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Helen K. Smith

BIOSYNTHESIS OF THE FUNGAL METABOLITE TENELLIN

Helen K Smith, MChem (Hons)

PhD Thesis

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DECLARATION

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For Dad

Abstract

Biosynthesis of the fungal metabolite tenellin

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This thesis concerns the biosynthesis of tenellin, a bright yellow secondary metabolite of the fungus *Beauveria bassiana*. Several putative intermediates have been synthesised and feeding experiments performed in order to provide some insight into the biosynthetic pathway.

Tenellin was originally thought to be formed from the condensation of phenylalanine and a polyketide moiety, however recent studies have now shown that tyrosine is the more direct amino acid precursor. The biosynthesis is proposed to occur by rearrangement of tyrosine to 3-amino-2-(4-hydroxy)-phenylpropionic acid, in a similar manner to the rearrangement observed in alkaloid biosynthesis. The synthesis of this β-amino acid is described and the subsequent incorporation study using labelled material discussed. The result argues against the intermediacy of this compound in the biosynthesis of tenellin.

Consequently, it is proposed that the biosynthesis of tenellin does not include the rearrangement of tyrosine, but instead proceeds *via* a five membered tetramic acid, which is considered to undergo rearrangement and ring expansion to generate the pyridone ring of tenellin. The putative acyl tetramic acid precursor is synthesised and administered to cultures of *Beauveria bassiana*. Preliminary ^{2}H NMR spectra indicate a possible incorporation and that the tetramic acid has a role in tenellin biosynthesis. Further analysis by HPLC and LCMS is described.

The biosynthetic origin of H-6 of tenellin has never been established. Several experiments, including the synthesis of [2-^{2}H]-tyrosine, are described, but the origin of this proton remains unclear.

Acknowledgements

Firstly, I would like to acknowledge Prof David O'Hagan for his invaluable supervision and ideas during my PhD. I would also like to thank Dr Andrew Kohler for his enthusiasm and interest in the project, particularly during my placement at Sanofi-Synthelabo.

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Finally, I am indebted to Christoph for his continual support and encouragement during the preparation of this thesis.

Abbreviations

Ac	acetyl
ACP	acyl carrier protein
ADP	adenosine diphosphate
anal.	analysis
APCI	atmospheric pressure chemical ionisation
aq	aqueous
AT	acyl transerase
atm	atmosphere
ATP	adenosine triphosphate
Bn	benzyl
Boc	*tert*-butoxycarbonyl
i-Bu	*iso*-butyl
t-Bu	*tert*-butyl
C	Celsius
C.	*Cylindrocladium*
calcd	calculated
CAN	ceric ammonium nitrate
Cbz	benzyloxycarbonyl
CoA	coenzyme A
conc	concentrated
COSY	correlation spectroscopy
d	day
d	doublet
D-	*dextro*
Da	Dalton
DAHP	3-deoxy-D-*arabino-heptulosonate*-7-phosphate
L-DAP	L-2,3-diaminopropanoic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DEBS	6-deoxyerythronolide B synthase

DH	dehydratase
DHQ	3-dehydroquinate
DIBALH	di*iso*butylaluminium hydride
DMAP	4-dimethylaminopyridine
DMAPP	dimethylallyl pyrophosphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
E-	entgegen
EI	electron impact
EPSP	5-enolpyruvyl-shikimate 3-phosphate
equiv.	equivalent
ER	enoyl reductase
Et	ethyl
et al.	*et alia*
FAS	fatty acid synthase
FMN	flavin mononucleotide
h	hour
HPLC	high pressure liquid chromatography
Hz	Hertz
KR	ketoreductase
KS	ketosynthase
L-	*laevo*
LCMS	liquid chromatography mass spectrometry
LDA	lithiumdi*iso*propylamide
LHMDS	lithium hexamethyldisilazane
m-	*meta*
m	multiplet
Me	methyl
mg	milligram
mM	millimolar
μC_i	micro Curie
min	minute
mmol	millimole

MSAS	6-methyl salicylic acid synthase
MS-MS	mass spectrometry-mass spectrometry
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NIH	National Institute of Health
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
o-	*ortho*
ORF	open reading frame
q	quartet
p-	*para*
PAH	phenylalanine hydroxylase
PEP	phosphoenolpyruvate
PG	protecting group
Phth	phthalate
PKS	polyketide synthase
PLP	pyridoxal 5'-phosphate
PPTS	pyridinium *para*-toluene sulfonate
i-Pr	*iso*-propyl
n-Pr	*n*-propyl
RNA	ribonucleic acid
s	second
s	singlet
SAM	*S*-adenosylmethionine
t	triplet
TCA	tricarboxylic acid
temp.	temperature
TFA	trifluoroacetic acid
TFMSA	trifluoromethane sulfonic acid
THF	tetrahydrofuran

TMS	trimethylsilyl
Tos	tosyl
U	universal
Z	zusammen

Contents

Chapter 1

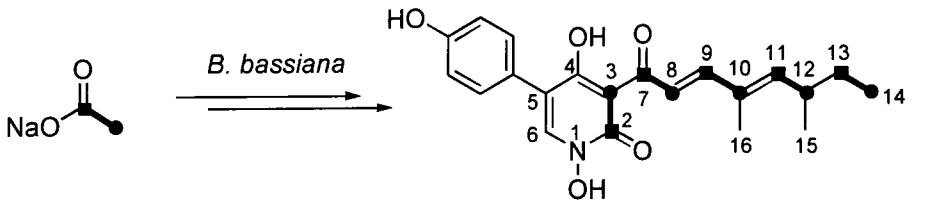
1 Introduction

1.1 Use of isotopes in biosynthetic studies

The study of the biosynthesis of metabolites made significant progress with the advent of radioisotopes and has been further developed by the use of stable isotopes. Putative precursors carrying one or more isotopic labels are administered to an organism and the isolated metabolite examined for evidence of isotopic incorporation. Such tracer studies have been fundamental to the majority of biosynthetic studies performed to date.
It is possible to use both radio-labelled, mainly ^{3}H and ^{14}C atoms, and the stable isotopes ^{2}H, ^{13}C, ^{15}N and ^{18}O in these experiments. The latter can be detected by nuclear magnetic resonance (NMR) and/or mass spectrometric techniques and the former by scintillation counting.

Radioisotope labelling has two main advantages. The isotopes are very sensitive to detection by scintillation counting and also have zero natural abundance so low enrichments can be detected. However, a high degree of radiochemical purity is needed in order to avoid errors from contamination of minor impurities.

The use of stable isotopes is more common and in particular, there have been many biosynthetic studies carried out with ^{13}C labelled substrates. Incorporation of the administered precursor can be detected by enhancement of the resonances in the ^{13}C NMR spectrum. Subsequent comparison to the natural abundance spectrum allows the enriched carbon/s to be readily determined. A common application of the ^{13}C label is in the form of doubly labelled acetate, [1,2-$^{13}C_2$]-acetate. Observation of the coupling constants in ^{13}C NMR arising from adjacent ^{13}C nuclei enables intact acetate units in the resultant metabolite to be identified. For example, feeding experiments[1] with [1,2-$^{13}C_2$]-acetate **2** were used to show the intact incorporation of five acetates during tenellin **1** biosynthesis in the fungus *Beauveria bassiana* (Scheme 1-1).



2a **1**

Scheme 1-1

In the ^{13}C NMR spectrum, satellite peaks positioned around the isolated ^{13}C resonances showed that C-2, C-3 and C-7 to C-14 all derive from intact C_2 units (Scheme 1-1). The use of doubly labelled ^{13}C acetate is especially useful in cases of low isotopic incorporation, since the satellites arising from the adjacent ^{13}C atoms allow lower enrichments to be observed (<0.1 %).

Deuterium (^2H) has a low natural abundance (0.015 %). As a consequence, negligible incorporations only slightly above the natural abundance level can be detected by ^2H NMR. In this respect, ^2H-labelling is 60 times more sensitive than ^{13}C labelling. Although the quadrupolar nature of the ^2H nucleus leads to line broadening in NMR, the advantages of a short relaxation time and no nuclear Overhauser effect (nOe) mean that determination of incorporation can be performed directly by peak integration. Such a feature can compensate for the poor resolution of deuterium spectra. Coupling constants for $J(^2H\text{-}^1H)$ are approximately one sixth of those for $J(^1H\text{-}^1H)$.

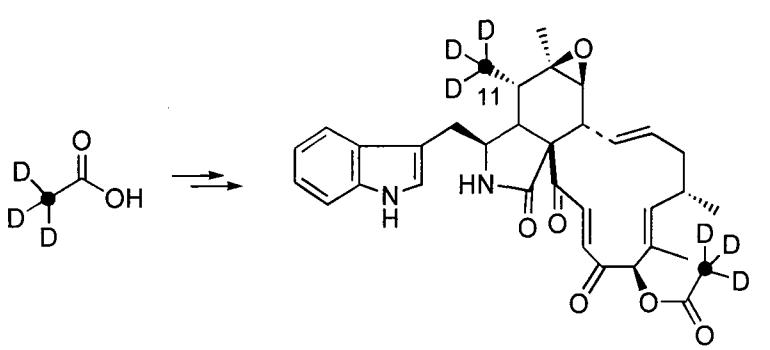
^{17}O has been used effectively in some studies[2] but it is not a routinely utilised isotopic label for exploring biosynthetic processes. Although the natural abundance of ^{17}O is low (0.037 %), the broadening of resonances by this quadrupolar nucleus yields poor quality spectra. ^{15}N, another less frequently used nucleus, is sometimes employed for the labelling of amino acids, however rapid transamination processes can give results that are not always reliable.

As well as the detection of atoms, incorporation studies can be used to trace the fate of bonds through biosynthetic pathways, for example C-H. It is possible to detect small changes in the ^{13}C chemical shift due to the presence of α- and β-substituted isotopes.

The α-shift, an upfield shift of between 0.3 and 0.6 ppm, occurs when a deuterium is directly attached to the carbon-13 atom. However, reduced signal to noise ratio due to poor relaxation, signal multiplicity from the nuclear spin of deuterium (I=1) and loss of nOe, mean that this technique is not always satisfactory for quantitative measurements of isotope incorporation.

The use of molecules with deuterium two bonds away from carbon, for example [1-^{13}C, 2-$^{2}H_3$]-acetate, leads to a β-shift effect, which is usually also an upfield shift. The magnitude of the β-shift is small, but is additive being approximately 0.04 ppm for each deuterium. Therefore, the more deuterium atoms retained, the greater the shift. The main advantage of this technique is that the level of incorporation can be determined directly from peak height as there is no β-coupling.

This phenomenon has been useful in investigating the retention and/or loss of ^{2}H from the polyketide backbone in biosynthetic studies of polyketides. The information obtained can yield the identification of chain starter units and the relative amounts of hydrogen exchange at given sites, both of which are invaluable in probing the mechanistic details of a biosynthesis. For example [2-^{13}C, 2-$^{2}H_3$]-acetate **2b** incorporation into 19-O-acetylchaetoglobosin A **3**, a member of the cytochalasans, was used to determine the starter unit of the polyketide chain (Scheme 1-2).[3] ^{13}C NMR showed strongly enhanced singlets for eight carbons, and two multiplet peaks for C-11 and the O-acetyl methyl group. Therefore, all but two of the carbons originating from C-2 of acetate lost most, if not all, of their deuterium label.



2b **3**

Scheme 1-2

The loss of one deuterium can occur during the normal dehydration process of polyketide synthesis, and exchange with the medium could account for the remaining loss. Thus, it is only the chain starter unit and the O-acetyl methyl group, which retain the majority of the deuterium incorporation.

The use of isotopes for studying biosynthetic pathways is generally very effective, but there are some limitations. For example, the administration of increased concentrations of the labelled precursor in order to maximise enrichments can lead to a perturbation of the growth of the organism. It is also possible for the substrate to be degraded *en route* by the organism or become very poorly incorporated due to difficulties of cell penetration.

1.2 Metabolism

1.2.1 Catabolism and anabolism

Metabolism is the term used to describe the chemical transformations performed on a substance by an organism from the time it enters the organism until it is eliminated as waste products. Cells extract energy from their surroundings and by metabolite degradation they create the building blocks, which can then be used in the synthesis of complex organic molecules. Metabolism can be divided into two categories; catabolism, which refers to the degradation processes of complex structures and the associated release in energy, and anabolism, which is concerned with biosynthesis, a process which requires energy. The two processes are inter-related, as the small molecules used by the anabolic pathways in the synthesis of larger biomolecules are often the intermediates formed from the breakdown of substrates by catabolic operations.

Although there are many reactions occurring in metabolism, the number of reaction types is relatively small and there are only a restricted number of molecules that play a central role in the biochemistry of all living organisms.

1.2.2 Primary and secondary metabolism

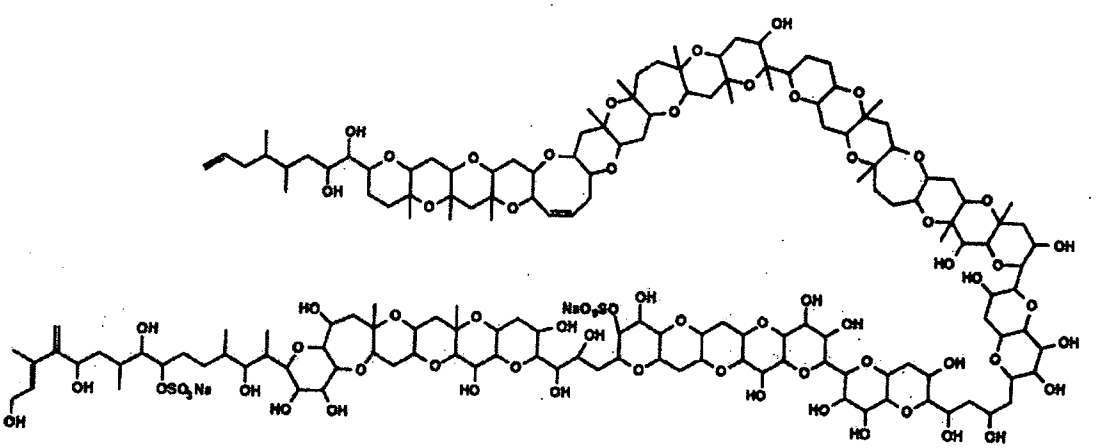
Natural products can be divided essentially into primary and secondary metabolites. Primary metabolites are common to all groups of living organisms and are essential to the survival of the cell. Secondary metabolites on the other hand, are compounds of limited distribution often produced by a single species and are generally peripheral to cell metabolism. However, they have greater significance to the well being of the organism as a whole. They may, for example, be a deterrent to other organisms, acting as defence agents or repellents.

Secondary metabolites are often structurally complex molecules, yet they are produced from a relatively small number of biochemical pathways, most commonly *via* acetate, mevalonate, shikimate and the amino acids.

1.3 Polyketides

1.3.1 Introduction to polyketides

Polyketides are a class of naturally occurring secondary metabolites most commonly produced by fungi but they are also represented in many bacteria. The route by which polyketides are synthesised is one of the most widespread in nature, accounting for compounds such as orsellinic acid,[4] a structurally unexceptional aromatic or at the other extreme, maitotoxin **4**,[5] at 3422 Da, the largest secondary metabolite known.



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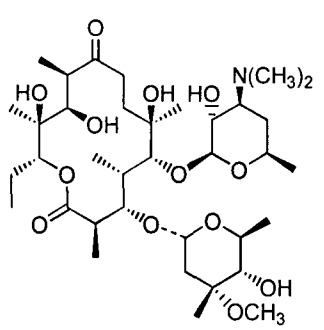
Bacteria, predominantly the Actinomycetes, are responsible for the production of many medicinally important polyketides including the antibiotic erythromycin A **5** first isolated in 1952 from *Saccharopolyspora erythraea.*[6] Tylosin **6**[7] is produced by *Streptomyces fradiae* and is widely used in veterinary medicine.



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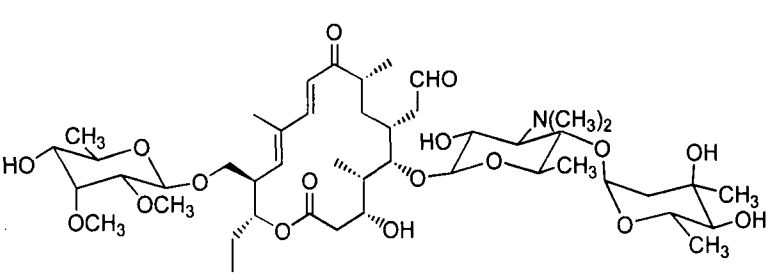

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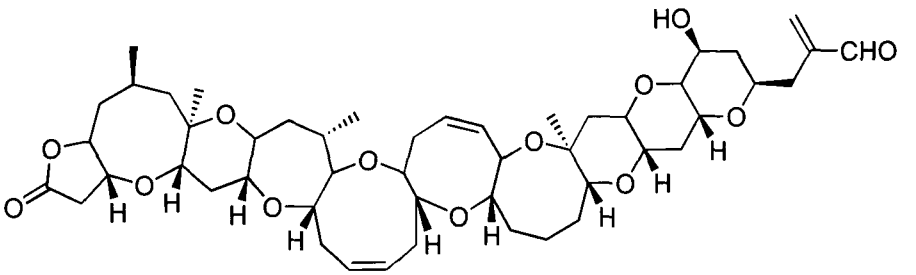
There have also been complex polyketides isolated from marine organisms (e.g. brevitoxin **7**[8]) and higher plants (e.g. anthraquinones).



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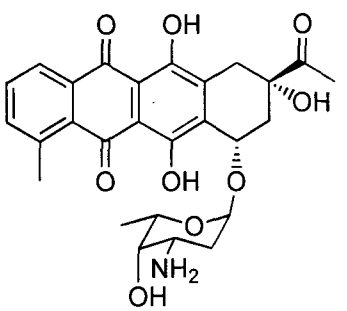
The polyketides have attracted considerable interest due to their broad range of biochemical activities as antibiotics (erythromycin A **5**), anti-cancer agents (daunomycin **8**[9]) and cholesterol-lowering agents (lovastatin **9**[10]).



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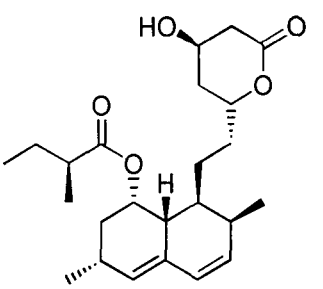
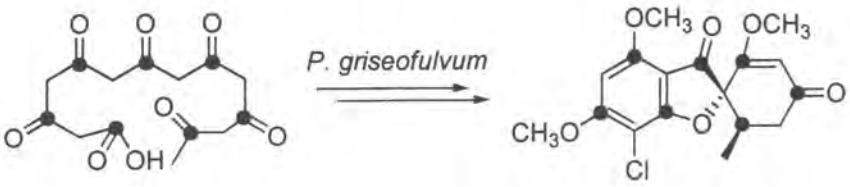


9

A fundamental understanding of the biosynthesis of the polyketides was realised by Collie in 1907.[11] The treatment of heptane-2,4,6-trione with base, led to the formation of a naphthalene derivative *via* a series of intermolecular aldol reactions. This was used as a template to establish the hypothesis for an analogous process of folding during the biosynthesis of aromatic compounds and polyphenols from similar polyketomethylene intermediates formed from head to tail coupling of acetyl units. Work carried out on the biosynthesis of a metabolite of the fungus *Penicillium griseofulvum*[12] was seminal in verifying this acetate hypothesis.[13] Incorporation of [1-^{14}C]-acetate into griseofulvin **10**, an antibiotic, showed a pattern consistent with the predicted folding of a heptaketide precursor (Scheme 1-3).



10

Scheme 1-3

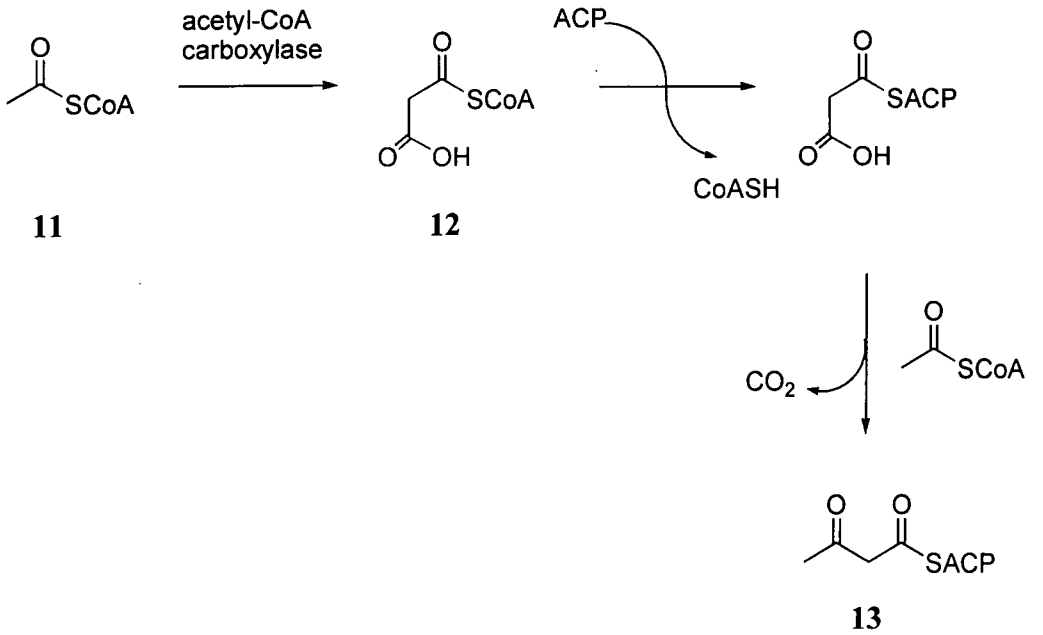
Since this research was performed, biosynthetic experiments using isotopically labelled acetates have been used in order to confirm the polyketide origin of many secondary metabolites.

Polyketides are closely related in origin to the fatty acids produced in primary metabolism and it is instructive to briefly review fatty acid biosynthesis.

1.3.2 Fatty acid biosynthesis

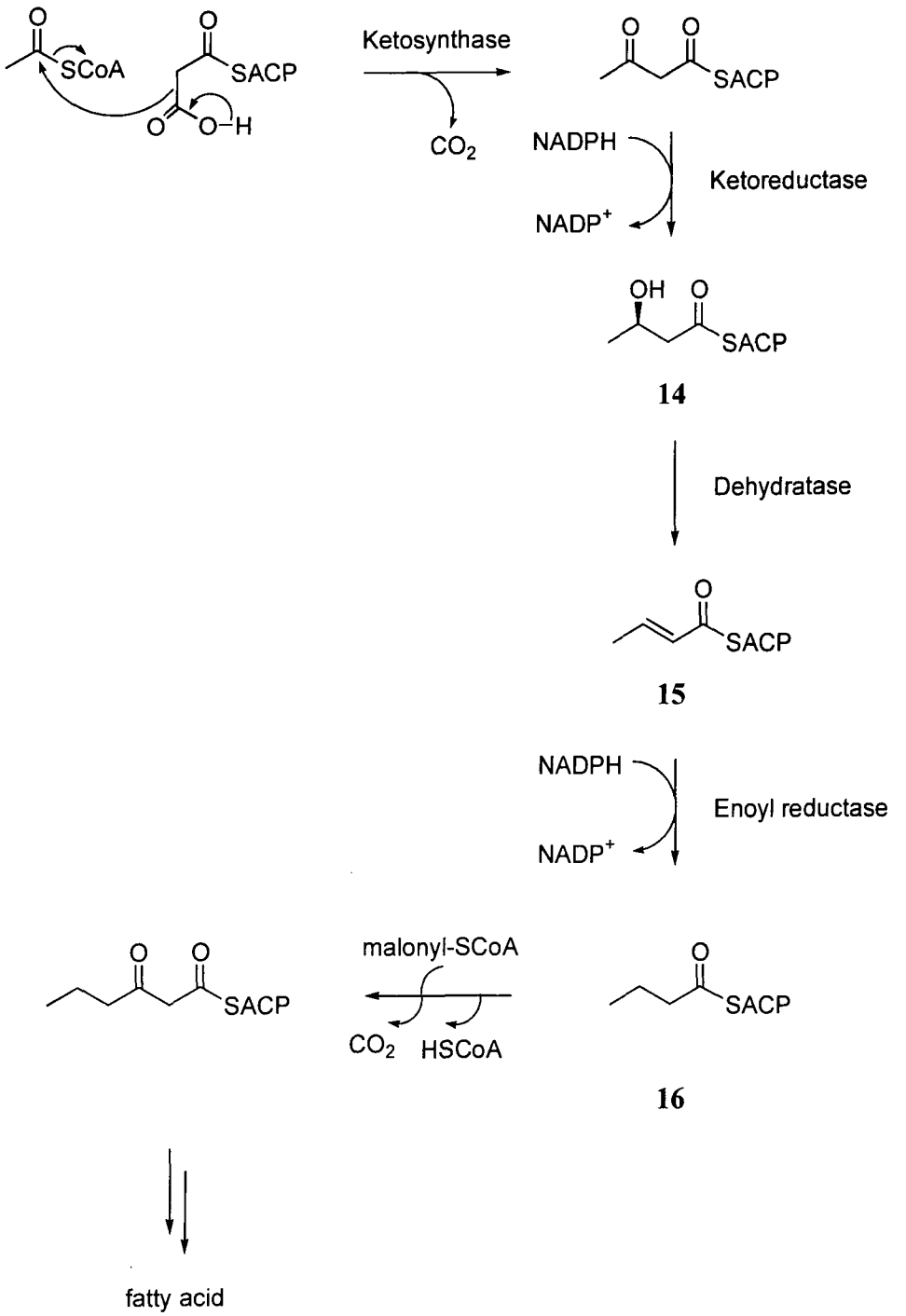
Fatty acids are widely distributed primary metabolites that have important cellular functions such as cell membrane assembly and energy storage. The polar carboxylic acid head groups and long lipophilic hydrocarbon tails are essential for their structural role in the formation of cell membranes. Glyceride degradation provides a pool of acetate, which can be used in biosynthetic processes or incorporated into the tricarboxylic acid (TCA) cycle for the construction of other building blocks. The biosynthesis of fatty acids is initiated from acetyl coenzyme-A **11** (acetyl-CoA) and malonyl-CoA **12** and is catalysed by an enzyme complex called the fatty acid synthase (FAS).[14]

The first step in fatty acid biosynthesis (Scheme 1-4) is the formation of malonyl-CoA **12** from acetyl-CoA **11**. This involves the action of acetyl-CoA carboxylase along with the co-factor biotin and an associated biotin carboxyl carrier protein. The second step is a decarboxylative condensation between acetyl-CoA and malonyl-CoA to generate an intermediate β-ketothioester **13**.



Scheme 1-4

All of the intermediates during fatty acid biosynthesis are anchored to an acyl carrier protein (ACP) which directs the biosynthetic steps by controlling the positioning of the intermediate acyl thioester around the catalytic sites of the FAS.[14] Similar to CoA, ACP is linked to a phosphopantetheine moiety, which is in turn covalently bound to a serine residue on the protein. The carbonyl of the β-ketoacyl-ACP **13**, formed from the acetyl and malonyl condensation by a ketosynthase (KS), is reduced by the action of a β-ketoacyl-ACP reductase (KR) to give D-3-hydroxyacyl-ACP **14**, which is subsequently acted upon by a dehydratase (DH), to give the α, β-unsaturated thioester **15**. The reduction uses nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and is stereospecific, with the *pro*-4*S* hydrogen acting as the hydride source to deliver the D-alcohol. *Syn* elimination of water produces the (*E*)-unsaturated olefin. Reduction of the α, β-unsaturated thioester **15** by an enoyl-CoA reductase (ER) generates butyryl-ACP **16**, the first saturated intermediate, and the end product of the first cycle in fatty acid biosynthesis. This is then transferred to the ACP of the FAS where another molecule of malonyl-CoA can attack in order to initiate the cycle of enzymic reactions once more. This sequence continues until the optimum chain length has been reached and the fatty acid is released from the FAS complex by a thioesterase (Scheme 1-5).



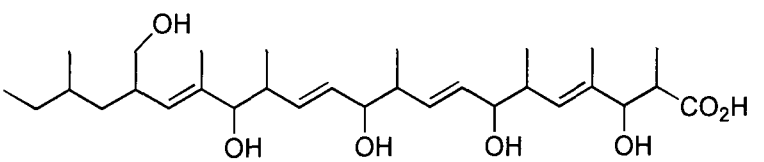
Scheme 1-5

There is essentially only one reaction sequence for the production of all saturated fatty acids, however two general types of FASs exist. Type I FASs, which are found in most higher organisms (except plants), consist of one multifunctional protein which includes the ACP, with all of the activities on a large protein. On the other hand, Type II FASs of higher plants and bacteria are made up of several discrete dissociable enzymes and an

acyl carrier protein. Fungi and yeast have characteristics of both Type I and Type II FASs.

1.3.3 Fungal polyketides

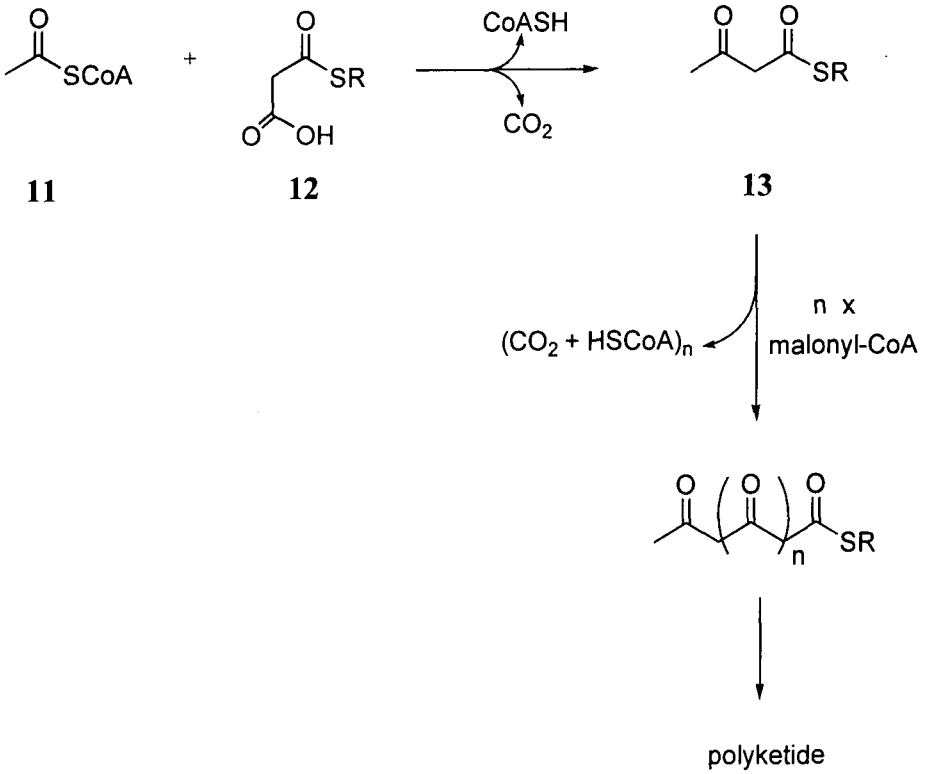
The majority of polyketides isolated from fungal sources are aromatic. Non-aromatic polyketides such as cubensic acid **17**[15] are rare. Cubensic acid is biosynthesised from eleven acetate units and eight methyl groups contributed by L-methionine (*vide infra*).



17

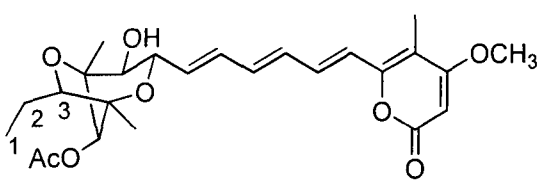
In a similar manner to fatty acid biosynthesis, there are two general types of polyketide synthases (PKS). Type I PKSs, are large multifunctional polypeptides containing all the necessary enzymic functions in distinct regions on a single large protein. Extensive studies on 6-methylsalicylic acid synthase (6-MSAS)[16] have shown that the gene order of catalytic domains is identical to that of mammalian FASs and that there is a single multifunctional protein in operation.

As described previously for FASs, PKSs catalyse a sequence of decarboxylative condensation reactions between acyl thioesters and malonyl-CoA followed by a cycle of ketoreduction, dehydration and enoyl reduction activities (Scheme 1-6). However, unlike fatty acid biosynthesis where the FAS always performs the complete reductive cycle, the PKS is highly selective and can control the manner of polyketide modifications generating keto, alcohol, double bond and saturated units. A wide range of structures can therefore be created by different programming sequences.



Scheme 1-6

In a similar approach to fatty acid biosynthesis, acetyl-CoA **11** is employed as a starter unit along with an extender unit, malonyl-CoA **12**, to generate a β-keto ester **13**. However, there are a few cases where other starter units, such as propionate and amino acids, can be used to initiate the PKS. One particularly interesting example is aurovertin B **18**, which can incorporate either propionate or a combination of acetate and the methyl group of methionine to generate the first three carbon atoms.[17]



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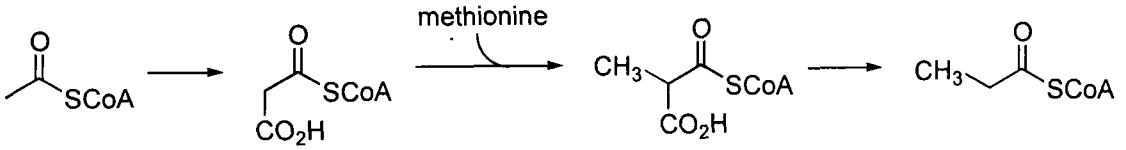
It is probable that propionate **20** is the true starter unit in aurovertin B biosynthesis and that methylmalonyl-CoA **19** is generated by methionine mediated methylation of malonyl-CoA **12** (Scheme 1-7).

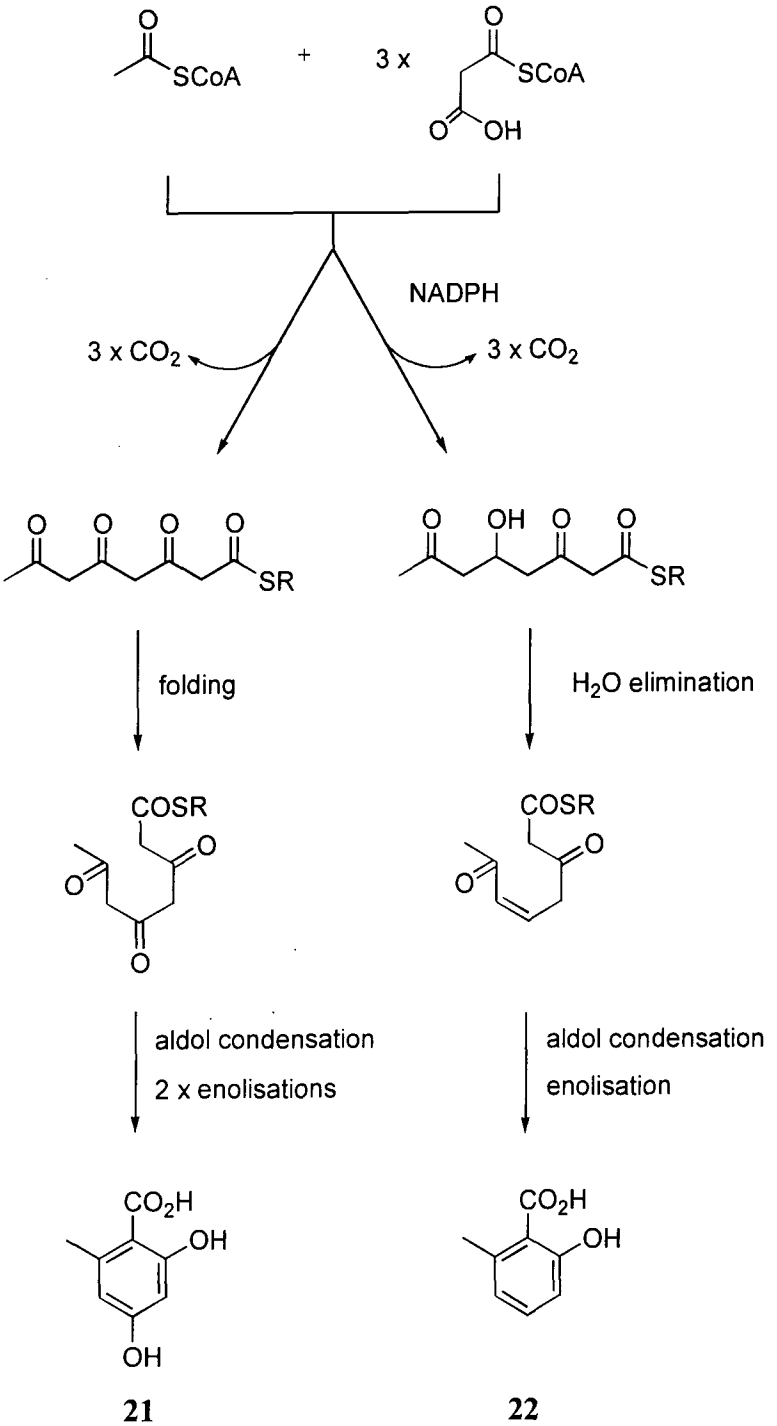


11 **12** **19** **20**

Scheme 1-7

Unlike bacterial systems, where propionate incorporation can occur at any stage, fungal PKSs can only utilise propionate as a chain starter unit. The use of various extender units, for example methylmalonate, introduces a further degree of structural complexity.

One of the simplest polyketide structures is orsellinic acid **21**. It is synthesised from an acetyl-CoA starter unit with three subsequent malonyl-CoA condensations (Scheme 1-8).[4] Cyclisation of the assembled poly β-keto ester *via* an aldol type condensation followed by dehydration and enolisation gives the aromatic product, orsellinic acid, after hydrolysis. It can be seen that there is no β-keto reductase activity present in this system. The biosynthesis of 6-MSA **22** is similar, however, an NADPH mediated carbonyl reduction results in the removal of the C-4 oxygen. Subsequent dehydration and enolisation steps generate **22**.

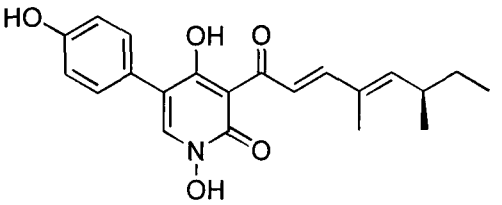


21 **22**

Scheme 1-8

A common feature of polyketide structures is the presence of methyl groups not accounted for by conventional polyacetate chain folding. The vast majority of these originate from the methyl group of L-methionine in which the donor methyl group is activated in the form of *S*-adenosyl-L-methionine (SAM). This was first shown by Birch[18] in 1958 during research into mycophenolic acid **23**. Feeding experiments

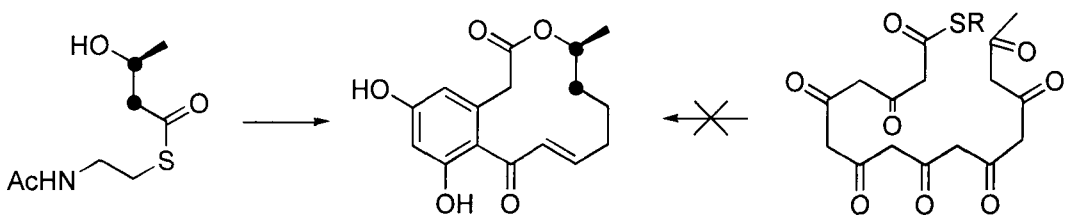
established that the C-5 methyl and the methoxyl carbon originate from the methyl group of L-methionine.



23

As previously discussed (p. 11), all of the methyl groups of cubensic acid **17** have also been shown to derive from SAM. Such C-methylations probably occur during polyketide assembly, by attack at the methyl group of SAM by an enolate intermediate.

Biosynthetic experiments involving elaborated fragments of polyketide structures have been used to glean information about the C-methylation process. The intact incorporation of thioester **24** into dehydrocurvularin **25**[19] (Scheme 1-9), produced by *Alternaria cinerariae*, strongly suggests that the direct reduction of poly-β-keto-thioester **26** is unlikely and that a mechanism whereby there is *processive* assembly of the polyketide is operating. The term processive describes the reduction of the β-carbonyl, formed after each malonate condensation, to the required oxidation level before each subsequent malonate condensation occurs.

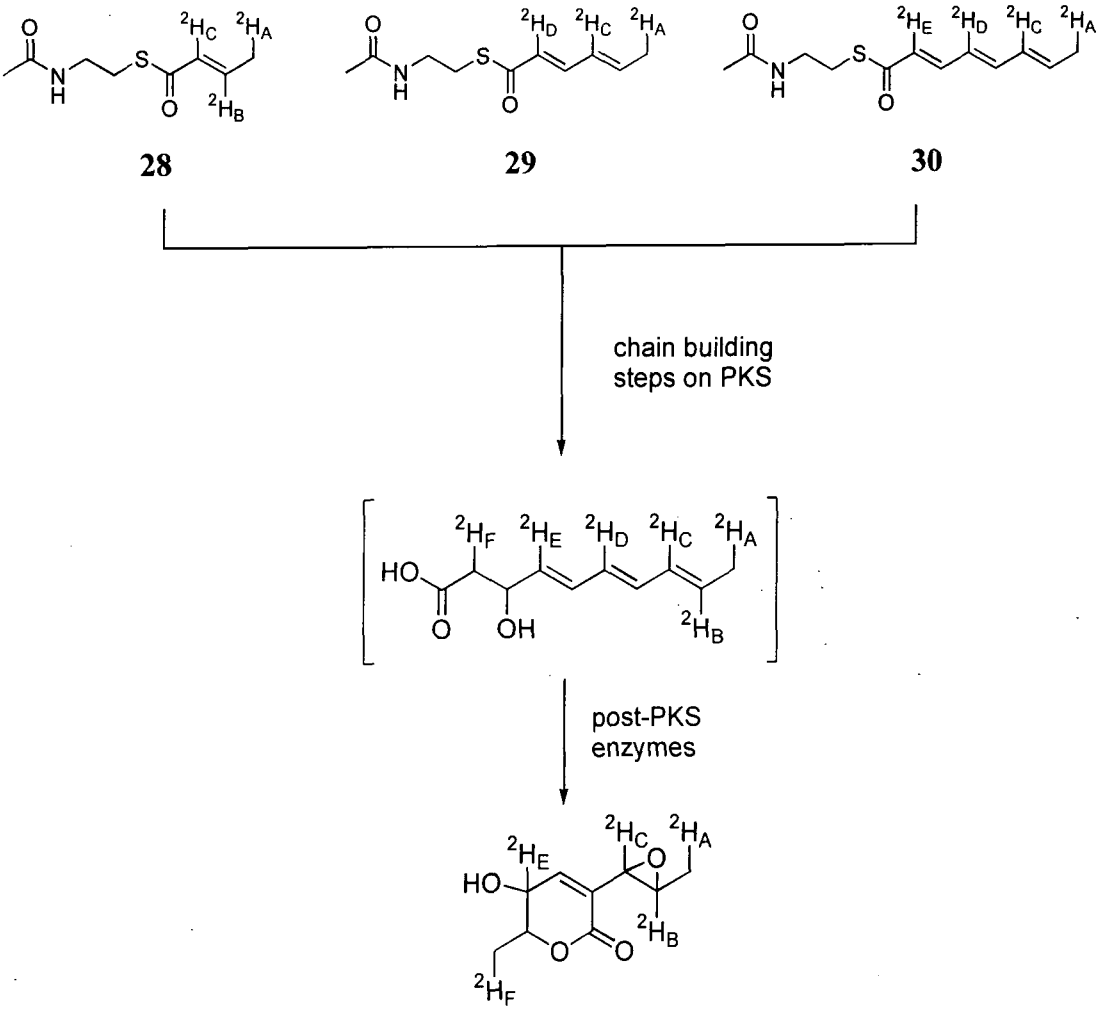


24 **25** **26**

Scheme 1-9

Other experiments have also been used to demonstrate a *processive* pathway for polyketide biosynthesis. Aspyrone **27** biosynthesis has been studied by NMR detection of ^{2}H incorporation of deuterium labelled intermediates (Scheme 1-10).[20] The results

are indicative of an assembly where the status of the carbonyl is adjusted before each subsequent condensation. The putative intermediates were fed to *Aspergillus melleus* in the form of acyl esters **28, 29** and **30**, which are able to transacylate with the ACP of the PKS. It is interesting to note that when the corresponding acids were fed, complete degradation to acetate occurred prior to any isotope incorporation.

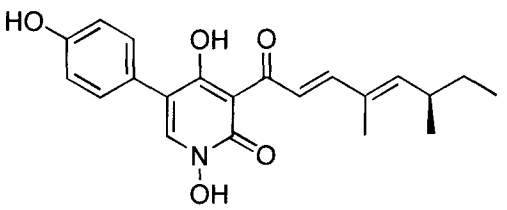


Scheme 1-10

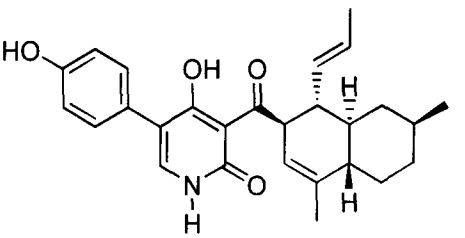
It is envisaged that O- and N- methylations take place towards the end of metabolic pathways.

A structurally different class of polyketide is derived from the combination of the usual polyacetate backbone with an amino acid. Tenellin **1**, which forms the subject of this

thesis, is a bright yellow metabolite produced by the fungus *Beauveria bassiana.*[1] Both tenellin and ilicicolin-H **31** from *Cylindrocladium ilicicola*[21] contain a 2-pyridone ring system derived from condensation between a polyketide component and phenylalanine. The structures also contain extra methyl groups, which have been shown to originate from L-methionine, presumably *via* SAM.

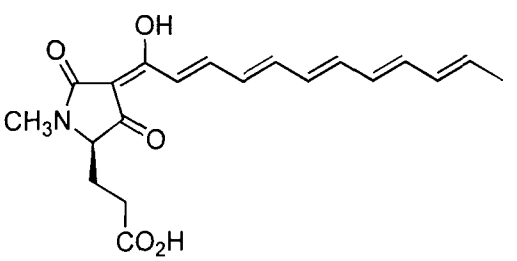


1



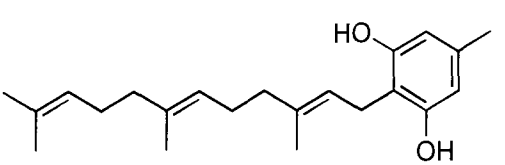
31

Other amino acids are involved in the biosynthesis of related polyketides; for example, glutamic acid condenses with a polyketide moiety to generate fuligorubin-A **32** in *Fuligo septica.*[22]



32

Another group of mixed origin polyketide metabolites are those which possess terpene moities. Pendent isoprenoid groups are most probably attached to phenol ring systems by electrophilic aromatic substitution of the corresponding isoprenoid pyrophosphate. Accordingly, grifolin **33**,[23] shown below, derives from farnesyl pyrophosphate and orcinol.

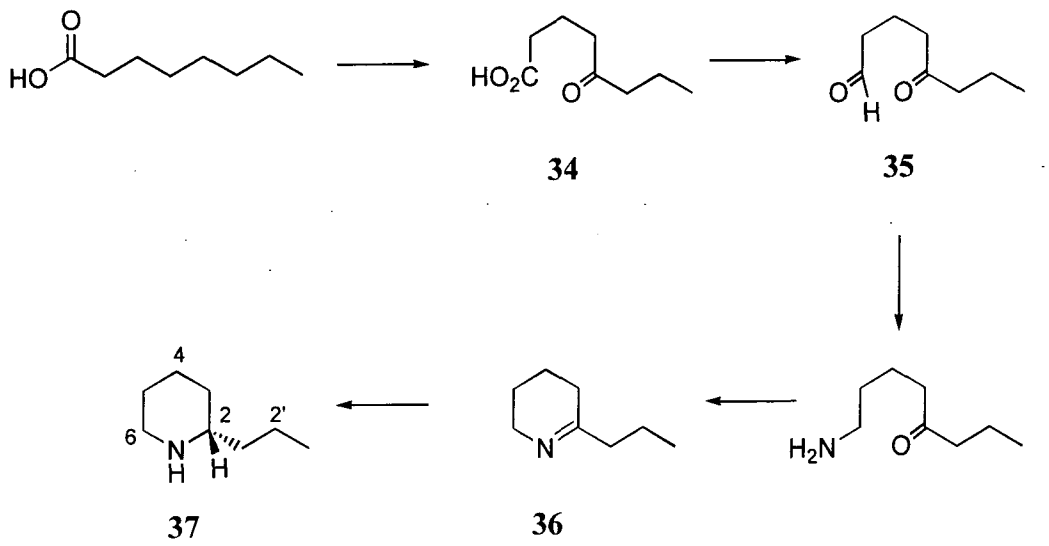


33

1.3.4 Plant polyketides

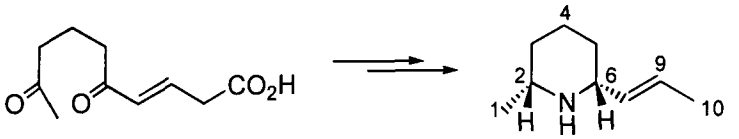
Alkaloids are largely biosynthesised from amino acids, however there are examples that have origins in polyketide chemistry. Isotopically labelled acetate is incorporated into coniine **37**,[24] a hemlock alkaloid, and pinidine **39**, an alkaloid of *Pinus jeffreyi*.[25]

The entire skeleton of coniine produced by *Conium maculatum* is derived from acetate. Labelling experiments have shown that C-2, C-2', C-4 and C-6 are enriched by [1-^{14}C]-acetate. 5-Oxo-octanoic acid **34**, 5-oxo-octanal **35** and γ-coniceine **36** are also readily incorporated into coniine **37** and appear to be advanced biosynthetic intermediates. The biosynthetic scheme shown in Scheme 1-11 has been proposed on the basis of these results.



