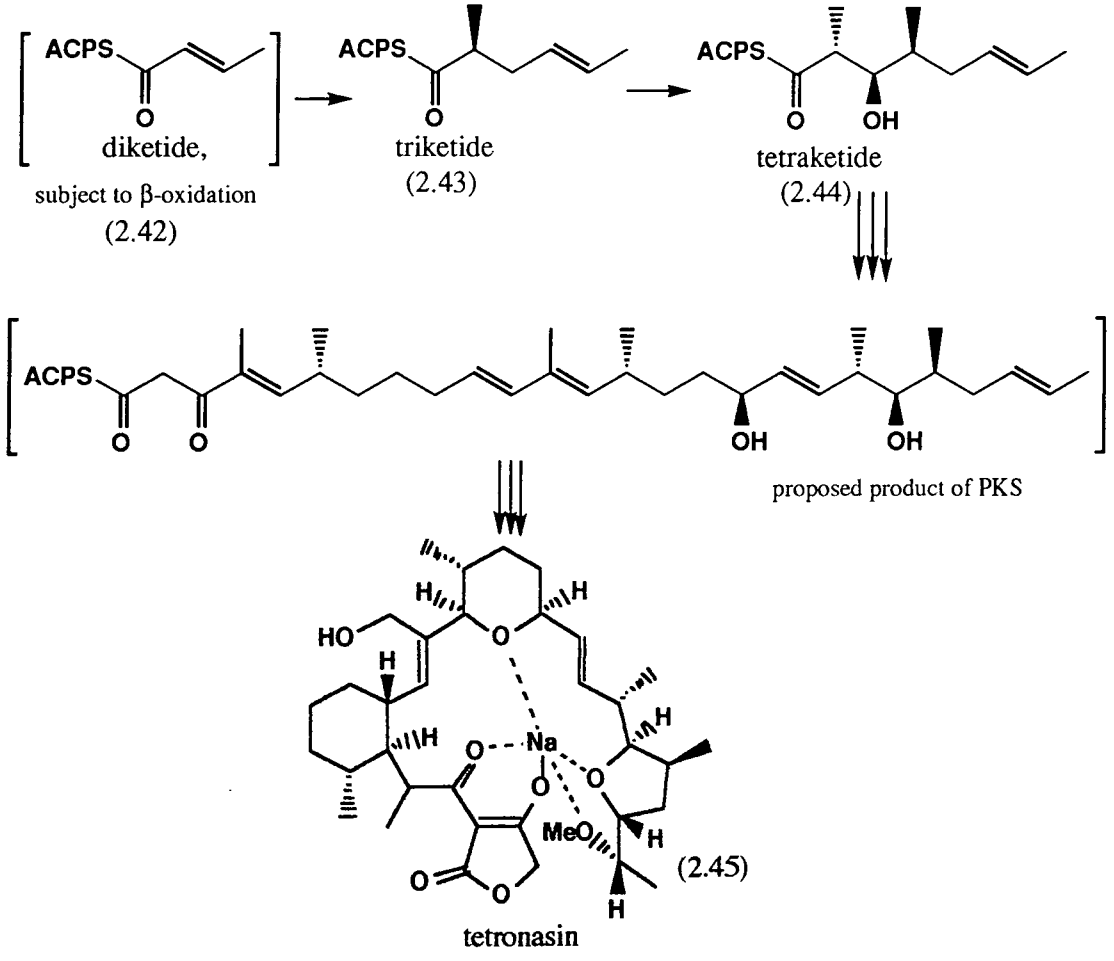


Figure 2-25 Intact incorporation of tri- and tetraketide intermediates into tetronasin

When N-propionylcysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate was administered to senescent cells (pulse fed, to final concentration 5.0 mmolar), the deuterium signal observed was weaker, but only one peak was observed in the aliphatic region. This was at 0.89 ppm, and could correspond to enrichment of tenellin at C-15. No enrichment of CH_3-14, resulting from β-oxidation of the intermediate and incorporation of labelled acetate was observable, consistent with the inactivation of the enzymes of fatty acid degradation. However after rigorous HPLC purification of the 'crude' tenellin no ^2H signals were observable. Presumably the enrichment at 0.89 ppm, was again due to contamination with [2-*methyl*-$^2H_3$]-2-methylbutyrate or a related metabolite.

Again in this experiment, a large impurity peak was observed at 8.2 ppm in the crude tenellin. This labelled impurity peak was also observed in the ^2H NMR of 'crude' tenellin recovered after feeding [C^2H_3]-acetate, and it was suggested that this is due to labelling of an aromatic polyketide produced by *B. bassiana.* Deuterium labelled acetate derived from β-oxidation of [2-*methyl*-$^2H_3$]-2-methylbutyrate is presumably the cause of the labelling of this metabolite. Thus it appears that some fatty acid degradation to acetate did occur during this experiment. If this is the case, then tenellin production during the latter production phase must have been low, as no acetate labelling of tenellin is observable. It is therefore unlikely that we will observe any intact incorporation of [2-*methyl*-$^2H_3$]-2-methylbutyrate into tenellin at this stage of cell growth.

2.3.7 Feeding of N-propionylcysteamine-[$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoate to *Beauvaria bassiana*

Although it might be presumed that it is increasingly difficult to intercept the PKS with increasingly advanced intermediates, these intermediates if correctly chosen, may be more immediately recognisable by the PKS, and conversely less acceptable substrates for fatty acid degradation. This was concluded in the tetronasin studies. The deuterium labelled diketide precursor was not incorporated intact in contrast to the tri- and tetraketide precursors at any stage in the growth cycle.[24] For the tenellin studies, the putative intermediate triketide, N-propionylcysteamine-[$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoate (2.37) was pulse fed to *B. bassiana* cultures to a final concentration of 3.0 mM. After purification by HPLC, ^2H NMR of the isolated tenellin did not show incorporation of ^2H from [$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoate. Interestingly there

was no observable enrichment of tenellin from the catabolic products of [$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoate. Perhaps the precursor was unable to cross the cell membrane of the fungi, in sufficient amounts to observe incorporation.

2.4 Discussion

Attempts to establish a processive mechanism for polyketide assembly in tenellin, involving early reduced, methylated intermediates have been unsuccessful. Incorporations, when observed were inconclusive. The weight of evidence supports a processive mechanism for the biosynthesis of non-methylated reduced polyketides, and the failure to detect incorporation only highlights the difficulties which plague such feeding experiments with advanced precursors. The problems are multiple: lack of solubility and cell permeability of substrates, rapid β-oxidation of substrates that do penetrate into the cell, difficulty in intercepting the polyketide synthase. All these factors result in low incorporation levels, and in this case probably below the detection limits of the techniques used.

However, similar successful experiments led the field in understanding the nature of polyketide synthases. More recently it has been genetic approaches to understanding polyketide synthases, that have continued to make important advances. Although the majority of this work has concerned bacterial systems, the mevinolin PKS from *Aspergillus terreus* has been sequenced.[25] Further results from this research will be of particular interest as mevinolin is both reduced and has a methionine derived methyl group.

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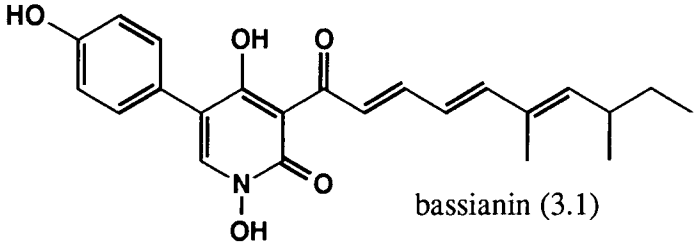
3. Investigations into a Putative Tetramic acid Intermediate

3.1 Introduction and Background

3.1.1 The 2-pyridones

Tenellin is one of a small group of fungal metabolites which possess a 2-pyridone ring. These metabolites belong to a larger group of metabolites, shown to be derived from a polyketide and an amino acid moiety. This group also includes the tetramic acids[1] (discussed below), and the cytochalasins (discussed in Chapter 4).[2]

Cultures of *Beauvaria bassiana* may also produce a yellow pigment differing from tenellin only in the possession of an extra trans ethylene group in the side chain. This 2-pyridone is known as bassianin (3.1), and is co-produced with tenellin in certain cultures of *Beauvaria tenella*.[3] Perversely, tenellin is produced predominantly by *B. bassiana* and bassianin by *B. tenella*.

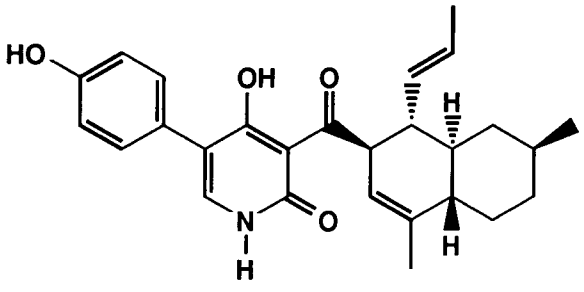


bassianin (3.1)

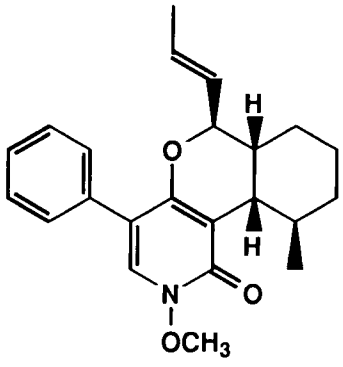
Figure 3-1 The structure of bassianin

A closely related metabolite to tenellin is ilicicolin H (3.2) produced by *Cylindrocladium ilicicocola*. This metabolite is also substituted at the 5 position of the pyridone ring with a 4-hydroxyaryl group. Isotopic labelling studies on this metabolite have established the biosynthesis to be analogous to tenellin (Tanabe *et al.*[4]). Carbon-13 enriched acetates labelled the polyketide moiety in the usual way, and pendent methyl groups were enriched by [$^{13}CH_3$]-methionine. Furthermore ^{15}N-phenylalanine was shown to label the nitrogen of the pyridone ring. In the ^{13}C-NMR of the sample labelled with nitrogen-15, ^{13}C-^{15}N spin-spin coupling could be detected with an observed coupling constant of 12.5 Hz. However, contrary to the claims of Tanabe *et al.* this observation does not eliminate the possibility that phenylalanine is metabolised to phenylpyruvic acid prior to incorporation, with nitrogen-15 becoming *re-incorporated* at a later stage. A more rigorous experiment

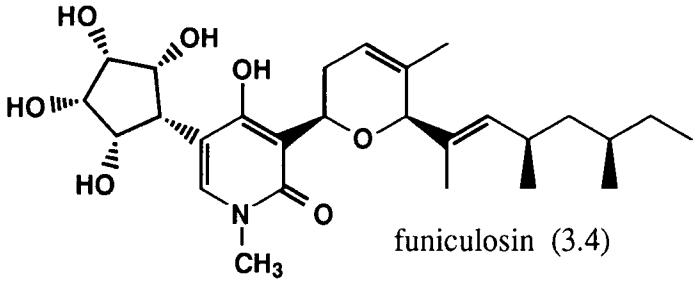
would have involved the intact incorporation of double-labelled [2-$^{13}C^{15}N$]-phenylalanine. This possibility was investigated for tenellin biosynthesis and is discussed in Chapter 4. Other closely related metabolites in this group are leporin A (3.3) (produced by *Aspergillus leporis*) which has an aryl ring at carbon-5 of the pyridone ring,[5] and funiculosin (3.4) (from *Penicillium funiculosum*) bearing an interesting tetrahydroxy cyclopentane group at this position.[6]



ilicicolin H (3.2)



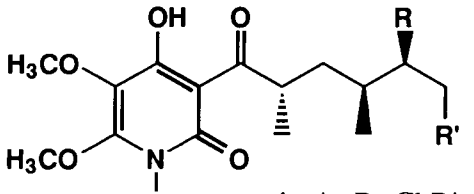
leporin A (3.3)



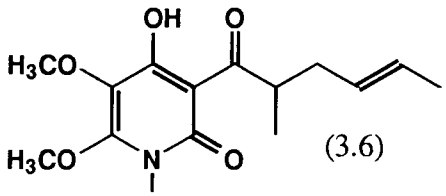
funiculosin (3.4)

Figure 3-2 Pyridone metabolites related to tenellin

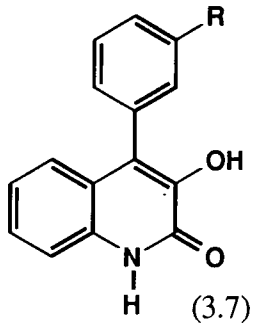
The 2-pyridones also include the atpenins (3.5) (*Penicillium sp. FO125*),[7] harzianopyridone (3.6) (*Trichoderma harzianum*)[8] viridicatin and viridicatol (3.7) (*Penicillum discolor*),[9] and the efrotomycin antibiotics (3.8) (*Streptomyces goldinensis*).[10] The polyketide origin of the side chain of harziopyridone was established by ^{13}C-labelled acetate feeding experiments, again consistent with tenellin biosynthesis, and the methoxyl carbons were labelled predictably by [$^{13}CH_3$]-methionine. However the origin of the remainder of the pyridone ring is not unambiguously established, although there is some evidence to suggest that aspartic acid may be a precursor.



(3.5) atpenin A_4 R=Cl R'=H
atpenin A_5 R=Cl R'=Cl
atpenin B R=H R'=H



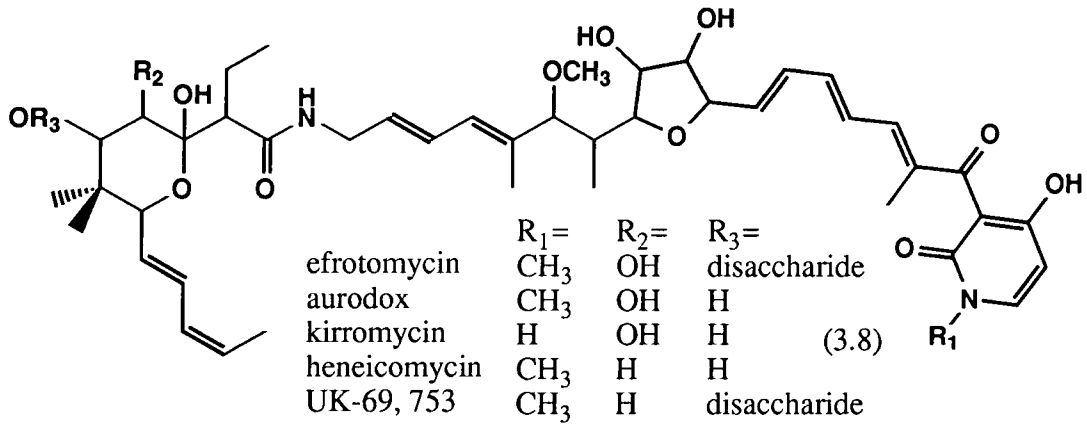
(3.6) harzianopyridone



(3.7)
viridicatin R=H
viridicatol R=OH

Figure 3-3 Pyridone metabolites

The biosynthesis of the pyridone ring of aurodox (3.8) is more enigmatic.[11] As anticipated for a bacterial polyketide the backbone of the structure is derived from the linear combination of acetate, propionate and butyrate units. However [2-^{13}C]-acetate failed to become incorporated into position-3 (or position-5) of the pyridone ring, suggesting that there is another pathway by which nature may construct such a pyridone. Interestingly, *Nocardia lactamdurans*, which produces efrotomycin, has been shown to catabolyze uracil *via* the reductive pathway. The end product of this pathway, β-alanine, appears to be incorporated into the pyridone ring of efrotomycin. [5,6-$^{3}H_2$]-Uracil is found to label efrotomycin, and carbon-13 coupling at C4 and C5 of efrotomycin is observed after feeding resting cells with [4,5-^{13}C]-uracil.[12] Novel members of the efrotomycin family, ganefromycin[13] and L-681,217[14] have been isolated that lack the pyridone moiety, the polyketide chain terminating with a carboxylic acid group, suggesting attachment of the pyridone moiety may be a late stage modification.



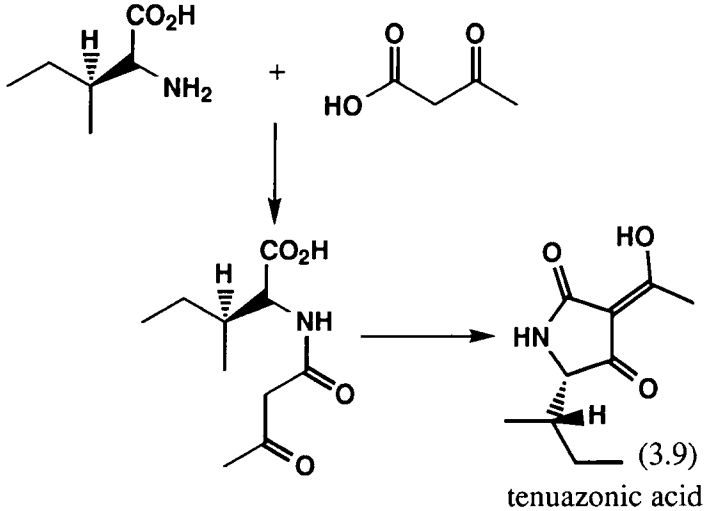
	R_1=	R_2=	R_3=
efrotomycin	CH_3	OH	disaccharide
aurodox	CH_3	OH	H
kirromycin	H	OH	H
heneicomycin	CH_3	H	H
UK-69, 753	CH_3	H	disaccharide

(3.8)

Figure 3-4 The efrotomycin family

3.1.2 The acyl-tetramic acids

The tetramic acids (2,4-pyrrolidinediones) occur in both bacteria and fungi. The majority of natural tetramic acids are 3-acyl derivatives, the simplest being tenuazonic acid (3.9), first isolated from *Alternaria tenuis*.[15] Isotopic labelling studies have confirmed that the biosynthesis of the ring occurs *via* the cyclisation of N-acetoacetyl-L-isoleucine, itself derived from the condensation of L-isoleucine with a diketide.[16]



(3.9)
tenuazonic acid

Figure 3-5 Biosynthesis of tenuazonic acid

Tetramic acids bearing a 1-oxopentadienyl substiuent at carbon-3 of the ring are classified as dienoyltetramic acids, the first to be isolated being streptolydigin (3.10) from *Streptomyces lydicus*. Isotopic labelling studies indicated that acetate, propionate, D-glucose, and glutamic acid are all incorporated into streptolydigin. Closely related

acyltetramic acids are tirandalydigin (3.11) and the tirandamycins (3.12), also from Streptomyces sp.

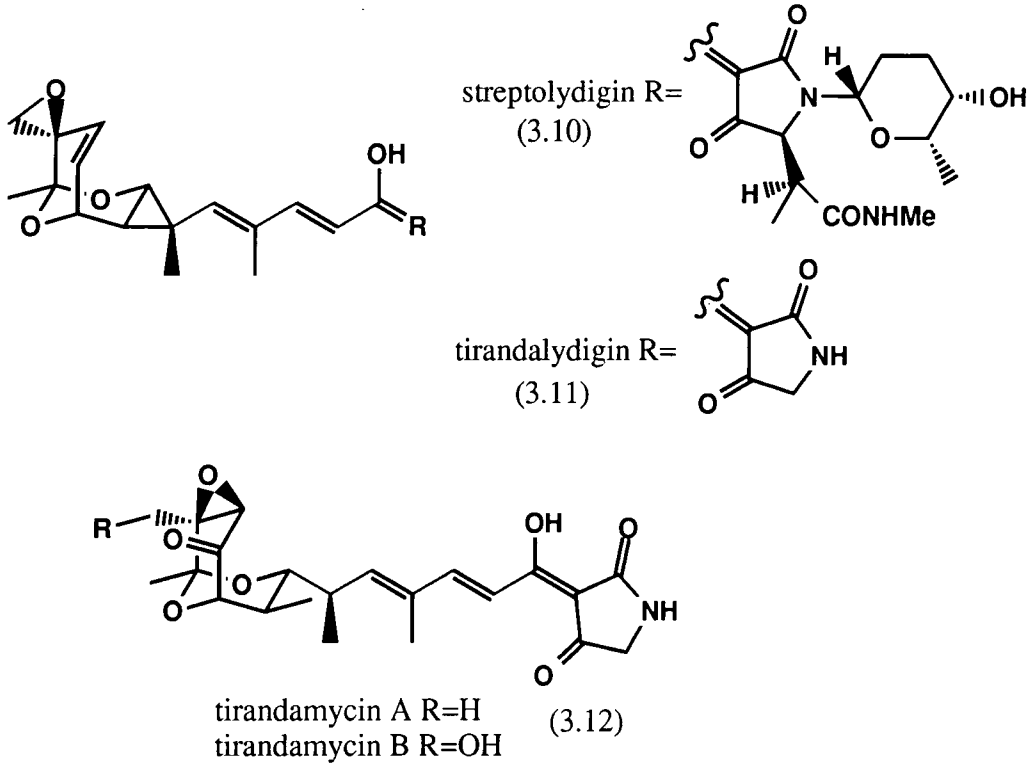


Figure 3-6 Streptolydigin and related tetramic acids

A number of polyenoyl tetramic acids have been isolated, the first of which, erythroskyrine (3.13), was the principal pigment of *Penicillium islandicum*. It appears to be derived from L-valine and acetate, but the origin of tetramic acid ring carbons-2 and 3 and the N-methyl group have not been secured.

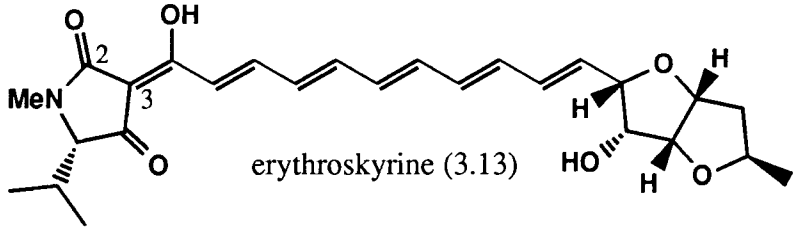


Figure 3-7 Structure of erythroskyrine

A group of orange / yellow coloured pigments (3.14) have been isolated from the slime mold *Leocarpus fragilis*.[17] These compounds clearly derive from tyrosine and a polyketide moiety.

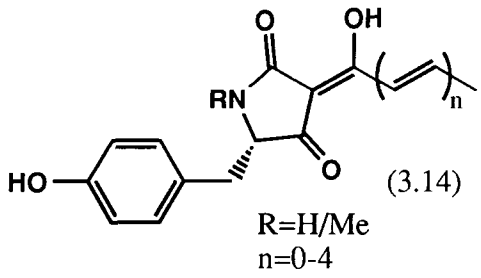
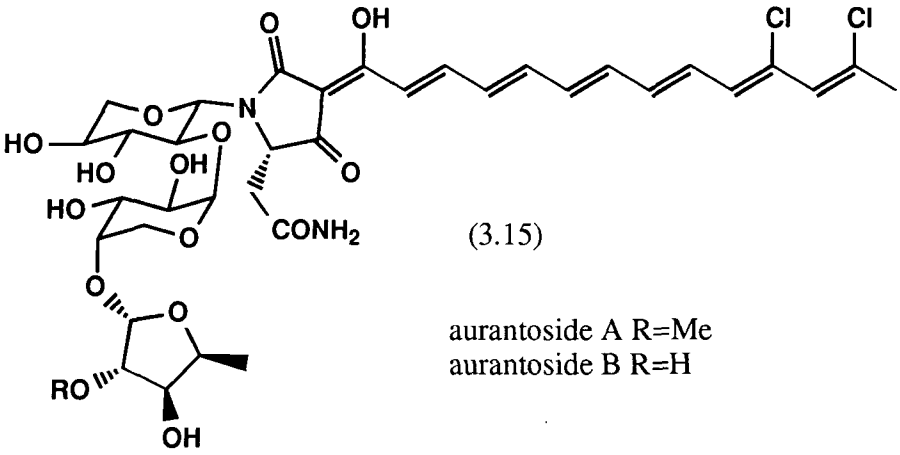


Figure 3-8 tyrosine derived tetramic acids

The marine sponges also produce a range of acyltetramic acids including the orange, chlorine-containing aurantosides (3.15) from the Theonella sp.,[18] and cylindramide (3.16) from *Halichondria cylindrata.*[19] Both show cytotoxic activity, against leukemic cells and melanoma cells respectively.



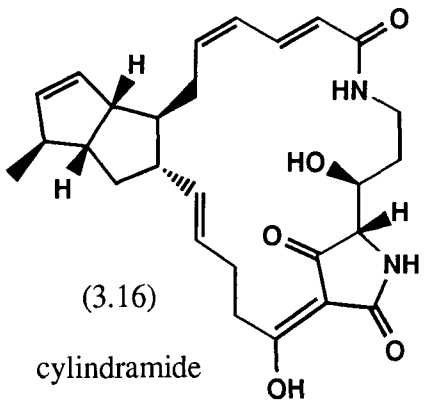


Figure 3-9 Marine tetramic acids

3.1.3 Vining's hypothesis for the biosynthesis of tenellin

As discussed in Chapter 1 the biosynthesis of the pyridone ring of tenellin (3.18) necessitates an intriguing rearrangement. The pyridone ring is derived from the terminal

acetate of the polyketide chain and a moiety resulting from an intramolecular rearrangement of a phenylpropanoid (phenylalanine derived) unit. The intramolecular nature of this rearrangement was elegantly demonstrated by Leete *et al.*, by a feeding experiment with DL-[1,3-$^{13}C_2$]-phenylalanine (3.17). Tenellin was found to be enriched in positions 4 and 5 of the ring, with clear ^{13}C-^{13}C coupling observed. An intermolecular migration of the carbonyl group would not exhibit this coupling.

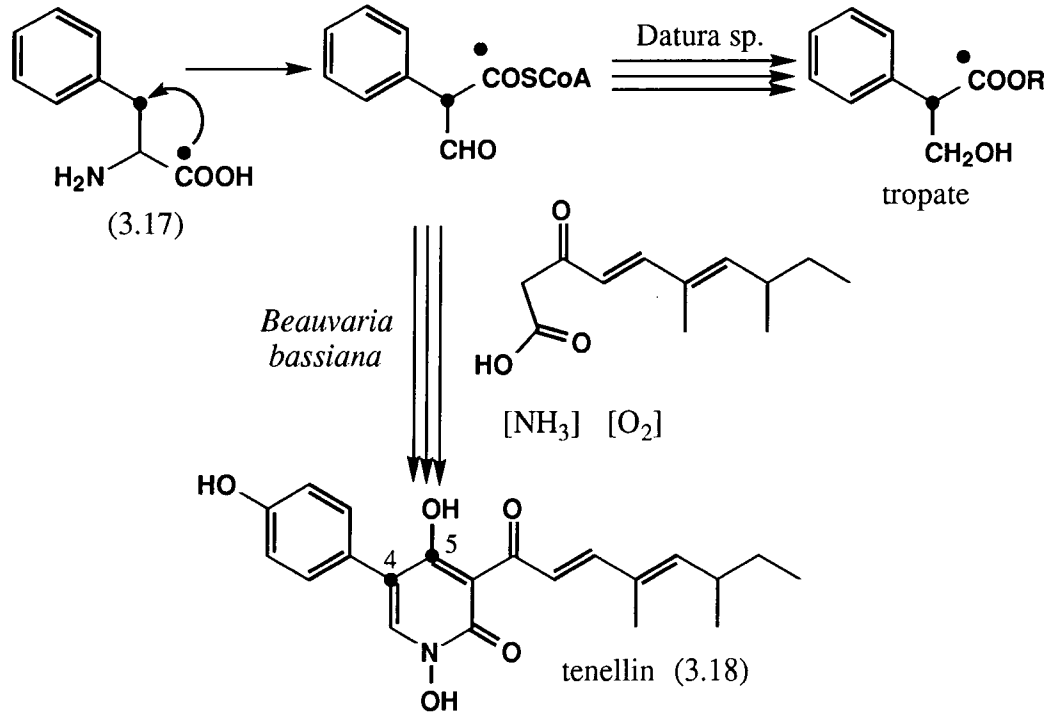


Figure 3-10 Leete's hypothesis for the biosynthesis of tenellin

A similar type of rearrangement had previously been observed in the biosynthesis of tropic acid, the acid moiety of the Datura alkaloids, scopalamine and hyoscyamine. Leete and Vining initially proposed α-formyl phenylacetyl coenzyme A as a plausible intermediate in both biosyntheses.[20]

However Vining's subsequent investigations with L-[^{15}N]-phenylalanine strongly indicated that phenylalanine was incorporated with little or no loss of nitrogen into tenellin. The extent of the incorporation of nitrogen-15 was comparable with that obtained for carbon-13 labelled phenylalanines. Vining also investigated the possibility that tyrosine might be a more direct precursor to tenellin. L-[U-^{14}C]-Tyrosine was shown to have a sharply reduced incorporation (1.6%) compared to L-[β-^{14}C]-phenylalanine (8.4%), suggesting that although tenellin possesses a *para*-hydroxy group, phenylalanine

is the more direct precursor. Their hypothesis required a *para*-hydroxylation step after the polyketide-phenylpropanoid condensation step and had circumstantial support in that such hydroxylations readily occur in fungi. These results and the widespread occurrence of the tetramic acids in fungi, led Vining to propose the alternative process for the observed rearrangement. Phenylalanine initially undergoes a condensation with the fully assembled polyketide (3.19) resulting in an intermediate tetramic acid (3.20). Rearomatization of the methylenequinoid (3.21) formed by *para*-hydroxylation, promotes ring expansion to the pyridone ring of tenellin.[21]

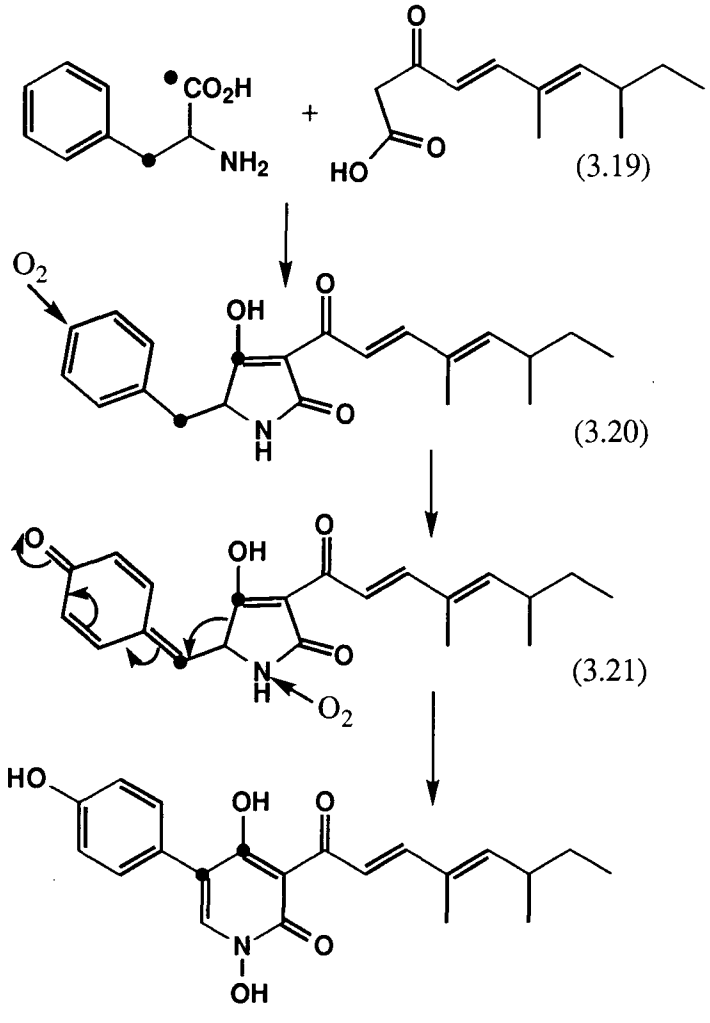


Figure 3-11 Vining's hypothesis for the biosynthesis of tenellin

Vining's hypothesis is attractive as acyl tetramic acids are ubiquitous in the fungi, and numerous biosynthetic investigations have confirmed their derivation from the condensation of amino acids with the terminal residue of a polyketide moiety.[1] The mechanism proposed could also account for the biosynthesis of ilicicolin H, which has similarly been shown to arise from phenylalanine and a polyketide chain. However the

related pyridones, funiculosin and leporin A, lack a *para*-phenol group. Clearly ring expansion cannot be the result of rearomatisation of an intermediate methylenequinoid in these cases. An alternative mechanism may involve the generation of a free radical at the C-5 methylene carbon. For tenellin, bassianin, ilicicolin and leporin A, this would be a relatively stable benzyl radical. This could involve a cytochrome P-450 enzyme, which mediate some rearrangement reactions as well as hydroxylations, oxidations, desaturations and bond cleavage reactions.

3.1.4 Rearrangment reactions of cytochrome-P-450 enzymes

Cytochrome P-450 enzymes catalyse the oxidations of many diverse groups of compounds. Some P-450s will only oxidise a specific group of substrates, but others (e.g. the inducible P-450s utilised in xenobiotic oxidation) appear to have broad range activity. Within a single organism there may be more than thirty genes coding for these enzymes.[22] Enzymes of this type possess a heme prosthetic group, and an axially bound cysteine residue.[23]

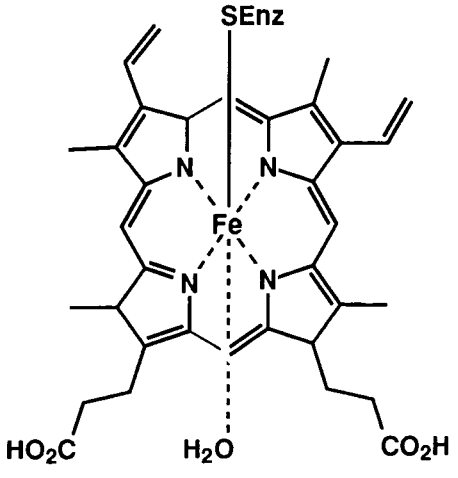


Figure 3-12 Structure of the heme prosthetic group in cytochrome P-450 enzymes

The following stoichiometry is characteristic of monooxygenases.

$$NAD(P)H + O_2 + RH \rightarrow NAD(P)^+ + H_2O + ROH$$

The mechanism may be thought of as two stages: oxygen activation and substrate oxidation.

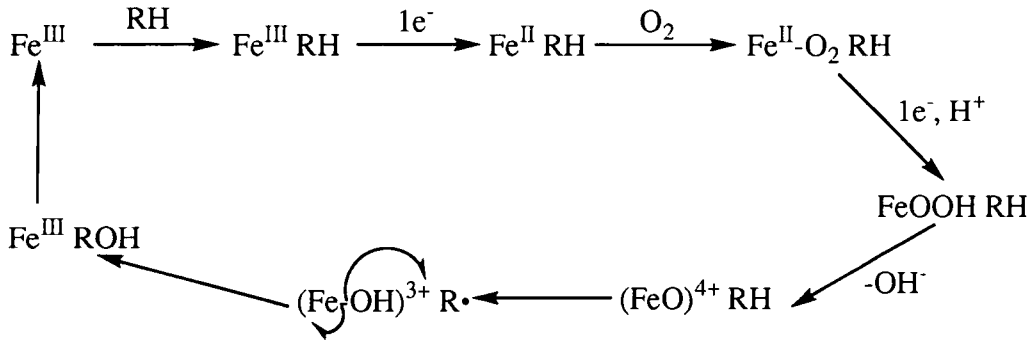


Figure 3-13 Mechanism of cytochrome P-450 action

The substrate is bound near the site of oxygen binding, and the cycle proceeds with reduction of iron (III) followed by oxygen binding. The following three steps are putative, deduced from model work and inference. The $(FeO)^{3+}$ radical then abstracts hydrogen from the substrate, to generate a substrate radical, followed by quenching of the substrate radical by an hydroxyl radical from the iron complex. This process has been termed oxygen rebound, and is characteristic of P-450 mechanisms.

The product does not always appear as a simple alcohol, as rearrangement of intermediate radicals may also occur.[24] Moreover unusual reactions such as dehydrogenation, ring expansion,[25] N-hydroxylation, exchange of protons with solvent may occur, all dependent on the substrate presented to the enzyme. Hakamatsuka *et al.* have demonstrated that many reactions in the biosynthesis of natural products can be reasonably explained by P-450 reactions associated with migration or bond cleavage.[26] For example the rearrangement of flavanone to isoflavone which involves a 1,2 aryl migration[27] has been unequivocally shown to involve a cytochrome P-450 enzyme, and a radical mechnism has been proposed (Figure 3-14).

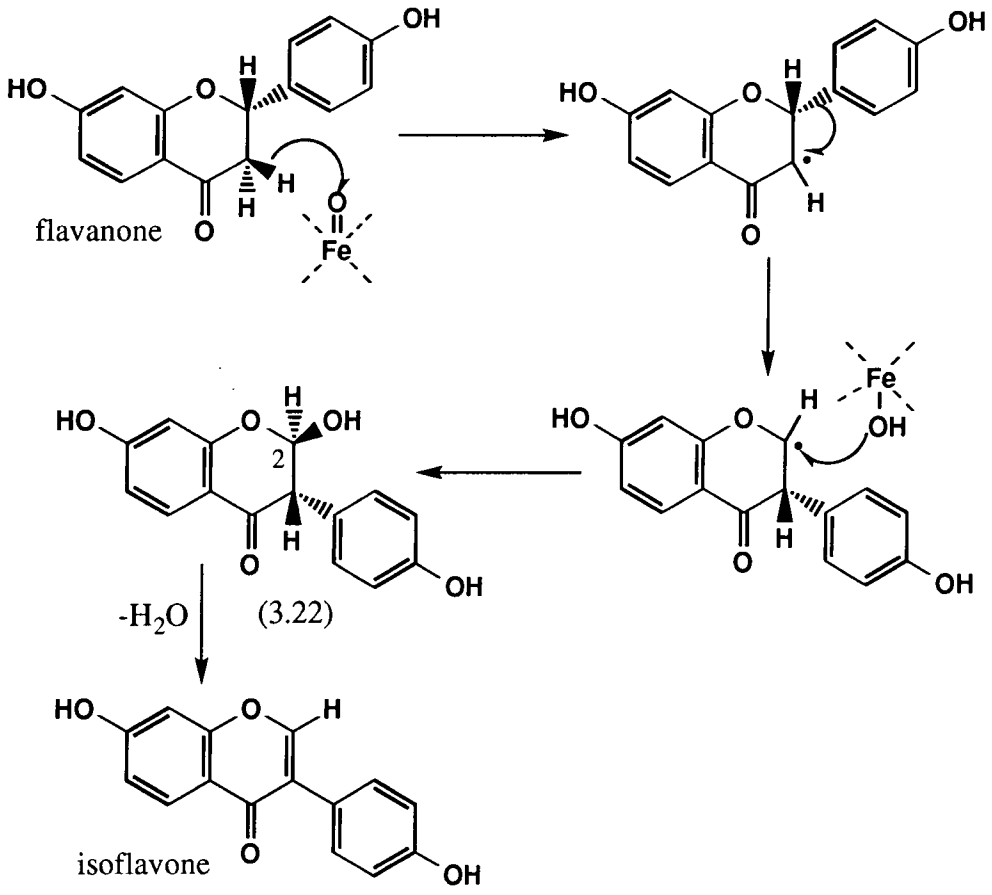


Figure 3-14 Proposed mechanism for the P-450 catalysed rearrangement of flavanone to isoflavone

The precise details of the mechanism of this rearrangement are still the subject of some debate and modified mechanisms have been suggested by Crombie and Whiting which also take account of the stabilising effect of the para-hydroxyl of the aryl migrating group.[28] However intermediate (3.22) was identified in a cell free study and became labelled with oxygen-18 (~30%) when the reaction was conducted under $^{18}O_2$.[29]

In an extension of this hypothesis Cox[30] proposed a plausible cytochrome P-450 mediated radical mechanism for tenellin biosynthesis proceeding *via* a tetramic acid intermediate shown below. However this mechanism is not without difficulties as investigations with phenylalanine labelled with deuterium at the α-position did not result in the labelling of tenellin with deuterium at carbon-5 of the pyridone ring. This is discussed in Chapter 4.

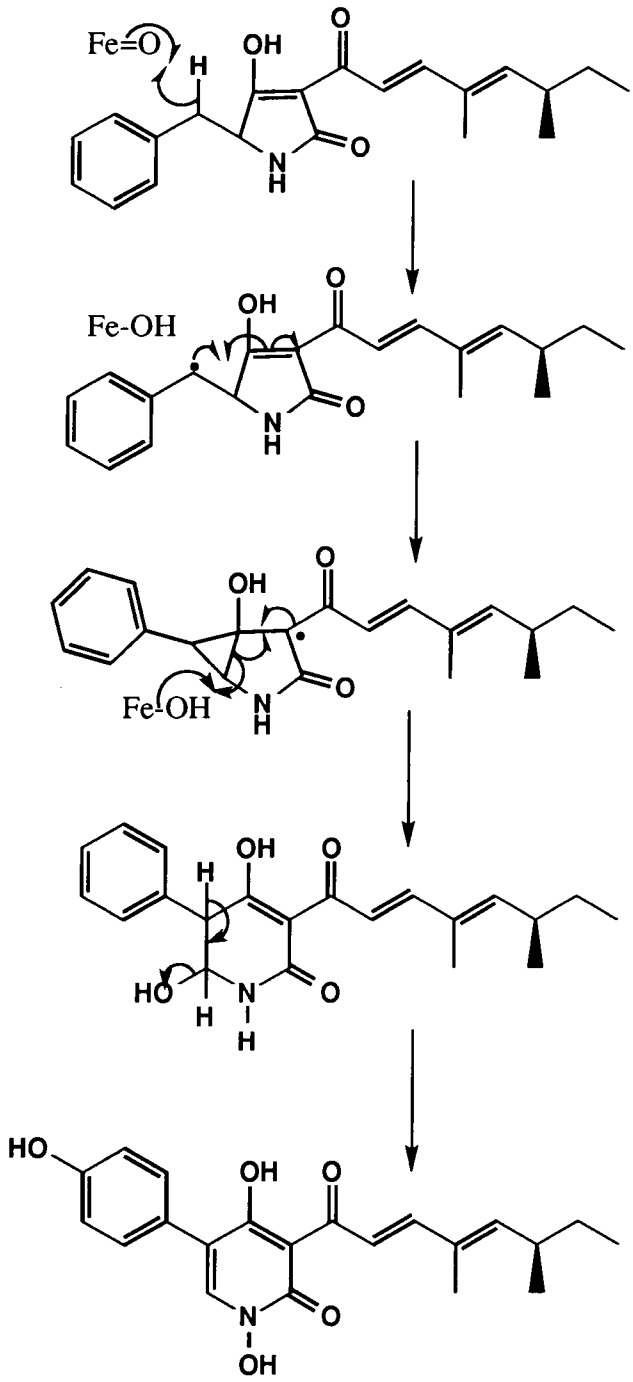
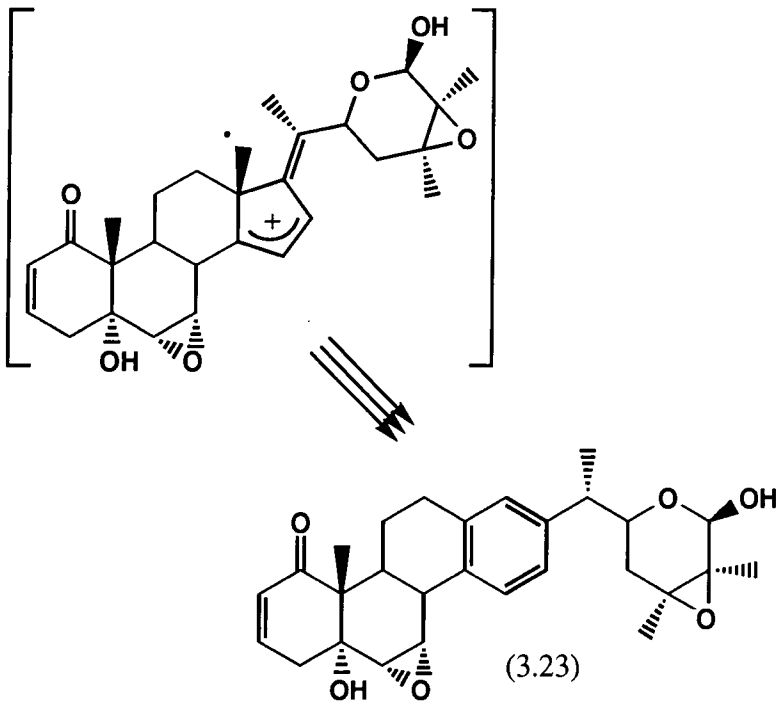


Figure 3-15 Possible P-450 catalysed mechanism for rearrangement of putative tetramic acid intermediate in tenellin biosynthesis

Recently it has been suggested that the biogenenesis of the unique ring-D-aromatic phytosteroids (3.23) from *Nicandra physaloides* involves a ring expansion of a five membered ring-D *via* a cytochrome P-450 generated radical on the methyl group.[31]



(3.23)

Figure 3-16 Proposed ring expansion in biosynthesis of the phytosteroids

A key objective of this present study was to investigate the intermediacy of Vining's putative tetramic acid (3.20) in tenellin biosynthesis. Accordingly it was synthesised bearing isotopic labels, for feeding experiments to *Beauvaria bassiana*. It was anticipated that the synthetic material could also be used as a reference for HPLC and LCMS analysis of the cultures to try to identify its presence in the broth.

3.2 Synthesis of Vining's Proposed Intermediate

3.2.1 Synthetic routes to acyltetramic acids

The majority of the naturally occuring tetramic acids that have been isolated exhibit biological activity, and the syntheses of such compounds has attracted a great deal of interest. Within this group of natural products there are members which are potent antibiotics and antiviral agents, members which exhibit antiulcerative properties, cytotoxicity, mycotoxicity, inhibition of tumours, fungicidal action, as well as those that are responsible for the pigmentation of certain molds and sponges.[32]

3-Acyltetramic acids are acidic having pK_a values in the range 3.0-3.5.[33] Analysis using proton NMR indicates that these systems are completely enolized, and that they exist in several tautomeric forms. The tautomeric behaviour can be explained in terms of two pairs of rapidly interconverting internal tautomers.

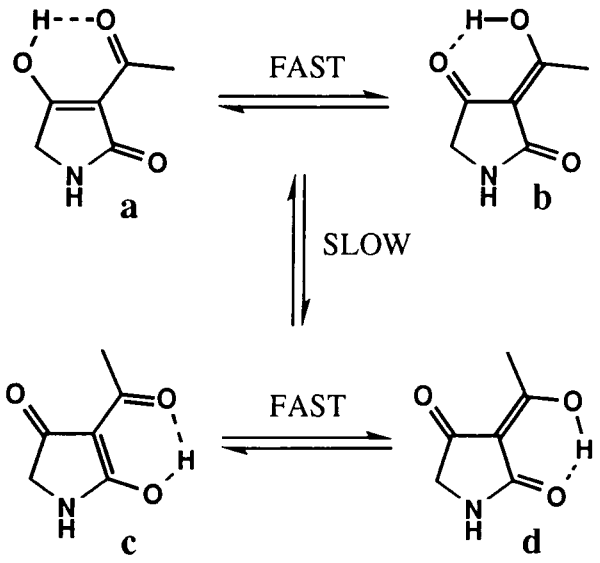


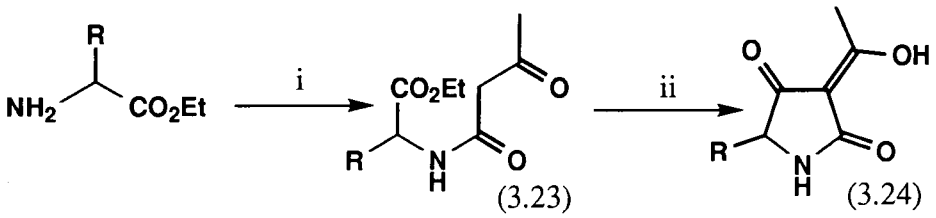
Figure 3-17 Tautomeric behaviour of 3-acyl tetramic acids

Within each pair interconvertion is rapid, arising from proton transfer along the intramolecular hydrogen bond. Interconversion between the pairs (external tautomers) is slow and can be detected on the NMR time scale, and the population ratio calculated. The observed chemical shifts and coupling constants represent the weighted population averages of the corresponding internal tautomers.[34] The enolic forms b and d predominate, as shown by the predominance of the lower frequency carbon-6 enolic

carbon resonance. The *exo*-enol d is the main tautomeric form as the higher frequency C-2 hydrogen bonded carbonyl signal predominates.

The polarity of acyl tetramic acids renders them very difficult to purifiy. For this reason it is expedient to construct the 2,4-dioxopyrrolidine ring at a late stage in the synthesis. A convergent synthetic strategy is also attractive as it restricts the number of synthetic steps necessary with the isotopically labelled precursors, and reduces loss of overall yield. Clearly it is also desirable to be able to construct enantiomerically pure tetramic acids, which can be achieved be using the naturally occuring amino acids in the construction of the ring.

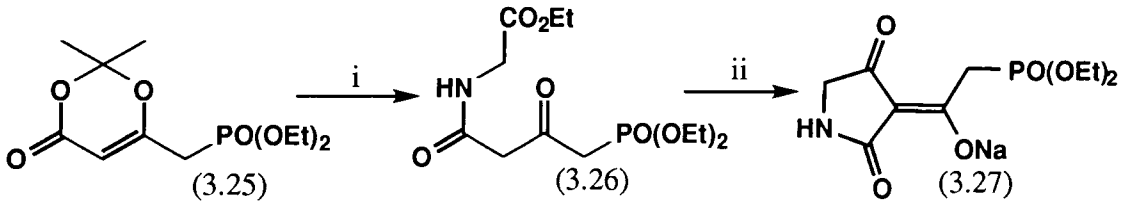
Many stategies have been applied to the synthesis of tetramic acids, and that most widely adopted for the construction of the tetramic acid ring of 3-acyltetramic acids is Lacey's modified Dieckmann cyclisation.[32] The initial method involved the condensation of an α-aminoester with diketene ($(CH_2CO)_2$) to give the N-acetoacetyl-α-aminoester (3.23), which on treatment with sodium ethoxide was cyclized to 3-acyltetramic acid (3.24).[35]



(i) Diketene, (ii) NaOMe

Figure 3-18 Lacey's modified Dieckmann cyclisation

The advantage of this method is its flexibility, enabling preparation of 3-acyltetramic acids with a diversity of substituents, although a potential drawback is that the use of basic conditions may lead to racemisation of vunerable stereogenic centers. The more complex the sidechain, the more problematical it is to construct the β-keto amide intermediate, and decomposition of substrates often results in capricious yeilds. Boeckman[36] circumvented this problem by the synthesis of a tetramic acid nucleus with the 3-acyl group funtionalised with a phosphonate group for further elaboration with aldehydes. Ring opening of 2,2-dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one (3.25) with ethyl glycinate, followed by Dieckman cyclisation of the phophonoacetoacetyl-α-amino ester (3.26) gave rise to the sodium salt of the tetramic acid.



(i) $NH_2CH_2CO_2Et$, PyH^+Tos^-, (ii) NaOMe.

Figure 3-19 Boeckman's tetramic acid synthesis

This tetramic acid phoshonate reagent (3.27) was used by both DeShong and Boeckman[37] in their syntheses of tirandamycin (3.28). Wadsworth-Emmons olefination of the tetramic acid with aldehydes requires protection of the ring nitrogen, and the 2,4-dimethoxybenzyl group was selected by Schlessinger *et al.* for its ease of removal under acid conditions in their total synthesis of tirandamycin.[38]

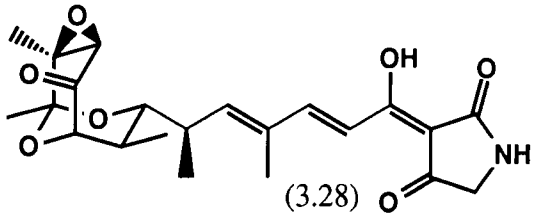
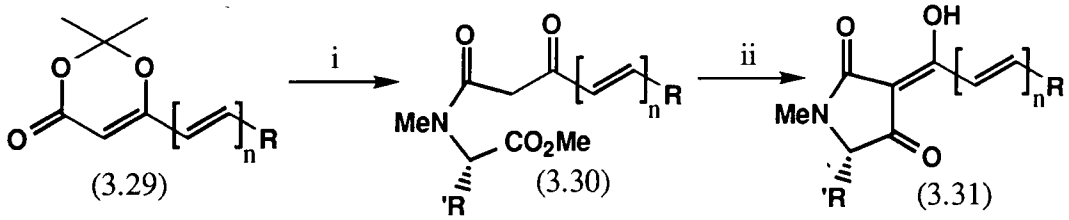


Figure 3-20 Tirandamycin

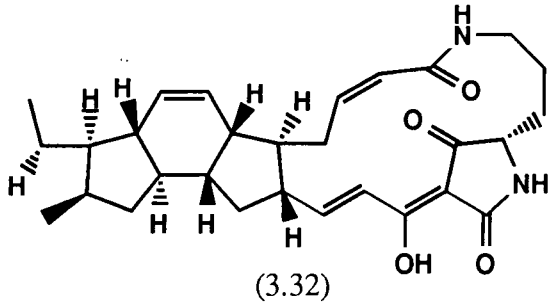
An alternative to chain extension of phosphonoacetyl tetramic acid derivatives for the synthesis of 3-(poly)enoyltetramic acids (3.31) has been developed by Jones.[39] 2,2,6-Trimethyl-1,3-dioxen-4-one (acetone-diketene adduct) was converted *via* the alkylchloride to the 6-phosphonomethyl derivative (3.25). Wadsworth-Emmons olefination with saturated and unsaturated aldehydes gave the (poly)ene dioxinones (3.29) which could be reacted with the methyl ester of amino acids to give the β-ketoamides (3.30), and cyclised to tetramic acids under Dieckmann cyclisation conditions.



(i) R'CH(NMeH)(CO_2Me), ptps, toluene (ii) K^tOBu, ^tBuOH.

Figure 3-21 Jones' methodology using polyene dioxones

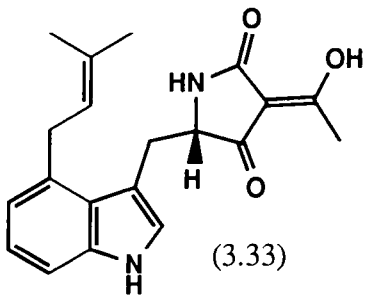
The advantage of this strategy is that it is a mild method which generates the highly polar and acidic heterocycle in the final step. This sequence has been used in the landmark synthesis of the macrocyclic enoyltetramic acid, ikarugamycin (3.32).[40]



(3.32)

Figure 3-22 Ikarugamycin

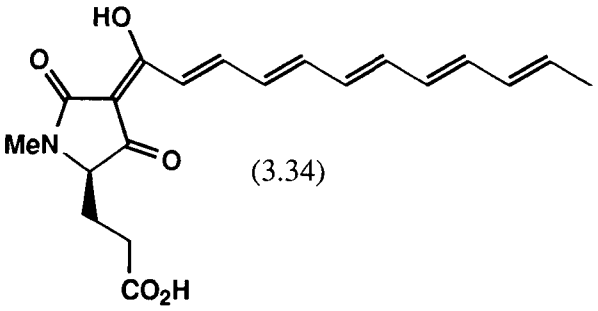
Despite the problems associated with the base induced cyclisation, the Lacey-Dieckmann method has been the principal method for the preparation of chiral tetramic acids. This is because it lends itself so well to the use of chiral amino acids to introduce the asymmetry. Lacey-Dieckman cyclisations have been carefully evaluated to establish the degree of racemisation under various conditions. Studies have shown that racemisation does occur to a degree dependent on base concentration and reaction time.[41] Incredibly, however, β-cyclopiasonic acid (3.33) has been prepared in optically pure form, through exposure of the β-keto amide intermediate to methoxide in refluxing benzene for ten hours![42]



(3.33)

Figure 3-23 β-Cyclopiasonic acid

Enantio-selective Lacey-Dieckmann cyclisations are usually accomplished under mild conditions; potassium tert-butoxide in tert-butanol at room temperature for minimal reaction times. Such conditions were used in the total synthesis of fuligorubin A (3.34) by Ley,[43] and that of ikarugamycin (3.32) by Boeckman and Paquette.[40]



(3.34)

Figure 3-24 Fuligorubin

The optical rotation of the synthetic material was virtually identical to that of the natural samples. It was argued that the steric bulk of the base may be a significant factor as the stereogenic center is more hindered in comparison to the active methylene group.

3.2.2 Synthesis of the proposed tetramic acid in tenellin biosynthesis

The synthetic strategy for the construction of the putative acyl tetramic acid intermediate in tenellin biosynthesis was based on the methodology of Jones, and first attempted by Cox.[30] The disconnections are shown below.

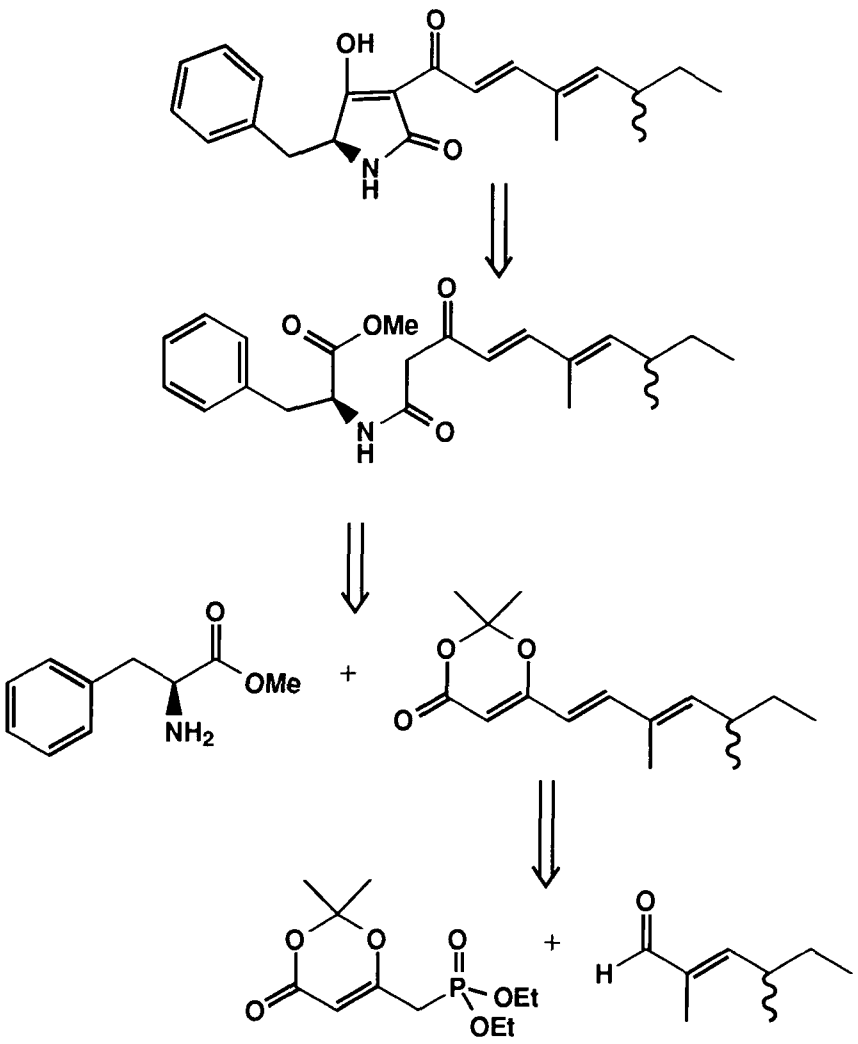
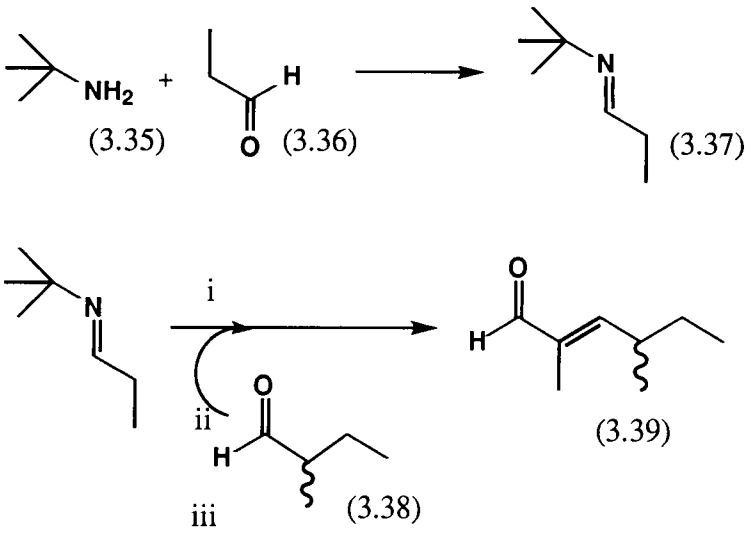


Figure 3-25 Synthetic strategy for the construction of the putative tetramic acid intermediate in tenellin biosynthesis

3.2.3 Synthesis of (E)-2,4-dimethylhex-2-enal



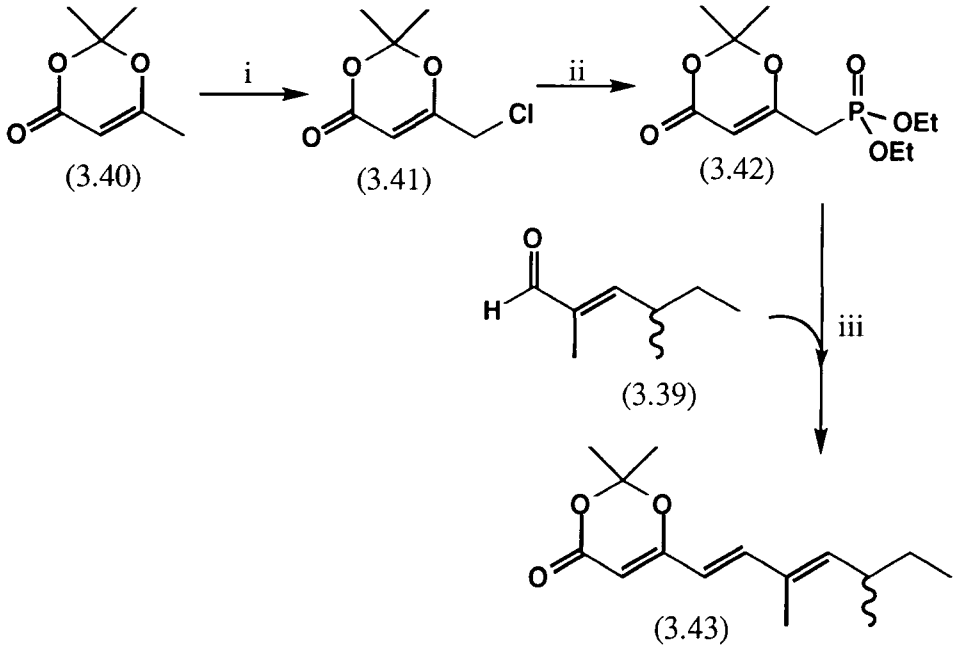
(i) BuLi, -78°C, (ii) -78°C to 0°C, (iii) $(CO_2H)_2$, H_2O, 25°C.