Scheme 1-11

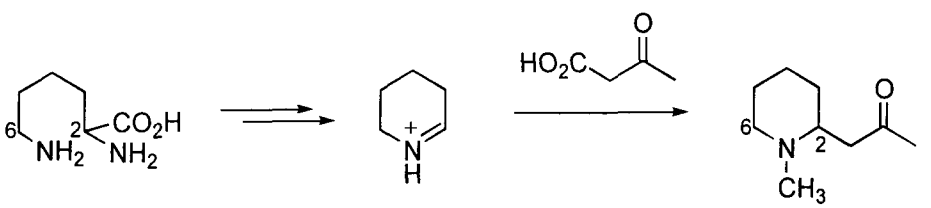
Pinidine **39** has a similar structure to coniine and also derives from the condensation of acetate units. The positions at C-2, C-4, C-6 and C-9 are labelled by [1-^{14}C]-acetate. The carboxyl group at C-10 of the pentaketide precursor **38** is lost during the biosynthesis (Scheme 1-12).



38 **39**

Scheme 1-12

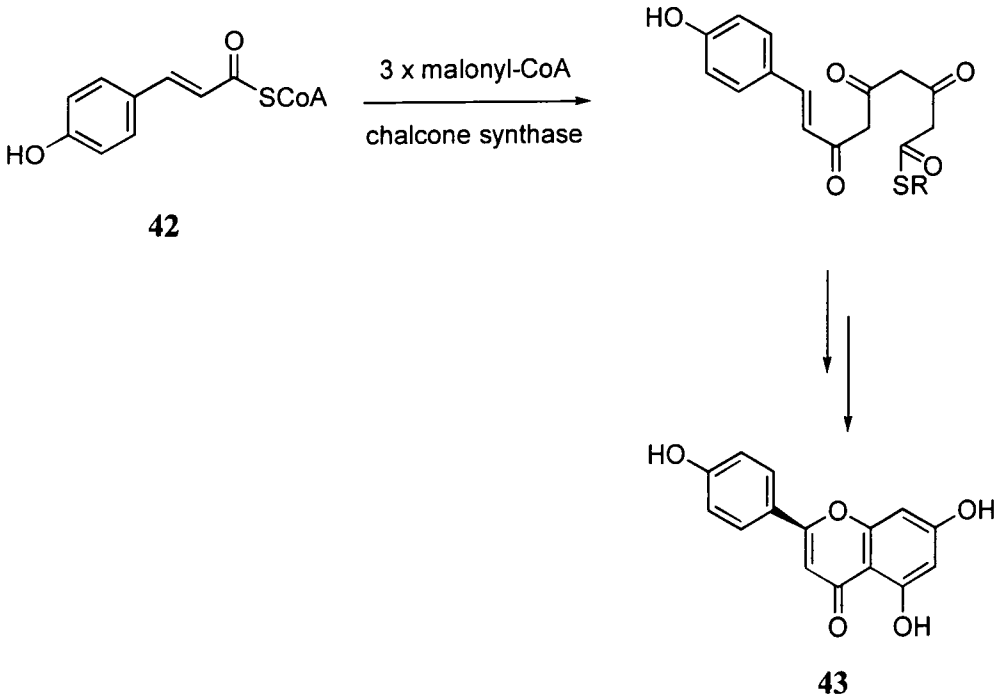
Coniine and pinidine are not characteristic of the piperidine alkaloids. More generally, the piperidine ring of these compounds derives from lysine **40** and acetate is utilised exclusively to provide parts of the metabolite that are not delivered by the amino acid, for example, in the side-chain of N-methylpelletierine **41**.[26] [^{15}N]-Lysine incorporation studies have suggested that the ring formation of **41** involves retention of the amino nitrogen group at C-6 and loss of the nitrogen at C-2 (Scheme 1-13).



40 **41**

Scheme 1-13

The flavonoids and related compounds are a ubiquitous and structurally diverse class of metabolite, which have a variety of functions, such as antifeedants, pigmentation and molecular communication. The condensation of three malonyl-CoA units onto a 4-coumaryl-CoA **42** starter unit provides flavone **43**, the key building block in flavanoid biosynthesis (Scheme 1-14).[27] The initial step in the biosynthesis is mediated by the enzyme chalcone synthase, a single protein that has no co-factor requirement. This enzyme is one of only two polyketide-condensing enzymes that do not contain an ACP and utilise the CoA thioesters of the substrates directly by transacylation onto specific cysteine thiol sites.



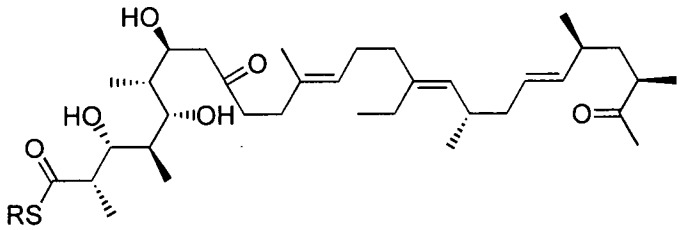
42 **43**

Scheme 1-14

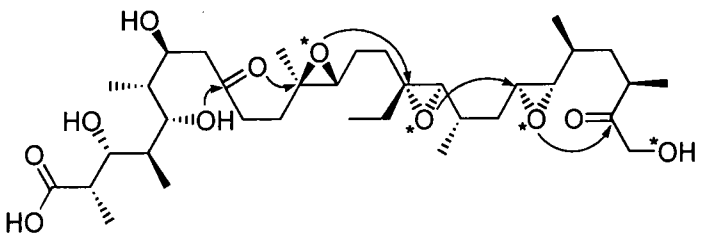
1.3.5 Bacterial polyketides

The Actinomycetes are the most prolific polyketide producers of all bacterial systems. Like fungal polyketides, many of the structures are aromatic. Many of those that are not feature the incorporation of propionate and butyrate derived extender units during polyketide assembly, results in greater alkyl functionality along the carbon backbone, and hence some of the most complex secondary metabolites known.

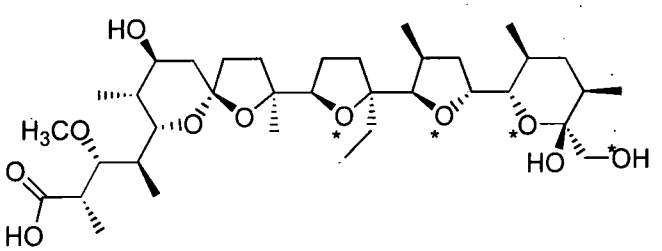
Monensin-A **46**[28] is a polyether derived from five acetate, seven propionate and one butyrate subunit.[29] Cascade cyclisation of a triepoxide **45** formed by epoxidation of an intermediate triene, and addition of molecular oxygen could account for the structure shown (Scheme 1-15).



44



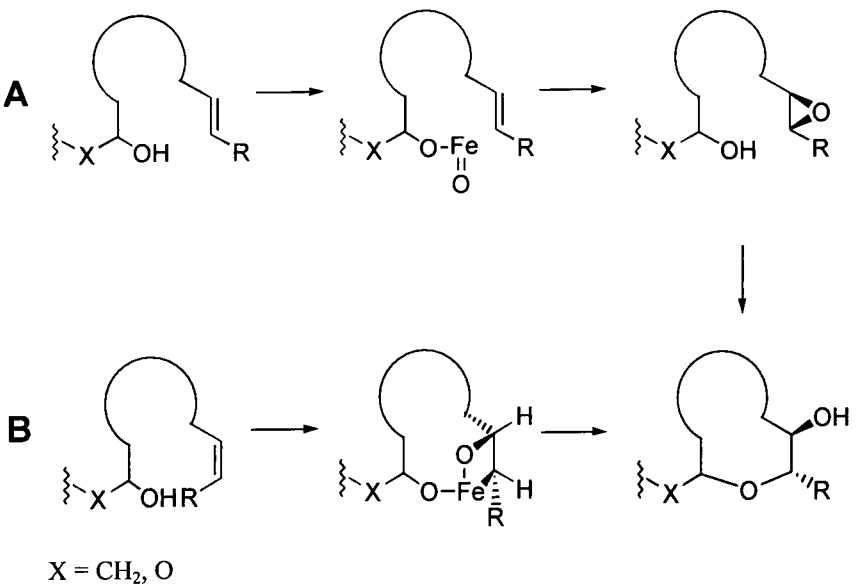
45



46

Scheme 1-15

It has been suggested that the all (*E*)-triene, premonensin **44**, is formed by standard polyketide assembly and is then epoxidised to the triepoxide **45**. However, all attempts to observe premonensin **44** in the culture broth or to cyclise synthetic material administered to *S. cinnamonensis* were unsuccessful. Therefore, the (*Z*)-triene, although not expected from usual polyketide biosynthesis, has also been considered as a valid intermediate. Two alternative mechanisms involving iron-mediated oxidative cyclisation have been proposed for polyether biosynthesis (Scheme 1-16).[30] These are epoxidation *via* oxygen transfer (Path **A**) and [2+2] cycloaddition of oxygen from a ferryl species (Path **B**).



$X = CH_2, O$

Scheme 1-16

A Type II PKS, a series of enzymes, which associate into a multi-enzyme complex, performs the assembly of many of the bacterial polyketides. However, the higher polyketides such as the polyethers and macrolides have a Type I PKS. Although the systems are different, it is essentially the same enzyme activities that perform the elongation and modification processes in both types of polyketide synthase.

In the last decade, there has been a great deal of interest in the isolation and sequencing of genes for FAS and PKS systems. It emerges that there are similarities at the genetic level between the two enzyme systems, however, there are genes, for example those expressing enzymes for cyclisations in polyketide synthesis, which have no counterpart in FASs.

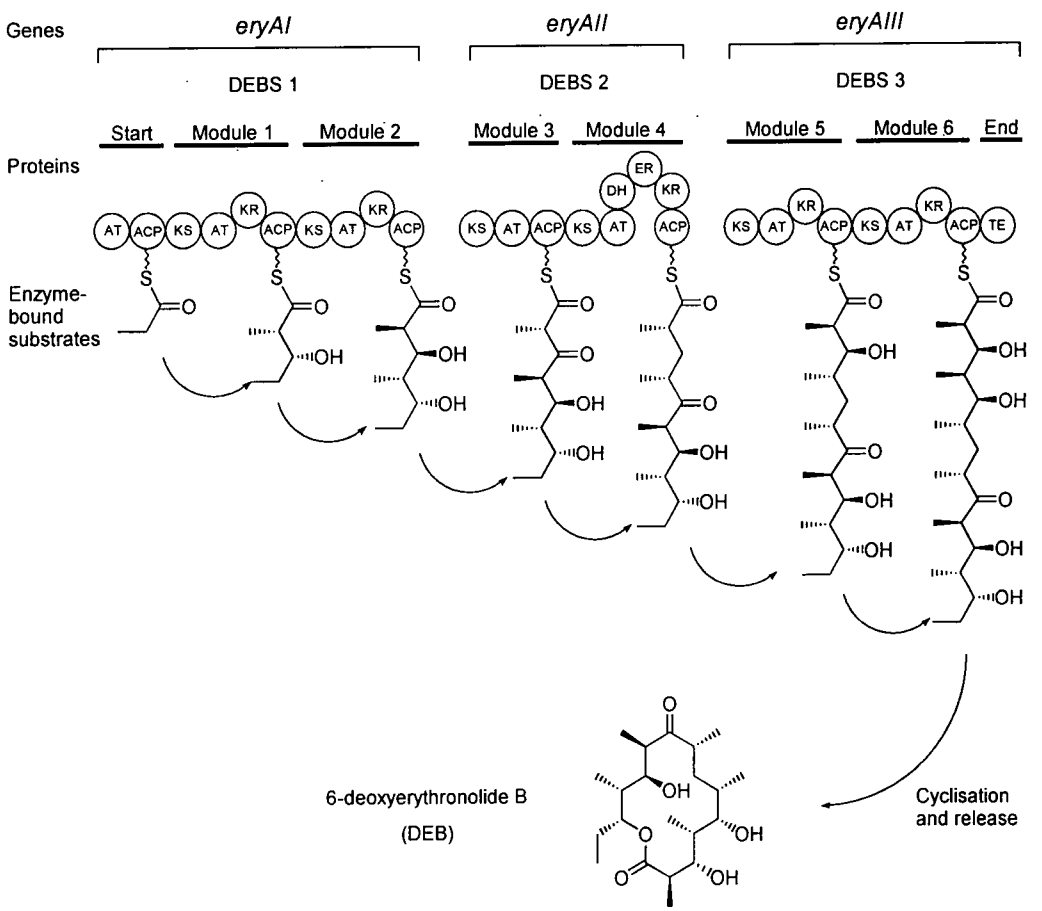
1.3.6 Genetic Studies

Genetic studies on PKSs have mainly been performed on bacterial systems, especially the antibiotics of the Actinomycetes, which are formed by a Type II PKS. These are coded for by discrete genes rather than by large open reading frames (ORFs) containing multifunctional PKS activities as in Type I PKSs. Actinorhodin, from *Streptomyces*

coelicolor, which has a Type II PKS, was the first system to be extensively studied.[31] The results identified seven ORFs. Genes from the *act* system have been used to probe other PKS systems.

The complete sequencing of 6-MSAS from *Penicillium patulum*[32] reveals a Type I PKS. The gene sequence contains an ORF, which is 5322 base pairs in length and encodes for 1774 amino acids. The domains for the four common polyketide activities, acyl and malonyl transferase, ACP and 2-β-ketoacylsynthase, were identified along with a β-ketoreductase activity.

The most studied Type I PKS system is that of 6-deoxyerythronolide-B synthase (DEBS), which produces the polyketide moiety of erythromycin. Three multifunctional proteins, identified as DEBS 1, 2 and 3, have been coded and characterised (Figure 1-1).[33]

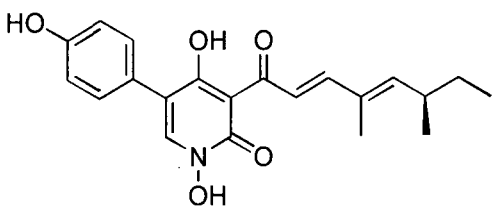


47

Figure 1-1 Biosynthesis of DEB **47**

Each cassette has two modules with the intermediates remaining bound to the PKS throughout the whole sequence of biosynthetic transformations until cyclisation and release of 6-deoxyerythronolide-B **47**.

Tenellin **1** is proposed to be assembled in a processive manner by a Type I PKS. It is probable that, in a similar manner to DEBS, the transformations take place in a sequence of enzyme cassettes. The first cassette would perform the condensation of acetyl-CoA and malonyl-CoA, followed by methylation at the β-keto stage and subsequent carbonyl reduction. An extender malonate unit would then be added, another methylation performed and the ketone reduced before delivery to the second cassette. Condensations with a further two malonyl-CoA units would increase the chain length to the pentaketide required.



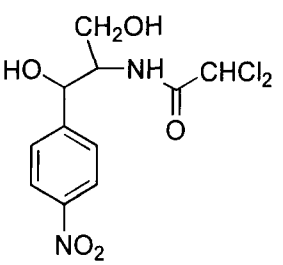
1

Chapter 2

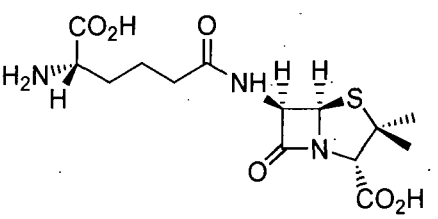
2 Investigations into the rearrangement of phenylalanine/tyrosine in tenellin biosynthesis

2.1 Amino acids in secondary metabolism

The amino acids are involved in the biosynthesis of a wide variety of secondary metabolites. The alkaloids, which are derived from the aliphatic amino acids ornithine and lysine, or the aromatic amino acids phenylalanine, tyrosine and tryptophan, are found in higher plants and make up a large number of known compounds. However, there are still many other amino acid derived metabolites produced by bacteria and fungi such as chloramphenicol **48**,[34] or derivatives of peptides, for example isopenicillin N **49**.[35]



48



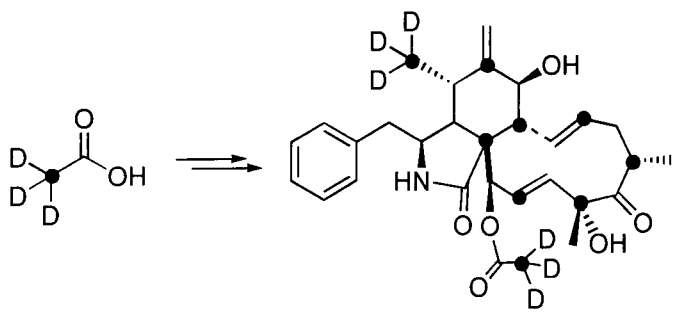
49

2.1.1 Amino acid and polyketide derived structures

Many secondary metabolites incorporate fragments originating from different metabolic pathways. These have been termed 'mero-metabolites'. Examples include the mevalonate-shikimate derived isoprenoid quinones, the acetate-shikimate derived flavonoids and the ergot alkaloids, such as strychnine,[36] which is produced from tryptophan and mevalonate. Polyketides that contain an amino acid fragment are well known and some of these are discussed below.

The cytochalasans[37] are a class of metabolites that are inhibitors of cytoplasmic cleavage and consist of a highly substituted hydrogenated isoindolone ring fused to an 11- to 14-membered macrocycle. The most biologically active structures are found to contain an exocyclic double bond in the isoindolone ring and an allylic alcohol functionality.

There are three basic types of cytochalasan: the cytochalasins, the chaetoglobosins and the aspochalasins. All are thought to evolve from similar biosynthetic origins involving an amino acid and an acetate derived polyketide backbone. Any additional C_1 residues are proposed to arise from methionine. Cytochalasin D **50** produced by *Zygosporium masonii* is found to consist of phenylalanine, nine intact acetate units and three methionine delivered methyl groups.[38] The biosynthesis probably involves initial formation of the nonaketide, followed by N-acylation of phenylalanine. Examination of deuterium incorporation from [2-^{13}C, 2-$^{2}H_3$]-acetate **2b** gave the expected ^{13}C enrichments but no observed deuterium labelling apart from incorporation at C-11 and the methyl of the O-acetyl group (Scheme 2-1).[39] Therefore, exchange of most, if not all, of the deuterium from C-2 of acetate occurs during the biosynthesis of cytochalasin D. Only the polyketide chain starter unit and the acetyl methyl group retain an observable level of the isotopic label.

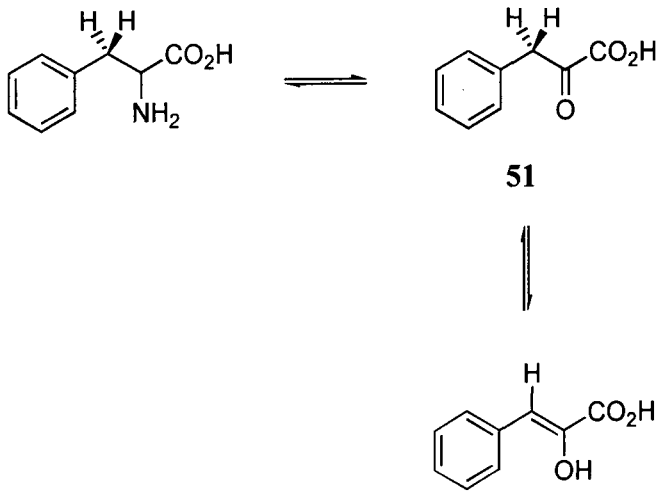


2b **50**

Scheme 2-1

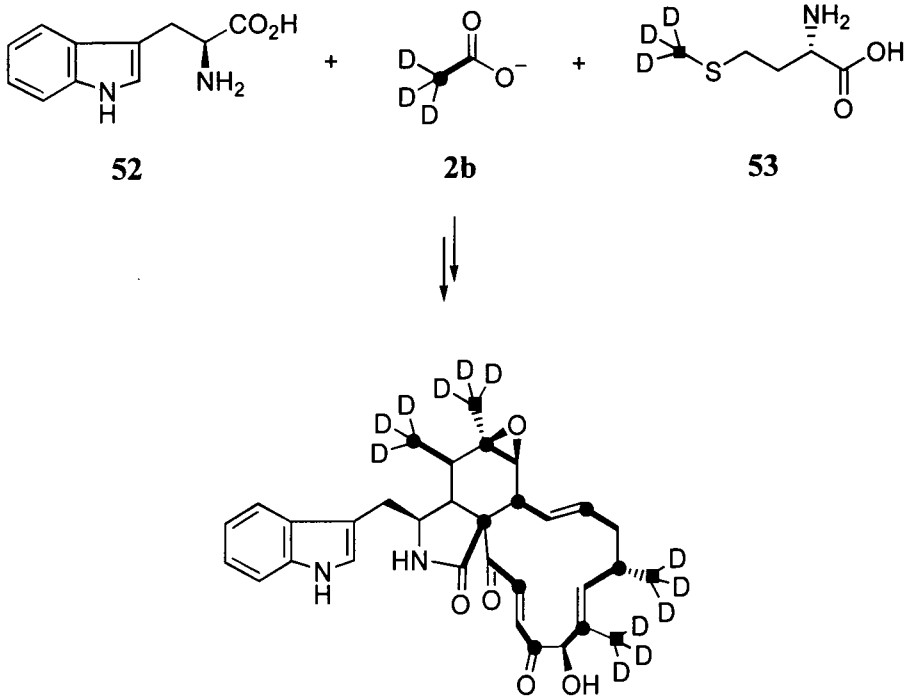
There is no rearrangement of phenylalanine during the biosynthesis, however appropriate biosynthetic investigations have revealed loss of the α-H-atom and the α-amino group of phenylalanine in the final cytochalasin structure.[40] It is therefore likely that nitrogen exchange (transamination) reactions, due to the presence of amino acid oxidases and aminotransferases, occur in the amino acid pool before incorporation.

[U-^{14}C]-Phenylpyruvic acid was incorporated into **50** at a level of 6.5 %, a similar level to that found for phenylalanine, indicating that transamination can occur. On the basis of the structure of the metabolite, it remains probable that phenylalanine is the more direct precursor. The transamination process could also account for the significant loss of tritium from administered [3-^{3}H]-phenylalanine since β-proton exchange can occur in the free phenylpyruvic acid **51** (Scheme 2-2).



Scheme 2-2

Chaetoglobosin A **54** produced by *Chaetomium globosum* is a closely related metabolite to cytochalasin D and has a biosynthesis similar to that of the cytochalasins in which tryptophan **52** replaces the phenylalanine residue (Scheme 2-3).[41] Nine acetate, three methionine and one tryptophan unit are responsible for the complete structure. Biosynthetic experiments using [2-^{13}C, 2-$^{2}H_3$]-acetate **2b** identified the chain starter unit as this was the only carbon that showed retention of all three deuterium atoms in ^{13}C NMR. Studies with [^{13}C, $^{2}H_3$]-L-methionine **53** indicated that all three of the deuterium atoms were retained during incorporation into the three methyl groups of the polyketide moiety.

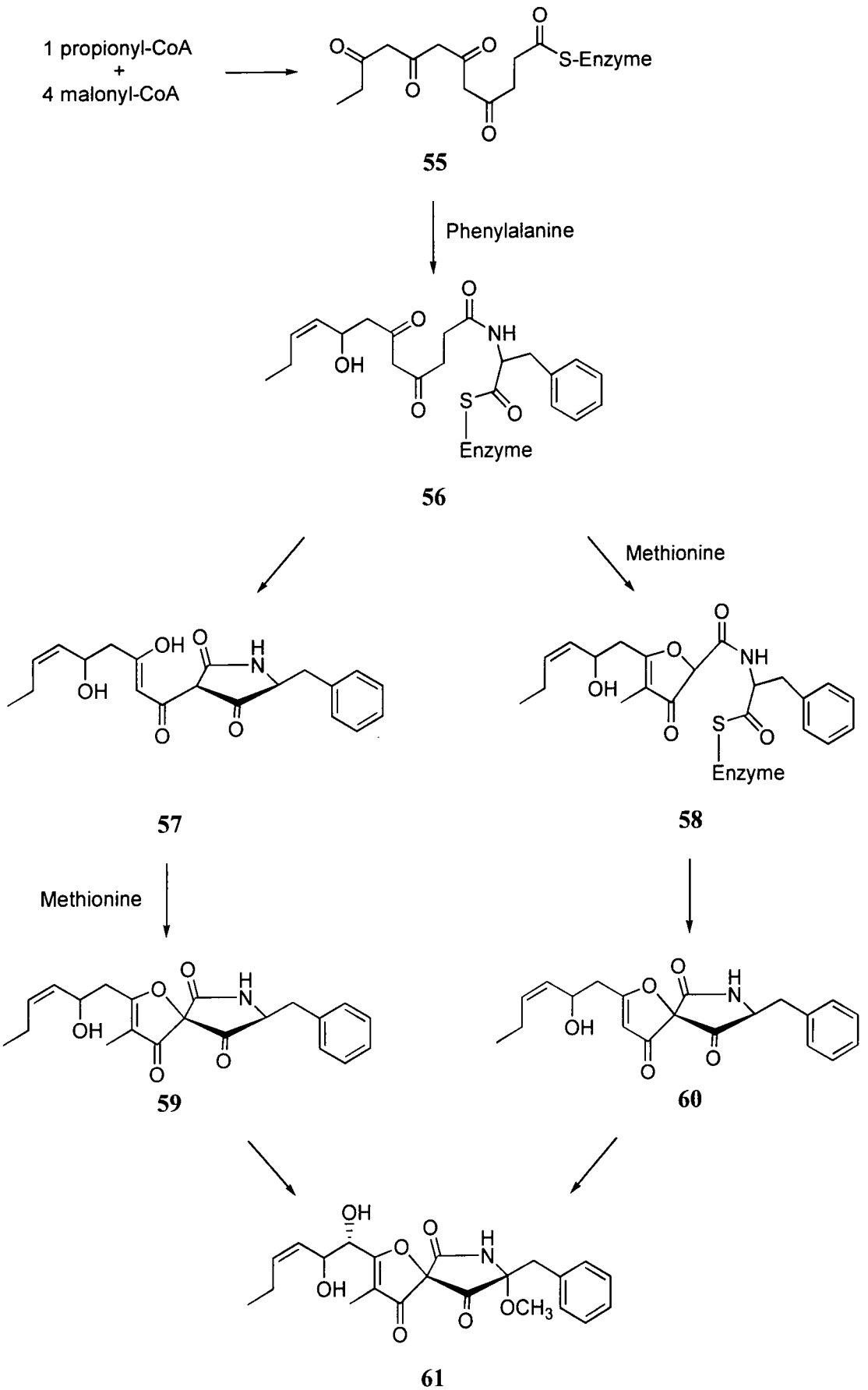


52 **2b** **53**

54

Scheme 2-3

The pseurotins are a class of metabolites characterised by an 1-oxa-7-aza-spirol[4.4]non-2-ene-4,6-dione skeleton. They are secondary metabolites isolated from *Pseudeurotium ovalis* differing from each other by the functionalised side chain attached to the bicyclic ring. Mohr *et al.*[42] proposed that condensation of a phenylalanine unit with a polyketide moiety could account for the biogenetic origin of all the atoms. Pseurotin A **61** has been the subject of a biosynthetic study,[42] and feeding experiments with labelled acetates, malonate, propionate, methionine and phenylalanine revealed a plausible biosynthetic route. The building blocks for pseurotin A **61** were found to be one propionate unit, four malonate units, phenylalanine and two methionine units. All three deuterium atoms from [^{13}C, $^{2}H_3$]-L-methionine were retained in both O- and C-methylation steps. A biosynthetic route has been proposed to account for the results of the incorporation experiments (Scheme 2-4). Initially, propanoyl-CoA condenses in four successive cycles with malonyl-CoA to give a C_{11}-polyketide unit **55**, which can then combine with phenylalanine to generate the enzyme bound structure **56**.

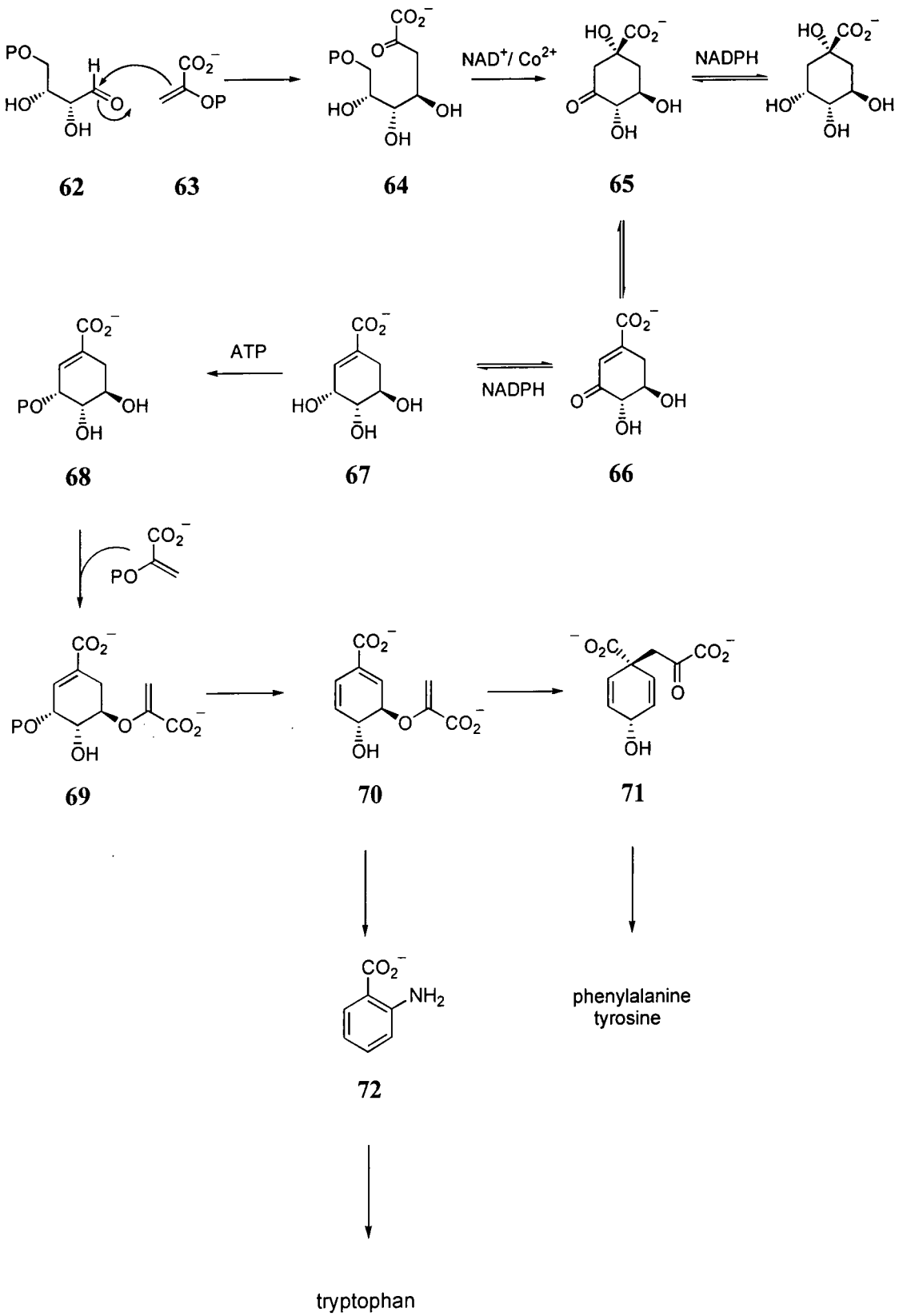

1 propionyl-CoA
+
4 malonyl-CoA
S-Enzyme
55
Phenylalanine
NH
OH
Enzyme
56
Methionine
57
58
Methionine
59
60
OCH3
61

Scheme 2-4

At this point, one of two pathways can be followed depending on whether the nitrogen- or oxygen-containing ring is assembled first. In the former case, production of the tetramic acid heterocycle **57** would be followed by formation of the spiro compound **59**, probably *via* generation of an electrophilic site at C-5. In the second route, initial formation of the furenidone **58** would precede cyclisation to tetramic acid **60**. Oxidation at C-8, C-10 and the benzylic C-atom are thought to occur as late stage transformations.

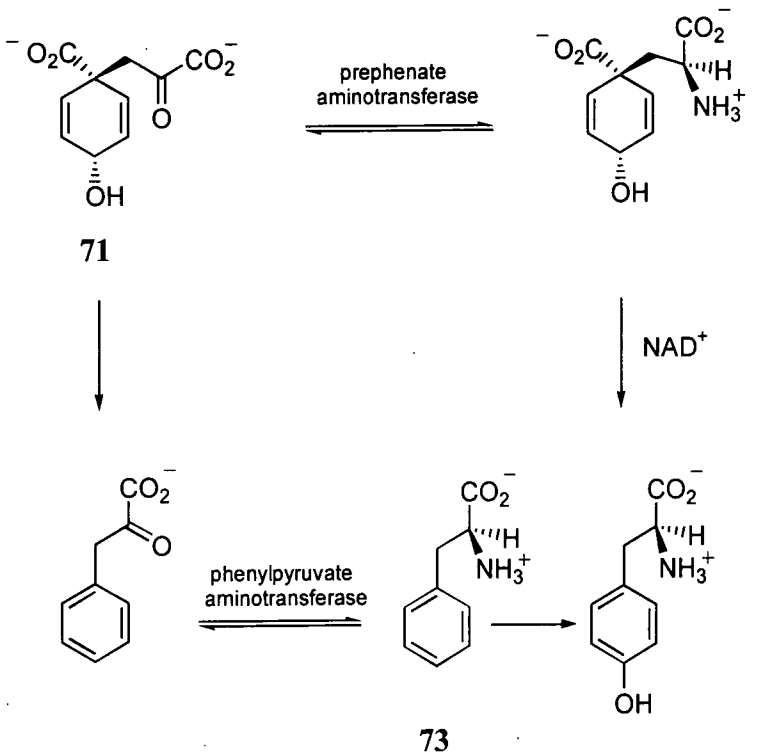
2.1.2 The shikimate pathway

The aromatic amino acids, phenylalanine, tyrosine and tryptophan, are biosynthesised *via* the shikimate pathway (Scheme 2-5).[43] The initial step is the condensation of D-erythrose 5-phosphate **62** with phosphoenolpyruvate (PEP) **63** to yield 3-deoxy-D-arabino-heptulosonate-7-phosphate **64** (DAHP). Loss of the phosphoryl group and subsequent cyclisation gives 3-dehydroquinate (DHQ) **65**, which undergoes a 3-dehydroquinase catalysed dehydration to 3-dehydroshikimate **66**. An NADPH mediated reduction provides shikimate **67,** which is then converted to shikimate-3-phosphate **68** by the action of shikimate kinase with ATP acting as co-substrate. Reaction of the phosphate **68** with another PEP molecule gives 5-enolpyruvyl-shikimate 3-phosphate **69** (EPSP), which undergoes *anti*-1,4 elimination of phosphate and the 6-*pro*R proton to yield chorismate **70**. This is performed by chorismate synthase, which has a requirement for reduced flavin mononucleotide (FMN). A radical mechanism is suggested whereby loss of the phosphate group is mediated by donation of an electron from the co-factor with subsequent proton loss leading to the formation of chorismate and regeneration of FMN. Chorismate **70** then undergoes either a chorismate mutase catalysed rearrangement to prephenate **71**, the precursor to phenylalanine and tyrosine, or transformation to anthranilate **72**, the first step in tryptophan production.


NAD+/ Co2+
NADPH
ATP
NADPH
phenylalanine
tyrosine
tryptophan

Scheme 2-5

Depending upon the producing organism, the synthesis of phenylalanine **73** from prephenate **71** can be performed by two different pathways (Scheme 2-6).

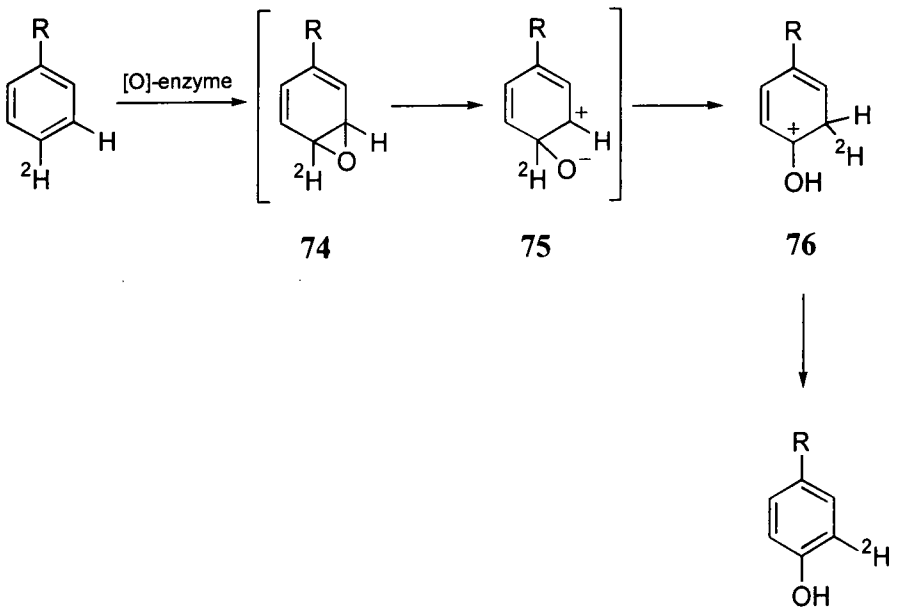


71 **73**

Scheme 2-6

2.1.3 The NIH shift

In some organisms, including mammals, phenylalanine is converted to tyrosine by the action of a phenylalanine hydroxylase (PAH). A tetrahydrobiopterin co-factor is also present and is oxidised to 4α-hydroxytetrahydropterin by molecular oxygen during the transformation. PAH is a non-haem iron containing enzyme, which has an unclear mechanism, although a pathway which includes the formation of an arene oxide intermediate **74** has been postulated (Scheme 2-7). It was found that on treatment of [4-^{3}H]-phenylalanine with PAH, the tritium is lost from the 4-position and is transferred to position-3 *ortho* to the inserted oxygen.[44] This process has been termed the NIH shift as it was discovered at the National Institute of Health laboratories in Bethesda, Maryland.



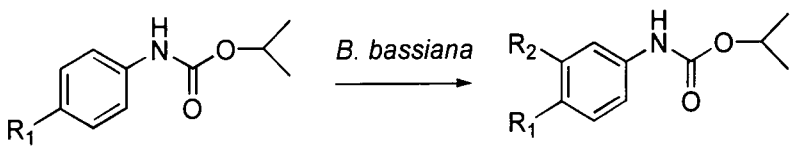
Scheme 2-7

The proposed mechanism shows the migration of the 4-position hydrogen after the formation of carbocation **75** produced by the opening of an arene oxide **74**. The 1,2-sigmatropic rearrangement gives **76**, which then aromatises. Studies have shown that it is a kinetic isotope effect rather than a stereoselective loss of the non-migratory species that is in operation. For [2,3,5,6-$^2H_4$]-phenylalanine,[45] where a proton migrates, the deuterium incorporation measured is the same as for [4-^{2}H]-phenylalanine where a deuteron is migrating. This was corroborated by results from feeding studies with [4-^{3}H]-phenylalanine where the amount of tritium retention was higher than that for deuterium.

2.1.4 Hydroxylations by *Beauveria bassiana*

The selective hydroxylation of aromatic compounds in synthetic organic chemistry is difficult to accomplish, but the use of organisms such as *B. bassiana* make such reactions readily achievable.

The *para*-methylated **77** and -deuterated **78** derivatives of Propham, an isopropyl N-phenyl carbamate insecticide, were subjected to *B. bassiana* mediated hydroxylation,[46] yielding benzyl alcohol **79** and the *para*-hydroxylated compound **80** respectively (Scheme 2-8). The latter case showed a 46 % ^2H content at the *meta* position, indicating a 72 % overall retention of deuterium and a process consistent with the formation of an arene oxide followed by an NIH shift. As expected the *para*-methylated substrate did not show any evidence for the operation of an NIH shift. In both cases it is interesting to note, that hydroxylation was regiospecific and occurred exclusively at the *para* position of the aromatic ring.



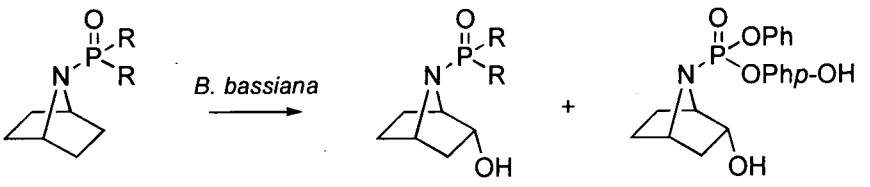
77 $R_1 = CH_3$ **79** $R_1 = CH_2OH$; $R_2 = H$

78 $R_1 = {}^2H$ **80** $R_1 = OH$; $R_2 = {}^2H$

Scheme 2-8

Another set of experiments demonstrated that N-benzyloxycarbonyl protected piperidines were hydroxylated with greater regio-selectivity than the corresponding N-benzoyl analogues.[47] It is thought that the selectivity in *B. bassiana* is not only determined by the distance between the carbonyl and the site of hydroxylation, but additionally by the distance to the aromatic side chain. This suggests that there is a defined aromatic binding pocket within the active site of the hydroxylation enzyme in *B. bassiana*.

The hydroxylation of unactivated methylene carbons in N-phosphinyl azabicyclic structures **81** with *Beauveria bassiana* (Scheme 2-9) has been shown to occur in fair to good yields.[48] The ability of these compounds to act as good substrates was thought to be due to the P=O bond acting as a mimic of carbonyl and sulfonyl groups by anchoring the substrate at the active site.



R = OEt, OPh, Ph

81 **82** **83**

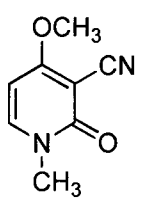
Scheme 2-9

All three derivatives underwent hydroxylation to yield structures **82**. However, the phenoxy derivative was also hydroxylated to some extent at the *para*-position of the aromatic ring to give compound **83**. A time course study showed that production of the monohydroxylated product **82** occurred initially and that the dihydroxylated product **83** formed by conversion of the monohydroxylated species. The products could be deprotected with trifluoroacetic acid in dichloromethane (TFA-DCM) and used in the syntheses of natural products.

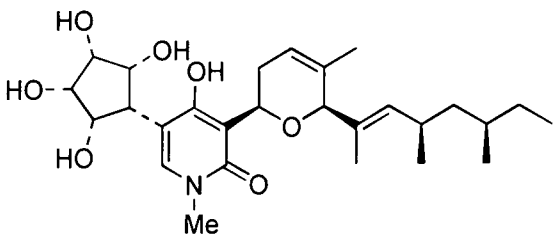
2.2 The 2-pyridone ring system

2.2.1 Tenellin and other 2-pyridones

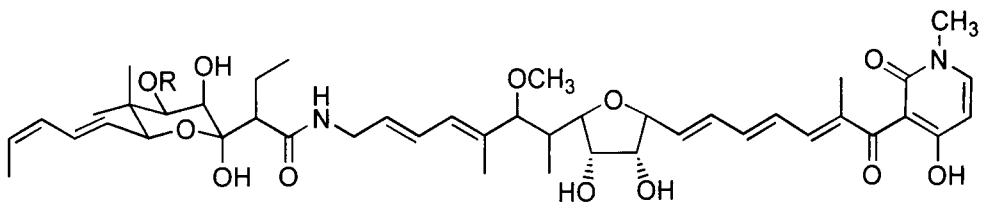
Since the isolation of tenellin and related 2-pyridone metabolites, the biosynthetic pathways to these structures have been widely investigated. This family of compounds varies in complexity from ricinine **84**,[49] to funiculosin **85**,[50] elaborated by the fungus *Penicillium funiculosum*, to aurodox **86**[51] produced by the bacterium *Streptomyces goldiniensis*.



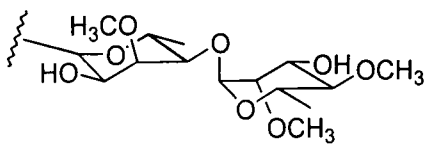
84



85



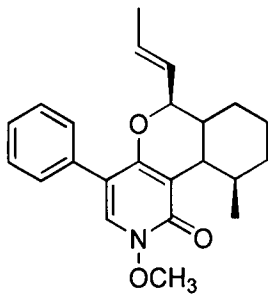
R = H R =



86 **87**

Some members of this group of 2-pyridones have been shown to display antibiotic activity, which has made them attractive targets for synthesis and biological evaluation. In this context, the family of elfamycin antibiotics, including aurodox **86** and efrotomycin **87**, have been the subject of a total synthesis by Nicolaou and co-workers.[52]

Leporin A **88**[53] from *Aspergillus leporis* contains an unusual N-methoxypyridone, and a polyketide derived ring system.



88

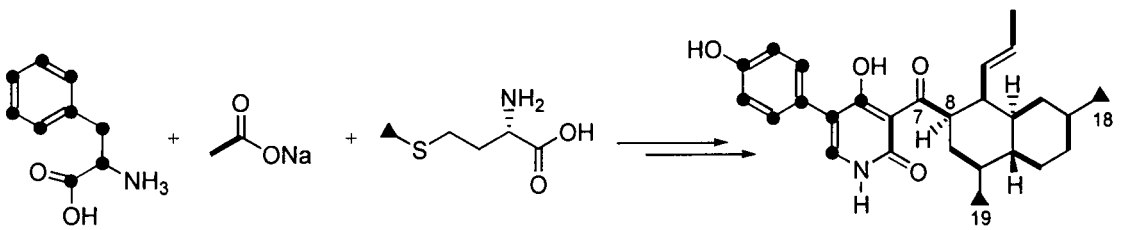
Notably the aromatic ring of the non-polyketide moiety is not phenolic. This differs from tenellin **1** and ilicicolin H **31** suggesting that the presence of the phenol is not essential for the generation of the pyridone ring system. One hypothesis for the formation of the pyridone ring in tenellin **1** postulates the rearrangement of a tetramic acid *via* a quinoid intermediate (*vide infra*). This hypothesis could clearly not be used to explain the biosynthesis of leporin A **88** as it does not contain a *para*-phenol moiety.

2.2.2 Pyridone ring biosynthesis

The biosynthesis of ilicicolin H **31**, a metabolite of the fungus *Cylindrocladium ilicicola*, has been investigated.[54] The metabolite contains two structurally significant moieties; a substituted pyridone ring system identical to that of tenellin and a polyketide derived bicyclic decalin moiety. Usual polyketide head to tail coupling of one acetate and seven malonyl units accounts for the octaketide backbone, with L-methionine **53** *via* SAM providing methylation of the polyketide at two sites to contribute the C-18 and C-19 methyl groups (Scheme 2-10). The decalin fragment can then be formed by a series of aldol reactions to give the bicyclic structure.

The average incorporation of [1,2-$^{13}C_2$]-acetate **2a** into the polyketide derived carbons was 3 % and the enrichment into the methyl groups from methionine was measured at

3.7 %. Ilicicolin H **31** recovered after administration of [U-^{14}C]-phenylalanine **73a** to *C. ilicicola* had a specific activity of 13.0 μCi/mmol giving an incorporation of 0.5 %.

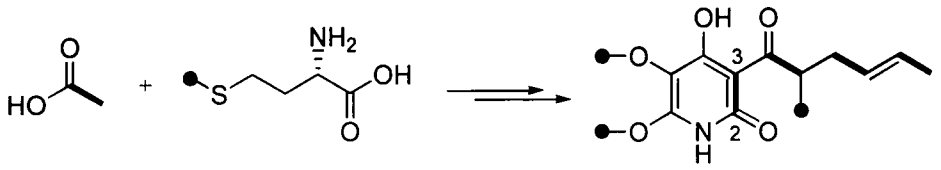


73a **2a** **53** **31**

Scheme 2-10

Feeding studies performed with [2-^{13}C, ^{15}N]-phenylalanine gave a key insight into the biosynthetic pathway. Mass spectrometric analysis of an ion containing the pyridone ring and formed from the cleavage of C-7 to C-8 bond, showed a 3 % ^{15}N enrichment. Further evidence for intact phenylalanine incorporation came from NMR where a ^{13}C-^{15}N spin-spin-coupling constant of 12.5 Hz was detected. This value is consistent with a one bond coupling of an sp^2 hybridised carbon attached to a nitrogen. The authors propose therefore, that transamination to phenyl pyruvate does not occur as a requirement of the biosynthesis and that phenylalanine is incorporated as an intact unit.

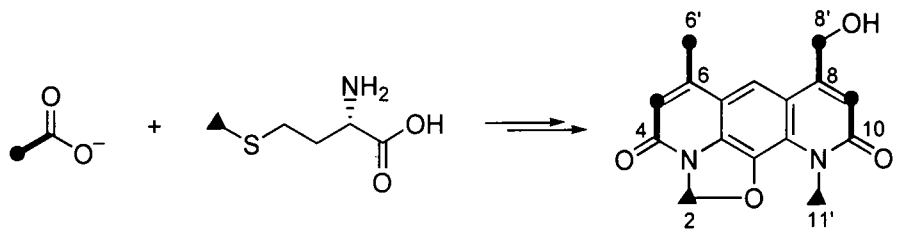
Harzianopyridone **89** from the fungus *Trichoderma harzianum* possesses a 4-hydroxy-5,6-dimethoxypyridone ring substituted at C-3 with a side chain of polyketide origin.[55] Incorporations from [1,2-$^{13}C_2$]-acetate **2a** revealed the involvement of a tetraketide unit, which forms the hexenyl chain and C-2 and C-3 of the pyridone ring (Scheme 2-11). A feeding experiment with [*methyl*-^{13}C]-methionine **53** established the origin of the extra carbons in the methyl group of the side chain and the methoxyl groups of the ring system. The remaining three carbons of the pyridone ring may derive from aspartic acid. [U-^{14}C]-Aspartic acid was incorporated into **89** at a level 0.03 %, well below the level at which ^{13}C NMR could be used to detect incorporation. Therefore, this proposal remains inconclusive and is only indicative of a possible precursor relationship.



2a **53** **89**

Scheme 2-11

The structure of nybomycin **90** has been shown to contain two pyridone rings. The biosynthesis was elucidated by feeding studies with [1-^{13}C]-acetate which showed that C-4, C-6, C-8 and C-10 were all labelled to an equal extent.[56] The carbons at C-6' and C-8' were labelled by [2-^{13}C]-acetate. The methyl group attached to nitrogen, C-11', and the methylene group forming part of the five-membered oxazoline ring, C-2, are both delivered by methionine **53** (Scheme 2-12).

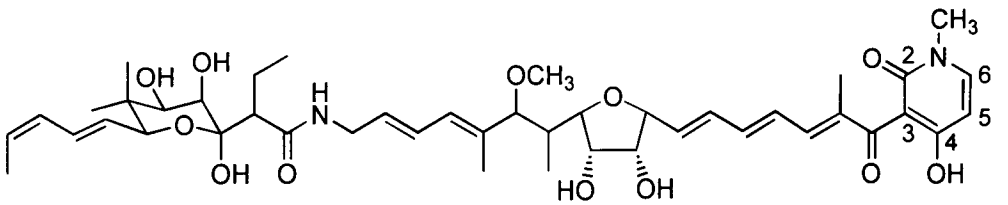


53 **90**

Scheme 2-12

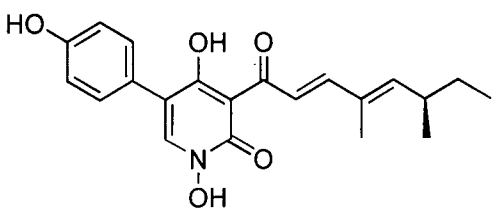
The central portion of the structure is thought to originate from shikimate or a related biosynthetic intermediate.[57] Sodium [2-^{13}C]-pyruvate and D-[6-^{13}C]-glucose are metabolised to acetate, labelled at C-1 and C-2 respectively, and hence label the outer carbons of the pyridone rings as expected. Incorporation into C-2 and C-11' also occurs from the diversion of labelled atoms into the 'one carbon' metabolic pool.

Studies on the biosynthetic origin of the 2-pyridone moiety in aurodox **86** have shown that C-2, C-4 and C-6 are enriched by [1-^{13}C]-acetate, but C-3 and C-5 are not labelled by [2-^{13}C]-acetate. The C_2 units are therefore *not* incorporated *via* a conventional polyketide pathway and a unique mode of assembly must be in operation.



86

Tenellin **1**, which forms the subject of this thesis, also contains a pyridone ring system. The pyridone is known to derive from phenylalanine/tyrosine, however there is not a straightforward incorporation of these units and a structural rearrangement of the amino acid precursor at some stage along the biosynthetic pathway is required.



1

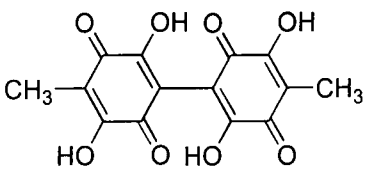
2.3 Biosynthesis of tenellin

2.3.1 Metabolites of *Beauveria* fungi

Beauveria bassiana is a widely distributed soil-borne fungus and was the first microorganism to be recognised as an agent of animal disease.[58] Heavy losses of larval silkworms in the 16th and 17th centuries, prompted an Italian scientist, Agostino Bassi de Lodi, to show that the deaths of the insects were caused by a fungus that multiplied in and on the body of the insect.[58] The fungus is a saprophyte and known to attack a broad range of insects from aphids to grasshoppers. There is considerable variation in host range and virulence between the different strains, but all attack directly through the insect's skin, secreting enzymes that attack and dissolve the cuticle. The fungus can also live endophytically in the vascular tissue of corn plants thereby suppressing the population of corn borer pests. *B. bassiana* is sold as a microbial insectide, and is available on the market as Naturalis® and Mycotrol®.

Vuillemin[59] established the genus *Beauveria* in 1912, as a general name for fungi previously known as *Isaria*, *Sporotrichum* or *Botrytis*. There are two widely recognised subdivisions, *B. bassiana* and *B. tenella*, distinguished by the size and shape of their spores. Red, yellow or green pigmentation is characteristic among these fungi.

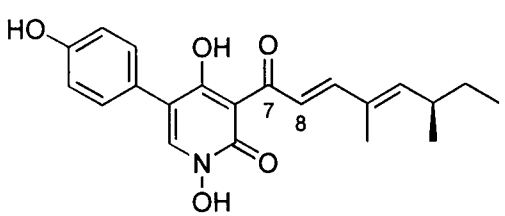
The red colouration in cultures of *Beauveria* is due to oosporein **91,**[60] a dibenzoquinone, thought to be produced by oxidative dimerisation of a benzenoid intermediate formed from acetate and malonate condensation.[61]



91

Inhibition of organisms including *Staphylococcus aureus* and *Bacillus subtilis* by oosporein may account for the antibiotic activity of the *Beauveria* species.[62]

Tenellin **1**, a yellow metabolite, was first isolated from *Beauveria bassiana* by El Basyouni *et al.* in 1968.[63] The structure was elucidated later by Vining[64] using spectroscopic and degradative techniques. In concurrent research, a structurally similar metabolite to tenellin **1**, differing only by an additional CH=CH unit between C-7 and C-8, was found to be produced by *Beauveria tenella*. This metabolite, also yellow in colour, was termed bassianin **92**.



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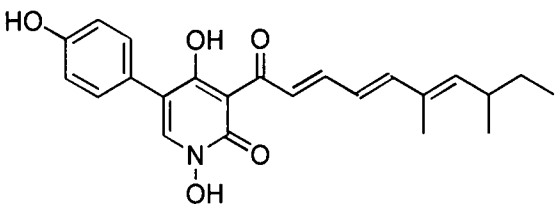
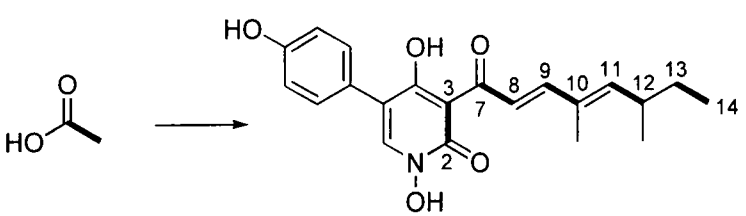


92

2.3.2 Structure elucidation of tenellin

The structure of tenellin was solved by spectroscopic analysis and a series of comprehensive labelling studies.[64,65,66,67] The polyketide nature of tenellin was deduced by acetate feeding experiments,[67] which gave the expected pattern of isotope incorporation. After the addition of [1,2-$^{13}C_2$]-acetate **2a** to *B. bassiana* cultures, tenellin labelled at five two-carbon units was isolated. Analysis by ^{13}C NMR showed pairs of intact C_2 units corresponding to C-2 and C-3, C-7 and C-8, C-9 and C-10, C-11 and C-12, and finally C-13 and C-14 (Scheme 2-13).

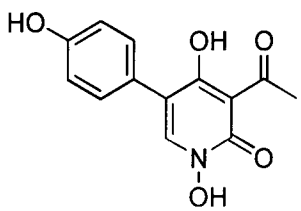


2a **1**

Scheme 2-13

Examination of tenellin by ^{13}C NMR isolated after growing cultures with $K^{15}NO_3$ as the only nitrogen source established that only C-6, C-2 and C-3 were coupled to nitrogen. The relative values of the $J_{13C\text{-}15N}$ coupling constants, 15.0, 11.0 and 9.2 Hz respectively, indicate that nitrogen is geminal to C-3 and directly bonded to C-6 and C-2. The signal for H-6 had a $J_{1H\text{-}15N}$ coupling constant of 1.0 Hz.

Structural analysis of tenellin was supported by chemical evidence.[66] Alkaline peroxidation yielded *p*-hydroxybenzoic acid and retro-aldol cleavage in refluxing potassium hydroxide gave 2-methylbutanal and 2,4-dimethylhex-2-enal, which were characterised as the 2,4-dinitrophenylhydrazones. A crystalline product, **93**, was also obtained from these degradation experiments. Compound **93** had a similar spectrum to tenellin, but with resonances for C-8 to C-14 replaced by a single acetyl methyl peak.



93

The results from the labelled acetate and nitrate feeding experiments, in conjunction with the chemical degradative studies, indicated that the structure of tenellin must contain a 1-hydroxy-2-pyridone moiety with a substituted acyl side-chain. The hydrogen at position-6 and the *p*-phenol group were shown by an nOe study to be on adjacent carbons, the chemical shift of C-5 in ^{13}C NMR consistent with the deshielding effect of the aromatic ring. The chemical shifts of C-3, C-4 and C-7 in ^{13}C NMR, in addition to the detection of a hydrogen bonded hydroxyl group in the ^{1}H NMR, indicated the presence of the enol form of a β-diketone.

The ^{1}H and ^{13}C NMR for tenellin are shown (Figure 2-1 and Figure 2-2).

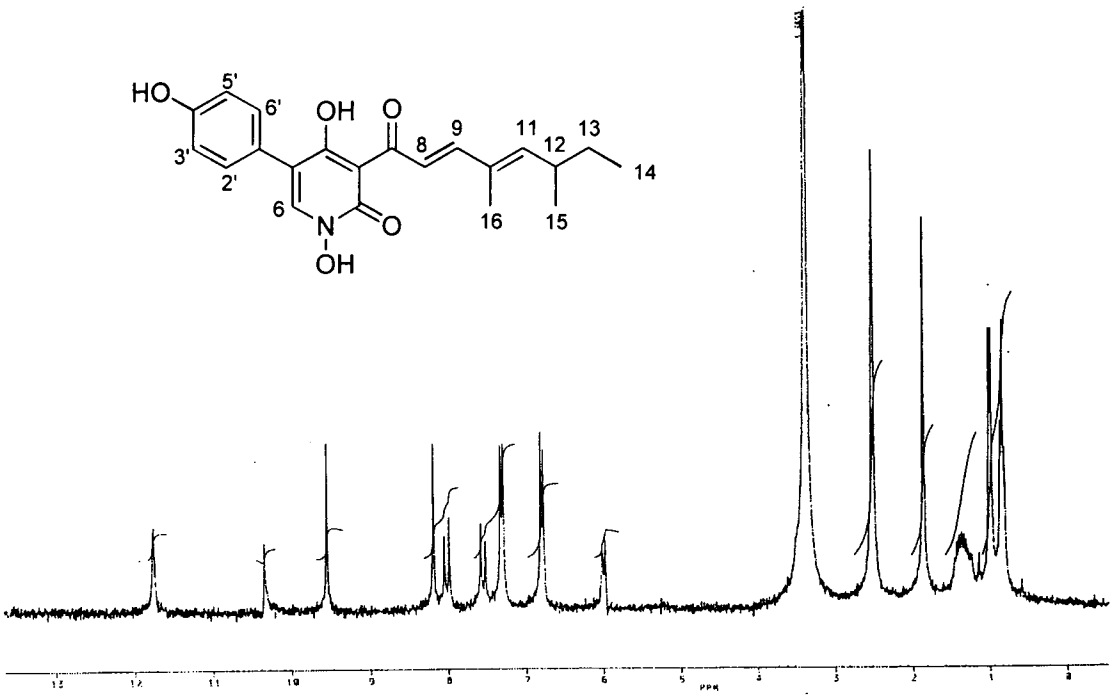


Figure 2-1 ^{1}H NMR of tenellin **1**

Table 2-1 ^{1}H chemical shifts (δ) and coupling constants (J Hz) for tenellin **1**

Proton	multiplicity	δ
6	s	8.19
8	d, J 15.4	8.00
9	d, J 15.3	7.54
11	d, J 9.5	6.00
12	m	2.50
13	m	1.37
14	t, J 7.4	0.84
15	d, J 6.4	0.99
16	bs	1.86
2', 6'	d, J 8.1	7.30
3', 5'	d, J 8.2	6.79
N-OH	s	11.75
PhOH	s	9.55

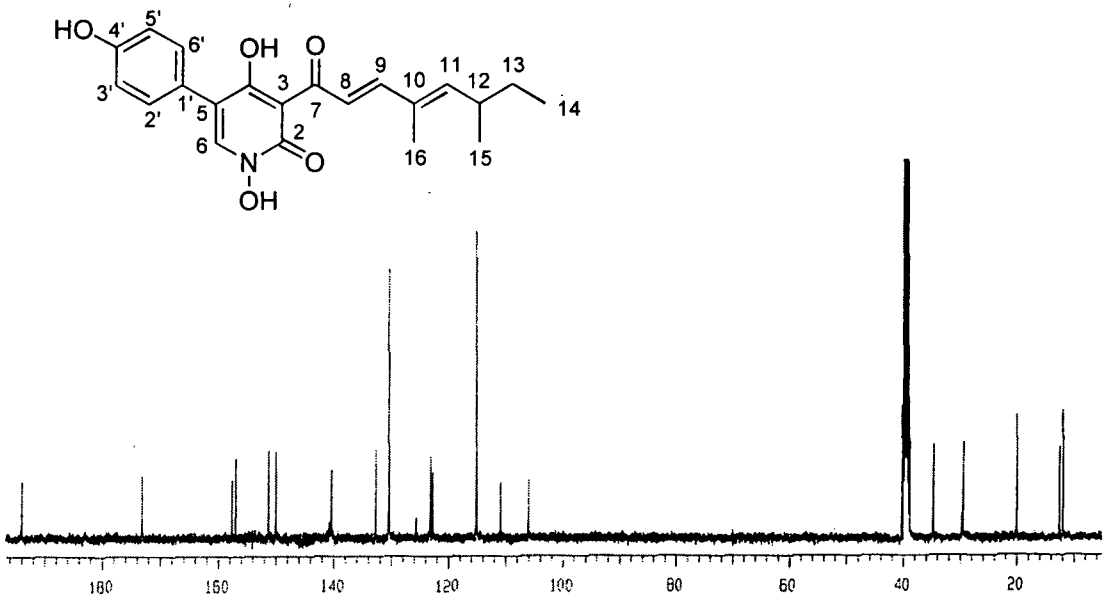
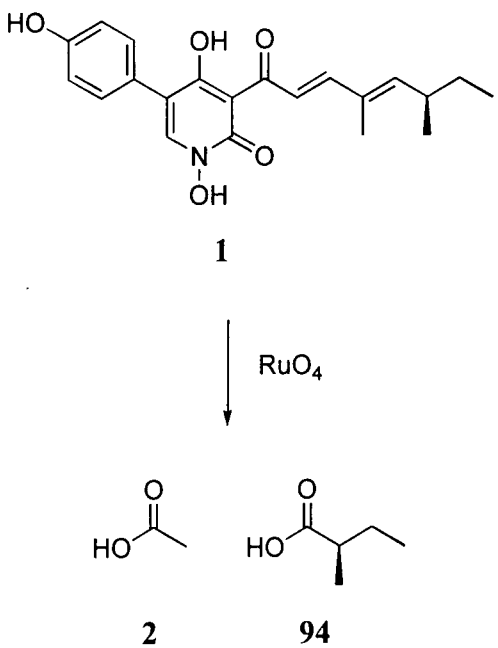


Figure 2-2 ^{13}C NMR of tenellin **1**

Table 2-2 ^{13}C chemical shifts (δ) for tenellin **1**

Carbon	δ
2	157.6
3	105.9
4	173.0
5	110.9
6	140.2
7	194.0
8	122.6
9	149.8
10	132.7
11	151.3
12	34.6
13	29.4
14	11.9
15	20.0
16	12.3
1'	123.2
2', 6'	130.2
3', 5'	115.0
4'	157.0

The absolute configuration of tenellin was determined by a degradative process.[68] Catalytic ruthenium tetroxide oxidation of a sample of tenellin yielded a mixture of carboxylic acid sodium salts, which were purified by acidification and lyophillisation to generate a mixture of acetate **2** and 2-methylbutyrate **94** (Scheme 2-14). After treatment with gaseous HCl under deuteriochloroform, the resultant solution was analysed by ^1H NMR in the presence of a chiral base. The chemical non-equivalence of the protons induced by the addition of (*R,R*)-(-)-1,2-diphenyldiamino-ethane[69] to the solution was used to assess the absolute stereochemistry of the diastereomeric complex formed. Comparison of the degradative sample with reference standards of (*R*)- and (*S*)-2-methylbutyric acids revealed that C-12 of tenellin has the *R*-configuration.



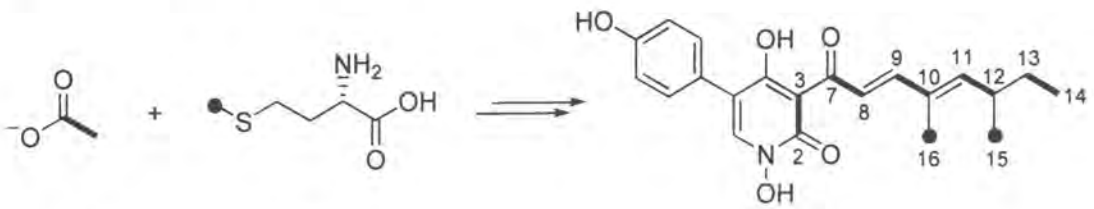
1 **2** **94**

Scheme 2-14

2.3.3 Biosynthetic studies on tenellin

Early biosynthetic experiments[1,64] on tenellin **1** showed that acetate **2a** efficiently labelled carbons 2 and 3 and carbons 7-14 indicating a polyketide origin (*vide supra).* Isotopically labelled phenylalanines were shown to contribute the rest of the pyridone

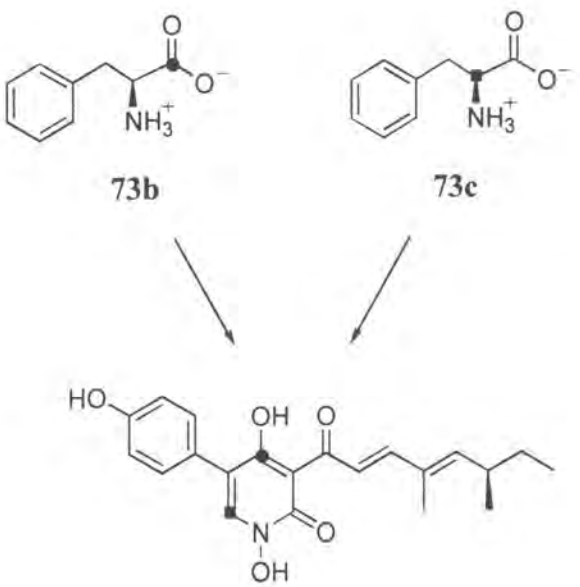
and the aryl ring, and labelled methionine **53** was used to show that the two methyl groups, C-15 and C-16, derive from SAM (Scheme 2-15).



2a **53** **1**

Scheme 2-15

Labelling studies[1] showed that C-4 and C-6 of tenellin **1** are enriched by [1-^{13}C]-phenylalanine **73b** and [2-^{13}C]-phenylalanine **73c** respectively (Scheme 2-16).



73b **73c**

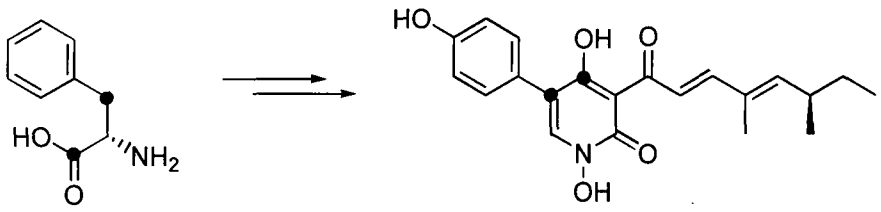
1

Scheme 2-16

The intact incorporation of a phenylalanine unit would indicate that a bond cleavage and rearrangement process must occur during the biosynthesis.

Further support for the rearrangement hypothesis was obtained from ^{13}C NMR analysis after administration of doubly labelled [1,3-$^{13}C_2$]-phenylalanine **73d** (Scheme 2-17).[70]

The recorded ^{13}C NMR spectrum showed ^{13}C-^{13}C coupling exclusively between C-4 and C-5. The contiguity of the labelled carbons and the almost equal enrichments, at C-4 and C-5 clearly suggests that the rearrangement is an intramolecular process.



73d **1**

Scheme 2-17

The first study investigating the incorporation of ^{15}N into tenellin from L-[^{15}N]-phenylalanine[65] suggested intact incorporation of the amino acid and that transamination to the corresponding keto acid does not occur. However, experiments with DL-[2-^{13}C, ^{2}H, ^{15}N]-phenylalanine[71] contradicted this. The lack of coupling to ^{15}N in the ^{13}C NMR spectrum and the absence of any indication of an intact $^{13}C^{2}H$ bond suggests that transamination to phenylpyruvate is a facile process and that previous results were misinterpreted. This could occur if the nitrogen pool is significantly enriched with ^{15}N from the administered precursor and that the isotope is reincorporated at a later stage.

2.3.4 Phenylalanine *versus* tyrosine

Tenellin has a C-5 phenol ring attached to the pyridone ring system, however, it is interesting to note that the initially reported level of radiolabelled phenylalanine incorporation into tenellin was significantly higher than that for tyrosine (Table 2-3).[65] Accordingly, the authors proposed that tyrosine is not a direct precursor of tenellin and that hydroxylation of the aromatic ring occurs *after* condensation of phenylalanine with the polyketide fragment and possibly concomitantly with ring expansion.

Table 2-3 % ^{14}C Incorporation levels of ^{14}C-labelled phenylalanine and tyrosine into tenellin[65]

	Substrate		Tenellin	
	mM	μCi/mmol	μCi/mmol	%[a]
L-[2-^{14}C]-Phenylalanine	2	11.6	0.98	8.4
L-[2-^{14}C]-Phenylalanine	2[b]	11.6	0.88	7.5
L-[U-^{14}C]-Tyrosine	1	7.33	0.122	1.6

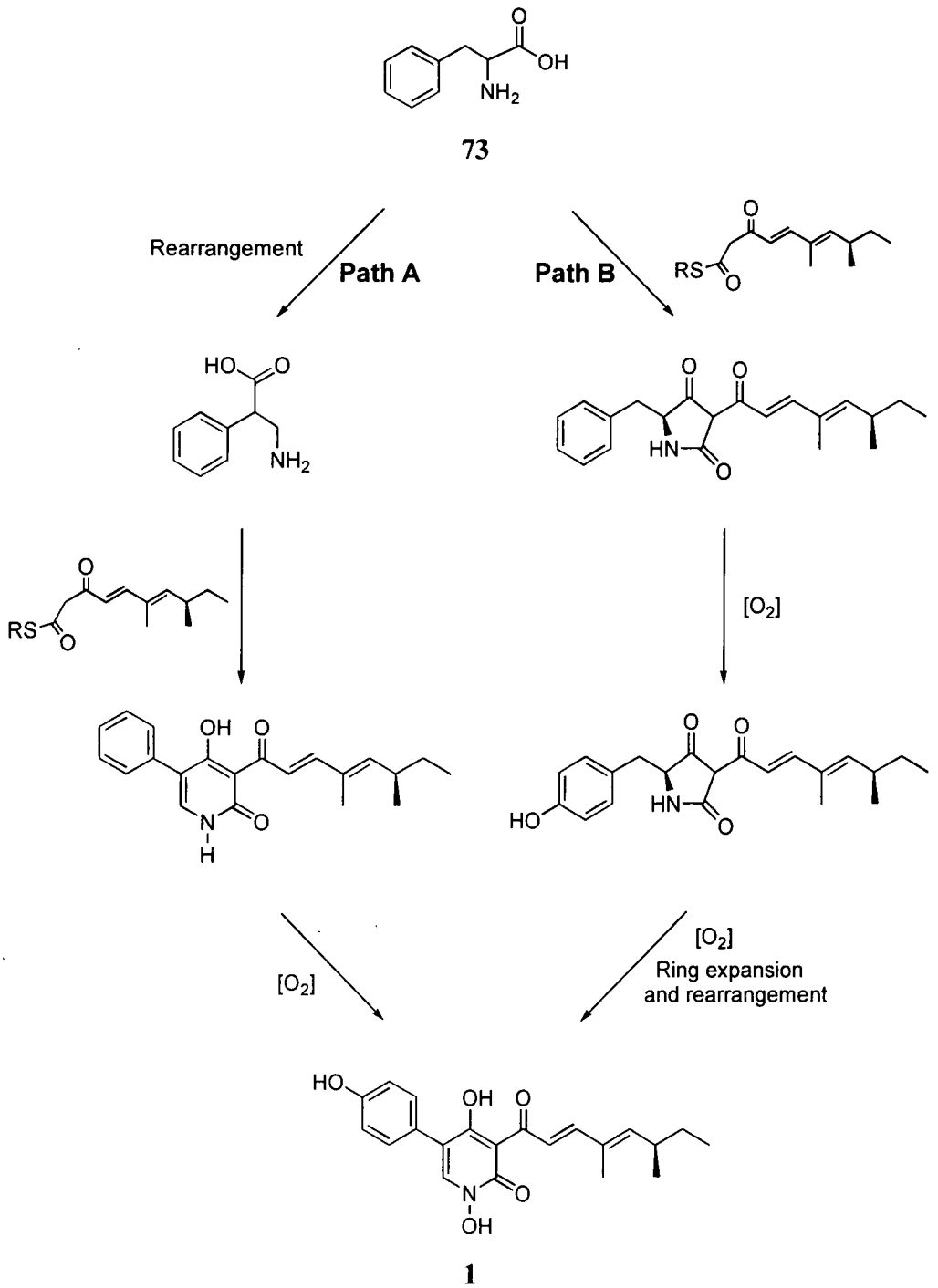
[a] Specific incorporation estimated as 100 times specific activity of tenellin divided by specific activity of substrate

[b] The substrate was added in equal portions on the second, fourth and sixth days to give a final concentration of 2 mM.

2.4 Rearrangement of phenylalanine in tenellin biosynthesis

2.4.1 Two possible pathways for tenellin formation

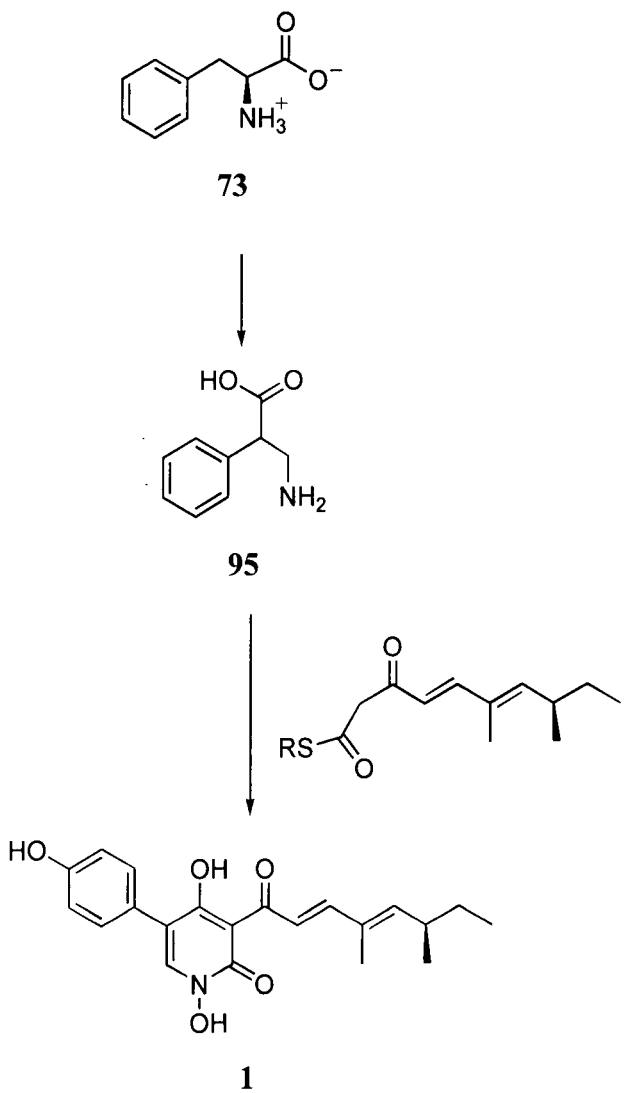
The focus of this study was to examine the details of the rearrangement of phenylalanine in tenellin biosynthesis. Two possible pathways have been proposed for the generation of the 2-pyridone ring system of tenellin (Scheme 2-18). Path A suggests that phenylalanine **73** undergoes a direct isomerisation to 3-amino-2-phenylpropionic acid **95**, which then couples with a polyketide moiety to form the pyridone ring. Subsequent hydroxylation of the nitrogen and at the *para*-position of the aromatic ring would deliver tenellin. However, a process whereby a tetramic acid intermediate is formed would also satisfy the observed labelling experiments (path B). *Para*-oxygenation to a quinone intermediate with subsequent ring expansion and rearrangement is then postulated. This 5-membered heterocyclic intermediate was proposed by Vining[65] and is discussed in Chapter 3.


73
Rearrangement
Path A
Path B
[O2]
[O2]
[O2]
Ring expansion
and rearrangement
1

Scheme 2-18

2.4.2 Hypothesis for tenellin *via* a direct phenylalanine rearrangement

The hypothesis suggested in Path A was initially considered. It was proposed that a 1,2-carboxyl shift of phenylalanine **73** would generate 3-amino-2-phenylpropionic acid **95**, which could then condense with a polyketide moiety to give the pyridone ring system. Hydroxylation of the aromatic ring by a PAH and delivery of an hydroxyl to the nitrogen would generate tenellin **1**.



73

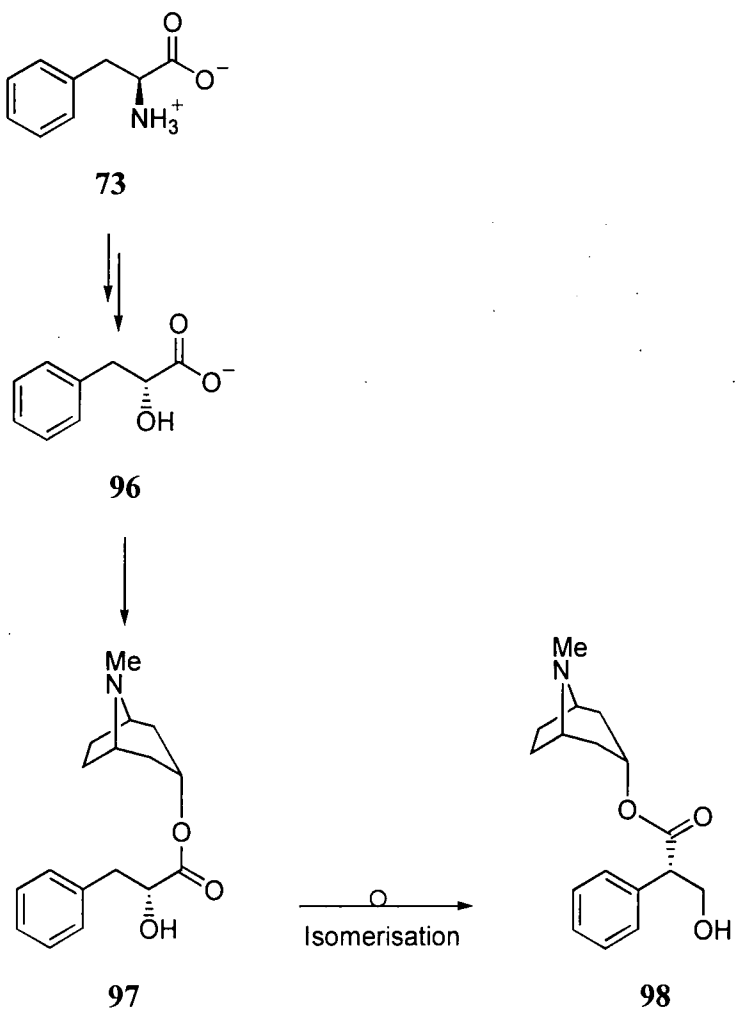
95

1

Scheme 2-19

2.4.3 Other rearrangements in secondary metabolism

A similar phenylalanine type rearrangement to that proposed for tenellin in path A is found in the biosynthesis of the tropate ester moiety of alkaloids, such as hyoscyamine and scopolamine. The tropate skeleton is generated by a rearrangement of a phenylalanine derived phenylpropanoid moiety, which has been shown to be phenyllactate **96**.[72] Importantly, it was found that the rearrangement does not occur until after condensation of phenyllactate and the tropate moiety to generate littorine, and that hyoscyamine **98** is formed from the direct isomerisation of littorine **97** (Scheme 2-20).[73]

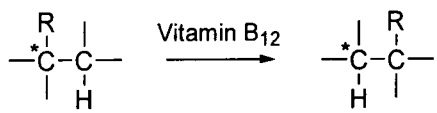


73 **96** **97** **98**

Scheme 2-20

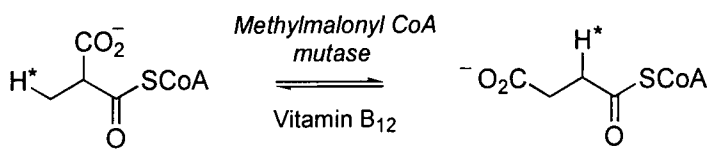
There are structural similarities between this process and rearrangements involving co-enzyme-B_{12} enzymes, however the occurrence of vitamin-B_{12} in higher plants is not proven and this is unlikely to be a B_{12} mediated process.

Co-enzyme B_{12} plays a role in enzymic rearrangments that involve the vicinal interchange of a hydrogen atom and a substituent on an adjacent carbon (Scheme 2-21). It is thought that hydrogen abstraction from the substrate generates a radical, which may then undergo direct rearrangement to give the product. The hydrogen is then returned to the other migration terminus.



Scheme 2-21

One co-enzyme B_{12} system that has been studied in some detail is methylmalonyl-CoA mutase (Scheme 2-22).[74]



Scheme 2-22

This reaction is initiated by the formation of an adenosyl radical, which abstracts a hydrogen atom from the methyl group of the substrate. The study of methylmalonyl-CoA mutase using a labelled substrate showed that the hydrogen that was removed was not scrambled with water during the rearrangement, but became relocated at C-2 of succinyl-CoA. Analogous reactions involving 1,2-migrations of vinyl groups are known to proceed *via* cyclopropylmethyl radical intermediates, so it is likely then that the thioester group in the methylmalonyl-CoA mutase system migrates *via* a similar intermediate. Thus, coenzyme B_{12} appears to act as a reversible free radical carrier.

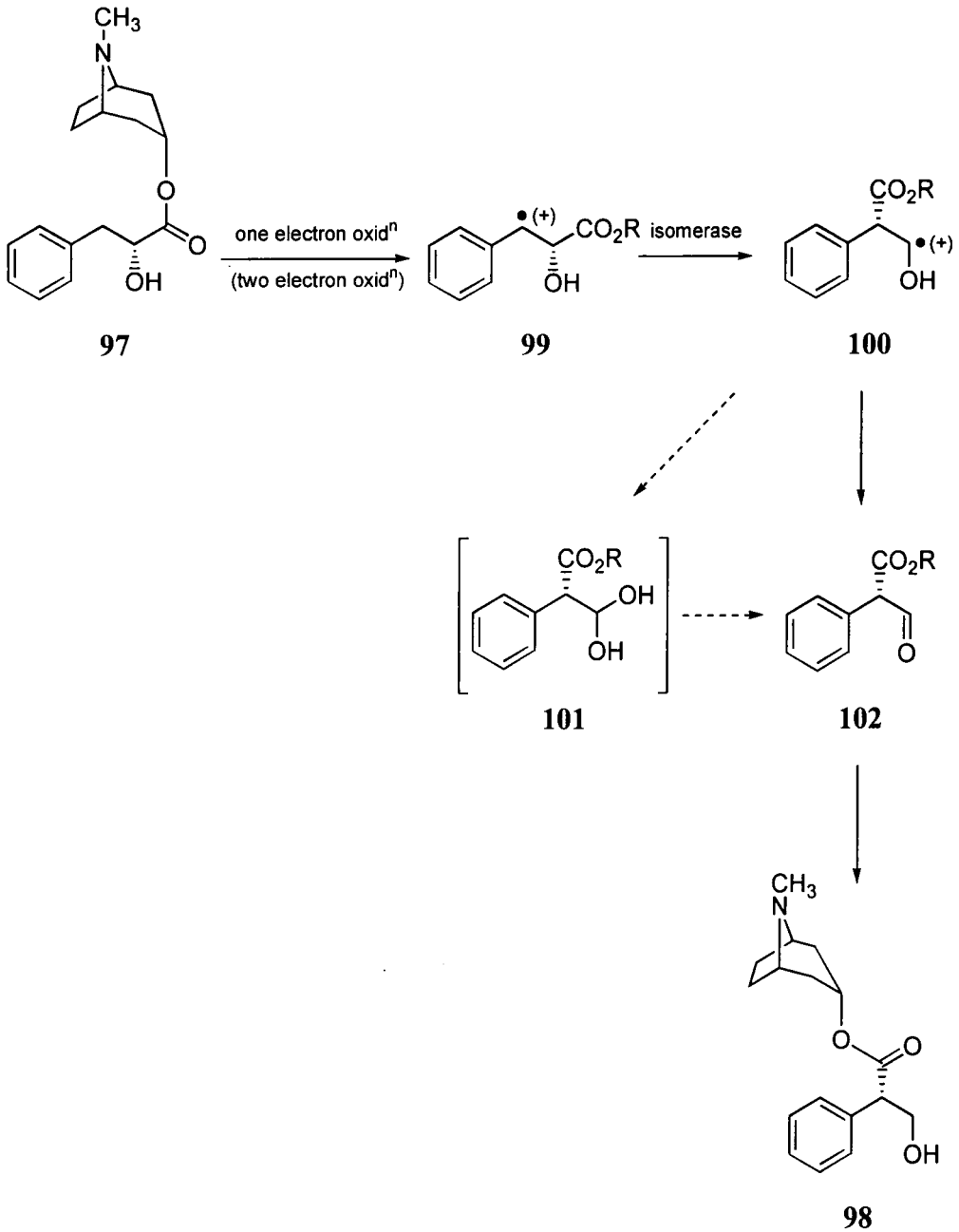
Experiments on tropic acid biosynthesis gave conflicting evidence on the role of coenzyme B_{12}. An early feeding study[75] using radiolabelled phenyllactate reported a

vicinal interchange process, where the hydrogen removed from C-2 was apparently relocated at the original carboxyl site, suggesting a co-enzyme B_{12} type mechanism. However, a more recent experiment[76] using stable isotopes and ^{13}C NMR analysis, saw no evidence for a vicinal interchange process. Leete has also indicated an absence of co-enzyme B_{12} in *Datura* plants and therefore it would appear unlikely that this co-factor is involved in the rearrangement of littorine to hyoscyamine during tropic acid biosynthesis.

The rearrangement may be mediated by a cytochrome P_{450} enzyme. The generation of radicals by iron-oxo species is not unknown, and it has recently been shown that the conversion of littorine to hyoscyamine is inhibited by chlotrimazole, a recognised P_{450} inhibitor. Oxygen rebound mechanisms are a feature of such systems and the fate of oxygen in the tropic acid system has been studied.

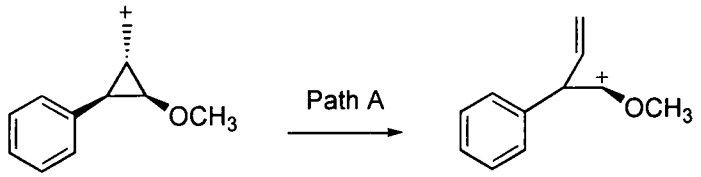
In a feeding experiment with [2-^{2}H, ^{18}O]-hyoscyamine,[77] it was found that 71-75 % of the ^{18}O was retained. The loss of label can be rationalised in several ways from Scheme 2-23, however none of the explanations account for a loss of 25-29 %. Stereospecific collapse of diol **101** favouring loss of oxygen from the rebound hydroxyl over that from the phenyllactate would give 100 % retention, whereas loss of the hydroxyl from phenyllactate would give 100 % loss. Non-stereospecific collapse would give a retention of 50 %. If there is no oxygen rebound mechanism operating, then aldehyde **102** would form directly and there would be no loss of the ^{18}O label. Therefore, the level of retention of ^{18}O is thought to occur by partial stereospecific collapse of the diol or from exchange of the aldehyde with the medium.

Accordingly, it is proposed that abstraction of the 3'-*pro-R* hydrogen by an Fe(IV)-O· species generates radical **99** which then undergoes rearrangement to **100**. Formation of aldehyde **102** then occurs, either by quenching of the radical with a hydroxyl group from Fe(IV)-OH (oxygen rebound) to form hydrate **101**, followed by stereospecific loss of the rebound hydroxyl, or directly, by dehydration of **100**. The action of a dehydrogenase then delivers hyoscyamine.

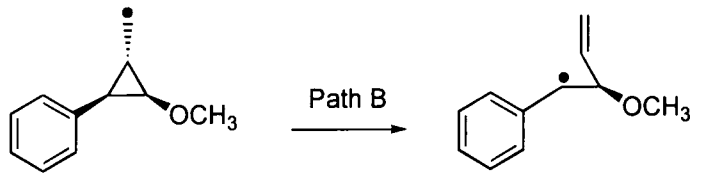


Scheme 2-23

P_{450} enzymes usually favour radical processes, however there is precedence for the involvement of cations emerging from Newcomb's study on substituted methylcyclopropane ring opening reactions (Scheme 2-24).[78] In model studies, cation induced ring opening of **103**, followed path A where the resultant positive charge in **104** became stabilised by the methoxyl group, rather than the phenyl ring. Conversely, radical induced ring opening of **105**, followed path B, with ring opening favouring the benzyl radical **106**.



103 **104**



105 **106**

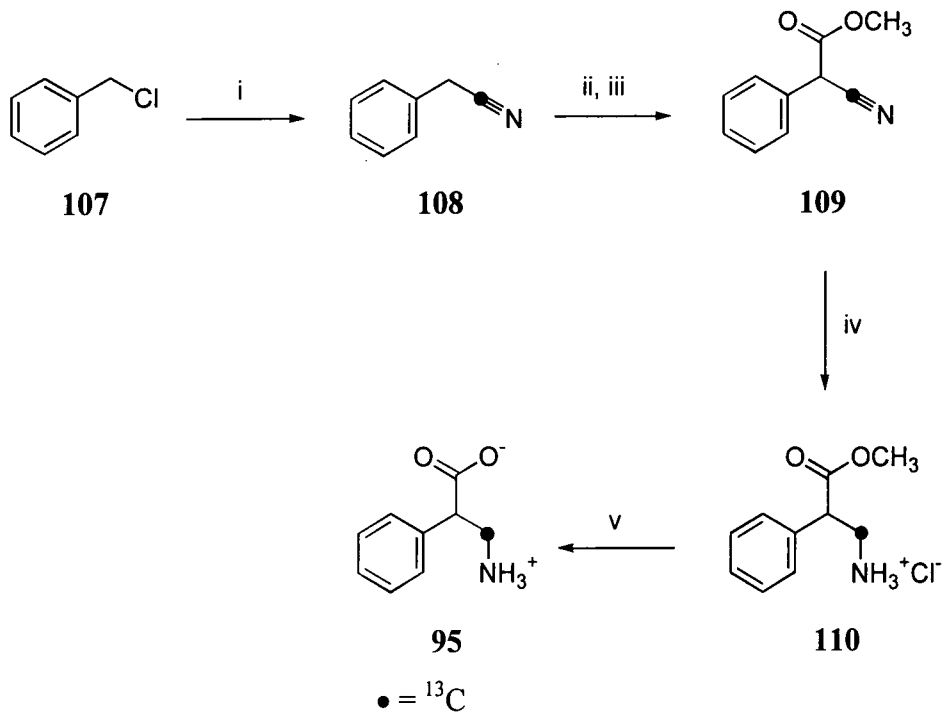
Scheme 2-24

The process occurring during littorine (tropate) isomerisation has the positive charge or radical moving from a benzyl position towards a methyl position and is therefore more suggestive of a cation type process.

For tenellin biosynthesis, the possibility that phenylalanine rearranges in such a manner has been explored. DL-[3-^{13}C]-3-Amino-2-phenylpropionic acid **95** was synthesised[79] in a previous study (Russell Cox) (Scheme 2-25) and then administered to cultures of *B. bassiana*.

2.4.4 Synthesis and feeding of DL-[3-^{13}C]-3-amino-2-phenylpropionic acid 95[79]

The isotopic label was introduced by treatment of benzyl chloride **107** with potassium [^{13}C]-cyanide. The benzyl cyanide **108** produced was converted to the phenylcyano-acetic acid methyl ester **109** and then hydrogenated to yield the amine hydrochloride **110**. Base hydrolysis of the ester gave the target compound **95**, which was purified by ion exchange chromatography.



Scheme 2-25 *Reagents and conditions:* i, [^{13}C]-KCN, 18-crown-6, acetonitrile, 18 °C, 12 h, 90 %; ii, BuLi in THF, then solid CO_2 and acidify with 10 % H_2SO_4, 38 %; iii, CH_2N_2; iv, H_2-PtO_2 in EtOH-$CHCl_3$ (10:1), 2.5 atm, 18 °C, 18 h, 40 %; v, NaOH then H^+-Dowex, 58 %.

Analysis of tenellin isolated from a feeding experiment with DL-[3-^{13}C]-3-amino-2-phenylpropionic acid **95** did not show any incorporation of label at C-5. It was concluded from this experiment that **95** is not an early intermediate in tenellin biosynthesis.

2.5 Tyrosine as a precursor in tenellin biosynthesis

2.5.1 Tyrosine *versus* phenylalanine

As described earlier, Vining *et al.*[65] reported that radiolabelled phenylalanine became incorporated into tenellin at considerably higher levels than tyrosine. However, subsequent feeding experiments in the Durham laboratory (Caragh Moore) with stable isotopes have shown that tyrosine is incorporated at *similar* levels to phenylalanine.[80] [1-^{13}C]-Phenylalanine was incorporated into tenellin at a level of 8.5 % and [3-^{13}C]-tyrosine at 6.1 %.

In the current study, these experiments have been repeated and a similar set of incorporation rates obtained (Table 2-4).

Table 2-4 % Incorporation of phenylalanine and tyrosine into tenellin

	Days after inoculation when precursor was fed	Level of incorporation into tenellin (%)[a]
DL-[1-^{13}C]-phenylalanine	0	5.9
	3	6.4
	4	4.8
DL-[3-^{13}C]-tyrosine	3	5.8
	4	3.8
	5	3.8

[a] Tenellin was extracted 7 days after inoculation of cultures.

Figure 2-3 shows the ^{13}C NMR spectra for one of each of the feeding experiments with [1-^{13}C]-phenylalanine and [3-^{13}C]-tyrosine. Tenellin extracted after a feeding experiment with [1-^{13}C]-phenylalanine (middle spectrum) showed incorporation of label at δ 173 corresponding to C-4 of tenellin. Incorporation of [3-^{13}C]-tyrosine (bottom spectrum) into tenellin was indicated by enrichment at the C-5 resonance at δ 111.