Figure 3-26 Synthesis of (E)-2,4-dimethylhex-2-enal

(E)-2,4-Dimethylhex-2-enal (3.39) was prepared following literature methods reported in the synthesis of racemic tenellin by Williams and Sit[44] using the directed aldol condensation first described by Wittig.[45] Propionaldehyde (3.36) was condensed with tert-butylamine (3.35) at 0°C to give (1,1-dimethyl-N-propylidene) ethylamine (3.37), and left to stand over potassium hydroxide pellets, enabling the separation of the aqueous layer. It was necessary to distil the product three times over potassium hydroxide pellets to obtain it free from tert-butylamine and water.[46] Decomposition of the product stored under nitrogen at 0°C occured within a few weeks, and therefore it was always redistilled prior to use.

The imine (3.37) was lithiated using butyllithium at -78°C, and condensed with 2-methylbutyraldehyde (3.38). The lithiated aldimine adduct could be decomposed with an aqueous slurry of oxalic acid to the thermodynamically more stable *trans* olefin, (E)-2,4-dimethylhex-2-enal (3.39). This was relatively stable to storage at -20°C under nitrogen if first purified over silica. However further purification was necessary prior to use.

3.2.4 Synthesis of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one.



(3.40) (3.41) (3.42) (3.39) (3.43)

(i) LDA, THF, -78°C, C_2Cl_6, -50°C, (ii) $(EtO)_2PHO$, K^{t}OBu, DMF,
(iii) LHMDS, THF.

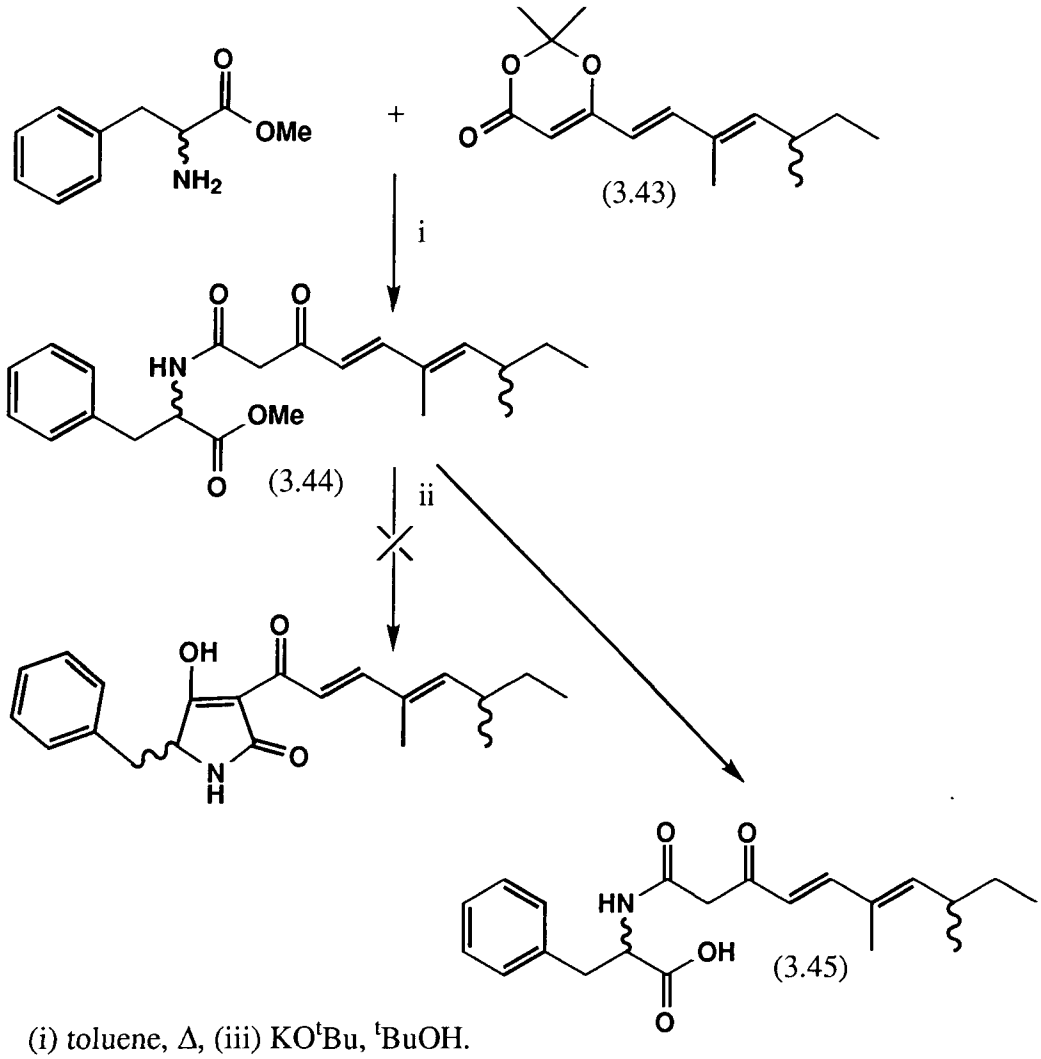
Figure 3-27 Synthesis of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one

Thermolysis of the acetone diketene adduct (3.40) to β-acetyl ketene[47] in the presence of a nucleophile such as an alcohol or amine results in the formation of β-keto esters and amides. The diethyl phosphonate is a more versatile activated β-keto ester or amide equivalent. It was first prepared by Boeckman and Thomas[36] for use in the synthesis of tetramic acids, permitting Wadsworth-Emmons olefination under mild conditions.

A modification of this initial synthesis was followed[48] involving lithiating freshly distilled acetone diketene adduct (3.40) in tetrahydrofuran at -78°C, and quenching the yellow precipitate thus formed with a solution of hexachloroethane in tetrahydrofuran. To obtain high yields it was necessary to transfer the precipitate *via* Teflon tubing in a dropwise manner to the hexachloroethane solution at -50°C. The chlorinated acetone diketene adduct (3.41) could be purified over silica. This was treated with a solution of potassium diethylphosphite in dimethylformamide to give the Wadsworth-Emmons reagent (3.42). Excess diethyl phosphite was removed by distillation, and the product could be purified by flash chromatography and stored at 0°C under nitrogen.

Wittig olefination of this phosphonate (3.42) was accomplished by first lithiating the phosphonate using lithium hexamethyldisilazide[39] in tetrahydrofuran at 0°C. The blood red solution was quenched by the addition of 2,4-dimethylhex-2-enal (3.39) to give the all-E triene. The product was stable to storage under nitrogen at 0°C after purification over silica. This key intermediate dioxenone (3.43) could be thermolysed and trapped with various protected amino acids to giving β-keto amide intermediates.

3.2.5 Attempted preparation of acyl tetramic acids using phenylalanine methyl ester



(i) toluene, Δ, (iii) KO^{t}Bu, ^{t}BuOH.

Figure 3-28 Attempted preparation of acyl tetramic acids using phenylalanine methyl ester

When the dioxenone (3.43) was treated with one equivalent of phenylalanine methyl ester and heated in refluxing toluene the β-keto amide (3.44) was formed in high yield. After purification over silica, Dieckmann cyclisation was attempted using freshly sublimed

potassium tert-butoxide in tert-butanol for the relatively short period of ten minutes. These mild conditions were selected for the cyclisation step, as the higher temperature required for deprotenation by sodium methoxide in methanol resulted in large scale decomposition and racemisation.

In the event the orange oil obtained, after submitting the β-keto ester (3.44) to these conditions, was found to be the corresponding acid (3.45), formed by the hydrolysis of the methyl ester. There was no evidence that cyclisation to the tetramic acid had occured. The results could be rationalised if deprotonation was occurring at the amide nitrogen. Delocalisation of the negative charge onto the amide oxygen could be followed by cyclisation by nucleophilic displacement of the methyl ester. The heterocycle (3.46) would be readily opened by nucleophilic attack of water during the acidic work-up, resulting in the formation of the acid.

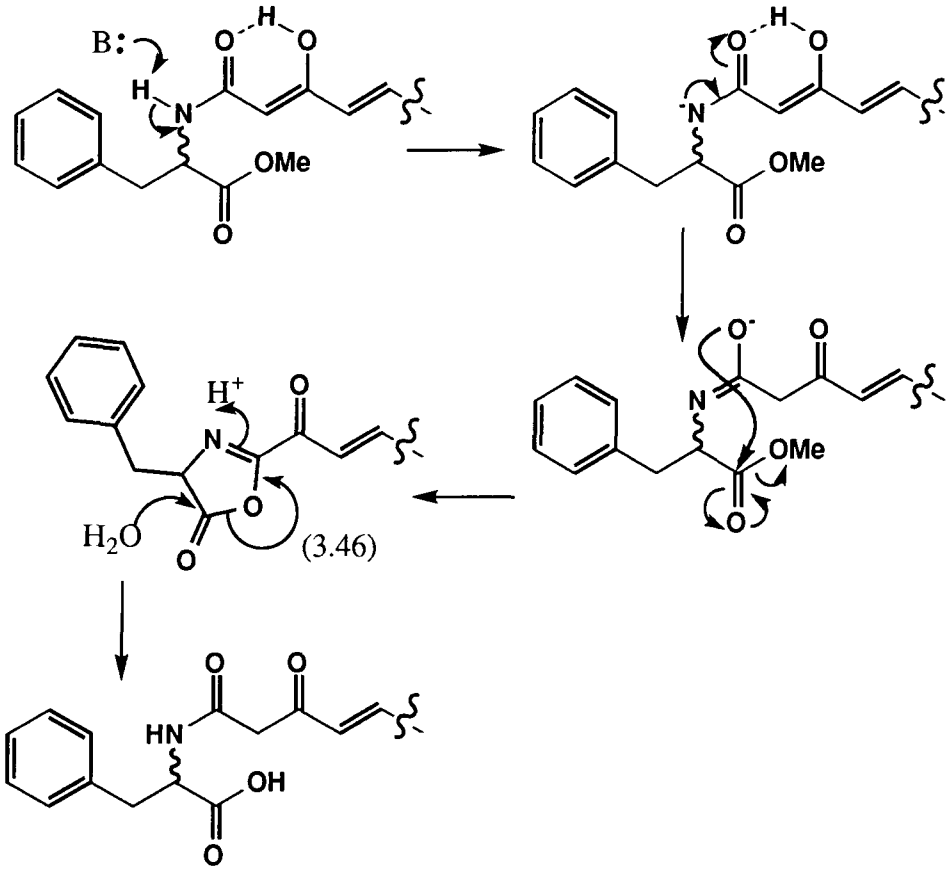


Figure 3-29 Rationale for the failed cyclisation step

3.2.6 The requirement for a protecting group

Problems with the cyclisation to generate the acyltetramic acid were reported in the total synthesis of malonomycin (3.57), depending on the nature of the acyl substituent. An alternative strategy was adopted in this case, involving coupling of the peptide side chain to the pyrolidine-2,4-dione ring after cyclisation.[49] However, there was precedent for such cyclisations preceding smoothly with less complex short chain tetramic acids (both in the literature[50] and in the work of our research group) without necessitating the protection of the nitrogen of the amide. In our case it was evident that protection of the nitrogen was necessary. In the reported total syntheses of more complex tetramic acids such as tirandamycin and ikarugamycin, N-protection has been generally used.[51] In such syntheses the acid labile 2,4-dimethoxybenzyl group is a common choice for protection of the amine. Schlessinger[52] first described the use of this protecting group in tetramic acid syntheses. It has the advantage of being readily removed by either mild oxidative or acidic conditions. Brief treatment with neat trifluoroacetic acid at room temperature has proven effective for such deprotections.[37]

In the light of the literature precedence it seemed prudent to repeat our synthesis with the N-protected phenylalanine methyl ester.

3.2.7 Synthesis of N-(2,4-dimethoxybenzyl) phenylalanine methyl ester

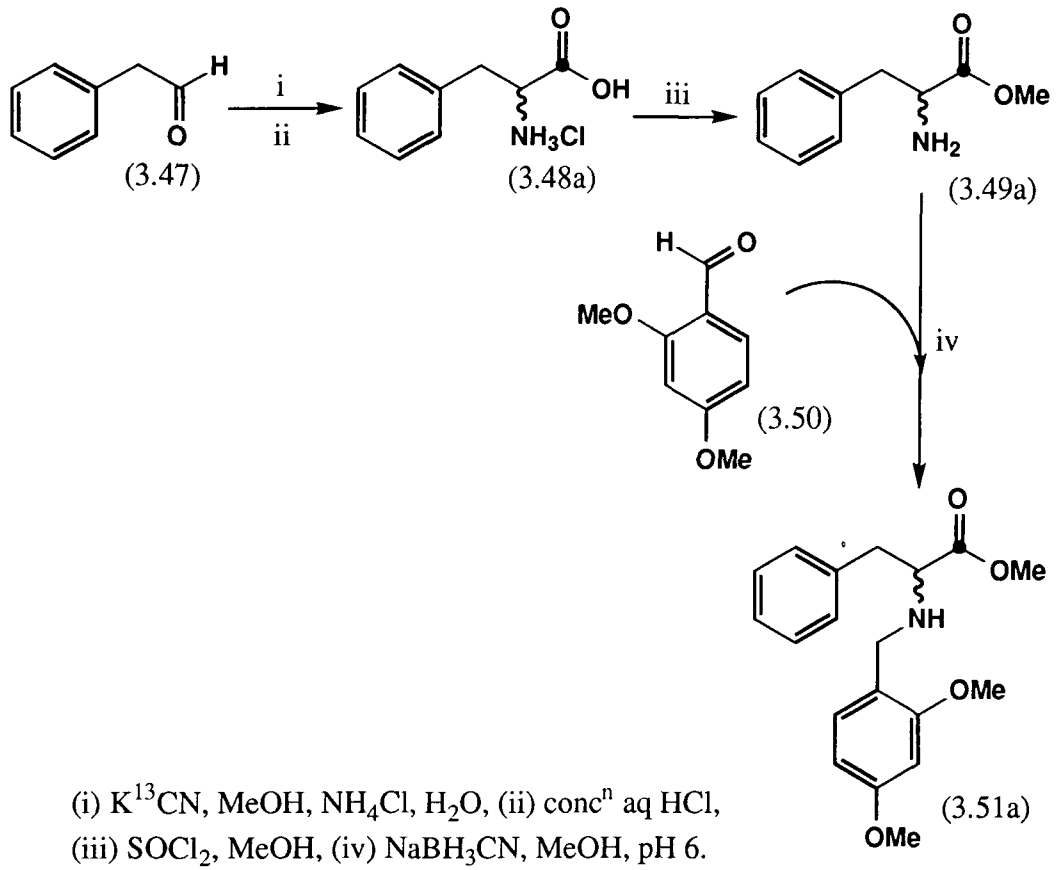


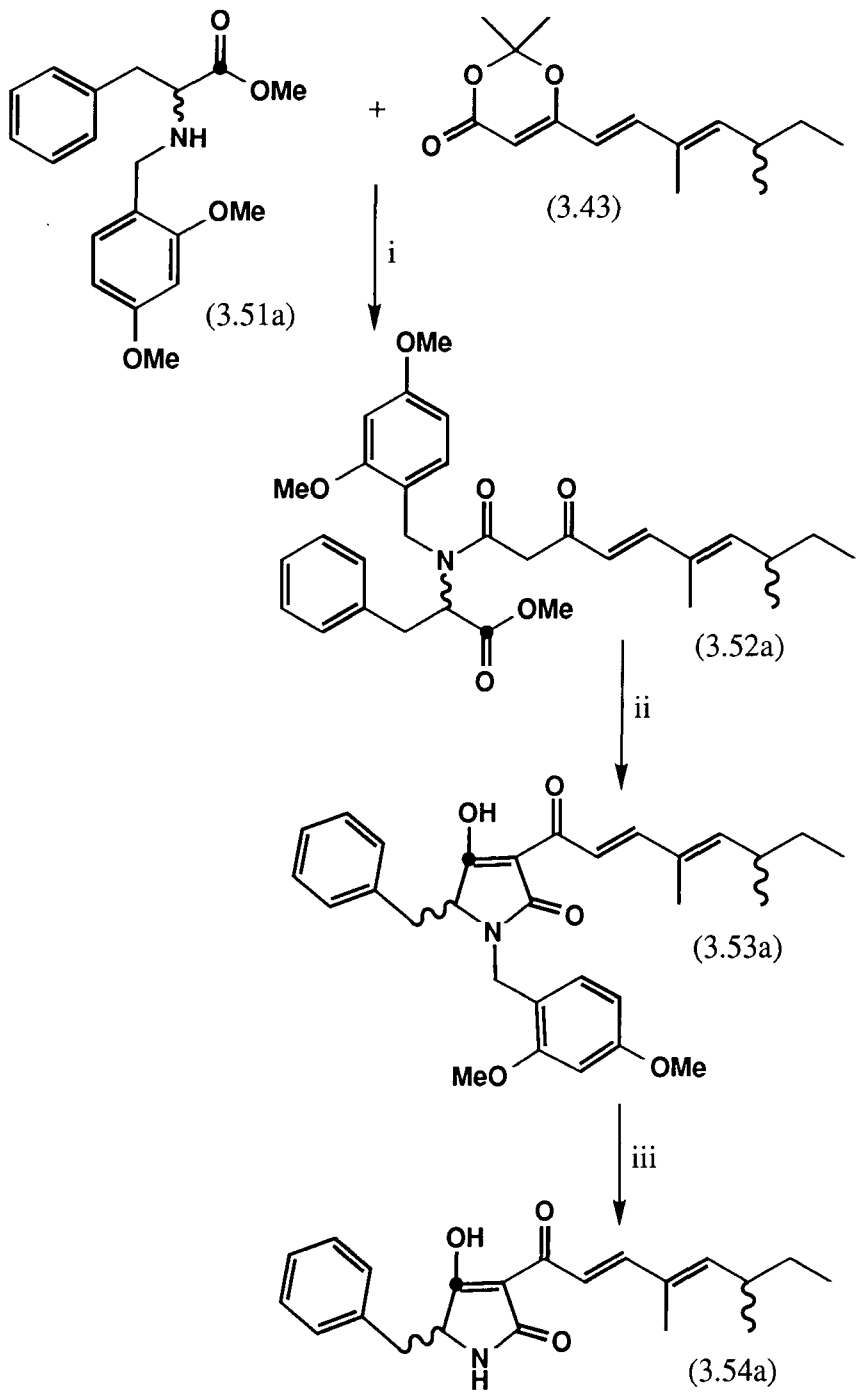
Figure 3-30 Synthesis of N-(2,4-dimethoxybenzyl) phenylalanine methyl ester

The synthesis of [$^2H_5$]-labelled protected amino acid started from commercially available [$^2H_5$-*aryl*]-(L)-phenylalanine. [1-^{13}C]-Phenylalanine was synthesised by a modified Strecker synthesis[53] as it enabled the introduction of a ^{13}C label at the carboxyl group. Phenylacetaldehyde (3.47) was treated with [^{13}C]-potassium cyanide and ammonium chloride in methanol. The [1-^{13}C]-2-amino-3-phenylpropionitrile intermediate required refluxing in concentrated aqueous hydrochloric acid for two days to complete hydrolysis of the nitrile, giving [1-^{13}C]-phenylalanine hydrochloride (3.48a). The amino acid could be methylated using an excess of thionyl chloride in methanol,[54] and then treatment with sodium bicarbonate solution released the free amine (3.49a), which was used immediately as it was prone to polymerisation on standing.

Conversion of (3.49) to its N-(2,4-dimethoxybenzyl)-derivative (3.51a) was achieved by treatment of phenylalanine methyl ester with 2,4-dimethoxybenzaldehyde (3.50) in methanol at pH 6 and at ambient temperature. Addition of sodium cyanoborohydride to the reaction mixture effected the selective reduction of the imine formed. Impurities of

2,4-dimethoxybenzylalcohol were removed by distillation and the residue purified by silica gel column chromatography to give the protected amino acid as a colourless viscous oil.

3.2.8 Preparation of acyl tetramic acids using N-(2,4-dimethoxybenzyl) phenylalanine methyl ester



(i) toluene, ptps, reflux, (ii) KO^{t}Bu, ^{t}BuOH, (iii) CF_3COOH.

Figure 3-31 Preparation of acyl tetramic acids using N-(2,4-dimethoxybenzyl) phenylalanine methyl ester

First attempts at the condensation of N-(2,4-dimethoxybenzyl) phenylalanine methyl ester (3.51a) with 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (3.43) by thermolysis in refluxing toluene were unsuccessful. Unreacted N-(2,4-dimethoxybenzyl) phenylalanine methyl ester and a mixture of breakdown products of the protected ketene could be recovered from the reaction mixture. Although uncatalysed thermolysis is the preferred method of reaction of nucleophiles with dioxenones, mild acid catalysts such as pyridinium *para*-toluene sulphonate (ptps) may be used to assist the opening of the acetal as described by Jones *et al.* for the synthesis of 3-polyenoyltetramic acids.[39] It was rationalised that as the nucleophilicity of the amine was now reduced, due to the bulky protecting group, use of a mild catalyst might assist the reaction to proceed in the desired manner. It was encouraging to find that this indeed was the case, and the N-protected β-keto amide (3.52a) was a major product of the acid catalysed thermolysis. Although there was a loss in yield compared with the unprotected phenylalanine methyl ester, the recovery was perfectly acceptable at approximately 50%, particularly as the final two steps were anticipated to be high yielding reactions.

It was gratifying when the Dieckman cyclisation step using potassium *tert*-butoxide in *tert*-butanol proceeded smoothly giving the [4-^{13}C]-N-(2,4-dimethoxybenzyl)-protected tetramic acid (3.53a) in excellent yield. ^{1}H and ^{13}C NMR showed clearly the two slowly interconverting external tautomers in ratio of 84 : 16. Ring carbon-5 and the attached proton showed a particularly large difference in chemical shift between the tautomers in the ^{13}C and ^{1}H NMR spectra respectively, as is commonly observed for tetramic acids.[55] Deprotection was readily accomplished in under five minutes using the standard conditions; neat trifluoroacetic acid at room temperature yielding the target tetramic acid (3.54a). NMR analysis confimed that the product was sufficiently pure to use in subsequent feeding experiments. Again two external tautomers could be seen, this time in the ratio 85 : 15. In the case of the ^{13}C labelled tetramic acid, only two labelled peaks were observed in a ratio of 85 : 15 consistent again with the two tautomers of the acid. There were no labelled impurities.

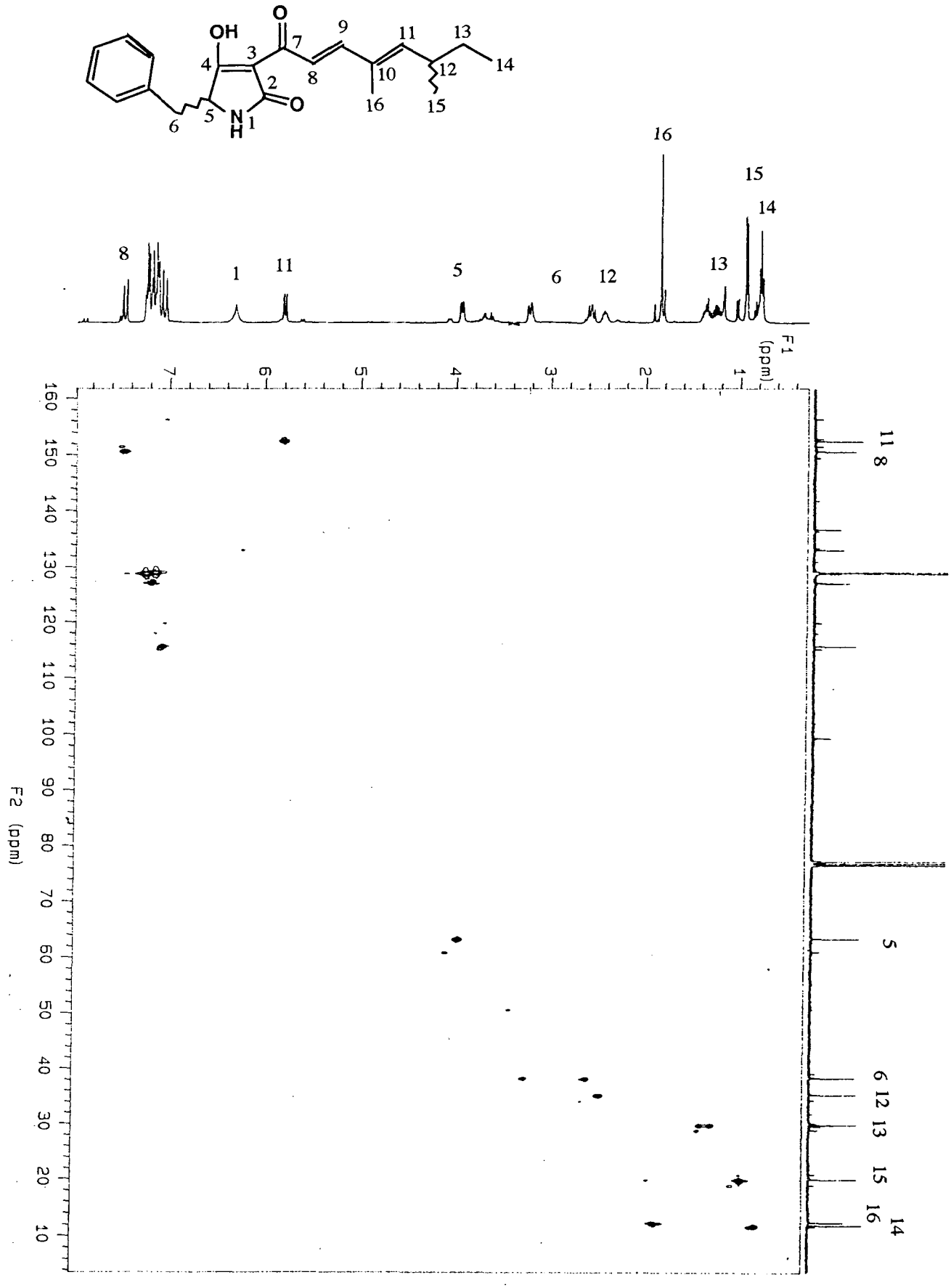
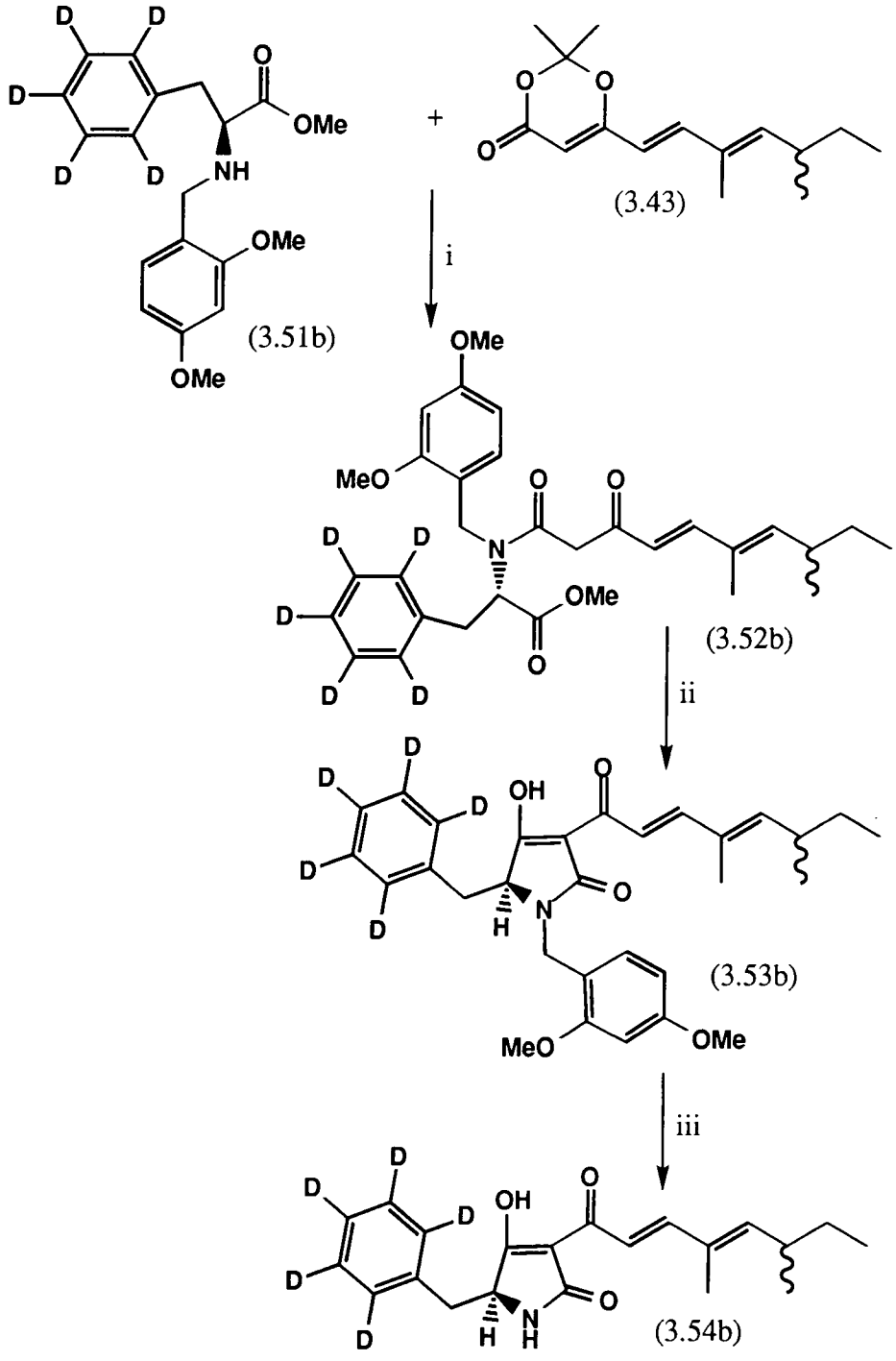


Figure 3-32 HETCOR NMR of unlabelled tetramic acid

After completion of the synthesis of the ^{13}C labelled tetramic acid (3.54a), the same route was exploited to prepare the [$^{2}H_5$]-derivative (3.54b) for further experiments. In this case, with lower cost of deuterium labelled materials, the synthesis was readily amenable to providing a gram of final product; ample for feeding experiments to *Beauvaria bassiana*.



(i) toluene, ptps, reflux, (ii) KO^{t}Bu, ^{t}BuOH, (iii) CF_3COOH.

Figure 3-33 Route to deuterium labelled tetramic acid (3.54b)

[$^2H_5$-aryl]-L-Phenylalanine was used to initiate the synthesis. Reaction time for the cyclisation was kept to a minimum to limit racemisation of the enantiomerically pure centre. A number of attempts were made to establish the enantiomeric purity of the product, using chiral HPLC. However the peaks were too broad to be resolved. An attempt was made to measure the specific rotation of the deuterated tetramic acid, however, optical rotation measurements proved impossible to record due to the deep yellow colour of the solutions in ethanol.

3.3 Biosynthetic Investigations

3.3.1 Feeding of acyl tetramic acids to *Beauvaria bassiana*

Initially the ^{13}C-labelled putative tetramic acid precursor 5(RS)-[4-^{13}C]-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54a) was pulse fed to *Beauvaria bassiana* cultures to a final concentration 2 mM, immediately after tenellin production had commenced. As the labelled compound was insoluble in water it was pulse fed in the minimum volume of ethanol.[56] The cultures developed an uncharacteristic brown colouration, possibly due to the toxicity of ethanol, but a reasonable quantity of tenellin was never the less produced and extracted for analysis. ^{13}C NMR of the crude tenellin showed the presence of a considerable amount of the labelled precursor, and no observable enrichment of carbon-4 of the pyridone ring of tenellin at 173 ppm. The extract was analysed by HPLC, and the large labelled impurity confirmed to co-elute with the synthetically prepared tetramic acid precursor. Preparative HPLC separated the pure tenellin for more accurate analysis, and established that within the sensitivity of the ^{13}C NMR experiment, there was no ^{13}C enrichment, and that the tetramic acid recovered had not been metabolised by the cells.

Such a result, although disappointing was not surprising. Firstly it must be considered that the size and insolubility of the proposed intermediate poses problems to such feeding experiments. In general large, polar molecules are notoriously resistant to crossing cell membranes, and thus incorporation levels were anticipated to be low from the outset. However intact incorporations of a deuterium labelled tetramic acid (3.56) intermediate have been observed into the smaller tetramic acid antibiotic, malonomycin II (3.57),[57] and this clearly provided a nice precedent.

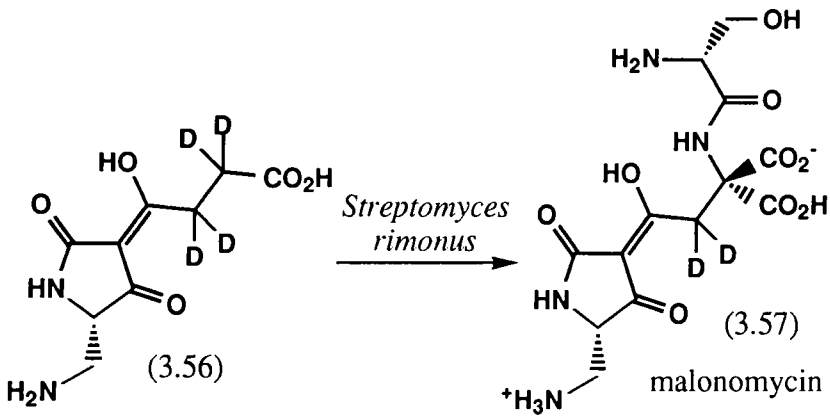


Figure 3-34 Incorporation of tetramic acid precursor into malonomycin

The experiment was repeated with the deuterium labelled precursor, particularly as ^{2}H NMR analysis will increase the sensitivity of the experiment due to the low natural abundance (0.015%) of deuterium.

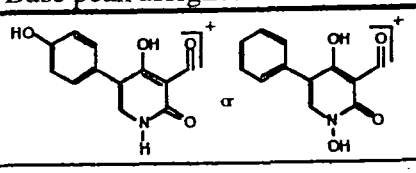
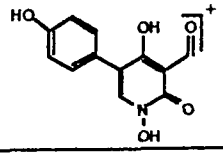
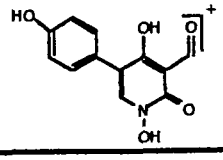
Accordingly 5(S)-[$C_6{}^{2}H_5$-*benzyl*]-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54b) was pulse fed to *Beauvaria bassiana* cultures to a final concentration 1.5 mM. In one experiment the labelled compound was pulse fed in the minimum volume of ethanol, and in a repeat experiment dimethylsulphoxide[58] was used as the solvent. After HPLC purification and ^{2}H NMR analysis of the resultant tenellin no incorporation of the tetramic acid was observable in either case.

Other more violent techniques, such as lysing the cells to release the enzymes for the supposed pathway, and then attempting a biotransformation of our putative intermediate, were considered, but no similar biotransformations have to our knowledge been attempted with *B. bassiana*. A more elegant experiment would be to load the cultures with radioactive ^{14}C-acetate. HPLC analysis combined with ^{14}C-scintillation counting would be a very sensitive probe of any labelled tetramic acid produced. Before attempting such an experiment however, other results (discussed below) indicated that the proposed tetramic acid was an unlikely intermediate in tenellin biosynthesis, and so experiments with this putative intermediate were discontinued.

3.3.2 HPLC-analysis of *Beauvaria Bassiana* cultures

HPLC with UV detection and LCMS were used in attempts to identify the formation of intermediates in the biosynthesis of tenellin. Using LCMS it was possible to speculate on the nature of minor metabolites, the more abundant of which could be purified by preparative HPLC, and analysed by ^{1}H NMR. Furthermore now with the synthetically prepared putative acyl tetramic acid (3.54) as a reference compound, a specific search could be made in the cultures for this metabolite. Pyridones and tetramic acids are notoriously difficult to purify due to their polar nature. However a technique was successfully developed using reverse-phase HPLC with acetonitrile or methanol and water as solvents, acidified with 0.1% trifluoroacetic acid. Without the acid the peaks were very broad and could not be separated. 'Tailing' of the peaks was difficult to overcome, but could be diminished dramatically on an analytical scale by use of a BDS-protected reverse-phase column. In general excellent separations were accomplished in this way.

Tenellin was found to elute at 5.9 minutes. A small shoulder, often resolved into a separate peak, preceded the main peak at 5.6 minutes. This was subsequently shown to be a tautomer of tenellin, the two tautomers being in approximate ratio 1 : 10. If the fractions were collected, and resubmitted to HPLC an identical trace was obtained for each, again each showing the two peaks in the ratio 1 : 10. Thus it was concluded that although tautomerism was a slow process, even allowing separation on the HPLC time scale, the tautomers could equilibrate readily over time. Both peaks were confimed to have identical mass spectra, with an MH^+ of 370. Another peak was frequently observed, eluting earlier at 3.2 minutes. LCMS confirmed this to have a MH^+ of 354. Daughter ion spectra were obtained for each of the peaks. The base peak for tenellin and its tautomer had m/e 246, which could be assigned to a fragment ion $C_{12}H_8NO_5^+$. Its structure is shown in the table below (Figure 3-35). The minor metabolite with MH^+ 354 had a similar fragmentation pattern but each peak had m/e 16 units less than that for tenellin. Its base peak at 230 could then be assigned to an ion $C_{12}H_8NO_4^+$. Thus we conclude that this is the reduced precursor to tenellin, prior to hydroxylation at the nitrogen of the pyridone ring, or prior to hydroxylation on the phenyl ring.

Peak at	M+1	Base Peak	Base peak assigned to
3.2min	354	230 ($C_{12}H_8NO_4$)	
5.6min	370	246 ($C_{12}H_8NO_5$)	
5.9min	370	246 ($C_{12}H_8NO_5$)	

(3.18)

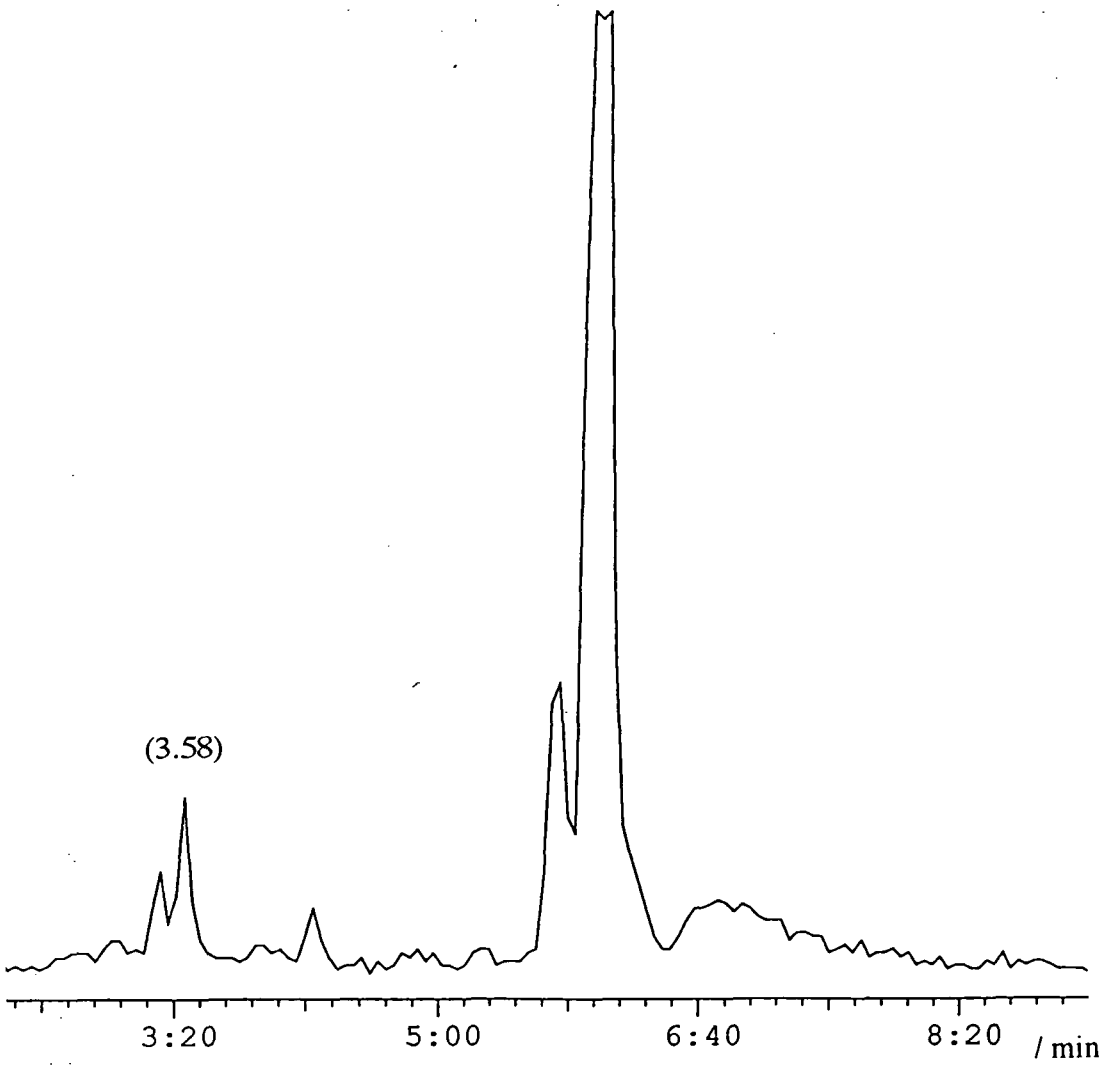


Figure 3-35 HPLC of crude tenellin

Using preparative HPLC a sample of this minor metabolite was collected in sufficient quantity (5.8mg) to obtain a ^{1}H NMR. Although this sample was impure, the spectrum was very similar to that of tenellin. Most informatively a para-substitution pattern of coupling was evident in the phenyl region. Thus it was concluded that the structure is the pyridone(H) (3.58). This compound is tenellin devoid of the hydroxamic acid OH.

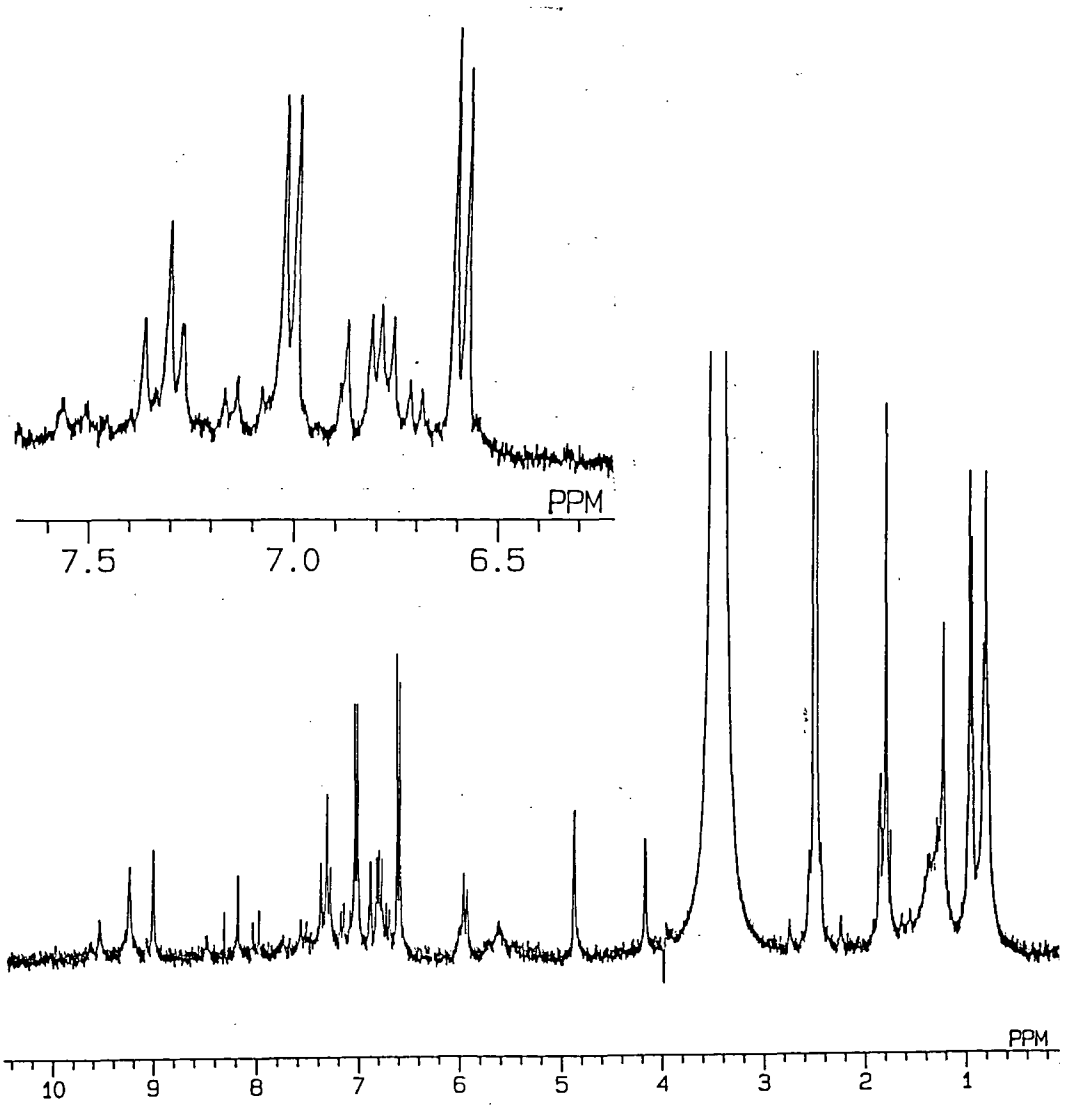
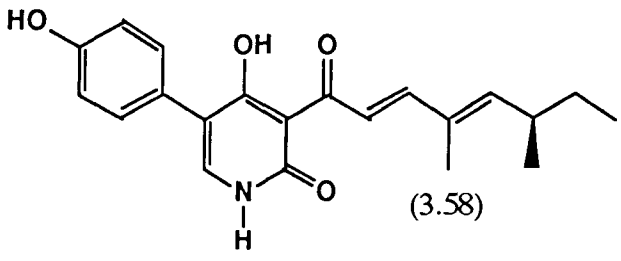


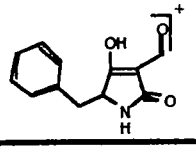
Figure 3-36 ^{1}H NMR of minor metabolite



(3.58)

Figure 3-37 Suggested structure of co-metabolite of tenellin in *B. bassiana*

The synthetic tetramic acid (3.54) was found to elute later from the HPLC column than tenellin, at 8.2 minutes. (M+1, 340). The daughter ion spectrum of this component had a base peak of m/e 216 corresponding to $C_{12}H_{10}O_3N$ as shown on the spectrum below.

Peak at	M+1	Base Peak	Base peak assigned to
8.2min	340	216 ($C_{12}H_{10}NO_3$)	

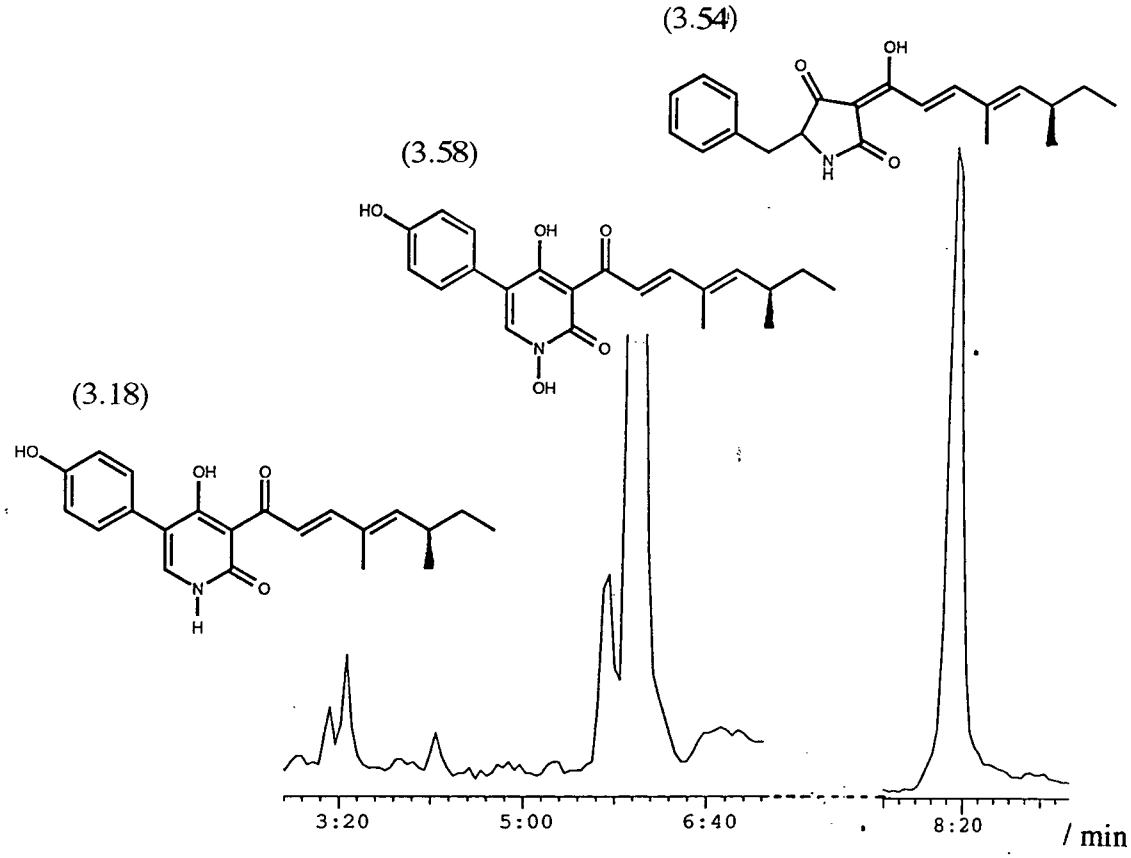
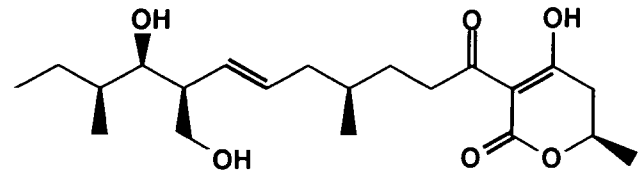


Figure 3-38 HPLC of synthetic tetramic acid and comparison with tenellin

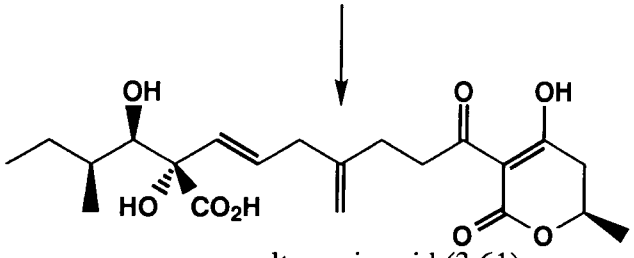
Cultures of *Beauvaria bassiana* were analysed by HPLC for traces of the putative tetramic acid intermediate, or any similar polar intermediate produced throughout the growth cycle. Precursors to tenellin would be expected to be evident earlier in the growth cycle. Cultures were sampled on days 1 to 9 after inocculation of fresh production media. The mycelia were extracted into acetone in a soxhlet thimble and the acetone extracts evaporated and analysed by LCMS. An exhaustive search was made for compounds with masses corresponding to tenellin, reduced precursors, the proposed tetramic acid, and other related hydroxylated tetramic acids. Daughter ion spectra were obtained on any interesting parent ions observed. Apart from the metabolites previously isolated no novel metabolites were identified and there was no evidence for any of the Vining's proposed tetramic acid in the *B. bassiana* cultures.

3.3.3 Use of P-450 inhibitors

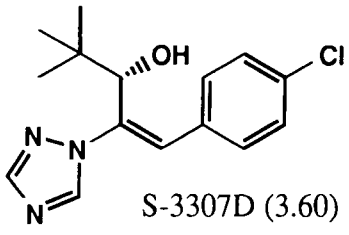
An alternative approach to finding plausible intermediates in the biosynthesis of secondary metabolites has employed the use of specific enzymatic inhibitors. As cytochrome P-450 enzymes are well known in numerous organisms, from bacteria to mammals, a number of cytochrome P-450 inhibitors have been developed. When the organism is grown in the presence of the inhibitor it is sometimes possible to isolate a less oxidised metabolite along the biosynthetic pathway by blocking reactions on that pathway which involve P-450 enzyme catalysis. In 1986 VanMiddlesworth *et al.* observed the accumulation of trichodiene when inhibitors were administered to the strain producing the mycoxin diacetoxyscirpenol.[59] Since then the technique has been applied to the elucidation of the biosynthesis of a number of secondary metabolites, including aphidicolin, nigericin, betaenone B, chaetoglobosin A, and alternaric acid (3.61).[60] For example when the fungus *Alternaria solani*, which produces alternaric acid, was incubated with the inhibitor S-3307D (3.60) a new metabolite, proalternaric acid (3.59) was isolated which is a plausible precursor to alternaric acid.[61]



proalternaric acid (3.59)



alternaric acid (3.61)

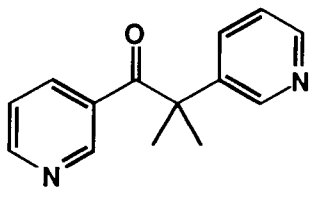


S-3307D (3.60)

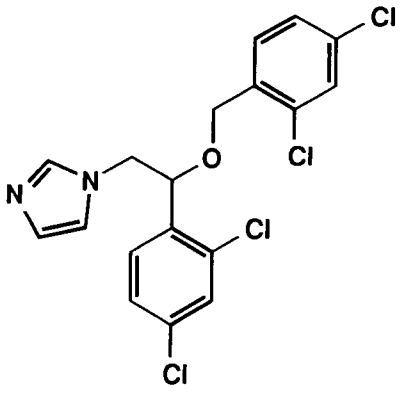
Figure 3-39 Plausible biosynthesis of alternaric acid

It has been shown that *Beauvaria bassiana* is able to metabolize diazepam, warfarin and testosterone *via* oxidative reactions such as hydroxylation and N-demethylation. Metabolism of each substrate can be inhibited by the cytochrome P450 inhibitors SKF-525A and metyrapone, consistent with the involvement of this enzyme system in the metabolism of these drugs by *B. bassiana*. Clearly this indicates that *B. bassiana* possesses a cytochrome P-450 oxidative metabolizing system.[62] As discussed above, a cytochrome P-450 dependent enzyme may be responsible for the observed rearrangement in the biosynthesis of tenellin. Also if tetramic acid (3.20) is indeed the substrate for this enzyme according to Vining's hypothesis, it may be possible to identify the tetramic acid intermediate, prior to hydroxylation, by incubating the cultures with cytochrome P-450 inhibitors.

Accordingly *Beauvaria bassiana* cultures were incubated with two P-450 inhibitors: metapyrone (3.62) and miconazole (3.63), at two concentrations (2 mM and 4 mM).



metapyrone (3.62)



miconazole (3.63)

Figure 3-40 Structures of metapyrone and miconazole, P450 inhibitors

The cultures were incubated for ten days and then the mycelia were extracted with acetone. The cultures incubated with miconazole appeared to produce tenellin normally, as indicated by the characteristic yellow colouration after four days of incubation, although tenellin production was reduced in the flask with the higher concentration of inhibitor. Metapyrone appeared to inhibit tenellin production to a greater extent. The cultures remained a creamish colour, indicating that tenellin production was substantially arrested. The extracts were analysed by LCMS to search for any tetramic acid (3.20) present. For the cultures incubated with miconazole the dominant peak was tenellin with m/e 370 (MH^+). A slight increase from 1% to 3% in the peak corresponding to the co-produced pyridone (3.58) was evident, relative to the tenellin peak. There was no evidence for the production of any tetramic acid (3.20). Cultures incubated with metapyrone showed low levels of tenellin with negligible amounts of the co-produced pyridone (3.58) precursor. It may be assumed that the tenellin detected was primarily residual from the innoculum, and produced in the previous sub-culture. Tenellin production was clearly suppressed but there is no evidence for the production of any tetramic acid or other major metabolites. This suggests that metapyrone had a general toxicity effect. However, a P-450 mechanism may be implicated at a much earlier stage in the biosynthesis of tenellin. This could involve the rearrangement of phenylalanine or a related metabolite, prior to condensation with the polyketide chain. This is discussed in Chapter 4.

3.4 Reinvestigation into the Intermediacy of Tyrosine

3.4.1 Feeding of [3-^{13}C]-tyrosine to *Beauvaria bassiana*

In the above investigation no intermediates were detected by LCMS and HPLC analysis which contained a phenyl group (from phenylalanine) prior to *para*-hydroxylation. This and the other results discussed in the following chapter led us to reconsider Vining's conclusion that tyrosine was not involved in tenellin biosynthesis. This ^{14}C study with tyrosine showed negligible incorporation (1.6%) compared with the high incorporation levels (8.4%) observed after ^{14}C-phenylalanine was administered to the cultures.[63] The experiment was re-investigated with ^{13}C labelled precursors (1-^{13}C-labelled phenylalanine and 3-^{13}C-labelled tyrosine) and both amino acids were incorporated into tenellin at similar levels. This is discussed fully in Chapter 4.