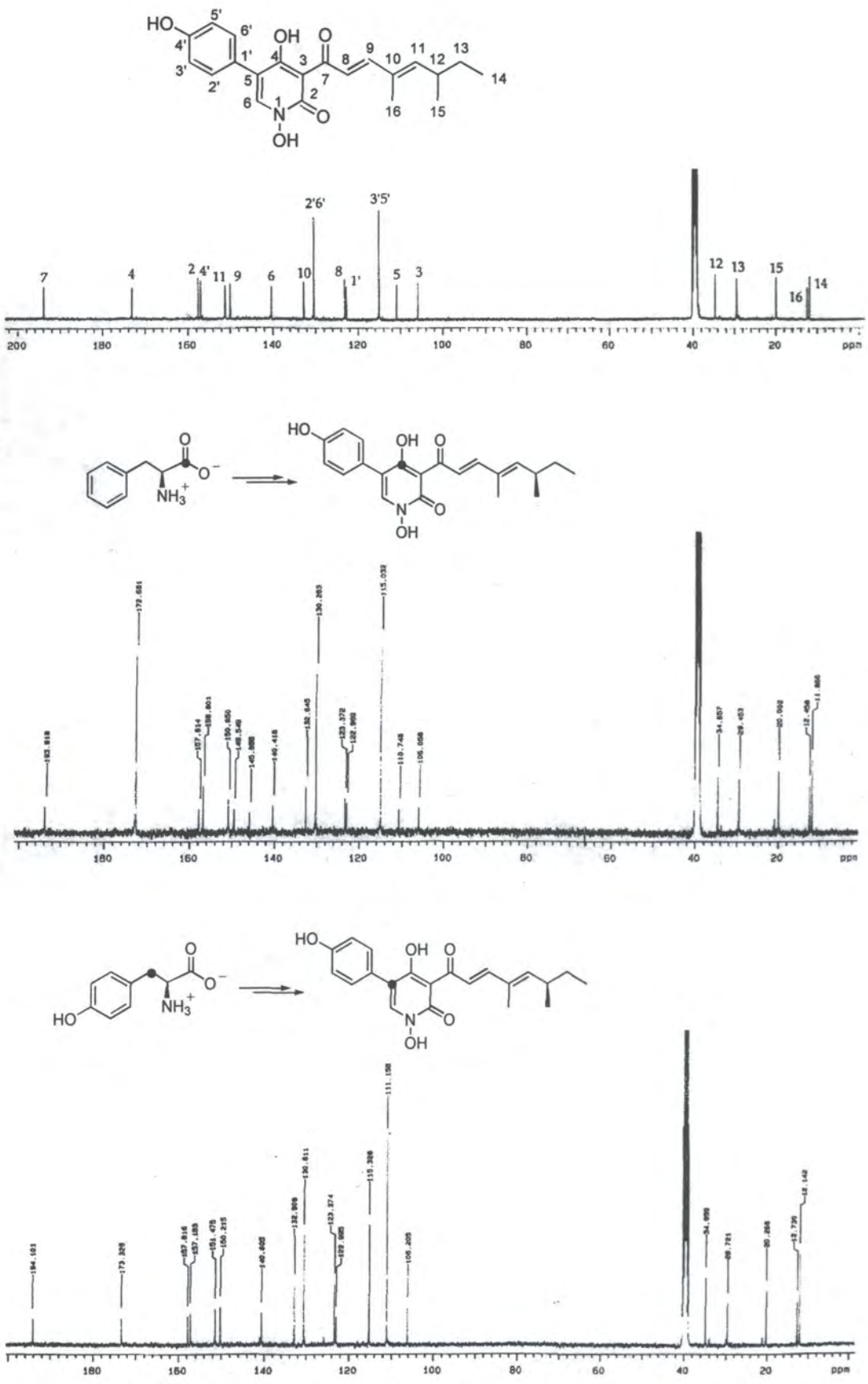
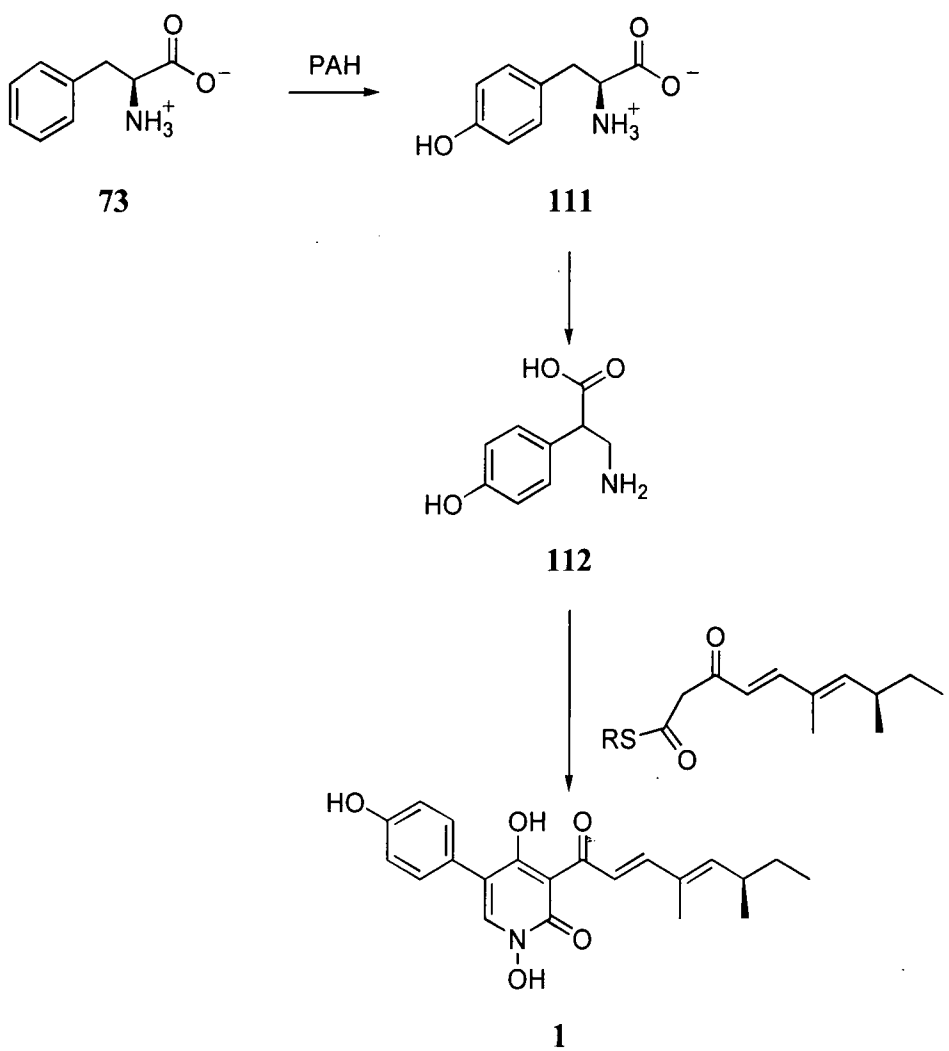


Figure 2-3 ^{13}C NMR spectra of tenellin : natural abundance (top), after feeding with [1-^{13}C]-phenylalanine (middle), after feeding with [3-^{13}C]-tyrosine (bottom)

The results suggest that tyrosine and phenylalanine are equally efficient precursors to tenellin and raises the possibility that perhaps it is tyrosine and not phenylalanine that undergoes a direct rearrangement during the early stages of tenellin biosynthesis. This is structurally satisfactory as tyrosine carries the phenol ring system found in tenellin.

2.5.2 The revised tyrosine hypothesis

The hypothesis for the biosynthesis of tenellin can be re-written to account for the hydroxylation of phenylalanine **73** to tyrosine **111**, by a phenylalanine hydroxylase (PAH), and subsequent rearrangement to the β-amino acid **112** (Scheme 2-26). Condensation with the polyketide unit would furnish tenellin as before.



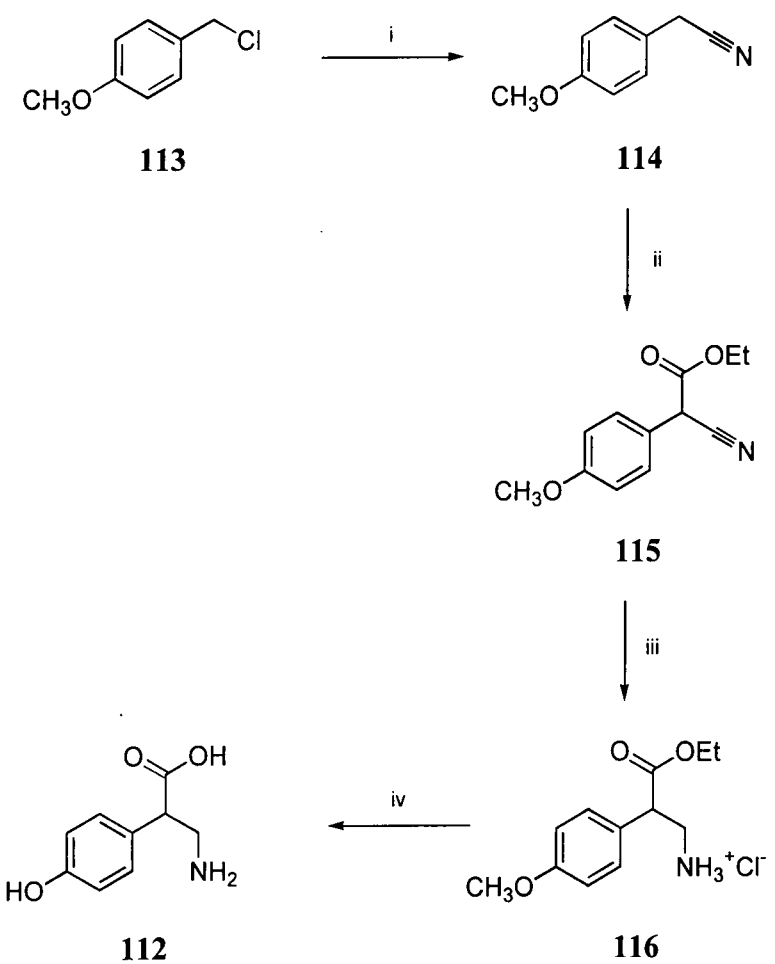
73 **111** **112** **1**

Scheme 2-26

In order to test the validity of this revised hypothesis, whereby tyrosine and not phenylalanine is the more immediate precursor to tenellin, it proved necessary to synthesise an isotopically labelled sample of the putative precursor 3-amino-2-(4-hydroxyphenyl)propionic acid **112** for use in a feeding experiment. Clearly, a synthetic route to **112** had to be considered.

2.5.3 Synthesis of [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid 112a

A similar strategy to that used by previous workers to prepare 3-amino-2-phenylpropionic acid **95**[79] was used (Scheme 2-27).



Scheme 2-27 *Reagents and conditions*: i, KCN, NaI, $(CH_3)_2CO$, reflux, 24 h, 94 %; ii, NaH, THF, 0 °C, then $CO(C_2H_5O)_2$, reflux, 1 h, 55 %; iii, H_2-PtO_2, $CHCl_3$, EtOH, 2.5 atm, 40 h, 94 %; iv, 1M BBr_3 in DCM, -78 °C to 0 °C, 12 h, then 1M NaOH then H^+-Dowex, 42 %.

p-Methoxybenzyl chloride **113** was reacted with sodium or potassium cyanide to yield 4-methoxybenzyl cyanide **114** in high yield. The use of this starting material obviated the requirement for protection of the phenolic hydroxyl group later in the synthesis. Deprotection of aromatic methyl ethers[81,82,83] is widely reported and with many different reagents, therefore it was anticipated that the cleavage of this ether would not prove too difficult. The resultant nitrile **114** was purified by column chromatography over silica gel. Treatment of **114** with NaH and diethyl carbonate[84,85] gave the ethyl carbonate **115**. This reaction proved problematic and was performed a number of times (Table 2-5) until an optimal set of conditions was established. Only moderate yields could be obtained even after such optimisation. The deprotonation was carried out using a variety of reaction times and either sodium hydride or sodium ethoxide as base. The nitrile was stirred with a base and appropriate solvent for between 0 and 60 min before the addition of diethyl carbonate. Deprotonation with sodium hydride in tetrahydrofuran for 30 min was found to give the highest yields. Variable product to starting material ratios could be obtained by altering the reflux time. The results suggested a decarboxylation, as longer reaction times appeared to decrease the product yield. In all cases, some starting material was recovered from the reaction, although this could be minimalised by using a five-fold excess of diethyl carbonate. To show that there was no difficulty with deprotonation, D_2O was added to quench the anion. The presence of the deuterium atom was then determined by ^1H NMR analysis. The geminal proton at C-2 could be seen as a triplet at δ 3.57 in the ^1H NMR spectrum indicating coupling to a deuterium atom. In the ^{13}C NMR spectrum, C-2 was also coupled to the deuterium as evidenced by a triplet for this signal. Therefore, it was concluded that the problems experienced are due to either the low reactivity of the electrophile or a decarboxylation. Reactions were also attempted with ethyl chloroformate[86] and solid CO_2 (entries 8-10), but these were unsuccessful. Thus, work on the diethyl carbonate reactions was continued in order to improve the yield.

114 **115**

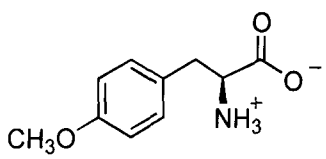
Scheme 2-28

Table 2-5 Reaction conditions employed for the conversion of **114** to **115**

No.	Reagent	Deprotonation (0°C)	Conditions	Yield (%)
1	diethyl carbonate	NaH, THF, 30 min	reflux 4 h	64
2	diethyl carbonate	NaH, THF, 30 min	reflux 2 h	55
3	diethyl carbonate	NaH, THF, 0 min	reflux 4 h	0
4	diethyl carbonate	NaH, THF, 45 min	reflux 8 h	0
5	diethyl carbonate	NaH, THF, 1 h	reflux 8 h	0
6	diethyl carbonate	Na, $(C_2H_5)_2O$	reflux 2h	10
7	diethyl carbonate	Na, $(C_2H_5)_2O$	reflux 8 h	8
8	ethyl chloroformate	NaH, THF, 30 min	reflux 4 h	0
9	ethyl chloroformate	NaH, THF, 0 min	reflux 4 h	0
10	carbon dioxide	NaH, THF, 30 min	stir 6 h	0
11	diethyl carbonate	NaH, THF, 30 min	reflux 1 h	53

The next step required the conversion of the nitrile **115** to an amine **116**. Hydrogenation of the nitrile was initially attempted using a palladium on carbon catalyst. ^{1}H NMR analysis showed a complex spectrum indicating a mixture of products, so the use of this catalyst was abandoned. Fortunately, utilisation of platinum oxide[87] under hydrogen gas at 2.5 atm gave the desired product. If the reaction was carried out using chloroform as a co-solvent with ethanol, the resultant amine was generated directly as the hydrochloride salt. This proved ideal for purification as the product was a white powder and could be collected after filtration of the catalyst and evaporation of the solvent.

The remaining step in the synthesis required the cleavage of the methyl ether. To examine the efficacy of the various possible ether cleavage reagents, test reactions were carried out on O-methyl tyrosine **117**.



117

It emerged that the methyl ether could be cleaved using hydriodic acid[88] in acetone and methanol, when treated under pressure in a Carius tube at 200 °C. However, on several occasions the tube ruptured and the product was lost. The harsh conditions also resulted in heat induced decomposition and significant purification of the product was required, resulting in low yields. Reactions with trimethylsilylbromide (TMSBr) or trimethylsilyl-iodide (TMSI),[89] produced *in situ* from sodium iodide and trimethylsilylchloride (TMSCl) were unsuccessful. Hydrogen bromide (48 % solution in acetic acid)[81] proved effective but product yields were low.

Eventually, the most efficient reagent for the deprotection of **116** was found to be boron tribromide (BBr_3).[90] Time constraints led to this reagent being tested directly on **116**. When a solution of **116** in dichloromethane and BBr_3 was stirred at -78 °C and allowed to warm to room temperature overnight, cleavage of the ether occurred. Subsequent stirring in 1M sodium hydroxide solution was required in order to hydrolyse the ethyl

ester and give the desired product in a moderate yield. Purification of the resultant β-amino acid was performed by ion-exchange chromatography over H^+-Dowex.

This first series of experiments was performed with unlabelled sodium cyanide in order to verify the feasibility of the synthetic route. Once efficient protocols had been established, labelled potassium [^{13}C]-cyanide was used in the synthesis. The reactions were carried out as described for the unlabelled material to produce [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid **112a** in 20.2 % overall yield.

The coupling of the methylene protons with the ^{13}C isotopic label can be seen by ^{1}H NMR (Figure 2-4). The pair of doublet of doublets at δ 2.85 and δ 3.0 corresponding to the protons at C-3 is coupled to another pair of doublet of doublets at δ 3.35 and δ 3.5, with a coupling constant of 145.7 Hz. The C-2 proton is superimposed on one of the methylene resonances at δ 3.5 and there is a characteristic pair of doublets at δ 6.75 and δ 7.04 indicating a *para*-substituted aromatic.

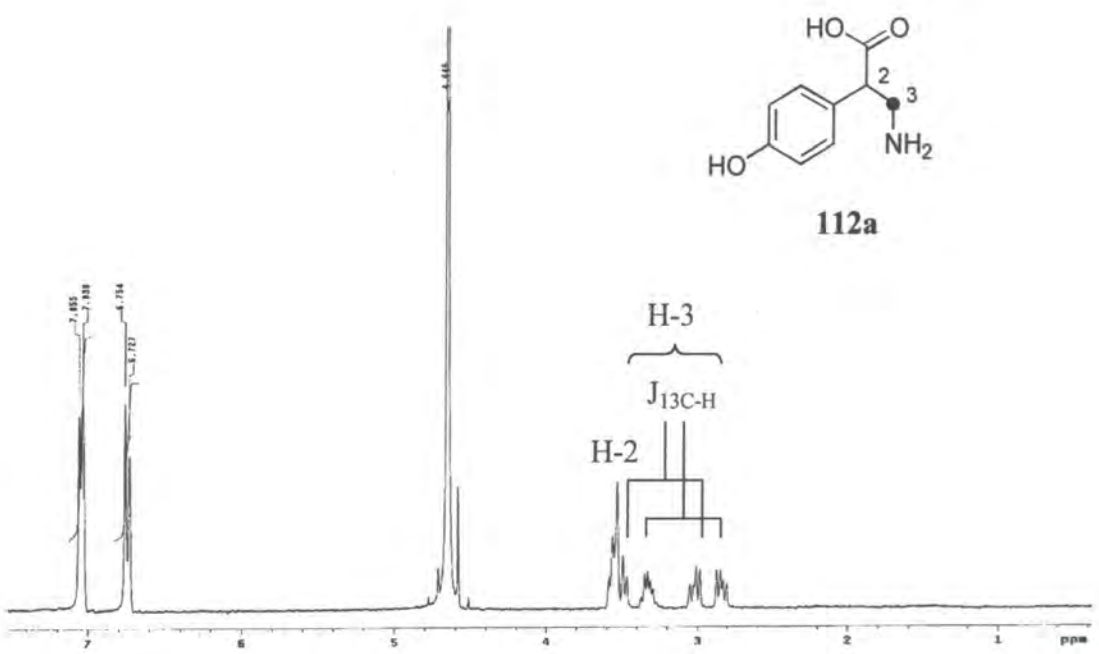


Figure 2-4 ^{1}H NMR of [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid **112a**

It can be seen that the integral of the aromatic signal at δ 6.75 is lower than the other aromatic resonance. This is possibly due to deuterium exchange with the NMR solvent.

In the ^{13}C NMR, the presence of the ^{13}C label in [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid is indicated by the intense peak at δ 42.5 (Figure 2-5). Unfortunately, the acquisition of the spectrum is terminated once one of the peaks reaches a certain height, so the unlabelled peaks are small.

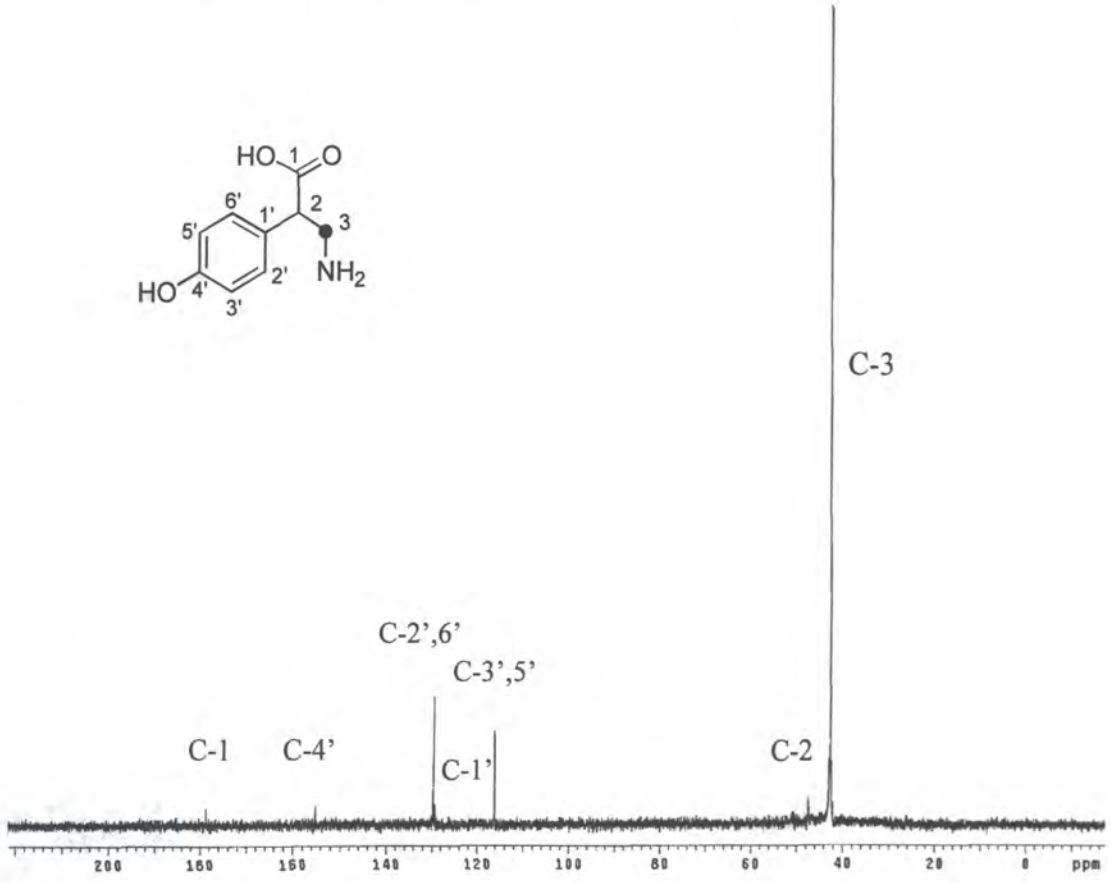
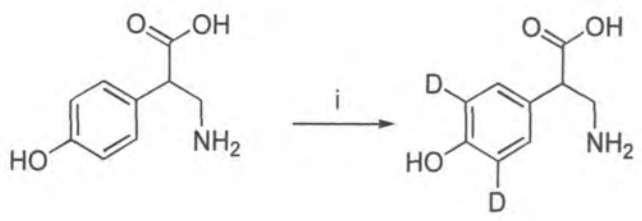


Figure 2-5 ^{13}C NMR of [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid **112a**

[3',5'-$^{2}H_2$]-3-Amino-2-(4-hydroxyphenyl)propionic acid **112b** was also prepared. Heating **112** in deuterated acid and water under pressure for 3 h produced **112b** in moderate yield. The product was found to be >95 % deuterated by ^{1}H NMR.



112 **112b**

Scheme 2-29 *Reagents and conditions*: i, DCl/D_2O, Carius tube, 80 °C, 3 h, 64 %.

It was also found that [3',5'-$^{2}H_2$]-3-amino-2-(4-hydroxyphenyl)propionic acid could be generated by utilising deuterated hydrobromic acid (DBr/CD_3CD_2OD) in the final ester hydrolysis step of the synthesis. This material was also found to be >95 % deuterated by ^{1}H NMR.

2.5.4 Feeding of [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid 112a to *Beauveria bassiana*

[3-^{13}C]-3-Amino-2-(4-hydroxyphenyl)propionic acid **112a** was administered to two culture flasks of *Beauveria bassiana* at a final concentration of 5 mmol. The bright yellow cultures were extracted with acetone on day 10 and tenellin (25 mg) was isolated. The ^{13}C NMR spectrum recorded was indicative of tenellin, but unfortunately, no ^{13}C incorporation of the labelled material could be detected.

2.5.5 Feeding of [3',5'-$^{2}H_2$]-3-amino-2-(4-hydroxyphenyl)propionic acid 112b to *Beauveria bassiana*

The deuterium labelled material **112b** was fed to cultures of *Beauveria bassiana* to a final concentration of 5 mmol. The mycelia produced by two flasks of fungus were combined and tenellin extracted in the usual manner. After Soxhlet extraction and work-up, tenellin (30 mg) was isolated as an orange/yellow powder. ^{2}H NMR analysis of the sample did not indicate any deuterium incorporation.

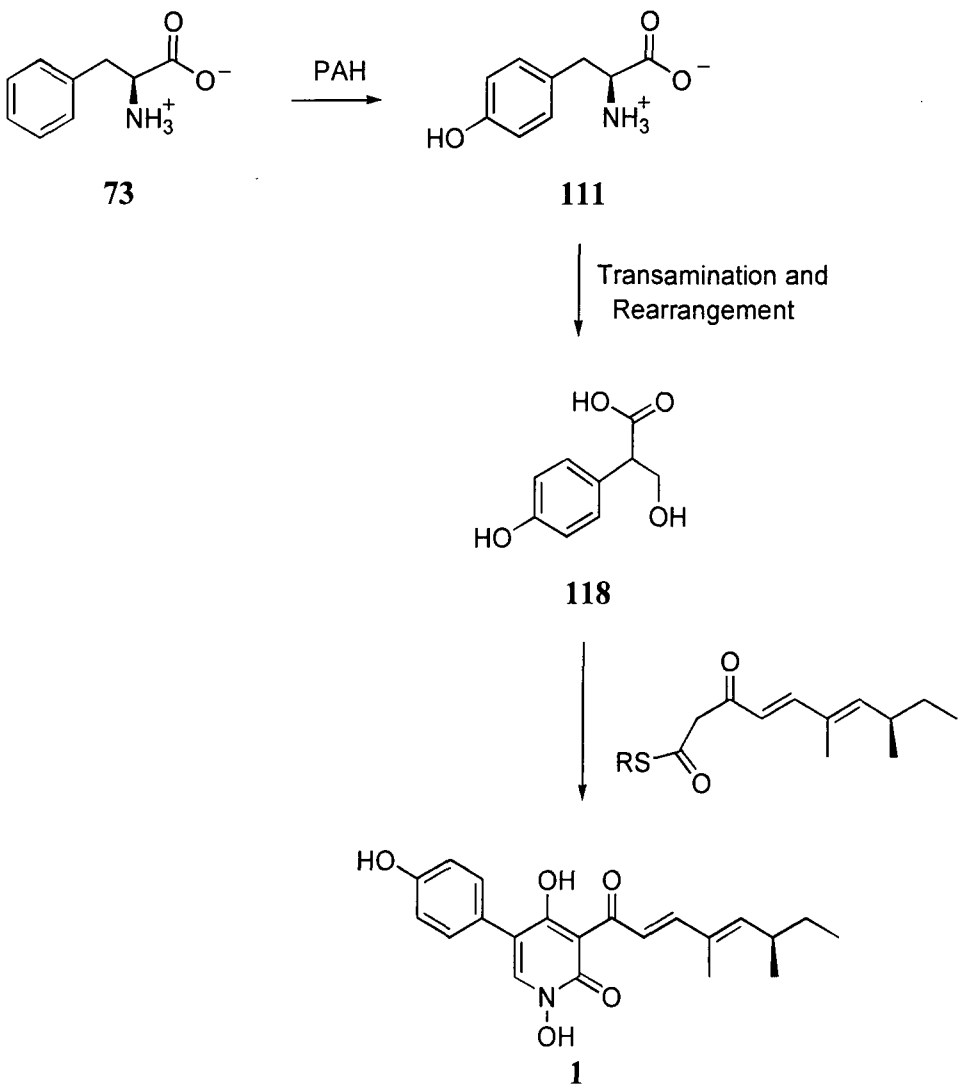
From these results it was concluded that 3-amino-2-(4-hydroxyphenyl)propionic acid **112** is not a relevant precursor in tenellin biosynthesis and the production of the secondary metabolite must be elaborated by another biosynthetic pathway.

2.6 A refined hypothesis involving tyrosine in tenellin biosynthesis

2.6.1 3-Hydroxy-2-(4-hydroxyphenyl)propionic acid 118

The hypothesis tested to this point assumes a direct rearrangement of tyrosine. In view of the failure of incorporation of the synthetic intermediates **95** and **112**, the possibility of a transamination occurring before rearrangement was now considered. It has been shown (Chap 2.3.3) that there was no intact incorporation of the isotopes from [2-^{13}C, ^{2}H, ^{15}N]-phenylalanine indicating a facile transamination process.

If we now consider the possibility of transamination occurring as a requirement before the rearrangement of tyrosine, then a new putative intermediate **118** is formed.



73 **111** **118** **1**

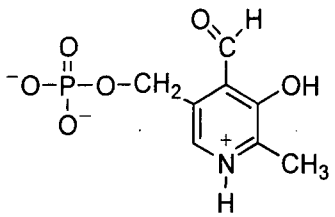
Scheme 2-30

The hydroxy acid, 3-hydroxy-2-(4-hydroxyphenyl)propionic acid **118**, was now examined as a putative precursor to tenellin and therefore emerged as a new target for synthesis.

The revised hypothesis is shown in Scheme 2-30.

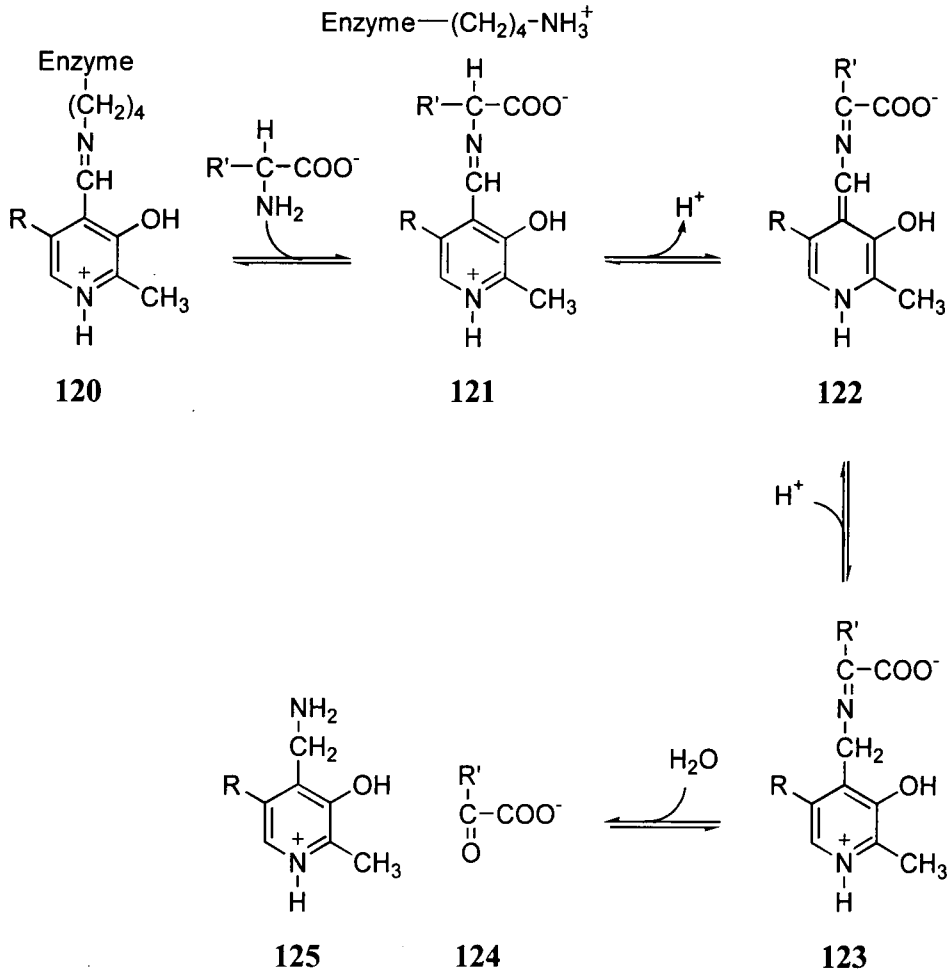
2.6.2 Transamination of phenylalanine

Pyridoxal 5'-phosphate (PLP) **119** is involved in all aminotransferase reactions of amino acids. It is a co-enzyme derived from vitamin B_6 (pyridoxine) which functions as an electron sink and can covalently attach amino acids *via* an aldehyde moiety at position C-4.



119

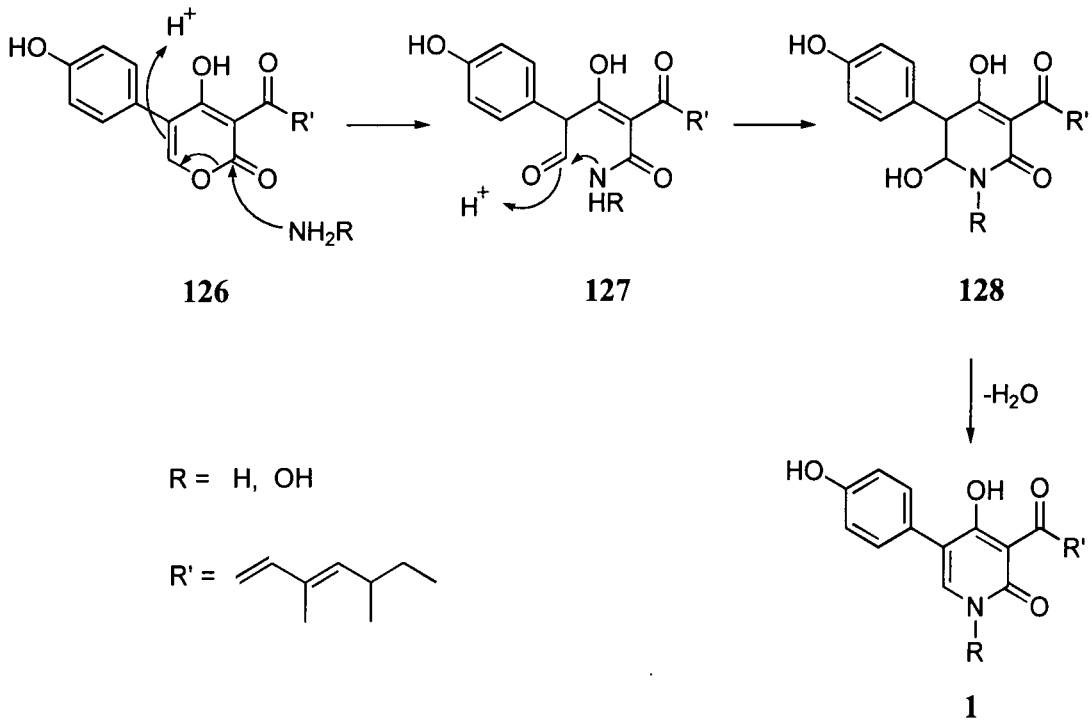
When no substrate is present, the aldehydic linkage is bound to the transaminase enzyme through a Schiff base **120** to the ε-amino group of a lysine residue at the active site. An α-amino group of an amino acid can displace the lysine forming an aldimine structure **121**. The addition of the amino acid increases the acidity of the α-proton, which is lost to give a quinonoid intermediate **122**, the pyridine ring acting as a sink for the electrons. Reprotonation produces a ketimine **123**, which can be hydrolysed to release the keto-acid **124** and pyridoxamine 5'-phosphate **125** (Scheme 2-31).



Scheme 2-31

2.6.3 Pyranone formation in tenellin biosynthesis

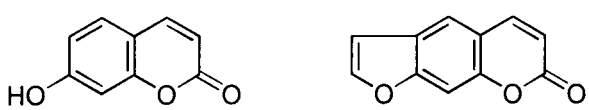
The proposal that 3-hydroxy-2-(4-hydroxyphenyl)propionic acid **118** is a precursor in tenellin biosynthesis implicates the pyranone structure **126** as an intermediate. If the hydroxy acid condenses with the polyketide unit then pyranone **126** will be formed. It can be envisaged that a nitrogen containing compound, possibly ammonia or hydroxylamine, could attack at the carbonyl and open the pyranone ring. Intramolecular nitrogen attack at the newly formed carbonyl in **127** could then form the six membered ring **128**, which could undergo loss of water to generate the pyridone ring system and deliver tenellin.



Scheme 2-32

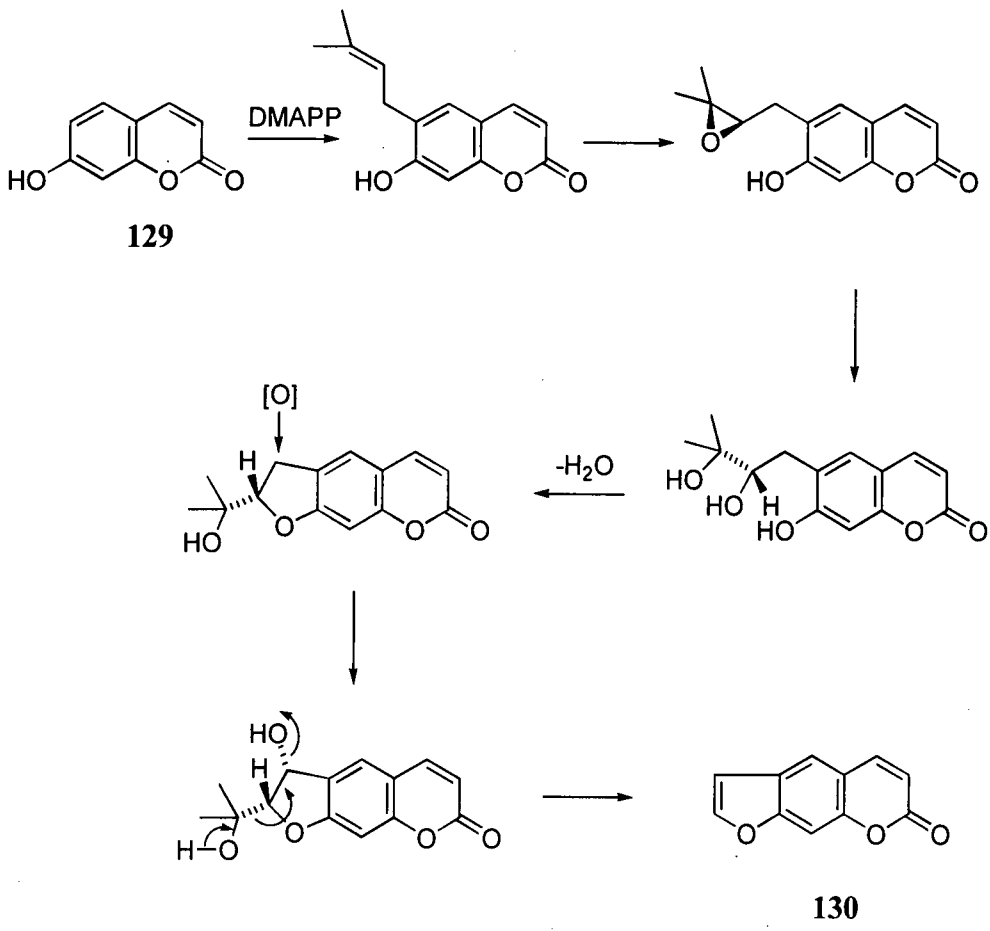
Many pyranone structures are known to occur in natural products. They can be α-or γ-pyranones, depending on whether the carbonyl is *ortho* or *para* to the heterocyclic oxygen.

The coumarins are widespread and contain an α-pyrone system. They are often evolved from shikimate *via p*-coumaric acid but can also derive from acetate. Umbelliferone **129**, the UV-absorbing component of many suntan preparations and psoralen **130**, a furanocoumarin, are shikimate derived.[23]



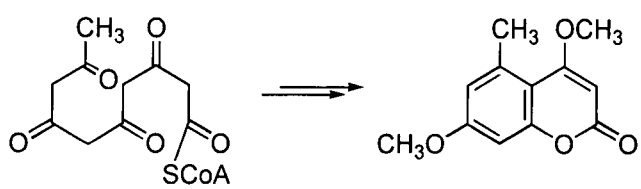
129 **130**

A fused furan ring is a common feature of these structures and in this case is derived from the alkylation of umbelliferone **129** with dimethylallyl pyrophoshate (DMAPP). Loss of three carbons then generates psoralen **129** (Scheme 2-33).



Scheme 2-33

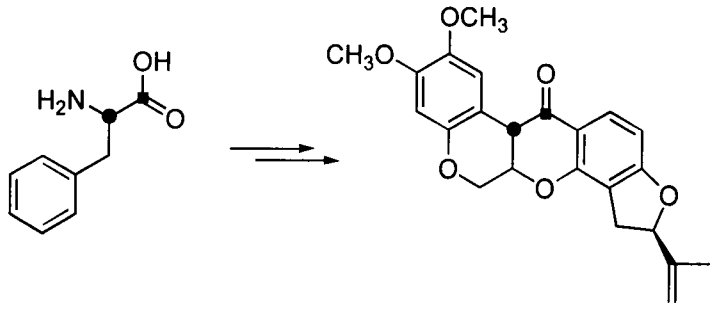
In contrast, the metabolite 4,7-dimethoxy-5-methylcoumarin **131** from the fungus *Aspergillus variecolor* is derived from acetate (Scheme 2-34).[91]



131

Scheme 2-34

The flavones and isoflavones, for example rotenone **132**, are γ-pyrones. The biosynthesis of rotenone from phenylalanine *via* cinnamic and *p*-coumaric acids has been shown in biosynthetic studies (Scheme 2-35).[92]

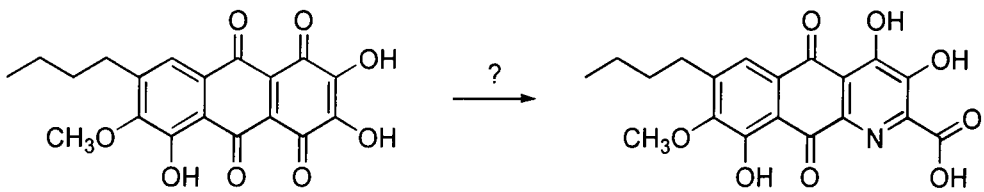


132

Scheme 2-35

2.6.4 Origin of the nitrogen atom

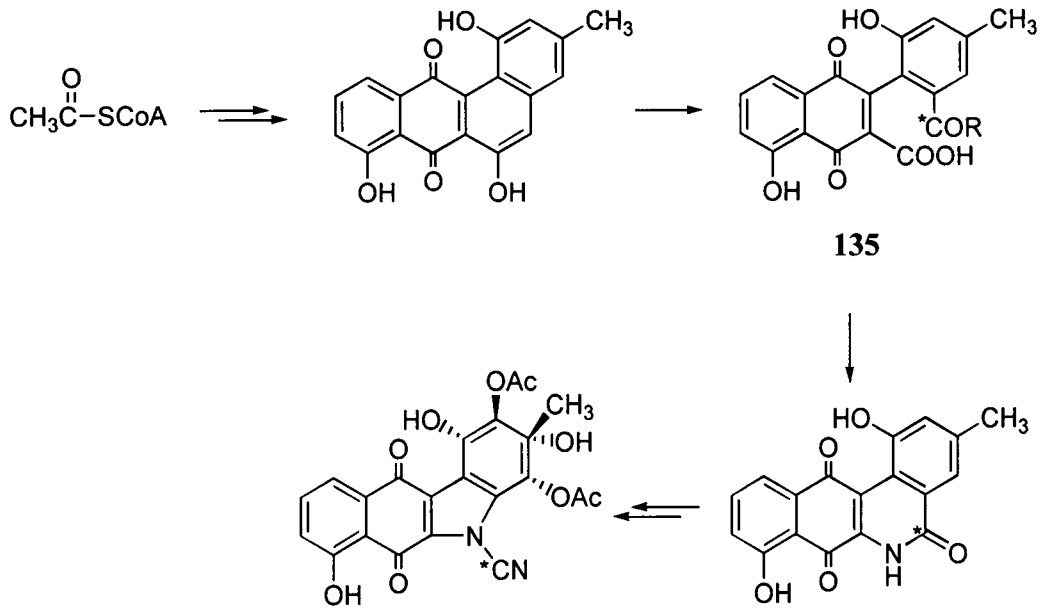
Tracing the fate of the nitrogen atom in natural products has been the focus of many studies. Transamination processes remove the nitrogen atom from amino acid donors, and re-incorporation of ammonia can occur at a significant level. For example, phomazarin **134** has a tricyclic structure and is derived from nine intact acetate units.[93] The nitrogen is thought to be incorporated into structure **133** (Scheme 2-36).



133 **134**

Scheme 2-36

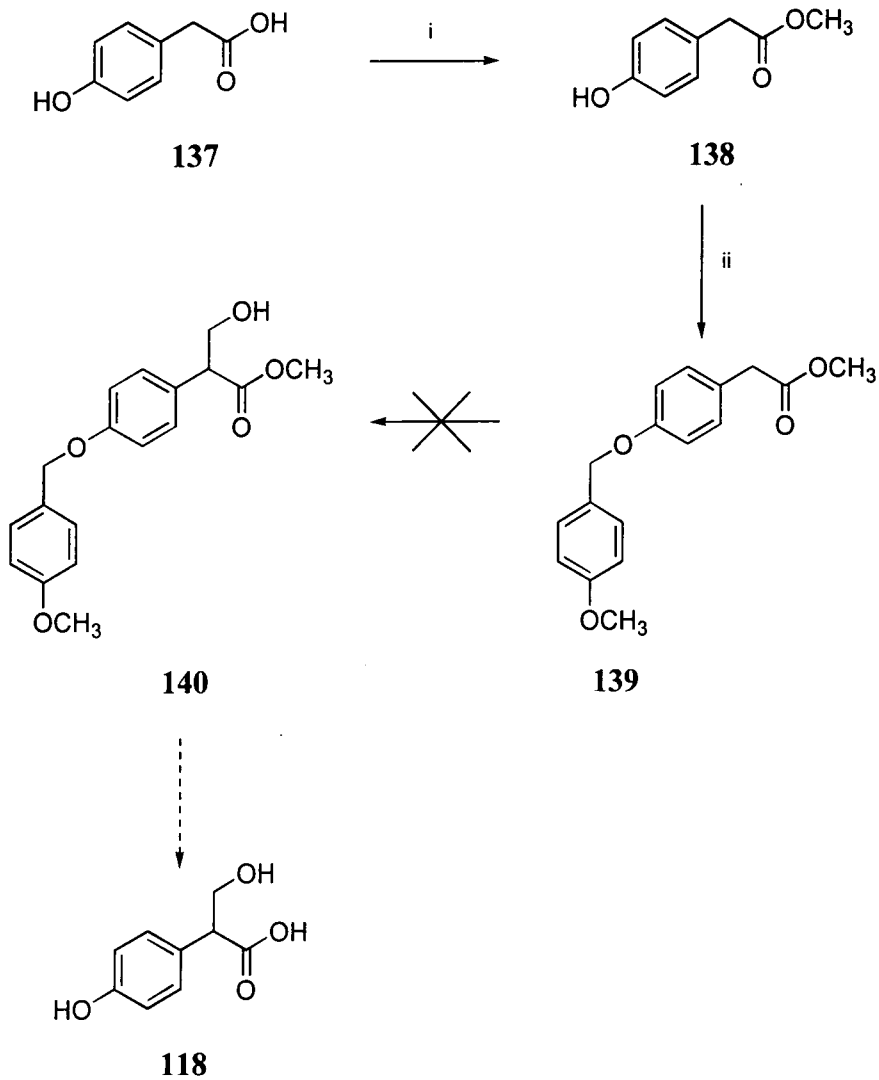
The insertion of nitrogen into a diacid precursor **135** is thought to account for the structure of the antibiotic kinamycin D **136**. Labelling studies have shown that C-6 is lost during the biosynthesis and C-5 (*) becomes the carbon of the N-cyano group (Scheme 2-37).[94]



Scheme 2-37

2.6.5 Synthesis of 3-hydroxy-2-(4-hydroxyphenyl)propionic acid 118

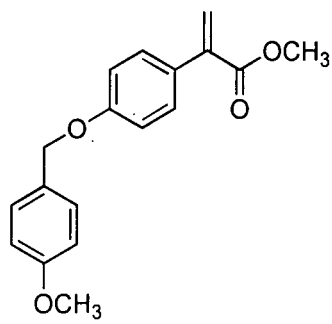
Two strategies were considered for the synthesis of 3-hydroxy-2-(4-hydroxyphenyl)propionic acid **118**. The first of these is shown below (Scheme 2-38).



Scheme 2-38 *Reagents and conditions*: i, CH_3OH, H_2SO_4, reflux, 12 h, 75 %; ii, 4-methoxybenzyl chloride, $Bu_4N^+I^-$, K_2CO_3, $(CH_3)_2CO$, 60 °C, 48 h, 57 %.

This route involved the protection of 4-hydroxyphenylacetic acid **137** followed by the introduction of a CH_2OH functional group at position-2 by condensation with paraformaldehyde. It was envisaged that deprotection would then generate the desired hydroxy acid.

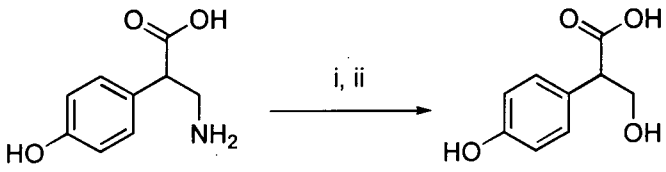
4-hydroxyphenylacetic acid methyl ester **138** could be generated in good yield from the reaction of the corresponding acid **137** with methanol and sulfuric acid. In order to protect the phenol, the ester was treated with 4-methoxybenzyl chloride and potassium carbonate in acetone,[95] giving the desired product **139** as a white crystalline material. The next step was to introduce the hydroxymethyl group adjacent to the carbonyl. Accordingly, the *p*-methoxybenzyl ether **139** was deprotonated with sodium hydride and then treated with paraformaldehyde.[96] Two products formed in a ratio of approximately 1:1 as determined by ^{1}H NMR. One was the desired hydroxymethyl product **140** and the other was the methyl acrylate **141**, which clearly arose after dehydration of **140**. Hydroboration of the acrylate **141** was now considered as a method for the synthesis of **140**. To perform the hydroboration, the mixture of products **140** and **141** was initially stirred in acid to convert all of the material to the alkene. However, during this process the material decomposed, so the hydroboration could not be performed.



141

The reaction was repeated with a shorter reaction time, and a mixture of starting material **139** and traces of product **140** was isolated. In view of the difficulty with this reaction, an alternative approach to **118** was considered.

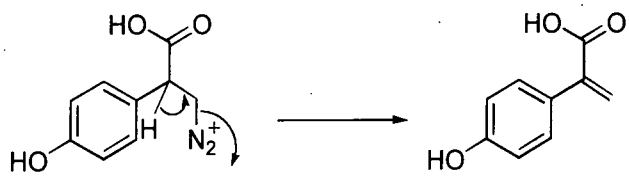
Since the β-amino acid 3-amino-2-(4-hydroxyphenyl)propionic acid **112** was already in hand, conversion of the NH_2 to OH by diazotisation would clearly provide a route to furnish **118** in a single step (Scheme 2-39).



112 **118**

Scheme 2-39 *Reagents and conditions:* i, 2M H_2SO_4, 2M $NaNO_2$, 0 °C, 3 h, then room temp., 12 h; ii, 2M NaOH, 2h, 41 %.

The diazotisation of α-amino acids to α-hydroxy acids is well documented.[97] It has been used in the preparation of α-hydroxy ketones,[98] which are useful as chiral synthons or stereodirecting groups in organic synthesis, and also in the synthesis of α-hydroxy carboxanilides.[99] No such reactions for β-amino acids could be found in the literature. It was not clear at the outset if this reaction would be successful particularly as loss of nitrogen from the diazo compound **118**, could clearly promote elimination. Alternatively, the initially formed protonated β-hydroxy acid may be prone to elimination also and could potentially generate **143** (Scheme 2-40).



142 **143**

Scheme 2-40

Nevertheless, the reaction was attempted under standard diazotisation conditions.[100] The amino acid was stirred in a mixture of 2M sulfuric acid and sodium nitrite at 0 °C for 3 h and then at room temperature overnight. After filtration and removal of solvent, a brown oil was produced.

It was found that after the diazotisation reaction, the hydroxy acid **118** was not the major product. The chemical shift of the methylene protons, a pair of doublet of doublets at δ 4.35 and 4.97 (Figure 2-6) was instead indicative of the formation of the β-lactone **144**.[101]

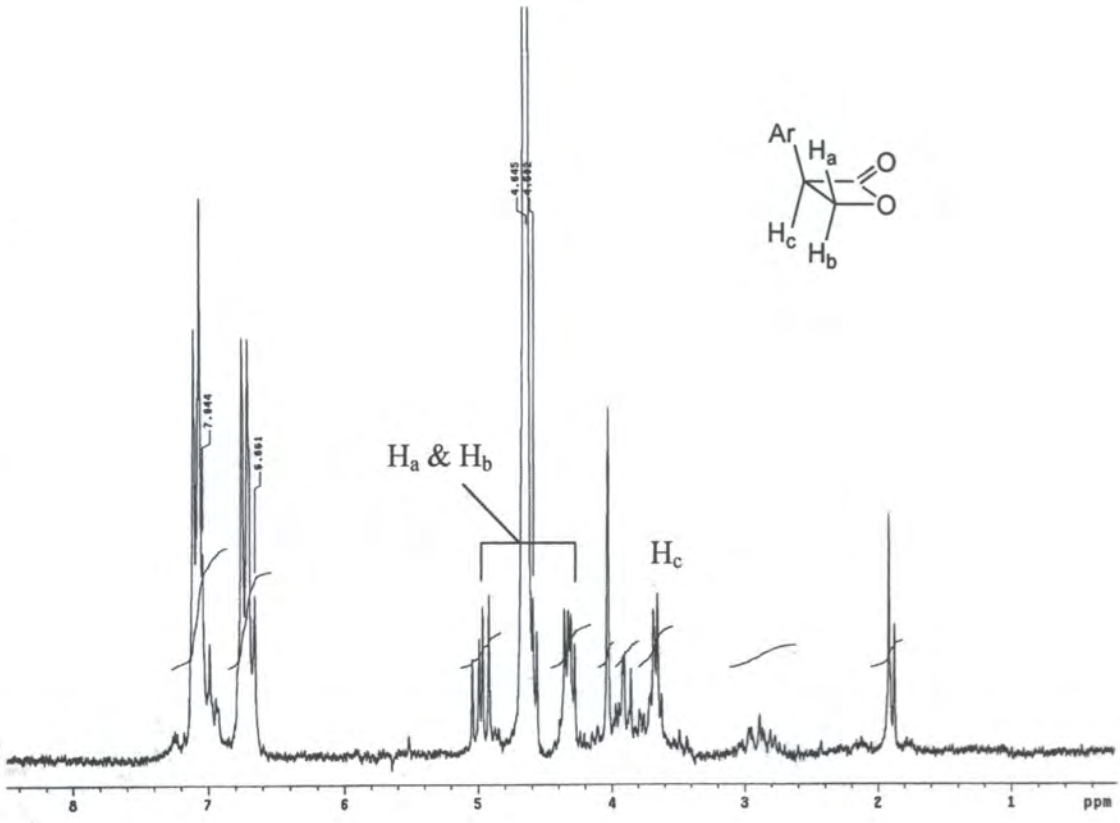


Figure 2-6 ^{1}H NMR of lactone **144**

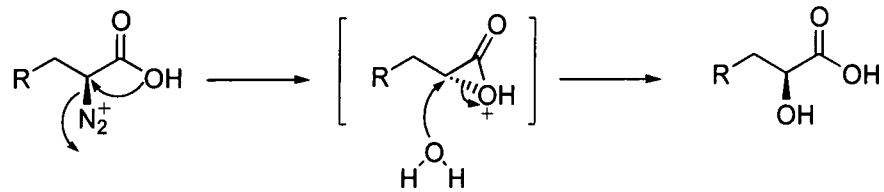
Presumably once the diazonium ion **142** has formed, an intramolecular attack of the carboxylate results in loss of nitrogen to yield the 4-membered lactone **144** (Scheme 2-41).

142 **144**

Scheme 2-41

The corresponding product, an α-lactone **145**, is a transient intermediate in α-amino acid diazotisations. However, this intermediate is short lived due to ring strain. Consequently, the α-lactone **145** is readily hydrolysed to the α-hydroxyacid **146** under aqueous acidic conditions (Scheme 2-42). This reaction proceeds with overall retention

of configuration as there are formally two inversions of configuration occurring during the process.



145 **146**

Scheme 2-42

In order to generate the desired hydroxy acid **118**, the β-lactone was stirred without purification in aqueous NaOH for 2 h. After this time ^{1}H NMR analysis showed an upfield shift, to δ 3.65 and 3.87, in the AB signal corresponding to the methylene protons (Figure 2-7) indicating that hydrolysis had occurred.

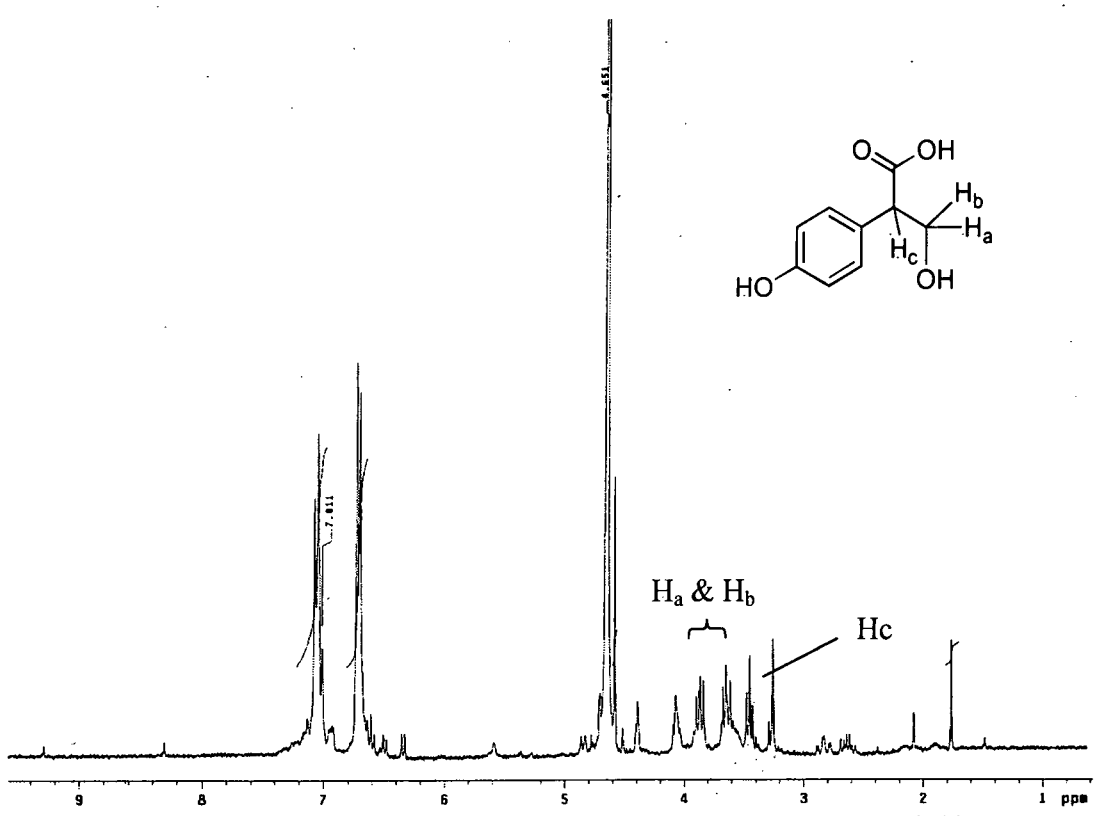
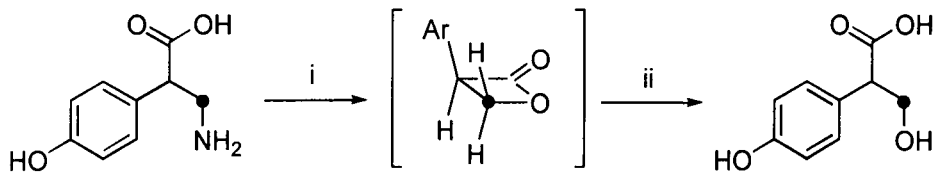


Figure 2-7 ^{1}H NMR of hydroxy acid **118**

This is consistent with ring opening and greater shielding of the protons. Further characterisation revealed that the correct product had been generated.

The synthesis was repeated with [3-^{13}C]-labelled amino acid, prepared as in Scheme 2-27, to give the corresponding ^{13}C labelled hydroxy acid (Scheme 2-43). The chemical shift of C-3 had shifted from δ 42 in the amino acid to δ 63 in the hydroxy compound.



112a **144** **118a**

Scheme 2-43 *Reagents and conditions:* i, 2M HCl, 2M $NaNO_2$, 0 °C, 3 h, then room temp., 12 h; ii, 1M NaOH, 2 h, 44 %.

2.6.6 Feeding of [3-^{13}C]-3-hydroxy-2-(4-hydroxyphenyl)propionic acid 118a to *Beauveria bassiana*

[3-^{13}C]-3-Hydroxy-2-(4-hydroxyphenyl)propionic acid **118a** was administered to *B. bassiana* at a final concentration of 3 mmol. Tenellin (31 mg) was extracted from the fungal mycelia and ^{13}C NMR spectra recorded. There was no enhancement of any peaks in the ^{13}C NMR spectrum and hence no evidence of incorporation of the administered material.

2.6.7 Summary

From the feeding studies performed with **112** and **118**, it is probable that both 3-amino-2-(4-hydroxyphenyl)propionic acid **112** and 3-hydroxy-2-(4-hydroxyphenyl)propionic acid **118** are not relevant intermediates in the early stages of tenellin biosynthesis.

However, it cannot be discounted that these labelled substrates were not able to permeate the cell membrane or were degraded before incorporation could occur. The absence of an incorporation from these two substrates together with the earlier study with 3-amino-2-phenylpropionic acid **95** indicates that the rearrangement process which occurs during tenellin biosynthesis is unlikely to involve a rearrangement similar to that occurring in tropic acid formation (littorine to hyoscyamine).

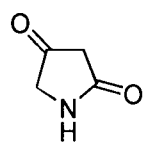
Chapter 3

3 A tetramic acid as an intermediate in tenellin biosynthesis

3.1 Introduction

3.1.1 The tetramic acids

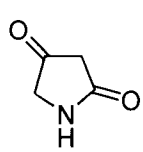
The tetramic acids are a family of structures incorporating the five-membered nitrogen containing ring **147**. They occur in many natural products and have received considerable attention due to the large number of molecules of this class that exhibit biological activity. The members of this group range from antibiotics and cytotoxins to fungicides and pigments.



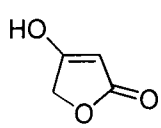
147

3.1.2 Properties of tetramic acids

Tetramic acid **147** has a pK_a = 6.4[102] and is a much weaker acid than the oxygen equivalent, tetronic acid **148**.[102] Therefore, it is less highly enolised, existing mainly in the 2,4-diketo form.



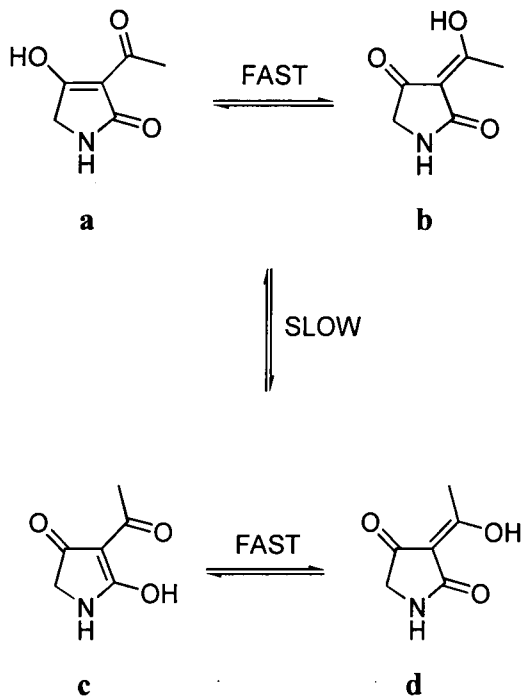
147



148

Derivatives with a 3-acyl substituent have lower pK_a values and show complete enolisation. They exist as two sets of rapidly interchanging internal tautomers (**a** and **b**),

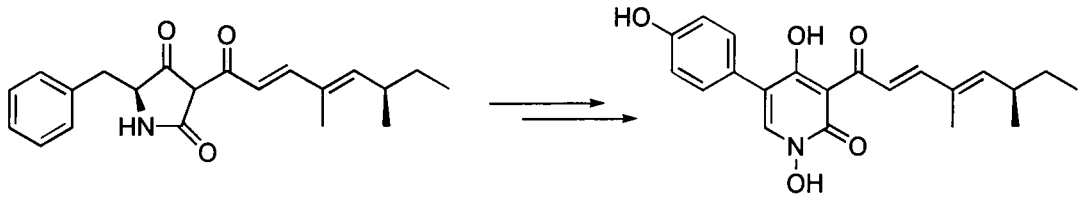
which arise from proton transfer within the molecule, and two sets of rapidly interconverting external tautomers (**c** and **d**) resulting from rotation of the side chain (Scheme 3-1).[102]



Scheme 3-1

Exchange between the two types of structures (**a b** and **c d**) is slow and can be observed on the NMR time-scale. The coupling constants and chemical shifts can be used to give information on the population of the tautomeric forms. Enolic carbons resonate at a lower frequency than the corresponding keto carbons, and hydrogen bonded carbonyl carbons at a higher frequency to free carbonyl groups.[103] The *exo*-enol **d** was found to be the most predominant tautomer[104] indicated by the presence of a lower frequency C-6 enol resonance and a C-2 hydrogen bonded carbonyl resonating at a higher frequency.

Tenellin **1** contains a 3-acylpyridone ring moiety. Vining[65] proposed that the biosynthesis of tenellin could proceed *via* tetramic acid **149** (Scheme 3-2) after condensation of phenylalanine and a polyketide fragment.

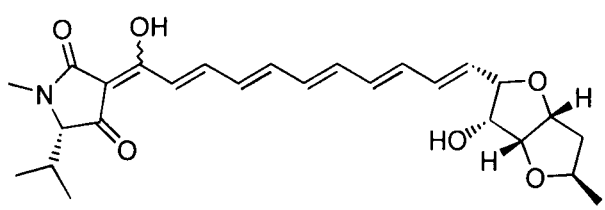


149 **1**

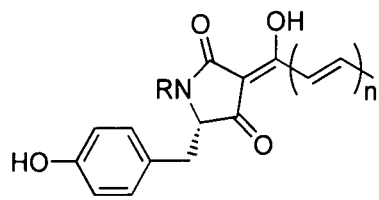
Scheme 3-2

3.1.3 Biosynthesis of the tetramic acid moiety

Tetramic acids are known to be derived from polyketides and L-amino acids. Erythroskyrine **150**, a polyenoyltetramic acid, which has antibiotic activity against some species of *Staphylococcus*, is derived from acetate, malonate and L-valine.[105] Attempts to identify the origin of the N-methyl group were inconclusive. Notably *S*-adenosylmethionine did not label this N-Me group. Several fuligorubin A related metabolites, such as the phenol substituted tetramic acids **151-153** from the slime mould *Leocarpus fragilis*,[106] are derived from L-tyrosine and an acetate generated polyketide component.



150

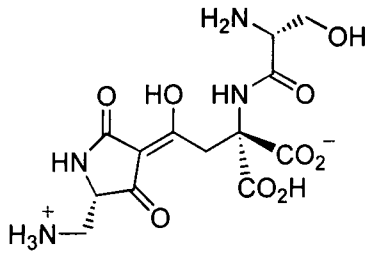


151 R = H, n = 0

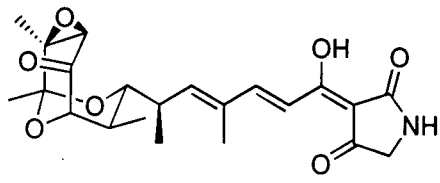
152 R = H, n = 3

153 R = CH_3, n = 3

In the antibiotic malonomicin **154**, the tetramic acid moiety is derived from one acetate unit and L-2,3-diaminopropanoic acid (L-DAP).[107] Succinic acid, carbon dioxide and L-serine contribute the side chain.



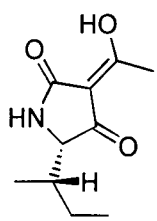
154



155

Other tetramic acid containing compounds include tirandamycin **155**,[108] which has attracted much synthetic interest due to its inhibitory action on bacterial RNA polymerase. The high activity of this compound above other 3-acyltetramic acids is thought to be a consequence of the 2,9-dioxabicyclo[3.3.1]none or the dienoyl functionality.

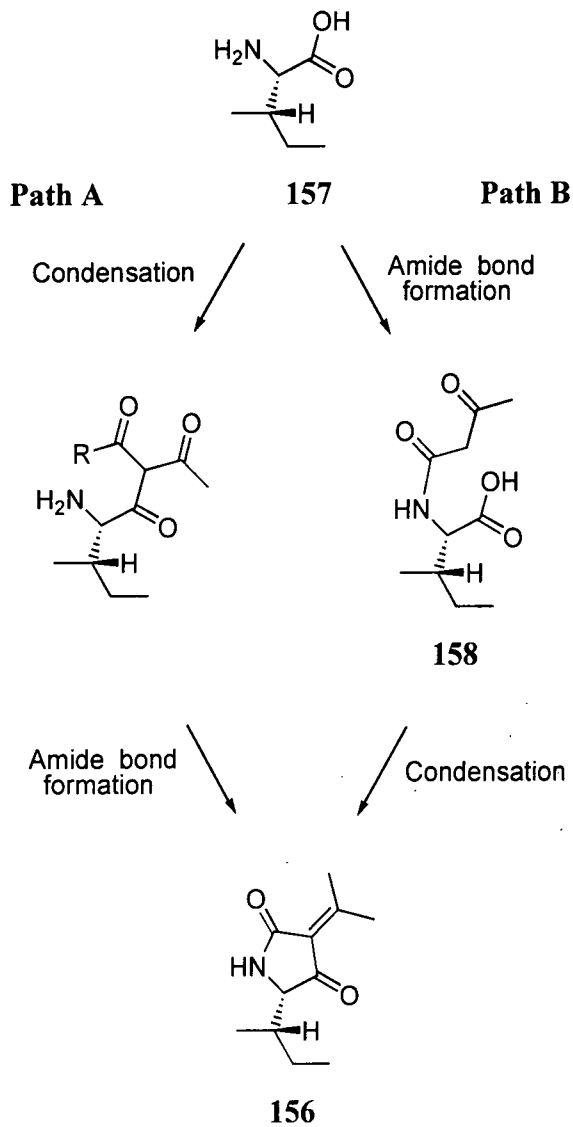
One of the simplest structures in this class is the 3-acyltetramic acid, tenuazonic acid **156**, which has been isolated from a number of fungal species.[109]



156

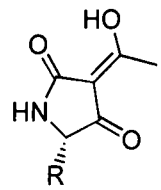
Degradation studies performed on **156**, gave L-isoleucine as product after ozonolysis and acidic hydrolysis. The authors[109] proposed that there are two potential pathways for the formation of tenuazonic acid **156** (Scheme 3-3). The first requires the condensation of the carboxyl group of L-isoleucine **157** with the α-methylene carbon of the β-ketoacid followed by ring closure as a result of amide bond formation (Path A). The second possibility is the generation of the amide bond initially, and condensation of the α-methylene carbon and carboxyl group occurring as the second step (Path B). The

trapping of N-acetoacetyl-L-isoleucine **158** indicates that the latter mechanism applies and that the initial step is the formation of the amide bond (Path B).



Scheme 3-3

Although tenuazonic acid has a wide range of biological activities,[110] its toxicity makes it of limited value. Isopropyl **159**, isobutyl **160**, and *n*-propyl **161** analogues of tenuazonic acid resulted from feeding studies with L-valine, L-leucine and L-norvaline respectively. These compounds were found to have similar, but less potent activity to tenuazonic acid itself.

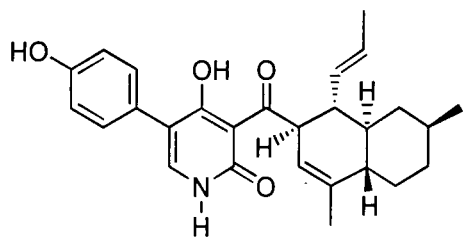


159 R= *i*-Pr,

160 R= *i*-Bu,

161 R= *n*-Pr

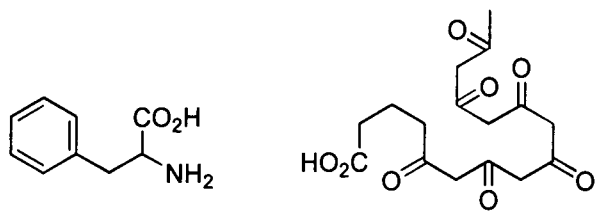
The antifungal antibiotic ilicicolin H **31** is known to derive from phenylalanine and a polyketide unit.[54] Feeding studies carried out to explore the origin of the atoms of ilicicolin H have already been discussed (Chap 2.2.2).



31

From the results obtained, it was proposed that the biosynthesis of ilicicolin H proceeds *via* a tetramic acid intermediate (Scheme 3-4) in a similar manner to that suggested for tenellin. This proposal is essentially derivative of that developed by Vining for tenellin (see Chap 3.1.4).

It was postulated that the decalin moiety of ilicicolin H **31**, formed from the condensation of a polyketide, couples with phenylalanine to afford the five-membered intermediate **162**. *Para*-oxygenation of the aromatic ring to the quinone intermediate **163** would provide the required oxygen at C-4'. A ring expansion and rearrangement would then be necessary to afford the pyridone ring structure of ilicicolin H. The results from incorporation studies with labelled acetate, methionine and phenylalanine gave a pattern consistent with such a hypothesis.



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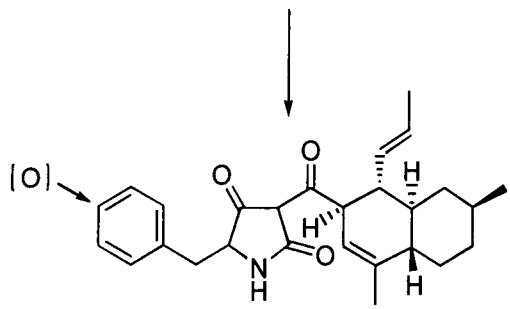
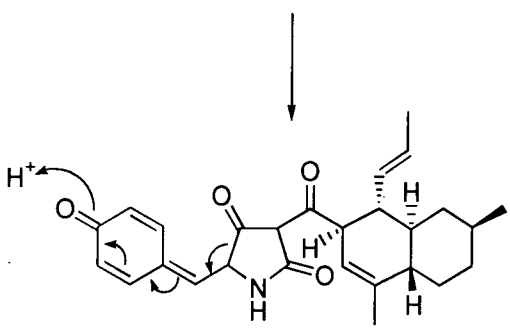


162



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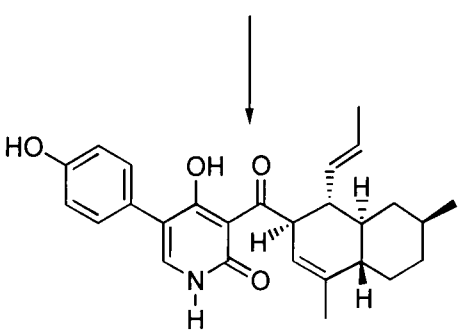
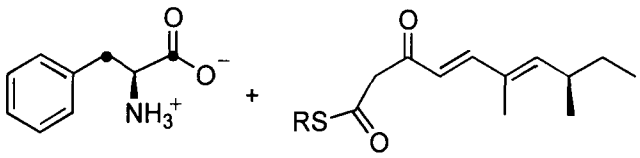


31

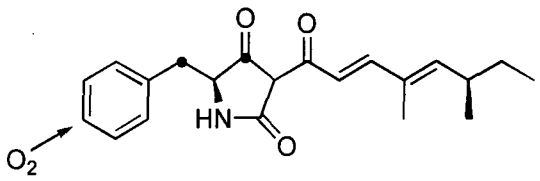
Scheme 3-4

3.1.4 Vining's hypothesis for tenellin biosynthesis

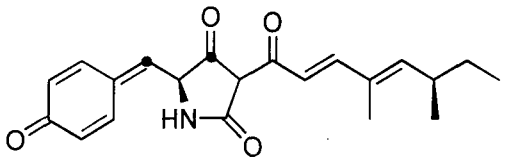
Vining proposed a hypothesis for the biosynthesis of tenellin as detailed below (Scheme 3-5).[65]



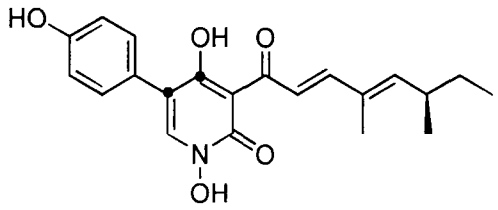
73



149



164



1

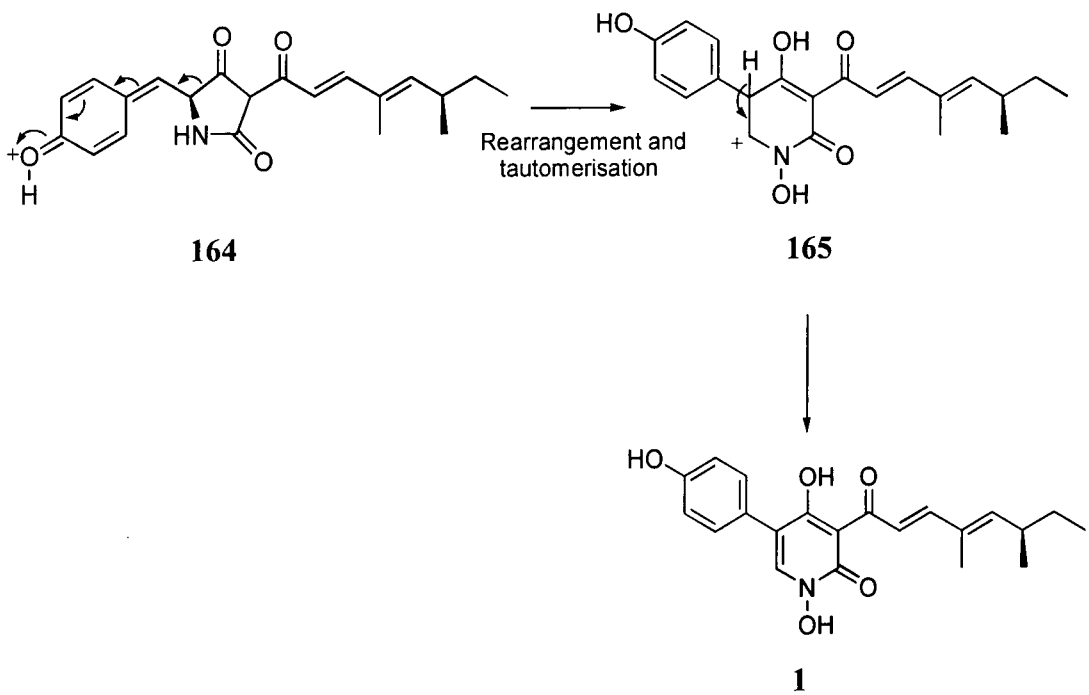
Scheme 3-5

The results from a feeding experiment using [1,3-$^{13}C_2$]-phenylalanine **73d** have previously been discussed (Chap 2.3.3). It was found that the ^{13}C labels from the administered precursor became incorporated at positions 4 and 5 in tenellin. The contiguous nature of these labels in the isolated metabolite indicates that a rearrangement of the phenylalanine has occurred.

Mechanisms that have been proposed for this hypothesis could involve either a cation or a radical process and are discussed in the next section (chap. 3.1.5)

3.1.5 Putative mechanisms for the proposed tetramic acid rearrangement in tenellin biosynthesis

The mechanism proposed by Vining[65] could proceed *via* a cationic species. *Para*-hydroxylation, by oxidation to quinone **164** primes migration of the C-4, C-5 bond yielding the dihydropyridone cation **165**. Loss of a proton from position-5 would then create the pyridone ring structure found in tenellin **1** (Scheme 3-6).

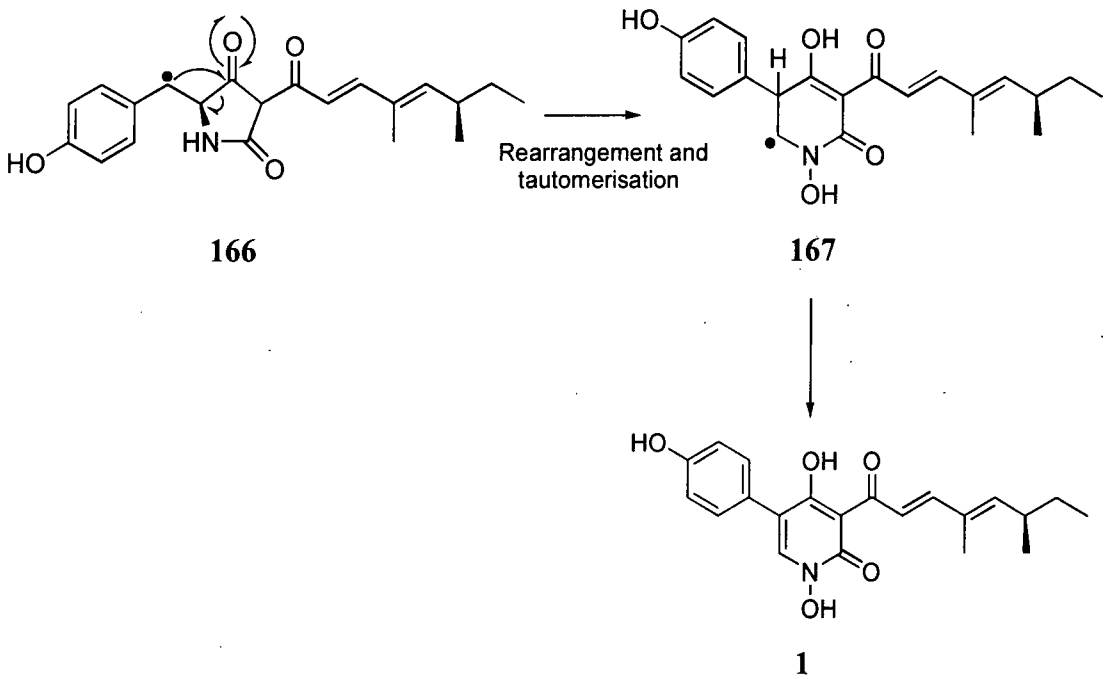


164 **165** **1**

Scheme 3-6

A weakness with this hypothesis is the formation of the unstabilised cation **165**. The lone pair of electrons on nitrogen is associated with the amide bond and will not obviously be able to stabilise the adjacent positive charge.

An alternative hypothesis for this rearrangement proposes a radical mechanism involving a one electron oxidation (Scheme 3-7).[68] The radical **166** is possibly more favourable than the cation **165** as stability could be derived from the aromatic ring. Attack of the radical at the carbonyl carbon followed by bond cleavage and rearrangement could yield the dihydropyridone radical **167**. Loss of a hydrogen atom from this species could then generate tenellin **1**.

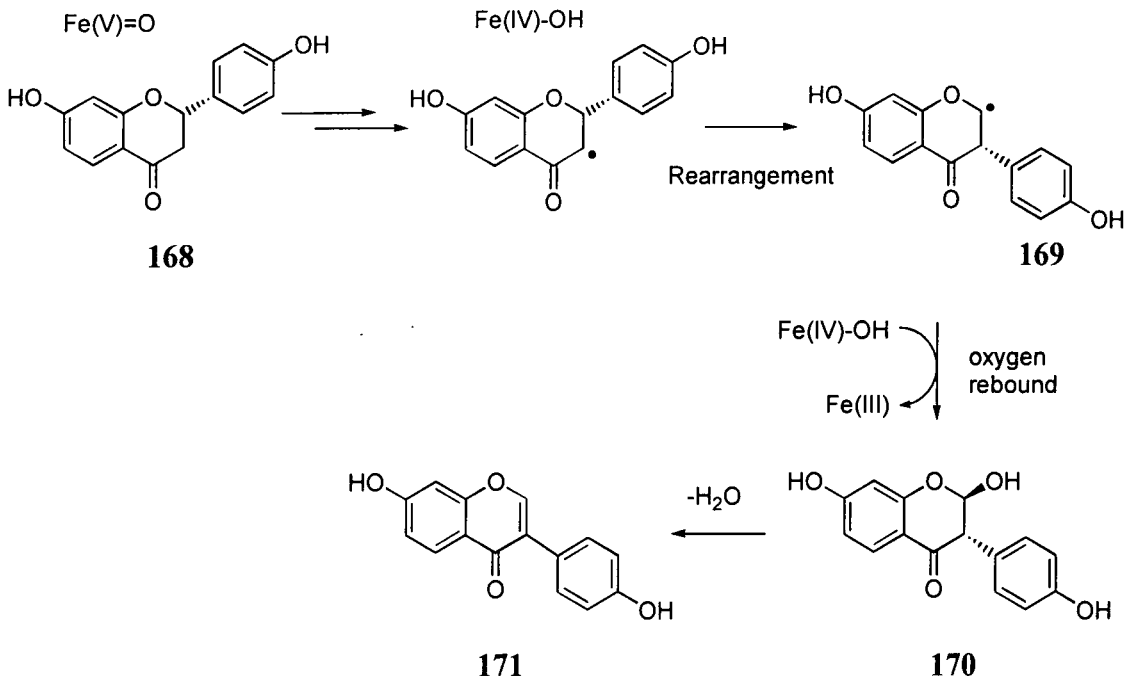


166 **167** **1**

Scheme 3-7

3.1.6 P_{450} mediated rearrangements

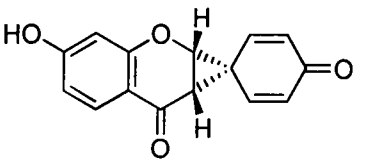
There may be some similarity between the mechanism of tenellin formation and the P_{450} mediated rearrangement of flavanones **168** to isoflavones **171**. 1,2-Aryl migration in this system is known to be initiated by isoflavone synthase, a P_{450} iron containing enzyme, which requires dioxygen and NADPH. An oxygen rebound mechanism has been proposed (Scheme 3-8)[111] whereby radical **169** is quenched by an hydroxyl radical from Fe(IV)-OH. A dehydratase then generates the isoflavanone **171**. Cell free extract studies showed that the hydroxyl group in **170** was labelled from $^{18}O_2$.



168 **169** **171** **170**

Scheme 3-8

Other researchers have suggested a different mechanism, which accounts for the presence of the free *p*-hydroxyl group in the migratory aromatic ring. In experiments with *p*-methoxylated substrates, it was found that demethylation of the ether was required before the rearrangement would occur. On these suppositions a new intermediate, spirodienone **172**, was postulated.[112]



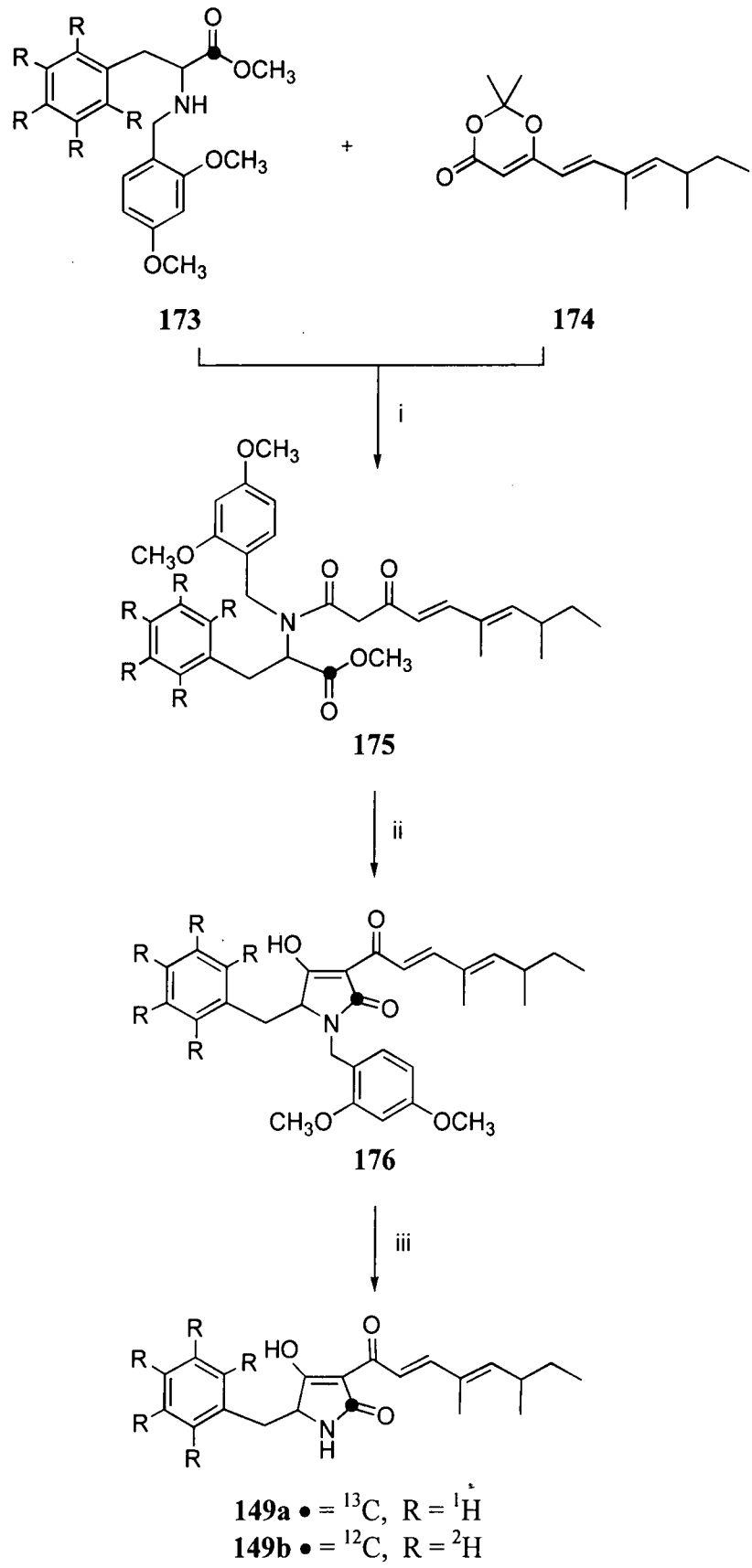
172

An oxygen rebound mechanism remains most probable with an Fe(III)-OH species attacking the spirodienone to give the corresponding hydroxyl compound and Fe(III), which can then be reactivated after the binding of another substrate molecule.

3.1.7 Testing of Vining's hypothesis

The validity of the hypothesis proposed by Vining[65] was tested in Durham by incorporation experiments with labelled tetramic acid **149**.[80] The synthesis (Scheme 3-9) of this compound involved the coupling of the protected phenylalanine **173** with an acetonide **174** to give the β-ketoamide **175**, which could then be cyclised under Dieckmann conditions to the tetramic acid **176**. Straightforward N-deprotection yielded the desired product **149** as a mixture of diastereoisomers.

[2-^{13}C]-Tetramic acid **149a** was then fed to cultures of *Beauveria bassiana* over several days. There was no evidence by ^{13}C NMR of any incorporation into C-4 of the isolated tenellin. In order to increase the sensitivity of the analysis by mass spectrometry, [*phenyl*-$^{2}H_5$]-tetramic acid **149b** was also prepared and administered to the fungus. Again, there was no detectable incorporation into tenellin. It was therefore concluded that tetramic acid **149** is not an intermediate on the biosynthetic pathway of tenellin.

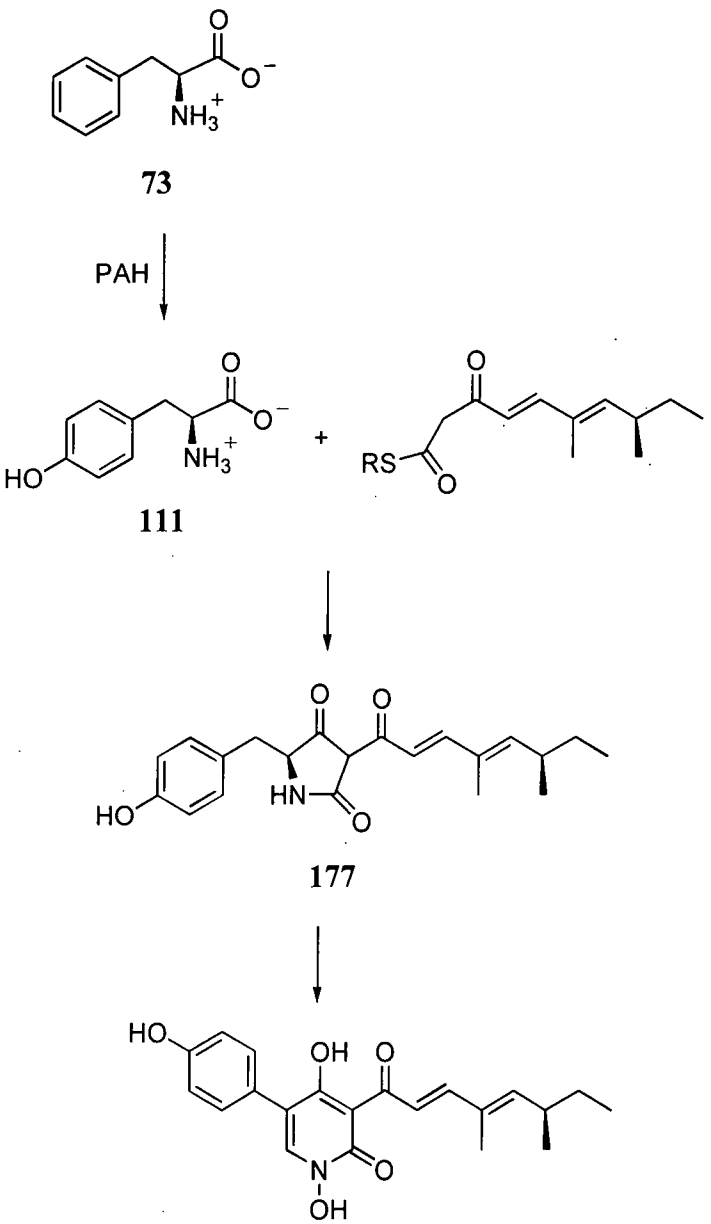


Scheme 3-9 *Reagents and conditions*: i, toluene, PPTS, reflux, 3h, 42 %; ii, *t*BuOK, *t*BuOH, 30 min, 92 %; iii, TFA, 5 min, 92 %.

These experiments were performed prior to the more contemporary discovery that tyrosine is an equally efficient precursor to tenellin as phenylalanine (see chapter 2.5.1). It is now concluded that the first step in the biosynthesis of tenellin is *para*-hydroxylation of the aromatic ring of phenylalanine, which therefore infers the *para*-hydroxylation of all ensuing substrates on the pathway, including tetramic acid **149**. Therefore in retrospect, it is not surprising that tetramic acid **149** is not accepted as an intermediate in tenellin biosynthesis. Tetramic acid **177** now emerges as the more relevant intermediate for study.

3.1.8 New tetramic acid hypothesis

Vining's hypothesis outlined in section 3.1.4 can now be modified to account for the new role of tyrosine **111** in the biosynthesis of tenellin. The revised hypothesis is as shown in Scheme 3-10. In the first step, phenylalanine **73** is hydroxylated by a phenylalanine hydroxylase to yield tyrosine **111**, which can then undergo condensation with a polyketide moiety to generate the tetramic acid derivative **177**. Ring expansion and rearrangement would then afford tenellin **1** (section 3.1.5).



Scheme 3-10

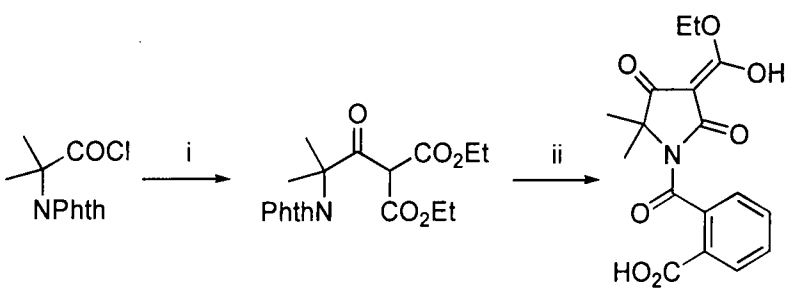
The tetramic acid **177** became a key synthetic target of this project in order to conduct a feeding study to test this modified hypothesis. Clearly, a synthesis amenable to isotopic labelling had to be developed.

A similar general strategy to that utilised for the synthesis of **149** was adopted.

3.2 Synthesis of tetramic acid 177

3.2.1 General synthetic methods towards tetramic acids

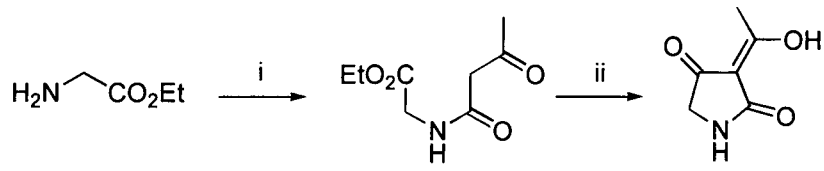
The first tetramic acid synthesis was completed by Gabriel[113] in 1914. Diethyl sodiomalonate was reacted with phthalimidoisobutyryl chloride **178** to give product **179** which cyclised on treatment with acid to the 3-ethoxycarbonyl tetramic acid **180** (Scheme 3-11).



178 **179** **180**

Scheme 3-11 *Reagents and conditions*: i, diethyl sodiomalonate; ii, conc. H_2SO_4.

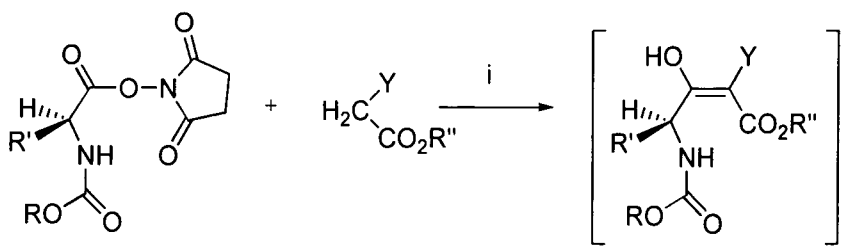
A popular technique for tetramic acid preparation involves Dieckmann cyclisation of an N-acetoacylated amino acid **182**, formed from an amino acid ester **181** and diketene, as shown in Scheme 3-12.[114] This is a particularly useful method for accessing 3-acyl derivatives, such as **183**, as it is advantageous to form the highly polar heterocycle towards the end of a synthetic route due to the difficulties associated with the manipulation and purification of these compounds.



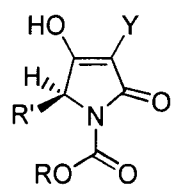
181 **182** **183**

Scheme 3-12 *Reagents and conditions*: i, diketene; ii, $NaOCH_3$.

A cyclisation procedure involving N-hydroxysuccinimide esters of N-protected amino acids **184** (Scheme 3-13) has also been reported for the synthesis of 3-substituted tetramic acids **186**.[115] Under basic conditions, the esters undergo *in situ* cyclisation after C-acylation with active methylene compounds **185**. Optically active tetramic acids have been obtained from L-amino acid precursors.



184 **185**



186

Y = $COCH_3$, COPr, COPh

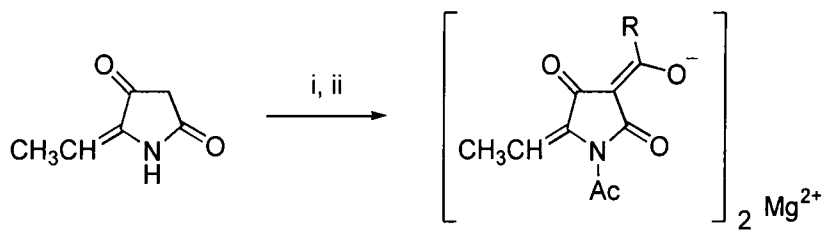
CO_2CH_3, CO_2Et, SO_2CH_3

R = Bn, $(CH_3)_3C$

R' = H, CH_3 R'' = CH_3, Et

Scheme 3-13 *Reagents and conditions*: i, NaH, benzene, room temp.

In the previous examples, it can be seen that elaboration of the molecule is performed initially and cyclisation occurs as the final step. However, it is also possible to form the 3-substituted products by acylation after formation of the heterocyclic ring. Kohl[116] was the first to attempt this strategy in the synthesis of magnesidin (**188**, **189**), a magnesium containing antibiotic. Acylation was achieved by reaction of the acyltetramic acid core **187** with an acyl chloride and boron trifluoride-etherate (Scheme 3-14). Unfortunately, loss of the product during the basic work-up led to low yields.



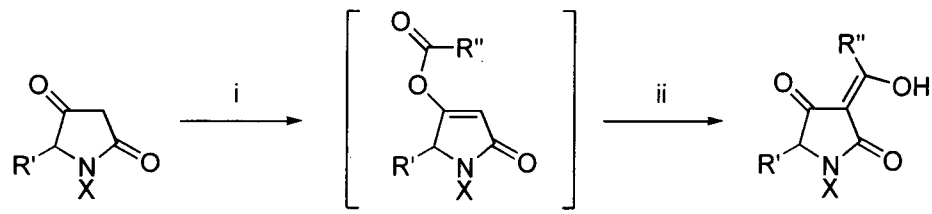
187 **188** R = $(CH_2)_4CH_3$

189 R = $(CH_2)_6CH_3$

Scheme 3-14 *Reagents and conditions*: i, RCOCl, $BF_3.OEt_2$; ii, Ac_2O, NaOAc, $MgCl_2$.

This methodology was investigated further by Jones[117] who improved the yield by introducing a neutral work-up procedure. The products were isolated as the boron difluoride complexes, which could then be released with methanol to give the 3-acyltetramic acids in 50-78 % yield. Titanium tetrachloride could also be used but poorer yields were obtained.

Yoshii[118] described an interesting reaction in which 3-acyltetramic acids were synthesised from the corresponding 4-O-esters (Scheme 3-15). Exposure of the O-acylated tetramic acids **190** to base, initiated rearrangement to the 3-substituted products **191**.