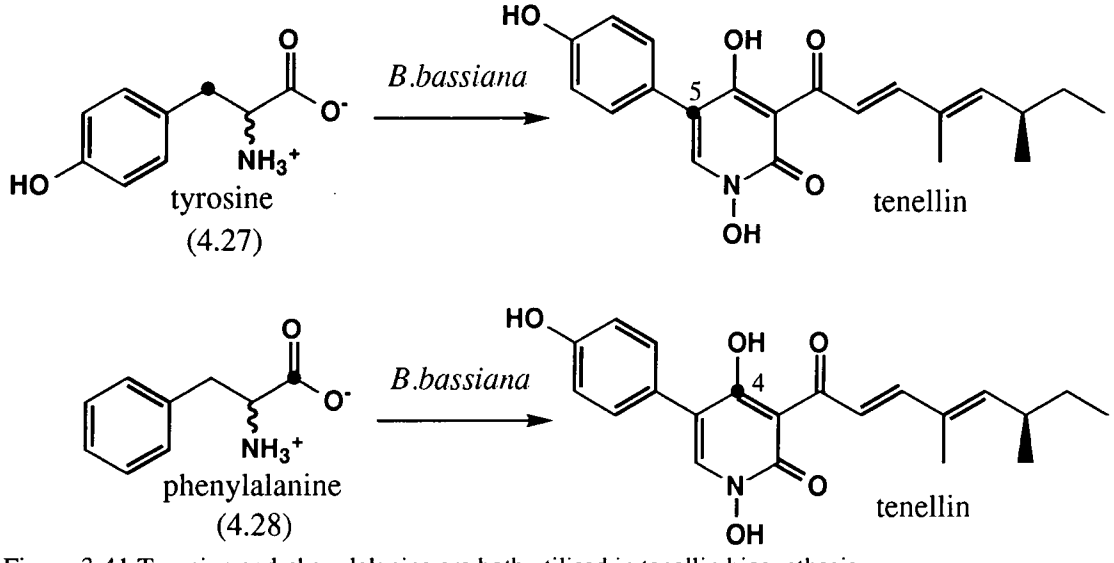
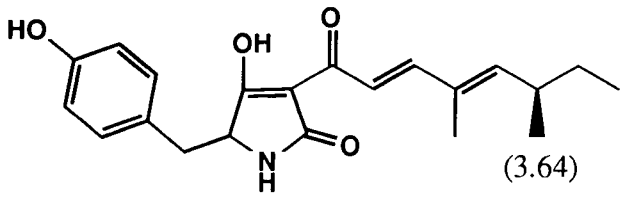


Figure 3-41 Tyrosine and phenylalanine are both utilised in tenellin biosynthesis

Phenylalanine may well first be *para*-hydroxylated to tyrosine, by a phenylalanine hydroxylase, and then used in the biosynthesis of tenellin. It is possible therefore that tyrosine condenses with the polyketide chain to form the tetramic acid intermediate shown below (3.64), which then undergoes the proposed rearrangement to the pyridone ring structure of tenellin.



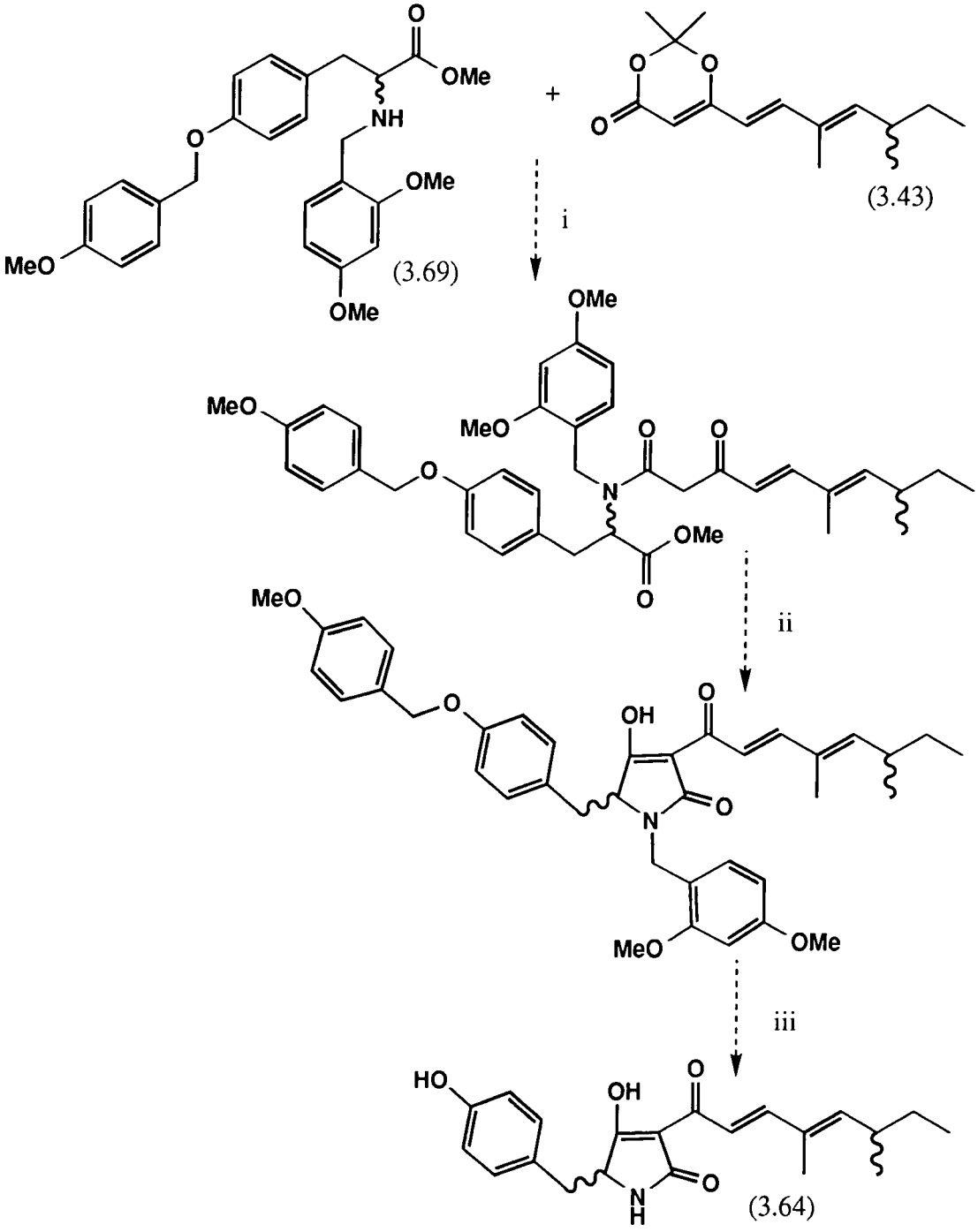
(3.64)

Figure 3-42 An alternative putative tetramic acid intermediate

Synthesis of this modified tetramic acid (3.64) became the new synthetic target, both for a reference compound in LCMS analysis, and for biosynthetic investigations such as those discussed above.

3.4.2 Attempted synthesis of p-hydroxylated tetramic acid intermediate

The proposed synthetic strategy involved a modification of that used in the synthesis of Vining's proposed intermediate (3.20), using a protected tyrosine as the starting material. An added complication is the requirement for protection of the phenol hydroxyl group.



(i) toluene, ptps, reflux, (ii) KO^tBu, ^tBuOH, (iii) CF_3COOH.

Figure 3-43 Proposed synthesis of p-hydroxylated tetramic acid intermediate

The group initially selected for the protection of the phenol was 4-methoxybenzyl. It was envisaged that this could be readily removed in the final deprotection step with trifluoroacetic acid, along with the N-2,4-dimethoxybenzyl group.

The methyl ester of tyrosine as its hydrochoride salt (3.66), was readily prepared using thionyl chloride in methanol.[64] Unlike the free amine this was stable to storage and could be prepared on a large scale. Protection of the amine with 2,4-dimethoxybenzaldehyde (3.50) was accomplished (70% yield) in methanol at room temperature with sodium cyanoborohydride affecting reduction of the imine. Work-up procedures proved to be problematic as the basic conditions, generated on the addition of water, deprotonated the phenolic group. However, quenching with 1 M hydrochloric acid circumvented this problem, but added the complication of hydrogen cyanide production during the process. Thus the procedure had to be effected with caution with the waste gases bubbled through concentrated sodium hydroxide solution to neutralise the hydrogen cyanide. The alternative protection of the phenolic group prior to protection of the amine was unsuccessfully attempted. The white crystalline hydrochloride product (3.67) was protected as the O-4-methoxybenzyl ether (3.69) by treating with two equivilents of potassium carbonate, 4-methoxybenzyl choride (3.68) and tetrabutyl ammonium iodide. This protection step was attempted many times in acetone, according to literature procedures,[65] over a number of days, with warming to reflux, but no reaction occurred. Alternative bases and solvents such as diisopropylethylamine in dichloromethane[66] were attempted, but the reagents appeared equally unwilling to react. Eventually the reaction was accomplished in high yield (92%) using dimethylformamide as the solvent, stirring at 60°C for three days. Presumable this was due to the increased solubility of the reactants in this solvent. The product (3.69), an oil, could be purified over silica.

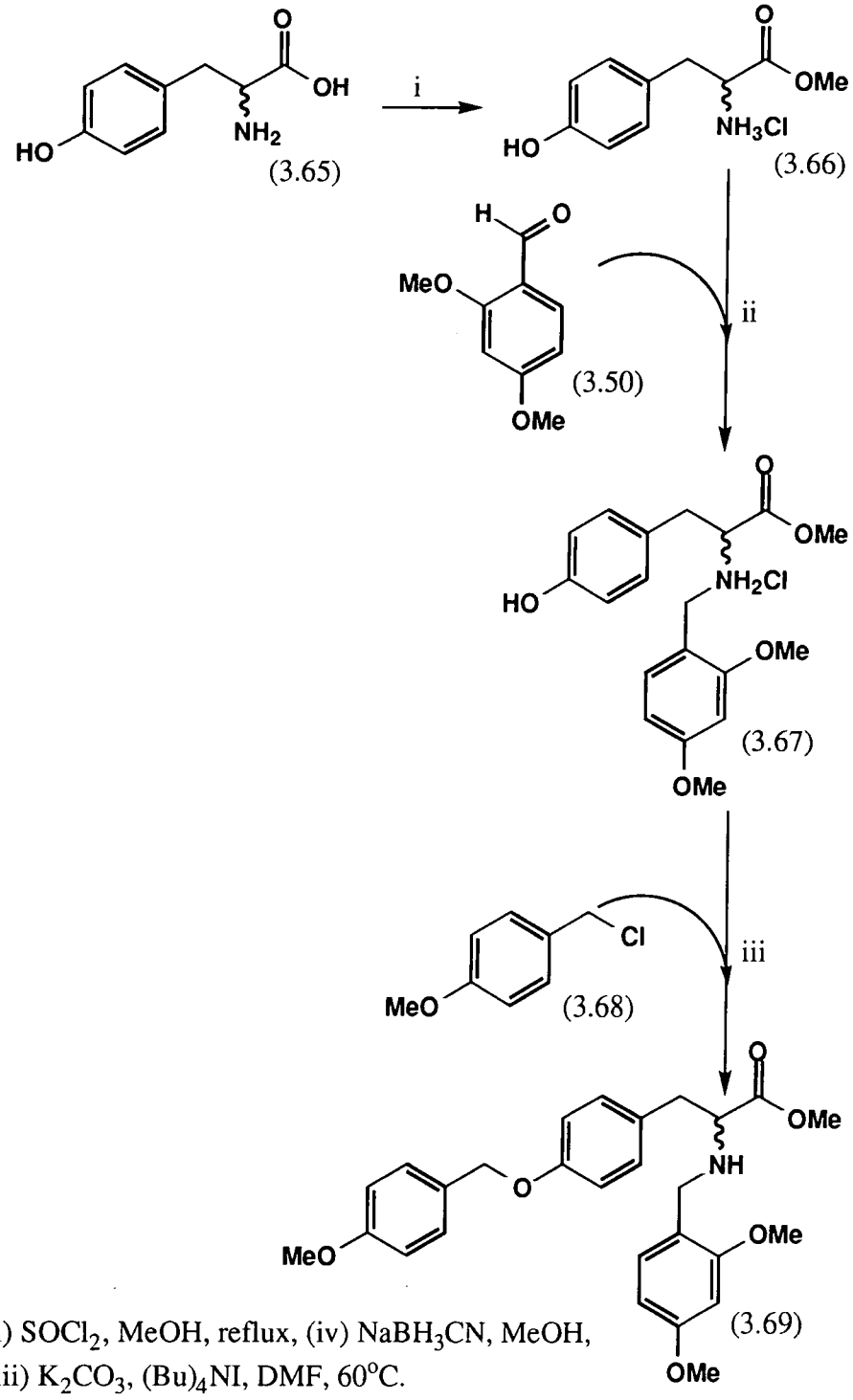


Figure 3-44 Synthesis of N-(2,4-dimethoxybenzyl)-O-(4-methoxybenzyl) tyrosine methyl ester

Attempts at the condensation of the protected tyrosine (N-2,4-dimethoxybenzyl)-O-(4-methoxybenzyl) tyrosine methyl ester (3.69) with 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (3.43) by thermolysis in refluxing toluene were unsuccessful. Unreacted (3.69) was recovered, along with a mixture of breakdown products from the protected ketene. Mild acid catalysis with pyridinium *para*-toluene

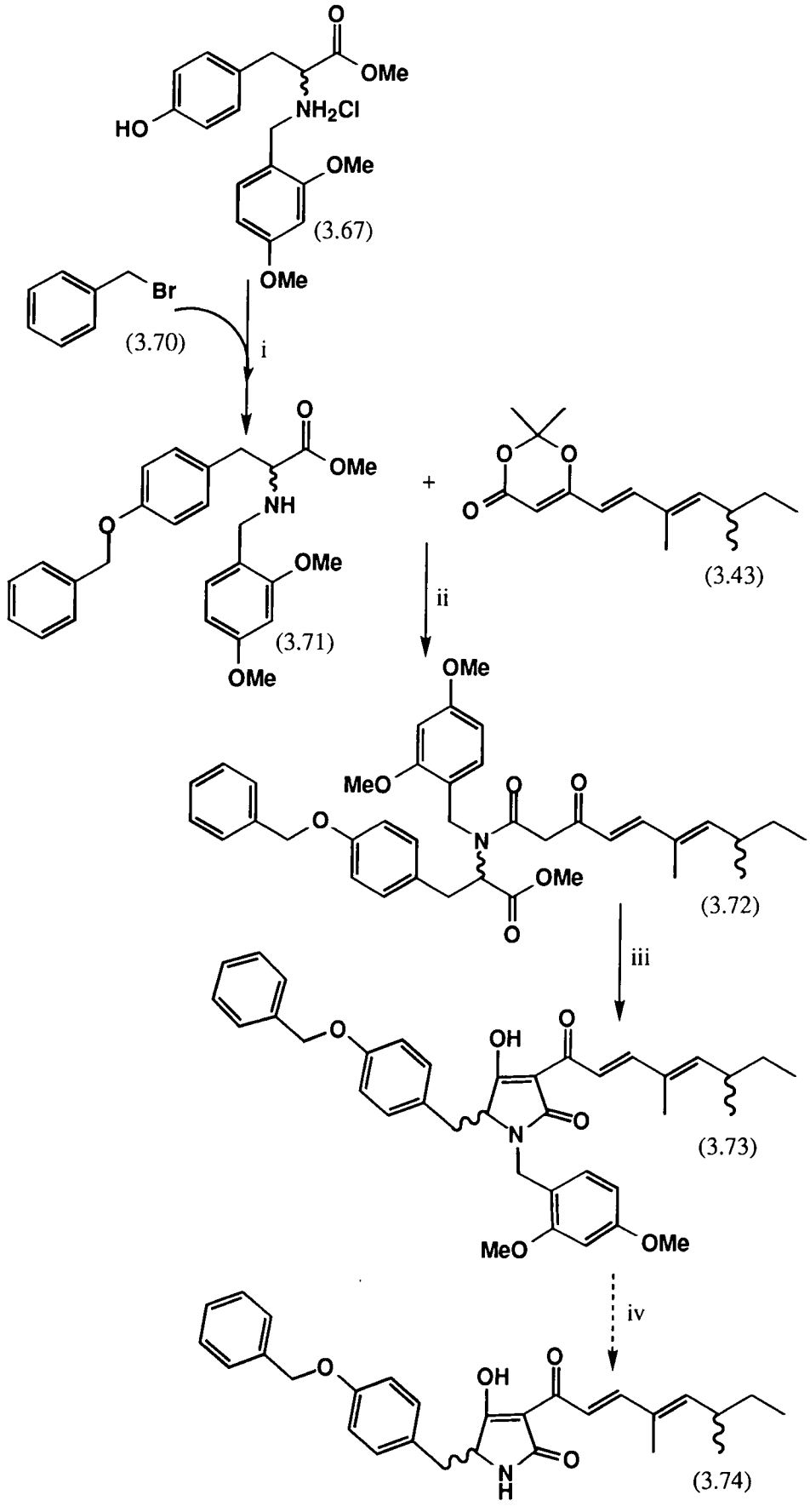
sulphonate was attempted. After separation over silica, an ion with m/e 657 corresponding to the molecular ion for the desired condensation product was detected by mass spectroscopy, in one fraction, however negligible product was detectable by NMR. Loss of the 4-methoxybenzyl group appeared to occur even under the mildly acidic conditions, and a variety of unwanted products were obtained. Phenolic benzyl ethers are in general more labile than phenyl protected amines. Attempts were made to catalyse the reaction with various Lewis acids: aluminium compounds, and boron trifluoride etherate, but without success. An alternative strategy was attempted which aimed first to deprotonate the amine of the protected tyrosine with sodium hydride, followed by reaction with the protected diketene. Again a mixture of products resulted, with no observable product. As the usual conditions (reflux in toluene with ptps) for condensation had been so successful for the phenylalanine derivative, the sensible approach was to change the protecting group used, so as to avoid unwanted deprotection under these conditions.

The benzyl group has been widely used in peptide synthesis to protect the phenolic group of tyrosine.[67] It is also cleaved by trifluoroacetic acid, although long reaction times are required. A further disadvantage is the migration of the benzyl cation to the 3-position of the ring, during cleavage, giving unwanted side products. 2,6-Dichlorobenzyl ethers have also been used as they give a lower incidence of alkylation at the 3-position of the benzene ring, but they are even more stable to trifluoroacetic acid deprotection.[68] Sensitivity of the tetramic acid to long treatment with such a powerful acid was envisaged to present a future problem. Side reactions, when deprotecting phenolic benzyl ethers, can be minimised if a cation scavenger is included in the reaction mixture. Favourites are dimethyl sulphide, thioanisole and pentamethylbenzene.[69] Pentamethylbenzene[70] was selected as it has the lowest incidence of side reactions and furthermore has been shown to increase the rate of deprotection.

Synthesis of the O-benzyl protected N-2,4-dimethoxylbenzyl tyrosine methyl ester (3.71) was accomplished with potassium carbonate and benzyl bromide (3.70)[71] in dimethyl formamide over two days at 60°C. Pyridinium *para*-toluene sulphonate catalysed thermolysis, in refluxing toluene, of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (3.43) with the protected tyrosine (N-2,4-dimethoxybenzyl)-O-(benzyl)

tyrosine methyl ester (3.71) did indeed result in the desired condensation product (3.72), in reasonable yield (55%). Even more gratifying was the ease at which the Dieckmann cylisation using potassium *tert*-butoxide in *tert*-butanol of this product proceeded smoothly, yielding the N- and O-protected tetramic acid (3.73) (81%).

The final hurdle was deprotection step. In anticipation of some problems, very small amounts were used in deprotection attempts, and the reactions monitored by reverse phase HPLC. After ten minutes stirring in neat trifluoroacetic acid containing ten equivalents of pentamethyl benzene, the trifluoroacetic acid was removed *in vacuo* from the bright red mixture. The resulting solid was dissolved in dichloromethane and washed with water. The majority of the pentamethylbenzene could be precipitated out from methanol : water, (85: 15), the solvent used for HPLC analysis. The filtrate was analysed by HPLC which showed predominantly one product. Isolation of this compound, followed by mass spectroscopy identified this as the N-deprotected, O-protected tetramic acid (3.74). Clearly longer reaction times were necessary for deprotection of the phenolic benzyl ether. The reaction was monitored over four hours during which time the benzyl ether remained intact! After seven hours the N-deprotected tetramic acid could no longer be detected by HPLC, and work-up confirmed, unsurprisingly, that decomposition had occured. As deprotections of the phenolic ether of tyrosine are accomplished within one and a half hours this was indeed a disappointing result. As a last resort attempts were made to deprotect the tetramic acid using an alternative reagent, trimethylsilyl iodide in acetonitrile. This has been effectively used to deprotect tyrosine benzyl ethers in peptide syntheses.[72] Yields are high and no formation of 3-benzyl tyrosine is generally observed. However with the relatively sensitive tetramic acid many side reactions occurred and no product was observable. Alternative protection / deprotection strategies will need to be explored for the successful synthesis of the tetramic acid (3.64). For example deprotection with trifluoromethanesulphonic acid is also known to remove benzyl and tert-butyl ethers, where reaction times are sometimes shorter than with trifluoroacetic acid deprotection.[73] The methyl ether may also be cleaved with trifluoromethanesulphonic acid.[74]



(i) K_2CO_3, DMF, 60°C, (ii) toluene, ptps, reflux, (iii) KO^{t}Bu, ^{t}BuOH, (iv) CF_3COOH.

Figure 3-45 Synthesis of 4-benzyloxy tetramic acid intermediate

3.5 Discussion

Vining's proposed tetramic acid intermediate in tenellin biosynthesis (3.20) was successfully synthesised in both ^{13}C labelled (3.54) and deuterium labelled (3.55) forms. Both of these compounds were fed to cultures of *Beauvaria bassiana*, but successful incorporation was not observed. Subsequent re-evaluation of the role of tyrosine in tenellin biosynthesis established that it is a good precursor to tenellin. Therefore Vining's tetramic acid (3.20) appears an unlikely intermediate in tenellin biosynthesis. The tyrosine derived tetramic acid (3.64) is a plausible intermediate, and its synthesis has been attempted. All but the final deprotection step were achieved. Deprotection of the phenol group will require an alternative protection / deprotection strategy to avoid the problems discussed above.

However, LCMS analysis of *Beauvaria bassiana* cultures did not reveal evidence for the phenylalanine derived tetramic acid (3.20) or the tyrosine derived tetramic acid (3.64), at any stage in the growth cycle. Additionally, the use of cytochrome P-450 inhibitors did not result in the accumulation of such intermediates. It is unlikely that these relatively stable putative intermediates should be present in amounts below the detection limits of such a technique. Rather, the results suggest that tenellin biosynthesis does not proceed *via* a tetramic acid intermediate at all. A rearrangement of phenylalanine / tyrosine may well occur at a much earlier stage in the biosynthesis, prior to condensation with the polyketide moiety. This is discussed in Chapter 4.

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4. Alternative Rearrangement Mechanisms for the Biosynthesis of the Pyridone ring of Tenellin

4.1 Introduction and Background

4.1.1 Phenylalanine and tyrosine metabolism

Phenylalanine and tyrosine are synthesed *in vivo, via* the shikimate pathway, the main steps of which are shown below. The branch point occurs at prephenate which undergoes decarboxylation and aromatisation to phenylpyruvate and 4-hydroxyphenylpyruvate. These are reversibly transaminated to phenylalanine and tyrosine respectively[1].

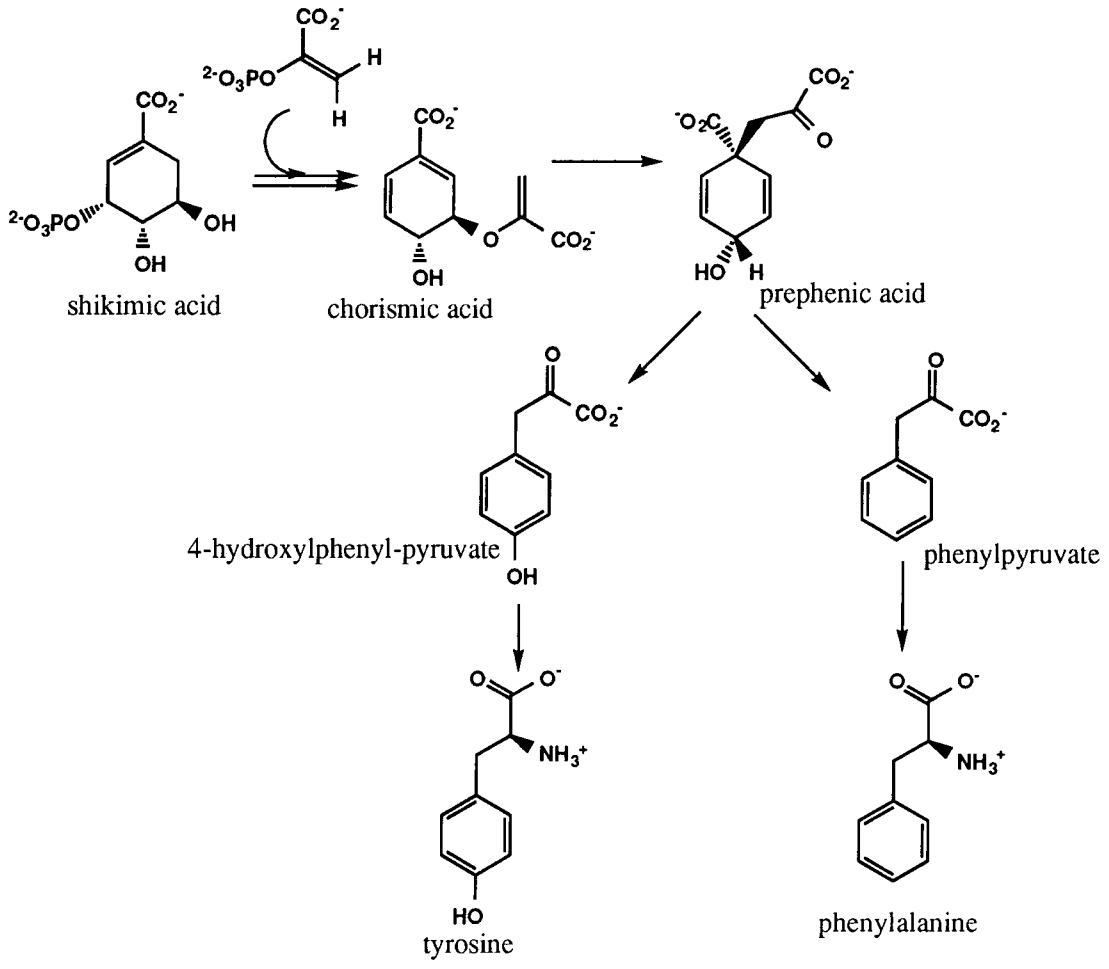
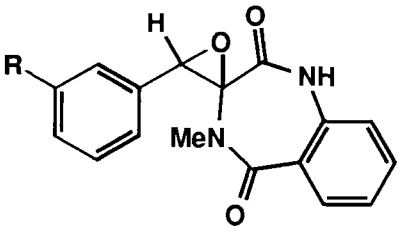


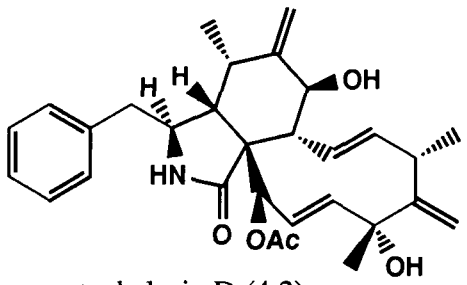
Figure 4-1 The shikimate pathway to tyrosine and phenylalanine

Reversible amination results in the interconversion of L and D forms of phenylalanine as for example in the case in the biosynthesis of cyclopenin and cyclopenol (4.1) by

Penicillium cyclopium.[2] and in the cytochalasin-D (4.2) producing fungus *Zygosporium masonii*.[3]



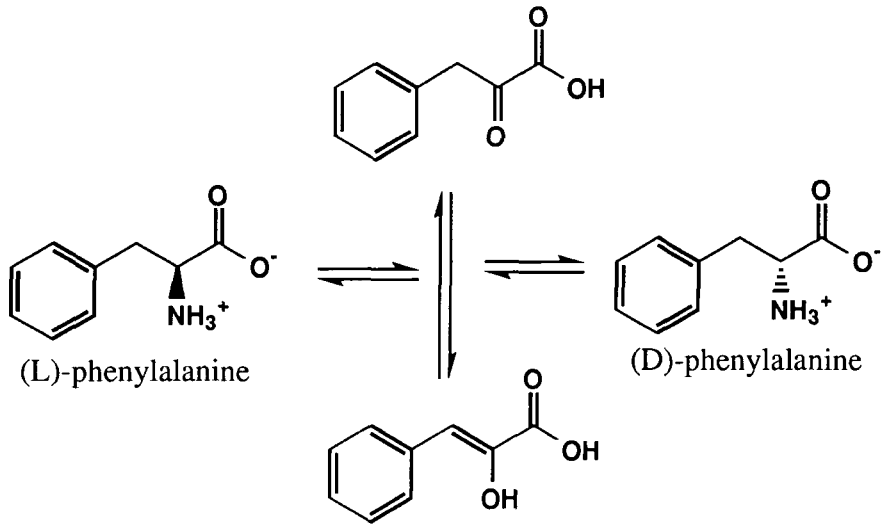
cyclopenin R=H (4.1)
cyclopenol R=OH



cytochalasin D (4.2)

Figure 4-2 D and L-Phenylalanine derived secondary metabolites

Both D- and L- phenylalanine are incorporated into the metabolites. Feeding experiments with stereospecifically labelled D-phenylalanine resulted in the complete loss of tritium from the α-position and extensive loss of tritium from the β-position, an observation consistent with the enolisation of phenylpyruvate. (Reversible transamination is also known to occur in many antibiotic biosyntheses, including the penicillins, cephalosporins, gramicidin S, actinomycin and gliotoxin.[3]



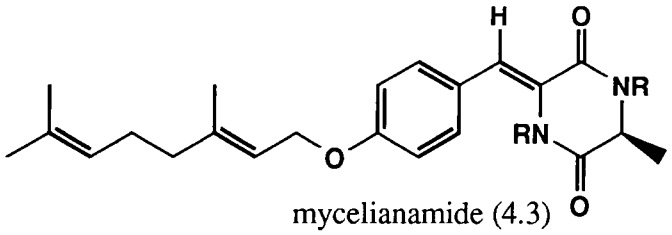
(L)-phenylalanine (D)-phenylalanine

phenylpyruvate

Figure 4-3 Interconversion of (D) and (L)-phenylalanine

Interestingly during the biosynthesis of mycelianamide (4.3) (from *Penicillium griseofulvum*), a metabolite related to cyclopenin and cyclopenol, no such interconversion between L- and D- tyrosine is observed prior to incorporation. High

retention of the 3-*pro*-S hydrogen of tyrosine and the complete loss of the 3-*pro*-R-hydrogen was observed as the result of a stereospecific dehydrogenation step.



mycelianamide (4.3)

Figure 4-4 Tyrosine derived mycelianamide

Phenylalanine and tyrosine may also undergo stereospecific *anti*-elimination of the 3-*pro* S hydrogen together with ammonia, to give cinnamic acid (4.4) and *para*-coumaric acid (4.5) (*para*-hydroxy cinnamic acid) respectively. The transformations are catalysed by the enzymes phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL).[4] Both of these enzymes are widespread in grasses and some fungi.[5] However most organisms seem to favour conversion of cinnamic acid into *para*-coumaric acid, rather than direct loss of ammonia from tyrosine. Cinnamic acids are key intermediates in the biosynthesis of the phenylpropanoids, the most common shikimate metabolites involved in the biosynthesis of many alkaloids and flavanoids.[6]

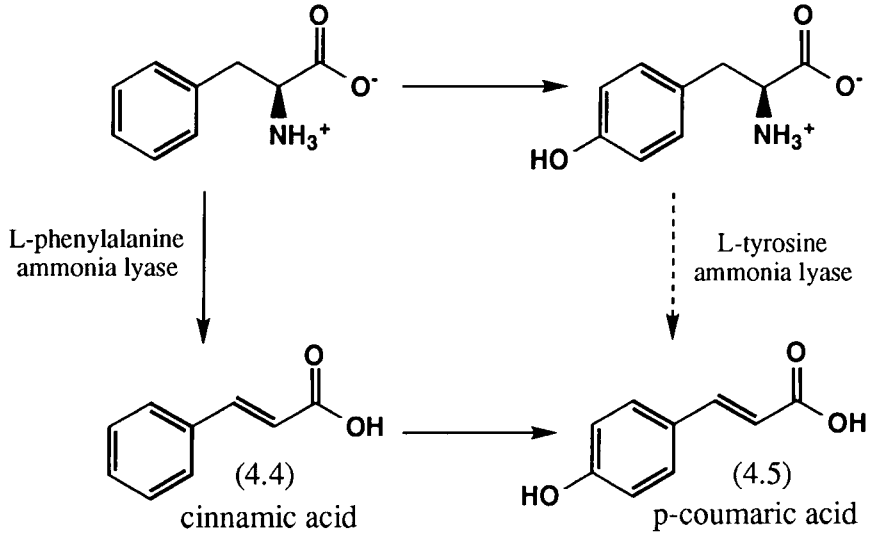


Figure 4-5 Enzymes involved in phenylalanine metabolism

Para-hydroxylation of phenylalanine to tyrosine is also the first step of phenylalanine degradation. This reaction may also be used to provide an additional source of tyrosine.

Such phenylalanine hydroxylases are widespread in microfungi, plants and higher animals.[7] The process is catalysed by an iron (III) containing phenylalanine hydroxylase (PAH), and requires the participation of the cofactor, biopterin.

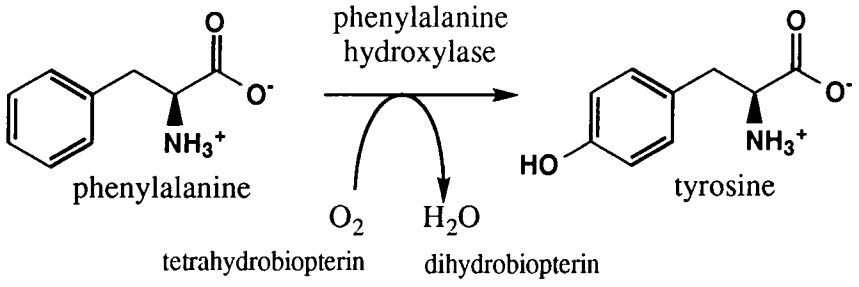


Figure 4-6 Action of phenylalanine hydroxylase

It has been postulated that the mechanism involves formation of an arene oxide (4.6), opening of which results in a carbocation at ring carbon-3. Migration of an hydride is observed from ring carbon-4 to carbon-3 (known as the NIH shift) which may result from the formation of an oxonium ion (4.7) followed by enolisation to tyrosine.[1] An epoxide intermediate was isolated after oxidising L-[2,5-H_2]-phenylalanine with PAH, consistent with this hypothesis.[8]

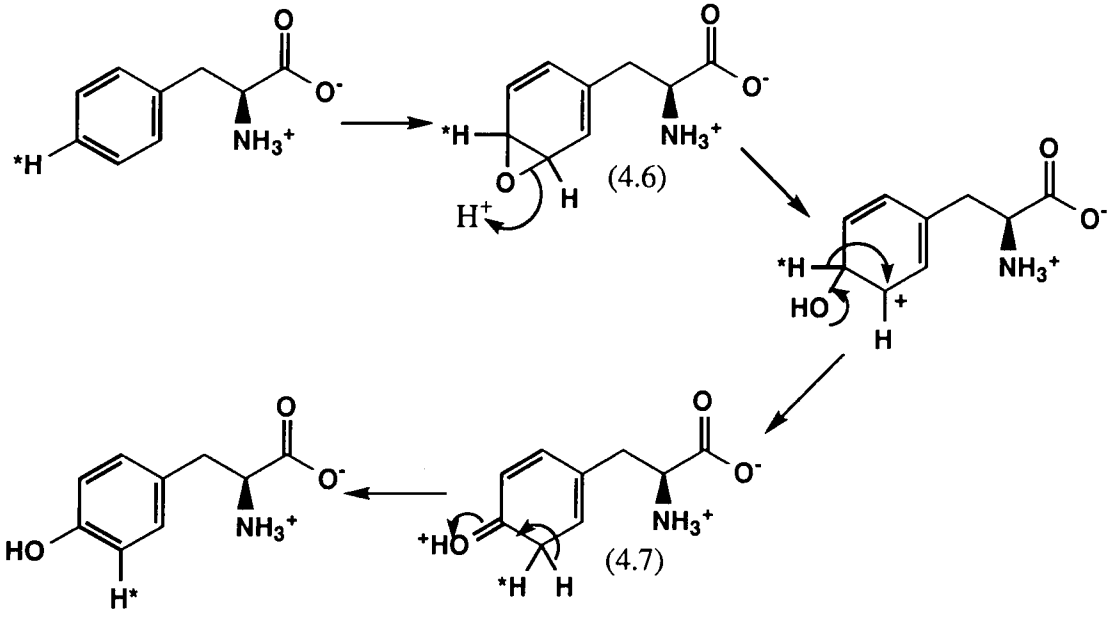


Figure 4-7 Proposed mechanism of the NIH shift

Contrary to mammalian PAH, it has recently been shown that phenylalanine hydroxylase from the bacterium *Chromobacterium violacium* does not require any redox active metal

such as iron for activity.[9] Investigations of this metal independent hydroxylation and suggests that its mode of action is similar to mammalian PAH. Clearly any mechanistic analogy to the iron mediated cytochrome P-450 mediated oxidations discussed below is unlikely. More recently measurements of the kinetic isotope effects on hydroxylation have brought into question the obligatory nature of the arene oxide intermediate.[10] The catalytic mechanism of PAH thus still remains undefined.

4.1.2 Hydroxylase activities in Beauvaria sp.

The Beauvaria are particularly well known for their hydroxylase activities. When hydroxylation of an aromatic ring is observed, *ortho*-hydroxylation is usually predominant. *Para*-hydroxylations, however, are also known amongst these biotransformations, notably in the species *Beauvaria sulfurescens*. This species has consequently been used in preparative organic chemistry as an alternative to chemical hydroxylation of aromatic rings. Such chemical transformations can often be problematic and are generally never achieved with complete *para*-regioselectivity. The mechanism of this hydroxylation has been shown to involve the NIH shift, consistent with the formation of an arene oxide intermediate, and thus mimics the mammalian pathway of detoxification.[11] A cytochrome P-450 mediated enzyme is implicated.

Griffiths *et al.* have recently shown that the fungus *Beauveria bassiana* is capable of metabolising diazepam, warfarin and testosterone *via* oxidative reactions such as hydroxylation and N-demethylation. This activity was shown to be inhibited by cytochrome P-450 inhibitors, suggesting involvement of this enzyme system in the metabolism of these drugs.[12] Thus it appears that *Beauveria bassiana* is able to employ a range of oxidative activities in its metabolism.

The mechanism of cytochrome P-450 catalysed aromatic hydroxylation continues to be an area of debate. Similarly to phenylalanine hydroxylase reactions, the NIH shift sometimes occurs, but isotope effects are generally not observed, except in the formation of *meta*-hydroxylated metabolites where large isotope effects may be observed.[13] Initial electrophilic attack of the cytochrome P-450 Fe(III)=O on the aromatic ring, followed by formation of the hydroxylated benzene, possibly *via* arearrangement of a keto product, is emerging as the most likely mechanism.[14]

4.1.3 Rearrangements involving 1,2-carboxyl migration

There are a number of known carbon skeleton rearrangements which are mediated by coenzyme B_{12}.[15] Two such processes are shown below which require the 1,2-migration of a carboxyl moiety, and involve the migration of the COSCoA group with concurrent 1,2-back migration of a hydrogen atom. These reactions are mediated by the enzymes methylmalonyl-CoA mutase[16] and isobutyryl-CoA mutase respectively[17]. In both cases the hydrogen replaces the COSCoA group with predominant retention of configuration.

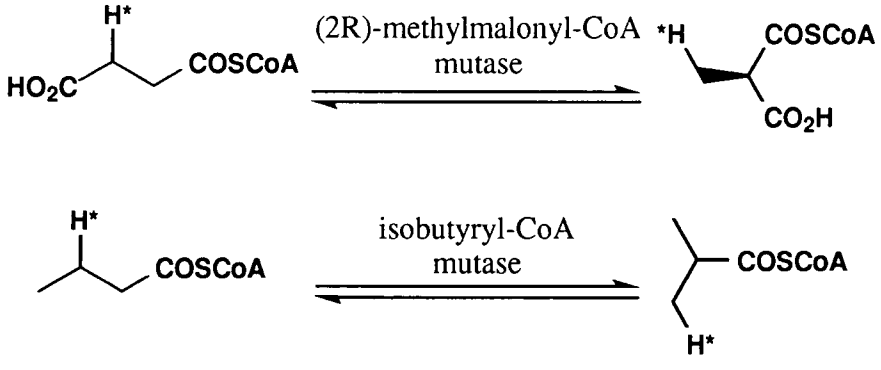
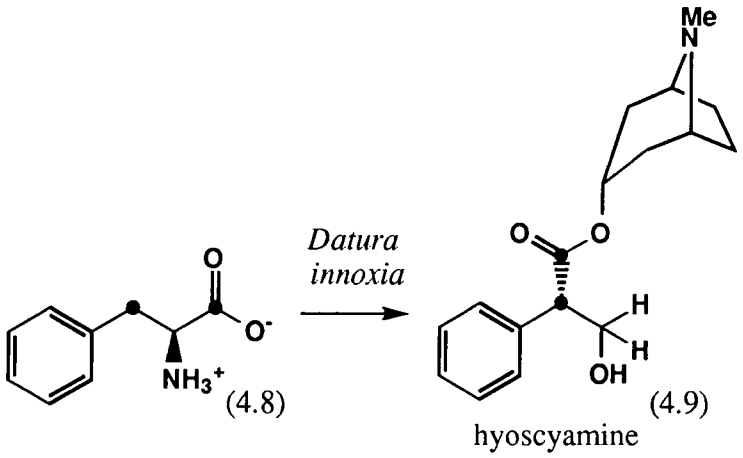


Figure 4-8 Coenzyme B_{12} mediated vicinal interchange processes

Clearly there is a superficial similarity between these vicinal interchange process and the observed 1,2-carboxyl migration of phenylalanine in tenellin biosynthesis.

4.1.4 The biosynthesis of tropic acid

As briefly discussed in Chapter 3, the biosynthesis of the tropic acid moiety of the Datura alkaloids, hyoscyamine and scopolamine, involves an intramolecular rearrangement of a phenylalanine derived phenylpropanoid unit. DL-[1,3-$^{13}C_2$]-Phenylalanine (4.8) was incorporated intact into hyoscyamine (4.9), the ^{13}C labelled atoms having become contiguous. The observed ^{13}C-^{13}C coupling in the ^{13}C-NMR unequivocally established the intramolecular nature of the rearrangement.[18] The apparent resemblance of this rearrangement to the rearrangement in tenellin biosynthesis was recognised by Leete and Vining.[19]



(4.8) (4.9)

hyoscyamine

Figure 4-9 Incorporation of phenylalanine into hyoscyamine

Considerable advances have been made in understanding this rearrangement.[20] Firstly D-phenyllactic acid (4.11) was established as a direct precursor to the tropate ester (4.12) on the basis of intact incorporation of DL-[2-$^{13}C^{2}H$]-phenyllactate.[21] The retention of the deuterium (as observed as an α-shifted ^{13}C component in the ^{13}C-NMR) eliminated the possibility that phenylpyruvic acid (4.10) is a closer precursor, although significant washout of deuterium was observed. Presumably this may be accounted for by the rapid interconversion of phenylpyruvate and phenyllactate. This interconversion also explains the observation that both ^{13}C-labelled D and L-phenyllactic acids are incorporated into tropate. DL-[2-$^{13}C^{2}H$]-Phenyllactate was resolved, and the separate enantiomers administered to *D. stramonium*. D-phenyllactic acid was incorporated into hyoscyamine with the $^{13}C^{2}H$ label intact, whereas its enantiomer had clearly lost its deuterium label prior to incorporation. D-Phenyllactic acid is thus the true precursor to tropate.[22]

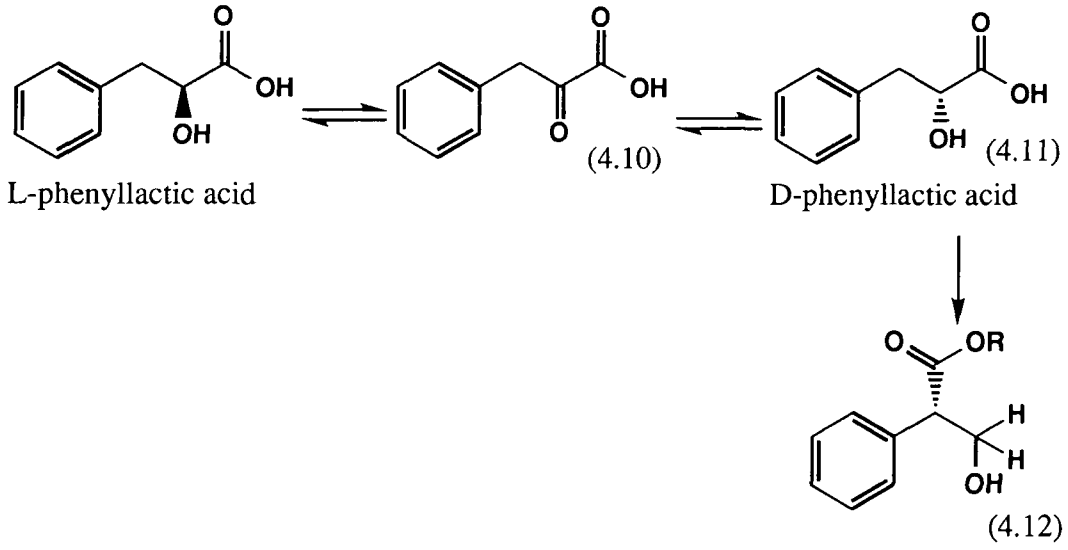


Figure 4-10 Incorporation of L- and D- phenyllactic acids into tropate esters

The stereochemical features of this rearrangement have been fully investigated.[23] It was demonstrated that an inversion of configuration is occuring at both migration termini, and that no vicinal interchange (back migration of hydrogen) was operating.[24] (Figure 4-11)

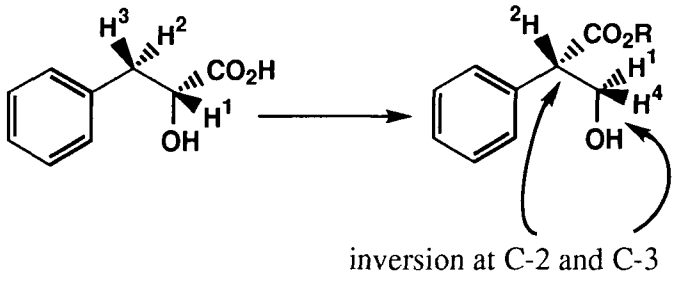
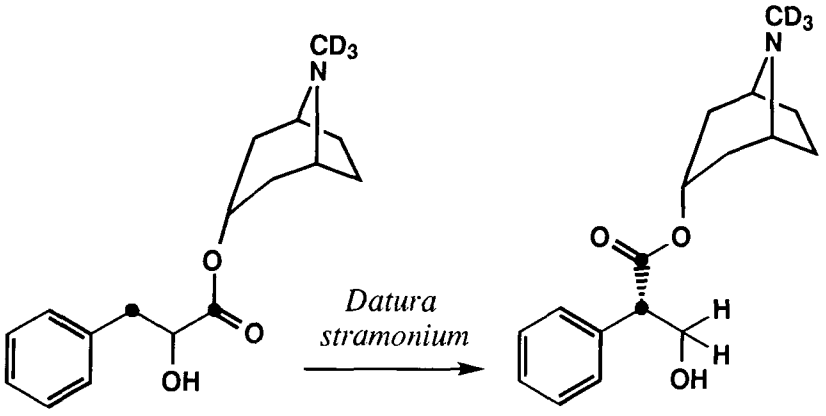


Figure 4-11 Overall stereochemistry of the phenyllactate to tropate rearrangement

Robins *et al.* have investigated the substrate for the rearrangement, and have demonstrated that littorine (4.13), the D-tropine ester of D-phenyllactate is rearranged directly to hyoscyamine (4.14). Littorine prepared from [*methyl*-$^{2}H_3$]-tropine and [1,3-$^{13}C_2$]-phenylacetic acid appears to be rearranged to hyoscyamine intact. The level of incorporation of the quintuply-labelled molecule was inconsistent with hydrolysis of the ester prior to incorporation.[25]



littorine (4.13) hyoscyamine (4.14)

Figure 4-12 The intact rearrangement of littorine to hyoscyamine

Now that the possibility of a vicinal interchange process has been discounted, investigations are focusing on the nature of the rearranging enzyme, and there is mounting evidence that a cytochrome P-450 enzyme is implicated. Labelling studies on a number of related minor alkaloids in the Datura species support this proposal,[26] and it has been recently demonstrated that the conversion of littorine to hyoscyamine appears to be inhibited by the P-450 inhibitor chlotrimazole.[27] Incorporation of [2-$^{13}C^{18}O^{2}H$]-phenyllactic acid was studied to examine the oxygen content in hyoscyamine relative to littorine. It was anticipated that half of the ^{18}O label would be lost on collapse of the aldehyde hydrate (4.17) if an oxygen rebound process was operating. However the retention of 71-75% of the ^{18}O did not support this. Model studies have been investigated by Newcombe[28] on methylcyclopropane ring opening reactions, with the stabilizing substituents, aryl and oxygen, similar to littorine and hyoscyamine. The methylcyclopropane cation opens towards oxygen, whereas the radical opens towards the aryl group, and this clearly supports a carbocation process and a two electron oxidation. Two electron oxidations have precedent in the P-450 mediated biosyntheses of thromboxane and prostacyclin.[29] Alternatively the oxygen-18 result may be accounted for by a radical process involving either direct disproportionation of the product radical[30] or a partially stereospecific breakdown of the aldehyde hydrate.

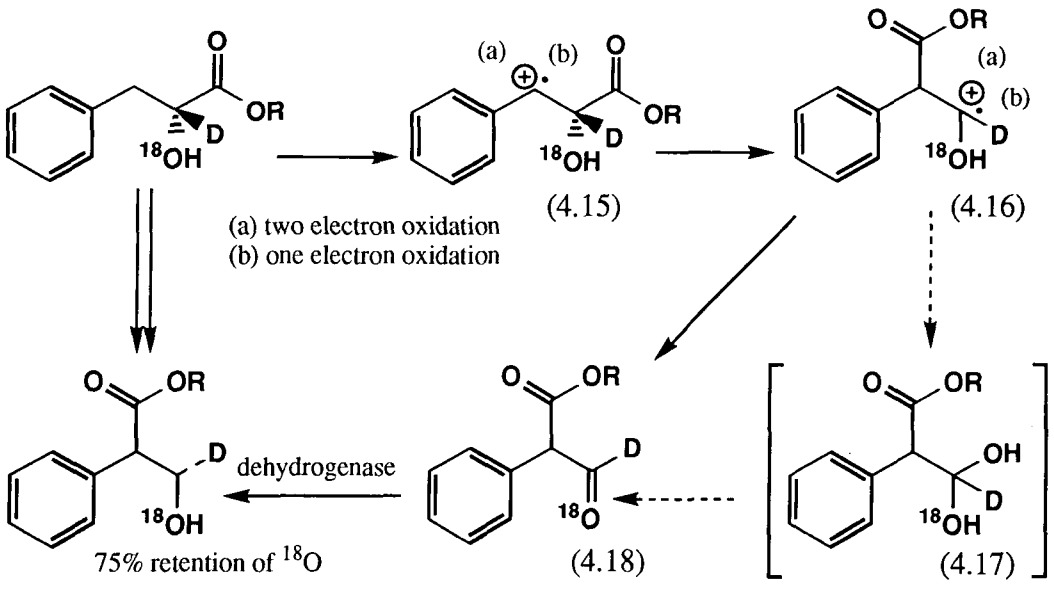


Figure 4-13 Proposed mechanism for the P-450 mediated rearrangement of littorine to hyoscyamine

4.2 Alternative Mechanistic Explanations for the Intramolecular Rearrangement in Tenellin Biosynthesis

4.2.1 A direct rearrangement of phenylalanine?

An alternative pathway to that proposed by Vining (discussed in Chapter 3) may be postulated for the biosynthesis of the pyridone ring of tenellin, which would also account for the observed intramolecular rearrangement. Phenylalanine or some closely related metabolite, could rearrange prior to condensation with the polyketide chain (Figure 4-14), giving the requisite carbon framework necessary to form the 6-membered pyridone ring directly.

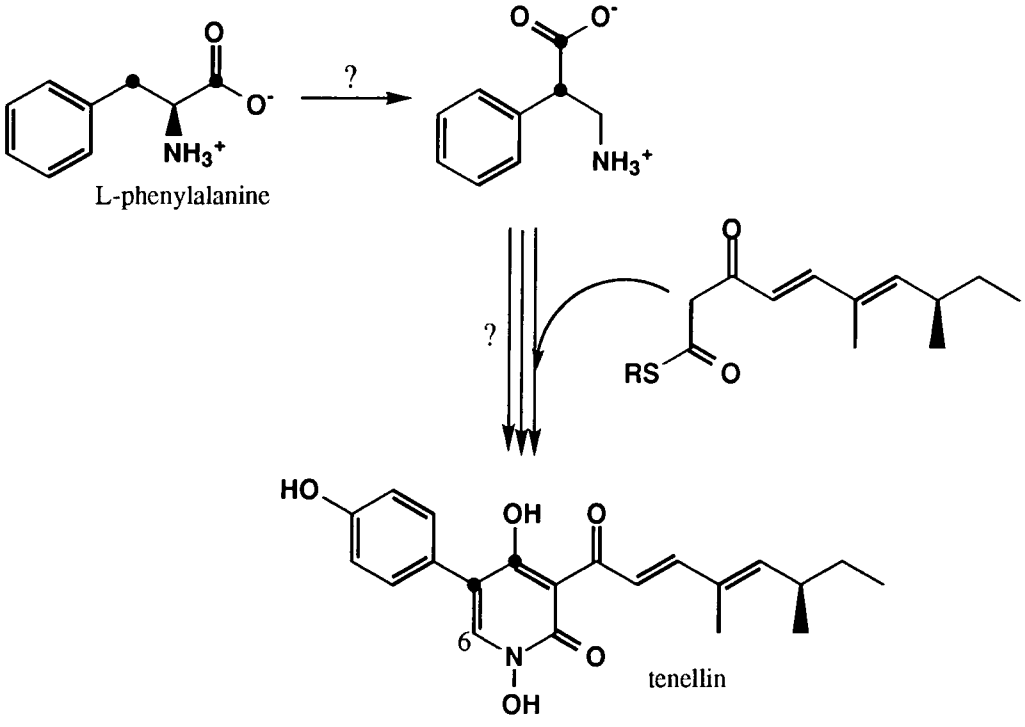


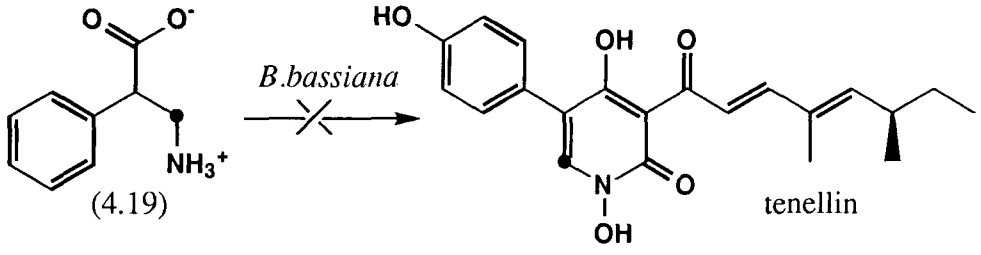
Figure 4-14 Alternative mechanism for tenellin biosynthesis involving the rearrangement of phenylalanine directly

Vining had attempted to rule out the possibility of any vicinal interchange mechanism involving a 1,2-hydride shift. When *B. bassiana* cultures were supplemented with non-stereospecifically labelled [1-^{14}C, 3-^{3}H]-L-phenylalanine the tenellin isolated had lost at least 96% of the original tritium[31]. It might be expected that tritium would be retained at position-6 on the pyridone ring. However if we consider that the rearrangement would proceed in a stereospecific manner, the loss of tritium can also be accounted for as follows. Tritium is transferred from the benzylic position by a 1,2-hydride shift to either

the *pro*-R or the *pro*-S aminomethylene carbon. One hydrogen from this carbon is lost in the construction of the pyridone ring and if this occurs in a stereospecific manner, then the tritium transfered from the benzylic position may be lost. The tenellin isolated would contain no tritium, and thus this experiment was not fail safe.

4.2.2 The direct rearrangement of phenylalanine investigated

Realisation that Vining's assertions were not conclusive led Cox[32] to investigate the possible intermediacy of the rearranged phenylalanine precursor in the biosynthesis of tenellin. A sample of 3-amino-2-phenylpropionic acid (4.19) bearing a ^{13}C label in the 3-position was prepared and administered to cultures of *B. bassiana*. However the tenellin isolated showed no evidence of incorporation of the carbon-label. Thus it was concluded that 3-amino-2-phenylpropionic acid is not a true intermediate, and that phenylalanine does not undergo a direct rearrangement prior to condensation with a polyketide chain.



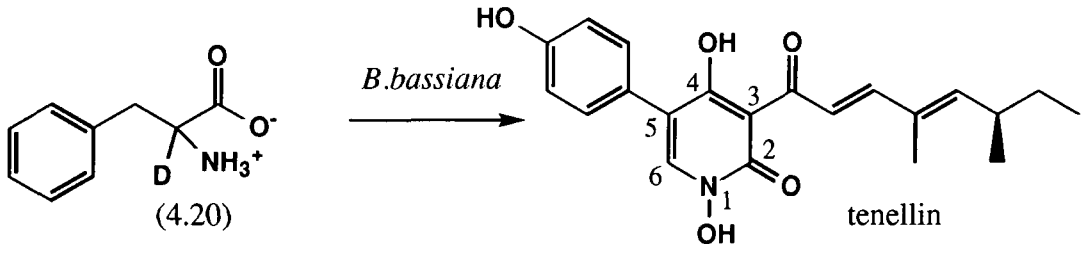
(4.19) tenellin

Figure 4-15 3-Amino-2-phenylpropionic acid is not a precursor to tenellin

4.3 Further Investigations with Phenylalanine and Phenyllactic acid

4.3.1 The origin of hydrogen-6 in the pyridone ring

Of all the hydrogen atoms in tenellin, the origin of hydrogen-6 in the pyridone ring remains unknown. The body of labelling data suggests that this will be retained from carbon-2 of phenylalanine. Accordingly, DL-[2-^{2}H]-phenylalanine (4.20) was pulse fed to cultures of *B. bassiana* to a final concentration of 6 mM. Tenellin (50mg) was isolated after a ten day incubation, and purified by HPLC. Analysis by ^{2}H NMR did not indicate any incorporation of a deuterium label, at the signal corresponding to C-6.

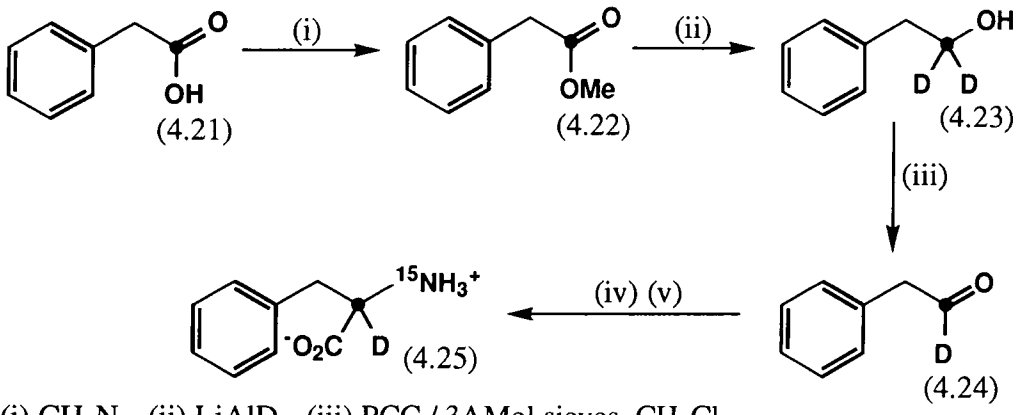


(4.20) tenellin

Figure 4-16 Loss of deuterium from [2-D]-phenylalanine in tenellin biosynthesis

This result is intriguing, but can be rationalised in at least two ways, (i) a process which requires the obligate removal of this hydrogen during formation of the pyridone, or (ii) a process which requires transamination of the amino acid resulting in the removal of the deuterium label before phenylalanine is used in the biosynthesis. The latter process would result in the scrambling of the nitrogen also. In an attempt to delineate these possibilities an experiment was conducted involving the synthesis and incorporation of triple labelled [2-^{13}C^{2}H^{15}N]-phenylalanine into tenellin.

4.3.2 Synthesis of DL-[2-$^{13}C^{2}H^{15}N$]-phenylalanine



(i) CH_2N_2, (ii) $LiAlD_4$, (iii) PCC / 3AMol sieves, CH_2Cl_2,
(iv) $^{15}NH_4Cl_{(aq)}$, KCN, MeOH, (v) conc^{n} HCl.