190 **191**

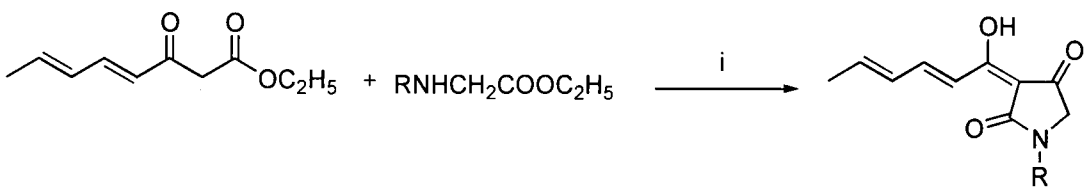
R' = H, ^{i}Pr, Bn

R''= ^{i}Pr, Bn, CH=CHCH=CHCH$_3$

X = H, Bn

Scheme 3-15 *Reagents and conditions*: i, R''CO_2H, DCC, DMAP; ii, Et_3N.

The syntheses of streptolydigin and tirandamycin **155** derivatives[119] have been performed by methods involving the cyclisation of the tetramic acid ring both prior and subsequent to the elaboration of the 3-acyl side chain. Low product yields were obtained from the route involving the acylation of unsubstituted 2,4-pyrrolidiones, so the cyclisation of β-ketoamides *via* an intramolecular Claisen condensation is preferred. Amino acid esters **193** were condensed with dienoylacetyl esters, for example **192**, produced from the corresponding dienoic acids, to give the 3-dienoyl tetramic acid derivatives **194** in moderate yield (Scheme 3-16).



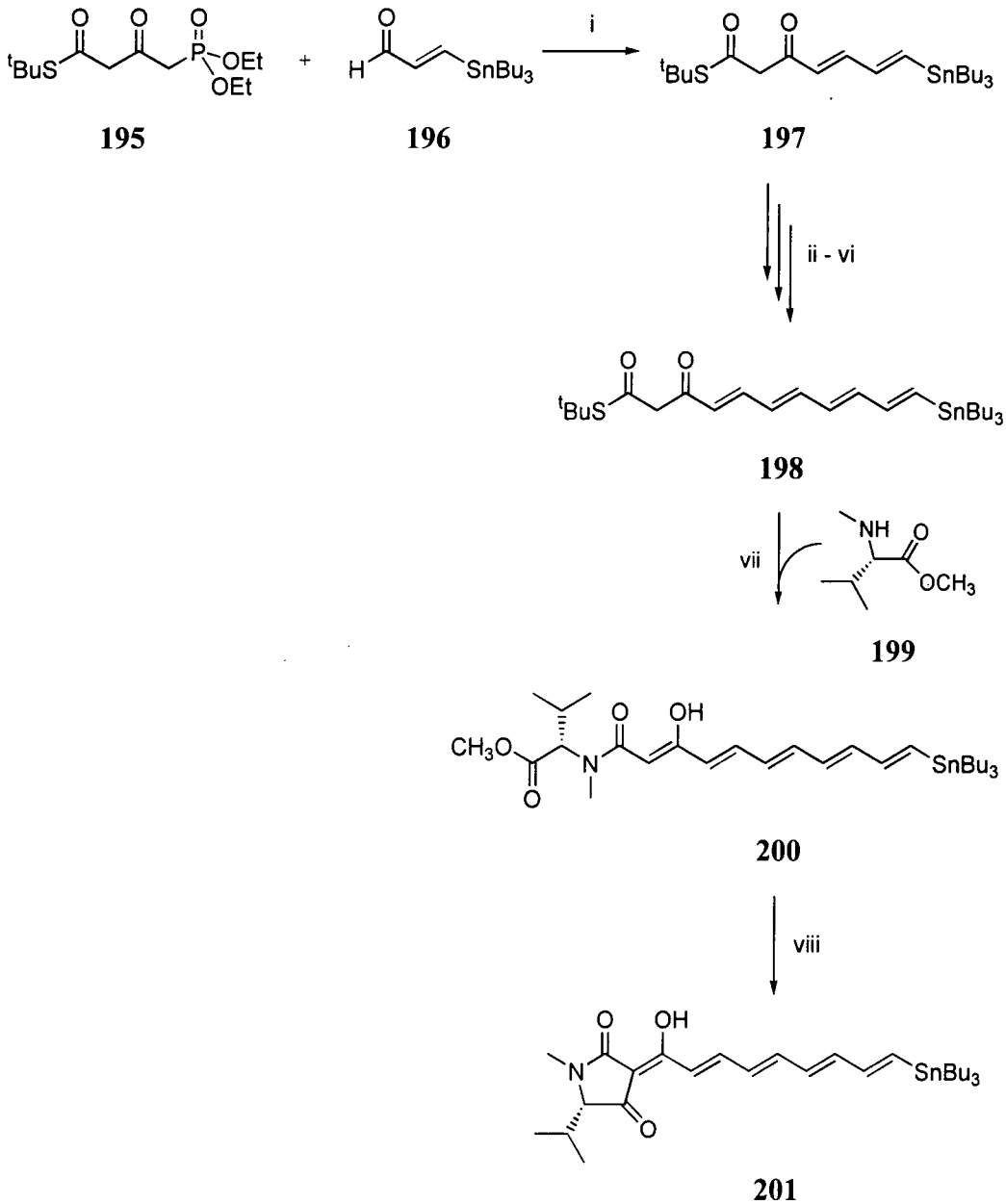
192 **193** **194**

R = $CH_2C_6H_5$, cyclohexyl

Scheme 3-16 *Reagents and conditions*: i, NaOEt or NaOMe.

The synthesis of the tetramic moiety of erythroskyrine **150**[120] was completed from N-methyl (*S*)-valine methyl ester **199** and a functionalised *t*-butyl thioester **198** (Scheme 3-17). The reaction between phosphonate ester **195** and aldehyde **196** produced diene **197** in >30:1, *E*:*Z* selectivity, which then underwent reduction with sodium borohydride and dehydration. A subsequent diisobutylaluminium hydride (DIBALH) reduction and Horner-Wadsworth-Emmons type reaction with phosphonate ester **195** yielded the

thioester **198**. Coupling with the valine derivative **199** and cyclisation under Lacey-Dieckmann conditions gave the tetramic acid **201**, which was found to exist as a mixture of enol forms in a 4:1 ratio.

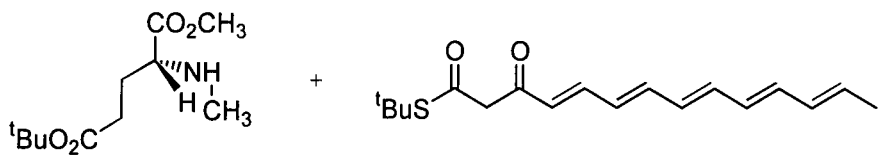


195 **196** **197** **198** **199** **200** **201**

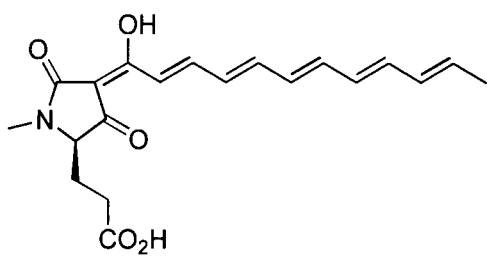
Scheme 3-17 *Reagents and conditions*: i, *t*-BuOK, THF, room temp. then **196**, -78 °C to 0 °C, 30 min; ii, $NaBH_4$, MeOH-*i*PrOH, -10 °C to -5 °C over 15 min; iii, Ac_2O, DMAP, DCM, room temp., 10 min; iv, DBU, THF, -30 °C to room temp. over 2 h; v, DIBALH, toluene, -78 °C, 10 min; vi, **195**, *t*-BuOK, THF, room temp. then aldehyde, -78 °C to 0 °C, 30 min; vii, **199**.HCl, Et_3N, CF_3COOAg, THF, 0 °C, 40 min; viii, CH_3ONa, MeOH, 25 °C, 90 s.

A Stille[121] coupling between the tetramic acid **201** and a suitably protected furan derivative, followed by subsequent deprotection in formic acid yielded the synthetic erythroskyrine **150**, which gave analytical data in good accordance with the natural product.

In a similar manner, the synthesis of fuligorubin A **32** was achieved (Scheme 3-18).[122] Condensation of the (*R*)-glutamic acid derivative **202** and a suitable polyene β-ketothioester **203** in the presence of silver(I) trifluoroacetate followed by cyclisation with potassium *t*-butoxide and deprotection with formic acid gave enantiomerically pure fuligurobin A **32**.



202 **203**

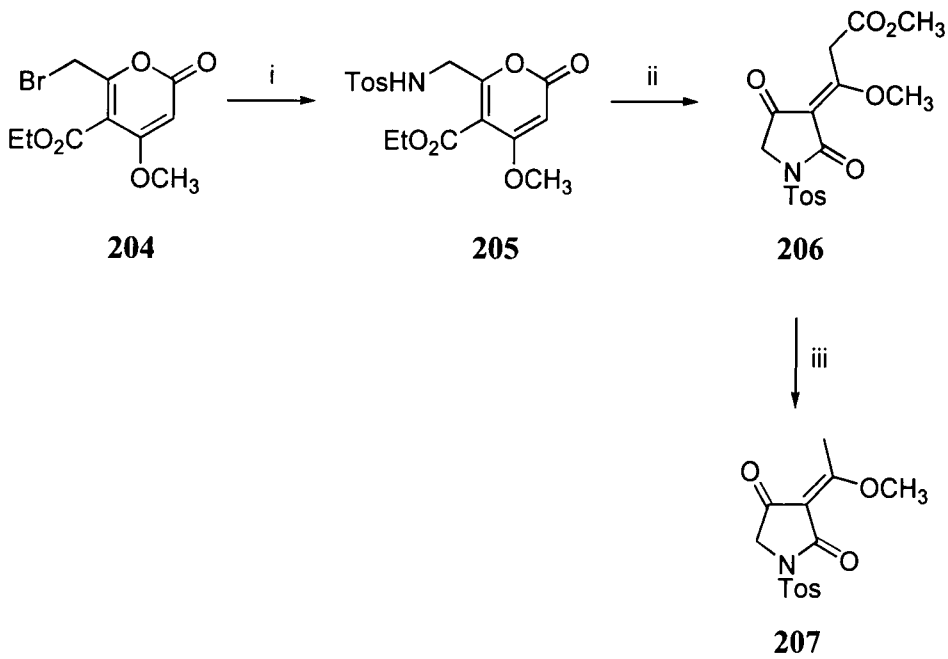


32

Scheme 3-18

The conversion of pyrones to tetramic acids has been achieved by reaction of halo-derivatives with sodium *p*-toluenesulfonamide and sodium methoxide (Scheme 3-19).[123] 6-Bromomethylpyrone **204** was treated with sodium *p*-toluenesulfonamide in tetrahydrofuran (THF) to generate sulfonamide **205**, which underwent fission of the pyrone ring and accompanying ring closure of the nitrogen heterocycle to give **206**. It is thought that the opening of the pyrone ring removes any steric constraints that could

hinder formation of the pyrrolidone ring. Base induced ester hydrolysis followed by decarboxylation in refluxing toluene gave the 3-acyltetramic acid **207**, which was found to exist in solution as the dienolic tautomer.

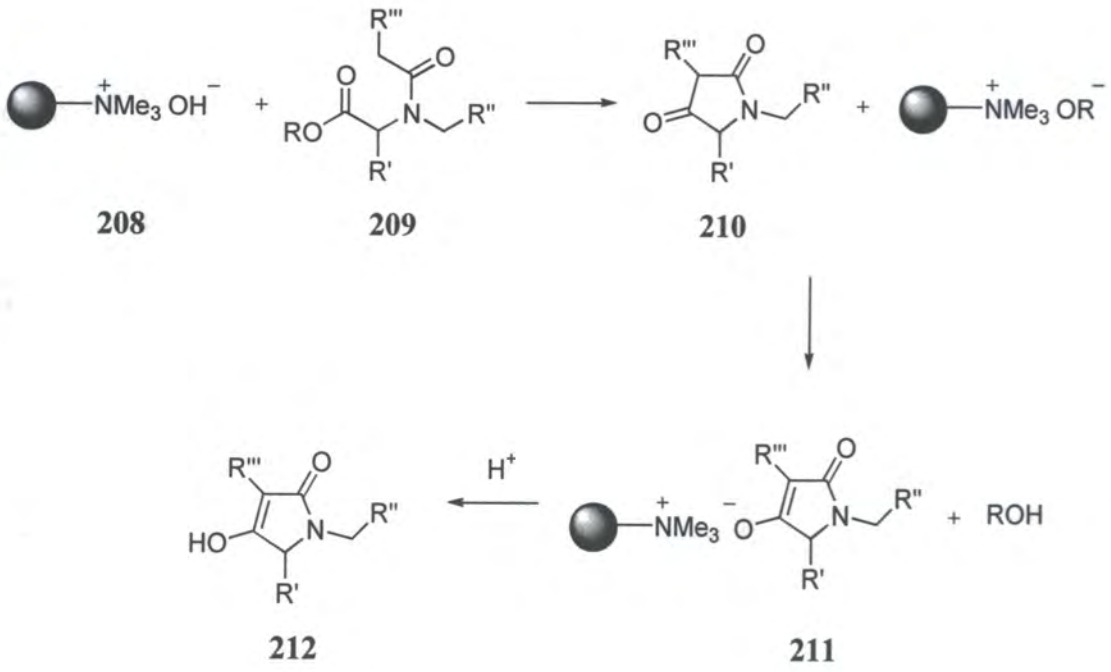


204 **205** **206** **207**

Scheme 3-19 *Reagents and conditions*: i, NaNHTos, THF, 20 °C; ii, NaOMe, MeOH, reflux; iii, NaOH (aq), 25 °C.

It is also possible to introduce the nitrogen atom using sodium azide. This yields an azidomethylpyrone intermediate, which can be reduced to an aminomethylpyrone by hydrogenation.[123] Protection as the tosylate gives **205**, which can then be treated in the same way as described above in order to deliver **207**.

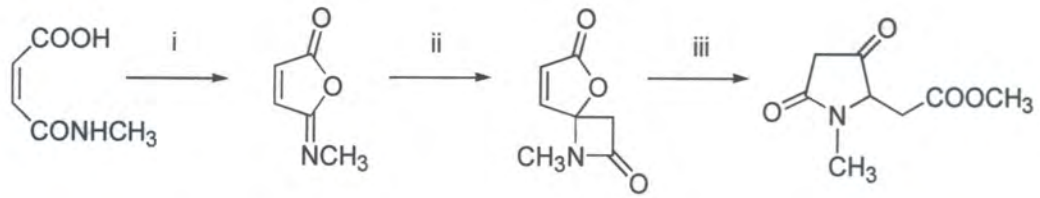
Combinatorial methods have also contributed to the wide range of tetramic acid syntheses (Scheme 3-20).[124] An Amberlyst resin (OH^- form) **208** was found to promote the cyclisation of acylated amino acid derivatives **209** to the corresponding pyrrolidinediones **210**. A particular advantage of this method is that the cyclised compounds remain tightly bound to the resin enabling other chemicals present to be washed away. Stirring of **211** in acid removed the bound material to give the free tetramic acid **212,** which was collected by filtrate evaporation.



208 209 210 212 211

Scheme 3-20

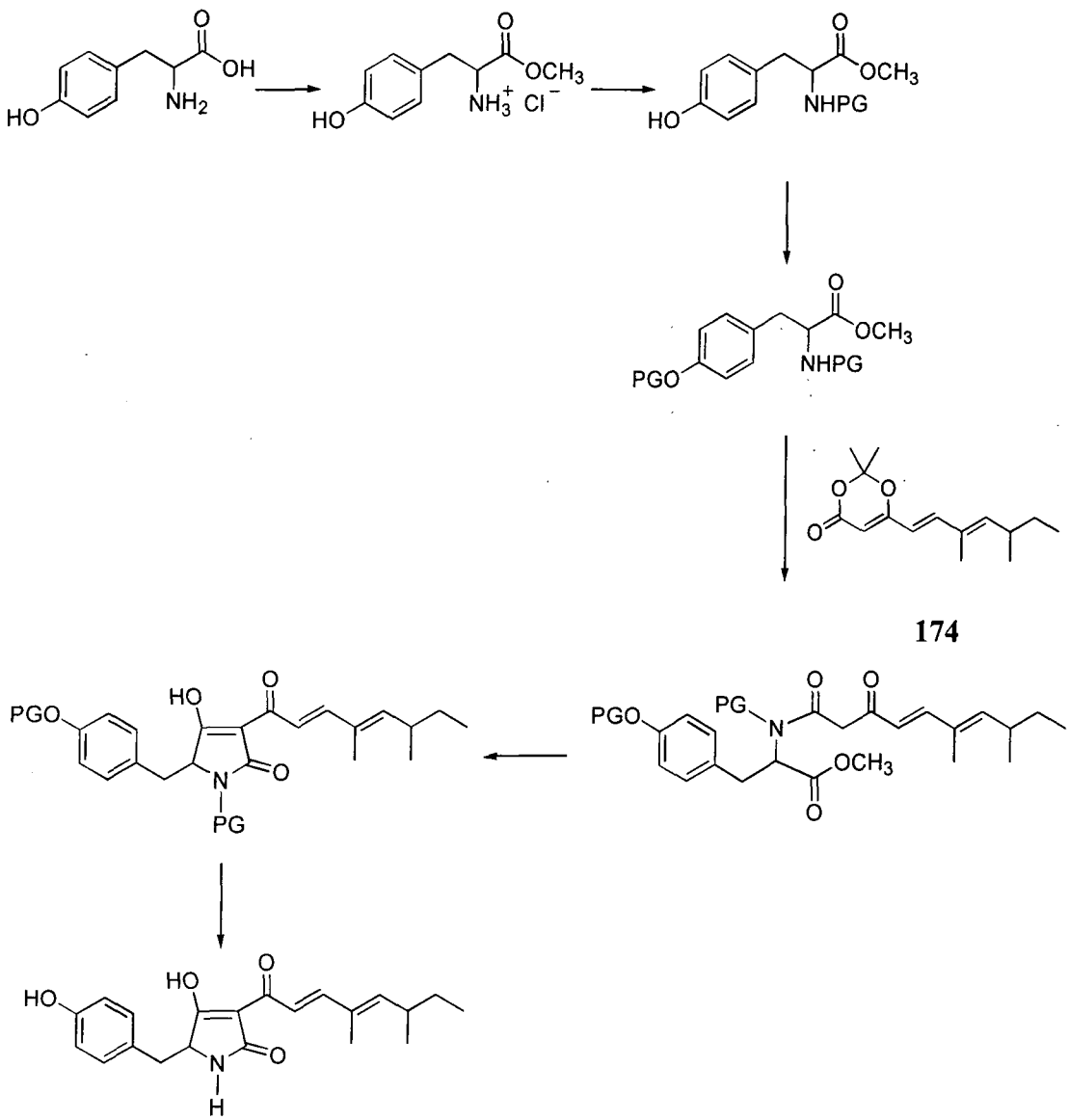
Other methods to tetramic acids include intramolecular Wittig reactions of γ-acylphosphonium ylides,[125] rearrangement of spiro β-lactams (Scheme 3-21)[126] and radical cyclisation of propargyl bromoamides.[127]



Scheme 3-21 *Reagents and conditions*: i, DCC, DCM; ii, CH_2=C=O, Ac_2O, 50 °C; iii, $NaOCH_3$, CH_3OH, reflux, 16 h.

3.2.2 Proposed synthetic route to tetramic acid 177

The route proposed for the synthesis of tetramic acid derivative **177** is outlined below (Scheme 3-22). This follows a common strategy towards the formation of this class of compound and has been used in the preparation of many 2,4-pyrrolidinedione ring systems. It involves the condensation of a protected α-amino acid and in this case the diketene derivative **174**[128] to give a β-ketoamine, which would then undergo cyclisation to the tetramic acid **177**.



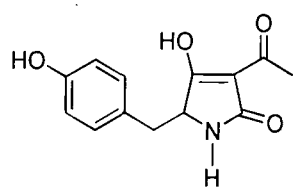
174

177 PG = protecting group

Scheme 3-22

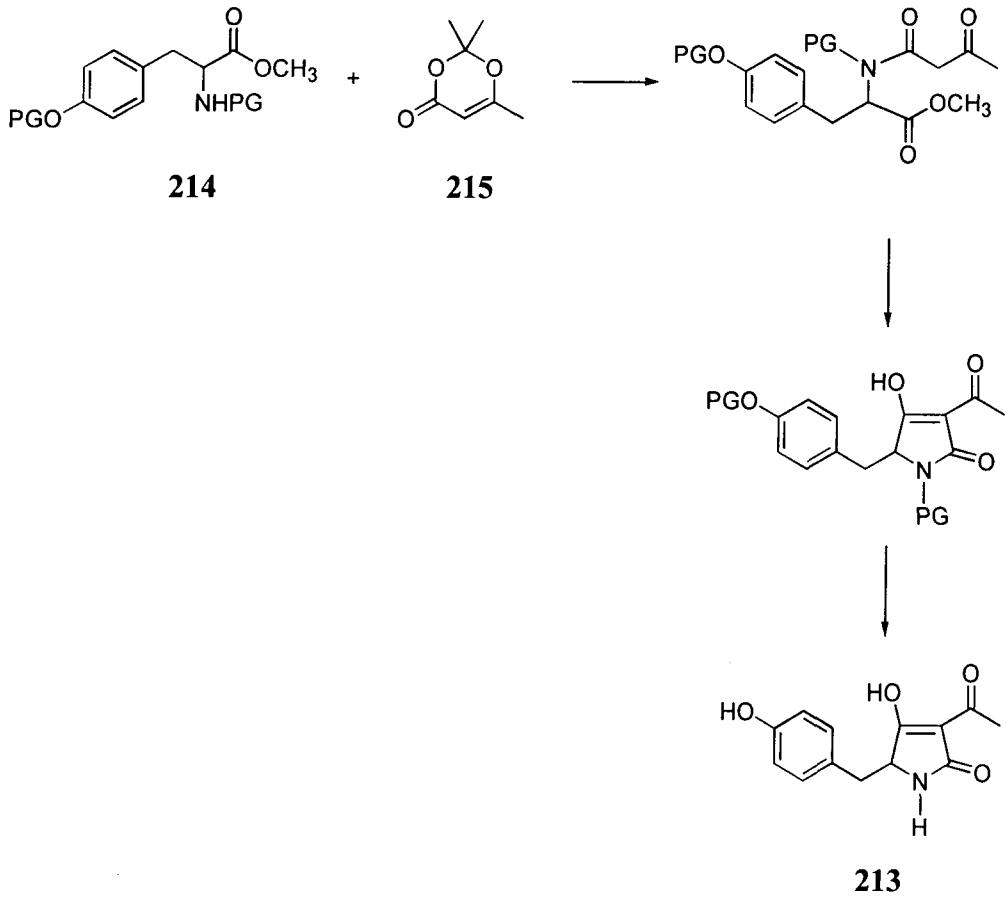
3.2.3 Protection strategy

An initial objective of the synthesis was to select suitable protecting groups for the route. These groups were required to be stable to all steps in the reaction sequence as well as being easily removed under mild conditions at the end of the synthesis. To test the suitability of different protecting groups a model system was selected. The tetramic acid derivative **213**, with a truncated acyl moiety containing a 3-acetyl group, emerged as an appropriate model target.



213

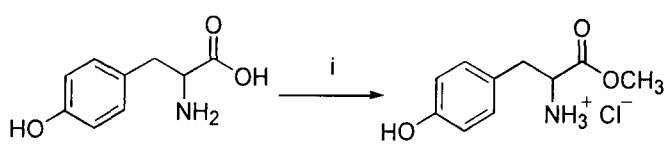
It was surmised that the absence of the polyketide moiety should not affect the chemistry at the nitrogen and phenolic oxygen centres. The synthesis of **213** was achieved in a similar manner to that shown in Scheme 3-22, by the reaction of the protected amino acid derivative **214** with 2,2,6-trimethyl-4H-1,3-dioxin-4-one **215** (Scheme 3-23).



214 **215** **213**

Scheme 3-23

In the first step, tyrosine **111** was readily converted to the methyl ester hydrochloride **216** by refluxing in methanol and thionyl chloride (Scheme 3-24).[129] After cooling, the addition of diethyl ether led to precipitation of the product, which could be collected by filtration to give **216** as a white powder in high yield.

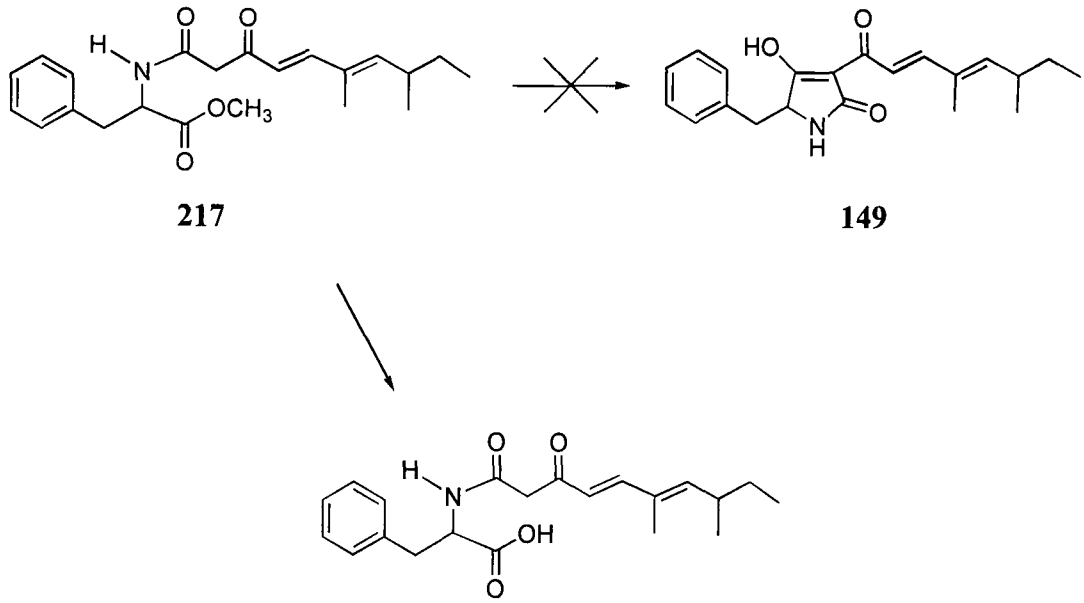


111 **216**

Scheme 3-24 Reagents and conditions: i, $SOCl_2$, MeOH, reflux, 3 h, 97 %.

The next step involved protection of the amine. It has been shown that short chain N-acylated derivatives can be cyclised to the corresponding tetramic acids without the use of a nitrogen protecting group. However, during the previous synthesis of tetramic acid

149,[80] the N-unprotected longer chain derivative **217** was found to undergo ester hydrolysis to **218** rather than cyclisation as expected (Scheme 3-25). For this series, cyclisation appears to demand nitrogen protection.

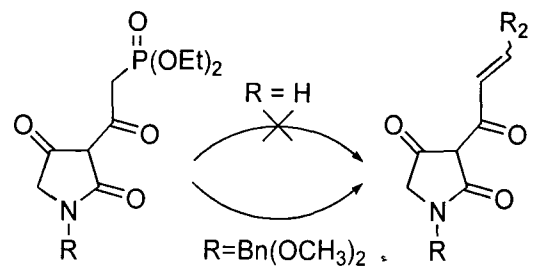


217 **149**

218

Scheme 3-25

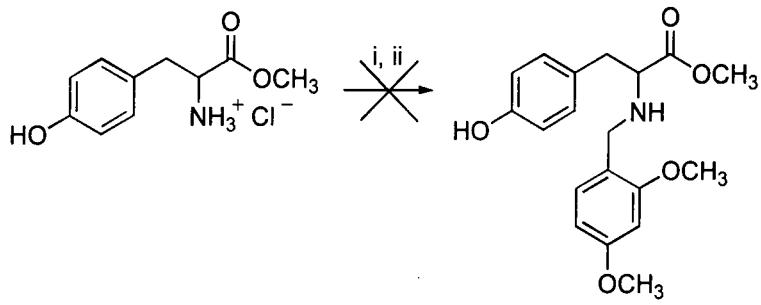
Similarly, during the formation of tetramic acids reported by DeShong *et al.*,[130] it was found that the condensation of phosphonate **219** derived anions with aldehydes was unsuccessful when the nitrogen was unprotected. N-benzyl derivatives readily underwent reaction with an appropriate aldehyde and potassium *t*-butoxide to afford the N-alkyl tetramic acids **220** (Scheme 3-26).



219 **220**

Scheme 3-26

Several syntheses of tetramic acids have reported the use of a 2,4-dimethoxybenzyl group for nitrogen protection.[131,132] Reaction of the amino nitrogen with 2,4-dimethoxybenzaldehyde forms an imine which can be reduced by sodium cyanoborohydride to the desired amine. Attempted reactions to generate **221** from tyrosine methyl ester hydrochloride **216** under these conditions were unsuccessful (Scheme 3-27).



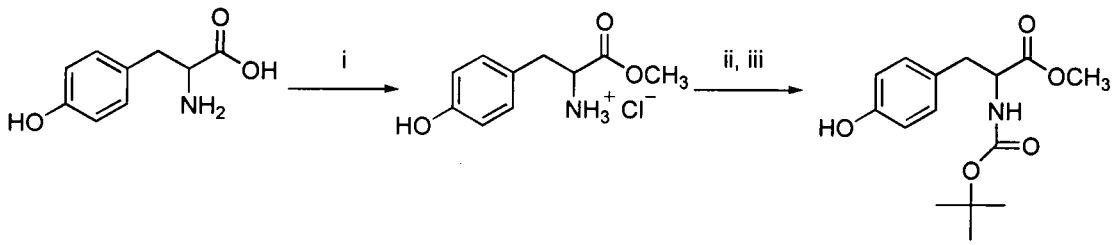
216 **221**

Scheme 3-27 *Reagents and conditions*: i, 2,4-dimethoxybenzaldehyde, MeOH, room temp., 30 min; ii, $NaCNBH_3$, room temp., 16 h, then HCl (aq).

This method was abandoned and other N-protecting groups were explored.

3.2.4 N-Boc-O-2,6-dichlorobenzyl protection

t-Butoxycarbonyl (Boc) is a common protecting group for amino acids,[133] particularly in the synthesis of peptides. Addition of di-*t*-butyl dicarbonate in *t*-butanol[134] to the free base of tyrosine methyl ester, generated the N-Boc derivative **222** in high yield (Scheme 3-28).

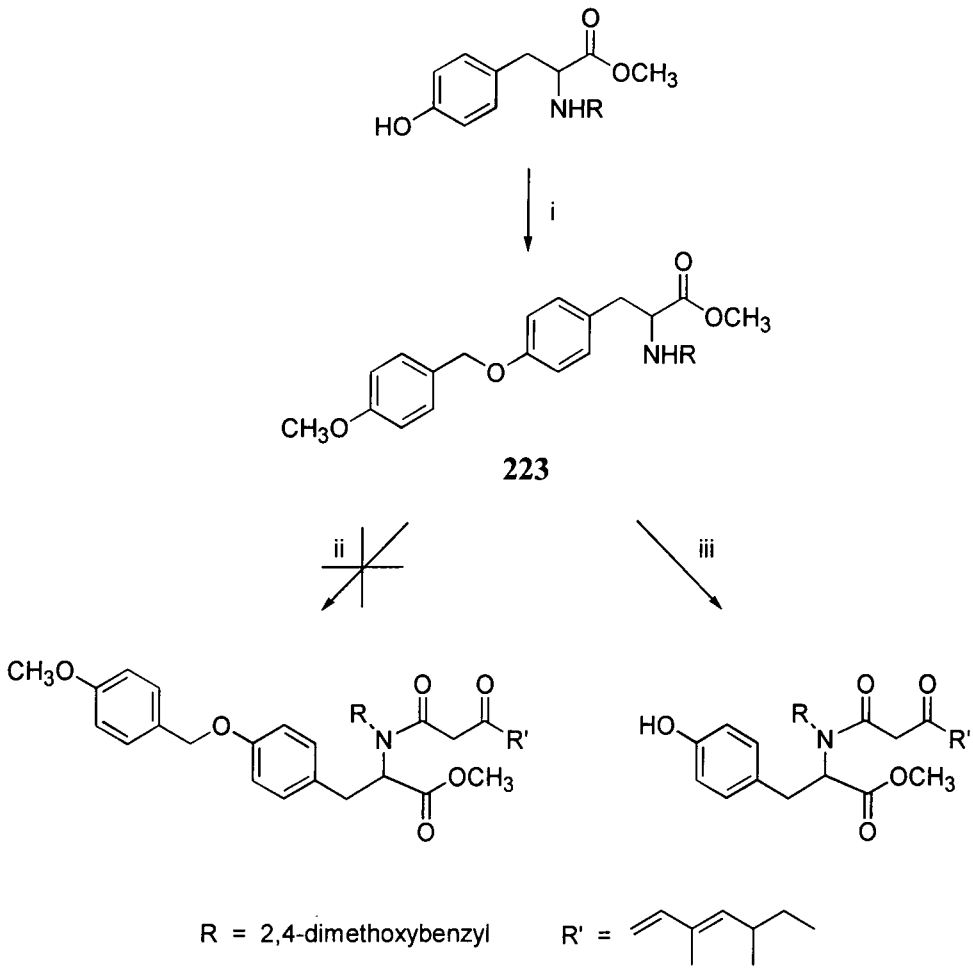


111 **216** **222**

Scheme 3-28 *Reagents and conditions*: i, $SOCl_2$, MeOH, reflux, 3 h, 97 %; ii, K_2CO_3 (aq); iii, $(Boc)_2O$, *t*-BuOH, Et_2O, room temp, 30 min, 92 %.

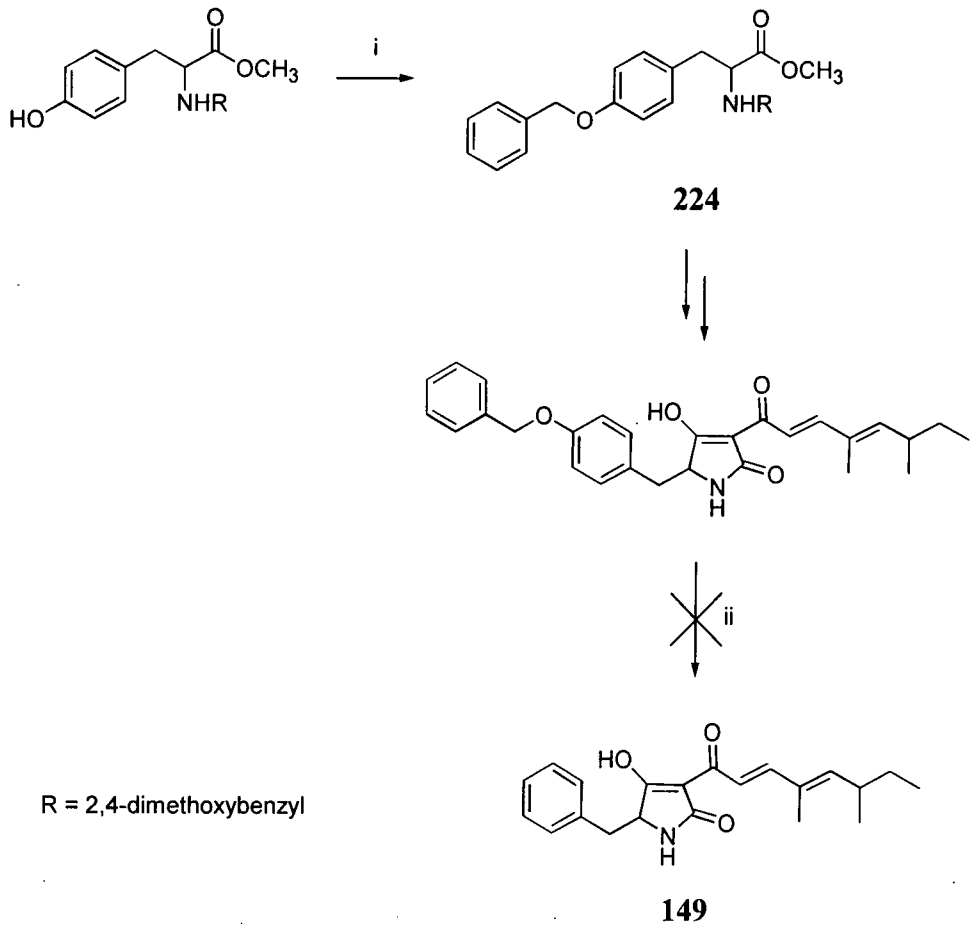
An oxygen protecting group was now required. A previous attempted synthesis of the tetramic acid derivative **177** in Durham**Error! Bookmark not defined.** had investigated the suitability of a *p*-methoxybenzyl and a benzyl group. Unfortunately, both protecting groups were found to be unsuitable. The reaction of N-protected tyrosine methyl ester **221** with *p*–methoxybenzyl chloride produced the desired protected amino acid **223** as required, however complete cleavage of the protected ether was observed in the subsequent reaction of **223** with the diketene adduct. The mild acidic conditions due to the presence of pyridinium *para*–toluene sulfonate (PPTS) were thought to be responsible. Condensations without PPTS were tried, but no reaction ensued (Scheme 3-29).

Deprotonation of the amine with sodium hydride prior to reaction with the diketene did not produce the desired product either.



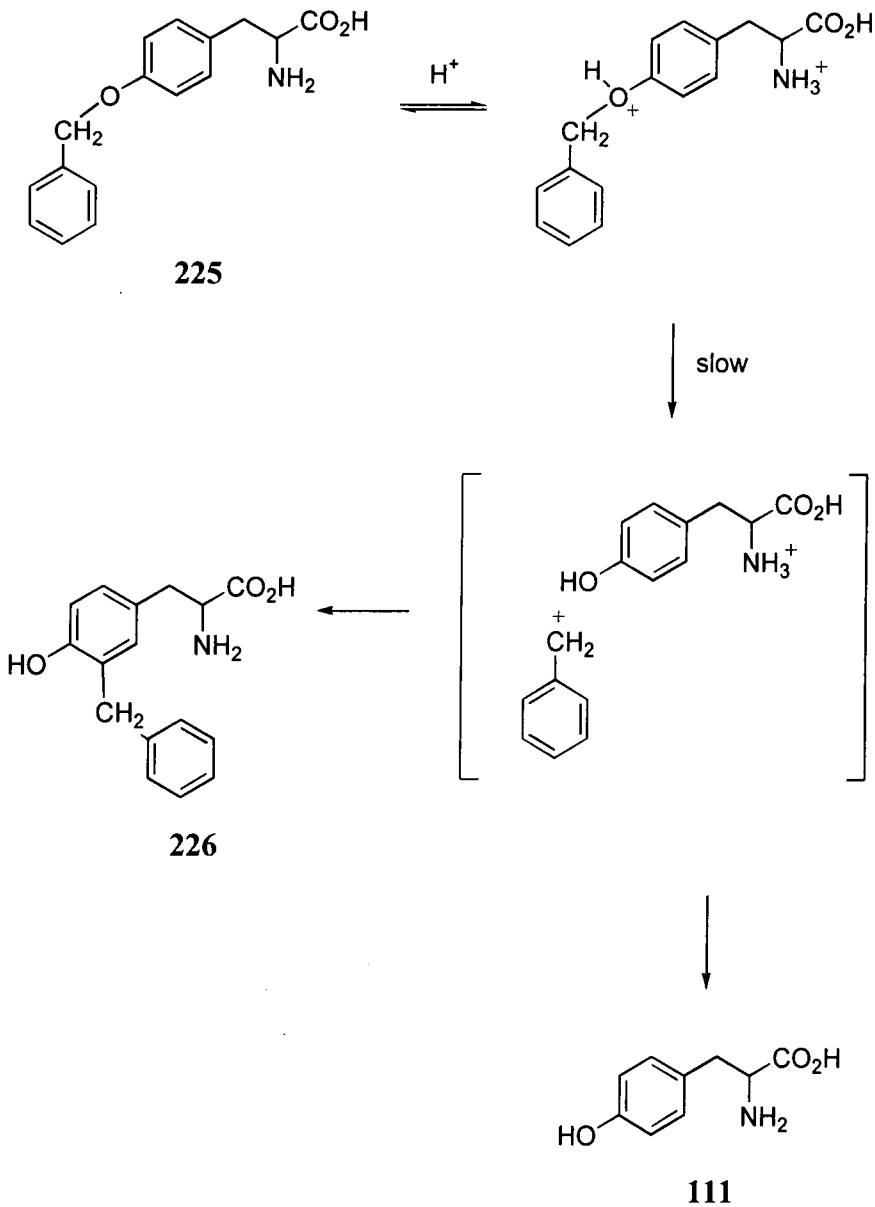
Scheme 3-29 *Reagents and conditions*: i, *p*-methoxybenzyl chloride, K_2CO_3, $(Bu)_4NI$, DMF, 60 °C, 3 d; ii, **174**, PPTS, toluene, reflux, 2 h; iii, **174**, toluene, reflux, 2 h.

The tyrosine methyl ester was then protected as the benzyl ether **224**, which was found to be stable to the acidic conditions of the reaction with the diketene adduct and also to all successive reactions in the synthesis. However, the benzyl group was insufficiently labile to be removed in the final step (Scheme 3-30). Extended deprotection times with TFA[81] led to decomposition. The presence of double bonds in the side chain of the tetramic acid excludes the use of hydrogenation, a frequently used method for the deprotection of benzyl ethers.[135]



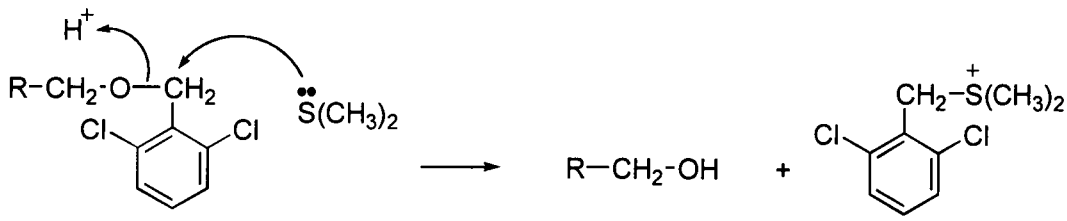
Scheme 3-30 *Reagents and conditions*: i, benzyl bromide, K_2CO_3, DMF, 60 °C, 2 d; ii, TFA, room temp., 30 min.

From these observations it was anticipated that a substituted aromatic having intermediate reactivity between a benzyl and *p*-methoxybenzyl group would fulfil the requirements needed for an effective protecting group. 2,6-Dichlorobenzyl groups have been used for the protection of tyrosine in other syntheses, chiefly because of their low incidence of rearrangement during cleavage,[136] a common feature of tyrosine chemistry, but also due to their ease of removal.[137] O-Benzyltyrosines **225** are frequently found to undergo intramolecular rearrangement to the corresponding 3-benzyltyrosines **226** during deprotection in acidic conditions (Scheme 3-31).



Scheme 3-31

Cation scavengers, for example thioanisole,[138] anisole[139] and dimethylsulfide[140] are often used in order to suppress these side reactions (Scheme 3-32).

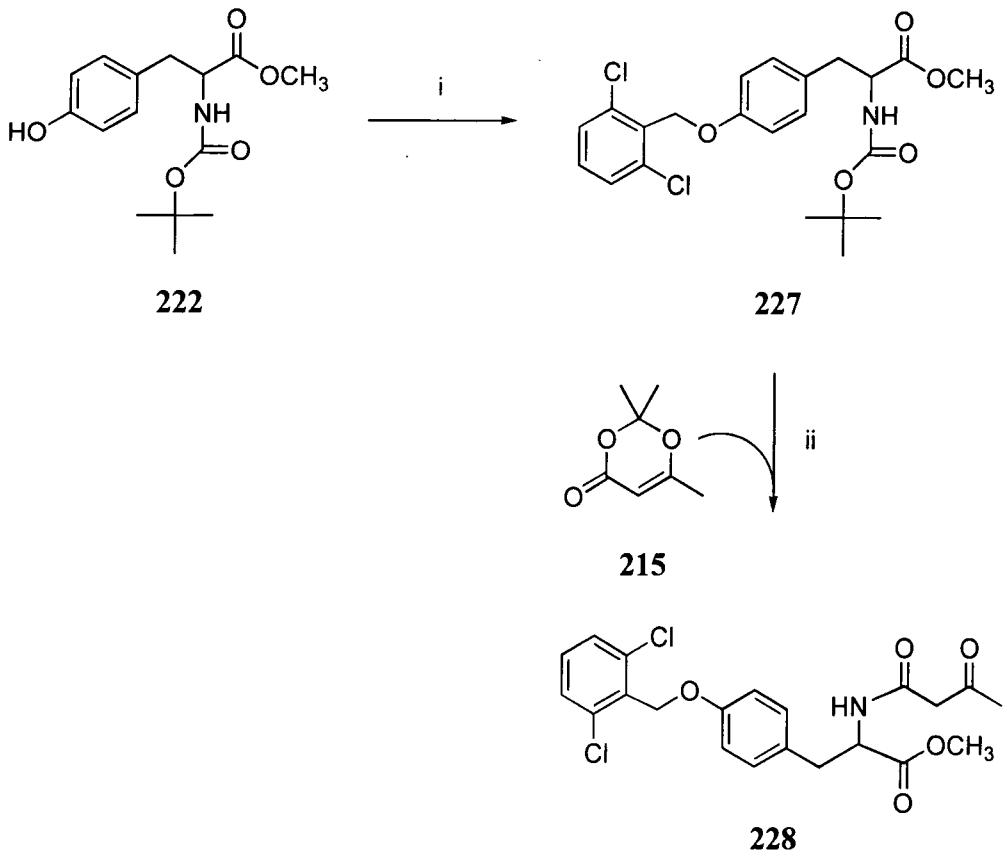


Scheme 3-32

Accordingly, the 2,6-dichlorobenzyl group was investigated as a potential phenol protecting agent in the synthesis of **177**.

The reaction of the N-*t*-butyloxycarbonyl tyrosine methyl ester **222** with 2,6-dichlorobenzyl chloride and potassium carbonate in dimethylformamide (DMF) afforded the protected amino acid **227** in good yield (85 %) (Scheme 3-33). The reaction was initially performed in acetone, but DMF was found to increase the solubility of the reagents and improve the yield. Washing the organic extracts repeatedly with water removed any residual traces of DMF.

Once the amino acid had been protected, acetoacylation of the nitrogen was required in order to form the β-ketoamine. Diketene adduct, 2,2,6-trimethyl-4H-1,3-dioxin-4-one **215**, was reacted with the protected tyrosine **227** and pyridinium *para*-toluene sulfonate for 3.5 h and the product subsequently purified by column chromatography. ^{1}H NMR analysis showed that the Boc group had almost entirely cleaved during the reaction. Although Boc groups are known to be acid labile, cleavage generally occurs under stronger acid conditions. A different N-protecting group was thus required.



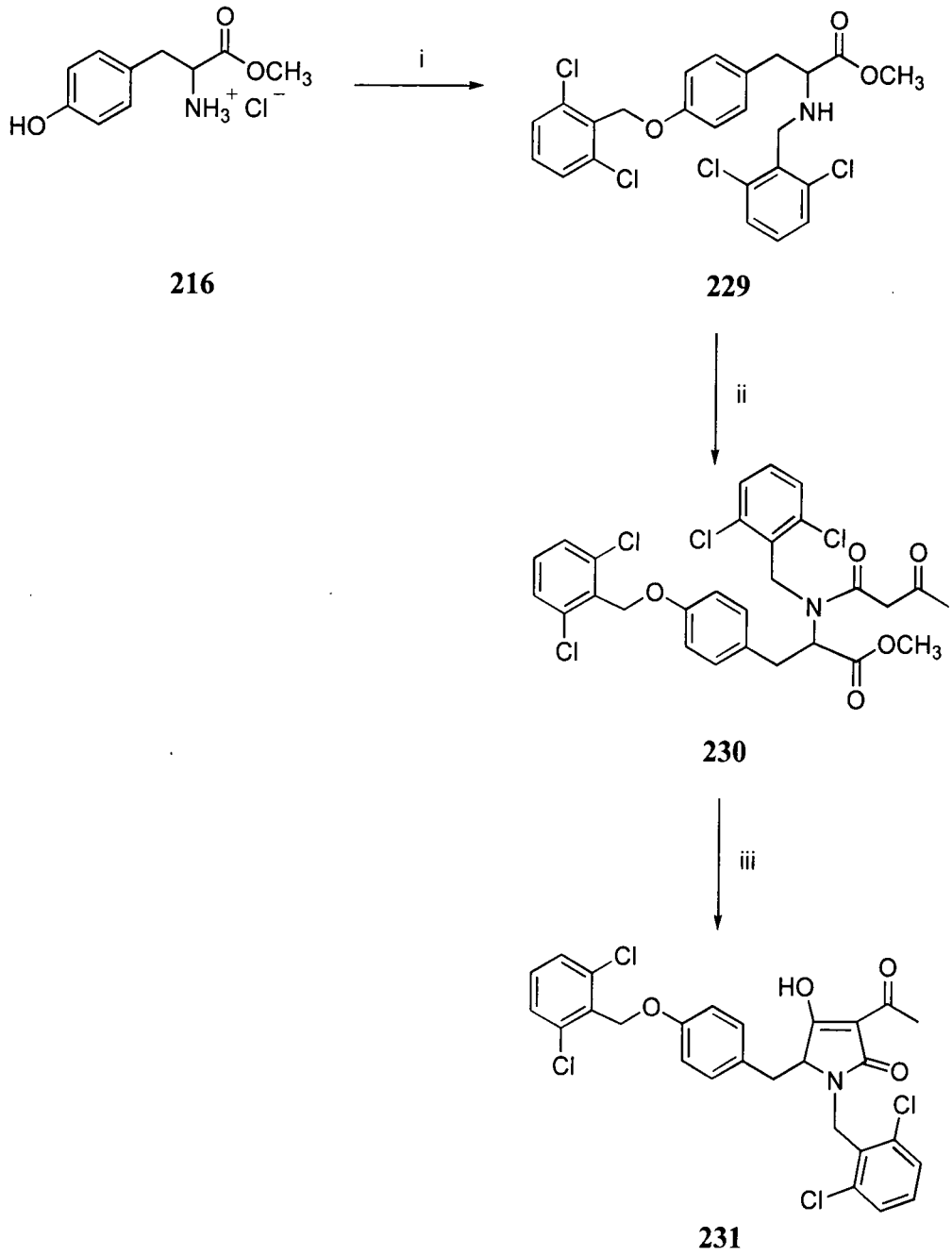
222 227 215 228

Scheme 3-33 *Reagents and conditions*: i, 2,6-dichlorobenzyl chloride, K_2CO_3, DMF, reflux, 48 h, 85 %; ii, 2,2,6-trimethyl-4H-1,3-dioxin-4-one, PPTS, toluene, reflux, 3.5 h.

3.2.5 N-2,6-dichlorobenzyl-O-2,6-dichlorobenzyl protection

At this stage of the synthesis the 2,6-dichlorobenzyl group was considered for both O- and N-protection. Initially, sole protection of the nitrogen was attempted using either triethylamine[141] or diisopropylethylamine[142] as the base, however reactions were unsuccessful resulting in the recovery of starting material only. Since reaction of the nitrogen alone proved difficult, a one step, dual protection strategy was adopted. The standard reaction conditions for O-protection were employed, but with 2 equivalents of 2,6-dichlorobenzyl chloride. After work-up, the major product was identified as N-2,6-dichlorobenzyl-O-2,6-dichlorobenzyl tyrosine methyl ester **229**. The yield of 60 % is moderate, but was acceptable as this reduced the synthetic scheme by one step and was equivalent to two protection steps of approximately 77 % yield.

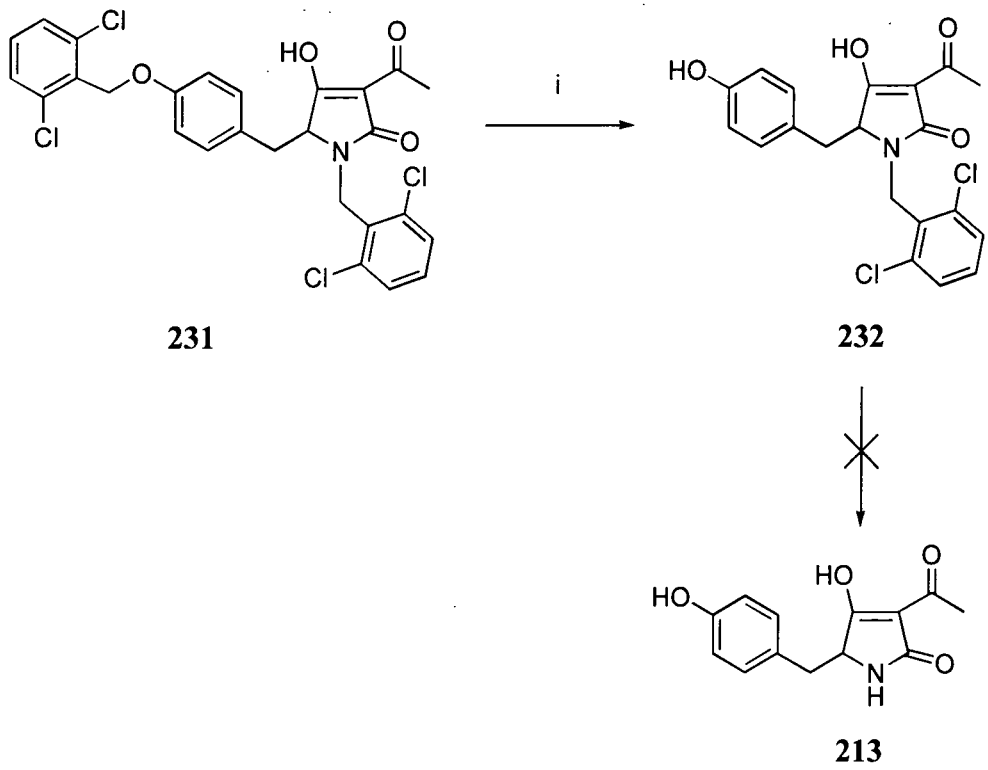
Following the previous protocol, the protected tyrosine **229** was reacted with the acetone diketene adduct **215** to give the N-acetoacylated compound **230**. Analysis of the product by ^{1}H NMR showed both protecting groups to have remained intact (Scheme 3-34).



Scheme 3-34 *Reagents and conditions*: i, 2,6-dichlorobenzyl chloride (2 equivalents), K_2CO_3, DMF, 60 °C, 12 h, 60 %; ii, 2,2,6-trimethyl-4H-1,3-dioxin-4-one, PPTS, toluene, reflux, 2 h, 74 %; iii, *t*BuOK, *t*BuOH, 25 °C, 30 min, 97 %.

Compound **230** was then cyclised *via* a Lacey-Dieckmann cyclisation[143] using potassium *t*-butoxide.[114] Lacey found that treatment of the DL-alanine ethyl ester with diketene yielded the corresponding acetoacetamide, which could then be cyclised to the acyltetramic acid with sodium ethoxide in ethanol/benzene. Cyclisation occurs very quickly and extended reaction times lead to racemisation.[144] In the event, an orange product **231** was isolated from the reaction mixture after work-up. Excess *t*-butanol was removed azeotropically with hexane.

The protected acyltetramic acid **231** was then treated with trifluoroacetic acid (TFA)[81] at room temperature in an attempt to cleave the dichlorobenzyl groups. After 1 h, NMR analysis showed that neither group had been removed. Other systems using TFA/thioanisole/trifluoromethylsulfonic acid (TFMSA),[145] and TFA/chloroform were also attempted. Again, no reaction had occurred. Longer reaction times or higher temperatures resulted in decomposition of the tetramic acid. Deprotections with TMSCl, [89] TMSBr,[89] ceric ammonium nitrate (CAN)[146] and acetic acid were also unsuccessful.



231 **232** **213**

Scheme 3-35 *Reagents and conditions*: i, 1M BBr_3 in DCM, -78 °C to room temp., 24 h.

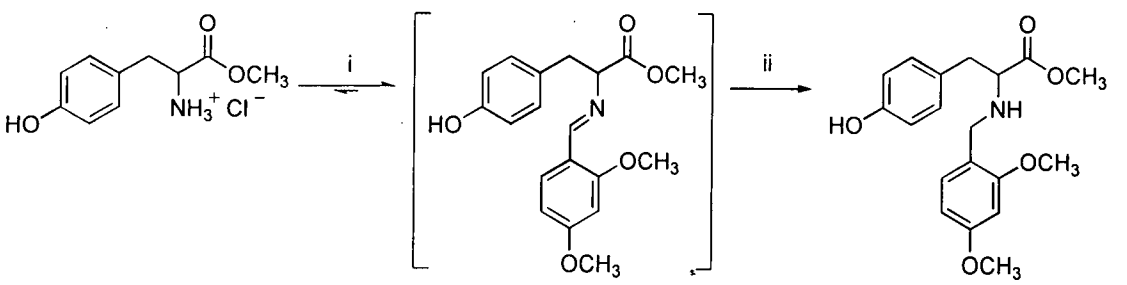
Finally, boron tribromide[90] was found to be effective for the removal of the phenol protecting group to yield compound **232**, but this reagent did not cleave the N-2,6-dichlorobenzyl group (Scheme 3-35). This could perhaps be due to steric hindrance from the two chlorine atoms, impeding the attack of a bromide ion in close proximity to the tetramic acid ring. The phenolic hydroxyl oxygen is clearly more accessible presumably facilitating ether cleavage.

Although the inability to cleave the nitrogen protecting group was disappointing, it was notable that an effective O-protection/deprotection strategy had been established. The 2,6-dichlorobenzyl group has been shown to be appropriate for the protection of the phenolic oxygen in this synthesis. It is easily introduced, is stable to all reaction steps, and can be readily removed in the final step with BBr_3 under mild conditions.

A nitrogen protecting group was however still required.

3.2.6 N-2,4-dimethoxybenzyl-O-2,6-dichlorobenzyl protection

At this point it appeared appropriate to re-examine 2,4-dimethoxybenzyl as a nitrogen protecting group. The reaction with 2,4-dimethoxybenzaldehyde was repeated as previously described but with the addition of magnesium sulfate ($MgSO_4$) to the mixture.



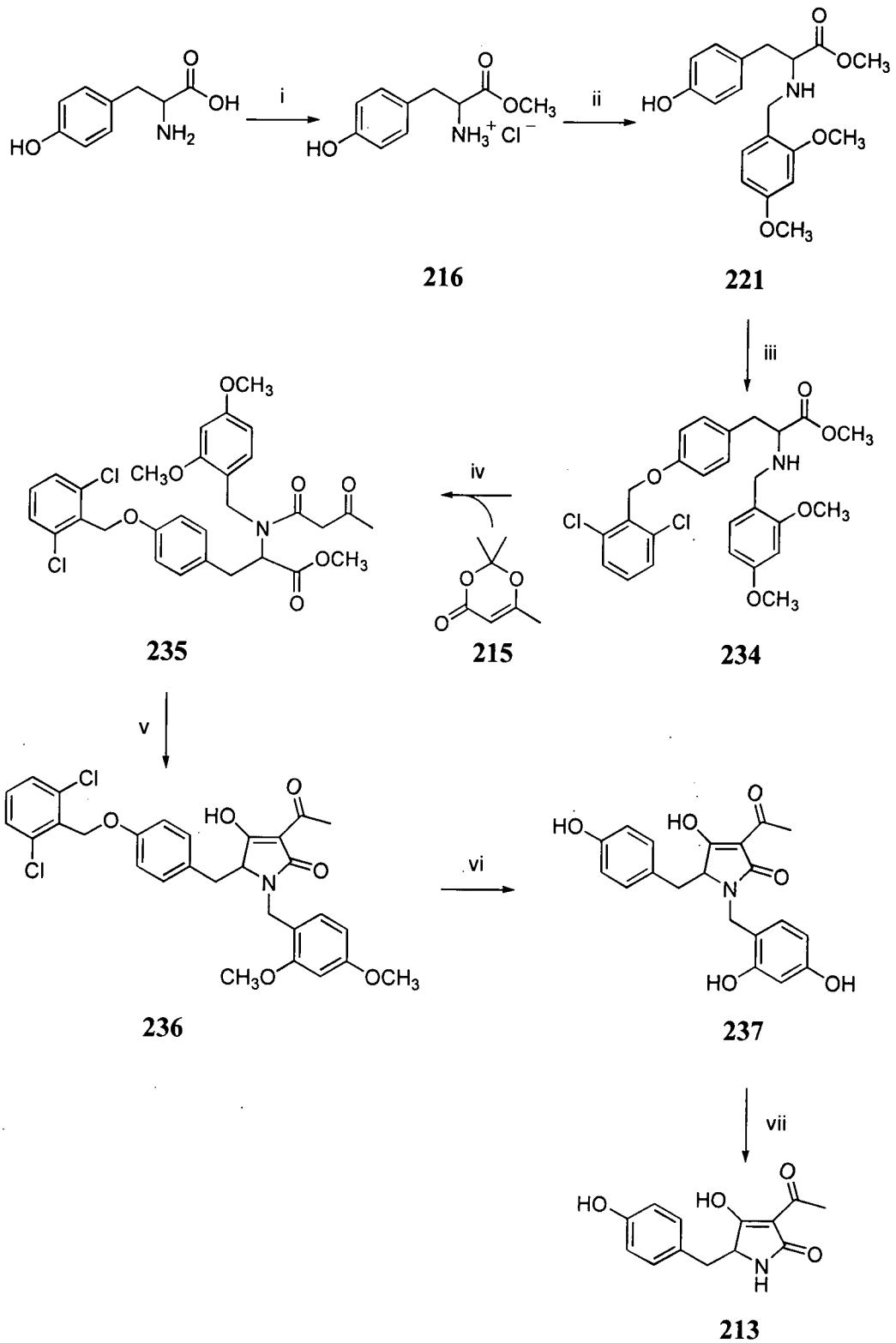
Scheme 3-36 *Reagents and conditions*: i, 2,4-dimethoxybenzaldehyde, $MgSO_4$, MeOH, room temp., 24 h; ii, $NaBH_4$, MeOH, 0 °C, 1 h, 66 %.

The removal of the water formed in the reaction was believed to shift the equilibrium in favour of the imine **233** (Scheme 3-36) and although yields were still not high, reproducible amounts of product **221** could now be obtained.
Previously, sodium cyanoborohydride had been used as the reducing agent, however the use of sodium borohydride removed the potential hazards associated with the production of hydrogen cyanide gas formed during the acidic work-up.

The successful synthesis of **221** proved a very important result as it allowed the project to move forward. Earlier work[71] had already established that the 2,4-dimethoxybenzyl group is stable to all reaction steps and can be readily cleaved at the end of the sequence by TFA. Therefore, in conjunction with a 2,6-dichlorobenzyl O-protecting group, an appropriate protection strategy appeared to be in place. It was anticipated that two separate deprotection steps, firstly using boron tribromide and then trifluoroacetic acid would give the desired product.

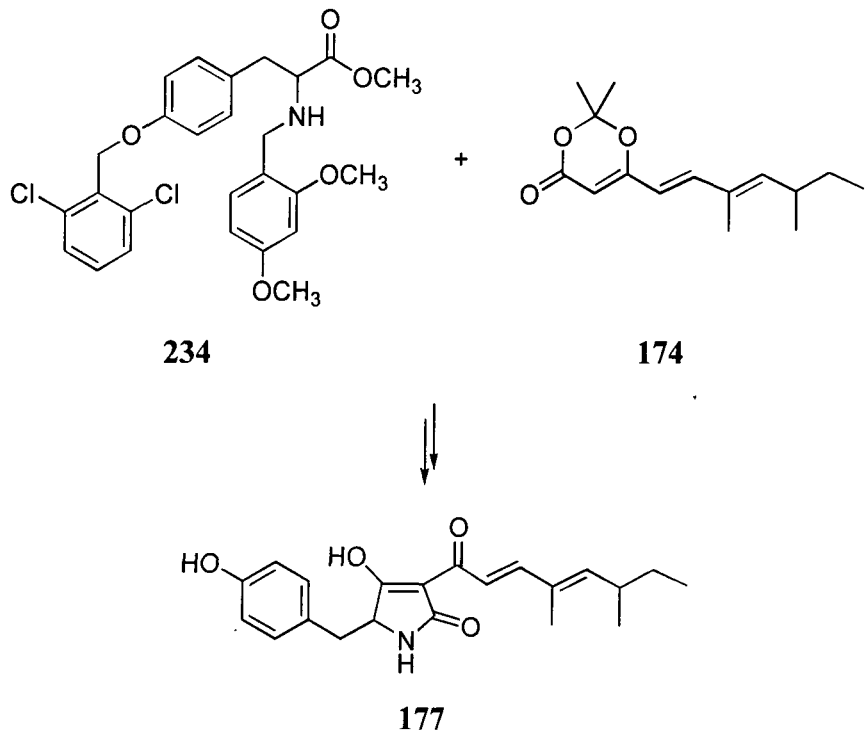
Accordingly, tyrosine methyl ester **216** was prepared as previously described and the product then stirred with 2,4-dimethoxybenzaldehyde and $MgSO_4$ in methanol. The resultant imine was reduced with sodium borohydride to give N-2,4-dimethoxybenzyl tyrosine methyl ester **221** as a white powder (Scheme 3-37).

Protection of the phenolic hydroxyl as before gave the protected tyrosine derivative **234**, which was then reacted with the acetone diketene adduct **215**. The product **235** was cyclised by treatment with potassium *t*-butoxide in *t*-butanol for 30 minutes at room temperature to give **236** as an orange solid. Due to the high polarity of this compound, no purification was attempted. Deprotection of the phenol with BBr_3 in DCM was complete in 6 h and analysis of the product concluded that substantial cleavage of the methyl ethers of the N-protecting group had also occurred. The N-2,4-dimethoxybenzyl protected tetramic acid **237**, which was then stirred for 1 hour in TFA[81] in order to yield the desired tetramic acid **213** in 33 % overall yield from tyrosine (Scheme 3-37).



Scheme 3-37 *Reagents and conditions*: i, $SOCl_2$, MeOH, reflux, 3 h, 97 %; ii, 2,4-dimethoxy-benzaldehyde, $MgSO_4$, MeOH, room temp., 12 h then $NaBH_4$, MeOH, 0 °C, 1 h, 66 %; iii, 2,6-dichlorobenzyl chloride, K_2CO_3, DMF, 60 °C, 24 h, 81 %; iv, 2,2,6-trimethyl-4H-1,3-dioxin-4-one, PPTS, toluene, reflux, 3 h, 79 %; v, *t*BuOK, *t*BuOH, 25 °C, 30 min, 98 %; vi, 1M BBr_3 in DCM, -78 °C to room temp., 6 h, 99 %; vii, TFA, room temp., 1 h, 85 %.

Now that suitable protection and deprotection strategies had been developed in the model system, a synthesis of the tetramic acid **177** could be addressed (Scheme 3-38). Clearly to succeed in the total synthesis, the acetone diketene adduct derivative **174** had to be prepared.



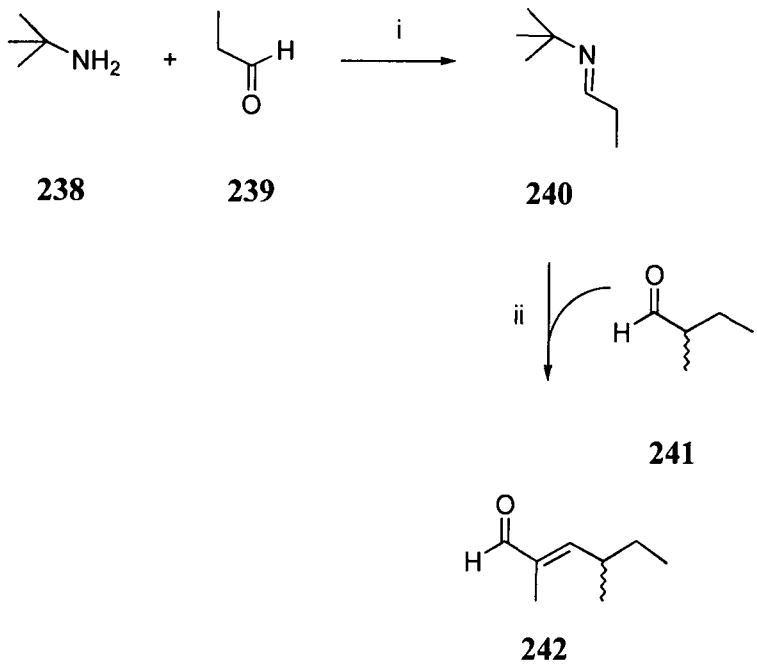
234 **174**

177

Scheme 3-38

3.2.7 Synthesis of acetone diketene adduct 174

The desired side chain had previously been prepared in Durham.[80] (*E*)-2,4-Dimethyl-hex-2-enal **242** was synthesised in two steps by an aldol condensation method reported by Wittig[147] (Scheme 3-39). Primarily the imine, (1,1-dimethyl-N-propylidene) ethylamine **240**,[148] was produced from the dropwise addition of propionaldehyde **239** to *t*-butylamine **238** followed by addition of potassium hydroxide and overnight refrigeration. The product was distilled from the reaction mixture to give a moderate yield of a clear, colourless oil. This compound proved unstable and it was generally prepared prior to use and if necessary stored over molecular sieves in the freezer.

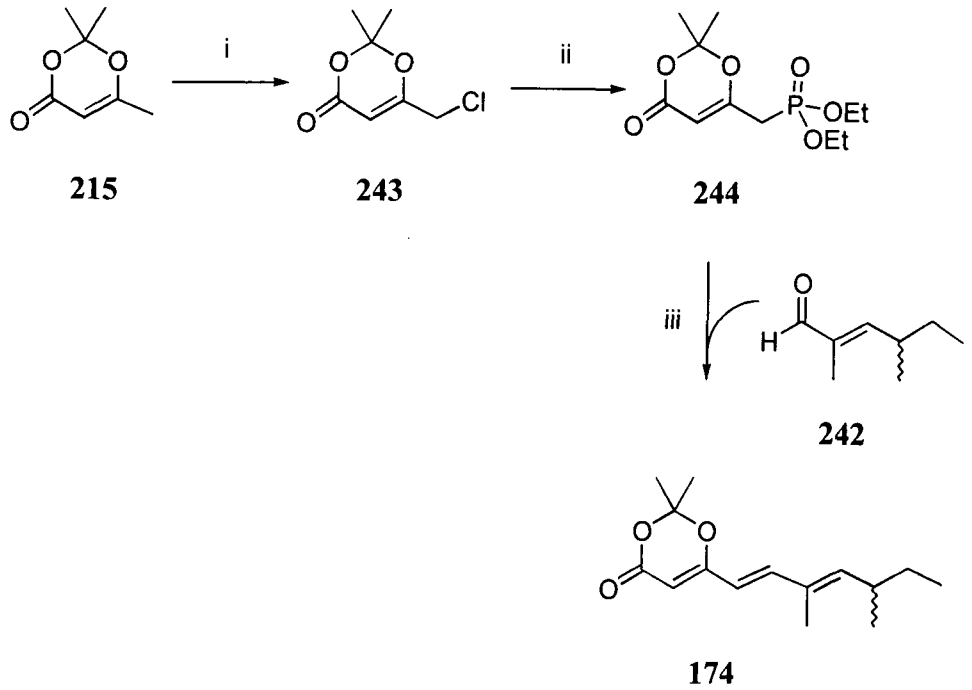


Scheme 3-39 *Reagents and conditions*: i, 0 °C then KOH, 5 °C, 12 h, 49 %; ii, BuLi, THF, -78 °C then **241**, 0 °C, 4 h; $(CO_2H)_2$, 72 h, room temp., 60 %.

The second step involved lithiation of imine **240** using butyllithium at -78 °C, followed by a condensation with 2-methylbutyraldehyde **241**. This gave the aldimine adduct, which decomposed on addition of oxalic acid to the more stable aldehydic *trans* olefin **242**[149] (Scheme 3-39). This aldehyde **242** was stable to storage at -10 °C but was generally purified just prior to use by column chromatography over silica gel.

The phosphonate ester **244**, which was required for the coupling reaction with aldehyde **242**, was synthesised from acetone diketene adduct **215** *via* the 4-chloro-derivative (Scheme 3-40).[150] Accordingly, deprotonation of 2,2,6-trimethyl-4H-1,3-dioxin-4-one **215** by lithium diisopropylamide (LDA) formed a yellow precipitate, which was transferred by cannula to a stirring mixture of hexachloroethane in THF at -55 °C. The resultant deep purple reaction mixture was quenched with acid and extracted into solvent to yield a brown oil after work-up. This material was purified by column chromatography utilising an increasing elution gradient of ethyl acetate in diethyl ether to give 2,2-dimethyl-6-chloromethyl-1,3-dioxin-4-one **243**[150] as a colourless oil. Early fractions from the column were found to contain excess unreacted hexachloroethane. For optimum yield the anion was rapidly transferred to the stirred mixture of

hexachloroethane in order to prevent decomposition from warming. Pre-cooling the cannula prior to use also proved beneficial.



215 **243** **244**

242

174

Scheme 3-40 *Reagents and conditions*: i, LDA, THF, -78 °C, C_2Cl_6, THF, -55 °C, 1h, 69 %; ii, $HP(O)(OEt)_2$, NaH, THF, 0 °C, 1 h 30, 52 %; iii, LHMDS, THF, -78 °C to room temp. over 4 h, then room temp., 8 h, 43 %.

Synthesis of the phosphonate ester **244** was not straightforward. Initial reactions were performed with potassium *t*-butoxide and diethyl phosphite in DMF, the latter two then being removed from the reaction mixture by distillation.[150] The reported reduced pressure could not be achieved, so the temperature had to be increased for distillation to occur. NMR analysis of the distillation residue showed that decomposition of the phosphonate ester, most likely due to the elevation in temperature, had occurred. Deprotonation of diethyl phosphite was subsequently accomplished using NaH in THF, a lower boiling solvent that could be removed easily at the rotary evaporator. 2,2-Dimethyl-6-chloromethyl-1,3-dioxin-4-one **243** was then added dropwise to the anion and the solution stirred at 0 °C for 30 min. After work-up, the product **244** was purified by column chromatography over silica gel. Absorption onto silica and washing with copious amounts of petroleum ether removed any remaining diethyl phosphite before elution of the product from the column with ethyl acetate. The low yield obtained could

be due to the formation of other products during the reaction. During studies on the alkylation of α, β-unsaturated carbonyl compounds, Smith[151] observed products from α- and γ-alkylation. Deprotonation of **215** and trapping with n-pentyliodide, gave the α- and γ-substituted products in a 2:3 ratio.

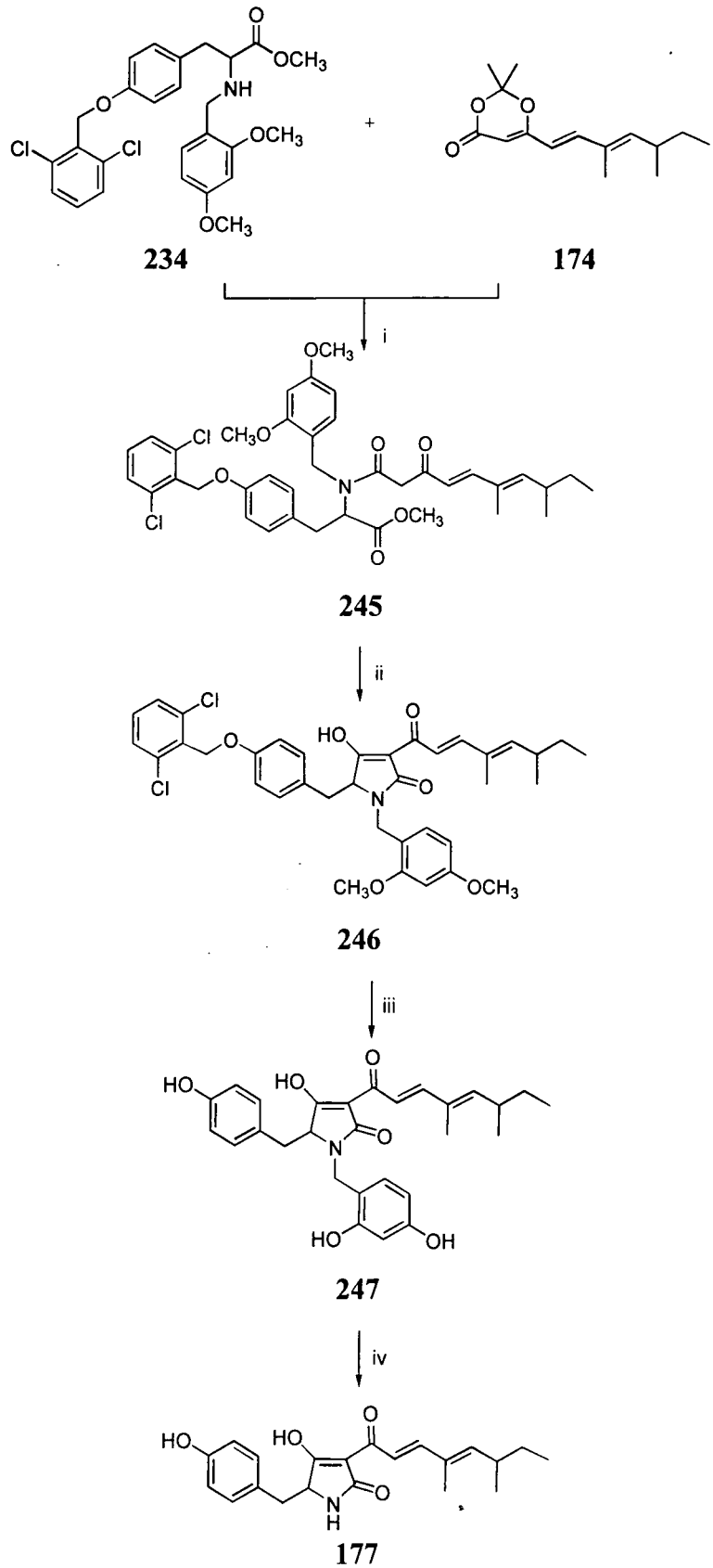
Phosphonate ester **244** was added to a solution of lithium hexamethyldisilazane (LHMDS) generated *in situ* from butyllithium and hexamethyldisilazane in THF.[114] The orange/red anion which formed was stirred for 20 min then freshly purified *E*-2,4-dimethyl-2-hexenal **242** was added and the mixture stirred until the reaction had gone to completion. Purification gave the product **174** as a yellow oil in moderate yield.

Although this synthesis required careful manipulation, an optimal protocol was developed and the synthesis of the long chain tetramic acid **177** could now be addressed.

3.2.8 Coupling of protected tyrosine 234 and polyketide moiety 174

The successful synthesis of the desired tetramic acid **177** was accomplished by the coupling of **174** with the protected tyrosine derivative **234** (Scheme 3-41).

Accordingly, 2,2-dimethyl-6-(*E,E*-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one **174** was reacted with N-2,4-dimethoxybenzyl-O-2,6-dichlorobenzyl tyrosine methyl ester **234** and pyridinium *para*-toluene sulfonate in toluene. The product was purified by column chromatography and could be seen by NMR to exist as a mixture of tautomers. The cyclisation was performed as before with potassium *t*-butoxide to give an orange product upon which no purification was attempted. Treatment with BBr_3 cleaved the dichlorobenzyl ether to give the N-protected phenol compound **247**, which was subsequently stirred in TFA to remove the 2,4-dimethoxybenzyl group. The desired tetramic acid, 5-(4-hydroxy)benzyl-3-(*E,E*-4,6-dimethyl-octa-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **177** was isolated as an orange oil.



Scheme 3-41 *Reagents and conditions*: i, PPTS, toluene, reflux, 2 h, 89 %; ii, *t*BuOK, *t*BuOH, 25 °C, 30 min, 79 %; iii, 1M BBr_3 in DCM, -78 °C to room temp., 6 h; iv, TFA, room temp., 1 h, 89 %.

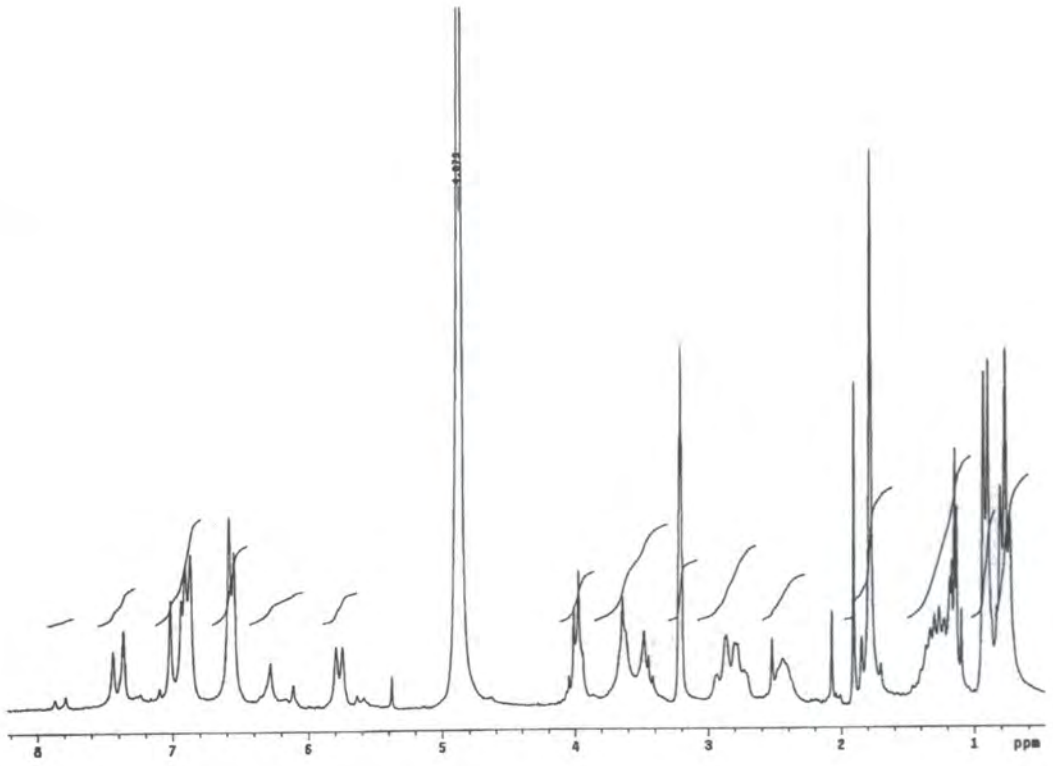
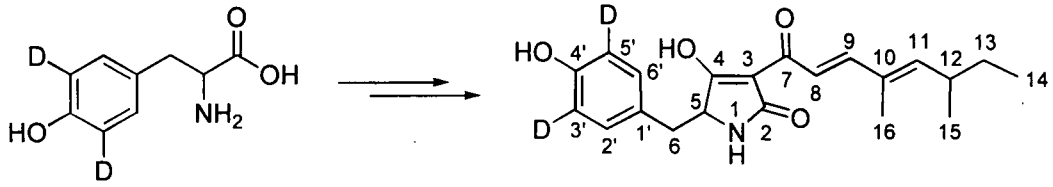


Figure 3-1 ^{1}H NMR spectrum of acyltetramic acid derivative **177**

The ^{1}H NMR spectrum of the acyltetramic acid derivative **177** is shown above (Figure 3-1). The hydrogens attached to saturated carbons can be seen on the right hand side, at lower chemical shifts, and the aromatic and olefinic protons at higher shifts. The *para*-substituted aromatic ring is indicated by the pair of doublets at δ 6.6 and 6.9. This spectrum is fully assigned in chapter 5.7.22.

3.2.9 Synthesis of labelled tetramic acid

Now that a route had been developed to the desired tetramic acid, it was necessary to carry out the synthesis incorporating an isotopic label. The same route as that employed for the production of **177** was followed, using commercially available [3',5'-$^{2}H_2$]-tyrosine as the starting material to produce the [3',5'-$^{2}H_2$] labelled tetramic acid **177a** (Scheme 3-42). The product was found by ^{1}H NMR to be approximately 85 % deuterated with an overall yield of 13.5 %.



177a

Scheme 3-42

The ^{1}H NMR spectrum of **177a** (Figure 3-2) is very similar to that recorded for the unlabelled sample (Figure 3-1). The major difference can be seen in the aromatic region. There has been some loss of deuterium label during the synthesis, indicated by the small doublet at δ 6.6, however the majority of material is deuterated, so the aromatic 2', 6' protons now resonate as a singlet at δ 6.9.

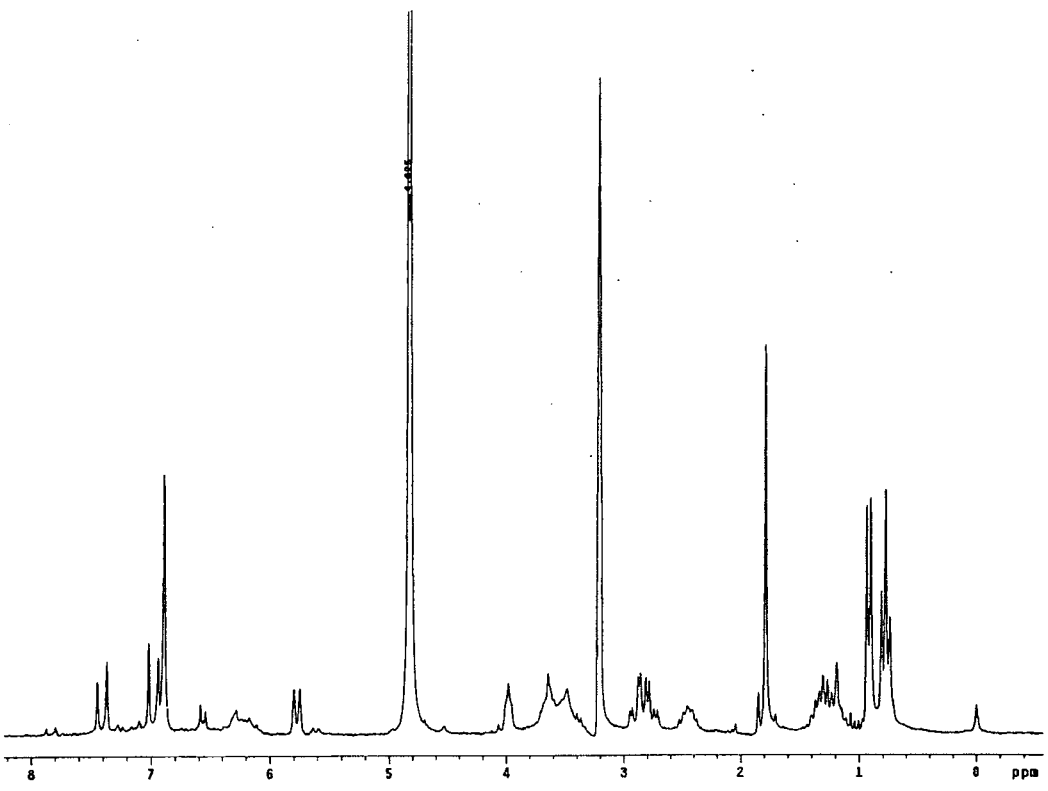


Figure 3-2 ^{1}H NMR spectrum of labelled tetramic acid derivative **177a**

^{1}H-^{1}H COSY analysis assisted in the interpretation of the 1-D proton spectrum. The crosspeaks allow adjacent hydrogens to be identified (Figure 3-3).

The protons resonating at the lowest frequency (highest field) can be seen as a triplet, indicating that they are adjacent to a CH_2 group and hence these protons belong to the methyl group of C-14. The crosspeak indicates coupling with the multiplet at δ 1.38, which is therefore due to the adjacent CH_2 at C-13. Coupling between the doublet at δ 0.99 and the multiplet at δ 2.52 shows that these are the CH_3 of C-16 and the CH of C-12. It is also possible to see coupling to a smaller signal (13 %) at δ 2.65, which arises from racemisation of the chiral centre. The singlet at δ 1.86, corresponding to the methyl group of C-15, couples to the doublet at δ 5.69, an olefinic resonance. There are no hydrogens on adjacent carbon atoms, so the coupling must be over 4 bonds. The doublet also couples to the proton at C-12, so therefore must be due to the proton at C-11.

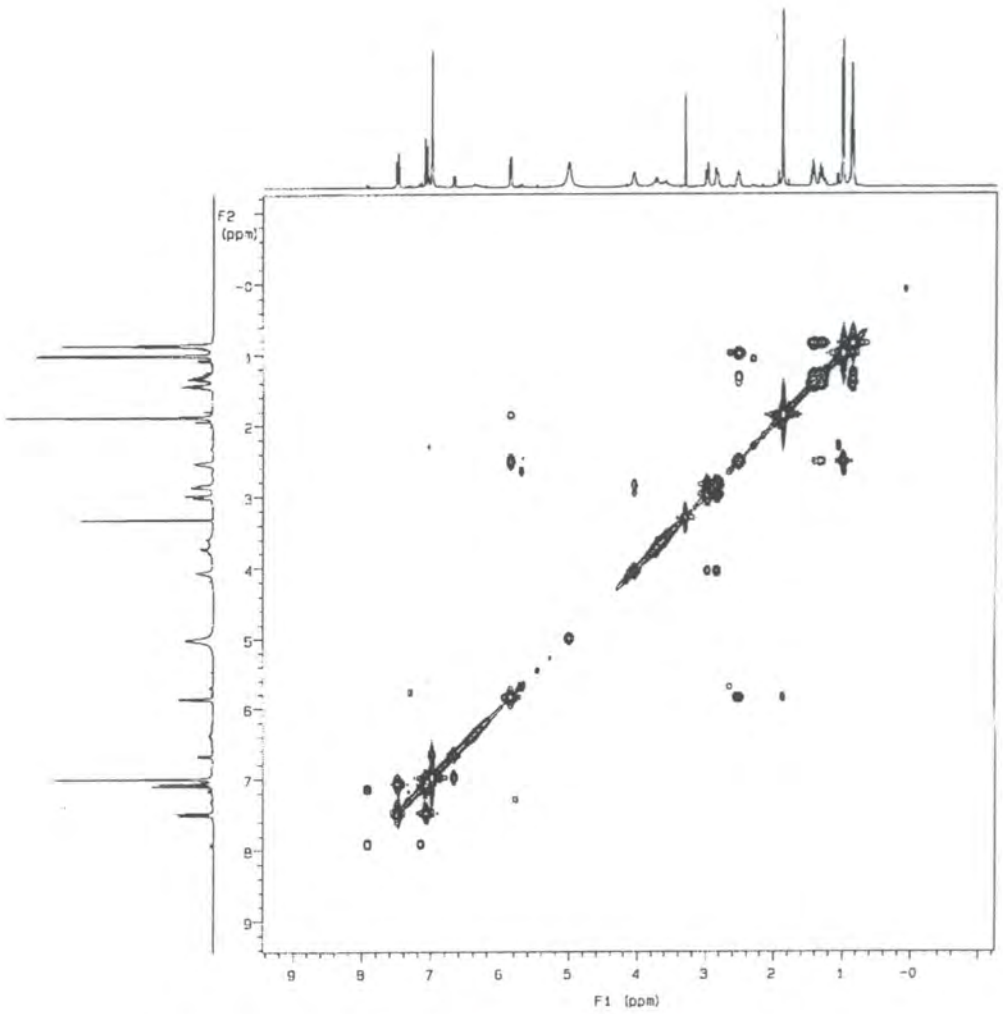


Figure 3-3 ^1H-^1H COSY of labelled tetramic acid **177a**

The doublet of doublets at approximately δ 3.0 show coupling to the broad singlet at δ 4.0 only. These are the methylene protons adjacent to the aromatic ring and the proton next to nitrogen respectively. The smaller doublet at δ 6.66 and the singlet at 6.98 are the aromatic protons. The majority of the compound is deuterated, however a small loss of the label (~15 %) has occurred during the synthesis. The two remaining protons of the molecule, on C-8 and C-9 of the double bond, resonate at δ 7.48 and 7.14, respectively and show coupling to each other only.

3.2.10 Feeding of labelled tetramic acid derivative 177a to *B. bassiana*

Labelled tetramic acid **177a** was administered to cultures of 3-day old *Beauveria bassiana*. On day 10 the mycelia were subjected to Soxhlet extraction and tenellin (25 and 17 mg) isolated as a yellow/orange powder. Two feeding experiments were performed simultaneously and each worked-up separately to give two samples of tenellin for analysis. The solid was then analysed by ^{2}H NMR.

The ^{2}H NMR spectra recorded of the two samples of isolated tenellin are shown in Figure 3-4 and Figure 3-5. The spectrum in Figure 3-4 shows a broad signal, which may contain two peaks with chemical shifts of δ 6.6 and δ 6.8. The ratio of these peaks is approximately 3:2 respectively. The second spectrum (Figure 3-5) also indicates two unresolved peaks in this region. The approximate ratio of the peaks is again about 3:2, however in this case favouring the downfield resonance. A possible explanation for the two peaks is the presence of both deuterium labelled tenellin and the administered deuterium labelled tetramic acid in the samples analysed. The respective chemical shifts for the proton resonances of the 3', 5'-phenyl protons in unlabelled tenellin and the unlabelled tetramic acid are δ 6.6 and δ 6.8 respectively.

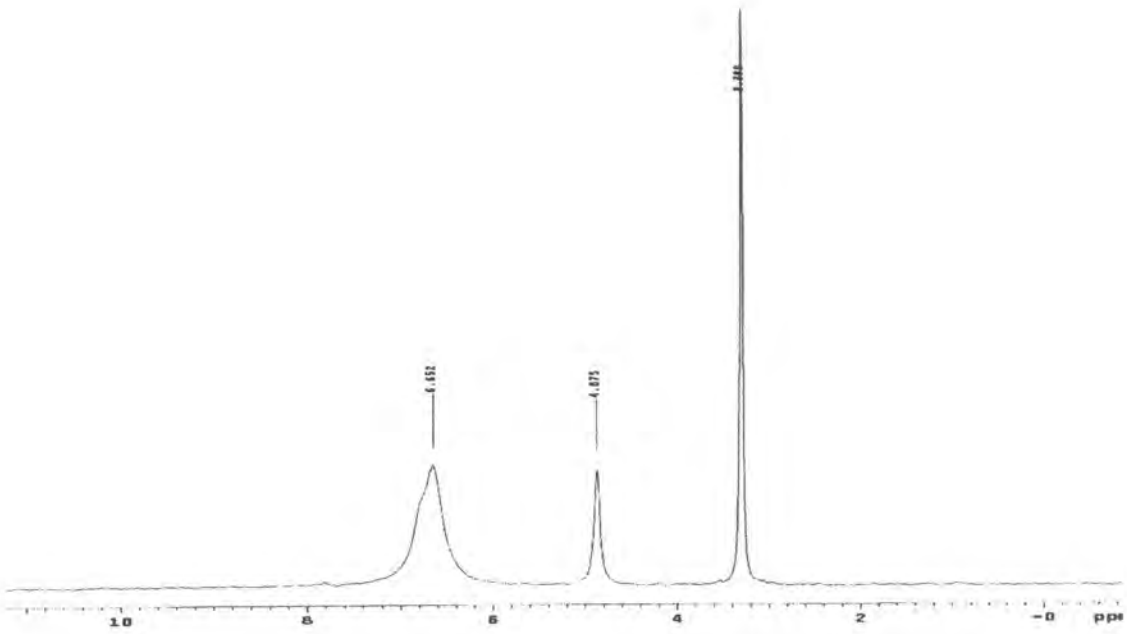


Figure 3-4 ^{2}H NMR spectrum of tenellin after incorporation experiment 1

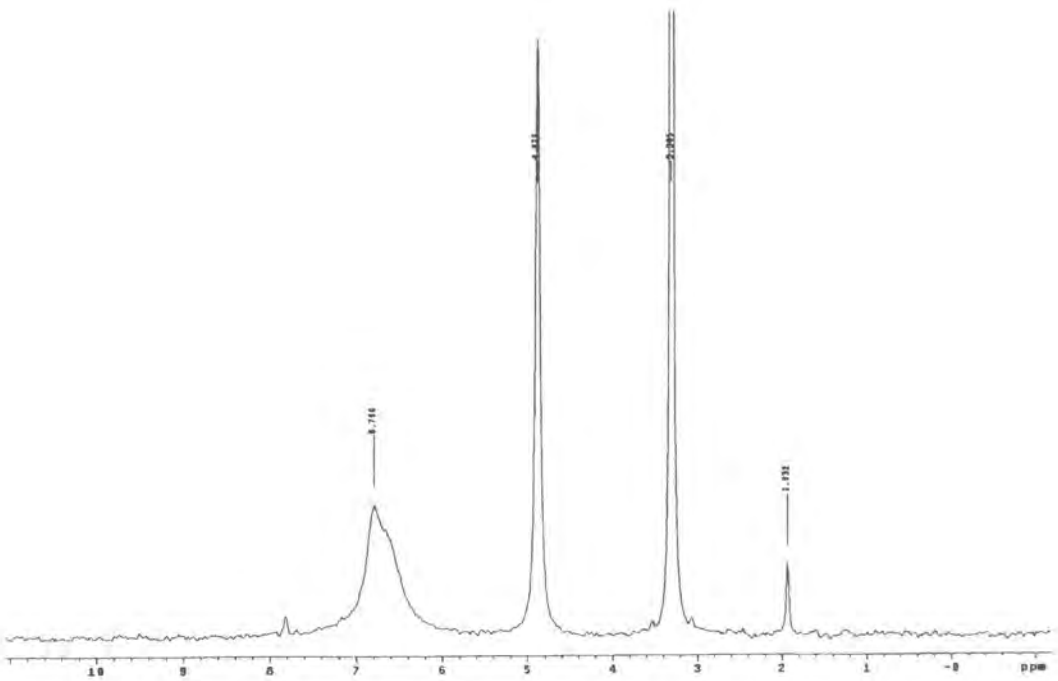


Figure 3-5 ^{2}H NMR spectrum of tenellin after incorporation experiment 2

Purification of these tenellin samples was attempted by reverse-phase preparative HPLC. Reference samples of purified tenellin were found to contain a number of peaks, thought to be due to several tautomeric forms, all of which eluted soon after injection, most probably with the solvent front. Peak separation was poor, so purification could

not be attempted using this system. Increasing the polarity of the solvent and reducing the flow rate had little effect on the chromatographic resolution. Therefore purification could still not be achieved. The injections of tenellin thus far had been performed in methanol. Using the mobile phase (water; methanol, 30:70) as the injecting solvent improved the separation of the peaks considerably. Some peaks still eluted after a short time, but a single peak thought to be tenellin eluted after 18 min. Samples of the synthetic tetramic acid were then analysed. Several small peaks thought to be impurities were eluted soon after injection with a main peak eluting after 16 min. The sample of tenellin from the feeding experiment with the labelled tetramic acid gave a similar profile to the reference sample of tenellin but with a peak at 16 min also. This additional peak was thought to be the synthetic tetramic acid.

An augmentation of the M+2 peak in the mass spectrum of tenellin from the feeding experiment would indicate incorporation of the administered tetramic acid. Analysis of these samples by electrospray mass spectrometry was then addressed. A peak at 370, corresponding to $[M+H]^+$ was dominant with two other major peaks at 354 and 330. Selective ion monitoring on these three peaks showed that fragments 354 and 330 were eluted early from the column and that the peak for 370 came after approximately 18 min, the retention observed for tenellin by HPLC. MS-MS of the molecular ion peak at 370, showed a major peak at 352. No peaks at 354 and 330 were detected, so it is deduced that these are impurities in the sample and do not arise from the fragmentation of the tenellin molecule. In general, the tenellin from the feeding experiments produced a similar MS profile to the standard tenellin sample. In addition to peaks from the unknowns of mass 330 and 354, and the peak (18 min) with mass 370, a peak at 16 min with a molecular weight of 358 was detected. This was tentatively assigned as $[M+H]^+$ for the administered labelled acyltetramic acid, which was expected to be residual in the sample. This is consistent with the two peaks observed in the ^2H NMR spectrum. LC-MS analysis of the labelled tetramic acid showed a peak of molecular weight 358 at 16 min supporting our assignment. This chromatogram did not show any indication of mass peaks at 330 or 354.