Figure 4-17 Synthesis of DL-[2-$^{13}C^{2}H^{15}NH_2$]-phenylalanine

The methyl ester of [1-^{13}C]-Phenylacetic acid (Aldrich) (4.21)was prepared in high yield using a solution of diazomethane in diethylether, generated from Diazald. After purification over silica the methyl ester (4.22) was reduced with a suspension of lithium aluminium deuteride in ether. Analytically pure [1-$^{13}C^{2}H_2$]-phenylethanol (4.23) was obtained in high yield after purification over silica. The oxidation of the alcohol to [1-$^{13}C^{2}H_2$]-phenylacetaldehyde (4.24) gave capricious yields with pyridinium chlorochromate and molecular sieves in dichloromethane. However previous experience in the group with this reaction had found these to be the optimum oxidative reaction conditions, and the yields were sufficient to progress material through the route.[21] The aldehyde was unstable to polymerisation on storage, and was routinely used immediately after isolation. The Strecker synthesis of [2-$^{13}C^{2}H^{15}$N]-phenylalanine (4.25) from [1-$^{13}C^{2}H_2$]-phenylacetaldehyde, using an aqueous solution of [^{15}N]-ammonium chloride and potassium cyanide in methanol, was low yeilding. However with commercial phenylacetaldehyde (which contains stabilizers) high yields could be achieved. Furthermore the volume of water used had to be kept to a minimum, to avoid the production of phenyllactic acid and this could be minimised if excess [^{15}N]-ammonium chloride was used. Hydrolysis of the intermediate nitrile required two days reflux in concentrated hydrochloric acid. [2-$^{13}C^{2}$H]-Phenyllactic acid (4.26) that was produced as a byproduct was separated and purified over Dowex (H^+) and also used in feeding experiments described below.

The isotopically enriched signal DL-[2-$^{13}C^{2}H^{15}N$]-phenylalanine in the $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ NMR are shown below The $^{13}C^{15}N$ coupling of 5.9 Hz is clearly seen. A similar labelling pattern is anticipated for carbon-6 of the pyridone ring of tenellin, if intact incorporation occurs.

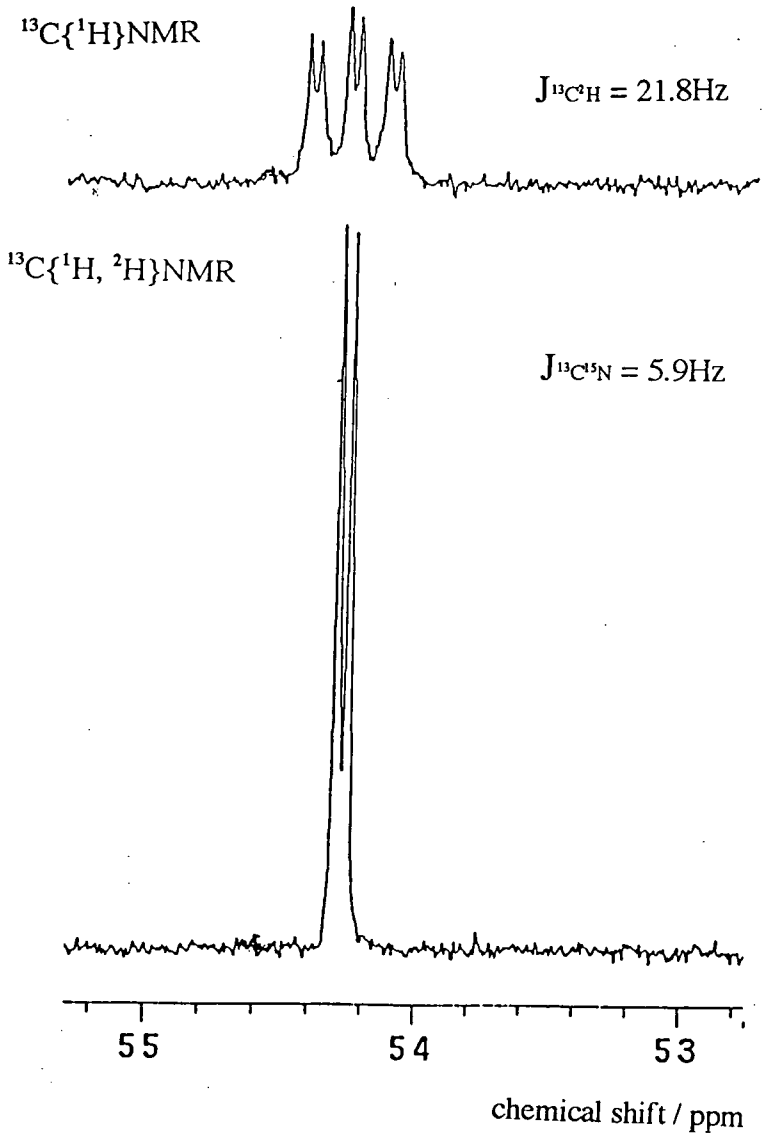


Figure 4-18 C-2 of ^{13}C NMR of [2-$^{13}C^{2}H^{15}N$]-phenylalanine

4.3.3 Feeding experiments with [2-$^{13}C^{2}H^{15}N$]-phenylalanine

DL-[2-$^{13}C^{2}H^{15}N$]-Phenylalanine was pulse fed to *Beauvaria bassiana* cultures to a final concentration of 4.5 molar during tenellin production. The resultant tenellin was purified and analysed by ^{13}C-NMR. The carbon-13 label had become incorporated into C-6 as expected. However there was no evidence of coupling to nitrogen-15 in the carbon-13 NMR spectrum, or of incorporation of an intact ^{13}CD unit (no observed α-shifted component). It is clear that the ^{13}C had become divorced from both ^{15}N and ^{2}H during the process. This reinforced the earlier result with [2-^{2}H]-phenylalanine and suggests a very facile transamination process operates. Alternatively a mechanism which requires these bonds to be broken in an obligate manner may operate.

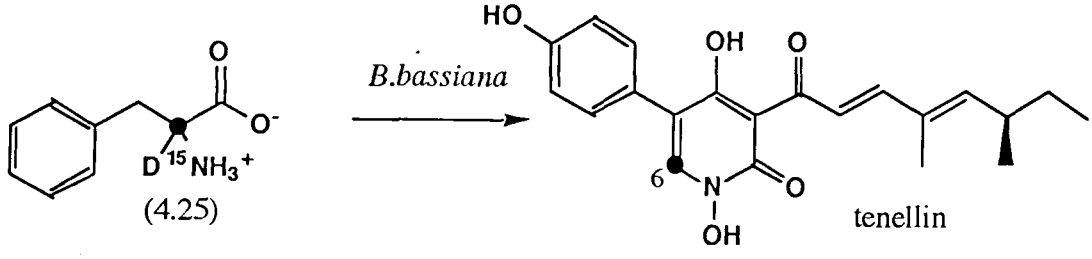


Figure 4-19 Incorporation of [2-$^{13}C^{2}H^{15}N$]-phenylalanine into tenellin

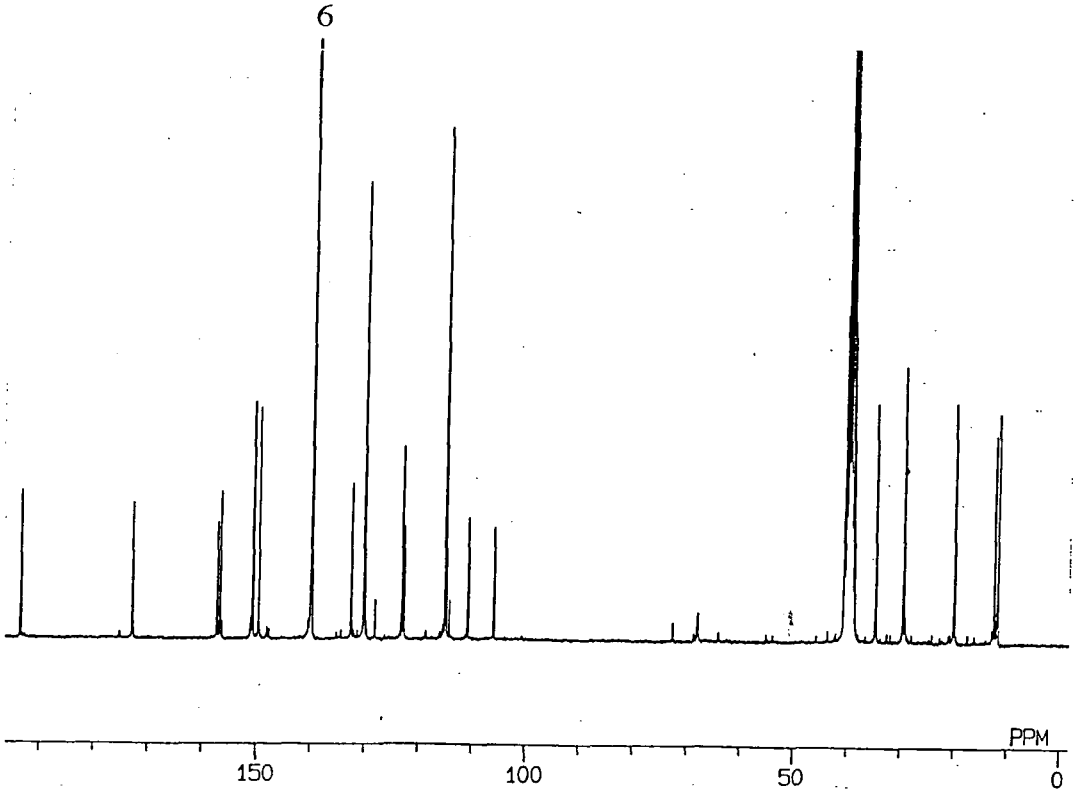


Figure 4-20 ^{13}C NMR of tenellin after feeding with DL-[2-$^{13}C^{2}H^{15}N$]-phenylalanine

It is pertinent to reflect on the early biosynthetic experiments on tenellin. Firstly it had been shown that ^{15}N-phenylalanine had become incorporated into tenellin and with the same efficiency as ^{13}C-phenylalanine. It had thus been concluded that phenylalanine is incorporated with the C-N bond remaining intact.[31] A similar conclusion had been drawn by Tanabe *et al.*[33] on the basis of his investigations into the biosynthesis of the fungal pyridone ilicicolin, discussed in Chapter 3. They observed ^{13}C-^{15}N coupling in the ^{13}C-NMR spectrum of ilicilolin after feeding the cultures with [^{15}N]-phenylalanine, and concluded that phenylalanine was incorporated without prior metabolism to phenylpyruvate.

However in the light of the triple labelled experiment with phenylalanine, these conclusions were almost certainly overinterpreted. When a large excess of labelled precursor is fed to the cells, the nitrogen pool is almost certainly flooded with ^{15}N from the labelled phenylalanine, and this can account for the credible incorporation. Razal *et al.*[34] have shown that during active phenylpropanoid metabolism in plants, the ammonia released by phenylalanine ammonia lyase and tyrosine ammonia lyase is efficiently recycled back to phenylalanine and tyrosine with glutamate serving as a shuttle. Clearly such a process, or the transamination to phenylpyruvate could be operating in *B. bassiana.* The triple labelling experiment does not support the intact incorporation of the C-N bond of phenylalanine.

4.3.4 Feeding experiments with [2-^{13}C^{2}H]-phenyllactic acid

In view of the similarity of the rearrangement during tenellin biosynthesis to that operating during the biosynthesis of tropic acid (Figure 4-12), phenyllactic acid (4.11) was investigated as a precursor to tenellin. Such a process would require transamination of phenylalanine to phenylpyruvate (4.10) followed by reduction by a phenylpyruvate dehydrogenase, and would account for the loss of ^{2}H and ^{15}N in the earlier experiment. Transamination would occur after condensation with the polyketide chain.

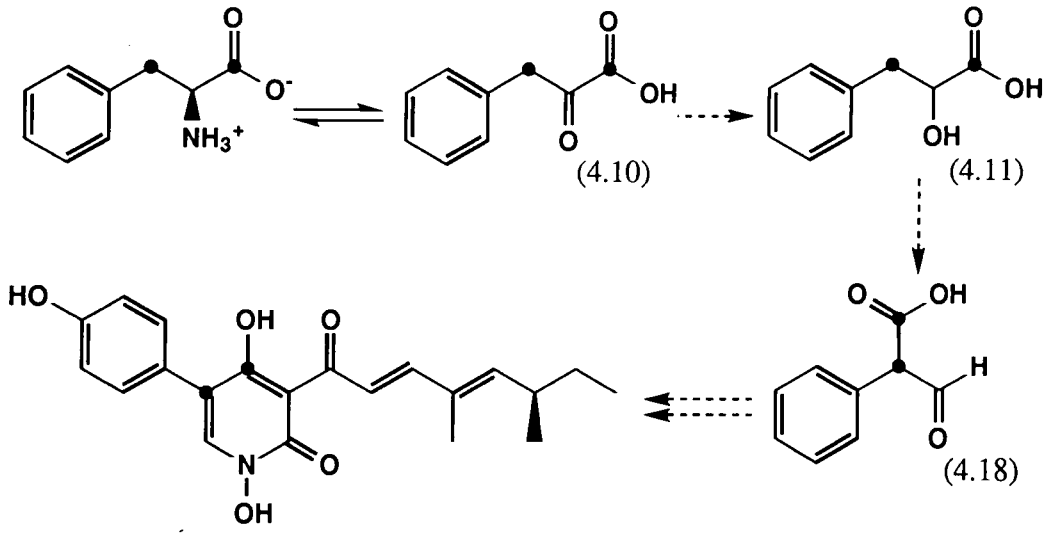


Figure 4-21 Alternative biosynthetic pathway for tenellin biosynthesis *via* phenyllactic acid

[2-$^{13}C^{2}H$]-Phenyllactic acid (4.26) was prepared as a byproduct of the synthesis of [2-$^{13}C^{2}H^{15}N$]-phenylalanine and was pulse fed to cultures of *B. bassiana* during tenellin production. In the event no incorporation was observed by ^{13}C NMR analysis of the isolated tenellin. Phenyllactic acid therefore, may be discounted as a true intermediate. Furthermore we may conclude from this negative result that *Beauvaria bassiana* does not have the requisite activities to convert phenyllactic acid (4.26) *via* phenylpyruvic acid (4.10), back to phenylalanine, a transformation which does appear to be reversible in the Datura species.[35]

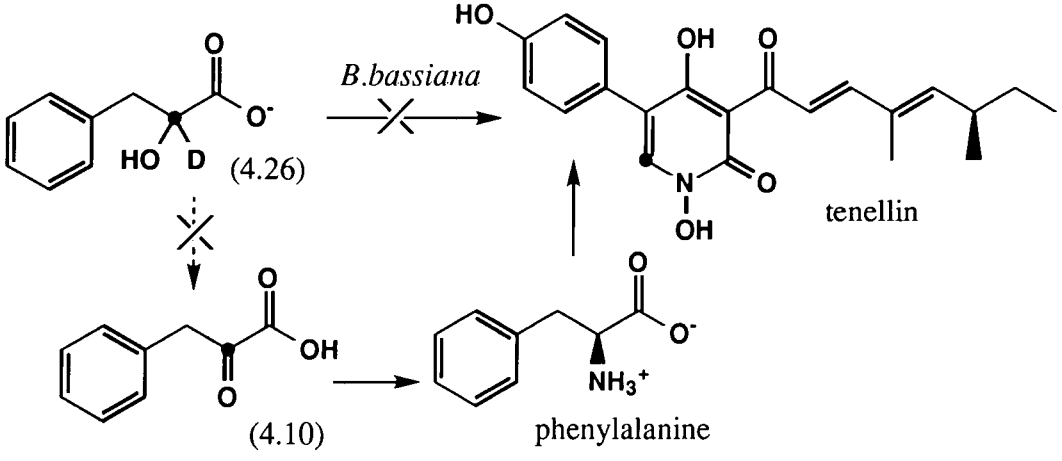


Figure 4-22 Phenyllactic acid is not incorporated *via* phenylpyruvic acid into tenellin

4.4 A Reinvestigation of the Intermediacy of Tyrosine

4.4.1 Feeding experiments with [3-^{13}C]-tyrosine

In the earlier biosynthetic studies on tenellin ^{14}C labelled tyrosine showed negligible incorporation when compared with the higher incorporation levels observed with ^{14}C-phenylalanine.[31] In retrospect, it is puzzling, that any incorporation of tyrosine should be observed at all if phenylalanine is the true precursor. Tyrosine is considerably less soluble than phenylalanine, and the results may have been a result of a solubility problem in its administration to the cultures. In the light of this, it was judged appropriate to repeat this feeding experiment, but with ^{13}C labelled tyrosine.

[1-^{13}C]-labelled phenylalanine (4.27) and [3-^{13}C]-labelled tyrosine (4.28) were administered to separate flasks of the same batch of *B. Bassiana* cultures, and at the same concentration (3.5 mM) in the media. Both amino acids required acidification followed by readjusting to pH6 to ensure solubility. Purification of the resultant tenellin and ^{13}C NMR analysis clearly showed that both amino acids had become incorporated into tenellin, phenylalanine at 8.5% and tyrosine at 6.1% enrichment.

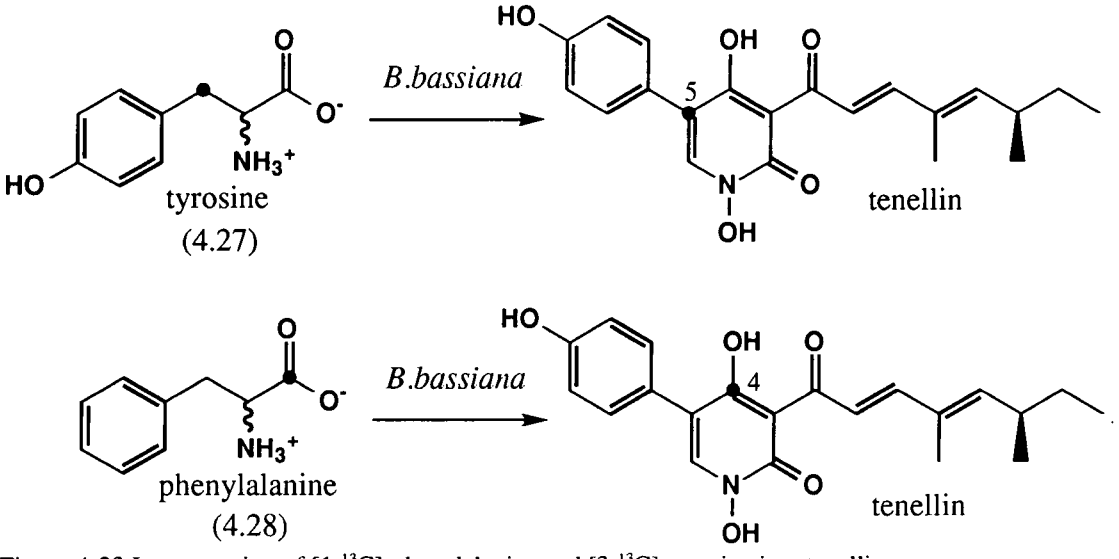


Figure 4-23 Incorporation of [1-^{13}C]-phenylalanine and [3-^{13}C]-tyrosine into tenellin

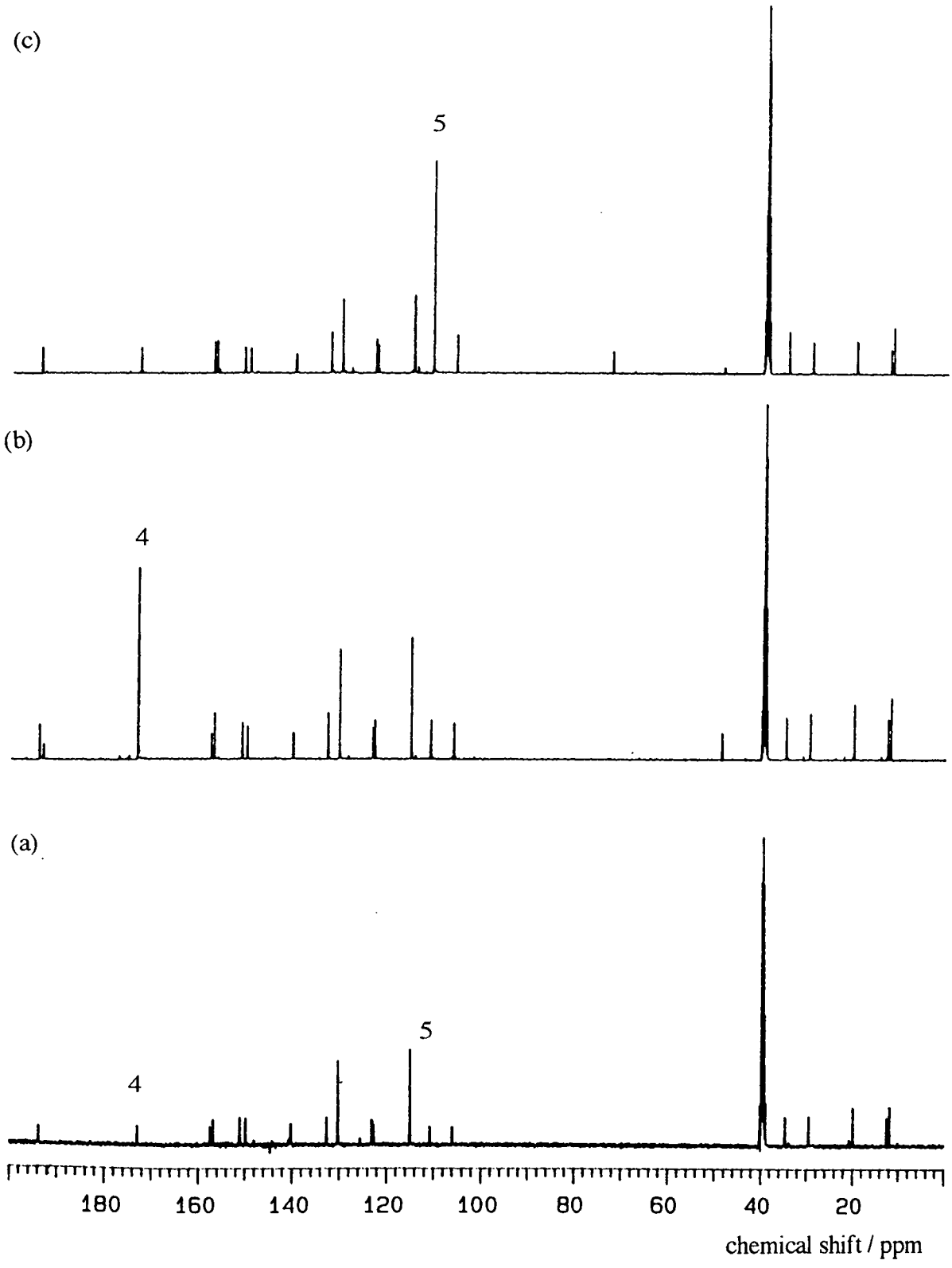
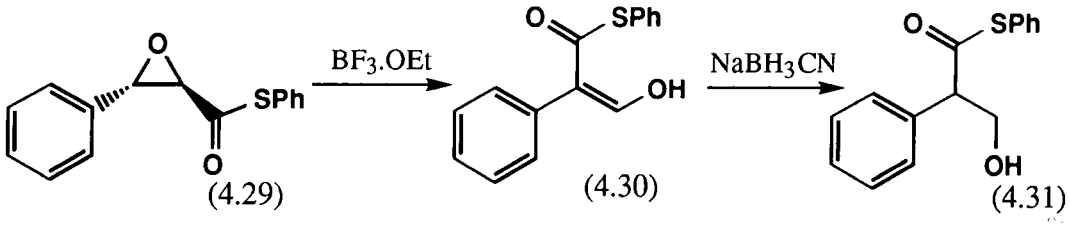


Figure 4-24 ^{13}C NMR of tenellin (a) natural abundance, (b) after feeding with [1-^{13}C]-phenylalanine, (c) after feeding with [3-^{13}C]-tyrosine

As discussed above, *para*-hydroxylation of phenylalanine to tyrosine is well documented in the fungi. Thus it may be concluded that phenylalanine is first converted to tyrosine prior to involvement in the biosynthesis of tenellin. This result opens a new spectrum of plausible intermediates not previously considered for tenellin biosynthesis. It is possible that tyrosine condenses directly with the polyketide chain to form the tetramic acid intermediate as discussed in the previous chapter. Alternatively however, it may rearrange prior to condensation with the polyketide chain. Such processes are discussed below.

4.4.2 *Para*-coumaric acid as a plausible intermediate?

The widespread occurrence of cinnamic acids in the biosynthesis of phenylpropanoids has led to the suggestion that they may be involved in the biosynthesis of tropic acid and tenellin.[36] It has been shown that the epoxycinnamate (4.29), when treated with boron trifluoride etherate, undergoes a rearrangement to α-formylphenylthiolacetate (4.30). Reduction with sodium cyanoborohydride generated the corresponding tropate ester (4.31).[37] It has been demonstrated that the thiol ester group migrates rather than the phenyl group.[38]

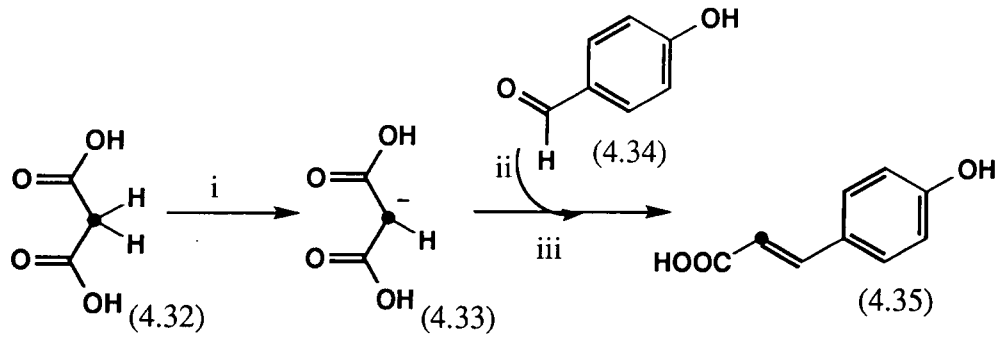


(4.29) (4.30) (4.31)

Figure 4-25 *In vitro* rearrangement of the epoxide of cinnamate

Feeding experiments to tropic acid in *Datura* species with the putative intermediates, cinnamic acid and its epoxide were unsuccessful.[39] However no such investigations have been reported with *Beauvaria bassiana*. In the light of a role for tyrosine, *para*-coumaric acid (*para*-hydroxycinnamic acid) emerged as a plausible intermediate.

4.4.3 Synthesis of *para*-[2-^{13}C]-coumaric acid and feeding to *B. bassiana*



(i) aniline, pyridine, 40°C, (ii) 4-hydroxybenzaldehyde, 65°C, (iii) conc^{n} HCl.

Figure 4-26 synthesis of [2-^{13}C]-*para*-coumaric acid

Para-[2-^{13}C]-hydroxycinnamic acid (*para*-coumaric acid) was synthesised *via* a standard Doebner reaction, according to the procedure of Adams and Bockstahler.[40] [2-^{13}C]-Malonic acid was dissolved in pyridine with a catalytic amount of aniline, and heated over sixteen hours with 4-hydroxybenzaldehyde. Aniline was found to be a superior base to piperidine[41] for condensations with *ortho-* and *para-* substituted benzaldehydes. After acidification the crude crystalline product could be purified by recrystallisation from ethanol / water.

An aqueous solution of the sodium salt of *para*-[2-^{13}C]-hydroxycinnamic acid (4.35) was prepared by neutralisation of the acid with dilute aqueous sodium hydroxide, for ease of admistration to *B. bassiana.* The solution was pulse fed to the cultures during tenellin production to a final concentration of 5 mM. ^{13}C NMR analysis of the tenellin harvested and purified after ten days showed no evidence of ^{13}C enrichment. Thus *para*-hydroxycinnamic acid does not appear to be an intermediate in the biosynthesis of tenellin, ruling out a sequence of events going from tyrosine to *para*-coumaric acid to epoxycinnamate.

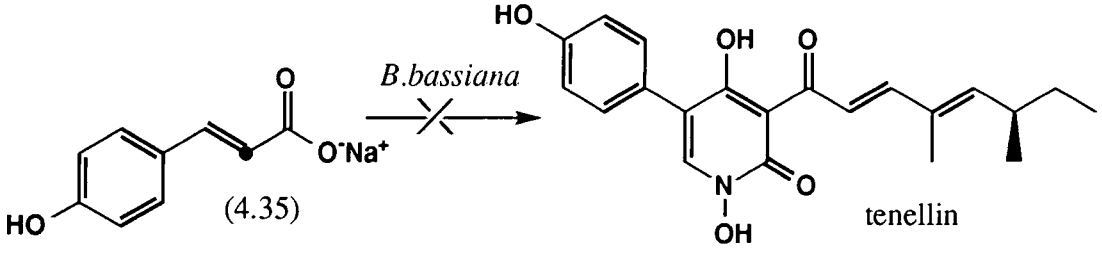
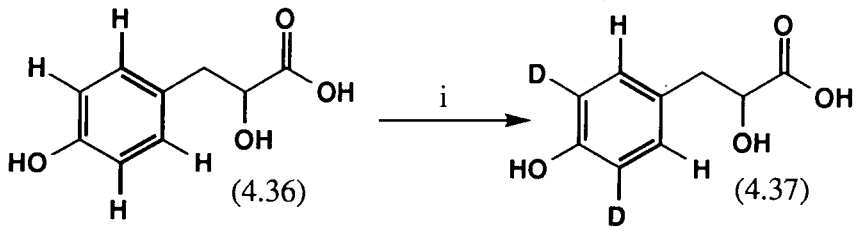


Figure 4-27 *Para*-[2-^{13}C]-coumaric acid is not incorporated into tenellin

4.4.4 *Para*-hydroxyphenyllactic acid as a plausible intermediate?

Although the intermediacy of phenyllactic acid in the biosynthesis of tenellin has been discounted, the apparent similarity of the rearrangement operating during the biosyntheses of tenellin and tropic acid remains intriguing (see Figure 4-12). Now that tyrosine has been shown to be a more immediate precursor than phenylalanine, *para*-hydroxyphenyllactic acid or an ester thereof, emerged as candidate substrates for the rearranging enzyme. It can be envisaged that tyrosine is transaminated to *para*-hydroxyphenylpyruvic acid which on reduction would give *para*-hydroxyphenyllactic acid. In an effort to test this hypothesis *para*-hydroxyphenyllactic acid was synthesised in two forms, with deuterium label both at the *meta*-position of the phenyl ring and also at carbon-2 of the propanoid moiety.

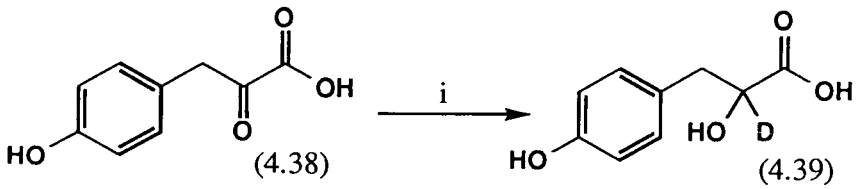
4.4.5 Syntheses of [*meta*-$^2H_2$]- and [2-^{2}H]- *para*-hydroxyphenyllactic acid



(i) 9 % DCl, D_2O, 80°C, sealed tube

Figure 4-28 Synthesis of DL -[*meta*-$^2H_2$]- *para* -hydroxy phenyllactic acid

DL-[*meta*-$^2H_2$]-*para* -hydroxy phenyllactic acid was synthesised from unlabelled DL-*para*-hydroxyphenyllactic acid by acid catalyzed exchange at the aromatic ring according to the published procedure of Brink and Jakobs.[42] Accordingly, DL-*para*-hydroxyphenyllactic acid was heated in a sealed tube with 9% deuterium chloride in deuterium oxide at 80°C. Extraction, followed by recrystallisation from ether, afforded the product in approximately 100% yield. ^{1}H NMR confirmed that deuterium exchange at the *meta*-position was almost quantitative.



(i) $NaBD_4$, H_2O

Figure 4-29 Synthesis of DL-[2-^{2}H]- *para* -hydroxyphenyllactic acid

DL-[2-^{2}H]-*Para*-hydroxyphenyllactic acid was synthesised by reduction of *para*-hydroxyphenylpuruvic acid using sodium borohydride. Although the yields were low using ethanol as the solvent, excellent yields could be obtained in aqueous media.

4.4.6 Feeding experiments with [*meta*-$^2H_2$]- and [2-^{2}H]- *para*-hydroxyphenyllactic acid

B. bassiana cultures were supplemented with [*meta*-$^2H_2$]- *para* -hydroxyphenyllactic acid (4.37) to a final concentration of 4.5 mM over four days. The resultant tenellin was isolated and purified by HPLC and subjected to ^{2}H NMR anaysis. It was particularly interesting that deuterium incorporation could readily be observed into tenellin, suggesting that this is indeed a true intermediate in the biosynthetic pathway.

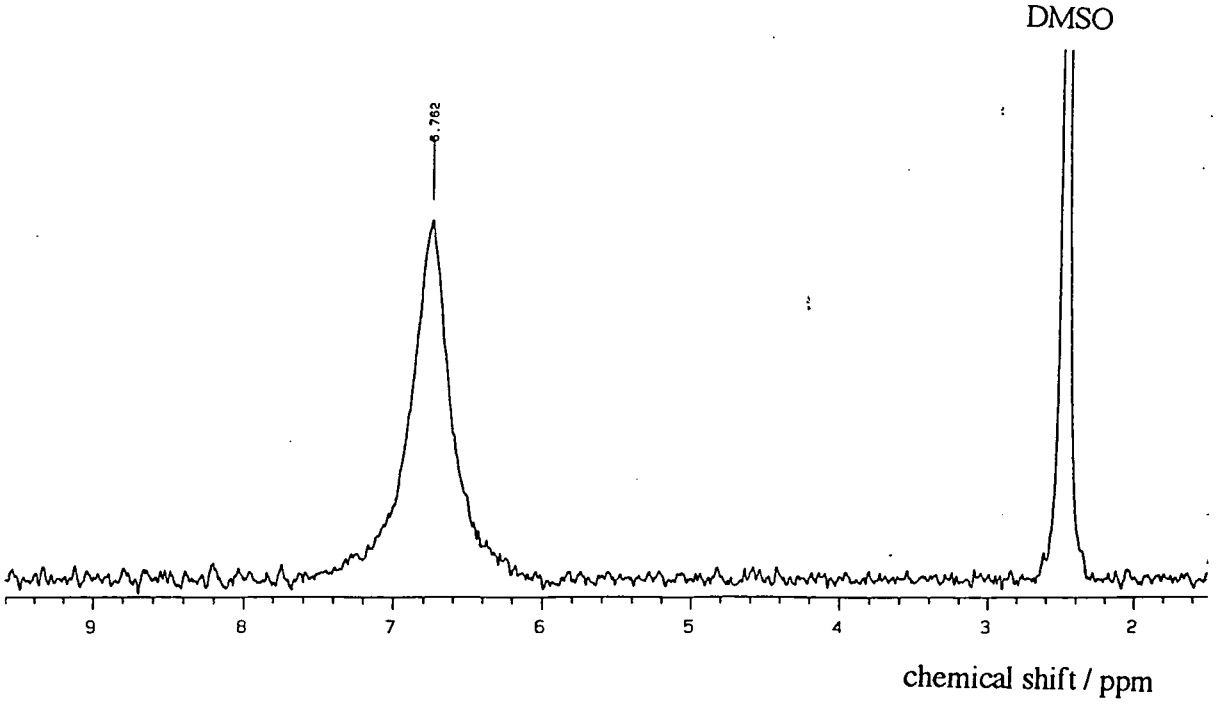


Figure 4-30 ^{2}H NMR of tenellin after feeding with [*meta*-$^2H_2$]-*para*-hydroxyphenyllactic acid

The results suggest that carboxyl group migration occurs in an analogous manner to tropic acid biosynthesis (see Figure 4-13). This may be after esterification with the polyketide chain, but prior to ring cyclisation. Alternatively another ester may be involved (route a, Figure 4-31). It cannot however be discounted that that *para*-hydroxyphenyllactic acid is first metabolised back to tyrosine (*via para*-hydroxyphenylpyruvic acid (4.41), route b) which is then incorporated into tenellin. This would involve the loss of the hydrogen attached to carbon-2 of *para*-hydroxyphenyllactic acid

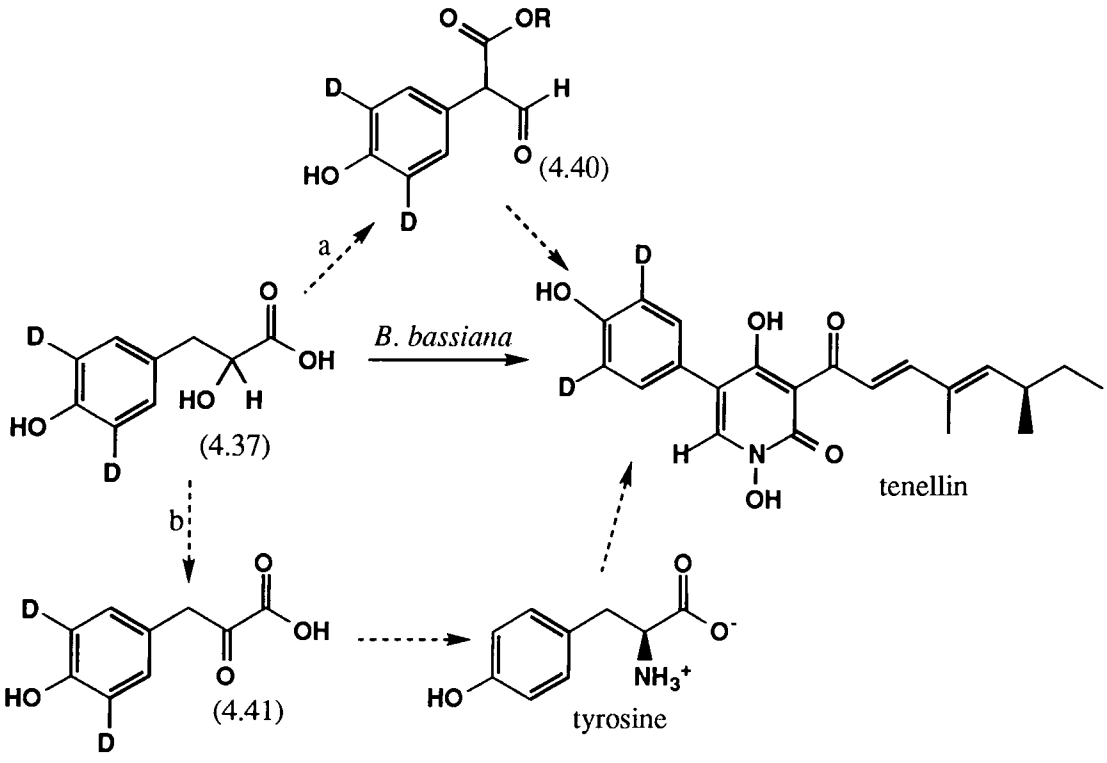


Figure 4-31 Incorporation of [*meta*-$^2H_2$]- *para* -hydroxyphenyllactic acid into tenellin

[2-^{2}H]-*Para* -hydroxyphenyllactic acid was then pulse fed to *B. bassiana* cultures, during tenellin production, to a final concentration of 5 mM. After isolation and purification of the resultant tenellin, analysis by ^{2}H NMR did not indicate any deuterium enrichment.

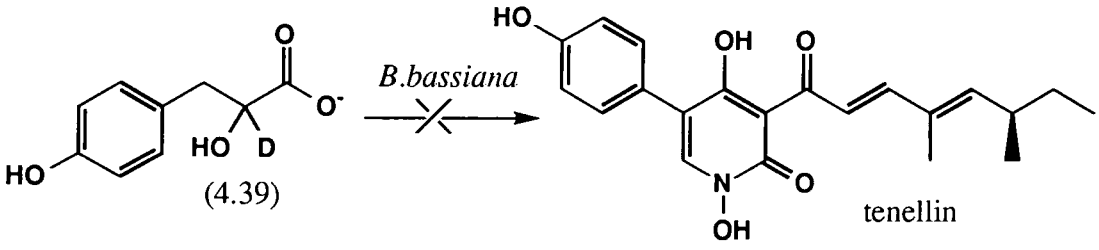


Figure 4-32 [2-^{2}H]-*Para* -hydroxyphenyllactic acid is not incorporated into tenellin

The incorporation of the deuterium label from [2-^{2}H]-*para*-hydroxyphenyllactic acid (4.39) would have established *para*-hydroxyphenyllactic acid as a direct intermediate, as metabolism to tyrosine prior to incorporation would result in the loss of this label. Rapid transamination to and from tyrosine *via* para-hydroxyphenylpyruvic acid (route b, Figure 4-31) may explain some of the loss of deuterium, but if *para*-hydroxyphenyllactic acid is a closer intermediate than tyrosine it is unlikely that all of the deuterium label would have been washed out. However, even though this hydrogen will be retained in a putative rearrangement process to (4.40) as illustrated in Figure 4-31, the deuterium may, infact, be lost in a later stage of tenellin biosynthesis, during the formation of the pyridone ring system.

4.4.7 A suggested mechanism for the biosynthesis of the pyridone ring

As described already for phenyllactate in tropic acid biosynthesis (Figure 4-13), a P-450 mediated carbocation or radical rearrangement process generates a rearranged aldehyde (4.18). A similar rearrangement may be envisaged for *para*-hydroxyphenyllactate as shown in Figure 4-33.

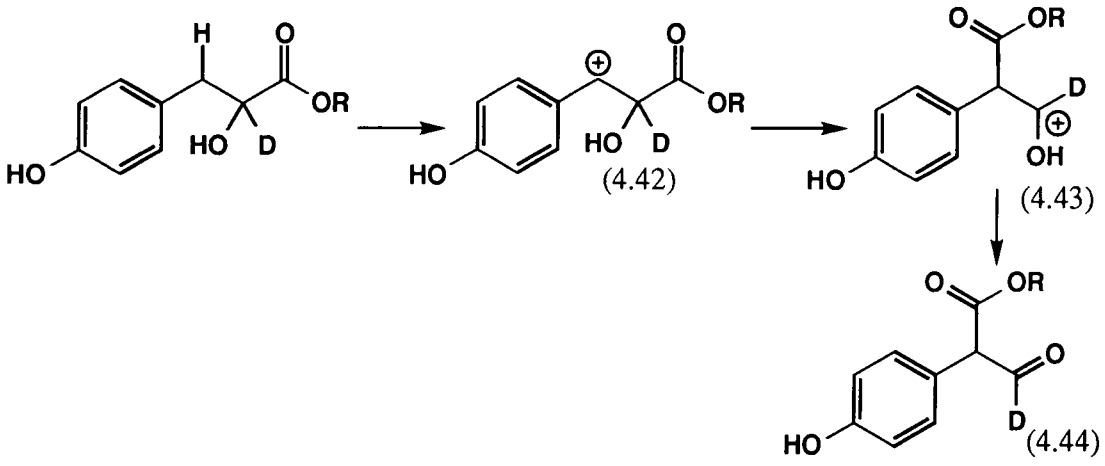


Figure 4-33 Putative mechanism for the P-450 mediated rearrangement of *para*-hydroxyphenyllactate

Transamination of the resultant aldehyde (4.44) would furnish the tyrosine isomer (4.45) which could condense with the polyketide moiety of tenellin (4.46) giving the reduced pyridone ring (4.47) directly. Deuterium should be retained up to this point. Dehydrogenation to the pyridone ring of tenellin (4.48) would however be stereoespecific and therefore may result in the specific loss of the deuterium at position-6 of the pyridone ring. Hydroxylation of the nitrogen of the ring completes the biosynthesis.

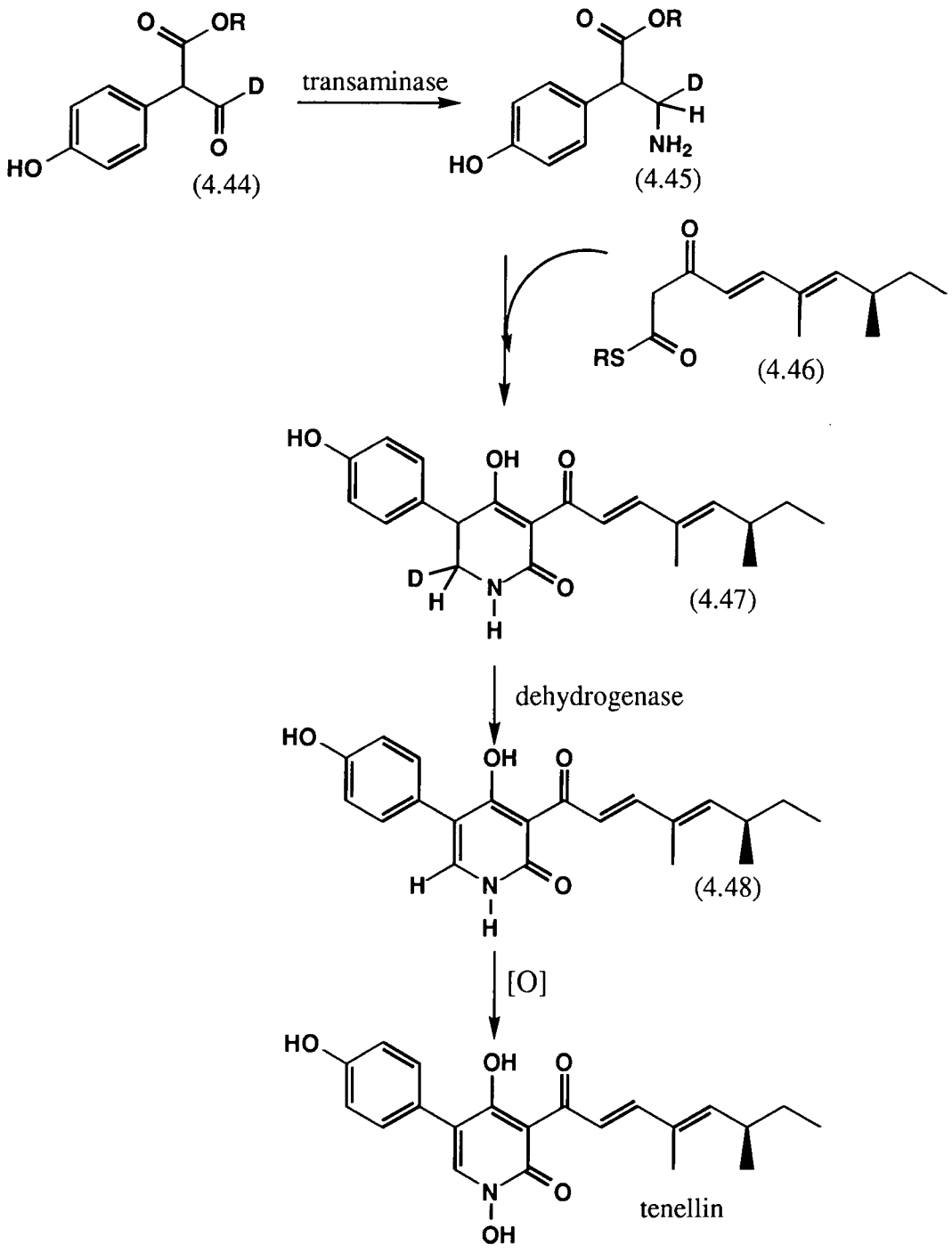


Figure 4-34 A biosynthetic pathway for the biosynthesis of tenellin from *para*-hydroxyphenyllactic acid, which fulfils all of the experimental data

4.5 Discussion

Although it remains possible that *para*-hydroxyphenyllactic acid is utilized only after metabolism to tyrosine (route b, Figure 4-31) it had previously been shown that no ^{13}C enrichment of tenellin was observed from [2-$^{13}C^{2}H$]-phenyllactic acid, implying that *B.bassiana* is unable to metabolise this *via* phenylpyruvic acid to phenylalanine (see Figure 4-22). In contrast, the incorporation of *para*-hydroxyphenyllactic acid suggests, at the very least, that *B. bassiana* does recognise and utilise this substrate in its phenylpropanoid metabolism.

Thus it is concluded that the mechanism elaborated in Figure 4-34 best explains the data to date. Further investigation is required to establish the role of *para*-hydroxy lactic acid. Firstly the relative incorporation levels of *para*-hydroxyphenyllactic acid and tyrosine should be compared. Although these may have differing solubilities and cell permeabilities it is likely to give an indication as to which is the closest percursor in tenellin biosynthesis. The successful incorporation of 3-amino-2-(*para*-hydroxyphenyl)propanoic acid (4.45) would provide further evidence for the suggested pathway, and this is now a target compound for labelled synthesis.

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PART 2

β-AMINOISOBUTYRATE METABOLISM

IN

STREPTOMYCES SP.

5. Aminoisobutyrate Metabolism in Streptomyces sp.

5.1 Introduction and Background

5.1.1 Bacterial polyketide biosynthesis

The Actinomycetes, and particularly the subclass Streptomyces, are soil bacteria which are able to produce a great variety of polyketides. These metabolites are generally structurally more complex than fungal polyketides due to the utilisation of propionate and butyrate units in addition to acetate units during biosynthesis. The incorporation of propionate and butyrate results in pendant methyl and ethyl groups respectively attached to the polyketide backbone.

Acetate, propionate, and butyrate units are activated by acyl-CoA carboxylases to give malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA respectively. The synthase enzymes are programmed to select each substrate in the correct order, and as the polyketide chain is modified by reducing enzymes the methyl and ethyl branches adopt either the D or L configuration.

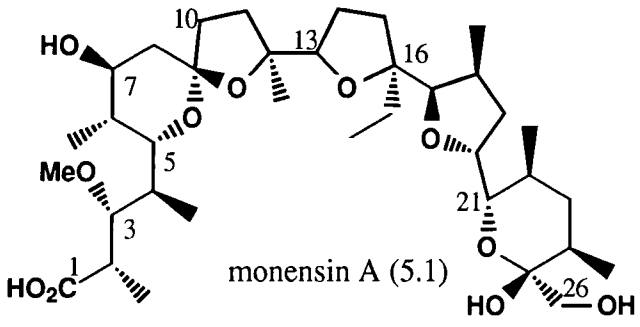
The malonyl-CoA precursors to the acetate, propionate and butyrate precursors from which the polyketide chain is assembled are also derived from the catabolism of various primary metabolites. Amino acids, fatty acids and the products of the citric acid cycle are known to contribute carbon atoms to the propionate and butyrate pools. Once cells enter the stationary stage of cell growth primary metabolites are broken down to these essential precursors and secondary metabolism can begin.

5.1.2 Biosynthesis of monensin A

Monensin-A (5.3) is a polyether antibiotic produced by *Streptomyces cinnamonensis*. It was first isolated in 1967.[1] Commercially it has been used as an antibiotic in the control of coccidiosis in poultry, and in the improvement of feed utilisation in ruminant livestock.[2] Polyether antibiotics consist of an array of tetrahydrofuranyl and tetrahydropyranyl rings, arranged in such a way that like crown ethers they are

ionophores, able to complex alkali metals. Their antibiotic activity is derived from this ability to complex sodium and potassium ions which results in the disruption of the Na^+ / K^+ balance across bacterial membranes. The polyethers are assembled from acetate, propionate and butyrate units, and it was suggested by Westley[3] that microbial epoxidation of diene or triene intermediates followed by a cascade cylisation could generate the tetrahydrofuranyl and tetrahydropyranyl rings.

The carbon atoms of monensin A (5.1) were first shown to derive from five acetate (5.2), seven propionate (5.3) and one butyrate (5.4) moiety after feeding experiments using ^{14}C-labelled precursors, followed by isolation and degradation of the isotopically labelled monensin.[4] The methoxyl carbon is derived from subsequent methylation of the hydroxyl functionality at C-3, by S-adenosyl methionine.



monensin A (5.1)

Figure 5-1 Monensin A

Monensin B, a co-product of monensin A from *S. cinnamonensis,* has a methyl in place of an ethyl group at C-16. This arises as a result of the incorporation of a propionate unit in place of butyrate during monensin biosynthesis.[5]

Carbon and proton NMR spectra of monensin A have been fully assigned allowing the use of ^{13}C and ^{2}H NMR spectroscopy to interpret the results of feeding experiments.[6] Cane *et al* , and Robinson *et al.* performed a series of feeding experiments with ^{18}O and ^{13}C labelled precursors identifying seven out of the eleven skeletal oxygen atoms as having their origins in the acetate, and propionate units.[7] When monensin A was biosynthesised under an atmosphere of $^{18}O_2$ it was found to be enriched at the other four sites.[8] Consistent with the Westley hypothesis, three of these enrichments can be rationalised if monensin assembly proceeds *via* an all-E-triene precursor (5.5) which

undergoes a series of epoxidations utilising molecular oxygen, to form a triepoxide (5.6). Attack of the C-5 hydroxyl at the C-9 carbonyl carbon would then initiate the cascade of ring closures to generate the polyether rings (Figure 5-2). The oxygen attached to C-26 was also shown to derive from atmospheric oxygen.[9] This hydroxylation is thought to be a post assembly modification, as a mutant has been isolated which produces 26-deoxymonensin.[10]

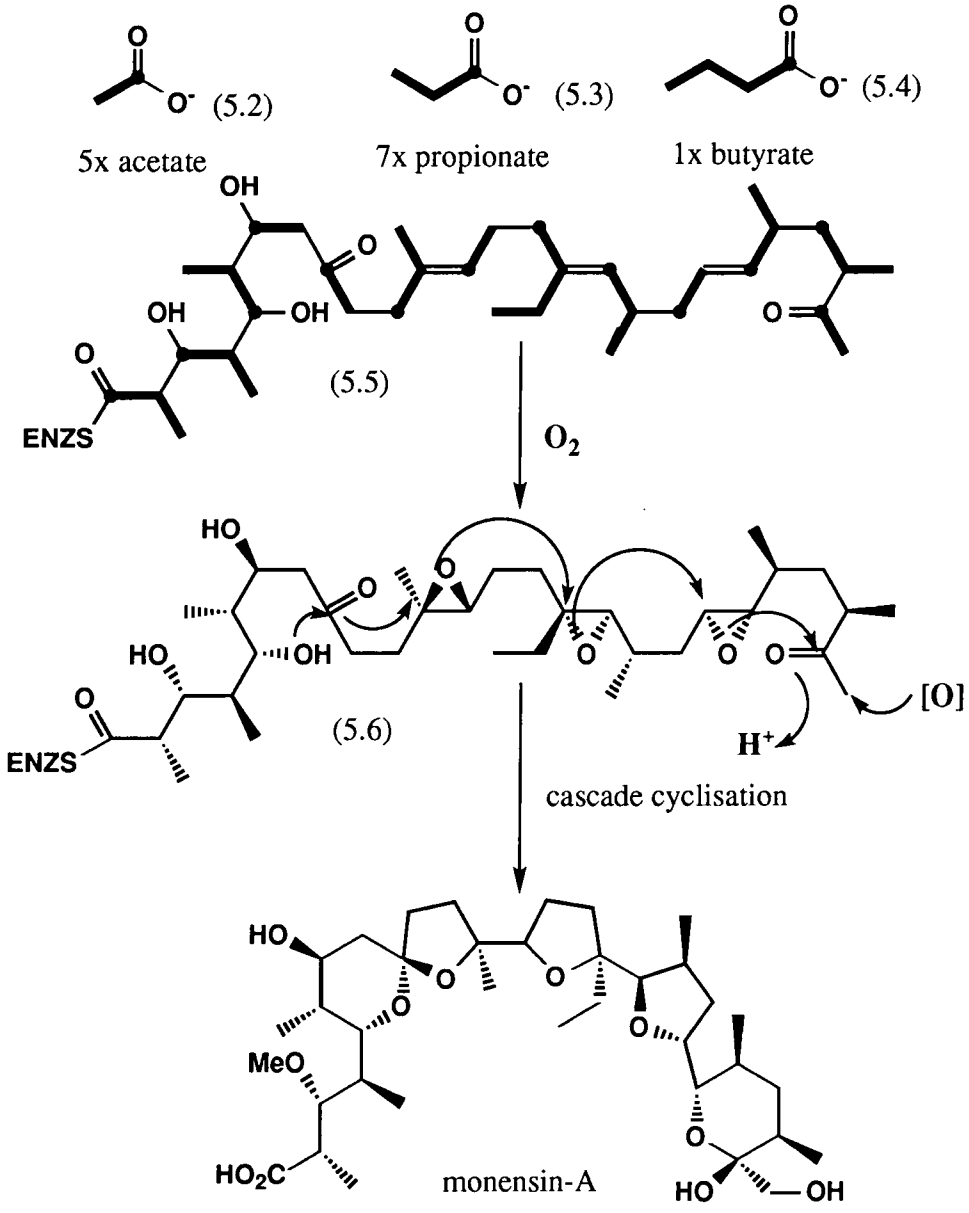


Figure 5-2 Proposal for monensin A biosynthesis from acetate, propionate and butyrate

5.2 Links between Primary and Secondary metabolism

5.2.1 Introduction

It is well established that the catabolic products of amino acids, fatty acids and the citric acid cycle are sources of the acetate, propionate and butyrate units necessary for polyketide biosynthesis. However the role of DNA bases in providing these units was only recently investigated, and is discussed below.

Studies on candicin production in *Streptomyces griseus*[11] and methylenomycin production in *Streptomyces coelicolor*[12] have demonstrated that the maximum concentration of antibiotic in the culture medium is reached when DNA synthesis ceases in the idiophase. It is therefore suggested that catabolic products of DNA may be utilised as substrates for secondary metabolism.

The role of the DNA base thymine as a contributor to the methylmalonyl-CoA pool (i.e. a precursor to the propionate units) and as a precursor to the butyrate units was first investigated by Sarah Rogers.[13] Although the relative importance of thymine as a source of methylmalonyl-CoA, as compared to other primary metabolites, was not investigated, it was demonstrated that both thymine and β-aminoisobutyrate are indeed efficiently incorporated into the propionate units and also the butyrate units of monensin-A.[14] Central to the following discussion are the catabolic pathways of L-valine and thymine.

5.2.2 The catabolism of L-valine

In both mammals and bacteria the amino acid L-valine contributes carbon atoms to the methylmalonyl-CoA and ethylmalonyl-CoA pools.[15] The importance of this catabolic pathway has been demonstrated for macrolide biosynthesis in actinomycetes.[16] L-Valine is catabolised to iso-butyryl-CoA (5.7) which contributes to the methylmalonyl-CoA (5.12) pool, presumably *via* the intermediacy of methacrylyl-CoA (5.10). Alternatively isobutyryl-CoA (5.7) is isomerised to butyryl-CoA (5.8) (discussed below), which is then activated to ethylmalonyl-CoA for incorporation into the polyketide.

It is also known that in mammals L-valine is catabolised to (S)-β-aminoisobutyric acid by the reversible transamination of S-methylmalonic semialdehyde (5.14),[20] as summarised in Figure 5-3 below.

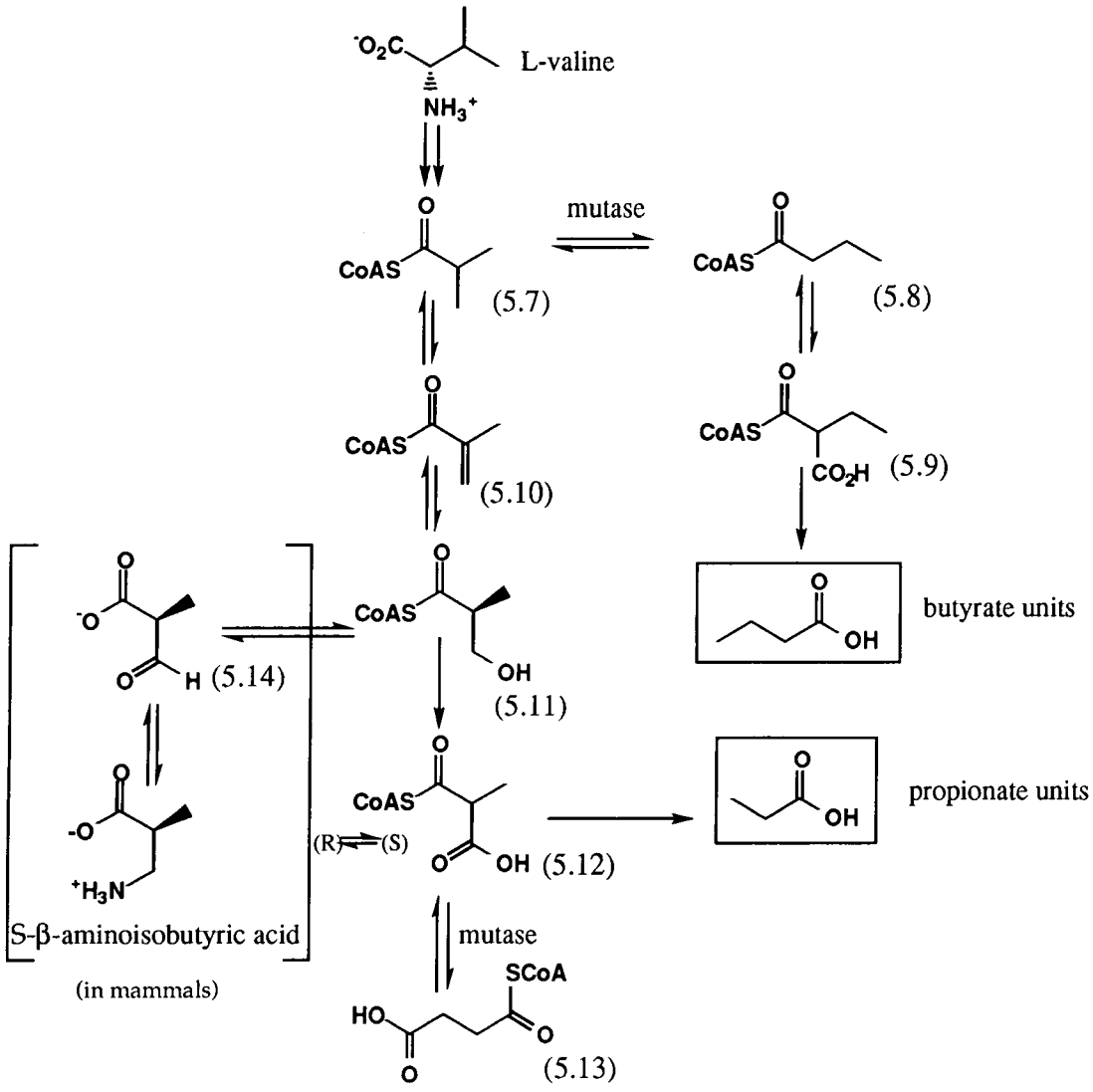


Figure 5-3 Catabolism of L-valine

Stereospecific interconversion of isobutyryl-CoA (5.7) and butyryl-CoA (5.8) is mediated by the co-enzyme-B_{12} dependent isobutyryl-CoA mutase. In an analogous manner to methylmalonyl-CoA mutase, this intramolecular rearrangement involves migration of the carboxyl carbon of isobutyryl-CoA to the 2-*pro*-S methyl group, with concomitant back migration of hydrogen, predominantly into the 3-*pro*-R position in butyrate. (Robinson *et al.* however, have shown recently that there is some loss of

stereospecificity in this process.[17]) This rearrangement is the pivotal link between straight and branched chain fatty acid metabolism in Streptomyces.[18]

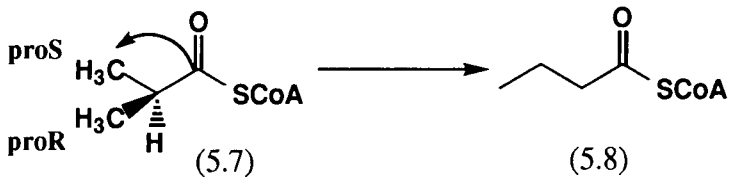


Figure 5-4 Action of isobutyryl-CoA mutase

The interconversion of the methylmalonyl-CoA (5.12) pool with succinyl Co-A (5.13) mediated by methylmalonyl-CoA mutase[19] also has important implications which will be considered in the following discussion of previous feeding experiments with isotopically labelled precursors.

5.2.3 The catabolism of thymine

Thymine (5.15) is catabolised to (2R)-β-aminoisobutyric acid (5.16) in mammalian systems as shown below.[20]

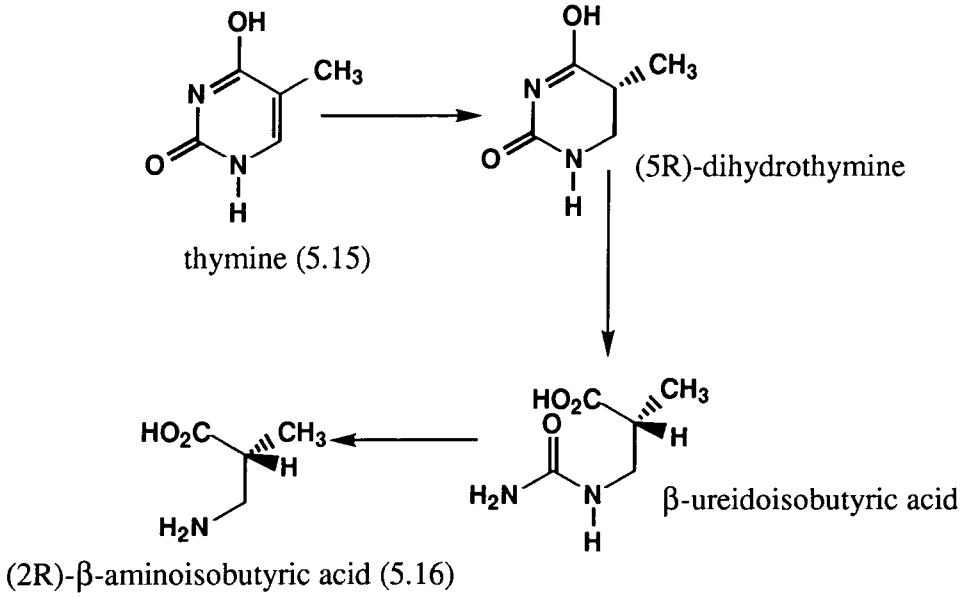
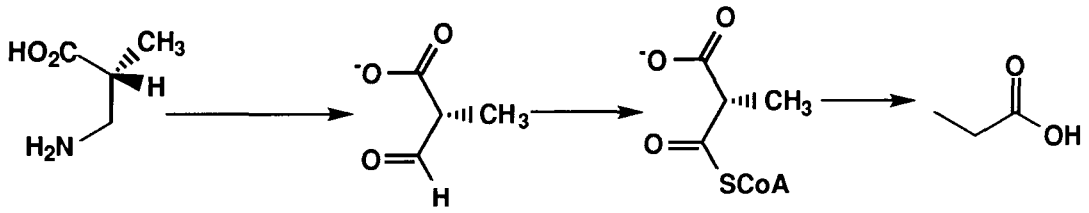


Figure 5-5 Catabolism of thymine in mammalian systems

A small number of microorganisms are known to degrade pyrimidines *via* this reductive pathway.[21] β-Aminoisobutyric acid may then be converted to (2S)-methylmalonyl-CoA (the activated form of propionate) *via* transamination to (R)-methylmalonic semialdehyde.



(2R)-β-amino-isobutyric acid | (R)-methylmalonic semialdehyde | (2S)-methylmalonyl-CoA | propionate units

Figure 5-6 Pathway from (2R)-β-amino-isobutyric acid to propionate units

Key intermediates of the thymine and valine catabolic pathways were synthesised by Rogers, labelled with ^{13}C and deuterium and used in feeding experiments to *S. cinnamonensis*.[13] The results of these studies are discussed below.

5.2.4 The results of previous feeding experiments

When [*methyl*- $^{13}C^{2}H_3$]-thymine was administered to cultures of *Streptomyces cinnamonensis* incorporation was observed not only into the propionate but also into the butyrate units.

It was clear from the $^{13}C\{^{1}H, ^{2}H\}$ NMR that there was a small peak corresponding to a $^{13}C^{2}H_3$, α-shifted to higher frequency from the natural abundance peak. This corresponds to carbon-33 of monensin A, resulting from the unexpected incorporation of a butyrate unit, labelled with an intact $^{13}C^{2}H_3$-*methyl* group. The enrichment was very small, (0.2 fold), but significant. The methyl groups derived from the propionate groups were also enriched (1-2 fold) but three components could be seen for each methyl group, α-shifted due to the attachment of one, two or three deuterium atoms, $^{13}C^{2}H^{1}H_2$, $^{13}C^{2}H_2{}^{1}H$, $^{13}C^{2}H_3$, in the ratio 1:2:3. There was clearly a partial loss of deuterium from the labelled methyl group occuring *en route* to the propionate units.

Propionate is derived *via* methylmalonyl-CoA (5.17) and the intramolecular rearrangement of (5.17) to succinyl-CoA (5.18), mediated by methylmalonyl-coA mutase, is well established. The operation of this enzyme results in hydrogen exchange and therefore readily explains the loss of deuterium from the methyl group of the propionate units. Conversely the intact incorporation of the $^{13}C^{2}H_3$-*methyl* into the butyrate units implies a pathway which does not involve the intermediacy of methylmalonyl-CoA.

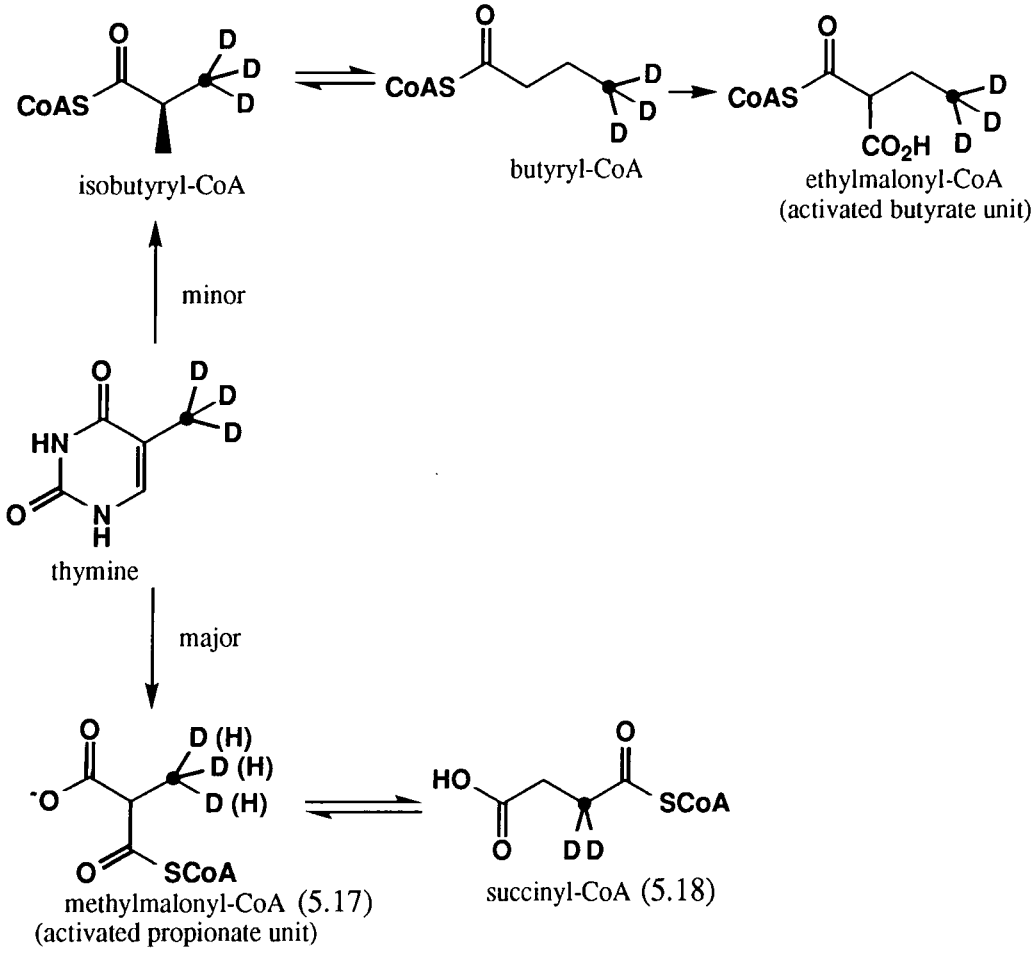


Figure 5-7 Origin of differently labelled propionate and butyrate units from [$^{13}C^{2}H_3$-*methyl*]-thymine

If thymine is degraded by a reductive pathway in Streptomycetes, similar to that known in mammals, it would expected that β-aminoisobutyrate would be similarly incorporated into monesin. This compound was synthesised bearing a ^{13}C label at the carboxyl group and also with a ^{13}C label at the methyl group, and administered to cultures of *S. cinnamonensis*. The results were consistent with those for thymine. (R,S)-[3-^{13}C]-β-Aminoisobutyrate labelled carbon-4 of the butyrate (1.5 fold) and carbon-3 of the

propionate groups (6-7 fold) in monensin A. The aminomethyl group becomes the *pro*-S methyl in isobutyrate.

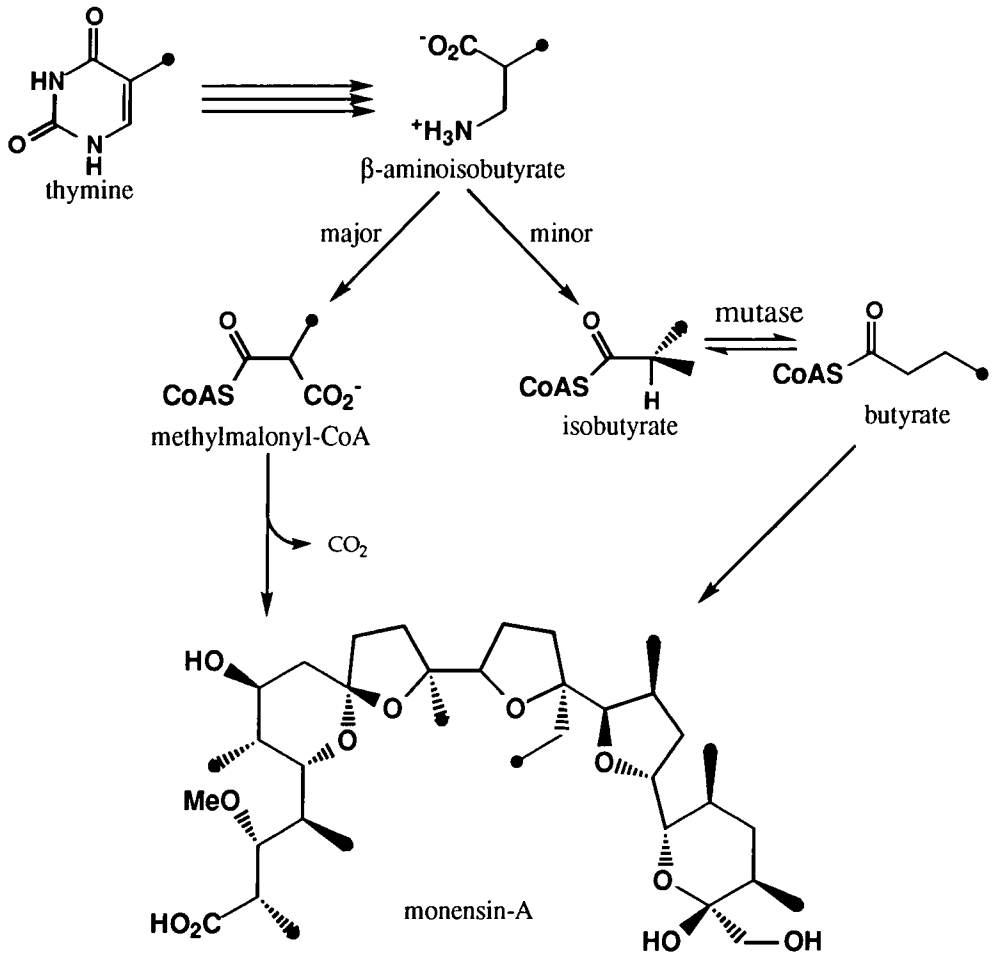


Figure 5-8 Incorporation of (R,S)-[3-^{13}C]-β-aminoisobutyrate into monensin A

(R,S)-[1-^{13}C]-β-Aminoisobutyrate labelled carbon-16 of monensin (2-3 fold), corresponding to carbon-1 of the butyrate unit, but was not observed to label the propionate units. This is consistent with the pathway outlined for the conversion of β-aminoisobutyrate to propionate *via* methylmalonyl-CoA, in which the amino-methyl carbon of β-aminoisobutyrate becomes the thioester carbonyl of methylmalonyl-CoA. Loss of the original carbonyl by decarboxylation results in loss of the isotope at carbon-1.

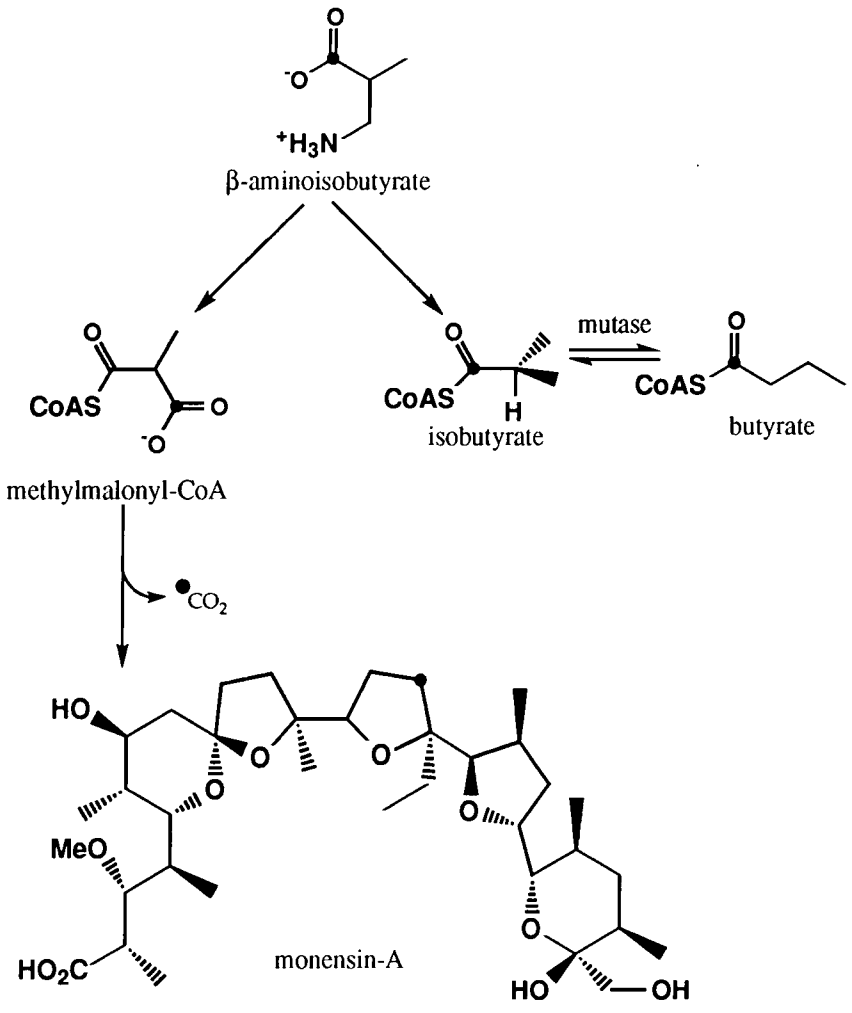


Figure 5-9 Incorporation of (R,S)-[1-^{13}C]-β-aminoisobutyrate into monensin A

Intermediates on the L-valine catabolic pathway, sodium (R,S)-[3-^{13}C]-isobutyrate and sodium [*methyl*-^{13}C]-methacrylate were also synthesised and administered to *S. cinnamonensis* cultures. (R,S)-Sodium [3-^{13}C]-isobutyrate was shown to enrich carbon-3 of all the propionate groups by a magnitude of 2.5 fold and carbon-2 and carbon-4 of the butyrate groups to a magnitude of 9 fold relative to the natural abundance signals. This is consistent with the catabolic pathway discussed for L-valine. Both carbon-2 and carbon-4 of the butyrate group in monensin A were enriched as the sodium [3-^{13}C]-isobutyrate used was a racemic mixture, half the molecules having the *pro*-R methyl labelled and half the *pro*-S methyl labelled.

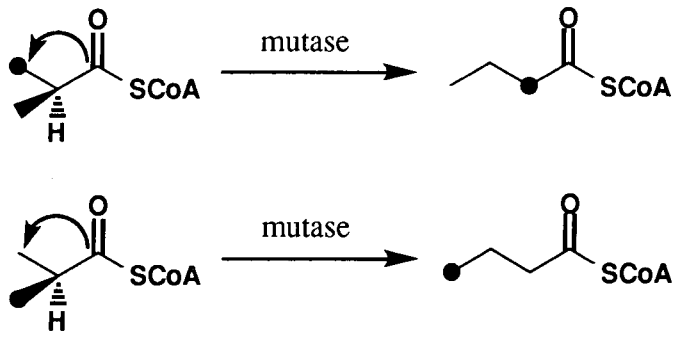


Figure 5-10 Action of mutase, resulting in differently labelled butyrate groups

^{13}C NMR analysis of the monensin-A isolated after feeding with sodium [*methyl*-^{13}C]-methacrylate showed carbon-3 of the propionate groups to be enriched by about 1.4 fold, and carbon-4 of the butyrate group of monensin A to be also enriched by a magnitude of 4.3 fold relative to the natural abundance signals. Methacrylyl-CoA must therefore be reduced in a stereospecific manner as previously described[22] to (R)-[3-^{13}C]-isobutyryl-CoA, followed by isomerisation to [4-^{13}C]-butyryl-CoA. Sodium [1-^{13}C]-methacrylate similarly showed incorporations into carbon-1 of the propionate groups with a 1 fold enrichment and into carbon-1 of the butyrate groups with about 3 fold enrichment relative to natural abundance signals. These results indicate that methacrylate is a true intermediate in valine catabolism in *S. cinnamonensis*, and that the interconversion of isobutyryl-CoA and methacrylyl-CoA is reversible. Methacrylate would appear to have been incorporated into β-hydroxyisobutyrate in *Pseudomonas putida* [22] and the labelling observed in the propionate units in monensin A suggest a simlar pathway in *Streptomyces cinnamonensis*. Of significance also, is the observation that isobutyrate and methacrylate feeding experiments give similar partition ratios for butyrate to propionate incorporation, approximately 3.5 : 1. This is consistent with the exogenously added materials intercepting the same metabolic pathway. Furthermore these labelling patterns are consistent with those observed for β-aminoisobutyrate.

A further question remains as to the route by which β-aminoisobutyric acid is incorporated into the butyrate unit of monensin-A. The above results indicate that methylmalonyl-CoA is not an intermediate, but rather that a more direct pathway, possibly *via* methacrylyl CoA to isobutyryl-CoA is operating. If this is the case then alternative pathways can thus be envisaged as shown in Figure 5-11.

- Route A, in which β-aminoisobutyrate is deaminated directly to methacrylate. This is attractive as it is a non redox process. (R)-β-Aminoisobutyrate, the catabolic product of thymine, could be the substrate for such a putative deaminase. However no β-aminoisobutyrate deaminase has been reported.

- Route B would proceed *via* methylmalonylsemialdehyde. This would be the reverse process of L-valine catabolism to (S)-β-aminoisobutyrate which occurs in mammals (Figure 5-3).

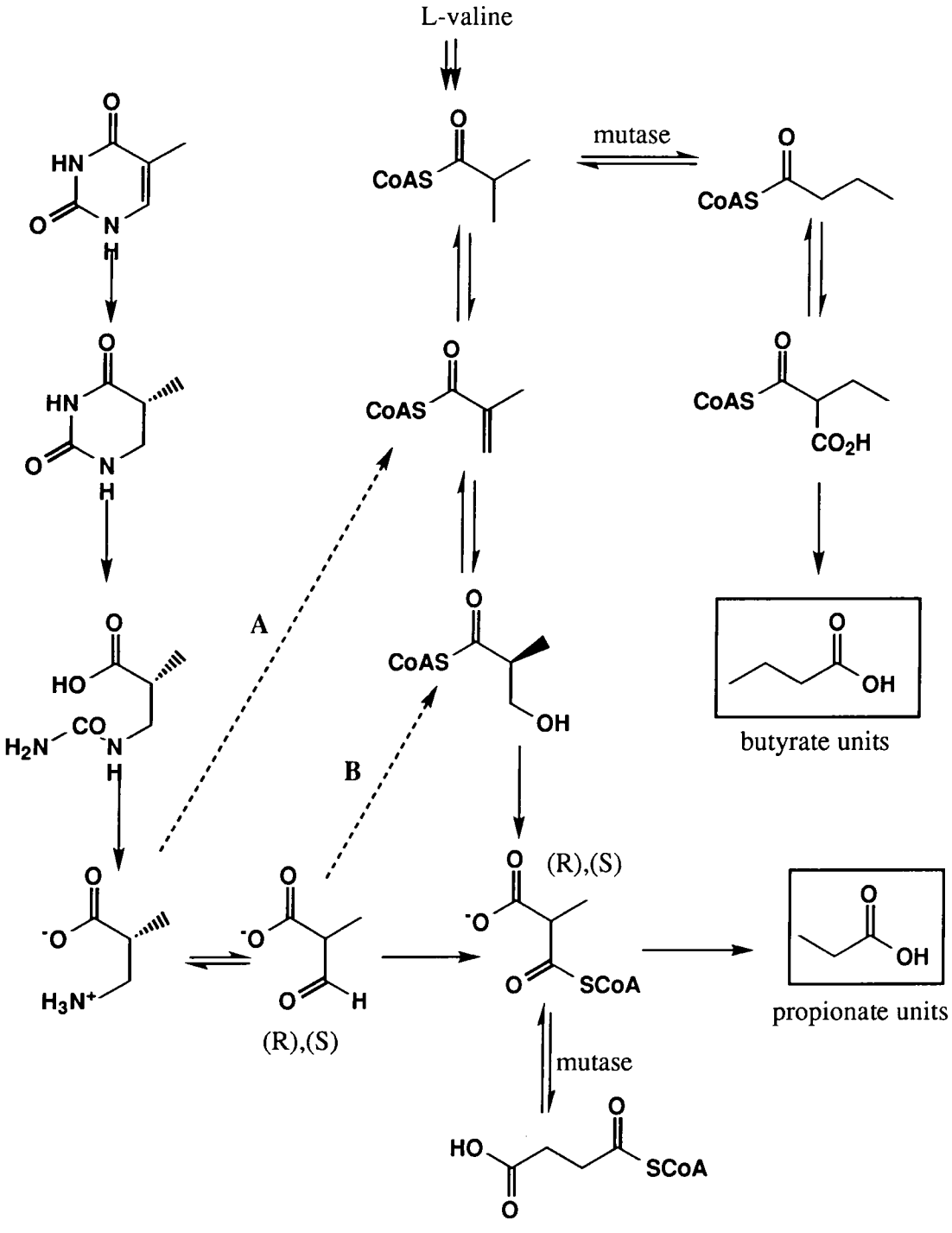


Figure 5-11 Links between thymine and valine catabolism and the propionate and butyrate units

The above figure summarises the links already established between thymine and L-valine catabolism and incorporation of the catabolic products into monensin-A.

5.3 Investigations to Delineate between Route A and Route B

5.3.1 The proposed strategy

In order to delineate between routes A and B, DL-[3-$^{13}C^2H_2$]-β-aminoisobutyric acid (5.22)was synthesised, and administered to *S. cinnamonensis*. Action of the deaminase enzyme on [3-$^{13}C^2H_2$]-β-aminoisobutyric acid will generate methacrylate *in vivo*, with retention of both deuterium atoms attached to the ^{13}C. Thus carbon-2 of butyrate would have a $^{13}C^2H_2$ label. Alternatively for route B only a single deuterium atom would be retained, as one deuterium atom would be lost during oxidation of β-aminoisobutyrate to methylmalonyl semialdehyde.

A complication immediately emerges however, as C-2 of butyrate, when incorporated into monensin-A, becomes C-16 of the antibiotic, a quaternary carbon, having lost both attached deuterium / hydrogen atoms from the precursor. However *S. cinnamonensis* also produces isopalmitic acid, an *iso*-C_{16} fatty acid which utilises isobutyryl-CoA as its starter unit. Analysis of incorporation of [3-$^{13}C^2H_2$]-β-aminoisobutyric acid into the starter unit of this fatty acid, rather than monensin A, should allow us to verify whether one or two deuterium atoms are retained, and thus delineate between pathways A and B.

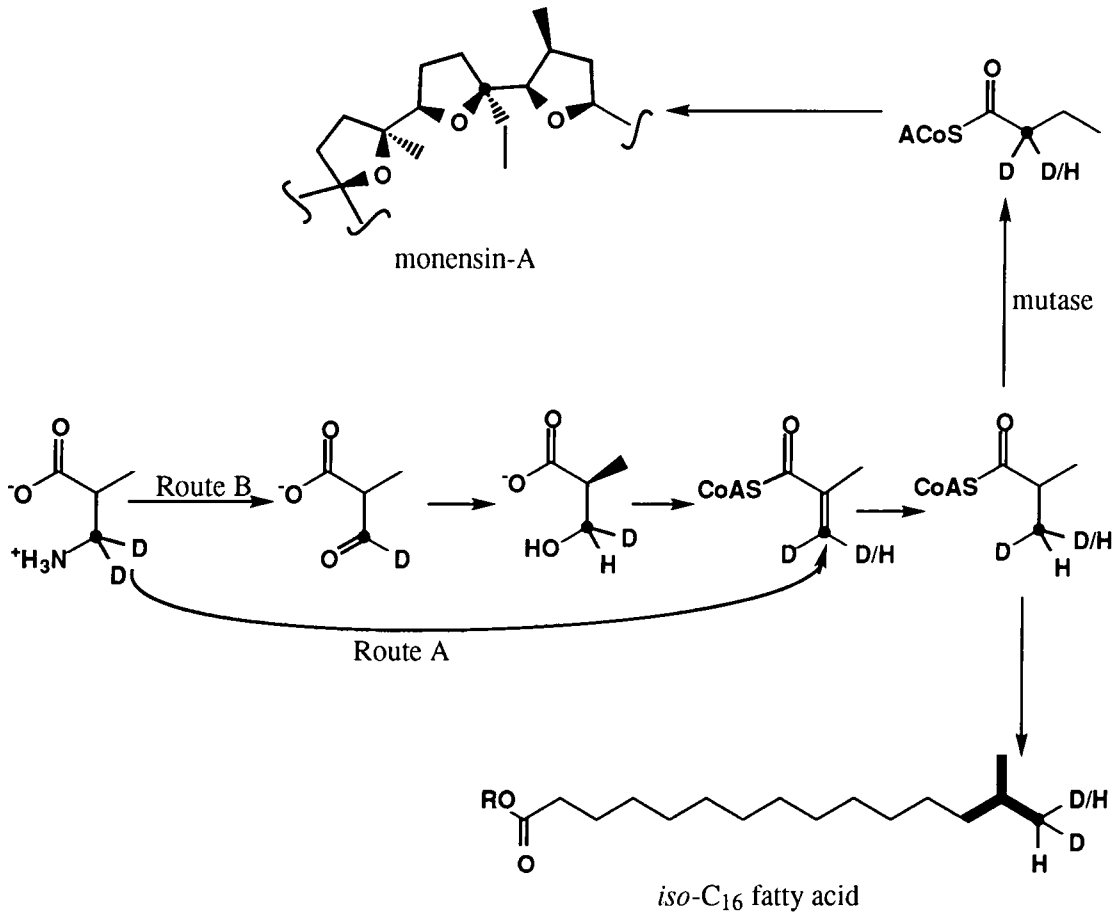


Figure 5-12 Incorporation of DL-[3-$^{13}C^{2}H_2$]-β-aminoisobutyric acid via route A into *iso*-C_{16} fatty acid and monensin-A

^{13}C-{^{1}H, ^{2}H} NMR of the fatty acid extract can be used to distingish between these routes. If both deuterium atoms are incorporated, an α-shift for the enriched methyl carbon corresponding to two directly bonded deuterium atoms will be observed (route A). If the pathway proceeds *via* route B then an α-shift corresponding to only one directly bonded deuterium atom will be observed. The coupling patterns observed in the ^{13}C-{^{1}H} NMR spectrum will further distinguish between incorporation of an $^{13}C^{2}H_2$ group or an $^{13}C^{2}H$ group. GCMS analysis of the fatty acid methyl esters (FAMEs) was used in the study, although there was concern at the outset that the anticipated low incorporations may be less than the threshold detection level for the M+2 peaks (due to natural abundance ^{13}C) by this method of analysis. Enrichment of the M+3 peaks may however be observable.

5.3.2 Culture maintenance of *Streptomyces cinnamonensis*

Production media of *S. cinnamonensis* were inoculated from frozen samples of production medium and cell growth was evident after a few days. Although monensin production was capricious branched chain fatty acids were consistently produced and it was possible to continue with these cultures for the DL-[β-$^{13}C^{2}H_2$]-β-aminoisobutyric acid feeding experiment.

5.3.3 Isolation of lipids from *Streptomyces cinnamonensis*

The production media was were first centrifuged and the pellet was washed thoroughly with phosphate buffer (pH7) before the lipid fraction was extracted. This was to ensure that all of the intracellular lipids were isolated, and the extracellular lipids discarded. 400 MHz ^{1}H NMR showed a clear doublet (0.8 ppm) corresponding to the terminal methyl groups of the branched lipids.

5.3.4 Analysis of the fatty acids by GCMS

Although GCMS proved too insensitive a technique to detect isotope incorporation accurately in the fatty acid fraction, it was used to confirm the presence of the *iso*-C_{16} lipid. The lipids first had to be prepared for analysis as methyl esters of the fatty acids (FAMEs). This required removal of the fatty acid chains from their triglycerides by saponification of the lipids with saturated KOH in methanol, followed by esterification with acidified methanol.

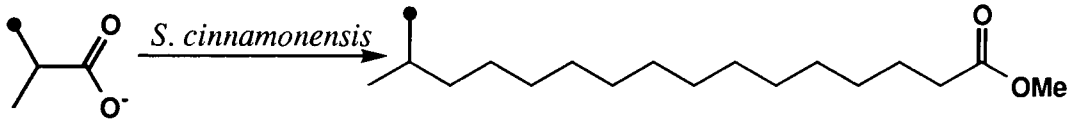
Mass spectroscopy under EI mode gave no observable molecular ion but comparison of the fragmentation patterns with library mass spectra confirmed the presence of methyl esters of fatty acids with a branched terminus. CI mode ionisation assigned two peaks from the gas chromatograph to have M^+ of 270, ($C_{17}O_2H_{34}$). Comparison with the EI spectra confirmed that the first of these to elute off the gas chromatography column was the iso-C_{16} fatty acid (isopalmitate). Straight chain palmitate eluted soon afterwards.

The M+2 ion of the isopalmitate peak was 3-5% of the natural abundance M+1 peak. To establish unambiguously accurate incorporation of deuterium alone would therefore

require at least 5% incorporation of the precursor into the fatty acid. However previous feeding experiments resulted in incorporations of only 4%, and thus the incorporations are below the reliable detection threshhold.

5.3.5 Assignment of the methyl groups for isopalmitate in the ^{13}C NMR

It was important at the outset to assign unambiguously the resonance in the ^{13}C NMR of the fatty acid extract corresponding to the methyl groups of the isobutyryl-CoA starter unit of iso-C16 fatty acid. This was achieved by a feeding experiment with sodium [3-^{13}C]-(R,S)-isobutyrate (5.19) which was pulse fed to *S. cinnamonensis* production medium cultures early in the growth cycle to a final concentration 10 mM. The isolated fatty acid extract was analysed by ^{13}C NMR, and incorporation of the label was clearly seen at 22.67 ppm as shown in Figure 5-14. A very high enrichment of the methyl groups of the branched chain fatty acid was observed with the peak height approximately 30 times greater than the natural abundance peak. The methyl esters of this fatty acid extract were also analysed by GCMS. An impressive 50% labelling of the M+1 peak of isopalmitate was observed.



(5.19)

Figure 5-13 Incorporation of sodium [3-^{13}C]-(R,S)-isobutyrate into isopalmitate

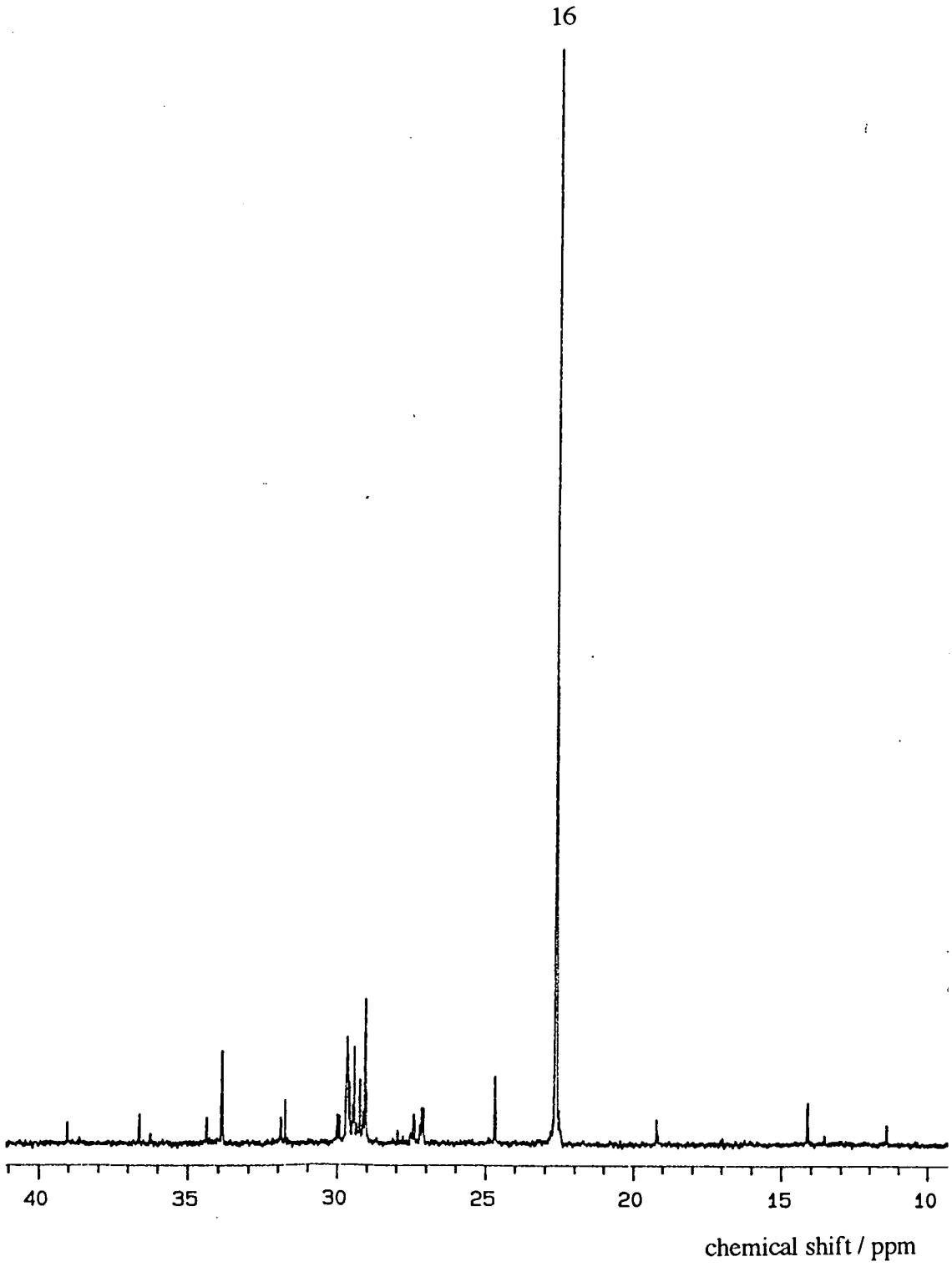
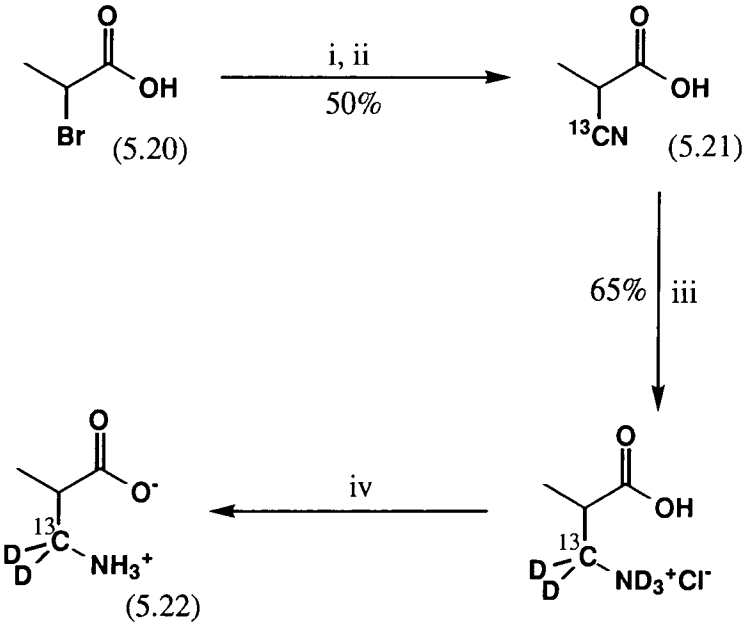


Figure 5-14 Section of the ^{13}C NMR of the fatty acid extract from *S. cinnamonensis* fed with sodium [3-^{13}C]-(R,S)-isobutyrate

5.3.6 Synthesis of DL-[3-$^{13}C^{13}H_2$]-β-aminoisobutyric acid

The synthetic route developed for DL-[3-$^{13}C^{2}H_2$]-β-aminoisobutyric acid (5.22) is shown in below.



(i)Na_2CO_3, CH_3CN, r.t., 3h, (ii)$K^{13}CN$, KOH, H_2O, 65-80°C, 4h, HCl(aq),
(iii) D_2, PtO_2 in MeOD-$CDCl_3$ (10 : 1), 1 atm, r.t., 18h, (iv) H^+ Dowex.

Figure 5-15 Synthesis of DL-[3-$^{13}C^{2}H_2$]-β-aminoisobutyric acid

α-Bromopropionoic acid (5.20) was first converted to its sodium salt, then treated with an excess of potassium [^{13}C]-cyanide in potassium hydroxide solution to yield [2-^{13}CN]-α-cyanopropanoic acid (5.21). The latter reaction gave only impure product as a yellow oil, and in a moderate 41% yield. The major impurity (7%) was the starting material, α-bromopropionoic acid. A minor impurity (2.5%), detectable by NMR, was α-hydroxypropionic acid formed by hydroxide rather than cyanide substitution. The yield of desired product could not be increased by extending the reaction time or increasing the temperature. To maximise the ratio of product to starting material successive syntheses were attempted using increased ratios of potassium cyanide to sodium α-bromopropionate. A ratio of 1.2 : 1 (mol : mol) gave a much improved yield of about 60%. Increasing the ratio of potassium cyanide to sodium α-bromopropionate even higher to 1.5 : 1 (mol : mol) gave a product with much increased purity, but reduced

yield. Thus it was decided to settle for a lower yield (50%) to obtain the product with only a small amount of starting material present. This crude product oil was then reduced using deuterium gas and PtO_2 as a catalyst at room temperature in deuterated solvents. With non-deuterated solvents hydrogenation rather than deuteration occurred, indicating that the reaction proceeds by a process in which solvent hydrogens are labilised by the catalyst. After purification by Dowex (H^+ form), impurities were still present in the oil. These could be removed by recrystallisation in ethanol affording essentially pure DL-[3-$^{13}C^2H_2$]-β-aminoisobutyric acid (5.22).

5.3.7 Feeding of DL-[3-$^{13}C^2H_2$]-β-aminoisobutyric acid to *S. cinnamonensis* lipids

DL-[β-$^{13}C^2H_2$]-β-Aminoisobutyric acid (5.22) was pulse fed to *S. cinnamonensis* production medium cultures to a final concentration of 5 mM, early in the growth cycle as the fatty acids are produced during the trophophase. $^{13}C\{^1H\}$ NMR of the fatty acid extract isolated from the cultures showed *no* observable α-shift associated with the ^{13}C resonance at 22.7 ppm. *No* enrichment of the peak at 22.7 ppm was observed either, as would be expected from incorporation of ^{13}C with loss of both deuteriums. $^{13}C\{^1H, ^2H\}$ NMR of the fatty acid extract further confirmed that there was no observable incorporation of a $^{13}C^2H$ group, and there was no observable difference between the $^{13}C\{^1H, ^2H\}$ NMR and the $^{13}C\{^1H\}$ NMR spectra (Figure 5-16).

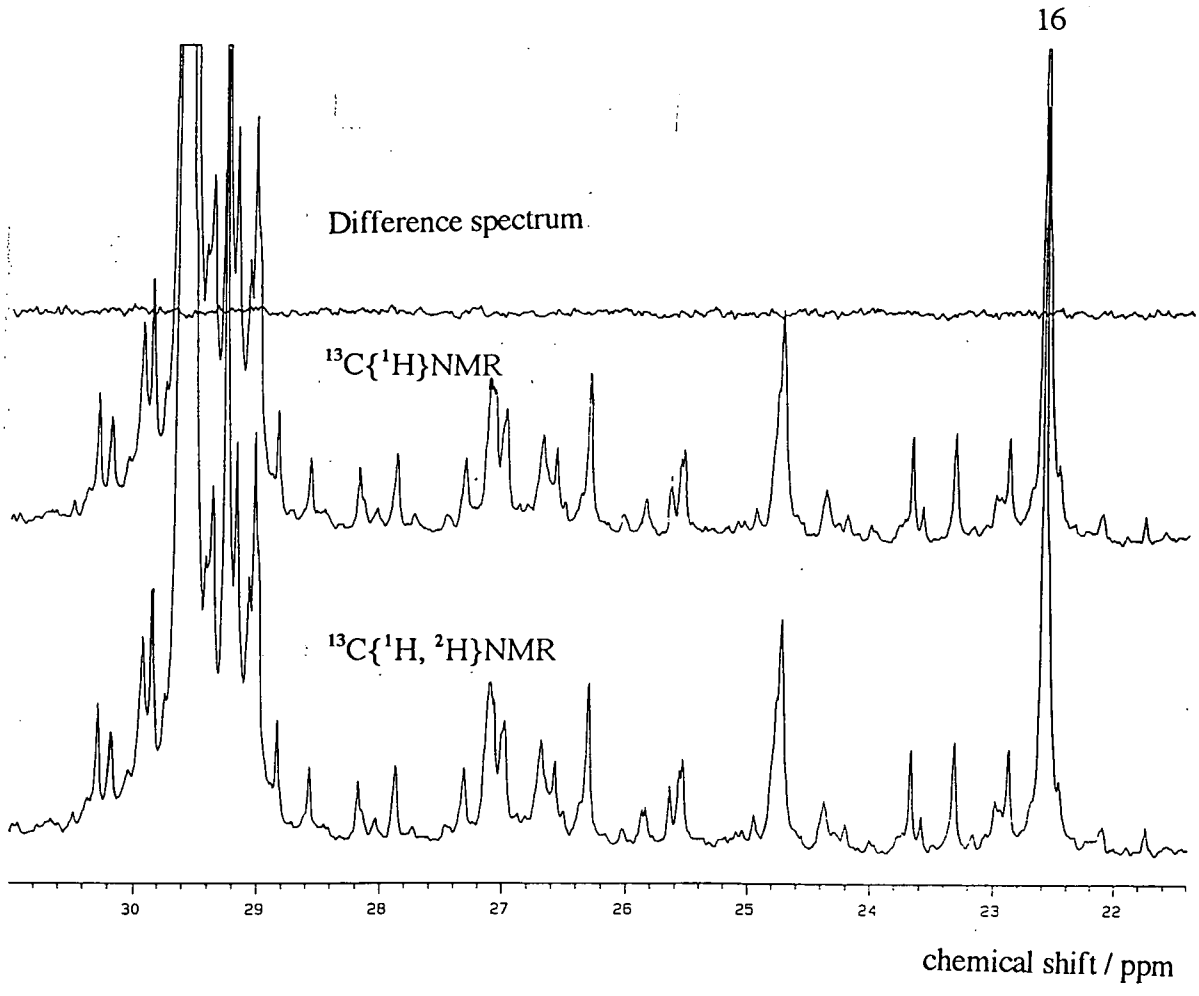


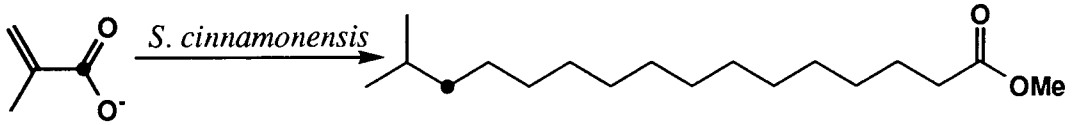
Figure 5-16 Section of the ^{13}C NMR of fatty acids extract from *S.cinnamonensis* fed with DL-[β-$^{13}C^{2}H_2$]-β-aminoisobutyric acid.

The absence of incorporation was somewhat unexpected, particularly as DL-[3-$^{13}C^{2}H_2$]-β-aminoisobutyric acid had previously been incorporated into the butyrate unit of monensin A at about 4%. The experiment was repeated using a higher concentration of DL-[β-$^{13}C^{2}H_2$]-β-aminoisobutyric acid (10mmolar), but again no incorporation was observed. The methyl esters of the fatty acid extracts fed with DL-[β-$^{13}C^{2}H_2$]-β-aminoisobutyric acid were analysed by GCMS. A tentative 4.5% labelling of the M+2 peak was observed, but this value is not within the expected limits of experimental error for such analyses.

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

Monensin-A is produced in the idiophase when cell growth is established, and it is at this stage that many of the primary metabolites produced in the trophophase are catabolised to provide the building blocks for construction of the secondary metabolites. As sodium [1-^{13}C]-methacrylate was shown previously to be a source of the butyrate units in monensin A, it was also pulse fed to production medium cultures of *S. cinnamonensis* early in the growth phase to establish whether earlier intermediates in the proposed pathway can be incorporated into the *iso*-C_{16} fatty acid.

Sodium [1-^{13}C]-methacrylate (5.23) was pulse fed to *S. cinnamonensis* production medium cultures to a final concentration of 6.5 mM, on days 0 to 3. $^{13}C\{^1H\}$ NMR of the fatty acid extract isolated from the cultures showed a clear enrichment (12%) at 38.03 ppm, as expected from labelling of the methylene adjacent to the isopropyl group of isopalmitate. This result suggests that the enzymes responsible for catalysis of the pathway are expressed in the trophophase. However incorporation levels of earlier intermediates may be too low to be observable. Thus another approach had to be taken to resolve this issue and this is discussed in the next section.



(5.23)

Figure 5-17 Incorporation of sodium [1-^{13}C]-methacrylate into isopalmitate

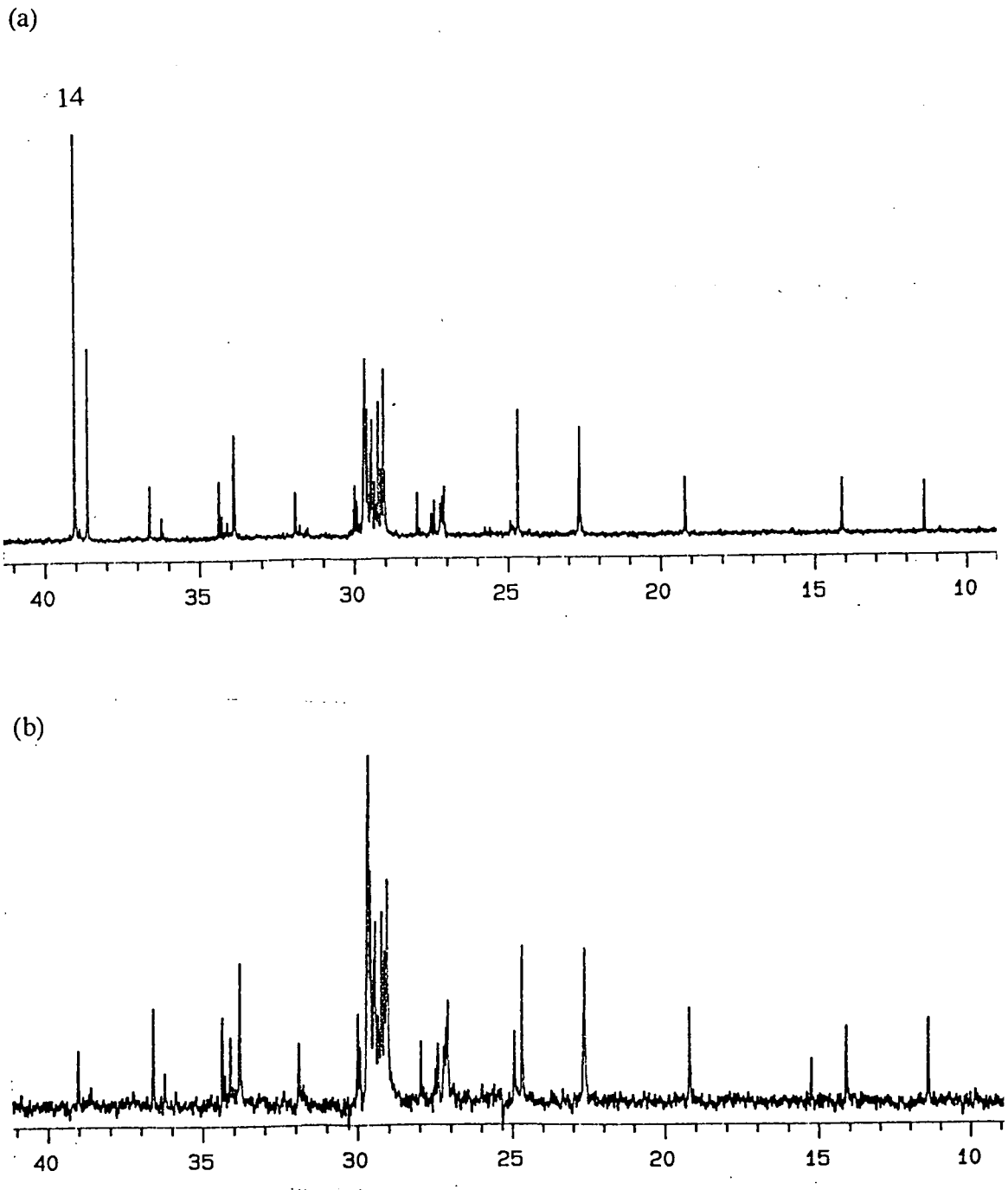
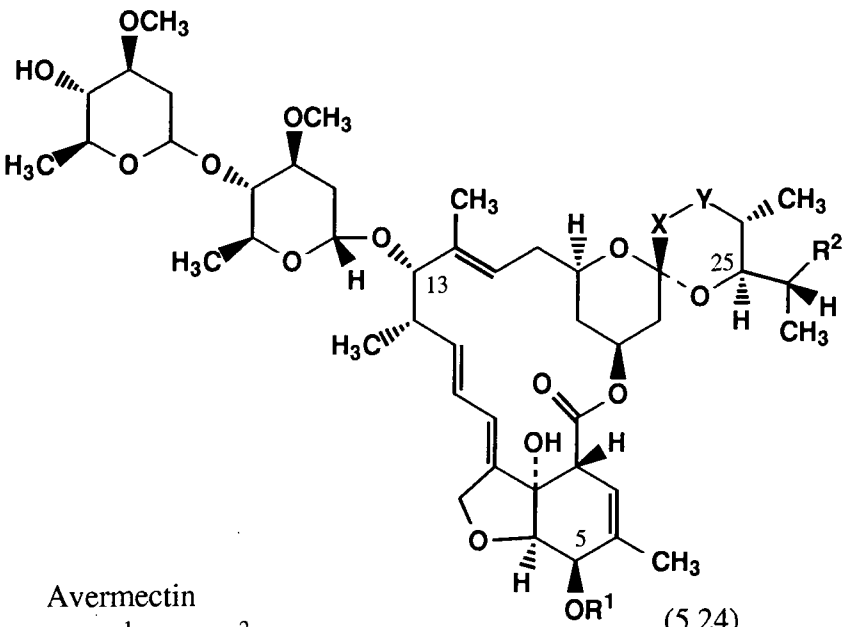


Figure 5-18 ^{13}C NMR of fatty acids extract from *S. cinnamonensis* fed with with (a) sodium [3-^{13}C]-methacrylate (5.23), and (b) natural abundance.

5.4 Studies with bkd negative *Streptomyces avermitilis*

5.4.1 Biosynthesis of the avermectins

The avermectins are a group of 16-membered macrolide polyketide metabolites produced by *Streptomyces avermitilis,* a gram positive filamentous soil bacteria. They consist of eight distinct, yet closely analogous structures, which can be subdivided into two groups of four. Group A generally predominate in bacterial fermentation and are derived from O-methylation at C-5 of the group B metabolites. They contain a spiroketal (C-17 - C-25), a feature common to many polyethers, as well as a 16-membered lactone, reminiscent of the macrolide antibiotics.



Avermectin (5.24)

A1a: $R^1 = CH_3$, $R^2 = C_2H_5$, X-Y = CH=CH
A1b: $R^1 = R^2 = CH_3$, X-Y = CH=CH
A2a: $R^1 = CH_3$, $R^2 = C_2H_5$, X-Y = CH_2-CH(OH)
A2b: $R^1 = R^2 = CH_3$, X-Y = CH_2-CH(OH)
B1a: R^1 = H, $R^2 = C_2H_5$, X-Y = CH=CH
B1b: R^1 = H, $R^2 = CH_3$, X-Y = CH=CH
B2a: R^1 = H, $R^2 = C_2H_5$, X-Y = CH_2-CH(OH)
B2b: R^1 = H, $R^2 = CH_3$, X-Y = CH_2-CH(OH)

Figure 5-19 Structure of avermectins

Despite their apparent structural similarities to the macrolide antibiotics e.g. tylosin, the avermectins appear to exhibit no antibacterial or antifungal activity. However they are

among the most potent anthelmintic, insecticidal and acaricidal compounds known. Avermectin B_{1a} is greater than 95% effective against gastrointestinal nematodes and lungworms of cattle at a single oral dose of 0.1 mg / kg, and is effective against hookworms of dogs at 0.005 mg / kg.[23]

As confirmed by incorporation experiments with ^{13}C and ^{18}O-acetates and propionates, the polyketide backbone is derived from seven acetate and five propionate units, extended from an α-branched chain fatty acid starter unit starter unit.[24] Incorporation of S(+)-α-methylbutyric acid gives rise to the major subgroup (a) and incorporation of isobutyric acid gives rise to the minor subgroup (b).

5.4.2 Sources of α-branched chain fatty acids

The primary metabolic route to the α-branched chain fatty acid starter units S(+)-α-methylbutyric acid and isobutyric acid, is the catabolism of the amino acids L-isoleucine and L-valine. This involves the action of branched-chain amino acid transaminase, followed by a branched chain α-keto acid dehydrogenase (BCDH) reaction. Branched chain fatty acids may also arise from branched chain α-keto acids produced by *de novo* synthesis. This is summarised in Figure 5-20.

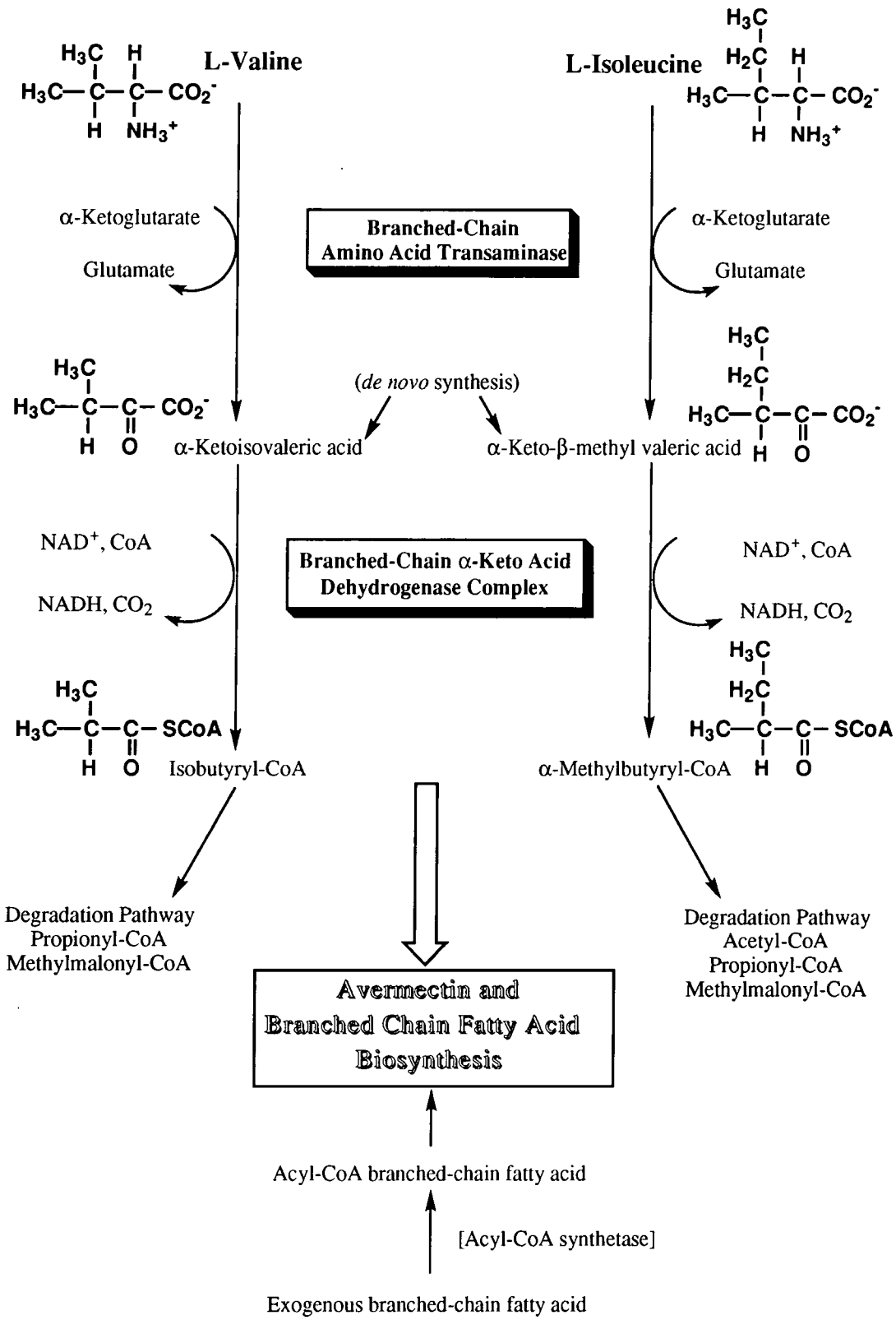


Figure 5-20 Pathways of L-valine and isoleucine catabolism and their relationship to avermectin biosynthesis

The branched-chain α-keto acid dehydrogenase (BCDH) complex is a multienzyme complex composed of four functional components: a BCDH and decarboxylase (E1α

and E1β), a dihydrolipoamide acyltransferase (E2), and a dihydrolipoamide dehydrogenase (E3). The BCDH complex catalyzes the oxidative decarboxylations of α-keto-isovalerate, α-keto-β-methylvalerate and α-ketoisocaproate (the deamination products of valine, isoleucine and leucine), generating the corresponding acyl-CoA analogs and NADH.

5.4.3 Mutagenesis of *S. avermitilis*

In order to test whether the degradative pathways for the 2-oxo acid analogues of isoleucine, leucine and valine provide the precursor acids required for avermectin biosynthesis, a mutant strain of *Streptomyces avermitilis* was isolated after chemical mutagenesis, in the Central Research Division of Pfizer. This mutant contained no functional branched chain α-keto acid dehydrogenase (BCDH) activity.[25] In contrast to its parent it was unable to grow with either a mixture of L-isoleucine, valine and leucine, or their α-keto derivatives, as sole carbon sources. The mutant grew nearly as well as the parent strain on glucose minimal medium or rich medium. Importantly it was incapable of synthesising natural avermectins in media lacking both S(+)-2-methylbutyric acid and isobutyric acid. Production of the corresponding four natural avermectins could be restored by supplementation with either of these acids. Thus it was concluded that branched chain 2-oxo acid degradation is not absolutely essential for acetate and propionate production, and that these precursors may be obtained from other sources, for example succinyl-CoA. Apparently this activity uniquely supplies the branched-chain fatty acid starter units. Again, supplementation with R(-)-2-methylbutyric acid yielded the novel isomeric avermectin, and several analogues of branched chain fatty acids have been shown to be similarly used by the mutant strain to synthesise novel avermectins[26]. Many of these new precursors are unlikely to be degraded to acetate and propionate lending support to the conclusion above that the cells can make these acids by at least one other route.

In order to understand further the relative importance of the BCDH-catalyzed reaction as a source of precursors for natural avermectin production, and to manipulate the production of these antibiotics, the gene cluster bkdFGH encoding the E1α, E1β and E2 components of an BCDH complex from *S. avermitilis* has subsequently been cloned and

characterised. Deletion of the genomic region comprising the 5' end of bkdF resulted in the construction of a mutant, which exhibited a typical Bkd- phenotype: it lacks E1 BCDH activity, and has accordingly lost the ability to grow on solid minimal medium containing L-isoleucine, leucine and valine as sole carbon source and is consequently unable to make the natural avermectins, or branched chain fatty acids.[27] Supplementation of the fermentation media with either one of these compounds restores production of the corresponding avermectin, bearing the isopropyl or (S)-*sec*-butyl group at C-25. This *bkd*F-disrupted strain is stable and can be used to generate and study the production of novel C-25 substituted antiparasitic avermectins in a large selection of avermectin production strains.

5.4.4 Thymine as a source of isobutyrate in avermectin biosynthesis?

Accordant with the studies with *Streptomyces cinnamonensis,* we predicted the catabolic products of thymine would provide an alternative source of isobutyrate in other Streptomyces species. β-Aminoisobutyrate should therefore be a precursor to isobutyrate, the starter unit in avermectin (b) biosynthesis. Feeding experiments with isotopically labelled β-aminoisobutyrate to *S. avermitilis bkd* mutant cultures became attractive as incorporation levels should be high. There will be no competition from unlabelled isobutyrate produced as a result of the catabolism of L-valine. Branched chain fatty acids produced by these mutants could also be analyzed for isotopic enrichments. Analysis of either the branched-chain iso-fatty acids or the avermectins after supplementation of the mutant cultures with DL-[3-$^{13}C^{2}H_{2}$]-β-aminoisobutyrate would enable us to differentiate between the two possible routes of incorporation: route A, implicating a deaminase enzyme or route B, proceeding *via* methylmalonylsemialdehyde. Incorporation of either a $^{13}C^{2}H$ unit or a $^{13}C^{2}H_{2}$ unit could be determined by GCMS analysis.

5.4.5 Feeding experiments to bkd-negative *S. avermitilis*

The following feeding experiments were performed by Dr Hamish McArthur at Pfizer Central Research Division, Groton, USA. GCMS analysis was undertaken by Dr Kevin

Reynolds at the Department of Pharmaceutical Science, University of Maryland, Baltimore.

Two flasks were innoculated with DL-[3-$^{13}C^{2}H_{2}$]-β-aminoisobutyrate to a concentration of 8.5 mM at the beginning of avermectin production, and the cultures were grown for 15 days, 29°C, 200rpm. Sample 1 was isolated from a culture grown in production medium. Sample 2 was isolated from a culture grown in particulate free production medium. These culture media were not free from potential precursors to branched chain fatty acids. For this reason a small level of iso-fatty acid and avermectin production is observed. However the levels of production are negligible, and production of the fatty acids and avermectins due to conversion of the putative intermediates administered to branched-chain fatty acid starter units would be expected to be considerable. In the event avermectin production was very low and only the fatty acid extracts were analysed for isotopic enrichments from DL-[3-$^{13}C^{2}H_{2}$]-β- aminoisobutyrate.

5.4.6 Analysis of the fatty acids

After work-up, both fatty acid extracts were converted to their methyl esters (FAMEs) for analysis by GCMS.

Two methods of MS anaysis were employed. a is more sensitive and therefore more accurate.

- a. MS was sweeping 240-280 AMU
- b. MS was sweeping 50-250 AMU

Significant incorporations were observed as evinced by the M+2 peaks of both isomyristate (*iso*-C_{14} fatty acid) and isopalmitate (*iso*-C_{16} fatty acid). There was no obvious increase in labelling of the M+1 peak for these fatty acids. Increase in labelling of the M+3 peaks could be attributed solely to natural abundance ^{13}C labelling of the M+2 species.

	M+2 labelling of isomyrisate *	M+2 labelling of isopalmitate *
Sample 1	29% (a) 28% (b)	12% (a) 15% (b)
Sample 2	12% (a) 14% (b)	14% (a) 15% (b)

* % values above natural abundance from reference spectra.

It is clear from these results that [3-$^{13}C^2H_2$]-β-aminoisobutyrate has been incorporated into the isobutyrate starter unit of the fatty acids, bearing only one deuterium atom attached to the ^{13}C. Enrichments were observed solely in the M+2 peak resulting from the incorporation of a $^{13}C^2H$ unit. This result is *inconsistent* with the action of a deaminase enzyme (route A) converting β-aminoisobutyrate directly to methacrylate. Rather the results strongly support route B. Thus it would appear that β-aminoisobutyrate is first oxidised to methylmalonyl semialdehyde, resulting in the loss of one deuterium atom, and then reduced to β-hydroxyisobutyric acid. This is the reverse of the pathway known to occur in mammalian L-valine catabolism.[28]

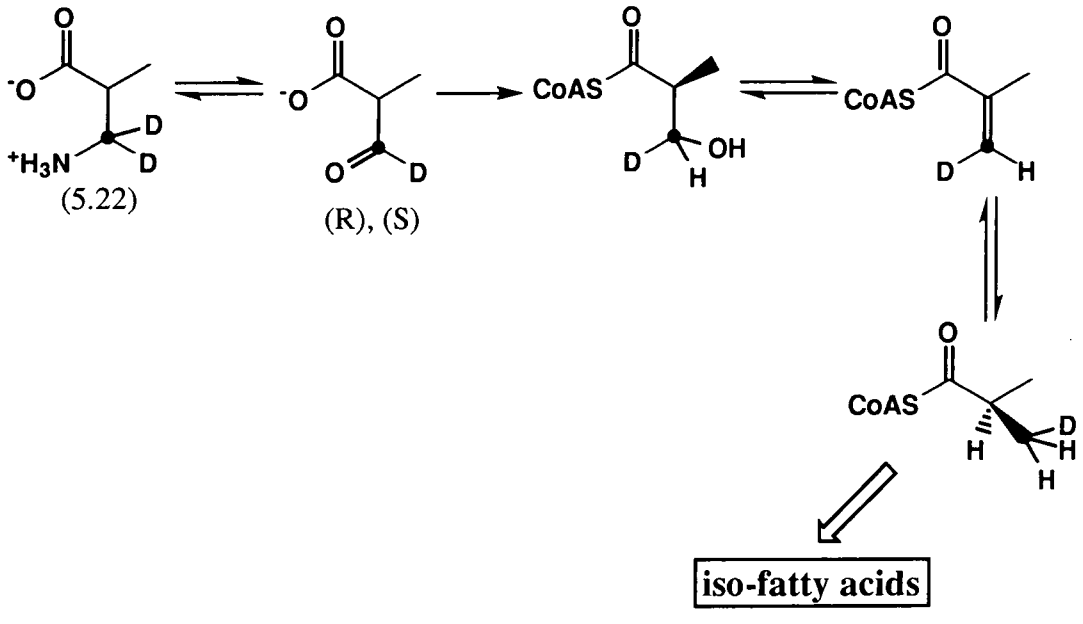


Figure 5-21 Route B: incorporation of β-aminoisobutyrate into fatty acids

5.5 Discussion

These results illustrate the potential of studies with mutant microorganisms when the incorporation levels of putative intermediates are expected to be low. The low level of iso-branched chain fatty acid production observed in this experiment however indicates that the pathway from β-aminoisobutyrate to isobutyrate is a very minor one. Thus it may be concluded that the catabolic products of thymine are unlikely to be major contributors to the isobutyrate and butyrate pools. The low enrichments observed into monensin may largely be due to overloading the cells with β-aminoisobutyrate, thus forcing the metabolism along an 'unnatural' pathway. According to the results of the Central Research Division of Pfizer, no natural avermectins or branched chain fatty acids are produced by the mutant strain of *S. avermitilis* unless the media is supplemented with the branched chain fatty acids starter units. Thus it appears there is normally no contribution from the thymine degradation route. It remains a possibility however that the production of monensin in *S. cinnamonensis* utilises these intermediates to a greater extent.

Despite the modest incorporations the experiment clearly distinguished between the two proposed routes, A and B, and rule out the the action of a novel deaminase enzyme. The alternative pathway, which appears to be in operation, *via* methylmalonyl semialdehyde requires transamination of β-aminoisobutyrate, followed by reduction to β-hydroxyisobutyric acid and is the longer pathway of the two. However, this is the reverse of the pathway occuring in mammalian valine catabolism. It is of interest that the end product of this pathway is (S)-β-aminoisobutyrate, whereas (R)-β-aminoisobutyrate is produced as the product of thymine catabolism in mammals. Presumably in *Streptomyces* both these enantiomers may be converted to methylmalonyl semialdehyde, allowing a link between the two catabolic pathways. Two separate enzymes may be involved. An alternative possibility is that only the (R)-enantiomer, from thymine catabolism is processed. It would be interesting to repeat the experiments with enantiomerically pure samples of β-aminoisobutyrate.

The discussion so far has considered only the possibility of the reductive catabolism of thymine. A relatively small number of microorganisms are known to degrade

pyrimidines along this pathway.[29] Only *Hydrogenomonas facilis* [30]and *Candida utilis* [31]are thought to utilise the carbon atoms from this degradative pathway. The results indicate that a degradative pathway from thymine to β-aminoiosbutyrate is also occuring in *Streptomyces*. However the operation of an oxidative catabolic pathway cannot be excluded. Uracil and thymine are known to be oxidised to barbituric acid and 5-methylbarbituric acid respectively by uracil dehydrogenase in bacteria able to grow aerobically. The thymine oxidising enzymes appear to be induced by growth on thymine.[32] Barbituric acid can further be degraded to urea and malonic acid by the action of barbiturase. The fate of 5-methylbarbituric acid is unknown. It is unaffected by the action of barbiturase, but a similar degradation to urea and methylmalonic acid may occur. This pathway would also account for the observed incorporations from thymine into the butyrate and propionate units of monensin. Incorporation of intermediates such as dihydrothymine from the reductive pathway are needed to test this.

The incorporation of both β-aminoisobutyrate and thymine suggest a reductive pathway, however. The products of this pathway may be used in Streptomyces sp. as precursors to the propionate butyrate units. This is summarised below in Figure 5-22.

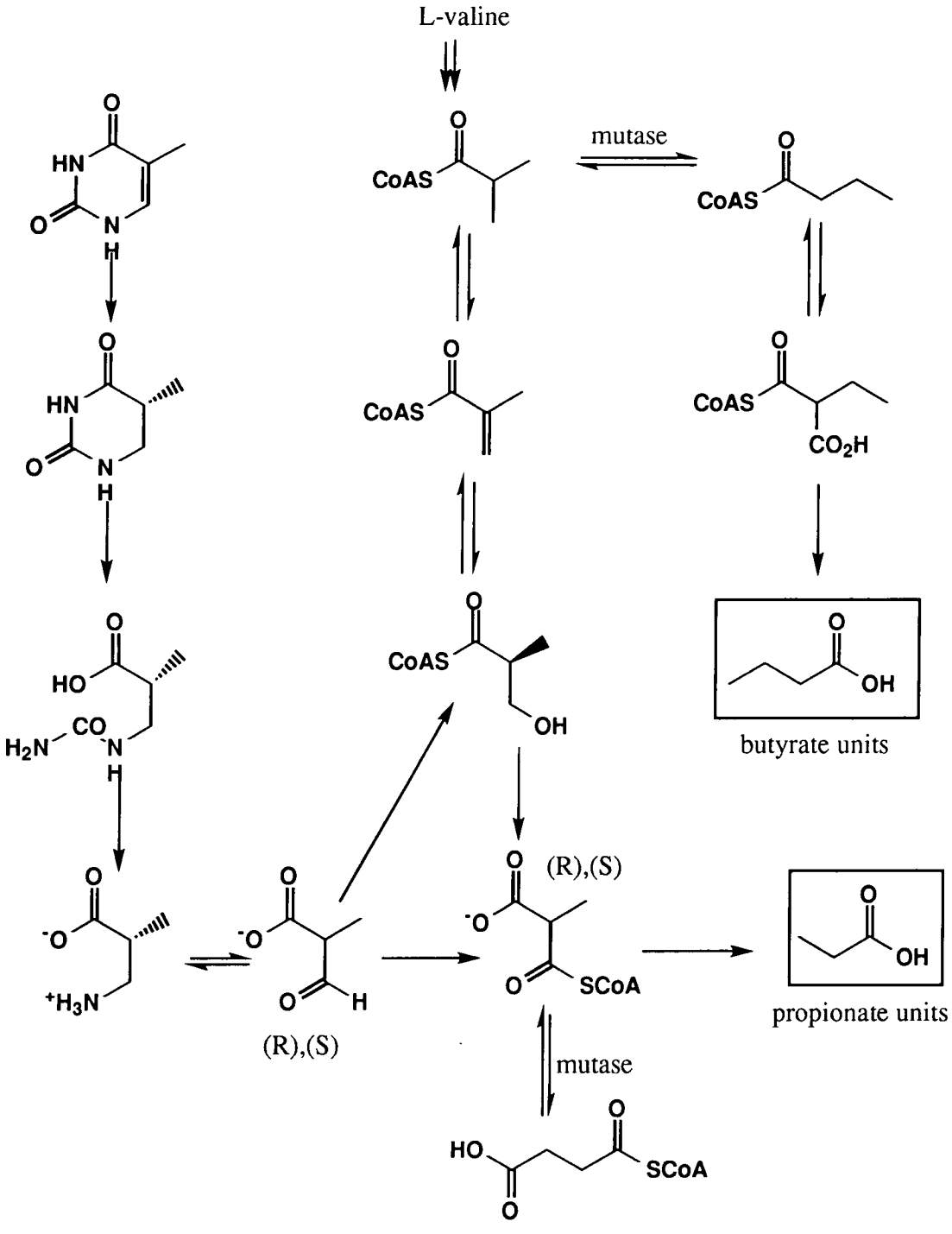


Figure 5-22 Suggested pathway for thymine catabolism in Streptomyces sp. and incorporation of catabolic products into butyrate and propionate units of polyketide metabolites

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6. Experimental

6.1 General

NMR spectra were recorded on Varian Gemini 200 MHz spectrometer (1H at 199 977 MHz, 13C at 50.30 MHz), Varian XL-200 (1H at 200.057 MHz), Varian VXR 400(S) (1H at 399.952 MHz, 13C at 100.577 MHz), Bruker AMX 500 (1H at 500.137 MHz, 13C at 125.759 MHz, 2H at 76.775 MHz), and Varian VXR-600 (University of Edinburgh) (13C at 150.869 MHz). Chemical shifts are quoted relative to TMS standard (δ=0). IR spectra were recorded on a Perkin-Elmer F. T. 1720X or 1600 spectrometer, or Perkin Elmer Paragon 1000 (Golden Gate single reflection). Low resolution mass spectra were recorded on a VG Analytical 7070E Organic mass spectrometer. High resolution mass spectra were recorded on a VG-ZAB spectrometer. HPLC analysis and purification was accomplished using a Varian Star 9012 solvent delivery pump, and peaks recorded on a Varian Star 9050 variable wavelength UV-VIS detector. LCMS analysis was recorded on a finnagan Matt MS TSQ700 or 7000 and samples were injected using a Gilson autosampler with a Hewlett Packard 1050 pump. Separations were over a Hypersil C-18 BDS-protected reverse-phase column (5μm, 250 x 4.6mm). Melting points were determined using a digital Gallenkamp melting point apparatus and are uncorrected. Solvents were routinely dried and distilled prior to use: tetrahydrofuran and diethylether (sodium benzophenone, under nitrogen), dichloromethane (calcium hydride), ethanol and methanol (dry magnesium turning, iodine). Reactions requiring anhydrous conditions were carried out under a nitrogen atmosphere. Flash chromatography was accomplished over Fluka silica gel-60 (35-70 μm) or Sorbsil-C60-H (40-60 μm).

PART 1

6.2 Production, Isolation and Analysis of Tenellin

6.2.1 Growth of *Beauvaria bassiana*

The tenellin producing culture of *Beauvaria bassiana* (Bals.) Vuill. (No 110.25), was obtained from the CBS culture collection, Oosterstrat, Delft, Netherlands. Growth was initiated onto corn meal agar plates from seven day old production cultures, and the plates incubated at 20°C, after which the cultures were initiated into seed media before subculturing into a production media.

Routinely, growth of *B. bassiana* was initiated by transfer of an aliquot of seven day old frozen production medium (2mL) into a 250mL Erlenmeyer flasks containing sterile production medium (50mL). This flask was shaken for seven days at 200rpm, 32°C in the dark. The resultant culture was used to initiate a batch of 250mL Erlenmeyer production medium flasks, each containing production medium (50mL), by the transfer of 2mL aliquots, and the cultures incubated under the same conditions for seven days. New production media flasks were subcultured from seven day old production medium flasks.

6.2.2 Seed medium

To distilled water (990mL) was added glucose (20g), ammonium tartrate (4.6g), KH_2PO_4 (1.0g), $MgSO_4.7H_2O$ (0.5g), NaCl (0.1g), $CaCl_2$ (0.1g) and a mineral ion solution (10mL) containing $CuSO_4$ (39.3mgL^{-1}), $B(OH)_3$ (5.7mgL^{-1}), $(NH_4)_6Mo_7O_{24}.4H_2O$ (3.68 mgL^{-1}), $MnSO_4.H_2O$ (6.1mgL^{-1}), $ZnSO_4.7H_2O$ (879mgL^{-1}) FeSO4.$7H_2O$ (99.6mgL^{-1}). The pH was adjusted to 7.0 prior to sterilisation. Portions (50mL) were sterilised in 250mL Erlenmeyer flasks, plugged with cotton wool.

6.2.3 Production medium

To distilled water (990mL) was added D-mannitol (50g), KNO_3 (5.0g), KH_2PO_4 (1.0g), $MgSO_4.7H_2O$ (0.5g), NaCl (0.1g), $CaCl_2$ (0.1g), $FeSO_4.7H_2O$ (20mg) and a mineral ion solution (10mL) containing $CuSO_4.5H_2O$ (40mgL^{-1}), $B(OH)_3$ (6.0mgL^{-1}),

$(NH_4)_6Mo_7O_{24}.4H_2O$ (4.0mgL^{-1}), $MnSO_4.H_2O$ (7.5mgL^{-1}), $ZnSO_4.7H_2O$ (880mgL^{-1}). 50mL portions were sterilised in 250 mL Erlenmeyer flasks plugged with cotton wool.

6.2.4 Isolation and purification of tenellin

The mycelia were obtained by centrifugation (45,000rpm, 45min, 4°C), washed with distilled water and exhaustively extracted into acetone in a soxhlet extractor. The yellow acetone extract was reduced to an orange solid residue *in vacuo* and re-dissolved into dichloromethane. This was repeatedly washed with brine to remove sugars and other water solubles. 10% aqueous HCl was added to assist separation of the layers. The organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* and the remaining yellow solid residue triturated with hexane to remove the lipids. Filtration afforded crude tenellin (30-50mg per flask) as a yellow solid. Tenellin could be purified by reverse phase HPLC (methanol : water, 85 : 15, 0.1% trifluoroacetic acid).

6.2.5 Analysis of tenellin

Tenellin was dissolved in DMSO-D_6 for ^1H and ^{13}C NMR, DMSO for ^2H NMR.

δ_H(DMSO-D_6, 399.96 MHz) 0.80 (3H, t, J 7.6, CH_3), 0.96 (3H, d, J 6.7, CH_3), 1.32 (2H, m, CH_2), 1.83 (3H, s, CH_3), 2.49 (1H, m, CH),), 5.96 (1H, bd, J 9.6, CH), 6.76 (2H, d, J 8.8, 2x CH), 7.28 (2H, d, J 8.8, 2x CH), 7.51 (1H, d, J 15.2, CH), 7.89 (1H, d, J 15.2, CH), 8.15 (1H, s, CH), 9.52 (1H, s, 0H), 11.73 (1H, bs, OH), 16.98 (1H, s, OH).

δ_D(DMSO, 76.774 MHz, natural abundance) 0.77 (bs, CH_3), 0.85 (bs,CH_3), 1.27 (bs,CH_2).

δ_C(DMSO-D_6, 100.58MHz) 11.8 (CH_3), 12.4 (CH_3), 19.9 (CH_3), 29.4 (CH_2), 34.6 (CH), 105.9 (C), 110.9 (C), 115.0 (2xPh), 122.7 (C), 123.1 (CH), 130.3 (2xPh), 132.6 (C), 140.3 (CH), 149.9 (CH), 151.1 (CH), 156.9 (COH), 157.5 (CO), 173.1 (COH), 193.8 (CO).

6.3 Synthesis of Putative Polyketide Intermediates

6.3.1 N, N'-Dipropionylcystamine (2.26)

Propionyl chloride (20g, 216mmol) was added dropwise, to an ice cooled solution of cystamine dihydrochloride (22.5g, 100mmol) in H_2O (20mL). Throughout the addition of propionyl chloride the reaction mixture was maintained above pH 8.2, by the addition of 12.5M KOH solution (35g of KOH in 50mL of H_2O). The resulting white precipitate was collected by vacuum filtration and washed thoroughly with cold water. After drying, the white solid of N,N'-dipropionyl cystamine (14.27g, 54.38mmol, 54.4%) was stored in the dessicator; m.p. 97-98°C; $\delta_H(CDCl_3)$ 1.17 (4H, t, J 7.6, CH_3), 2.26 (4H, q, J 7.6, CH_2), 2.84 (4H, t, J 6.5, CH_2), 3.58 (4H, dt, J 6.3 and 5.8, CH_2), 6.32 (2H, bs, NH); $\delta_C(CDCl_3)$ 10.31 (CH_3), 30.03 (CH_2), 38.32 (CH_2), 38.90 (CH_2), 175.09 (CO) ν_{max}(nujol)/cm^{-1} 3258, 3058, 1620, 1543; m/z (CI) 265 (M+1, 23.94%), 134 (100%).

6.3.2 3% Sodium amalgam

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

6.3.3 N-Propionyl cysteamine (2.27)

3% Sodium amalgam (43g) was added to a solution of N,N'-dipropionyl cystamine (4.39g, 16.7mmol) in dry methanol (100mL) under N_2, and the reaction mixture stirred for 90 minutes at room temperature. The remaining mercury was removed by filtration, and the resulting liquid added to dilute HCl and extracted into dichloromethane. Drying ($MgSO_4$) and concentration of the dichloromethane extract afforded N-propionyl cystamine (2.07g,15.7mmol, 47%), a colourless oil. $\delta_H(CDCl_3)$ 1.02 (3H, t, J 7.7, CH_3), 1.34 (1H, t, J 9.4, SH), 2.12 (2H, q, J 7.6, CH_2), 2.54 (2H, dt, J 8.4 & J 5.5, CH_2), 3.29 (2H, dt, J 7.9 and J 6.2, CH_2), 7.30 (1H, bs, NH); $\delta_C(CDCl_3)$ 10.35 (CH_3), 24.91 (CH_2), 29.99 (CH_2), 42.91 (CH_2), 174.94 (CO); ν_{max}(neat)/cm^{-1} 3297,

3079, 3078, 2977, 2937, 2878, 2549(w), 2240(w), 1644, 1547, 1233, 911, 732; m/z (EI) 134 (M+1, 100%).

6.3.4 Diethyl 2-ethyl-2-methylmalonate (2.21)

A suspension of sodium hydride (940mg, 36mmol) in tetrahydrofuran (20mL) was prepared from sodium hydride dispersion in mineral oils (1.5g), by extraction of the mineral oils into tetrahydrofuran. Diethyl malonate (3.76g, 20mmol), and methyl iodide (2.84g, 20mmol) were carefully added to this under nitrogen at 20°C. The solution was heated under reflux for 2 hours, cooled to room temperature and quenched with ethanol (10mL). The clear brown solution was added to water (20mL), and extracted into dichloromethane (2x 25mL). The combined organic extracts were dried ($MgSO_4$), reduced *in vacuo* to yield an orange oil, which was distilled under vacuum giving a colourless oil, diethyl 2-ethyl-2-methylmalonate, (b.p. 50°C, 0.1mbar, 2.20g, 10.9mmol, 54%). $\delta_H(CDCl_3)$ 0.63 (3H, t, J 6.0, CH_3), 1.00 (6H, t, J 7.1, 2x CH_3), 1.13 (3H, s, CH_3), 1.67 (2H, q, J 6.0, CH_2), 3.93 (4H, t, J 7.1, 2x CH_2). $\delta_C(CDCl_3)$ 8.8 (CH_3), 14.2 (2x CH_2), 19.4 (CH_3), 28.7 (CH_2), 54.2 (C), 61.1 (2x CH_2), 172.3 (CO) ν_{max} (neat)/cm^{-1} 2981, 2935, 1736, 1465, 1380, 1310; m/z (CI) 203 (M+1, 100%).

6.3.5 2-Ethyl-2-methylmalonic acid (2.22)

Diethyl 2-ethyl-2-methylmalonic acid (1.70g, 8.41mmol) was dissolved in aqueous potassium hydroxide (40mL, 1M) and the solution heated under reflux for 24 hours. After cooling to room temperature the solution was acidified by the dropwise addition of concentrated hydrochloric acid, and extracted into diethyl ether (5x 100mL), dried ($MgSO_4$), and the ether removed *in vacuo* yielding a white waxy solid, 2-ethyl-2-methylmalonic acid, (1.13g, 7.74mmol ,92%). $\delta_H(D_2O)$ 0.70 (3H, t, J 7.5, CH_3), 1.24 (3H, s, CH_3), 1.71(2H, q, J 7.5, CH_2).

6.3.6 Sodium 2-methylbutyrate (2.23)

2-Methyl-2-ethylmalonic acid (0.70g, 4.79mmol) was taken up in demineralised water (10mL) and transferred to a carius tube. The contents of the tube were frozen, the air evacuated and the tube sealed. The tube was heated in a steel bomb at 180°C for 3 hours.

After cooling the contents of the tube were acidified with dilute sulphuric acid, lyophalised to purify the product acid. The lyophilisate was adjusted to pH 8 with dilute aqueous sodium hydroxide and freeze dried to yield an amorphous white solid, sodium 2-methylbutyrate, (0.49g, 3.95mmol, 82%). m.p. 249.4°C; $\delta_H(D_2O)$ 0.72 (3H, t, J 7.0, CH_3), 0.91 (3H, d, J 7.0, CH_3), 1.27 (2H, m, CH_2), 2.08 (1H, q, J 7.0, CH); $\delta_C(D_2O)$ 14.4 (CH_3), 20.3 (CH_3), 30.2 (CH_2), 47.6 (CH), 190.2 (CO).

6.3.7 N-Propionyl cysteamine-2-methylbutyrate (2.29)

Sodium 2-methylbutyrate (0.30g, 2.42mmol) was dissolved in distilled H_2O (5mL), acidified with dilute HCl and extracted into diethyl ether (5x 50mL). The ether extracts were dried ($MgSO_4$), filtered and reduced *in vacuo* to give 2-methylbutyric acid, (0.24g, 97%) as a yellow oil. To a solution of 2-methylbutyric acid (0.20g, 2.0mmol) in dry ether (25mL), was added 1,3-dicyclohexylcarbodiimide (0.41g, 2.0mmol), 4-dimethylaminopyridine (0.24 g, 2.0mmol) and N-propionyl cysteamine (0.40g, 3.0mmol), and the reaction mixture was stirred under N_2 at room temperature for 12 hours. The white precipitate formed was removed by filtration and discarded, and the filtrate concentrated and purified over silica gel (chloroform : ethyl acetate, 70 : 30). The active fractions were combined and concentrated to afford N-propionylcysteamine-2-methylbutyrate (0.43g, 1.99mmol, 98.9%) as a colourless oil. This was stored in an evacuated flask at 4°C. $\delta_H(CDCl_3)$ 0.82 (3H, t, J 7.6, CH_3), 1.05 (3H, t, J 7.4, CH_3), 1.06 (3H, d,J 8.3, CH_3), 1.50 (2H, dm, J 7.6, CH_2), 2.14 (2H, q, J 7.2, CH_2), 2.50 (1H, m, CH), 2.95 (2H, t, J 6.8, CH_2), 3.34 (2H, dt, J 8.0 and 6.0, CH_2), 6.3 (1H, bs, NH); $\delta_C(CDCl_3)$ 10.2 (CH_3), 12.0 (CH_3), 17.6 (CH_3), 27.6 (CH_2), 28.5 (CH_2), 30.0 (CH_2), 40.1 (CH_2), 50.6 (CH), 174.6 (CO), 204.2 (CO); ν_{max}(neat) / cm^{-1} 3293, 3076, 2971, 2935, 2877, 2360 (w), 2341 (w), 1690, 1650, 1546, 1461,1231, 936, 755; m/z (CI) 218 (M+1, 44.69%), 190 (22.95%), 134(38.44%); Found 218.1214, $C_{10}H_{20}O_2SN$ (M+1) requires 218.1215.

6.3.8 Diethyl [2-*methyl*-$^2H_3$]-2-ethyl-2-methylmalonate (2.21)

Sodium hydride (1.49g, 62.08mmol), diethyl ethylmalonate (6.49g, 34.49mmol), and [$^2H_3$]-methyl iodide (5.0g, 34.39mmol) were used to prepare diethyl [2-*methyl*-$^2H_3$]-2-ethyl-2-methylmalonate by the method described for diethyl 2-ethyl-2-methylmalonate.

The product was distilled under vacuum as a colourless oil (b.p. 50°C, 0.1mmHg, 3.55g, 17.30mmol, 51%). $\delta_H(CDCl_3)$ 0.80 (3H, t, J 6.0, CH_3), 1.18 (6H, t, J 7.1, 2x CH_3), 1.81 (2H, q, J 6.0, CH_2), 4.10 (4H, t, J 7.1, 2x CH_2); $\delta_C(CDCl_3)$ 9.1 (CH_3), 14.5 (2x CH_2), 22.3 (m,CD_3), 28.8 (CH_2), 54.2 (C), 61.4 (2x CH_2), 172.3 (CO).

6.3.9 [2-*Methyl*-$^2H_3$]-2-ethyl-2-methylmalonic acid (2.22)

Diethyl [2-*methyl*-$^2H_3$]-2-ethyl-2-methylmalonate (3.50g, 17.05mmol) was dissolved in aqueous potassium hydroxide (80mL, 1M) and the esters hydrolysed and isolated as described for 2-ethyl-2-methylmalonic acid, yielding a white waxy solid, [2-*methyl*-$^2H_3$]-2-ethyl-2-methylmalonic acid (2.33 g, 15.63 mmol ,92%). $\delta_H(D_2O)$ 0.69 (3H, t, J 7.5, CH_3), 1.71 (2H, q, J 7.5, CH_2).

6.3.10 Sodium [2-*methyl*-$^2H_3$]-2-methylbutyrate (2.23)

[2-*methyl*-$^2H_3$]-2-ethyl-2-methylmalonic acid (2.33g, 15.63mmol) was decarboxylated as described for sodium 2-methyl-butyrate, yielding a white crystalline solid, sodium [2-*methyl*-$^2H_3$]-2-methylbutyrate, (1.63g, 12.79mmol, 82%). m.p. 249.0°C; $\delta_H(D_2O)$ 0.70 (3H, t, J 7.5, CH_3), 1.27 (2H, m, CH_2), 2.08 (1H, bq, J 7.5, CH). $\delta_C(D_2O)$ 14.4 (CH_3), 19.5 (m, CD_3), 30.1 (CH_2), 47.3 (CH), 190.2 (CO).

6.3.11 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate (2.29)

Sodium [$^2H_3$-*methyl*]-2-methylbutyrate (1.61g, 13mmol) was dissolved in distilled H_2O (20mL), acidified with dilute HCl and extracted into diethyl ether (5x 100mL). The ether extracts were dried ($MgSO_4$), filtered and reduced *in vacuo* to give [$^2H_3$-*methyl*]-2-methylbutyric acid (1.23g, 93%) as a yellow oil. To a solution of [$^2H_3$-*methyl*]-2-methylbutyric acid (1.16g, 11mmol) in dry ether (50mL), was added 1,3-dicyclohexylcarbodiimide (2.27g, 11mmol), 4-dimethylaminopyridine (1.34 g,11mmol) and N-propionyl cysteamine (1.85g, 14mmol), and the reaction mixture was stirred under N_2 at room temperature for 12 hours. The white precipitate formed was removed by filtration and discarded, and the filtrate was concentrated and purified over silica gel (chloroform : ethyl acetate, 70 : 30). The active fractions were combined and concentrated to afford N-propionylcysteamine-[$^2H_3$-*methyl*]-2-methylbutyrate (2.07 g,

9.39mmol, 85%) as a colourless oil. This was stored in an evacuated flask at 4°C. $\delta_H(CDCl_3)$ 0.91 (3H, t, J 7.4, CH_3), 1.14 (3H, t, J 7.6, CH_3), 1.60 (2H, m, CH_2), 2.20 (2H, q, J 7.7, CH_2), 2.55 (1H, bm, CH), 3.03 (2H, t, J 6.3, CH_2), 3.44 (2H, dt, J 6.5 and J 5.9, CH_2), 6.35 (1H, bs, NH); $\delta_C(CDCl_3)$ 10.2 (CH_3) 12.0 (CH_3), 16.5 (m, CD_3), 27.0 (CH_2), 28.6 (CH_2), 30.0 (CH_2), 40.1 (CH_2), 50.1 (CH), 174.7 (CO), 203.7 (CO); ν_{max}(neat) / cm^{-1} 3292, 3076, 2967, 2933, 2877, 2227 (w), 2070 (w), 1687, 1653, 1547, 1461,1231, 999, 872; m/z (CI) 221 (M+1, 85.91%), 134 (23.57%); Found 221.1403, $C_{10}H_{17}D_3O_2SN$ (M+1) requires 221.1406.

6.3.12 Ethyl 3-oxopentanoate (2.31)

A solution of Meldrum's acid (43.24g, 300mmol) in dried and distilled pyridine (60mL) was added dropwise to a solution of propionyl chloride (27.76g, 300mmol) in dried and distilled dichloromethane (125mL) at 0°C. This was left stirring at room temperature for 16 hours. The orange / red solution was poured into 2M hydrochloric acid (300mL), the lower layer separated and washed with distilled water (5x 100mL) and the organic layer was dried (Na_2SO_4), and reduced *in vacuo* yielding a brown oil which was refluxed in dry ethanol (200mL) for 5 hours. The ethanol was removed *in vacuo* and the product purified by distillation to give ethyl-3-oxo-pentanoate as a colourless oil, (b.p. 100°C, 20mmHg, 31.14g, 216mmol, 72%). $\delta_H(CDCl_3)$ 1.08 (3H, t, J 7.3, CH_3), 1.28 (3H, t, J 7.2, CH_3), 2.59 (2H, q, J 7.2, CH_2), 3.46 (2H, s, CH_2), 4.18 (2H, q, J 7.3, CH_2); $\delta_C(CDCl_3)$ 7.8 (CH_3), 14.4 (CH_3), 36.6 (CH_2), 49.3 (CH_2), 61.6 (CH_2), 167.7 (CO), 203.8 (CO); ν_{max}(neat)/cm^{-1} 2981, 2941, 2907, 1744, 1716, 1622, 1312, 1249, 1159; m/z (EI) 145 (M+1, 100%), 99 (21.57%), 57 (66.05%).

6.3.13 Ethyl 3-oxo-2-methylpentanoate (2.32)

Ethyl 3-oxo-pentanoate (5.04g, 35mmol) was added to a stirred solution of sodium ethoxide (2.38g, 35mmol) in dried and distilled ethanol (50mL) at 13°C. The solution was heated under reflux for 10 minutes, then methyl iodide (4.97g, 35mmol) was added dropwise, and the solution heated under reflux for a further 16 hours. After cooling the ethanol was removed *in vacuo* and the mixture added to diethyl ether (50mL), filtered to remove all insolubles, and reduced *in vacuo*. The yellow oil was purified by bulb to bulb distillation yielding a clear oil, ethyl 3-oxo-2-methyl pentanoate, (b.p. 95°C, 15mmHg,

3.70g, 23.4mmol, 67%). $\delta_H(CDCl_3)$ 1.08 (3H, t, J 7.3, CH_3), 1.27 (3H, t, J 7.2, CH_3), 1.34 (3H, d, J 7.2, CH_3), 2.58 (2H, m, CH_2), 3.54 (1H, q, J 7.3, CH), 4.19 (2H, q, J 7.3, CH_2); δ_C ($CDCl_3$) 7.76 (CH_3), 12.86 (CH_3), 14.05 (CH_3), 34.70 (CH_2), 53.00 (CH), 61.32 (CH_2), 170.74 (CO), 206.57 (CO); ν_{max}(neat)/cm^{-1} 2983, 2941, 1743, 1715, 1195; m/z (EI) 159 (M+1, 61.15%), 113 (29.25%), 57(100%).

6.3.14 Ethyl 2,4-dimethyl-3-oxohexanoate (2.33)

A slurry of sodium hydride (0.96g, 24mmol) in tetrahydrofuran (40mL) was prepared and stirred under nitrogen for 10 minutes at 0°C. To this ethyl 2-methyl-3-oxohexanoate (3.16g, 20mmol) in tetrahydrofuran (10mL) was added dropwise, at 0°C. Ten minutes after addition was complete a 1.6M hexane solution of butyllithium (15mL) was added, and the mixture stirred for 10 minutes at 0°C. To this bright yellow solution was added ethyl bromide (2.62g, 24mmol) in tetrahydrofuran (4mL), and stirring continued at room temperature for 30 minutes. The reaction was quenched with 10% hydrochloric acid (40mL), the organic layer separated, and washed with brine. The aqueous layer was further extracted into ether (2x 40mL), washed with brine, and the combined organic layers reduced *in vacuo* . The resulting yellow oil was distilled under vacuum to give ethyl 2,4-dimethyl-3-oxohexanoate as a clear oil, (b.p. 45°C, 0.2mmHg, 2.60g, 14mmol, 69.8%). $\delta_H(CDCl_3)$ 0.88 & 0.87 (3H, 2 xt, J 7.4, CH_3), 1.10 & 1.09 (3H, 2 xd, J 6.9, CH_3), 1.26 (3H, t, J 7.1, CH_3), 1.32 & 1.31 (2H, 2 xd, J 7.2), 1.2-1.7 (2H, m, CH_2), 2.70 (1H, m, CH), 3.68 & 3.67 (1H,2 x q,J 7.0, CH), 4.19 (2H, q, J 7.2, CH_2); ν_{max} (neat)/cm^{-1} 2970, 2938, 2877, 1744.53, 1714, 1460, 1196; m/z (EI) 187 (M+1, 100%).

6.3.15 Ethyl 2,4-dimethyl-3-hydroxyhexanoate (2.34)

Ethyl 2,4-dimethyl-3-oxohexanoate (1.86g, 10mmol) in dried and distilled ethanol (3mL) was added to a solution of sodium borohydride (0.19g, 5mmol) in dried and distilled ethanol (30mL) at 0°C. The mixture was stirred at 0°C for 30 minutes. Excess sodium borohydride was destroyed by the addition of 10% hydrochloric acid (10mL). The reaction mixture was reduced *in vacuo* to half the volume, and the product isolated by extraction with diethyl ether (3x 30mL), dried ($MgSO_4$), and reduced *in vacuo*. The product was purified over silica (ethyl acetate : hexane, 25 : 75), and alumina (ethyl

acetate : hexane 25 : 75) to give a colourless oil, ethyl 2,4-dimethyl-3-hydroxyhexanoate, (0.94g, 5mmol, 50%). δ_H ($CDCl_3$) 0.8-1.4 (11H, m, 3x CH_3, CH_2), 1.26 (3H, t, J 7.2, CH_3) 2.6 (1H, bs, OH), 3.58 (1H, m, CH), 4.10 (2H, q, J 7.2, CH_2); ν_{max}(neat)/cm^{-1} 3500 (br), 2963, 2935, 2877, 1731, 1715, 1461, 1377, 1185; m/z (EI) 189 (M+1, 71.18%), 171 (100%).

6.3.16 Ethyl (E)-2,4-dimethyl-2-hexenoate (2.35)

Ethyl 2,4-dimethyl-3-hydroxyhexanoate (376mg, 2.0mmol) was added to a mixture of aluminium ethoxide (324mg, 2.0mmol) and aluminium chloride (133.4mg, 1.0mmol) in tetrahydrofuran (10mL) under nitrogen. The heterogenous mixture was stirred at room temperature for 30 minutes. Lithium diisopropylamide was prepared in a nitrogen flushed dry flask from 1.6M butyllithium solution in hexane (3.75mL, 6.0mmol), and diisopropylamine (0.79mL, 6.0mmol). Tetrahydrofuran (6mL) was added to give a 1M solution, which was transferred to the heterogenous reaction mixture. The temperature was raised to 65°C for 1 hour. On cooling the resulting clear orange solution was quenched with 10% aqueous hydrochloric acid (20mL), diluted with diethyl ether (80mL), and the organic layer dried ($MgSO_4$) and reduced *in vacuo* yielding a yellow oil. This was chromatographed over silica (hexane : diethyl ether, 90 : 10) to give the desired unsaturated ester, ethyl (E)-2,4-dimethyl-2-hexenoate as a yellow oil, (0.15g, 0.88mmol, 44%). δ_H($CDCL_3$) 0.84 (3H, t, J 7.3, CH_3), 0.99 (3H, d, J 6.7, CH_3), 1.26 (3H, t, J 7.1, CH_3), 1.38 (2H, m, CH_2), 1.83 (3H, d, J 1.4, CH_3), 2.38 (1H, m, CH), 4.18 (2H, q, J 7.4, CH_2), 6.50 (1H, dq, J 10.7 and 1.4, CH); δ_C($CDCl_3$) 11.8 (CH_3), 12.5 (CH_3), 14.2 (CH_3), 19.6 (CH_3), 29.6 (CH), 34.8 (CH_2), 60.3 (CH_2), 126.5 (C), 147.8 (CH), 168.4 (CO); ν_{max} (neat)/cm^{-1} 2961, 2931, 2873, 1711, 1649, 1271, 1238, 734.

6.3.17 (E)-2,4-Dimethyl-2-hexenoic acid (2.36)

Ethyl (E)-2,4-dimethyl-2-hexenoate (100mg, 0.59mmol) was added to a solution of sodium hydroxide (300mg) in methanol (3mL). The solution was heated under reflux for 5 hours, cooled and acidified with 10% aqueous hydrochloric acid. The product was extracted into ether (5 x 50mL), dried ($MgSO_4$), filtered and reduced *in vacuo,* to give a yellow oil (E)-2,4-dimethyl-2-hexenoic acid (0.07g, 0.50mmol, 84%). δ_H($CDCl_3$) 0.82

(3H, t, J 7.3, CH_3), 0.97 (3H, d, J 6.7, CH_3), 1.35 (2H, m, CH_2), 1.81 (3H, d, J 1.4, CH_3), 2.40 (1H m, CH), 6.66 (1H, dq, J 10.1 & 1.4, CH); $\delta_C(CDCl_3)$ 12.4 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 30.0 (CH_2), 35.6 (CH), 126.2 (C), 151.1 (CH), 174.8 (CO); ν_{max}(neat)/cm^{-1} 3408 (b), 2960, 2929, 2873, 2657, 1686, 1644, 1457, 1420, 1290, 1280, 1218; m/z (EI) 143 (M+1, 64.17%), 125 (100%).

6.3.18 N-Propionyl cysteamine-(E)-2,4-dimethylhexenoate (2.37)

1,3-Dicyclohexylcarbodiimide (144mg, 0.70mmol), 4-dimethylaminopyridine (86mg, 0.70mmol) and N-propionylcysteamine (145mg, 1.1mmol) were added to a solution of (E)-2,4-dimethylhexenoic acid (100mg, 0.70mmol) in dry ether (6mL). The reaction mixture was stirred under dry N_2 at room temparture for 12 hours. A white precipitate formed which was removed by filtration and discarded. The filtrate was concentrated and the resultant oil was purified over silica (chloroform : ethyl acetate, 70 : 30). The active fraction were combined and concentrated to give a waxy solid, N-propionyl cysteamine-(E)-2,4-dimethyl hexenoate (113mg, 44mmol, 63%). NOE Studies confirmed this to be the (E) isomer. $\delta_H(CDCl_3)$ 0.80 (3H, t, J 7.0, CH_3), 0.95 (3H, d, J 6.5, CH_3), 1.07 (3H, t, J 7.5, CH_3), 1.31 (2H, m, CH_2), 1.81 (3H, d, J 1.2, CH_3), 2.13 (2H, dt, J 7.5 & 6.5, CH_2), 2.38 (1H, m, CH), 3.00 (2H, t, J 6.5, CH_2), 3.20 (2H, q, J 7.0, CH_2), 6.10 (1H, bs, NH), 6.46 (1H, dq,J 9.6 & 1.2, CH); $\delta_C(CDCl_3)$ 9.7 (CH_3), 11.8 (CH_3), 12.5 (CH_3), 19.4 (CH_2), 28.3 (CH_3), 29.5 (CH_2), 33.9 (CH), 35.0 (CH_2), 39.6 (CH_2), 134.5 (C), 147.4 (CH), 174.0 (CO), 194.2 (CO); ν_{max} (neat)/cm^{-1} 3318, 3075, 2970, 2927, 2874, 2851, 1656, 1547, 1437, 1241, 1035, 879; m/z (CI) 258 (M+1 100%), 125 (62.76%); Found $C_{13}H_{24}O_2NS$ (MH+) 258.1527, requires 258.1528.

6.3.19 Ethyl [2-*methyl*-$^2H_3$]-3-oxo-2-methylpentanoate (2.32)

Ethyl 3-oxo-pentanoate (9.95g, 69mmol) was added to a stirred solution of sodium ethoxide (4.70g, 69mmol) in dried and distilled ethanol (100mL) at 13°C. The solution was heated under reflux for 10 minutes, then [$^2H_3$]-methyl iodide (10g, 69mmol) was added dropwise, and the solution heated under reflux for a further 16 hours. After cooling the ethanol was removed *in vacuo* and the mixture added to diethyl ether (100mL), filtered to remove all insolubles, and reduced *in vacuo*. The yellow oil was purified by bulb to bulb distillation yielding a clear oil, ethyl [2-*methyl*-$^2H_3$]-3-oxo-2-

methyl pentanoate, (b.p. 97°C, 15mmHg, 8.20g, 50.87mmol, 74%). $\delta_H(CDCl_3)$ 1.05 (3H, t, J 7.3, CH_3), 1.24 (3H, t, J 7.1, CH_3), 2.52 (2H, m, CH_2), 3.48 (1H, bs, CH), 4.17 (2H, q, J 7.2, CH_2); $\delta_C(CDCl_3)$ 7.5 (CH_3), 11.9 (CD_3), 13.9 (CH_3), 34.5 (CH_2), 52.2 (CH), 61.1 (CH_2), 170.5 (CO), 206.3 (CO); ν_{max}(neat)/cm^{-1} 2982, 2941, 1743, 1715, 1189; m/z (EI) 162 (M+1, 100%), 116 (23.65%), 57(68.08%).

6.3.20 Ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-oxohexanoate (2.33)

A slurry of sodium hydride (1.44g, 60mmol) in tetrahydrofuran (100mL) was prepared and stirred under nitrogen for 10 minutes at 0°C. To this ethyl [2-*methyl*-$^2H_3$]-2-methyl-3-oxohexanoate (8.06g, 50mmol) in tetrahydrofuran (25mL) was added dropwise, at 0°C. Ten minutes after addition was complete of 1.6M hexane solution of butyllithium (38mL) was added, and the mixture stirred for 10 minutes at 0°C. To this bright yellow solution was added ethyl bromide (6.54g, 60mmol) in tetrahydrofuran (10mL), and stirring continued at room temperature for 30 minutes. The reaction was quenched with 10% hydrochloric acid (100mL), the organic layer separated, and washed with brine. The aqueous layer was further extracted into ether (2x 100mL), washed with brine, and the combined organic layers reduced *in vacuo*. The resulting yellow oil was distilled under vacuum to give ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-oxohexanoate as a clear oil, (b.p. 43°C, 0.2mmHg, 5.85g, 30.91mmol, 62%). $\delta_H(CDCl_3)$ 0.82 & 0.82 (3H, 2 x t, J 7.4, CH_3), 1.03 (3H, m, CH_3), 1.20 (3H, t, J 6.9, CH_3), 1.2-1.7 (2H, m, CH_2), 2.63 (1H, 2 x q, J 6.8 CH), 3.60 & 3.57 (1H,2 x bs, CH), 4.11 (2H, q, J 7.2, CH_2); ν_{max} (neat)/cm^{-1} 2968, 2937, 2877, 1745, 1714, 1461,1237, 1186; m/z (EI) 190 (M+1, 100%).

6.3.21 Ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-hydroxyhexanoate (2.34)

Ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-oxohexanoate (5.85g, 30.9mmol) in dried and distilled ethanol (10mL) was added to a solution of sodium borohydride (0.585g, 15.5mmol) in dried and distilled ethanol (90mL) at 0°C. The mixture was stirred at 0°C for 30 minutes. Excess sodium borohydride was destroyed by the addition of 10% hydrochloric acid (30mL). The reaction mixture was reduced *in vacuo* to half the volume, and the product isolated by extraction into diethyl ether (3x 100mL), dried ($MgSO_4$), and reduced *in vacuo*. The product was purified over silica (ethyl acetate :

hexane, 25 : 75), and alumina (ethyl acetate : hexane 25 : 75) to give a colourless oil, ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-hydroxyhexanoate, (3.60g, 18.82mmol, 61%). $\delta_H(CDCl_3)$ 0.41-0.90 (6H, m, 2x CH_3,) 1.10-1.80 (4H, m, 2 x CH, 1 x CH_2), 1.22 (3H, t, J 7.1, CH_3), 2.6 (1H, bs, OH), 3.58 (1H, m, CH), 4.10 (2H, q, J 7.2, CH_2); ν_{max}(neat)/cm^{-1} 3501 (br), 2966, 2935, 2876, 1717, 1464, 1374, 1184, 1034; m/z (EI) 192 (M+1, 87.69%), 174 (100%).

6.3.22 Ethyl [2-*methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoate (2.35)

Ethyl [2-*methyl*-$^2H_3$]-2,4-dimethyl-3-hydroxyhexanoate (3.60g, 18.8mmol) was added to a mixture of aluminium ethoxide (3.05g, 18.8mmol) and aluminium chloride (1.25g, 9.41mmol) in tetrahydrofuran (100mL) under nitrogen. The heterogenous mixture was stirred at room temperature for 30 minutes. Lithium diisopropylamide was prepared in a nitrogen flushed dry flask from 1.6M butyllithium solution in hexane (35.3mL, 56.5mmol), and diisopropylamine (7.40mL, 56.5mmol). Tetrahydrofuran (50mL) was added to give a 1M solution, which was transferred to the heterogenous mixture above. The temperature was raised to 65°C for 1 hour. On cooling the resulting clear orange solution was quenched with 10% aqueous hydrochloric acid (150mL), diluted with diethyl ether (300mL), and the organic layer dried ($MgSO_4$), reduced *in vacuo* yielding a yellow oil. This was chromatographed over silica gel (hexane : diethyl ether, 90 : 10) to give the desired unsaturated ester, ethyl [2-*methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoate as a yellow oil, (0.67g, 3.88mmol, 21%). $\delta_H(CDCl_3)$ 0.78 (3H, t, J 7.2, CH_3), 0.93 (3H, d, J 6.8, CH_3), 1.22 (3H, t, J 7.2, CH_3), 1.34 (2H, m, CH_2), 2.38 (1H, m, CH), 4.12 (2H, q, J 7.2, CH_2), 6.47 (1H, d, J 10.0, CH); δ_C ($CDCl_3$) 11.9 (CH_3), 14.3 (CH_3), 19.5 (CH_3), 29.5 (CH), 34.8 (CH_2), 60.3 (CH_2), 126.1 (CH), 146.9 (CH), 168.2 (CO); ν_{max}(neat)/cm^{-1} 2960, 2928, 2872, 1715, 1697, 1643, 1236, 1152, 727.

6.3.23 [2-*Methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoic acid (2.36)

Ethyl [2-*methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoate (0.66g, 3.15mmol) was added to a solution of sodium hydroxide (2.1g) in methanol (21mL). The solution was refluxed gently for 5 hours, cooled and acidified with 10% aqueous hydrochloric acid. The product was extracted into ether (4x 100 mL), dried ($MgSO_4$), filtered and reduced *in vacuo,* to give a yellow oil [2-*methyl*-$^2H_3$]-(E)-2,4-dimethyl-2-hexenoic acid (0.391g,

2.30mmol, 85%). $\delta_H(CDCl_3)$ 0.84 (3H, t, J 7.3, CH_3), 1.0 (3H, d, J 6.7, CH_3), 1.35 (2H, m, CH_2), 2.40 (1H m, CH), 6.68 (1H, d, J 10.1, CH); $\delta_C(CDCl_3)$ 12.3 (CH_3), 20.0 (CH_3), 30.0 (CH_2), 35.6 (CH), 126.1 (CH), 151.4 (CH), 174.7 (CO); ν_{max}(neat)/cm^{-1} 3000 (br), 2961, 2873, 2650, 1689, 1636, 1420, 1279; m/z (EI) 145 (M+, 33.90%), 73 (79.17%), 56 (100%).

6.3.24 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-(E)-2,4-dimethylhexenoate (2.37)

1,3-Dicyclohexylcarbodiimide (720mg, 3.40mmol), 4-dimethylaminopyridine (330mg, 2.68mmol) and N-propionylcysteamine (570mg, 4.29mmol) were added to a solution of [2-$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoic acid (390mg, 2.68mmol) in dry ether (25mL). The reaction mixture was stirred under dry N_2 at room temparture for 12 hours. A white precipitate formed which was removed by filtration and discarded. The filtrate was concentrated and the resultant oil was purified over silica gel (chloroform : ethyl acetate, 70 : 30). The active fraction were combined and concentrated to give a waxy solid, N-propionyl cysteamine-[2-$^2H_3$-*methyl*]-(E)-2,4-dimethyl hexenoate (326mg, 1.30mmol, 47%). $\delta_H(CDCl_3)$ 0.79 (3H, t, J 7.2, CH_3), 0.95 (3H, d, J 6.8, CH_3), 1.07 (3H, t, J 7.2, CH_3), 1.32 (2H, m, CH_2), 2.13 (2H, dt, J 7.6 & 6.8, CH_2), 2.37 (1H, m, CH), 3.00 (2H, t, J 6.8, CH_2), 3.39 (2H, q, J 7.2, CH_2), 6.15 (1H, bs, NH), 6.46 (1H, d,J 10.0, CH); $\delta_C(CDCl_3)$ 9.7 (CH_3), 11.8 (CH_3), 19.4 (CH_2), 28.2 (CH_3), 29.4 (CH_2), 33.9 (CH), 35.0 (CH_2), 39.5 (CH_2), 134.3 (C), 147.4 (CH), 174.0 (CO), 194.1 (CO); ν_{max}(neat)/cm^{-1} 3297, 3075.08, 2961, 2928, 2873, 1655, 1545, 1460, 1231, 1210, 1075, 860; m/z (CI) 261 (M+1 100%), 128 (28.87%).

6.4 Feeding of Putative Polyketide Intermediates

6.4.1 Growth of *Beauvaria bassiana* in 6.6% D_2O

Two production media flasks (50mL) prepared with 6.6% D_2O were innoculated with 7 day old production media (5mL), and shaken and 300 rpm, 32°C in the dark. On day 8 tenellin (20mg) was extracted and purified. $\delta_D(CDCl_3)$ 0.77 (CH_3), 0.92 (CH_3), 1.23 (CH_2), 1.79 (CH_3), 6.8 (Ph), 7.3 (Ph).

6.4.2 Sodium [$^2H_3$]-acetate

Sodium [$^2H_3$]-acetate was prepared from a solution of [$^2H_3$]-acetic acid in distilled water by neutralisation with 0.1M NaOH. The sodium salt (0.125g, 1.47mmol) was dissolved in distilled water (16mL). This was pulse fed to four production media flasks (50mL), through a micropore filter, on days 3, 4, 5 and 6, to give a final concentration 7.35 mmolar in each flask. Tenellin (72mg) was extracted and purified by HPLC on day 10. $\delta_D(CDCl_3)$ 0.72 (CH_3).

6.4.3 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate, during idiophase

N-Propionylcysteamine-[$^2H_3$-*methyl*]-2-methylbutyrate (204mg, 0.93mmol) was shaken with sterilised water (12mL) to form an emulsion. This emulsion was pulse fed to four production media cultures (50mL) of *B. bassiana* on days 1, 2 and 3 such that the final concentration was 4.6 mmolar. Tenellin (60 mg) was isolated and purified on day 7. No incorporation of deuterium was observable by ^2H NMR.

6.4.4 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate, during trophophase

N-Propionylcysteamine-[$^2H_3$-*methyl*]-2-methylbutyrate (271mg, 1.23mmol) was shaken with sterilised water (16mL) to form an emulsion. To increase the solubility ethanol (1mL) was added and this was pulse fed to four production media cultures (50mL) of *B. bassiana*, on days 3, 4, 5 and 6 such that the final concentration was 6.2mmolar. Tenellin (98mg) was isolated on day 10. δ_D (DMSO) (before HPLC purification) 1.79 (CH_3-16), 0.97 (CH_3-15), 0.74 (CH_3-14), ratio of peak intensities approx. 1 : 5 : 5. Impurity peak at $\delta_{D,H}$ 5.65; δ_C 54.90.

This experiment was repeated on a larger scale and the tenellin purified by reverse phase HPLC. N-Propionylcysteamine-[$^2H_3$-*methyl*]-2-methylbutyrate (551mg, 2.5mmol) was dissolved in a mixture of ethanol (8mL) and distilled water (10mL) and this was pulse fed to ten production media cultures (50mL) of *B. bassiana,* on days 3, 4, 5, 6 and 6 such that the final of concentration was 5.0mmolar. Crude tenellin (500mg) was subsequently isolated on day 10. Purification by reverse phase HPLC (methanol : water,

85 : 15) yielded tenellin as a yellow crystalline solid (290mg). δ_D(DMSO) 1.77 (CH_3-16), 0.85 (CH_3-15), 0.70 (CH_3-14), ratio of peak intensities 1 : 2 : 7.

6.4.5 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-2-methylbutyrate, to senescent cells

N-propionylcysteamine-[$^2H_3$-*methyl*]-2-methylbutyrate (230 mg, 1.04mmol) was shaken with sterilised water (16mL) to form an emulsion. To increase the solubility in the solution ethanol (1mL) was added and this was pulse fed to four production media cultures (50mL) of *B. bassiana* on days 9, 10, 11 and 12 such that the final concentration was 5.2 mmolar. Tenellin (50 mg) was isolated and purified on day 14. No incorporation of deuterium was observable by ^2H NMR.

6.4.6 N-Propionyl cysteamine-[2-*methyl*-$^2H_3$]-(E)-2,4-dimethylhexenoate

N-propionylcysteamine-[$^2H_3$-*methyl*]-(E)-2,4-dimethylhexenoate (300mg, 1.15mmol) was dissolved in a 1 : 1 mixture of ethanol : water (14mL) and this was pulse fed to 7 cultures (50mL) of *B. bassiana*, 0.5mL to each flask on days 3, 4, 5, 6, to give a final concentration in each flask of 4.6mmolar. Tenellin (80mg) was isolated on day 10, and purified by HPLC. No incorportion of deuterium was observable by ^2H NMR.

6.5 Synthesis of Putative Tetramic acid Intermediates

6.5.1 (1,1-Dimethyl-N-propylidene) ethylamine (3.37)

Propionaldehyde (29g, 0.5mol) was added dropwise over the course of 2 hours to ^{t}butylamine (36.5g, 0.5mol), stirred at 0°C. When addition was complete stirring was stopped and KOH pellets (20g) were added. The solution was left to separate at 0°C for 12 hours before the yellow upper layer was carefully decanted. This was distilled three times over KOH at atmospheric pressure and the title compound collected under dry N_2 as a clear colourless oil (25.4g, 224mmol, 45%, b.p. 103-105°C, 1 atm), which could be stored over molecular sieves, at 0°C. $\delta_H(CDCl_3)$ 0.96 (3H, t, J 7.6, CH_3), 1.10 (9H, s, 3x CH_3), 2.17 (2H, qd, J 7.6 & 4.9, CH_3), 7.52 (1H, t, J 4.9, CH); $\delta_c(CDCl_3)$ 11.2 (CH_3), 30.2 (3x CH_3), 55.9 (CH_2), 102.1 (C), 160.6 (CH); ν_{max} (neat)/cm^{-1} 2965, 2934, 2873, 1671, 1461, 1361, 1227, 1215; m/z (EI) 114 (M+1, 18.82%), 113 (M+, 30.20%), 98 (100%).

6.5.2 E-2,4-Dimethyl-2-hexenal (3.39)

A solution of *freshly distilled* (1,1-dimethyl-N-propylidene) ethylamine (5.65g, 50 mmol) in tetrahydrofuran (30mL) was stirred under dry N_2 and cooled to -78°C. Butyllithium (1.6M in hexanes, 34.5mL, 55mmol) was added *via* a dry N_2 flushed syringe, dropwise over ten minutes. The solution was warmed to 0°C, stirred for 20 minutes, then cooled to 78°C. 2-Methylbutyraldehyde (3.88g, 45mmol, distilled b.p.92°C, 1atm) was added dropwise *via* a dry N_2 flushed syringe and the solution allowed to warm to 0°C and stirred for 4 hours, at this temperature. An aqueous slurry of oxalic acid (30g in 110mL H_2O) was added and the biphasic mixture was vigorously stirred for 72h at room temperature. The mixture was filtered and the filtrate extracted into dichloromethane (3x75mL). The combined organic layers were dried ($MgSO_4$) and evaporated *in vacuo* to yield the crude aldehyde as a pale yellow aromatic oil (5.42g, 42.9mmol, 95%). This was purified over silica (CH_2Cl_2, R_f 0.75). The oil was stable for a few days at 20°C under dry N_2, but needed to be repurified prior to use. $\delta_H(CDCl_3)$ 0.84 (3H, t, J 7.3, CH_3), 1.02 (3H, d, J 6.6, CH_3), 1.40 (2H, m, CH_2), 1.71 (3H, d, J 1.4, CH_3), 2.58 (1H, m, CH), 6.22 (1H, dd, J 9.9 & 1.4, CH), 9.36 (1H, s, CHO); $\delta_c(CDCl_3)$ 11.3 (CH_3), 13.8 (CH_3), 21.4 (CH_3), 31.4 (CH_2), 37.1 (CH), 140.1 (C),

162.3 (CH), 197.4 (CO); ν_{max}(neat)/cm^{-1} 2963, 2929, 2875, 2817, 2707, 1689, 1641, 1459, 7349; m/z (EI) 126 (M+, 10.88%), 41 (100%); Found 126.1040, $C_8H_{14}O$ (M+) requires 126.1045.

6.5.3 2,2-Dimethyl-6-chloromethyl-1,3-dioxin-4-one (3.41)

Freshly distilled 2,2,6-trimethyl-1,3-dioxin-4-one (acetone diketene adduct) (7.1g, 50 mmol, b.p. 36°C, 0.03 mbar) was added dropwise to a solution of LDA (50mmol) stirred at -78°C under dry N_2. The bright yellow suspension which formed was stirred for 15 minutes. It was then carefully transferred dropwise, over a period of 30 minutes, *via* dry Teflon tubing to a stirred solution of hexachloroethane (17.7g, 75 mmol), in tetrahydrofuran (30 mL), which was cooled to -50 °C under dry N_2. The red solution thus formed was allowed to warm slowly to -20°C over 1 hour, after which the reaction mixture was quenched by addition of ice cold dilute aqueous HCl (150 mL). The acidic solution was shaken to dispel colour and extracted into diethyl ether (3x 100mL). The combined ether layers were washed with a saturated aqueous solution of $NaCO_3$ (2x 50mL), dried ($MgSO_4$), and reduced *in vacuo* to afford a yellow oil containing crystalline hexachloroethane. This was removed by trituration with hexane (2x 50 mL). The crude title compound (4.9g, 27.7 mmol, 55%) could be purified over silica (CH_2Cl_2), but was routinely used without further purification. δ_H($CDCl_3$) 1.66 (6H, s, 2x CH_3), 3.99 (2H, s, CH_2), 5.50 (1H, s, CH); δ_c($CDCl_3$) 26.7 (2x CH_3), 43.0 (CH_2Cl), 97.5 (CH), 109.4 (C), 162.3 (C), 166.6 (CO); ν_{max}(neat)/cm^{-1} 3103, 3000, 2946, 1729, 1642, 1391, 1377.66, 1274, 1203, 1016.

6.5.4 2,2-Dimethyl-6-(diethylphophonomethyl)-1,3-dioxin-4-one (3.42)

Diethylphosphite (20.7g, 150 mmol, distilled b.p. 27°C, 0.001mbar) was added dropwise to a solution of potassium ^{t}butoxide (16.8g, 150mmol) in dimethylformamide (100mL), cooled to 0°C under dry N_2. After 30 minutes, 2,2-dimethyl-6-chloromethyl-1,3-dioxin-4-one (5.50g, 31.14mmol) in DMF (25mL) was added dropwise over 20 minutes, and the deep purple solution generated was stirred at 0°C for a further 30 minutes. Dropwise addition of 10M HCl (6mL) discharged the colour, and the pale brown mixture was filtered through celite, and the celite pad washed with diethyl ether (100mL). The combined organic washings were evaporated *in vacuo* keeping the bath

temperature below 40°C. Excess dimethylformamide and diethyl phosphite were removed by vacuum distillation (0.01 mbar, <45°C) affording the crude title compound as a thick brown oil, which was diluted with ethyl acetate, left at 0°C overnight. The solution was decanted from the crystals thus formed, and reduced *in vacuo*. Purification by silica gel flash chromatography (ethyl acetate, R_f 0.19) yielded the title compound as a pale yellow oil (7.27g, 26.13mmol, 84%), which was stored under N_2 at 0°C. $\delta_H(CDCl_3)$ 1.28 (6H, t, J 7.0, 2x CH_3), 1.65 (6H, s, 2x CH_3), 2.75 (2H, d, J 22.5, CH_2), 4.07 (4H, m, 2x CH_2) 5.33 (1H, d, J 3.7, CH); $\delta_c(CDCl_3)$ 16.2 (d, J_{13CP} 6.57, 2x CH_3), 24.5 (2x CH_3), 31.0 (d, J_{13CP} 132.00, CH_2), 61.9 (d, J_{13CP} 6.58, 2x CH_2), 95.5 (d, J_{13CP} 8.81, CH), 106.6 (C), 159.9 (C), 164.3 (CO); ν_{max} (neat)/cm^{-1} 3400 (br), 2984, 2934, 2911, 1730, 1634, 1444, 1376, 1259, 1025.

6.5.5 2,2-Dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (3.43)

A solution of 2,2-dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one (4.17g, 15mmol) in THF (30mL) was added, *via* a dry N_2 flushed syringe, to a solution of lithium hexamethyldisilazide (15 mmol) in tetrahydrofuran (100mL), cooled to 0°C, and stirred under dry N_2. The resultant red solution was stirred for 20 minutes before cooling to -78°C. Freshly purified E-2,4-dimethyl-2-hexenal (1.89g, 15mmol) was added and the solution allowed to warm to room temperature over a period of 4 hours, followed by stirring for a further 8 hours at this temperature. Solvent was removed by evaporation *in vacuo* (bath temperature below 40°C), and the resulting red solid dissolved in dichloromethane (30mL). Colloidal inorganics were removed by vacuum filtration through glass wool, and the filtrate reduced *in vacuo*. The title compound was obtained by purification over silica (dichloromethane, R_F0.21) as a pale yellow oil (1.59g, 6.37mmol, 43%). $\delta_H(CDCl_3)$ 0.80 (3H, t, J 7.0, CH_3), 0.94 (3H, d, J6.25, CH_3), 1.32 (2H, m, CH_2) 1.66 (6H, s, 2x CH_3), 1.75 (3H, s, CH_3), 2.41 (1H, m, CH), 5.25 (1H, s, CH), 5.60 (1H, d, J 9.0, CH), 5.84 (1H, d, J 14.5, CH), 6.92 (1H, d, J 14.5, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.3 (CH_3), 20.2 (CH_3), 24.9 (CH_3), 25.0 (CH_3), 30.0 (CH_2), 34.9 (CH), 93.5 (CH), 106.1 (C), 117.0 (CH), 131.7 (C), 143.3 (CH), 148.0 (CH), 162.1 (C), 164.1 (CO); ν_{max}(neat)/cm^{-1} 2961, 2927, 2871, 1720, 1624, 1388, 1375, 1272, 1203, 1017; m/z (CI) 251 (M+1, 44.2%), 167 (100%); Found 251.1647, $C_{15}H_{23}O_3$ (MH+) requires 251.1647.

6.5.6 (DL)-Phenylalanine hydrochloride (3.48)

A solution of NH_4Cl (2.67g, 50mmol) in water (30mL) was slowly added to a stirred solution of phenylacetaldehyde (3g, 25mol) in methanol (60 mL) cooled to -5°C. After 10 min stirring, KCN (1.63g, 25mmol) was added and stirring continued at 20°C for 24 h. Methanol was removed *in vacuo* and the aqueous solution extracted into methylene dichloride, dried ($MgSO_4$) and evaporated *in vacuo* to afford a brown oil. This was heated under reflux with concentrated aqueous hydrochloric acid (100mL) for 48 hours. Solvent was removed *in vacuo* and the resulting solid washed with cold acetone (100mL) leaving a white amorphous solid, (DL)-phenylalanine hydrochloride (4.40g, 21.8mmol, 87%). m.p 230-230.5°C (lit. 230°C)[1] $\delta_H(D_2O)$ 3.07 (1H, dd, J_{gem} 14.6, J_{vic} 7.6, CH_2), 3.23 (1H, dd, J_{gem} 14.6, J_{vic} 5.9, CH_2); 4.24 (1H, dd, J_{vic} 7.8, J_{vic} 5.6, CH), 7.27 (5H, m, Ph); $\delta_c(D_2O)$ 40.0, (CH_2), 58.6 (CH), 132.5 (Ph CH), 133.7 (Ph CH), 133.9 (Ph CH), 138.4 (Ph C), 175.9 (CO); ν_{max}(neat)/cm^{-1} 2060 (br), 1732, 1601, 1486, 1413, 1226, 1194, 731, 699; m/z (CI) 166 (M+1, 69.94%), 120 (100%); Found 166.0868, $C_9H_{11}NO_2$ (MH+) requires 166.0868.

6.5.7 (DL)-Phenylalanine methyl ester (3.49)

Thionyl chloride (1.78g, 15mmol) was added dropwise to a solution of (DL) phenylalanine hydrochloride (2.02g, 10mmol) in methanol (15mL) and cooled to 0°C. The suspension was stirred at room temperature until product formation was complete, as observed by t.l.c. (silica plates eluted with nPrOH : NH_3 (aq con^c), 7 : 3, (developed with ninhydrin). Solvent was removed *in vacuo*, and the solid residue redissolved in saturated aqueous $NaHCO_3$ (25mL). The aqueous solution was extracted into diethyl ether (5x 75mL) and the organic extracts were dried ($MgSO_4$) before removal of the solvent *in vacuo* to afford the title compound as a pale yellow oil (1.66g, 9.26mmol, 93%). $\delta_H(CDCl_3)$ 1.55 (2H, bs, NH_2), 2.84 (1H, dd, J_{gem} 14.9, J_{vic} 7.8, CH_2), 3.08 (1H, dd, J_{gem} 14.9, J_{vic} 5.3, CH_2), 3.69 (3H, s, $COOCH_3$), 3.69 (1H, m, CH), 7.22 (5H, m, Ph); $\delta_c(CDCl_3)$ 43.1 (CH_2), 54.0 (OCH_3), 57.8 (CH), 128 8 (Ph CH), 130.6 (Ph CH), 131.3 (Ph, CH), 139.2 (Ph C), 177.4 (CO); ν_{max}(neat)/cm^{-1} 3619 (br), 3379, 3312, 3028, 2951, 2853, 1953 (w), 1737, 1603, 1495, 1454, 1437, 1198, 1174, 746, 702; m/z (CI) 180 (M+1, 100%).

6.5.8 (L)-Phenylalanine methyl ester (3.51)

(L)-phenylalanine (4g, 24.21mmol) was methylated as above affording the title compound as a pale yellow oil (4.33g, 24.16mmol, 99%). $\delta_H(CDCl_3)$ 1.71 (2H, bs, NH_2), 2.82 (1H, dd, J_{gem} 14.9, J_{vic} 7.8, CH_2), 3.06 (1H, dd, J_{gem} 14.9, J_{vic} 5.3, CH_2), 3.69 (3H, s, $COOCH_3$), 3.69 (1H, m, CH), 7.20 (5H, m, Ph).

6.5.9 (DL)-N-(E,E-3-Oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.44)

A solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.25g, 1.0mmol) and (DL)-phenylalanine methyl ester (0.20g, 1.12mmol) in toluene (10mL) was heated under reflux temperature for 1hour. After cooling, solvent was removed *in vacuo* and the resultant red oil purified by silica gel flash chromatography (chloroform : methylene dichloride, 25 : 75, R_f 0.11) yielding the title compound as an orange oil (0.20g, 53.8mmol, 54%). $\delta_H(CDCl_3)$ 0.85 (3H, t, J 7.3, CH_3), 0.98 (3H, d, J 6.1, CH_3), 1.33 (2H, m, CH_2), 1.78 (3H, s, CH_3), 2.43 (1H, m, CH), 3.11 (2H, m, CH_2), 3.53 (2H, s, CH_2), 3.70 (3H, s, CH_3), 4.87 (1H, m, CH), 5.8 (1H, d, J 10.0, CH), 6.10 (1H, d, J 16.0, CH), 7.25 (6H, m, Ph & CH), 7.51 (1H, bd, J 8.4, NH); $\delta_c(CDCl_3)$ 13.9 (CH_3), 14.4 (CH_3), 22.0 (CH_3), 31.9 (CH_2), 37.2 (CH), 39.9 (OCH_3), 48.6 (CH_2), 54.3 (CH_2), 55.5 (CH), 125.6 (CH), 129 1 (Ph), 130.6 (Ph), 131.3 (Ph), 133.9 (C), 137.9 (Ph), 152.9 (CH), 153.7 (CH), 167.8 (CON), 173.7 ($\underline{C}OOCH_3$), 197.2 (CO); ν_{max}(neat)/cm^{-1} 3329 (br) 3029, 2960, 2927, 2873, 1746, 1588, 1454, 1416, 1360, 1259, 1215, 1178, 701.

6.5.10 Attempted Dieckmann cyclisation of (DL)-N-(E,E-3-Oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.45)

A solution of (DL)-N-(E,E 3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (0.130g, 0.35mmol) in *tert*-butanol (1mL), was added to a stirred solution of freshly sublimed potassium *tert*-butoxide (0.091g, 0.81mmol) in *tert*-butanol (6mL) under nitrogen. After 30 minutes stirring the red solution was quenched with dilute aqueous hydrochloric acid (6mL). The organics were extracted into diethyl ether (1x 30mL), washed with water (1x 5mL), dried ($MgSO_4$) and evaporated *in vacuo* affording (DL)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine as an orange / red wax

(0.125g, 0.35mmol, 100%). $\delta_H(CDCl_3)$ 0.79 (3H, t, J 7.3, CH_3), 0.94 (3H, d, J 6.6, CH_3), 1.28 (2H, m, CH_2), 1.72 (3H, s. CH_3), 2.41 (1H, m, CH), 3.01 (1H, dd, J_{gem} 14.3, J_{vic} 8.1, CH_2), 3.28 (1H, dd, J_{gem} 14.3, J_{vic} 5.31, CH_2), 3.50 (2H, s, CH_2), 4.79 (1H, m, CH), 5.75 (1H, d, J 10.1, CH), 6.04 (d, J 15.8, CH), 7.18 (6H, m, Ph & CH), 7.72 (1H, bd, J 8.4, NH), 8.50 (1H, bs, COOH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.3 (CH_3), 19.9 (CH_3). 29.8 (CH_2), 35.2 (CH), 37.3 (CH_2), 46.0 (CH_2), 53.6 (CH), 123.3 (CH), 127.0 (Ph), 128.5 (Ph), 129.3 (Ph), 131.9 (C), 135.8 (Ph), 151.2 (CH), 152.1 (CH), 166.8 (CON), 174.5 (COOH), 195.4 (CO).

6.5.11 (DL)-N-(2,4-Dimethoxybenzyl) phenylalanine methyl ester (3.51)

Methanolic HCl was added to a stirred solution of phenylalanine methyl ester (1.59g, 8.87mmol) in methanol (50mL) to adjust to pH 6. 2,4-Dimethoxybenzaldehyde (1.50g, 9.03mmol) was added and the solution stirred at room temperature for 30 minutes. $NaBH_3CN$ (0.69g, 11.0mmol) was then added and the reaction stirred at room temperature for a further 16 hours. Methanol was removed *in vacuo,* distilled water (20mL) added and the aqueous mixture extracted into diethyl ether (3x 50mL). The organic extracts were combined, washed with an aqueous solution of $FeSO_4$, dried ($MgSO_4$), filtered and reduced *in vacuo,* affording the crude product as a pale yellow oil. Impurities of 2,4-dimethoxybenzylalcohol were removed by distillation under reduced presure (furnace temparature 110°C, 0.1mmHg). The product could be further purified over silica (hexane: ethylacetate, 50 : 50) (1.94g, 5.89mmol, 66%). $\delta_H(CDCl_3)$ 2.1 (1H, bs, NH), 2.95 (2H, m, CH_2), 3.50 (1H, m, CH), 3.60 (3H, s, $COOCH_3$), 3.63 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.75 (2H, d, J 9.6, CH_2), 6.36 (2H, m, Ph), 7.14 (6H, m, Ph); $\delta_c(CDCl_3)$ 41.6, (CH_2), 49.3 (NCH_2), 53.6 ($COO\underline{C}H_3$), 57.1 (OCH_3), 57.3 (OCH_3), 64.0 (CH), 100.4 (Ph CH), 105.6 (Ph CH), 122.0 (Ph CH), 128.6 (Ph CH), 130.4 (Ph CH), 131.2 (Ph CH), 132.4 (Ph C), 139.5 (Ph C), 160.6 (Ph CO), 162.2 (Ph CO), 176.9 (CO); ν_{max}(neat)/cm^{-1} 3250 (br), 3025, 2999.46, 2947, 2835, 2240 (w), 2028 (w), 1731, 1611, 1587, 1505, 1453, 1437, 1286.92, 1260, 1205, 1154, 1130, 1032, 699; m/z (EI) 330 (M+1, 100%), 329 (M+, 50.26%), 328 (M-1, 100%); Found 329.1627, $C_{19}H_{23}NO_4$ (M+) requires 329.1627.

6.5.12 (L)-N-(2,4-Dimethoxybenzyl) phenylalanine methyl ester (3.51)

(L)-Phenylalanine methyl ester (4.33g, 24.16mmol) was N-protected with the 2,4-dimethoxybenzyl group as above affording the title compound as a colourless oil (3.71g, 11.26mmol, 47%), $[\alpha]_D^{21}$ = -65.22 (c 1.01, ethanol). $\delta_H(CDCl_3)$ 2.14 (1H, bs, NH), 2.89 (1H, dd, J_{gem} 13.8, J_{vic} 7.8, CH_2), 2.97 (1H, dd, J_{gem} 13.8, J_{vic} 6.5, CH_2), 3.49 (1H, dd, J_{vic} 7.8, J_{vic} 6.5, CH), 3.59 (3H, s, $COOCH_3$), 3.61 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.79 (2H, d, J 8.1, CH_2), 6.39 (2H, m, Ph), 7.16 (6H, m, Ph); $\delta_c(CDCl_3)$ 39.5, (CH_2), 47.1 (NCH_2), 51.5 ($COO\underline{C}H_3$), 55.0 (OCH_3), 55.2 (OCH_3), 61.8 (CH), 98.2 (Ph CH), 103.4 (Ph CH), 119.8 (Ph CH), 126.5 (Ph CH), 128.3 (Ph CH), 129.0 (Ph CH), 130.3 (Ph C), 137.3 (Ph C), 158.5 (Ph CO), 160.1 (Ph CO), 174.7 (CO).

6.5.13 (DL)-N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.52)

Pyridinium *para*-toluene sulphonate (0.251g, 1.00mmol) was added to a solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.250g, 1.00mmol) and (DL)-N-(2,4-dimethoxybenzyl)-phenylalanine methyl ester (0.335g, 1.02mmol) in toluene (10mL), and the mixture heated to reflux temperature for 3 hours with stirring. After cooling the solvent was removed *in vacuo* and the resultant red oil purified over silica (petroleum ether : ethyl acetate, 6 : 4, R_f 0.51) yielding the title compound as a yellow oil (0.217g, 0.416mmol, 42%). The NMR was complicated by the presence of both the enol and keto tautomers. $\delta_H(CDCl_3)$ 0.738 0.762 (3H, 2x t, J 7.4, CH_3), 0.87 0.91 (3H, 2x d, J 6.4, CH_3), 1.27 (2H, m, CH_2), 1.65 1.73 (3H, 2x s, CH_3), 2.35 (1H, m, CH), 3.05 3.29 (2H, m, CH_2), 3.49 3.52 (3H, 2x s, $COO\underline{C}H_3$), 3.64 3.65 (3H, 2x s, OCH_3), 3.68 3.69 (3H, 2x s, OCH_3), 3.74 (m, $CO\underline{C}H_2CO$), 3.90 4.27 (2H, m, CH_2), 4.03 4.42 (1H, m, CH), 5.10 (s, $CO\underline{C}H$=COH), 5.47 5.70 (1H, 2x d, J 9.6, CH), 5.67 6.14 (1H, 2x d J 15.6, CH), 6.28 (2H, m, Ph), 7.05 (7H, m, Ph & CH), 14.0 (0.5H, bs, OH) ; $\delta_c(CDCl_3)$ 11.8 (CH_3) 12.2 (CH_3), 20.0 20.2 (CH_3), 29.8 30.0 (CH_2), 34.6 35.0 (CH), 35.0 35.4 (CH_2), 46.5 47.6 (CH_2), 48.7 (CH_2), 51.8 52.0 ($\underline{C}OOCH_3$), 54.9 55.0 (OCH_3), 55.2 55.2 (OCH_3), 60.2 61.4 (CH), 89.3 ($CO\underline{C}H$=COH), 98.0 98.4 (Ph CH), 103.5 103.6 (Ph CH), 115.8 116.4 (Ph C), 120.3 123.5 (CH), 126.3 126.2 (Ph CH), 128.2 128.4 (Ph CH), 129.1 129.3 (Ph CH), 129.1

130.4 (Ph CH), 131.7 131.9 (Ph C), 137.9 138.2 (C), 141.1 149.8 (CH), 145.4 150.5 (CH), 157.7 (Ph $\underline{C}OCH_3$), 158.6 (Ph $\underline{C}OCH_3$), 160.2 160.8 (CON), 170.1 170.6 ($\underline{C}OOCH_3$), 172.8 (C=$\underline{C}$OH), 193.7 (CO); ν_{max}(neat)/cm^{-1} 2956, 2871, 2126, 1738, 1613, 1576, 1506, 1454, 1206; m/z (CI) 522 (M+1, 3.51%), 330 (100%); Found 522.2855, $C_{31}H_{40}NO_6$ (MH+) requires 522.2856.

6.5.14 (L)-N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.52)

(L)-N-(2,4-dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester was prepared as above from (L)-N-(2,4-dimethoxybenzyl)-phenylalanine methyl ester (0.412g, 1.25mmol), pyridinium *para*-toluene sulphonate (0.26g, 1.04mmol) and 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.261g, 1.04mmol) in toluene (6mL), yielding the title compound as a yellow oil (0.34g, 0.65mmol, 62%). The NMR was complicated by the presence of both the enol and keto tautomers. δ_H($CDCl_3$) 0.83 (3H, 2x t, J 7.4, CH_3), 0.96 (3H, 2x d, J 6.4, CH_3), 1.30 (2H, m, CH_2), 1.72 1.79 (3H, 2x s, CH_3), 2.42 (1H, m, CH), 3.11 3.37 (2H, m, CH_2), 3.56 3.59 (3H, 2x s, COO$\underline{CH}_3$), 3.72 3.73 (3H, s, OCH_3), 3.76 3.77 (3H, s, OCH_3), 3.80 (m, CO$\underline{CH}_2$CO), 3.94 4.34 (2H, m, CH_2), 4.09 4.50 (1H, m, CH), 5.15 (1H, s, CO$\underline{CH}$=COH), 5.54 5.77 (1H, 2x d, J 9.6, CH), 5.72 6.21 (1H, 2x d J 15.6, CH), 6.35 (2H, m, Ph), 7.16 (7H, m, Ph & CH), 14.2 (0.5H, bs, OH) ; δ_c($CDCl_3$) 11.9 (CH_3), 12.4 (CH_3), 20.1 20.3 (CH_3), 29.9 30.1 (CH_2), 34.8 35.1 (CH), 35.2 35.5 (CH_2), 46.5 47.8 (CH_2), 48.7 (CH_2), 52.0 52.1 (COO$\underline{C}H_3$), 55.1 (OCH_3), 55.3 (OCH_3), 60.2 60.5 (CH), 89.4 (CO$\underline{CH}$=COH), 98.2 98.5 (Ph CH), 103.7 (Ph CH), 116.0 116.6 (Ph C), 120.4 123.6 (CH), 126.4 (Ph CH), 128.3 128.5 (Ph CH), 129.3 129.4 (Ph CH), 130.5 129.1 (Ph CH), (Ph CH), 131.8 132.0 (Ph C), 138.0 138.3 (C), 141.2 150.0 (CH), 145.6 150.6 (CH), 157.8 (Ph $\underline{C}OCH_3$), 158.7 (Ph $\underline{C}OCH_3$), 160.3 160.9 (CON), 170.2 171.3 ($\underline{C}OOCH_3$), 172.9 (C=$\underline{C}$OH), 193.8 (CO).

6.5.15 5(DL)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.53)

To a stirred solution of freshly sublimed potassium *tert*-butoxide (0.047g, 0.42mmol) in *tert*-butanol (8mL), was added a solution of (DL)-N-(2,4-dimethoxybenzyl)-N-(E,E 3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (0.110g, 0.21mmol) in

tert-butanol (4mL). After 30 minutes stirring the resultant red solution was quenched with dilute aqueous hydrochloric acid (5mL). The organics were extracted into diethyl ether (1x 30mL), washed with water (1x 5mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a yellow oil (0.095g, 0.19mmol, 92%). Two tautomers in the ratio 84 : 16 were observable by NMR. $\delta_H(CDCl_3)$ 0.76 0.77 (3H, 2x t, J 7.3, CH_3), 0.92 (3H, d, J 6.6, CH_3), 1.30 (2H, m, CH_2), 1.76(16%) 1.81(84%) (3H, d, J 1.01, CH_3), 2.20(16%) 2.41(84%) (1H, m, CH), 3.08 (2H, d, J 4.7, CH_2), 3.70 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 3.78(84%) 3.94(16%) (1H, t, J 4.7, CH), 4.08(84%) 4.16(16%) (1H, d, J_{gem} 14.6, CH_2), 4.92(84%) 4.98(16%) (1H, d, J_{gem} 14.6, CH_2), 5.72 (1H, d, J 9.7, CH), 6.36 (2H, m, Ph), 7.13 (7H, m, Ph & CH), 7.36 (1H, d, J 15.4, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.1 (CH), 35.2 (CH_2), 38.4 (CH_2), 55.3 (OCH_3), 55.3 (OCH_3), 62.2(16%) 64.8(84%) (CH), 98.4 (Ph CH), 100.3 (C), 104.2 (Ph CH), 115.9 (CH), 116.0 (Ph C), 126.7 (Ph CH), 128.3 (Ph CH), 129.5 (Ph CH), 131.3 (Ph CH), 133.0 (Ph C), 135.6 (C), 149.5 (CH), 151.5 (CH), 158.5 (Ph C), 160.7 (Ph C), 173.8 (CON) 173.8 (COH), 194.3(84%) 203.6(16%) (CO); ν_{max}(neat)/cm^{-1} 3028, 2960, 2927, 2871, 2837, 2360 (w), 2341 (w), 1695, 1614, 1571, 1508, 1458, 1294, 1262, 1209, 1037, 614; m/z (CI) 490 (M+1, 9.14%), 151 (100%); Found 490.2593, $C_{30}H_{36}NO_5$ (MH+) requires 490.2593.

6.5.16 5(L)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.53)

5(L)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine was prepared as above from (L)-N-(2,4-dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (0.161g, 0.31mmol) and freshly sublimed potassium *tert*-butoxide (0.165mL of 1M solution in *tert*-butanol, 0.615mmol) in *tert*-butanol (12mL) affording the title compound as a yellow oil (0.152g, 0.31mmol, 100%). Two tautomers in the ratio 84 : 16 were observable by NMR. $\delta_H(CDCl_3)$ 0.82 0.83 (3H, 2x t, J 7.5, CH_3), 0.97 (3H, d, J 6.5, CH_3), 1.31 (2H, m, CH_2), 1.84(16%) 1.88(84%) (3H, d, J 1.01, CH_3), 2.30(16%) 2.50(84%) (1H, m, CH), 3.14 (2H, d, J 4.6, CH_2), 3.72 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.85(84%) 3.99(16%) (1H, t, J 4.3, CH), 4.14(84%) 4.21(16%) (1H, d, J_{gem} 14.6, CH_2), 4.97(84%) 4.98(16%) (1H, d, J_{gem} 14.8, CH_2), 5.78 (1H, d, J 10.0, CH), 6.41 (2H, m, Ph), 7.13 (7H, m, Ph & CH), 7.42 (1H, d, J 15.9, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3),

12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.2 (CH), 35.2 (CH_2), 38.4 (CH_2), 55.3 (OCH_3), 55.4 (OCH_3), 62.6(16%) 64.9(84%) (CH), 98.4 (Ph CH), 100.3 (C), 104.3 (Ph CH), 116.0 (CH), 116.1 (Ph C), 126.8 (Ph CH), 128.3 (Ph CH), 129.5 (Ph CH), 131.3 (Ph CH), 133.0 (Ph C), 135.7 (C), 149.5 (CH), 151.5 (CH), 158.5 (Ph C), 160.8 (Ph C), 173.8 (CON & COH), 194.4(84%) 204.6(16%) (CO).

6.5.17 5(DL)-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54)

5(DL)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (59.2mg, 0.12mmol) was dissolved in trifluoroacetic acid (5mL) and the bright red solution stirred under nitrogen for 5 minutes. The reaction mixture was quenched with ice, resulting in the formation of a pale yellow precipitate. This was extracted into dichloromethane (2x 20mL), washed with saturated aqueous $NaCO_3$ solution, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was redissolved in methanol (5mL), and filtered to remove white solid impurities. The filtrate was reduced *in vacuo* to give the title compound as a waxy yellow solid (36.6mg, 0.11mmol, 92%). Two tautomers in the ratio 85 : 15 were observable by NMR. $\delta_H(CDCl_3)$ 0.79(85%) 0.82(15%) (3H, t, J 7.2, CH_3), 0.95(85%) 1.04(15%) (3H, d, J 6.6, CH_3), 1.27 (2H, m, CH_2), 1.82(15%) 1.85(85%) (3H, s, CH_3), 2.43 (1H, m, CH), 2.58 (1H, m, CH_2), 3.23 (1H, m, CH_2), 3.96(85%) 4.08(15%) (1H, m, CH), 5.62(15%) 5.80(85%) (1H, d, J 9.6, CH), 6.32 (1H, bs, NH), 7.07 (1H, J 15.6, CH), 7.19 (5H, m, Ph), 7.48(85%) 7.99(15%) (1H, d, J 15.6, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.3 (CH), 38.4 (CH_2), 61.1(15%) 63.4(85%) (CH), 99.4 (C), 115.7 (CH), 127.1 (Ph CH), 128.8 (Ph CH), 129.1 (Ph CH), 133.0 (Ph C), 136.7 (C), 150.6 (CH), 152.5 (CH), 175.5 175.6 (CON & COH), 194.1 (CO); ν_{max}(neat)/cm^{-1} 3189 (br), 3063, 2959, 2923.24, 2871, 2853, 2345, 1701, 1655, 1612, 1571, 1431, 1289, 980, 694; m/z (EI) 339 (M+, 8.16%), 91 (100%), Found 339.1834, $C_{21}H_{25}NO_3$ (M+) requires 229.1834.

6.5.18 5(L)-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54)

Deprotection of 5(L)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.129g, 0.263mmol) was accomplished as above in

trifluoroacetic acid (5mL) to give the title compound as a waxy yellow solid (84.5mg, 0.249mmol, 95%). Two tautomers in the ratio 85 : 15 were observable by NMR. $\delta_H(CDCl_3)$ 0.84 (3H, t, J 7.3, CH_3), 1.00(85%) 1.11(15%) (3H, d, J 6.2, CH_3), 1.31 (2H, m, CH_2), 1.86(15%) 1.90(85%) (3H, s, CH_3), 2.49 (1H, m, CH), 2.65 (1H, m, CH_2), 3.26 (1H, m, CH_2), 3.99(85%) 4.10(15%) (1H, bm, CH), 5.66(15%) 5.85(85%) (1H, d, J 9.7, CH), 6.32 (1H, bs, NH), 7.12 (1H, J 15.6, CH), 7.22 (5H, bm, Ph), 7.52(85%) 7.96(15%) (1H, d, J 15.4, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.3 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.3 (CH), 38.2 (CH_2), 61.0(15%) 63.5(85%) (CH), 99.4 (C), 115.7 (CH), 127.0 (Ph CH), 128.7 (Ph CH), 129.1 (Ph CH), 133.0 (Ph C), 136.7 (C), 150.5 (CH), 152.3 (CH), 175.4 175.6 (CON & COH), 194.3 (CO).

6.5.19 (DL)-[1-^{13}C]-Phenylalanine hydrochloride (3.48a)

A solution of NH_4Cl (1.60g, 29.91mmol) in water (18mL) was slowly added to a stirred solution of phenylacetaldehyde (1.82g, 15.15mmol) in methanol (35mL) cooled to -5°C. After 10 min stirring, K^{13}CN (1g, 15.13mmol) was added and stirring continued at 20°C for 24 h. Methanol was removed *in vacuo* and the aqueous solution extracted into methylene dichloride , dried ($MgSO_4$) and evaporated *in vacuo* to afford a brown oil. This was heated under reflux with concentrated aqueous hydrochloric acid (50mL) for 48 hours. Solvent was removed *in vacuo* and the resulting solid washed with cold acetone (75mL) leaving a white amorphous solid, (DL)-[1-^{13}C]-phenylalanine hydrochloride (2.74g 13.52mmol, 83%). $\delta_H(D_2O)$ 3.08 (1H, ddd, J_{gem} 14.8, J_{vic} 7.8, J_{H13C} 4.6, CH_2), 3.22 (1H, ddd, J_{gem} 14.8, J_{vic} 5.6, J_{H13C} 4.8, CH_2); 4.18 (1H, dd J_{H13C} 13.2, J_{vic} 5.6, CH), 7.24 (5H, m, Ph); $\delta_c(D_2O)$ 35.4 (CH_2), 54.1 (d, J 58.7, CH), 127.8 (Ph CH), 129.0 (Ph, CH), 129.2 (Ph CH), 133.8 (Ph C), 171.5 (CO, enriched); ν_{max}(neat)/cm^{-1} 2852 (br), 1690, 1599, 1484, 1398, 1222, 1185, 724, 699; m/z (CI) 167 (M+1, 100%), 120 (11.01%).

6.5.20 (DL)-[1-^{13}C]-Phenylalanine methyl ester (3.49a)

Thionyl chloride (0.892g, 7.5mmol) was added dropwise to a solution of (DL)-[1-^{13}C]-phenylalanine hydrochloride (1.10g, 5mmol) in methanol (8mL) cooled to 0°C. The suspension was stirred at room temperature until product formation was complete, as observed by t.l.c. (silica plates eluted with nPrOH : NH_3 (aq con^c) 7 : 3, (developed with

ninhydrin). Solvent was removed *in vacuo*, and the solid residue redissolved in saturated aqueous $NaHCO_3$ (15mL). The aqueous solution was extracted into diethyl ether (5x 30mL) and the organic extracts were dried ($MgSO_4$), before removal of the solvent *in vacuo* to afford the title compound as a pale yellow oil (0.649g, 3.60mmol, 72%). $\delta_H(CDCl_3)$ 1.54 (2H, s, NH_2), 2.82 (1H, ddd, J_{gem} 14.9, J_{vic} 7.81, J_{H13C} 3.6, CH_2), 3.06 (1H, ddd, J_{gem} 14.7, J_{vic} 5.3, J_{H13C} 4.0, CH_2), 3.68 (3H, d, J_{H13C} 3.7, $COOCH_3$), 3.68 (1H, m, CH), 7.24 (5H, m. Ph); $\delta_c(CDCl_3)$ 43.1 (CH_2), 53.9 (d, J 3.9, OMe), 57.8 (d, J 58.7, CH), 128.8 (Ph CH), 130.5 (Ph CH), 131.3 (Ph CH), 139.2 (Ph C), 177.4 (CO, enriched); ν_{max} (neat)/cm^{-1} 3599 (br), 3378, 3313, 3027, 2950, 2854, 1954 (w), 1693, 1603, 1495, 1454, 1434, 1195, 1156, 755, 701; m/z (CI) 181 (M+1, 100%).

6.5.21 (DL)-[1-^{13}C]-N-(2,4-Dimethoxybenzyl) phenylalanine methyl ester (3.51a)

Methanolic HCl was added to a stirred solution of (DL)-[1-^{13}C]-phenylalanine methyl ester (0.598g, 3.32mmol) in methanol (20mL) to adjust to pH 6. 2,4-Dimethoxybenzaldehyde (0.565g, 3.40mmol) was added and the solution stirred at room temperature for 30 minutes. $NaBH_3CN$ (0.252g, 4.01mmol) was then added and the reaction stirred at room temperature for a further 16 hours. Methanol was removed *in vacuo,* distilled water (10mL) added and the aqueous mixture extracted into diethyl ether (3x 50mL). The organic extracts were combined, washed with an aqueous solution of $FeSO_4$, dried ($MgSO_4$), filtered and reduced *in vacuo,* affording the crude product as a pale yellow oil. Impurities of 2,4-dimethoxybenzylalcohol were removed by distillation under reduced presure (furnace temparature 110°C, 0.1mmHg). The product could be further purified over silica gel flash chromatography (hexane: ethylacetate, 50 : 50) (0.877g, 2,65mmol, 80%). $\delta_H(CDCl_3)$ 2.20 (1H, bs, NH), 2.95 (2H, m, CH_2), 3.50 (1H, m, CH), 3.59 (3H, d, J_{H13C} 3.7, $COOCH_3$), 3.61 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.70 (2H, d, J 9.0, CH_2), 6.35 (2H, m, Ph), 7.17 (6H, m, Ph); $\delta_c(CDCl_3)$ 41.6, (CH_2), 49.3 (NCH_2), 53.6 (d, J 2.9, $COO\underline{C}H_3$), 57.1 (OCH_3), 57.3 (OCH_3), 63.8 (d, J 59.2, CH), 100.3 (Ph CH), 105.6 (Ph CH), 122.0 (Ph CH), 128.6 (Ph CH), 130.4 (Ph CH), 131.2 (Ph CH), 132.4 (Ph C), 139.5 (Ph C), 160.6 (Ph CO), 162.2 (Ph CO), 176.9 (CO, enriched); ν_{max}(neat)/cm^{-1} 3334 (br), 3027, 3001, 2949, 2835,

2251 (w), 2065 (w), 1693, 1613, 1588, 1507, 1463, 1289, 1261, 1208, 1157, 1037, 734, 701; m/z (EI) 331 (M+1, 34.23%), 151 (100%).

6.5.22 (DL)-[1-^{13}C]-N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.52a)

Pyridinium *para*-toluene sulphonate (0.415g, 1.65mmol) was added to a solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.409g, 1.63mmol) and (DL)-[1-^{13}C]-N-(2,4-dimethoxybenzyl)-phenylalanine methyl ester (0.628g, 1.90mmol) in toluene (15mL), and the mixture heated to reflux temperature for 3 hours with stirring. After cooling the solvent was removed *in vacuo* and the resultant red oil purified over silica (petroleum ether : ethyl acetate, 6 : 4, R_f 0.51) yielding the title compound as a yellow oil (0.323g, 0.618mmol, 38%). The NMR was complicated by the presence of both the the enol and keto tautomers. $\delta_H(CDCl_3)$ 0.85 (3H, 2x t, J 7.4, CH_3), 0.98 (3H, 2x d, J 6.7, CH_3), 1.30 (2H, m, CH_2), 1.74 1.82 (3H, 2x s, CH_3), 2.45 (1H, m, CH), 3.14 3.39 (2H, m, CH_2), 3.56 3.59 (3H, 2x d, J_{H13C} 3.9, COO<u>C</u>H_3), 3.74 3.75 (3H, 2x s, OCH_3), 3.78 3.79 (3H, 2x s, OCH_3), 3.80 (m, CO<u>C</u>H_2CO), 4.10 4.37 (2H, m, CH_2), 4.09 4.40 (1H, m, CH), 5.14 (1H, s, CO<u>C</u>H=COH), 5.56 5.79 (1H, 2x d, J 9.6, CH), 5.74 6.24 (1H, 2x d J 15.6, CH), 6.39 (2H, m, Ph), 7.20 (7H, m, Ph & CH), 14.0 (0.5H, bs, OH); $\delta_c(CDCl_3)$ 11.8 (CH_3) 12.2 (CH_3), 20.0 20.2 (CH_3), 29.8 30.0 (CH_2), 34.6 35.0 (CH), 35.0 35.4 (CH_2), 46.5 47.6 (CH_2), 48.7 (CH_2), 51.8 52.0 (<u>C</u>OOCH_3), 54.9 55.0 (OCH_3), 55.2 55.2 (OCH_3), 60.2 61.4 (2x d, J 67.0, CH) 89.4 (CO<u>C</u>H=COH), 98.0 98.4 (Ph CH), 103.5 103.6 (Ph CH), 115.8 116.4 (Ph C), 120.3 123.5 (CH), 126.3 126.2 (Ph CH), 128.2 128.4 (Ph CH), 129.1 129.3 (Ph CH), 130.4 129.1 (Ph CH), 131.7 131.9 (Ph C), 137.9 138.2 (C), 141.1 149.8 (CH), 145.4 150.5 (CH), 157.7 (Ph <u>C</u>OCH_3), 158.6 (Ph <u>C</u>OCH_3), 160.2 160.8 (CON), 170.1 170.6 (<u>C</u>OOCH_3, enriched),. 176.6 (C=<u>C</u>OH), 193.7 (CO); ν_{max}(neat)/cm^{-1} 2957, 2927, 2871, 2361, 1698, 1630, 1579, 1465, 1456, 1209, 529; m/z (CI) 523 (M+1, 17.34%), 331 (100%).

6.5.23 5(DL)-[4-^{13}C]-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta- 2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.53a)

To a stirred solution of freshly sublimed potassium *tert*-butoxide (0.139g, 1.24mmol) in *tert*-butanol (20mL), was added a solution of (DL)-[4-^{13}C]-N-(2,4-dimethoxybenzyl)-N-

(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (0.323g, 0.618mmol) in *tert*-butanol (10mL). After 30 minutes stirring the resultant red solution was quenched with dilute aqueous hydrochloric acid (5mL). The organics were extracted into diethyl ether (1x 50mL), washed with water (1x 10mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a yellow oil (0.298g, 0.61mmol, 99%). Two tautomers in the ratio 84 : 16 were observable by NMR. $\delta_H(CDCl_3)$ 0.75 (3H, 2x t, J 7.3, CH_3), 0.90 (3H, d, J 6.6, CH_3), 1.30 (2H, m, CH_2), 1.76(16%) 1.79(84%) (3H, s, CH_3), 2.40 (1H, m, CH), 3.08 (2H, dd, J 4.7 J_{H13C} 4.7, CH_2), 3.68 (3H, s, OCH_3), 3.69 (3H, s, OCH_3), 3.78(84%) 3.94(16%) (1H, m, CH), 4.08(84%) 4.16(16%) (1H, d, J_{gem} 14.7, CH_2), 4.90(84%) 4.96(16%) (1H, d, J_{gem} 14.9, CH_2), 5.70 (1H, d, J 9.9, CH), 6.34 (2H, m, Ph), 7.12 (7H, m, Ph & CH), 7.36 (1H, d, J 15.6, CH); $\delta_c(CDCl_3)$ 14.0 (CH_3), 14.5 (CH_3), 22.1 (CH_3), 32.0 (CH_2), 37.1 (CH), 37.3(CH_2), 40.5 (CH_2), 57.4 (2x OCH_3), 64.6(16%) 66.4(84%) (d, J 40.5, CH), 100.4 (Ph CH), 102.4 (d, J 63.4, C), 106.4 (Ph CH), 118.0 (CH), 118.2 (Ph C), 128.8 (Ph CH), 130.4 (Ph CH), 131.6 (Ph CH), 133.3 (Ph CH), 135.0 (Ph C), 137.7 (C), 151.6 (CH), 153.5 (CH), 160.6 (Ph C), 162.8 (Ph C), 174.4 (dd, CON) 175.9 (d, COH), 196.1(84%) 204.8(16%) (CO, enriched), 209.2(84%) 211.4(16%) (CO); ν_{max}(neat)/cm^{-1} 2958, 2925, 2871, 1664, 1608, 1561, 1507, 1450, 1291, 1208, 1157, 1035, 612, 490; m/z (CI) 151 (100%).

6.5.24 5(DL)-[4-^{13}C]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54a)

5(DL)-[4-^{13}C]-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.296g, 0.603mmol) was dissolved in trifluoroacetic acid (10mL) and the bright red solution stirred under nitrogen for 5 minutes. The reaction mixture was quenched with ice, resulting in the formation of a pale yellow precipitate. This was extracted into dichloromethane (2x 50mL), washed with saturated aqueous $NaCO_3$ solution, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was redissolved in methanol (10mL), and filtered to remove white solid impurities. The filtrate was reduced *in vacuo* to give the title compound as a waxy yellow solid (0.161g, 0.472mmol, 79%). Two tautomers in the ratio 85 : 15 were observable by NMR. $\delta_H(CDCl_3)$ (very broad) 0.78 (3H, t, J 7.2, CH_3), 0.93 (3H, d, J 6.4, CH_3), 1.27 (2H, m, CH_2), 1.83 (3H, s, CH_3), 2.43 (1H, m, CH), 2.57 (1H, m, CH_2), 3.20 (1H, m,

CH_2), 3.93 (1H, m, CH), 5.76 (1H, d, J 8.4, CH), 6.32 (1H, bs, NH), 7.16 (6H, m, CH & Ph) 7.43 (1H, d, J 15.2); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.2 (CH), 38.3 (CH_2), 62.7 (d, J 40.0, CH), 99.4 (d J 59.0 C), 116.2 (br, CH), 126.8 (Ph CH), 128.6 (Ph CH), 129.1 (Ph CH), 133.0 (Ph C), 136.8 (C), 149.4 (br, CH), 151.6 (br, CH), 174.8 175.9 (br, CON & COH), 194.1(85%) 202.3(15%) (CO, enriched); ν_{max}(neat)/cm^{-1} 3188 (br), 3064, 2959, 29259, 2871, 2345, 1678, 1645, 1609, 1567, 1432, 1288, 981, 695; m/z (EI) 340 (M+, 33.03%).

6.5.25 (L)-[C_6D_5]-Phenylalanine methyl ester (3.49b)

Thionyl chloride (1.05g, 8.82mmol) was added dropwise to a solution of (L)-[C_6D_5]-phenylalanine (1.0g, 5.88mmol) in methanol (10mL) cooled to 0°C. The suspension was stirred at room temperature until product formation was complete, as observed by t.l.c. (silica plates eluted with nPrOH/NH_3 (aq con^c) 7 : 3, (developed with ninhydrin). Solvent was removed *in vacuo*, and the solid residue redissolved in saturated aqueous $NaHCO_3$ (20mL). The aqueous solution was extracted into diethyl ether (5x 30mL) and the organic extracts were dried ($MgSO_4$), before removal of the solvent *in vacuo* to afford the title compound as a pale yellow oil (0.919g, 4.99mmol, 85%). $\delta_H(CDCl_3)$ 1.60 (2H, bs, NH_2), 2.83 (1H, dd, J_{gem} 13.9, J_{vic} 7.7, CH_2), 3.05 (1H, dd, J_{gem} 13.9, J_{vic} 5.1, CH_2), 3.68 (3H, s, $COOCH_3$), 3.68 (1H, m, CH).

6.5.26 (L)-[C_6D_5]-N-(2,4-Dimethoxybenzyl) phenylalanine methyl ester (3.51b)

Methanolic HCl was added to a stirred solution of (L)-[C_6D_5]-phenylalanine methyl ester (0.919g, 4.99mmol) in methanol (20mL) to adjust to pH 6. 2,4-Dimethoxybenzaldehyde (0.947g, 5.70mmol) was added and the solution stirred at room temperature for 30 minutes. $NaBH_3CN$ (0.440g, 7.00mmol) was then added and the reaction stirred at room temperature for a further 16 hours. Methanol was removed *in vacuo*, distilled water (10mL) added and the aqueous mixture extracted into diethyl ether (3x 30mL). The organic extracts were combined, washed with an aqueous solution of $FeSO_4$, dried ($MgSO_4$), filtered and reduced *in vacuo*, affording the crude product as a pale yellow oil. Impurities of 2,4-dimethoxybenzylalcohol were removed by distillation under reduced presure (furnace temparature 110°C, 0.1mmHg). The product could be further purified

by silica gel flash chromatography (hexane: ethylacetate, 50 : 50). R_f 0.60 (1.459g, 4.36mmol, 87%) $[\alpha]_D^{21}$ =-69.23 (c 1.02, ethanol). $\delta_H(CDCl_3)$ 2.12 (1H, bs, NH), 2.89 (1H, dd, J_{gem} 13.5, J_{vic} 7.6, CH_2), 2.97 (1H, dd, J_{gem} 13.5, J_{vic} 6.2, CH_2), 3.49 (1H, dd, J_{vic} 7.6, J_{vic} 6.26, CH), 3.59 (3H, s, $COOCH_3$), 3.61 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.80 (2H, d, J 8.1 CH_2), 6.37 (2H, m, Ph), 7.00 (1H, d, J 8.1, Ph); $\delta_c(CDCl_3)$ 39.4, (CH_2), 47.2 (NCH_2), 51.5 ($COO\underline{C}H_3$), 55.0 (OCH_3), 55.2 (OCH_3), 61.9 (CH), 98.3 (Ph CH), 103.4 (Ph CH), 119.8 (Ph CH), 127.4-129.0 (m, Ph CD), 130.3 (Ph C), 137.1 (Ph C), 158.5 (Ph CO), 160.1 (Ph CO), 174.7 (CO); ν_{max}(neat)/cm^{-1} 3335 (br), 2999, 2949, 2836, 2362 (w), 2274 (w), 1734, 1612, 1588, 1506, 1460, 1207, 1156, 1036; m/z (EI) 333 (M-1, 1.98%).

6.5.27 (L)-[C_6D_5]-N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (3.52b)

Pyridinium *para*-toluene sulphonate (0.851g, 3.40mmol) was added to a solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.851g, 3.40mmol) and (L)-[C_6D_5]-N-(2,4-dimethoxybenzyl)-phenylalanine methyl ester (1.34g, 4.00mmol) in toluene (15mL), and the mixture heated to reflux temperature for 3 hours with stirring. After cooling the solvent was removed *in vacuo* and the resultant red oil purified over silica (petroleum ether : ethyl acetate, 6 : 4 R_f 0.51) yielding the title compound as a yellow oil (0.947g, 1.80mmol, 53%). The NMR was complicated by the presence of both the the enol and keto tautomers. $\delta_H(CDCl_3)$ 0.83 (3H, 2x t, J 7.4, CH_3), 0.96 (3H, 2x d, J 6.4, CH_3), 1.31 (2H, m, CH_2), 1.72 1.80 (3H, 2x s, CH_3), 2.42 (1H, m, CH), 3.11 3.37 (2H, m, CH_2), 3.56 3.59 (3H, 2x s, $COO\underline{C}H_3$), 3.72 3.73 (3H, s, OCH_3), 3.76 3.77 (3H, s, OCH_3), 3.80 (m, $CO\underline{C}H_2CO$), 3.95 4.32 (2H, m, CH_2), 4.10 4.51 (1H, m, CH), 5.14 (1H, s, $CO\underline{C}H$=COH), 5.54 5.77 (1H, 2x d, J 9.6, CH), 5.73 6.21 (1H, 2x d J 15.6, CH), 6.35 (2H, m, Ph), 6.91 6.97 (1H, 2x d, J 8.6, Ph), 7.06 7.29 (1H, 2x d, J 15.6, CH), 14.0 (0.5H, bs, OH); $\delta_c(CDCl_3)$ 11.9 (CH_3) 12.4 (CH_3), 20.0 20.3 (CH_3), 29.9 30.1 (CH_2), 34.7 35.1 (CH), 35.1 35.4 (CH_2), 46.5 47.7, (CH_2), 48.7 (CH_2), 52.0 52.1 ($COO\underline{C}H_3$), 55.1 55.1 (OCH_3), 55.2 55.3 (OCH_3), 60.2 60.5 (CH), 89.4 ($CO\underline{C}H$=COH), 98.2 (Ph CH), 98.5 (Ph CH), 103.6 (Ph CH), 116.0 116.6 (Ph C), 120.4 123.5 (CH), 129.2 130.4 (Ph CH), 131.8 132.0 (Ph C), 137.8 138.1 (C), 141.2 149.9 (CH), 145.6 150.6 (CH), 157.8 (Ph $\underline{C}OCH_3$), 158.7 (Ph $\underline{C}OCH_3$), 160.3 160.9 (CON), 170.2 171.3 ($\underline{C}OOCH_3$), 172.9 (C=$\underline{C}$OH), 193.8 (CO);

ν_{max}(neat)/cm^{-1} 2962, 2934, 2871, 2362, 1738, 1613, 1583, 1506, 1454, 1497, 1206, 1156, 1118, 1032; m/z (CI) 527 (M+1, 6.85%), 335 (100%).

6.5.28 5(L)-[C_6D_6]-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.53b)

To a stirred solution of freshly sublimed potassium *tert*-butoxide (2.91mL of 1M solution in *tert*-butanol, 2.92mmol) in *tert*-butanol (30mL), was added a solution of (L)-[C_6D_5]-N-(2,4-dimethoxybenzyl)-N-(E,E-3-oxo-6,8 dimethyldeca-4,6-dienoyl) phenylalanine methyl ester (0.792g, 1.46mmol) in *tert*-butanol (20mL). After 15 minutes stirring the resultant red solution was quenched with dilute aqueous hydrochloric acid (10mL). The organics were extracted into diethyl ether (1x 60mL), washed with water (1x 15mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as a yellow oil (0.72g, 1.46mmol, 100%). Two tautomers in the ratio 84 : 16 were observed in the NMR. δ_H($CDCl_3$) 0.82 0.83 (3H, 2x t, J 7.3, CH_3), 0.97 (3H, d, J 6.7, CH_3), 1.30 (2H, m, CH_2), 1.84(16%) 1.86(84%) (3H, d, J 0.81, CH_3), 2.44 (1H, m, CH), 3.14 (2H, d, J 4.9, CH_2), 3.75 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.85(84%) 3.99(16%) (1H, t, J 4.7, CH), 4.14(84%) 4.22(16%) (1H, d, J_{gem} 14.6, CH_2), 4.96(84%) 5.05(16%) (1H, d, J_{gem} 14.9, CH_2), 5.77 (1H, d, J 9.7, CH), 6.41 (2H, m, Ph), 7.01 (1H, d, J 9.7, Ph), 7.04 (1H, d, J 15.4, CH), 7.42(84%) 7.43(16%) (1H, d, J 15.4, CH); δ_c($CDCl_3$) 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.1 (CH), 35.2 (CH_2), 38.4 (CH_2), 55.3 (OCH_3), 55.4 (OCH_3), 62.6(16%) 64.9(84%) (CH), 98.4 (Ph CH), 100.3 (C), 104.3 (Ph CH), 116.0 (CH), 116.1 (Ph C), 127.5-129.4 (m, 3x Ph CD) 131.3 (Ph CH), 133.0 (Ph C), 135.5 (C), 149.5 (CH), 151.5 (CH), 158.5 (Ph C), 160.8 (Ph C), 173.8 (CON) 174.7 (COH), 194.4(84%) 202.8(16%) (CO); ν_{max}(neat)/cm^{-1} 2959, 2928, 2874, 2270, 1609, 1565, 1506, 1450, 1290, 1261, 1207, 1156, 1100; m/z (CI) 495 (M+1, 8.34%), 151 (100%).

6.5.29 5(L)-[C_6D_5]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (3.54b)

5(L)-[C_6D_5]-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.671g, 1.36mmol) was dissolved in trifluoroacetic acid (25mL) and the bright red solution stirred under nitrogen for 5 minutes. The reaction mixture was quenched with ice, resulting in the formation of a pale yellow precipitate. This was

extracted into dichloromethane (2x 75mL), washed with saturated aqueous $NaCO_3$ solution, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was redissolved in methanol (20mL), and filtered to remove white solid impurities. The filtrate was reduced *in vacuo* to give the title compound as a waxy yellow solid (0.457g, 1.33mmol, 98%). Two tautomers in the ratio 85 : 15 were observable by NMR. $\delta_H(CDCl_3)$ 0.86 (3H, t, J 7.2, CH_3), 1.01(85%) 1.11(15%) (3H, d, J 6.8, CH_3), 1.34 (2H, m, CH_2), 1.86(15%) 1.92(85%) (3H, s, CH_3), 2.50 (1H, m, CH), 2.65 (1H, m, CH_2), 3.32 (1H, m, CH_2), 4.01(85%) 4.18(15%) (1H, m, CH), 5.70(15%) 5.87(85%) (1H, d, J 10.0, CH), 6.28 (1H, bs, NH), 7.14 (1H, J 15.6, CH), 7.55(85%) 7.96(15%) (1H, d, J 15.6, CH); $\delta_D(CDCl_3)$ 7.41 (bs, Ph); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.9 (CH_2), 35.3 (CH), 38.3 (CH_2), 61.5(15%) 63.4(85%) (CH), 99.4 (C), 115.7 (CH), 128.5 (m, Ph CD), 133.1 (Ph C), 136.6 (C), 150.7 (CH), 152.5 (CH), 175.5 175.6 (CON & COH), 194.1(85%) 202.7(15%) (CO); ν_{max}(neat)/cm^{-1} 3185 (br), 3063, 2967, 2954, 2922, 2871, 1659, 1620, 1569, 1428, 1293, 979, 722; m/z (EI) 344 (M+, 18.25%).

6.5.30 (DL)-Tyrosine methyl ester, hydrochloride (3.66)

Thionyl chloride (8.33g, 70mmol) was added dropwise to dry distilled methanol (50mL) and stirred rapidly at -5°C. (DL)-Tyrosine (12.7g, 70mmol) was then added and the solution heated under reflux for 3 hours. After cooling the solution was diluted with dry diethyl ether (200mL), resulting in precipitation of the product. The product was collected by filtration, washed with diethyl ether and dried *in vacuo*. Recrystallisation from ethanol afforded the product (15.1g, 65.18mmol, 93%) as shiny white crystals. m.p. 189.5-190°C (lit. 189-190°C)[2] $\delta_H(CDCl_3)$ 2.74 (1H, dd, J_{gem} 13.1, J_{vic} 7.9, CH_2), 2.98(1H, dd, J_{gem} 13.1, J_{vic} 5.3, CH_2), 3.55 (3H, bs & m, NH_2 & CH), 3.67 (3H, s, $COOCH_3$), 6.60 (2H, d, J 8.5, Ph), 6.91 (2H, d, J 8.3, Ph); $\delta_c(CDCl_3)$ 41.70 (CH_2), 54.22 (OCH_3), 57.52 (CH), 117.80 (Ph CH), 129.73 (Ph C), 132,34 (Ph CH), 157.43 (Ph COH); ν_{max} (neat)/cm^{-1} 3301, 3256, 3044, 2884, 2858, 2608, 1742, 1607, 1588, 1511, 1447, 1222.

6.5.31 (DL)-N-(2,4-Dimethoxybenzyl) tyrosine methyl ester, hydrochloride (3.67)

2,4-Dimethoxybenzaldehyde (2.74, 16.50mmol) was added to a stirred solution of (DL)-tyrosine methyl ester, hydrochloride salt (3.48g, 15.0mmol) in methanol (70mL) and the reaction mixture stirred at room temperature for 30 minutes. $NaBH_3CN$ (1.13g, 18.0mmol) was then added and the reaction stirred at room temperature for a further 16 hours. 10% hydrochloric acid was carefully added *via* a pressure equalising dropping funnel until there was no further evolution of gas, and the solution remained at pH 1. The evolved gases were bubbled through KOH solution in a fume hood to neutralise the HCN. The aqueous solution was extracted with ethyl acetate (3x 70mL), dried ($MgSO_4$) and reduced *in vacuo* to give a the title compound as a white amorphous solid, which could be recrystallised from acetonitrile (3.94g, 10.32mmol, 69%). $\delta_H(CDCl_3)$ 3.18 (2H, m, CH_2), 3.57 (3H, s, COOMe), 3.69 (1H, m, CH), 3.80 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 4.10 (3H, bm, CH_2 & NH), 6.60 (2H, m, Ph), 6.74 (2H, d, J 8.5, Ph), 6.99 (2H, d, J 8.4, Ph), 7.44 (1H, d, J 7.0, Ph); $\delta_c(CDCl_3)$ 34.6 (CH_2), 43.9 (NCH_2), 52.5 ($COO\underline{C}H_3$), 55.4 (OCH_3), 55.7 (OCH_3), 59.6 (CH), 98.2 (Ph CH), 105.0 (Ph CH), 110. 8 (Ph CH), 115.3 (Ph CH), 124.2 (PH C), 130.2 (Ph CH), 133.3 (Ph C), 156.7 (Ph COH), 159.0 (Ph $\underline{C}OCH_3$), 161.8 (Ph $\underline{C}OCH_3$), 168.5 (CO); ν_{max}(neat)/cm^{-1} 3224 (br), 3943, 2833, 2789, 2361, 1748, 1615, 1590, 1510, 1445, 1422, 1211.59, 1159, 1133, 1034; m/z (CI), 346 (M+1, 15.72%), 196 (96.74%), 151 (100%); Found 346.1654, $C_{19}H_{24}NO_5$ (MH+) requires 346.1654.

6.5.32 (DL)-N-(2,4-Dimethoxybenzyl)-O-(4-methoxybenzyl) tyrosine methyl ester (3.69)

(DL)-N-(2,4-Dimethoxybenzyl) tyrosine methyl ester, hydrochloride (1.75g, 4.58mmol), K_2CO_3(1.38g, 10.0mmol), 4-methoxybenzyl chloride (0.832g, 5.31mmol) and $(Bu)_4NI$ (1.85g, 5.01mmol) were dissolved in DMF (50mL) and stirred at 60° for 3 days. After cooling to room temperature, water (50mL) was added and the organics extracted into diethyl ether (3x 50mL). The ether extracts were washed with 1N NaOH solution and brine, dried ($MgSO_4$), and reduced *in vacuo* to give a colourless oil. This was purified over silica (diethyl ether: hexane, 70 : 30 R_f 0.39) to give the title compound as a viscous, colourless oil (1.96g, 4.21mmol, 92%). $\delta_H(CDCl_3)$ 2.90 (1H, dd, J_{gem} 13.6, J_{vic} 7.8, CH_2), 3.04 (1H, dd, J_{gem} 13.6, J_{vic} 7.6, CH_2), 3.54 (1H, m, CH), 3.75-

3.90 (4H, m, 2x CH_2), 3.71 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 5.81 (1H, bs, NH), 6.42 (2H, m, Ph), 6.68 (2H, d, J 7.2, Ph), 6.79(2H, d, J 7.2, Ph), 6.87(2H, d, J 7.2, Ph), 7.12(2H, d, J 7.2, Ph), 7.15 (1H, d, J 8.0, Ph); $\delta_c(CDCl_3)$ 32.7 (CH_2), 47.5 (CH_2), 51.1 ($COO\underline{C}H_3$), 53.9 (CH_2), 55.1 (OCH_3), 55.2, (OCH_3), 55.3 (OCH_3), 62.8 (CH), 98.1 (Ph CH), 103.9 (Ph CH), 113.4 (Ph CH), 114.9 (Ph CH), 119.7 (Ph, C), 129.7 (Ph, CH), 130.3 (Ph C), 130.4 (Ph CH), 131.6 (Ph C) 154.1 (Ph $\underline{C}OCH_2$), 158.3 (Ph $\underline{C}OCH_3$), 158.7 (Ph $\underline{C}OCH_3$), 159.5 (Ph $\underline{C}OCH_3$), 173.4 (CO); ν_{max}(neat)/cm^{-1} 3389 (br), 2949, 2835, 1727, 1611, 1586, 1507, 1454, 1438, 1240, 1206, 1154, 1105, 1032, 814; m/z (CI) 466 (M+1, 6.87%), 151 (100%); Found 466.2230, $C_{27}H_{32}NO_6$ (MH+) requires 466.2230.

6.5.33 (DL)-N-(2,4-Dimethoxybenzyl)-O-benzyl tyrosine methyl ester (3.71)

(DL)-N-(2,4-Dimethoxybenzyl) tyrosine methyl ester, hydrochloride (2.13g, 5.59mmol), K_2CO_3 (1.71g, 12.35mmol) and benzyl bromide (1.54g, 9.02mmol) were dissolved in DMF (50mL) and stirred at 60° for 2 days. After cooling to room temperature water (50mL) was added and the organics extracted into diethyl ether (3x 50mL). The ether extracts were washed with 1N NaOH solution and brine, dried ($MgSO_4$), and reduced *in vacuo* to give a colourless oil. This was purified over silica (diethyl ether: hexane, 70 : 30) R_f 0.42, to give the title compound as a viscous, colourless oil (1.87g, 4.29 mmol, 77%). $\delta_H(CDCl_3)$ 2.92 (1H, dd, J_{gem} 13.9, J_{vic} 7.8, CH_2), 3.06 (1H, dd, J_{gem} 13.9, J_{vic} 7.3, CH_2), 3.65 (1H, m, CH), 3.67-4.05 (4H, m, 2x CH_2), 3.72 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 5.45 (1H, bs, NH), 6.42 (2H, m, Ph), 6.69 (2H, d, J 8.6, Ph), 6.89 (2H, d, J 8.5, Ph) 7.22 (6H, m, Ph); $\delta_c(CDCl_3)$ 36.9 (CH_2), 49.9 (CH_2), 53.2 (CH_2), 56.6 (OCH_3), 57.2, (OCH_3), 57.4 (OCH_3), 65.0 (CH), 100.2 (Ph CH), 106.0 (Ph CH), 116.7 (Ph CH), 121.7 (Ph C), 128.7 (Ph CH), 130.0 (Ph CH), 130.7 (Ph CH), 132.4 (Ph CH) 132.5 (Ph CH), 132.6 (Ph C), 141.7 (Ph C), 156.1 (Ph $\underline{C}OCH_2$), 160.8 (Ph $\underline{C}OCH_3$), 161.7 (Ph $\underline{C}OCH_3$), 175.4 (CO); ν_{max}(neat)/cm^{-1} 3406 (br), 2948, 2834, 1715, 1611, 1587, 1506, 1453, 1437, 1287, 1262, 1206, 1154, 1110, 1036, 823, 728, 697; m/z (CI) 436 (M+1, 9.92%), 151 (100%); Found 436.2124, $C_{26}H_{30}NO_5$ (MH+) requires 436.2124.

6.5.34 (DL)-N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-0-benzyl tyrosine methyl ester (3.72)

Pyridinium *para*-toluene sulphonate (0.251g, 1.00mmol) was added to a solution of 2,2-dimethyl-6-(E,E-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one (0.250g, 1.00mmol) and (DL)-N-(2,4-dimethoxybenzyl)-0-benzyl tyrosine methyl ester (0.615g, 1.41mmol) in toluene (6mL), and the mixture heated to reflux temperature for 2 hours with stirring. After cooling the solvent was removed *in vacuo* and the resultant red oil purified over silica (petroleum ether : ethyl acetate, 6 : 4) yielding the title compound as a yellow oil (0.345g, 0.55mmol, 55%). The NMR spectra were complicated by keto and enol forms, and could not be fully assigned. $\delta_H(CDCl_3)$ 0.92 0.99 (3H, 2x t, J 7.4, CH_3), 1.70 1.21 (3H, 2x s, CH_3), 1.40 (2H, m, CH_2), 1.73 1.79 (3H, 2x s, CH_3), 2.42 (1H, m, CH), 2,96-3.08 (2H, m, CH_2), 3.10-3.39 (2H, m, CH_2), 3.61 3.63 (3H, 2x s, OCH_3), 3.70 3.73 (3H, 2x s, OCH_3), 3.79 (3H, s, $COO\underline{C}H_3$), 3.95 ($CO\underline{C}H_2CO$), 3.60-4.60 (3H, m, CH & CH_2), 5.13 (s, $CO\underline{C}H{=}COH$), 5.58 5.80 (1H, 2x d, J 9.6, CH), 5.67 6.20 (1H, 2x d J 15.6, CH), 6.15-7.20 (14H, m, Ph & CH); $\delta_c(CDCl_3)$ 11.9 (CH_3) 12.4 (CH_3), 20.0 20.3 (CH_3), 29.6 30.1 (CH_2), 34.3 34.6 (CH), 34.8 35.1 (CH_2), 47.6 51.1 (CH_2), 52.1 ($\underline{C}OOCH_3$), 55.10 (OCH_3), 55.3 (OCH_3), 60.5 61.8 (CH), 61.6 63.0 (CH_2), 88.9 ($CO\underline{C}H{=}COH$), 98.1 (Ph CH), 103.9 (Ph CH), 114-140 (Ph) 120.2 123.3 (CH), 141.7 151.0 (CH), 146.0 151.5 (CH), 145.4 150.5 (CH), 154.2 (Ph $\underline{C}OCH_3$), 154.8 (Ph $\underline{C}OCH_3$), 158.7 159.6 (CON), 170.8 171.2 ($\underline{C}OOCH_3$), 172.9 173.3 ($C{=}\underline{C}OH$), 193.8 (CO); ν_{max}(neat)/cm^{-1} 3371, 2959, 2932, 2358, 1738, 1614, 1578, 1451, 1205; m/z (CI) 436 (36%) 151 (100%).

6.5.35 5(DL)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-(O-benzyl)-4-hydroxybenzyl-2,4-dioxopyrrolidine (3.73)

To a stirred solution of freshly sublimed potassium *tert*-butoxide (1molar solution in tert-butanol), (0.74mL, 0.739mmol) in *tert*-butanol (10mL), was added a solution of (DL)-N-(2,4-dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-0-benzyl tyrosine methyl ester (0.238g, 0.379mmol) in *tert*-butanol (5mL). After 20 minutes stirring the resultant red solution was quenched with dilute aqueous hydrochloric acid (5mL). The organics were extracted into diethyl ether (1x 30mL), washed with water (1x 5mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford the title compound as an orange oil (0.182g, 0.306mmol, 81%). Two tautomers in the ratio 77 : 23 were observable by

NMR. $\delta_H(CDCl_3)$ 0.85 (3H, t, J 7.3, CH_3), 1.00 (3H, d, J 6.4, CH_3), 1.29 (2H, m, CH_2), 1.87 (3H, s, CH_3), 2.43 (1H, m, CH), 3.05 (2H, d, J 4.7, CH_2), 3.6-4.0 (10H, 2xCH_2 & 2xOMe), 5.22(77%) 5.40(23%) (1H, m, CH), 5.82 (1H, d, J 9.7, CH), 6.30-7.40 (13H, Ph & CH), 7.43 (1H, d, J 15.4, CH); $\delta_c(CDCl_3)$ 11.9 (CH_3), 12.4 (CH_3), 20.0 (CH_3), 29.7 (CH_2) 29.9 (CH_2), 34.6 (CH), 35.3 (CH_2), 43.7 (CH_2), 55.4 (2x OMe). 64.5 (CH), 98.1 (C), 115.7 (CH), 135.4 (C), 150.4 (CH), 152.3 (CH), (8x Ph CH & 6xPh C), 173.90 & 174.15 (COH & CON), 194.1 (CO); ν_{max}(neat)/cm^{-1} 3332, 2960, 2921, 2870, 2338 (w), 1610, 1565, 1506, 1448, 1205, 1154, 1035.

6.5.36 5(DL)-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-(O-benzyl)-4-hydroxybenzyl-2,4-dioxopyrrolidine (3.74)

5(DL)-N-(2,4-Dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-5-(O-benzyl)-4-hydroxybenzyl-2,4-dioxopyrrolidine (64.9mg, 0.10mmol) was dissolved in a solution of pentamethylbenzene (0.148g, 1.0mmol) in trifluoroacetic acid (2mL) and the bright red solution stirred under nitrogen, for 10minutes. The trifluoroacetic acid was removed *in vacuo* and the resulting solid dissolved in dichloromethane (10mL) and washed with water (2x 10mL). After removal of the solvent *in vacuo*, the majority of the pentamethylbenzeme was precipitated out in methanol : water (85 : 15) and the filtrate containing the crude product, a red solid, was analysed by HPLC. MS and ^1H NMR analysis of the crude product indicated only N-deprotection had occured even after four hours in trifluoroacetic acid, and therefore further purification was not attempted. $\delta_H(CDCl_3)$ 0.75 (3H, t, J 7.3, CH_3), 0.99 (3H, d, J 6.7, CH_3), 1.35 (2H, m, CH_2), 1.85 (3H, s, CH_3), 2.43 (1H, m, CH), 3.18 (2H, m, CH_2), 3.8-4.1 (3H, m, CH & CH_2), 5.8 (1H, d, J 9.6, CH), 6.5-7.5 (11H, Ph & 2x CH) m/z (EI) 445 (M+, 28%), 91 (100%).

6.6 Feeding of Putative Tetramic acid Intermediates

6.6.1 5(DL)-[4-^{13}C]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine

5(DL)-[4-^{13}C]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.136g, 0.40mmol) was dissolved in ethanol (8mL) and pulse fed to four production media cultures, through a micropore filter on days 4, 5, 6 and 7, to give a final

concentration 2.0 mmolar in each flask. Tenellin (70mg) was isolated and purified on day 11.^{13}C NMR showed no observable enrichment.

6.6.2 5(L)-[C_6D_5]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine

5(DL)-[C_6D_5]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.103g, 0.30mmol) was dissolved in ethanol (4mL) and pulse fed to four production media cultures, through a micropore filter on days 4, 5, 6 and 7, to give a final concentration 1.5 mmolar in each flask. Tenellin (80mg) was isolated and purified on day 11.^{2}H NMR showed no observable enrichment.

5(DL)-[C_6D_5]-3-(E,E-4,6-Dimethylocta-2,4-dienoyl)-5-benzyl-2,4-dioxopyrrolidine (0.103g, 0.30mmol) was dissolved in DMSO (2mL) and pulse fed as above. Tenellin (65mg) was isolated and purified on day 11.^{2}H NMR showed no observable enrichment.

6.7 Attempted Isolation of Tetramic acid Intermediate

6.7.1 LCMS analysis of *Beauvaria bassiana* cultures

Ten production media flasks (50mL) were innoculated from 7 day old production media (5mL) and shaken at 200rpm, 32°C in the dark. One flask was removed on each of days 1-9. The mycelia were obtained by centrifugation and exhaustively extracted into acetone. The acetone extracts were reduced *in vacuo* and redissolved in methanol to a concentration of 1mg/mL, and subjected to LCMS analysis, methanol : water, 85 : 15, 0.1% trifluoroacetic acid, over a Hypersil C-18 BDS-protected column (5μm, 250 x 4.6mm).

6.7.2 Growth *Beauvaria bassiana* with P450-inhibitors

Two production media flasks (50mL) were incubated with metapyrone and two with miconazole at two concentrations of 2 mmolar and 4 mmolar respectivley. After 10 days growth the mycelia were obtained by centrifugation and exhaustively extracted into acetone. The acetone extracts were reduced *in vacuo* and subjected to LCMS analysis,

methanol : water, 85 : 15, 0.1% trifluoroacetic acid over a Hypersil C-18 BDS-protected column (5μm, 250 x 4.6mm).

6.8 Synthesis of Phenylpropanoid Metabolites

6.8.1 Methyl phenylacetate (4.22)

Phenylacetic acid (2.5g, 18.36mmol) in diethyl ether (60mL) was quenched with a solution of diazomethane (25mmol) in ether (100mL) generated from Diazald (5.35g, 25mmol) in ether (80mL) and potassium hydroxide (2g, 35.71mmol) in ethanol (20mL). Excess diazomethane was quenched by the dropwise addition of glacial acetic acid such that the yellow colour disappeared. The solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified over silica (dichloromethane) to give methyl phenylacetate (2.70g, 17.99mmol, 98%) as a clear colourless oil. $\delta_H(CDCl_3)$ 3.66 (2H, s, CH_2), 3.71 (3H, s, OCH_3), 7.32 (5H, m, Ar); $\delta_c(CDCl_3)$ 41.7 (CH_2), 52.5 (OCH_3), 127.6 (CH), 129.1 (CH), 129.8 (CH), 134.5 (C), 172.6 (CO); ν_{max} (neat)/cm^{-1} 3031, 2953, 1738, 1258, 1161, 732; m/z (EI) 150 (M+, 57.99%), 91 (100%).

6.8.2 Phenylethanol (4.23)

Methyl phenylacetate (1.00g, 6.66mmol) was added dropwise to a stirred suspension of lithium aluminium hydride (1.01g, 26.64mmol) in diethyl ether (30mL). The mixture was heated under reflux for 2h under dry N_2. After cooling to 20°C the reaction was quenched by the addition of wet diethyl ether (20mL), and poured into 5% H_2SO_4 (30mL). The ether layer was separated and the aqueous layer extracted into diethyl ether (2x 30mL).The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was over silica (CH_2Cl_2) to give 2-phenylethanol (0.67g, 5.46mmol, 82%) as a clear colourless oil. $\delta_H(CDCl_3)$ 1.73 (1H, bs, OH), 2.87 (2H, t, J 6.6, CH_2), 3.86 (2H, t, J 6.6), 7.26 (5H, m, Ar); $\delta_c(CDCl_3)$ 39.7 (CH_2), 64.2 (CH_2), 127.0 (CH), 129.1 (CH), 129.6 (CH), 139.0 (C); ν_{max}(neat)/cm^{-1} 3355 (br), 3027, 2942, 2877, 2246, 1603, 1496, 1453, 1046, 733, 699; m/z (EI) 122 (M+, 40.56%), 91 (100%).

6.8.3 Phenylacetaldehyde (4.24)

Pyridinium chlorochromate (2.16g, 10mmol), dried molecular sieves (35g) and *freshly distilled* dichloromethane were added to a dry flask under dry N_2. 2-Phenylethanol (0.61g, 5mmol) was added dropwise and the mixture was stirred vigorously for 2h. The dark brown reaction mixture was filtered through silica (10cm depth) in a wide sintered glass funnel under reduced pressure. The column was washed with dichloromethane (400mL) and the filtrate reduced *in vacuo* to give phenylacetaldehyde as a pale yellow oil (0.47g, 3.91mmol, 78%). As the compound is unstable to storage it was routinely used without further purification. $\delta_H(CDCl_3)$ 3.69 (2H, d, J 1.0, CH_2) 7.30 (5H, m. Ar), 9.75 (1H, t, J 1.2); $\delta_c(CDCl_3)$ 51.1 (CH_2), 127.9 (CH), 129.5 (CH), 130.1 (CH), 130.2 (C), 199.9 (CO); ν_{max} (neat)/cm^{-1} 3030, 2823, 2729, 1724, 1702, 911, 733, 700.

6.8.4 (DL)-Phenylalanine hydrochloride (4.25)

To a stirred solution of phenylacetaldehyde (0.415g, 3.45mmol) in methanol (15mL) cooled to -5°C, was slowly added a solution of NH_4Cl (0.267g, 5mmol) in water (8mL). After 10 min stirring, KCN (0.326g, 5.00mmol) was added and stirring continued at 20°C for 24 hours. Methanol was removed *in vacuo* and the aqueous solution extracted into dichloromethane (3x 15mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford a brown oil. This was heated under reflux with concentrated aqueous hydrochloric acid (10mL) for 48 hours. Solvent was removed *in vacuo* and the resulting solid washed with cold acetone (20mL) leaving a white amorphous solid, (DL)-phenylalanine hydrochloride (0.335g, 1.66mmol, 49%). m.p 230-230.5°C (lit. 230°C)[1] $\delta_H(D_2O)$ 3.07 (1H, dd, J_{vic} 7.6, J_{gem} 14.6, CH), 3.23 (1H, dd, J_{vic} 5.9, J_{gem} 14.6, CH); 4.24 (1H, dd, J_{vic} 7.8, J_{vic} 5.6, CH), 7.27 (5H, m, Ar); $\delta_c(D_2O)$ 40.0 (CH_2), 58.6 (CH), 132.5 (C), 133.7 (CH), 133.9 (CH), 138.4 (C), 175.9 (CO); ν_{max}(neat)/cm^{-1} 2060 (br), 1732, 1600, 1486, 1412, 1226, 1194, 731.47, 699; m/z (CI+) 166 (M+1, 69.94%), 120 (100%); Found 166.0868, $C_9H_{12}O_2N$ (M+1) requires 166.0868; GCMS (O-, N-trimethylsilyl derivative) 218 (M-91, 100%).

6.8.5 [1-^{13}C]-Methyl phenylacetate (4.22)

[1-^{13}C]-Phenylacetic acid (1.0g, 7.29mmol) in diethyl ether (25mL) was quenched with a solution of diazomethane (15mmol) in diethyl ether (60mL) generated from Diazald

(3.21g, 15mmol) in diethyl ether (50mL) and potassium hydroxide (1.2g, 21.4mmol) in ethanol (30mL). Excess diazomethane was quenched by the dropwise addition of glacial acetic acid such that the yellow colour disappeared. The solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified over silica (dichloromethane) to give [1-^{13}C]-methyl phenylacetate (0.99g, 6.55mmol, 89%) as a clear colourless oil. $\delta_H(CDCl_3)$ 3.61 (2H, d, J_{13CH} 12.1, CH_2), 3.71 (3H, d, J_{13CH} 3.9, OCH_3), 7.31 (5H. m. Ph); $\delta_c(CDCl_3)$ 41.7 (d, J 57.7, CH_2), 52.5 (d, J 2.6, OCH_3), 127.6 (C), 129.1 (CH), 129.8 (CH), 134.6 (C), 172.5 (CO, enriched); ν_{max} (neat)/cm^{-1} 3031, 2952, 1738, 1258, 1161, 732; m/z (EI) 151 (M+, 13.47%), 91 (100%).

6.8.6 [1-^{13}C^{2}H$_2$]-Phenylethanol (4.23)

[1-^{13}C]-Methyl phenylacetate (0.93g, 6.19mmol) was added dropwise to a stirred suspension of lithium aluminium deuteride (1.00g, 23.82mmol) in diethyl ether (30mL). The mixture was heated under reflux for 2h under dry N_2. After cooling to 20°C the reaction was quenched by the addition of wet diethyl ether (20mL), and poured into 5% H_2SO_4 (30mL).The ether layer was separated and the aqueous layer extracted into diethyl ether (2x30mL).The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified over silica (dichloromethane) to give [1-^{13}C^{2}H$_2$]-phenylethanol (0.68g, 5.41mmol, 87%) as a clear colourless oil. $\delta_H(CDCl_3)$ 2.59 (1H, bs, OH), 2.86 (2H, d, J_{13CH} 5.5, CH_2) 7.28 (5H, m Ph); $\delta_c(CDCl_3)$ 39.5 (d, CH_2), 63.3 (enriched, p, CD_2), 126.9 (C), 129.1 (CH), 129.6 (CH), 139.2 (C); ν_{max}(neat)/cm^{-1} 3346 (br), 3027, 2931, 2190, 2089, 1603, 1496, 1453, 1103, 1081, 744, 699; m/z (EI), 125 (M+), (42.25%), 91 (100%).

6.8.7 [1-^{13}C^{2}H]-Phenylacetaldehyde (4.24)

Pyridinium chlorochromate (2.16g, 10mol), dried molecular sieves (35g) and *freshly distilled* dichloromethane were added to a dry flask under dry N_2. [1-^{13}C^{2}H$_2$]-Phenylethanol (0.54g, 4.32mmol) was added dropwise and the mixture was stirred vigorously for 2h. The dark brown reaction mixture was filtered through silica (10cm depth) in a wide sintered glass funnel under reduced pressure. The column was washed with dichloromethane (400mL) and the filtrate reduced *in vacuo* to give phenylacetaldehyde as a pale yellow oil (0.44g, 3.60mmol, 83%). As the compound is

unstable to storage it was routinely used without further purification and analysis. $\delta_H(CDCl_3)$ 3.69 (2H, d, J_{13CH} 7.0, CH_2) 7.30 (5H, m. Ar); $\delta_c(CDCl_3)$ 52.1 (d, J 31.7, CH_2), 129.4 (CH), 131.0 (CH), 131.6 (CH), 131.7 (C), 201.8 (enriched, t, CDO).

6.8.8 [2-$^{13}C^2H^{15}N$]-(DL)-Phenylalanine hydrochloride (4.25)

To a stirred solution of [1-$^{13}C^2H$]-phenylacetaldehyde (0.478g, 3.91mmol) in methanol (15mL) cooled to -5°C was slowly added a solution of $^{15}NH_4Cl$ (0.427g, 7.83mmol) in water (6mL). After 10 min stirring, KCN (0.260g, 4.0mmol) was added and stirring continued at 20 °C for 24 hours. Methanol was removed *in vacuo* and the aqueous solution extracted into dichloromethane (3x 20mL), dried ($MgSO_4$) and evaporated *in vacuo* to afford a brown oil. This was heated under reflux with concentrated aqueous hydrochloric acid (10mL) for 48 hours. Solvent was removed *in vacuo* and the resulting solid washed with cold acetone (50mL) leaving a white amorphous solid, [2-$^{13}C^2H^{15}N$]-(DL)-phenylalanine hydrochloride (0.139g, 0.68mmol, 17%). $\delta_H(D_2O)$ 3.16 (2H, m, CH_2), 7.30 (5H, m, Ph); $\delta_c(D_2O)$ 40.02 (CH_2), 60.14 (td, J_{13CD} 21.82, J_{13C15N} 5.9, $CD^{15}NH_2$), 132.41 (C), 133.54 (CH), 134.00 (CH), 138.48 (C), 175.96 (CO); GCMS (O-, N-trimethylsilyl derivative) 221 (M-91, 100%).

6.8.9 *Para*-hydroxycinnamic acid (4.35)

Malonic acid (0.25g, 2.40mmol) and aniline (0.017mL) were dissolved in pyridine (1mL) and warmed gently until dissolved. *Para*-hydroxybenzaldehyde (0.342g, 2.80mmol) was added and the mixture stirred at 65°C for 16 hours under N_2. After cooling, the reaction mixture was added to a mixture of concentrated hydrochloric acid (2mL) and ice (4g). The brown precipitate was collected by filtration. The filtrate was extracted into diethyl ether (2x 50mL), reduced *in vacuo* and combined with the precipitate. The precipitate was treated with 5% sodium hydrogen carbonate (100mL) to take up the acids, and washed with diethyl ether (2x 20mL). The alkaline aqueous solution was reduced *in vacuo* to 20 mL, and added dropwise to a mixture of concentrated hydrochloric acid (5mL) and ice (20g). The precipitate thus formed was collected by filtration, dried and recrystallised from ethanol / water to give *para*-hydroxycinnamic acid as a white solid (0.28g, 1.71mmol, 71%). m.p. 211-212°C (lit. 210-211°C[3]) δ_H(DMSO-d6) 6.30 (1H, d, J15.9 , CH), 6.80 (2H, d, J 8.4 ,Ph), 7.51

(1H, d, J 15.6, CH), 7.52 (2H, d, J 8.5, Ph), 10.00 (1H, s, OH), 12.18 (1H, bs, OH); δ_C(DMSO-d6) 117.1 (CH), 117.5 (Ph CH), 127.0 (Ph C), 131.9 (Ph CH), 146.0 (CH), 161.4 (Ph C), 169.8 (COOH); ν_{max}(neat)/cm^{-1} 3357 (br), 3027, 2818, 1666, 1626, 1600, 1589, 1510, 1447, 1311, 1241, 1211, 1171, 977, 828; m/z 164 (M+, 100%), 147 (44.80%); Found 164.0473, $C_9H_8O_3$ (M+) requires 164.0473.

6.8.10 [2-^{13}C]-*Para*-hydroxycinnamic acid (4.35)

[2-^{13}C]-Malonic acid (0.25g, 2.38mmol) and aniline (0.017mL) were dissolved in pyridine (1mL) and warmed gently until dissolved. *Para*-hydroxybenzaldehyde (0.342g, 2.80mmol) was added and the mixture stirred at 65°C for 16 hours under N_2. After cooling the reaction mixture was added to a mixture of concentrated hydrochloric acid (2mL) and ice (4g). The brown precipitate was collected by filtration. The filtrate was extracted into diethyl ether (2x 50mL), reduced *in vacuo* and combined with the precipitate. The precipitate was treated with 5% sodium hydrogen carbonate (100mL) to take up the acids, and washed with diethyl ether (2x 20mL). The alkaline aqueous solution was reduced *in vacuo* to 20 mL, and added dropwise to a mixture of concentrated hydrochloric acid (5mL) and ice (20g). The precipitate thus formed was collected by filtration, dried and recrystallised from ethanol / water to give [2-^{13}C]-para-hydroxycinnamic acid as a white solid (0.28g, 1.70mmol, 72%). m.p. 211-212°C δ_H(DMSO-d6) 6.27 (1H, dd, J_{13CH} 136.7, J 15.6, CH), 6.77 (2H, d, J 8.0, Ph), 7.48 (1H, d, J 16.8, CH),7.50 (2H, d, J 8.0, Ph), 9.97 (1H, s, OH), 12.13 (bs, OH); δ_C(DMSO-d6) 115.3 (CH, enriched), 115.8 (Ph CH), 125.3 (Ph C), 130.1 (d, J 4.5, Ph CH), 144.2 (d, J 70.2, CH), 159.6 (Ph), 168.0 (d, J 73.2, COOH), m/z (EI) 165 (M+, 100%), 148 (42.91%), 121 (36.07%).

6.8.11 [*meta*-$^2H_2$]-(DL)-*Para*-hydroxyphenyllactic acid (4.37)

(DL)-*Para*-hydroxyphenyllactic acid (100mg, 0.548mmol) was dissolved in 9% DCl in D_2O (4mL) and heated at 80°C for 6 hours in a sealed tube. After cooling the solution was saturated with NaCl and extracted with ethyl acetate (4x 10mL). The combined extracts were dried over $MgSO_4$, filtered and evaporated. The product was purified by recrystalisation from diethyl ether (78.7mg, 42.7mmol, 78%). m.p. 130-132.5°C (lit. 133-135°C)[4]; δ_H($CDCl_3$) 2.76 (1H, dd, J_{gem} 14.2, J_{vic} 7.4, CH_2), 2.91 (1H, dd, J_{gem}

14.2, J_{vic} 4.7, CH_2), 4.32 (1H, dd, J_{vic} 7.3, J_{vic} 4.9, CH), 7.01 (2H, s, Ph); $\delta_c(CDCl_3)$ 41.1 (CH_2), 73.7 (CH), 117.5 (t, J 24.2, Ph CD), 131.0 (Ph C), 133.1 (Ph CH), 156.6 (Ph COH), 179.5 (CO); ν_{max}(neat)/cm^{-1} 3464, 3290, 2950, 2927, 2568, 2463, 2274, 2209, 1711, 1602, 1472, 1420, 1293, 1174, 1072; m/z (CI) 202 (M+18, 6.42%), 184 (M+, 3.57%), 109 (100%).

6.8.12 (DL)-*Para*-hydroxyphenyllactic acid (4.39)

Para-hydroxyphenyl pyruvic acid (0.721g, 4.0mmol) as an emulsion in water (30mL) was added dropwise to sodium borohydride (0.227g, 6.0mmol) in water (15mL) stirred at 0°C. Stirring was continued under N_2 for 1.5 hours at room temperature. Excess sodium borohydride was destroyed by addition of 10% aqueous hydrochloric acid. The aqueous solution was reduced *in vacuo* to 10mL and extracted into ethyl acetate (3x 50mL). The organic extracts were dried ($MgSO_4$) and reduced *in vacuo* to give an off white solid. This was washed with diethyl ether to give pure (DL)-*para*-hydroxyphenyllactic acid, as a white solid (0.471g, 2.74mmol, 65%). m.p. 129-130°C (lit. 133-135°C)[4]; $\delta_H(D_2O)$ 2.73 (1H, dd, J_{gem} 14.1, J_{vic} 7.2, CH_2), 2.88 (1H, dd, J_{gem} 14.1, J_{vic} 4.7, CH_2), 4.30 (1H, dd, J_{vic} 7.4, J_{vic} 4.9, CH), 6.67 (2H, d, J 8.6, Ph), 6.98 (2H, d, J 8.5, Ph); $\delta_c(D_2O)$ 43.1 (CH_2), 75.8 (CH), 119.8 (Ph CH), 133.0 (Ph C), 135.2 (Ph CH), 158.7 (Ph COH), 181.3 (CO); ν_{max}(neat)/cm^{-1} 3468, 3173 (br), 2363, 2261, 1714, 1616, 1599, 1447, 1194, 1173, 1104, 1173; m/z (CI) 182 (29.90%, M+), 165 (39.46%), 147 (100%), Found 182.0817, $C_9H_{12}NO_3$ ($M+NH_4^+-H_2O$) requires 182.0817.

6.8.13 [2-C^2H]-(DL)-*Para*-hydroxyphenyllactic acid (4.39)

Para-hydroxyphenyl pyruvic acid (0.721g, 4.0mmol) as an emulsion in water (40mL) was added dropwise to sodium borodeuteride (0.293g, 7.0mmol) in water (20mL) stirred at 0°C. Stirring was continued under N_2 for 1.5 hours at room temperature. Excess sodium borodeuteride was destroyed by addition of 10% aqueous hydrochloric acid. The aqueous solution was reduced *in vacuo* to 10mL and extracted into ethyl acetate (3x 50mL). The organic extracts were dried ($MgSO_4$) and reduced *in vacuo* to give an off white solid. This was washed with diethyl ether to give pure [2-C^2H]-(DL)-*para*-hydroxyphenyllactic acid, as a white solid (0.732 g, 4.0mmol, 100%). $\delta_H(D_2O)$ 2.72

(1H, d, J_{gem} 14.2, CH_2) 2.88 (1H, d, J_{gem} 14.2, CH_2), 6.67 (2H, d, J 8.4, Ph), 6.98 (2H, d, J 8.4, Ph); $\delta_c(CDCl_3)$ 42.3 (CH_2), 74.7 (t, CD), 119.0 (Ph CH), 132.2 (Ph C), 134.4 (Ph CH), 157.9 (Ph COH), 180.8 (CO); $\delta_D(D_2O)$ 4.25 (CD); ν_{max} (neat)/cm^{-1} 3473, 3197 (br), 2408, 2260, 1731, 1613, 1600, 1432, 1185, 1113; m/z (CI) 201 (100%, M+18), 183 (0.71%).

6.9 Feeding of Phenylpropanoid Metabolites

6.9.1 [2-^{2}H]-(DL)-Phenylalanine

[2-^{2}H]-(DL)-Phenylalanine (0.30g, 1.80mmol) was dissolved in distilled water (24mL) and pulse fed to six production media cultures, through a micropore filter on days 3, 4, 5 and 6, to give a final concentration 6.0 mmolar in each flask. Tenellin (50mg) was isolated and purified on day 10. ^{2}H NMR showed no observable enrichment.

6.9.2 [2-$^{13}C^2H^{15}N$]-(DL)-Phenylalanine

[2-$^{13}C^2H^{15}N$]-(DL)-Phenylalanine (0.135g, 0.661mmol) was dissolved in distilled water (12mL) and pulse fed to three production media cultures, through a micropore filter on days 3, 4, 5 and 6, to give a final concentration 4.4 mmolar in each flask. Tenellin (50mg) was isolated and purified on day 10. δ_c(DMSO) 140.3 (^{13}CH, enriched). No observable incorporation of ^{2}H or ^{15}N.

6.9.3 [2-$^{13}C^2H$]-(DL)-Phenyllactic acid

[2-$^{13}C^2H$]-(DL)-Phenyllactic acid (0.532g, 0.320mmol) was dissolved in distilled water (12mL) and pulse fed to three production media cultures, through a micropore filter on days 3, 4, 5 and 6, to give a final concentration 6.0 mmolar in each flask. Tenellin (35mg) was isolated and purified on day 10. ^{13}C NMR showed no observable enrichment.

6.9.4 [1-^{13}C]-(DL)-Phenylalanine

[1-^{13}C]-(DL)-Phenylalanine (0.114g, 0.686mmol) was dissolved in distilled water (40mL) and pulse fed to four production media cultures, through a micropore filter on

days 3, 4, 5 and 6, to give a final concentration 3.5 mmolar in each flask. Tenellin (110mg) was isolated and purified on day 10. δ_c(DMSO) 173.1 (^{13}CO, enriched).

6.9.5 [3-^{13}C]-(DL)-Tyrosine

[3-^{13}C]-(DL)-Tyrosine (0.125g, 0.686mmol) was dissolved in distilled water (40mL) and pulse fed to four production media cultures, through a micropore filter on days 3, 4, 5 and 6, to give a final concentration 3.5 mmolar in each flask. Tenellin (106mg) was isolated and purified on day 10. δ_c(DMSO) 110.9 (^{13}C, enriched).

6.9.6 [2-^{13}C]-*Para*-hydroxycinnamic acid

[2-^{13}C]-*Para*-hydroxycinnamic acid (0.165g, 1.00mmol) was dissolved in distilled water (1mL), neutralised with 0.1M NaOH, and made up to 16mL with distilled water. This was pulse fed to four production media cultures, through a micropore filter on days 4, 5, 6 and 6, to give a final concentration 5.0 mmolar in each flask. Tenellin (70mg) was isolated and purified on day 10. ^{13}C NMR showed no observable enrichment.

6.9.7 [*Meta*-$^2H_2$]-(DL)-*para*-hydroxyphenyllactic acid

[*Meta*-$^2H_2$]-(DL)-*para*-hydroxyphenyllactic acid (0.079g, 0.428mmol) was dissolved in distilled water (12mL) and pulse fed to four production media cultures, through a micropore filter on days 3, 4, 5 and 6, to give a final concentration 4.3 mmolar in each flask. Tenellin (50mg) was isolated and purified on day 10. δ_D(DMSO) 6.8 (Ph CD, enriched).

6.9.8 [2-C^{2}H]-(DL)-*Para*-hydroxyphenyllactic acid

[2-C^{2}H]-(DL)-*Para*-hydroxyphenyllactic acid (0.275g, 1.50mmol) was dissolved in distilled water (24mL) and pulse fed to six production media cultures, through a micropore filter on days 4, 5, 6 and 6, to give a final concentration 5.0 mmolar in each flask. Tenellin (190mg) was isolated and purified on day 10.^{2}H NMR showed no observable enrichment.

PART 2

6.10 Production, Isolation and Analysis of Iso-fatty acids

6.10.1 Growth of *Streptomyces cinnamonensis*

Growth of *S. cinnamonensis* was initiated by transfer of aliquots (3mL) of seven day old frozen production medium into two 500mL Erlenmeyer flasks each containing sterile seed medium (100mL). These flasks were shaken for ten days at 200 rpm, 32° C in the dark. The resultant cultures were used to initiate a batch of 500mL Erlenmeyer production medium flasks, each containing production medium (100mL), by the transfer of aliquots (3mL), and the cultures incubated under the same conditions for seven days. New production media flasks were routinely initiated from seven day old production medium flasks.

6.10.2 Seed medium

A solution of glucose (3.36g), soybean flour (2.1g), $CaCO_3$ (0.42g), $FeSO_4.7H_2O$ (0.77g), MgCl (0.004g) in distilled water (140mL) was prepared. 70mL portions were sterilized in 500mL Erlenmeyer flasks plugged with cotton wool.

6.10.3 Production medium

A solution of glucose (45g), soybean flour (13.5g), $CaCO_3$ (2.7g), $FeSO_4.7H_2O$ (0.94g), MgCl (0.027g) in distilled water (900mL) was prepared. 100mL portions were sterilised in 500mL Erlenmeyer flasks plugged with cotton wool.

6.10.4 Isolation of lipids

Two flasks of seven day old production medium were centrifuged at 14 000 rpm for 30 min. The pellets (cell mass) were washed in pH 7 buffer and re-centrifuged twice. The pellets were suspended in methanol (100mL), homogenised and filtered. The residue was resuspended in methanol (100mL), homogenised and filtered. The supernatant was reduced in volume to 150mL *in vacuo*. This was extracted into dichloromethane (2x 150mL), dried ($MgSO_4$), and concentrated. The resultant brown coloured oil was

triturated with distilled hexane (15mL) to extract the lipids. Filtration and concentration of the filtrate afforded the lipid fraction (30mg) which was dried under vacuum to remove any hexane. This fraction was suitable for analysis by ^{1}H and ^{13}C NMR (400 MHz).

6.10.5 Analysis of fatty acids by GCMS

The lipid fraction (30mg) was refluxed in saturated KOH in methanol (15mL) for 16 hours. The reaction mixture was added to H_2O (85mL), acidified with concentrated HCl and extracted into ether. the ether extracts were washed (H_2O), dried ($MgSO_4$) and the ether removed *in vacuo* to yield the glycerol-free fatty acids.

The fatty acids were methylated by heating under reflux for 16 hours in methanol (10mL), acidified with *para*-toluene sulphonic acid (200mg, 1.43mmol). Methanol was removed *in vacuo* and the residue redissolved in diethyl ether (100mL) and washed with H_2O (3x 10mL). The ether was removed *in vacuo* leaving a pale yellow oil. This was dissolved diethyl ether (1mL) and was analysed by GCMS, under EI and CI mode.

6.11 Synthesis of β-Aminoisobutyrate

6.11.1 α-Cyanopropanoic acid (5.20)

Sodium carbonate (2.12g, 20mmol) was added to a solution of α-bromopropionic acid (6.12g, 40mmol) in acetonitrile (40mL), and the reaction stirred at room temperature for 3 hours. Precipitated sodium α-bromopropionate was filtered, washed with ether and dried under reduced pressure. A solution of this salt (6.52g, 37.3mmol), NaOH (0.10g, 2.50mmol), and excess NaCN (2.74g, 55.9mmol) was heated in H_2O (20mL) between 65°-80°C for 4 hours. After cooling, the reaction mixture was carefully acidified in a well ventilated fume hood, with dilute $HCl_{(aq)}$. The HCN gas evolved was bubbled into KOH solution. $HCl_{(aq)}$ was added dropwise until there was no further evolution of HCN. The organics were extracted into ethyl acetate, dried ($MgSO_4$), and concentrated to give α-cyanopropanoic acid as a yellow oil, (1.74g, 47%). $\delta_H(CDCl_3)$ 3.64 (1H, q, J 7.4, CH), 1.55 (3H, d, J 7.4, CH_3); δ_C ($CDCl_3$) 170.60 (CO), 117.91 (CN), 32.04 (CH),

15.51 (CH_3); ν_{max} (neat) / cm^{-1} 3103.9 (b), 2952, 2615, 2259, 1739, 1459.7, 1266, 1202, 1111, 811; m/z (EI) 100 (M+1,35.54%), 54, (100%).

6.11.2 (DL)-β-Aminoisobutyric acid (5.22)

Adam's catalyst, PtO_2 (200mg) was added to a solution of α-cyanopropionic acid (0.50g, 5.05mmol) in a mixture of dried and distilled ethanol : chloroform (10 : 1) (33mL), and stirred vigorously under hydrogen at room temperature and atmospheric pressure for 18 hours. Filtration of the reaction mixture through a Celite pad followed by concentration of the filtrate afforded an oil. This was purified by Dowex (H^+ form), eluted with NH_4OH, concentrated and recrystallised with ethanol affording β-aminoisobutyric acid (0.34g, 65%). m.p. 177.5-178°C (lit. 176.5-177.5°C)[5] δ_H (D_2O) 2.90 (2H, m, CH_2), 2.48 (1H, m, CH), 1.03 (3H, d, J 7.2, CH_3); δ_C(D_2O) 183.41 (CO), 44.90 (CH_2), 41.48 (CH), 17.05 (CH_3); ν_{max} (KBr disc)/cm^{-1} 3363.2, 3401.1, 2968.3, 1676.7, 1627.7, 1569.66, 1572.2, 1418.9; m/z (CI) 104 (M+1, 100%), 61 (22.27%).

6.11.3 [2-^{13}CN]-α-Cyanopropanoic acid (5.20)

Sodium carbonate (4.24g, 40mmol) was added to a solution of α-bromopropionic acid (12.2g, 80mmol) in acetonitrile (80mL), and the reaction stirred at room temperature for 3 hours. Precipitated sodium α-bromopropionate was filtered, washed with ether and dried under reduced pressure. A solution of this salt (4.72g, 27mmol), KOH (0.14g, 2.50mmol), and K^{13}CN (2.00g, 30.3mmol) was heated in H_2O (20mL) between 75° C for 4 hours. After cooling, the reaction mixture was carefully acidified in a well ventilated fume hood, with dilute HCl until there was no further evolution of H^{13}CN. The organics were extracted into ethyl acetate, dried ($MgSO_4$), and concentrated to give [1-^{13}C]-α-cyanopropanoic acid as a yellow oil, (1.69g, 56%). δ_H($CDCl_3$) 10.05 (1H, bs, OH), 3.71 (1H, dq, J_{CH} 10.6 and J 7.4, CH), 1.64 (3H, dd, J_{CH} 6.1 and J 7.4, CH_3); δ_C ($CDCl_3$) 171.40 (CO), 117.51 (^{13}CN), 32.62 (CH), 15.57 (CH_3); ν_{max}(neat)/cm^{-1} 3001 (b), 2951, 2607, 2207, 1939, 1459, 1267, 1209, 1110, 811; m/z (EI) 101 (M+1, 100%), 55 (75.22%).

6.11.4 (DL)-β-[3-$^{13}C^2H_2$]-Aminoisobutyric acid (5.21)

Adam's catalyst, PtO_2 (200mg) was added to a solution of α-cyanopropanoic acid (0.60g, 6.00mmol) in a mixture of dried and distilled MeOD : $CDCl_3$ (10 : 1 v/v) (33mL), and stirred vigorously under deuterium gas at room temperature and atmospheric pressure for 18 hours. Filtration of the reaction mixture through a celite pad followed by concentration of the filtrate afforded an oil. This was purified by Dowex (H^+ form), eluted with NH_4OH,concentrated and recrystallised with ethanol affording (DL)-[β-$^{13}C^2H_2$]-β-aminoisobutyric acid (0.32g, 50%). δ_H (D_2O) 2.48 (1H, m, CH), 1.03 (3H, dd, $^3J_{CH}$ 4.9 andJ 7.3, CH_3); δ_C(D_2O) 183.41 (CO), 44.90 (m, $^{13}CD_2$), 41.48 (CH), 17.05 (CH_3); m/z (CI) 107 (M+1, 100%).

6.12 Feeding Experiments to *Streptomyces cinnamonensis*

6.12.1 Sodium [3-^{13}C]-(DL)-isobutyrate

[3-^{13}C]-(R,S)-isobutyrate (221mg, 2.0mmol) was dissolved in sterilised water (6mL). This was pulse fed to 2x *S. cinnamonensis* production medium cultures on days 0, 1,and 2 to give final concentration 10mmolar in each flask. The fatty acids (13mg) were isolated on day 7. δ_C($CDCl_3$) 22.67 ($^{13}CH_3$ enriched). The fatty acid methyl ester were formed (as above). GCMS analysis confirmed 50% labelling of M+1 peak of isopalmitate.

6.12.2 (DL)-β-[3-$^{13}C^2H_2$]-Aminoisobutyric acid

(DL)-(β-$^{13}C^2H_2$)-β-aminoisobutyric (0.22g, 2.06mmol) was dissolved in distilled water (16mL) and pulse fed to 4x *S. cinnamonensis* production medium cultures on days 0, 1, 2, 3 of growth to give a final concentration 5.15mmolar in each flask. The fatty acids were isolated on day 7. δ_C($CDCl_3$) No incorporation of ^{13}C or ^2H observed.

6.12.3 (DL)-β-[3-$^{13}C^2H_2$]-Aminoisobutyric acid (increased concentration)

(DL)-(β-$^{13}C^2H_2$)-β-aminoisobutyric (0.21g, 2.00mmol) was dissolved in distilled water (16mL) and pulse fed to 4x *S. cinnamonensis* production medium cultures on days 0, 1, 2, 3 of growth to give a final concentration 10.0 mmolar in each flask. The fatty acids

were isolated on day 7. δ_C ($CDCl_3$) No incorporation of ^{13}C or ^{2}H observed. The fatty acid methyl ester were formed (as above). GCMS analysis showed a tentative 4.5% labelling of M+1 peak of isopalmitate.

6.12.4 Sodium [1-^{13}C]-methacrylate

[1-^{13}C]-Sodium methacrylate (0.21g, 1.94mmol) was dissolved in distilled water (12mL) and pulse fed to 3x *S. cinnamonensis* production medium cultures on days 0, 1, 2, 3 of growth to give a final concentration of 6.46 mmolar in each flask. The fatty acids were isolated on day 7. δ_C($CDCl_3$) 38.03 (^{13}CH enriched).

6.13 Feeding Experiments to bkd- *Streptomyces avermitilis*

6.13.1 (DL)-β-[3-$^{13}C^{2}H_2$]-Aminoisobutyric acid

The following feeding experiments were performed by R. W. Fedechko, Central Research Division, Bioprocess Research, Pfizer Inc., Eastern Point Road, Groton, CT 06340, USA.

Cells were grown for 3 days, 29°C, 200rpm, in seed medium (8mL) in a 1x 6" glass tube. These tubes were inoculated by transfer of 0.5mL from a 20% glycerol stock. After 3 days, 2x 300mL Erlenmeyer flasks, one containing 25mL of production medium, and one containing 25mL of 'particulate free' production medium were inoculated with preform growth (2mL) and grown for 15 days at 29°C, 200rpm, after which the metabolites were isolated and sent for analysis. The flasks were examined for avermectin production and it appeared to be occuring.

(DL)-β-[3-$^{13}C^{2}H_2$]-Aminoisobutyrate (22.5mg, 0.21mmol) was added to each flask at the beginning of the production phase of avermectin, to give a final concentration of 8.5mmolar. At the end of the fermentation, these flasks were tested for sterility (BHI streak), and confirmed to be sterile. In preparation for FAME derivitisation , the cells were collected by centrifugation (10,000 rpm/ Sorvall SS34/ 15 minutes) and then frozen at -20°C.

6.13.2 FAME derivitisation and GCMS analysis of fatty acids

Cells were removed from -20°C, and allowed to warm to room temperature. An aliquot (1g) was removed and Reagent 1 (45g NaOH, 150 mL HPLC-grade MeOH, 150 mL distilled water) (2.5 mL) was added to each. The tubes were vortexed, incubated for 5 minutes at 100°C, vortexed again, and incubated for an additional 25 minutes followed by cooling to room temperature. Methylation reagent (325mL 6N HCl, 5mL hplc-grade MeOH) (5mL) was added, followed by heating for 10 minutes at 80°C. After the tubes had cooled, extraction reagent (200mL hexane, 200mL MTBE) (3.25mL) was added. These tubes were gently mixed by inversion for 10 minutes, followed by removal of most of the lower aqueous layer using a Pasteur pipette. Base wash (10.8g NaOH in 900mL distilled water) (7.5 mL) was added, and the tubes mixed gently by inversion for 5 minutes. The upper, organic layer was then removed and evaporated to dryness.

GCMS analysis was performed by K. E. Reynolds, Department of Pharmaceutical Sciences, 20 North Pine Street, University of Maryland, Maryland 21202-1180, Baltimore, USA.

The above samples were analysed by GCMS with the mass spectrometer sweeping 240-280 AMU, and again with the mass spectrometer sweeping 50-350 AMU. In both samples labelling of isopalmitate and isomyristate with an M+2 species was observed.

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[3] R. Adams and T. E. Bockstahler, *J. Am. Chem. Soc.*, 1952, **74,** 5346.

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[5] R. F. Dietrich, *J. Org. Chem.*, 1979, **44**, 1894.

APPENDIX

Research Conferences Attended

21st Century Heterocyclic Chemistry, University of Sunderland, 7th May 1997.

Royal Society of Chemistry Bio-organic Group Postgraduate Symposium, University of Liverpool, 16th December 1996, (oral presentation).

Royal Society of Chemistry International Interdisciplinary Conference. Polyketides: Chemistry, Biology and Molecular Genetics, University of Bristol, 1st-3rd April 1996, (poster presentation).

Royal Society of Chemistry Bio-organic Group Postgraduate Symposium, University of Southampton, 18th December 1995.

Royal Society of Chemistry Perkin Regional Meeting, University of Durham, 8th December 1995.

Biological Challenges for Organic Chemistry, University of St Andrews, 9th-13th July 1995.

Symposium on Recent Progress in Polyketide Biosynthesis, University of Cambridge, 4th April 1995.

SCI Novel Organic Chemistry: 6th Graduate Symposium, University of York, 1st March 1995.

Recent Developments in Stereochemistry, University of Sheffield, December 20th 1994.

Royal Society of Chemistry Bio-organic Group Postgraduate Symposium, University of Durham, 12th December 1994.

Departmental Colloquia

1994

October 5 — Prof. N. L. Owen, Brigham Young University, Utah, USA
Determining Molecular Structure - the INADEQUATE NMR way

October 19 — Prof. N. Bartlett, University of California
Some Aspects of Ag(II) and Ag(III) Chemistry

November 2 — Dr P. G. Edwards, University of Wales, Cardiff
The Manipulation of Electronic and Structural Diversity in Metal Complexes-New Ligands

November 10 — Dr M. Block, Zeneca Pharmaceuticals, Macclesfield
Large-scale Manufacture of ZD 1542, a Thromboxane Antagonist Synthase Inhibitor

November 16 — Prof. M. Page, University of Huddersfield
Four-membered Rings and β-Lactamase

November 17 — Dr Cairns-Smith, Unversity of Glasgow
Clay Minerals and the Origin of Life

November 23 — Dr J. M. L. Williams, University of Loughborough
New Approaches to Assymetric Catalysis

1995

January 25 — Dr D. A. Roberts, Zeneca Pharmaceuticals
The Design and Synthesis of Inhibitors of the Renin-angiotensin System

January 1 — Mrs S.Owen, Northumberland Water
Trace Organics in the Environment

February 1 — Dr T. Cosgrove, Bristol University
Polymers do it at Interfaces

February 16 — Prof. H. Kroto, Sussex University
C_{60}-The Cellestial Sphere that Fell to Earth

February 22 — Prof. E. Schaumann, University of Claustal
Silicon- and Sulphur-mediated Ring-opening Reactions of Epoxides

March 23	Prof. Riess, Nice University Highly Fluorinated Compounds and Systems for Biomedical Uses
May 4	Prof. A. J. Kresge, University of Toronto *The Ingold Lecture* Reactive Intermediates: Carboxylic-acid Enols and Other Unstable Species
May 9	Prof. Townsend, Unilever *IOM Lecture* Polymers for the Year 2000: The Challenges Ahead.
July 7	Dr Kevin Reynolds, University of Maryland at Baltimore The Molecular Biology and Biochemisty of Fatty Acids from Streptomyces
October 18	Prof. A. Alexakis, Univ. Pierre et Marie Curie, Paris Synthetic and Analytical Uses of Chiral Diamines
October 25	Dr D. Martin Davies, University of Northumbria Chemical Reactions in Organised Systems
November 1	Prof. W. Motherwell, UCL London New Reactions for Organic Synthesis
November 3	Dr B. Langlois, University Claude Bernard-Lyon Radical Anionic and Pseudo Cationic Trifluoromethylation
November 8	Dr D. Craig, Imperial College, London New Strategies for the Assembly of Heterocyclic Systems

1996

January 10	Dr Bill Henderson, Waikato University, NZ Electrospray Mass Spectroscopy - A New Sporting Technique
February 14	Dr J. Rohr, Univ. Gottingen, FRG Goals and Aspects of Biosynthetic Studies on Low Molecular Weight Natural Products
February 28	Prof. E. W. Randall, Queen Mary & Westfield College New Perspectives in NMR Imaging

March 6 — Dr Richard Whitby, University of Southhampton
New Approaches to Chiral Catalysts: Induction of Planar and Metal Centred Asymmetry

March 13 — Prof. Dave Garner, Manchester University
Mushrooming in Chemistry

1997

January 15 — Dr V. K. Aggarwal, University of Sheffield
Suphur Mediated Assymetric Synthesis

January 16 — Dr Sally Brooker, University of Otago, NZ
Macrocycles: Exciting yet Controlled Thiolate Coordination Chemistry

January 22 — Dr Neil Cooley, BP Chemicals, Sunbury
Synthesis and Properties of Alternating Polyketones

January 29 — Dr Julian Clarke, UMIST
What can we learn about polymers and biopolymers from computer-generated nanosecond movie clips?

February 5 — Dr A. Haynes, University of Sheffield
Mechanism in Homogenous Catalytic Carbonylation

February 6 — Prof. P. Bartlett, University of Southampton
Integrated Chemical Systems

February 12 — Dr Geert-Jan Boons, University of Birmingham
New Developments in Carbohydrate Chemistry

February 25 — Professor A. G. Sykes, University of Newcastle
The Synthesis, Structure and Properties of Blue Copper Proteins

February 26 — Dr Tony Ryan, UMIST
Making Hairpins from Rings and Chains

March 5 — Dr J. Staunton FRS, Cambridge University
Tinkering with Biosynthesis: Towards a New Generation of Antibiotics