In order to gain further information on a possible incorporation it was necessary to calculate the relative proportions of M to M+2 in both the standard tenellin sample and the tenellin extracted from the feeding experiment. The M+2 peak, 372, for the standard

sample was estimated to be 3.7 % of the molecular mass ion. This was a fairly good approximation to the theoretical value of 3.86 % calculated from known natural abundance isotope values. The sample of tenellin from the labelled feeding experiment showed an M+2 that was 6.83 % of the molecular mass ion.

Table 3-1 Values of M+2/M ions in mass spectrometric analysis of tenellin samples

	M+2/M (%)
Theoretical value	3.86
Tenellin Standard	3.70
Feeding experiment	6.83

Although there are errors to these values and the results are not conclusive there is some indication that incorporation could have occurred. The possible role of the acyltetramic acid **177** in the biosynthesis of tenellin remains uncertain and further study is required.

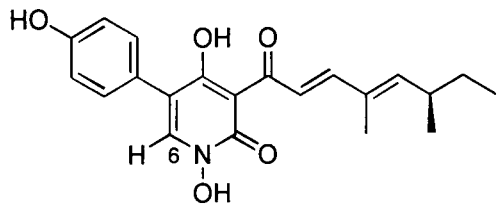
Chapter 4

4 Additional feeding experiments

4.1 Investigation into the origin of H-6 in tenellin

4.1.1 Feeding studies with phenylalanine and phenyllactic acids

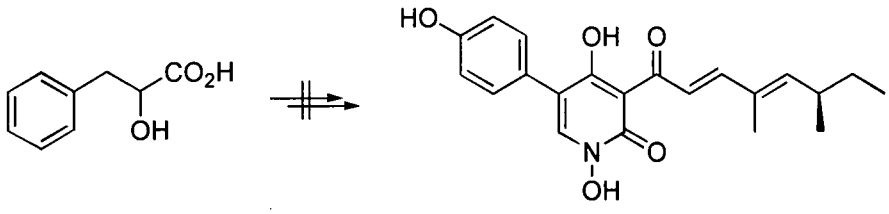
The biosynthetic origin of the hydrogen atom at position 6 of the pyridone ring of tenellin **1** has never been established.



1

The current understanding of the biosynthesis of tenellin suggests that H-6 arises from the proton attached to C-2 of tyrosine, an atom which should derive directly from phenylalanine. However, feeding studies[71] have shown that there is no incorporation of deuterium label from [2-^{13}C, ^{2}H, ^{15}N]-phenylalanine. This could be due to loss of the deuterium during the formation of the pyridone ring or because of transamination of the amino acid to phenylpyruvate during the biosynthesis. Claims for the latter explanation are to some extent substantiated from results of a [2-^{13}C, ^{2}H, ^{15}N]-phenylalanine feeding experiment that showed exclusive incorporation of the ^{13}C label into tenellin without ^{2}H or ^{15}N. Cleavage of the ^{13}C-^{15}N bond suggests that transamination is a significant process.

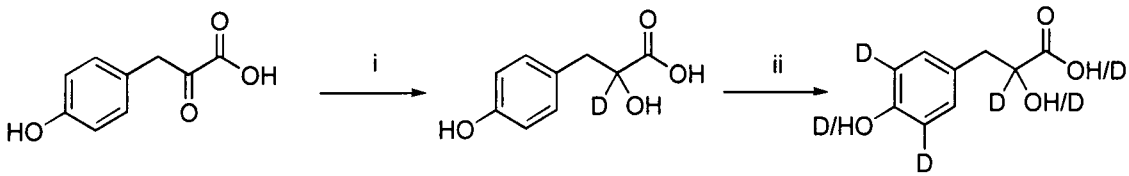
Previous feeding experiments have shown that phenyllactic acid **96** is not an intermediate in the biosynthesis of tenellin **1** (Scheme 4-1).



96 **1**

Scheme 4-1

However, if phenylalanine is hydroxylated to tyrosine before the postulated transamination process then the intermediate would be *p*-hydroxyphenyllactic acid. Therefore, isotopically labelled *p*-hydroxyphenyllactic acid was prepared in order to test the validity of this hypothesis (Scheme 4-2). The introduction of deuterium at the 2-position and at the 3' and 5'-positions of the aromatic ring was readily achieved.



248 **249a** **249c**

Scheme 4-2 *Reagents and conditions*: i, $NaBD_4$, H_2O, 0 °C, 1.5 h then 1M HCl, 86 %; ii, 35 % DCl in D_2O, Carius tube, 80 °C, 6 h, 99 %.

Deuterium was incorporated at C-2 by the reduction of hydroxyphenylpyruvate **248** with sodium borodeuteride (Scheme 4-2). Quenching, extraction and removal of the solvent gave the product **249a** in high yield as a white powder. Deuterium was also incorporated into the 3' and 5' positions of the aromatic ring by dissolving [2-^{2}H]-hydroxyphenyllactic acid **249a** in deuterated aqueous hydrochloric acid and heating for 6 hours in a sealed tube. Compound **249b** was generated in a very good overall yield.

A feeding study into tenellin was performed with these labelled hydroxyphenyllactic acids. Accordingly, solutions of **249a** and **249b** were administered portionwise to cultures of *Beauveria bassiana* over several days. Tenellin was extracted as a yellow

powder on day 10 and subjected to analysis by ^{2}H NMR. The tenellin isolated from cultures fed with **249b** showed a low incorporation of the deuterium atoms at positions 3' and 5' (broad signal at δ 6.8), but no enrichment from the 2-^{2}H atom. A peak at δ 8.2 consistent with [^{2}H]-formate, was also present (Figure 4-1). It was hypothesised that formate may be generated from oxidative cleavage of the aromatic ring.

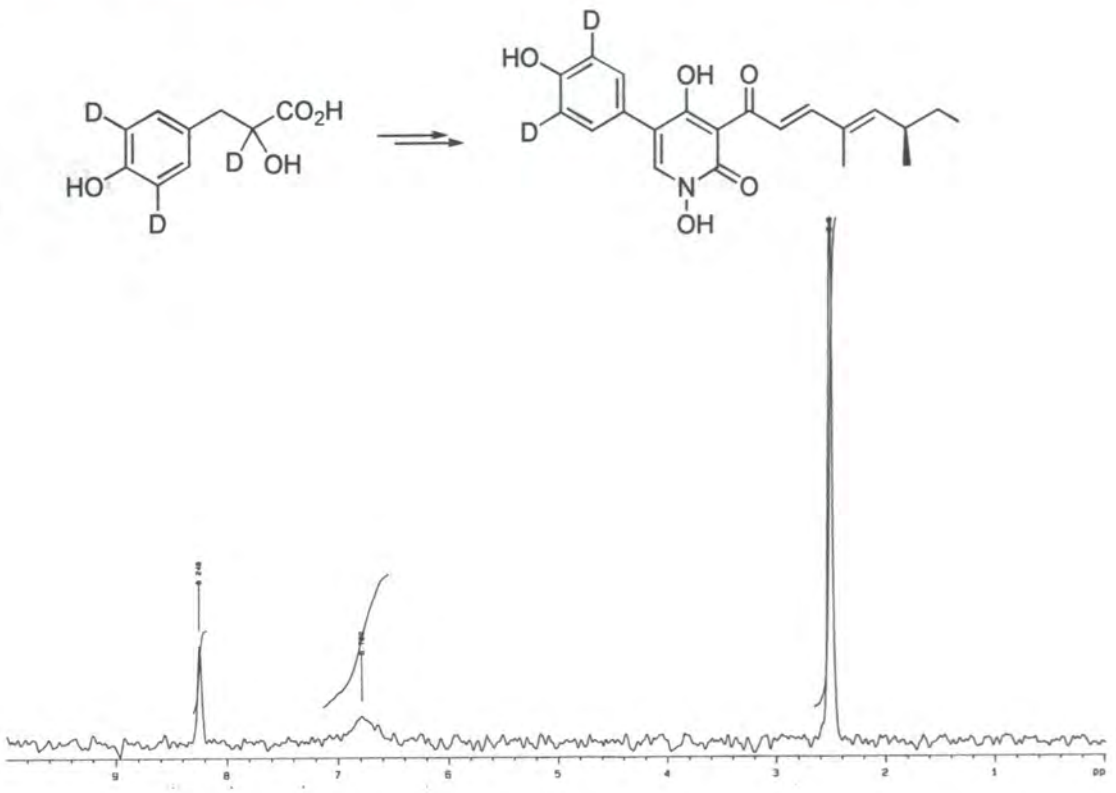


Figure 4-1 ^{2}H NMR of tenellin extracted from cultures fed with [2,3',5'-$^{3}H_2$]-*p*-hydroxyphenyllactic acid **249b**

There was no apparent deuterium incorporation into the tenellin from culture flasks fed with **249a**, suggesting that the deuterium at C-2 is lost during the biosynthesis.

The observed incorporation of deuterium from positions 3' and 5' indicates that *p*-hydroxyphenyllactic acid can become metabolised into tenellin. Loss of the α-deuterium atom can be explained by transamination back and forth to tyrosine *via p*-hydroxyphenyl pyruvate. However, it is perhaps unlikely that all of the label would have been lost by this process if *p*-hydroxyphenyllactic acid is a closer intermediate to

tenellin than tyrosine. It remains possible that this label is instead lost during the formation of the pyridone ring.

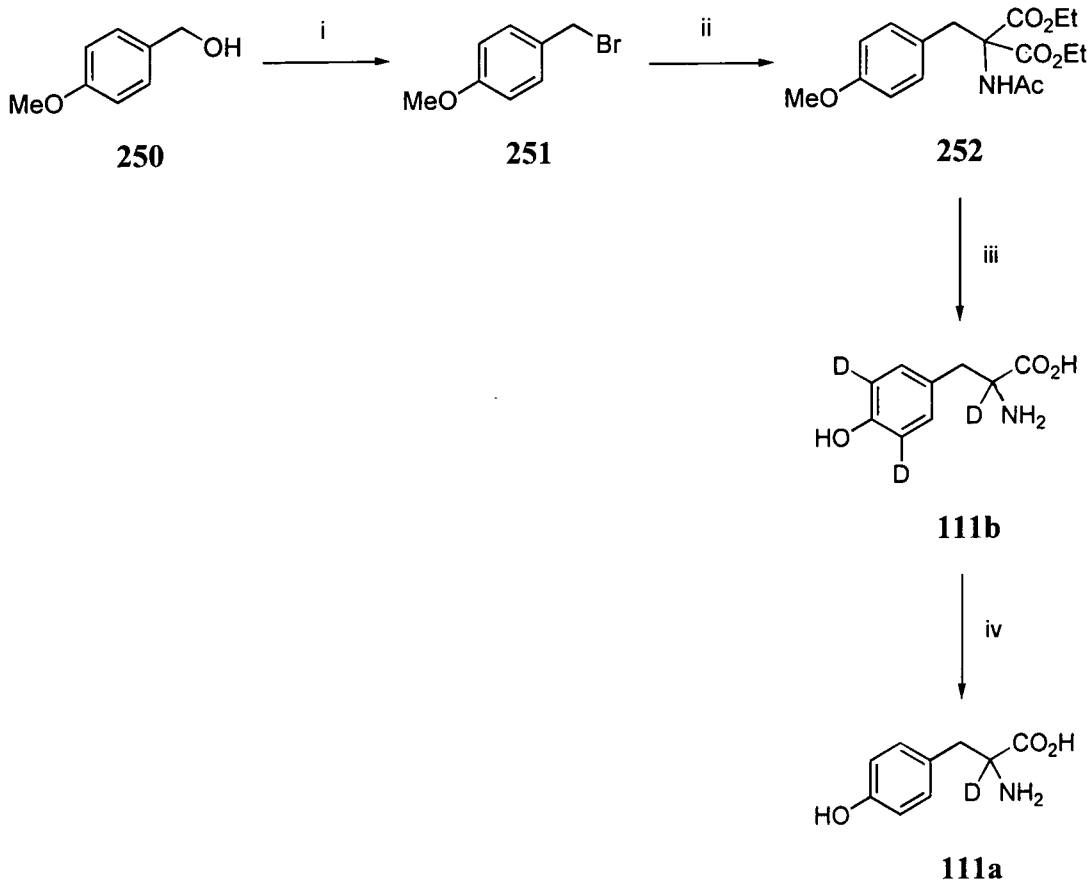
4.1.2 Feeding studies with labelled tyrosines

It has been established that both tyrosine and *p*-hydroxyphenyllactic acid are intermediates in the biosynthesis of tenellin although the more direct precursor of the two is uncertain. If a transamination process is responsible for the loss of deuterium, then the absence of an incorporation of deuterium from [2-$^2H_1$]-*p*-hydroxyphenyllactic acid would implicate tyrosine as the closer substrate.
[2-$^2H_1$]-DL-Tyrosine **111a** was synthesised in order to perform a feeding experiment.

4.1.3 Synthesis of [2-$^2H_1$]-DL-tyrosine 111a

[2-$^2H_1$]-DL-Tyrosine **111a** was synthesised as outlined (Scheme 4-3).

The addition of phosphorus tribromide (PBr_3) to *p*-methoxybenzyl alcohol **250** in pyridine and diethyl ether yielded the benzyl bromide derivative **251** as a colourless oil (Scheme 4-3).[152] This was used directly in the next step without further purification. Deprotonation of the diethyl acetamidomalonate was performed in a solution of sodium ethoxide produced from sodium and ethanol and then *p*-methoxybenzylbromide **251** was added. The addition of water precipitated the product as shiny, white crystals in high yield.[153] Hydrolysis of the ethyl esters and spontaneous decarboxylation of **252** was initiated by deuterium bromide, prepared *in situ* from D_2O and PBr_3. ^1H NMR analysis of the product **111b** indicated that deuterium had become exchanged into C-2 and at positions 3' and 5' in the aromatic ring. Refluxing **111b** in hydrochloric acid for an extended period induced exchange and washout of the aromatic deuterium labels to give the desired [2–$^2H_1$]-DL-tyrosine **111a**.



Scheme 4-3 *Reagents and conditions*: i, PBr_3, pyridine, Et_2O, room temp., 2 h, 87 %; ii, diethyl acetamidomalonate, Na, EtOH, 0 °C, 5 h, 91 %; iii, PBr_3, D_2O, 0 °C to room temp. then **252**, reflux, 2 h, 85 %; iv, 2M HCl, reflux, 37 h, 32 %.

4.1.4 Feeding of [2-$^2H_1$]-DL-tyrosine 111a to *Beauveria bassiana*

The [2-$^2H_1$]-DL-tyrosine **111a** prepared above was fed to cultures of *Beauveria bassiana*. After ten days, tenellin was extracted following the usual protocol to give a yellow solid (33 mg). ^1H NMR showed tenellin to be present in the sample, however ^2H NMR analysis of the resultant material did not indicate any incorporation of the deuterium label.

To corroborate this result, a feeding experiment with [3',5',2-$^2H_3$]-DL-tyrosine **111b** was also performed. Previous feeding studies indicated that the 3', 5' aromatic deuterium atoms of tyrosine are incorporated into tenellin at a sufficient level to be detected by ^2H NMR. Successful incorporation of the aromatic labels from this labelled

precursor would indicate that the lack of deuterium incorporation from the 2-position is due to loss of the proton during the biosynthesis and not due to the inability of the substrate to permeate the cells. Therefore, administration of the putative intermediate and extraction of the tenellin was performed as above. ^{2}H NMR analysis of the isolated tenellin showed incorporation of the aromatic deuterium atoms at δ 6.74, as expected (Figure 4-2). A very small amount of deuterium incorporation had also occurred into the polyketide starter unit, C-14. However, there was no detectable incorporation at C-6 in tenellin from the 2-^{2}H atom of **111b**.

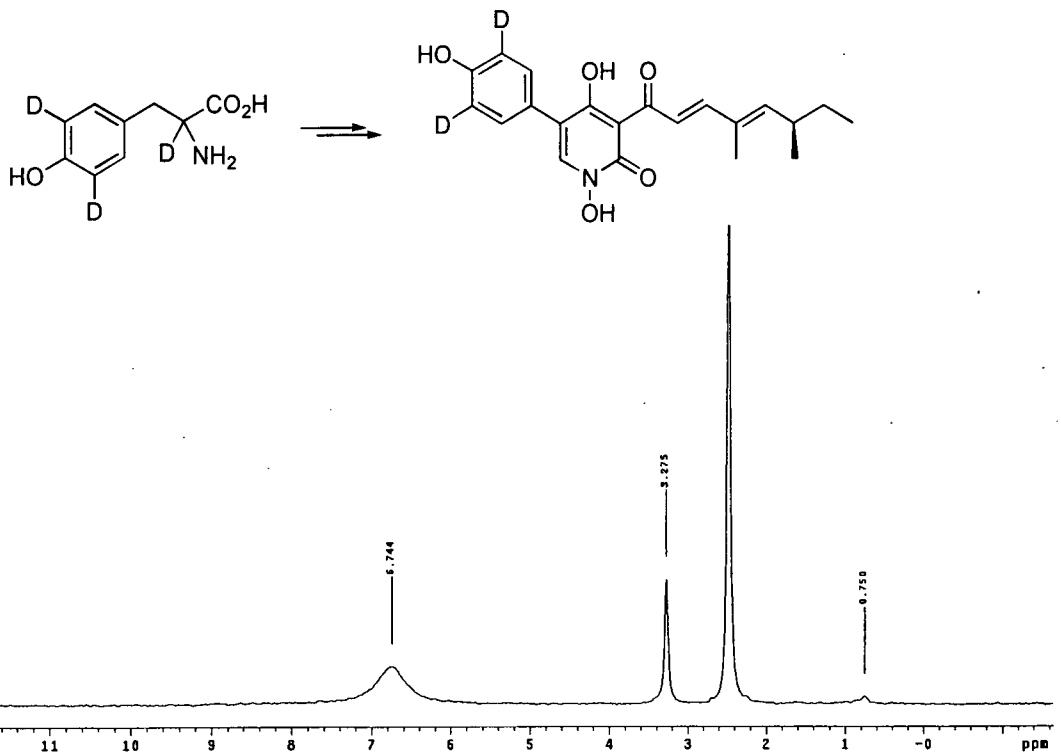
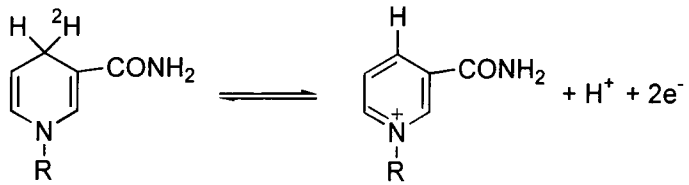


Figure 4-2 ^{2}H NMR of tenellin extracted from feeding experiment with [3',5',2-$^2H_3$]-tyrosine

The lack of incorporation at H-6 in tenellin from all of the described experiments is illustrative of a loss of hydrogen from this position during the formation of the pyridone ring. If this is the case, the hydrogen located at position-6 of tenellin must be delivered by some other route.

4.1.5 Delivery of H-6 by NADPH

The possible introduction of H-6 into tenellin from NADPH **253** was considered. NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate **254** ($NADP^+$) (Scheme 4-4). NADPH is an electron donor almost exclusively employed for reductive biosynthetic processes.



253 **254**

R=adenonine dinucleotide phosphate

Scheme 4-4

Sodium [$^2H_1$]-formate and [1-$^2H_2$, 2-$^2H_3$]-ethanol were added separately to cultures of *Beauveria bassiana* at the time of subculture in an attempt to label up the co-factor NADPH as $NADP^2H$. This should allow any hydride insertions from the co-factor into tenellin to be detected by ^2H NMR.

Tenellin extracted from cultures supplemented with [$^2H_1$]-sodium formate showed incorporation at δ 0.93 and 1.79 in the resultant ^2H NMR spectrum, indicative of isotopic labelling of the C-15 and C-16 methyl groups.

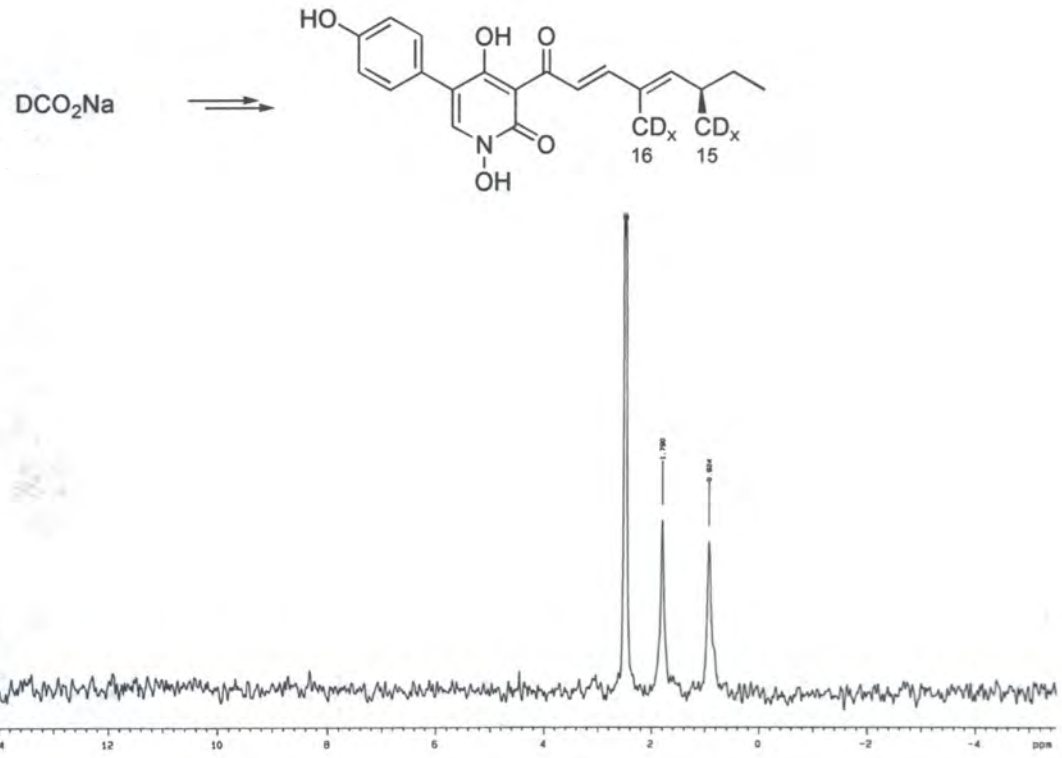
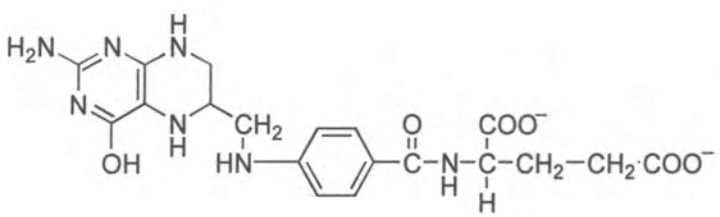


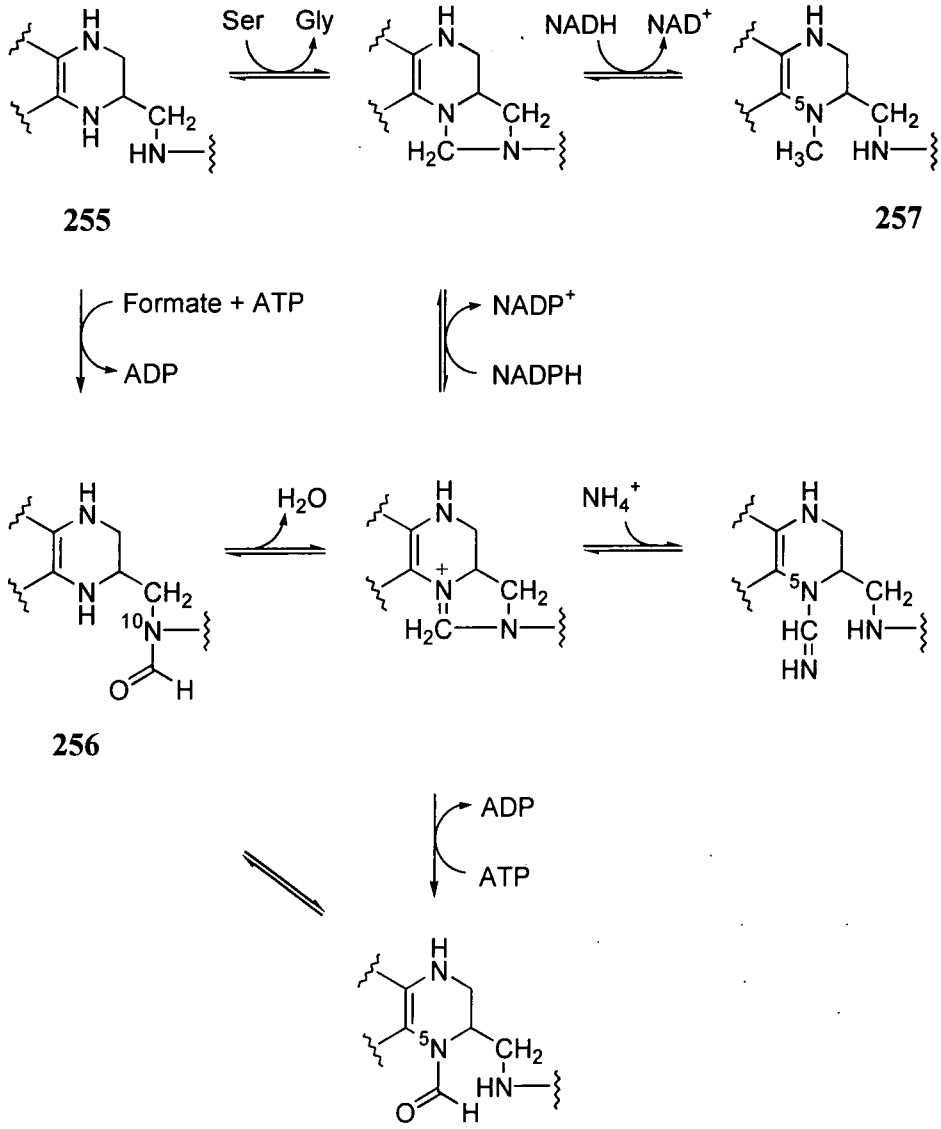
Figure 4-3 ^{2}H NMR of tenellin extracted from cultures fed with [$^{2}H_1$]-sodium formate

The methyl groups at C-15 and C-16 have previously been shown to originate from L-methionine (Chap 2.3.3), therefore the CH_3 group of methionine has become labelled from [$^{2}H_1$]-formate and then transferred to the polyketide backbone. This is most probably achieved *via* tetrahydrofolate **255**, a carrier of activated one carbon atom units in metabolism.



255

Formate can add to tetrahydrofolate **255** to give N^{10}-formyl-tetrahydrofolate **256**, which loses water and undergoes two NADPH reductions to N^{5}-methyl-tetrahydrofolate **257**.


Ser
Gly
NADH
NAD+
255
257
Formate + ATP
ADP
NADP+
NADPH
H2O
NH4+
256
ADP
ATP

Scheme 4-5

In contrast, those cultures supplemented with [1-$^2H_2$, 2-$^2H_3$]-ethanol showed deuterium incorporation at δ 0.73 in the resultant ^2H NMR of tenellin.

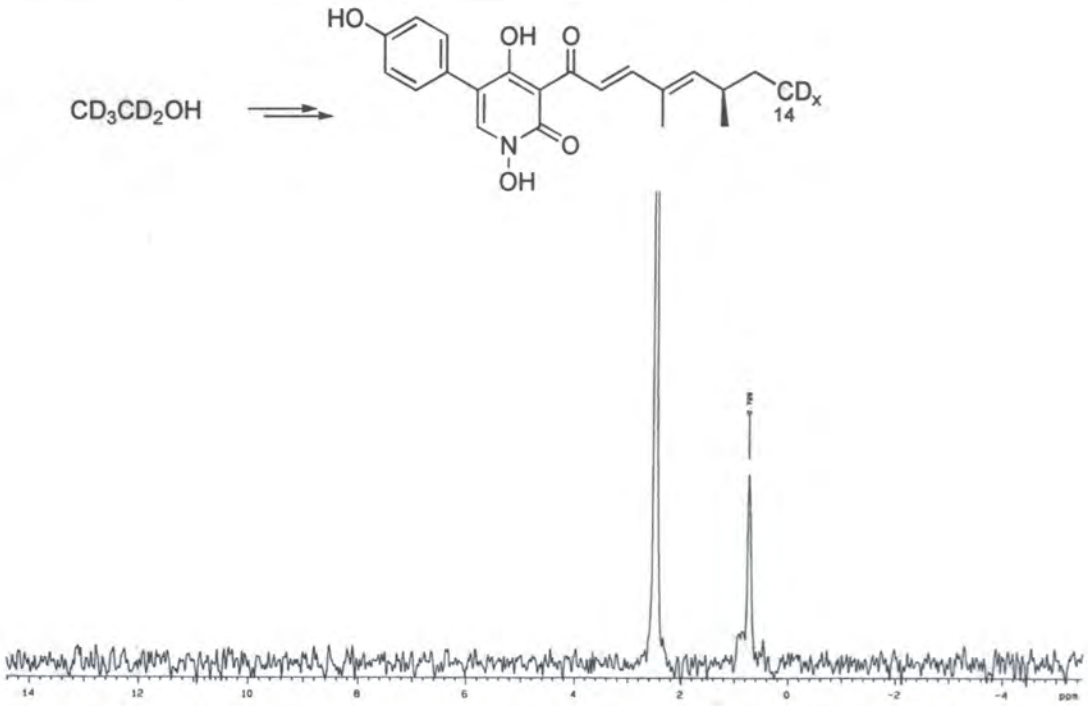
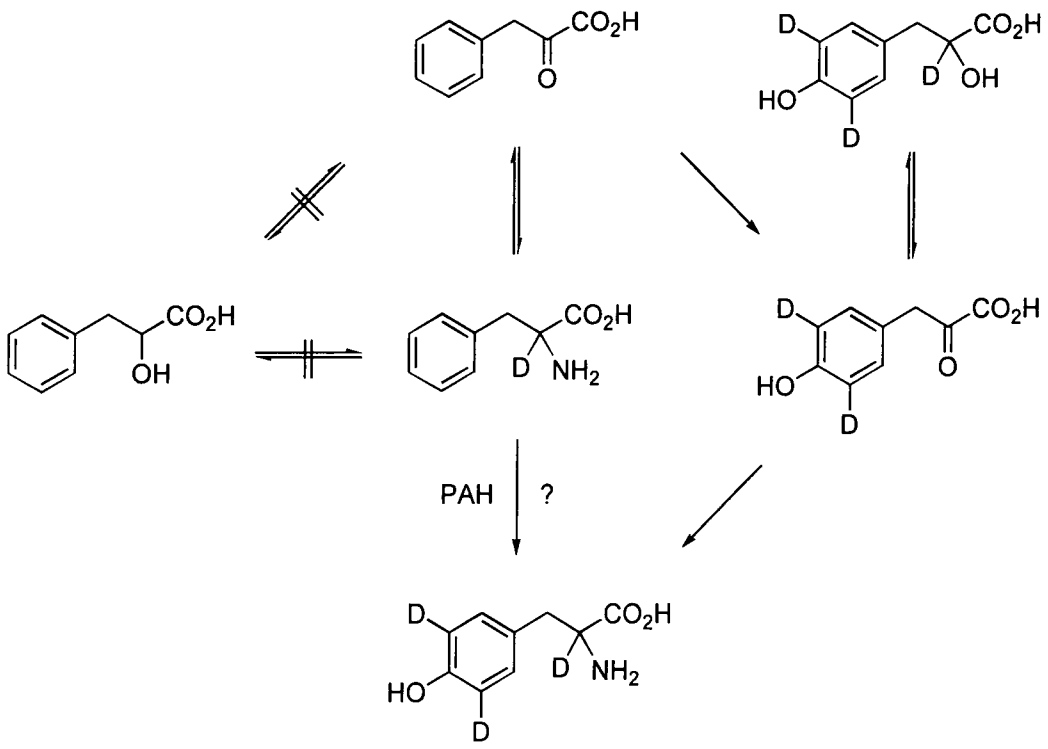


Figure 4-4 ^2H NMR of tenellin extracted from cultures fed with [1-$^2H_2$, 2-$^2H_3$]-ethanol

This enrichment is consistent with deuterium incorporation at C-14 of tenellin, the methyl group of the acetate starter unit of the polyketide backbone. Incorporation at this site implies metabolic oxidation of the administered ethanol to acetate. It is noteworthy that there is no incorporation into any of the other acetate derived carbons of the polyketide chain. The requirement for activation to malonyl CoA in those cases perhaps effects rapid exchange of the acidic deuterium atoms with the medium and hence lead to isotope wash-out.

Although some interesting results have been obtained from the experiments described in this chapter, the biosynthetic origin of the H-6 proton of tenellin remains unclear.



Scheme 4-6

In light of the feeding experiments with phenylalanine, tyrosine, phenyllactate and *p*-hydroxyphenyllactate, a current working hypothesis describing the metabolic relationships between these metabolites can be formulated as summarised in Scheme 4-6. The route by which phenylalanine is incorporated into tenellin *via* tyrosine does not appear to be straightforward and is an area that requires further study.

Chapter 5

5 Experimental

5.1 General methods

NMR spectra were recorded on either a Varian Gemini Mercury 200 (^{1}H at 199.991 MHz, ^{13}C at 50.288 MHz), a Varian Gemini Unity 300 (^{1}H at 299.908 MHz, ^{13}C at 75.412 MHz), a Varian VXR 400S (^{1}H at 399.970 MHz, ^{13}C at 100.572 MHz) or a Varian 500 (^{1}H at 499.779 MHz, ^{13}C at 125.683 MHz). Chemical shifts are quoted in ppm relative to $CDCl_3$, D_2O, DMSO or CD_3OD. Coupling constants are given in Hz. IR spectra were recorded neat or as KBr discs on a Perkin Elmer Paragon 1000 or FT 1720X machine. Mass spectra were acquired using a VG Analytical 7070E mass spectrometer operating at 70 eV. High resolution mass spectra were recorded on a VG-Zab spectrometer. HPLC analysis was performed on a Varian Star 9012 solvent delivery pump and peaks recorded by a Varian Star 9050 variable wavelength UV-VIS detector at 254 nm. LCMS analysis was performed by injection using a Gilson autosampler and a Hewlett Packard 1050 pump, and spectra recorded using a finnagan Matt MS TSQ7000. A Hypersil BDS C-18 5 μ column (250 x 4.6 mm) was used for HPLC and LCMS analysis. Melting points were recorded using Gallenkamp apparatus and are uncorrected. Reactions were performed under nitrogen gas. Solvents were routinely dried and distilled under nitrogen prior to use. THF and diethyl ether were distilled from sodium benzophenone, dichloromethane from calcium hydride and ethanol and methanol from magnesium turnings and iodine. Flash column chromatography was performed over Fluka silica gel-60 (35-70 μm) and ion exchange chromatography over 50WX8-400 Dowex-H^+.

5.2 Production, isolation and analysis of tenellin

5.2.1 Growth of *Beauveria bassiana*

The culture of *Beauveria bassiana* (Bals.) Vuill. (No. 110.25) was obtained from the CBS culture collection, Oosterstrat, Delft, Netherlands.

Regularly, growth was initiated by the transfer of a small amount of *Beauveria bassiana*, from a fungal slant stored at 4 °C, into a 250 ml Erlenmeyer flask containing 50 ml of sterile production medium. The flask was then shaken in the dark at 200 rpm (3 cm eccentricity) and 32 °C. After 10 days, a 2 ml aliquot of this production medium was used to inoculate another sterile production medium flask, which was incubated under the same conditions. New production medium flasks were subcultured from 10 day old production medium flasks. Labelled substrates were added to cultures on day three after inoculation, or on days 3, 4 and 5 for pulse feeding experiments, immediately prior to tenellin production indicated by a vivid yellow colouration. The cultures were incubated for another seven days before harvesting the tenellin produced.

5.2.2 Production medium

To distilled water (990 ml) was added D-mannitol (50 g), KNO_3 (5 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), NaCl (0.1 g), $CaCl_2$ (0.1 g), $FeSO_4.7H_2O$ (20 mg) and a mineral ion solution (10 ml) containing $CuSO_4.5H_2O$ (40 mgl^{-1}), $B(OH)_3$ (6 mgl^{-1}), $(NH_4)_6Mo_7O_{24}.4H_2O$ (4 mgl^{-1}), $MnSO_4.H_2O$ (7.5 mgl^{-1}) and $ZnSO_4.7H_2O$ (880 mgl^{-1}). 50 ml portions were sterilised in 250 ml Erlenmeyer flasks plugged with cotton wool.

5.2.3 Isolation of tenellin

The mycelia of *Beauveria bassiana* were obtained by centrifugation (14,000 rpm, 20 min, 20 °C), washed with water and then exhaustively extracted by Soxhlet extraction

with acetone. Reduction *in vacuo* of the yellow extracts yielded an orange solid, which was dissolved in dichloromethane and repeatedly washed with brine to remove sugars and other water solubles. 1M HCl was added to assist in the separation of the layers. The organic phase was dried ($MgSO_4$) and the dichloromethane removed at the rotary evaporator. The yellow/orange solid was then triturated with hexane and filtered to give tenellin as a yellow solid. 10-30 mg of tenellin were produced from each flask

5.2.4 Analysis of tenellin

NMR spectra of tenellin were recorded in DMSO-d_6 for ^1H and ^{13}C NMR and DMSO or CH_3OH for ^2H NMR.

δ_H(250.1 MHz; DMSO-d_6) 0.84 (3H, t, J 7.2, **H**-14), 0.99 (3H, d, J 6.4, **H**-15), 1.37 (2H, m, **H**-13), 1.86 (3H, s, **H**-16), 2.51 (1H, m, **H**-12), 6.00 (1H, d, J 9.3, **H**-11), 6.79 (2H, d, J 8.2, **Ph**), 7.30 (2H, d, J 8.1, **Ph**), 7.55 (1H, d, J 15.3, **H**-9), 8.01 (1H, d, J 15.4, **H**-8), 8.19 (1H, s, **H**-6), 9.55 (1H, s, O**H**), 10.35 (1H, s, O**H**), 11.75 (1H, bs, O**H**).

δ_C(100.6 MHz; DMSO-d_6) 11.9 (**C**-14), 12.5 (**C**-16), 20.0 (**C**-15), 29.7 (**C**-13), 34.7 (**C**-12), 106.0 (**C**-3), 111.0 (**C**-5), 115.2 (**C**-3', 5'), 123.1 (**C**-1'), 124.5 (**C**-8), 130.4 (**C**-2', 6'), 132.7 (**C**-10), 140.4 (**C**-6), 150.1 (**C**-9), 151.3 (**C**-11), 156.3 (**C**-4'), 157.6 (**C**-2), 172.2 (**C**-4), 193.9 (**C**-7).

5.3 Feeding experiments

5.3.1 Feeding of [1-^{13}C]-phenylalanine 73b

[1-^{13}C]-Phenylalanine (137 mg, 0.82 mmol) was added to distilled water and the pH adjusted to pH 6. An aliquot (5 ml) of the solution was added to a different production flask of *B. bassiana* on each of days 0, 3 and 4. Three batches of tenellin were extracted on days 10, 11 and 12. δ_C(DMSO-d_6) 172.7 (^{13}C-4, enriched). Incorporations were measured at 5.9, 6.4 and 4.8 % for tenellin isolated on days 10, 11 and 12 respectively.

5.3.2 Feeding of [3-^{13}C]-tyrosine 111d

[3-^{13}C]-Tyrosine (150 mg, 0.82 mmol) was added to distilled water and the pH adjusted to pH 6. An aliquot (5 ml) of the solution was added to a different production flask of *B. bassiana* on each of days 3, 4 and 5. Three batches of tenellin were extracted on days 10, 11 and 12 respectively. δ_C(DMSO-d_6) 111.1 (^{13}C-5, enriched). Incorporations were measured at 5.8, 3.8 and 3.8 % for tenellin isolated on days 10, 11 and 12 respectively.

5.3.3 Feeding of [3-^{13}C]-3-amino-2-(4-hydroxyphenyl)propionic acid 112a

[3-^{13}C]-3-Amino-2-(4-hydroxyphenyl)propionic acid (100 mg, 0.55 mmol) was administered to cultures of *Beauveria bassiana* on days 3, 4 and 5 to a final concentration of 5 mmol. Tenellin (25 mg) was extracted on day 10 and analysed by ^{13}C NMR. No incorporation could be seen in the spectra recorded.

5.3.4 Feeding of [3',5'-$^2H_2$]-3-amino-2-(4-hydroxyphenyl)propionic acid 112b

[3',5'-$^2H_2$]-3-Amino-2-(4-hydroxyphenyl)propionic acid (98 mg, 0.54 mmol) was fed to cultures on days 3, 4 and 5 to a final concentration of 5 mmol. The tenellin produced (30 mg) was extracted on day 10. ^2H NMR analysis of the metabolite showed no apparent deuterium incorporation.

5.3.5 Feeding of [3-^{13}C]-3-hydroxy-2-(4-hydroxyphenyl)propionic acid 118a

[3-^{13}C]-3-Hydroxy-2-(4-hydroxyphenyl)propionic acid (37 mg, 0.20 mmol) was administered to cultures on days 3, 4 and 5 after subculture to a final concentration of 3 mmol. After 10 days, tenellin (31 mg) was extracted and subjected to ^{13}C NMR. There was no enhancement of any peaks in the NMR spectrum and hence no incorporation of the synthesised material.

5.3.6 Feeding of 5-(4-(hydroxy)-[3',5'-$^2H_2$]-benzyl-3-(*E*,*E*-4,6-dimethylocta-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 177a

5-(4-(Hydroxy)-[3',5'-$^2H_2$]-benzyl-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one (25 mg, 0.07 mmol) was dissolved in ethanol and administered to cultures of 3-day old *Beauveria bassiana*. On day 10 the mycelia were subjected to Soxhlet extraction and the tenellin produced analysed by ^2H NMR. Two feeding experiments were performed simultaneously and each worked up separately to give two batches of tenellin (25 and 17 mg).

5.3.7 Feeding of [2-$^2H_1$]-4-hydroxyphenyllactic acid 249a

[2-$^2H_1$]-4-Hydroxyphenyllactic acid (95 mg, 0.52 mmol) was dissolved in distilled water (3 ml). The solution was pulse fed to two production medium cultures on days 3, 4 and 5 to a final concentration, in each flask, of 5 mmol. Tenellin (40 mg) was isolated after extraction on day 10. No observable enrichment could be seen by ^2H NMR.

5.3.8 Feeding of [3',5'-$^2H_2$]-4-hydroxyphenyllactic acid 249b

[3',5'-$^2H_2$]-4-Hydroxyphenyllactic acid (95 mg, 0.52 mmol) was dissolved in distilled water (3 ml). The solution was pulse fed to two production medium cultures on days 3, 4 and 5 to a final concentration, in each flask, of 5 mmol. Isolation on day 10 gave tenellin (59 mg) as an orange/yellow powder. δ_{2H}(DMSO-d_6) 6.79 (Ph C^2H, enriched).

5.3.9 Feeding of [2,3',5'-$^2H_3$]-4-hydroxyphenyllactic acid 249c

[2,3',5'-$^2H_3$]-4-Hydroxyphenyllactic acid (96 mg, 0.52 mmol) was dissolved in distilled water (3 ml). The solution was pulse fed to two production medium cultures on days 3, 4 and 5 to a final concentration, in each flask, of 5 mmol. Tenellin (44 mg) was isolated after extraction on day 10. δ_{2H}(DMSO-d_6) 6.79 (Ph C^2H, enriched). No incorporation of the proton at C-2 was detected.

5.3.10 Feeding of [2-$^2H_1$]-tyrosine 111a

[2-$^2H_1$]-Tyrosine (86 mg, 0.53 mmol) was added to distilled water (1 ml) and the pH adjusted to pH 6. The solution was pulse fed to two flasks of *B. bassiana* on days 3, 4,

5 and 6 to a final concentration in each flask of 4.5 mmol. After 10 days, tenellin (50 mg) was isolated. ^{2}H NMR indicated that no incorporation had occurred.

5.3.11 Feeding of [2,3',5'-$^{2}H_3$]-tyrosine 111b

[2,3',5'-$^{2}H_3$]-Tyrosine (95 mg, 0.52 mmol) was added to distilled water (1 ml) and the pH adjusted to pH 6. The solution was pulse fed to two flasks of *B. bassiana* on days 3, 4 and 5 to a final concentration in each flask of 4.5 mmol. After 10 days, tenellin (50 mg) was isolated. δ_{2H}(DMSO-d_6) 6.76 (Ph C^{2}H, enriched). No incorporation of the 2-^{2}H deuterium was indicated.

5.3.12 Growth of *Beauveria bassiana* with [$^{2}H_1$]-sodium formate

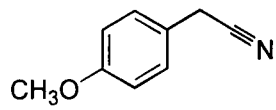
[$^{2}H_1$]-Sodium formate (35 mg, 0.5 mmol) was added to two production medium flasks at the time of subculture at a concentration of 10 mmol. On day 10, tenellin (31 mg) was isolated as an orange/yellow powder. δ_{2H}(DMSO-d_6) 0.92 (^{2}H-15, enriched), 1.79 (^{2}H-16, enriched).

5.3.13 Growth of *Beauveria bassiana* in [1-$^{2}H_2$, 2-$^{2}H_3$]-ethanol

[1-$^{2}H_2$, 2-$^{2}H_3$]-Ethanol (0.03 ml) was added to two production medium flasks at the time of subculture at a concentration of 10 mmol. On day 10, tenellin (20 mg) was isolated as an orange/yellow powder. δ_{2H}(DMSO-d_6) 0.73 (^{2}H-14, enriched).

5.4 Synthesis of 3-amino-4-(hydroxy)phenylpropionic acid 112

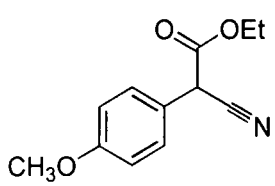
5.4.1 4-Methoxyphenylacetonitrile 114[154]



To a stirred solution of 4-methoxybenzyl chloride **113** (2.0 g, 12.8 mmol) in dry acetone (10 ml) was added finely ground and dried (105 °C) sodium cyanide (939 mg, 19.2 mmol) and sodium iodide (72 mg, 0.48 mmol). The mixture was heated to reflux for 12 h and then the solution was filtered to remove any unreacted cyanide. The acetone was removed *in vacuo* to yield a brown oil which was purified over silica gel (petroleum ether: diethyl ether, 3:1) to give a yellow oil (1.60 g, 85.2 %).

δ_H(200 MHz; $CDCl_3$) 3.69 (2H, s, C**H**$_2$), 3.81 (3H, s, OC**H**$_3$), 6.90 (2H, d, J 8.6, **Ph**), 7.25 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 22.8 (**C**H$_2$), 55.3 (O**C**H$_3$), 114.5 (Ph **C**H), 118.1 (**C**N), 121.7 (Ph **C**), 129.1 (Ph **C**H), 159.3 (Ph **C**OCH$_3$); υ_{max}(neat)/cm^{-1} 3036, 3003, 2958, 2936, 2837, 2248 (CN), 1613, 1586, 1513, 1251, 813; m/z (EI) 148 ([M+H]$^+$, 37.1 %), 147 ([M]$^+$, 100 %), 121 ([M-CN]$^+$, 65.6 %), 107 ([M-CH$_2$CN]$^+$, 17.3 %), 78 ([PhH]$^+$, 40.3 %), 77 ([Ph]$^+$, 65.6 %).

5.4.2 Cyano–(4-methoxyphenyl)acetic acid ethyl ester 115[84]

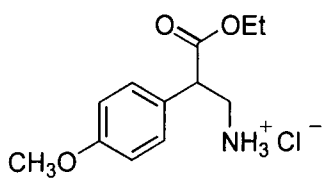


To a stirred suspension of NaH (60% in mineral oil, 272 mg, 6.80 mmol) in dry tetrahydrofuran (THF) (20 ml) at 0 °C was added 4-methoxyphenylacetonitrile **114** (500 mg, 3.40 mmol) and the mixture stirred for 30 min. Diethyl carbonate (522 mg, 4.42

mmol) was then added and the reaction mixture heated to reflux for 4 h. The reaction was quenched with 1M HCl (50 ml) and the THF removed *in vacuo*. The aqueous solution was extracted with ethyl acetate (3x 30 ml), washed with brine (3x 15 ml) and dried ($MgSO_4$). Evaporation gave the crude product, which was purified over silica gel (hexane:diethyl ether, 4:3) to yield cyano-(4-methoxyphenyl)acetic acid ethyl ester as a yellow oil (480 mg, 64.4 %).

δ_H(200 MHz; $CDCl_3$) 1.28 (3H, t, J 7.0, CH_2CH_3), 3.82 (3H, s, $ArOCH_3$), 4.22 (2H, q, J 7.4, CH_2CH_3), 4.65 (1H, s, **CH**), 6.90 (2H, d, J 8.8, **Ph**), 7.38 (2H, d, J 8.6, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 13.9 (CH_2CH_3), 42.9 (**CH**), 55.3 (OCH_3), 63.2 (CH_2CH_3), 114.6 (Ph **CH**), 115.9 (**CN**), 121.8 (Ph **C**), 129.1 (Ph **CH**), 160.1 (Ph **C**OCH_3), 165.2 (**CO**); υ_{max}(neat)/cm^{-1} 2982, 2833, 2251 (CN), 1737 (CO), 1608, 1584, 1510, 1248, 1026; m/z (EI) 219 ($[M]^+$, 15.3 %), 147 ($[M+H-CO_2Et]^+$, 33.1 %) 146 ($[M-CO_2Et]^+$, 100.0 %), 132 ($[M+H-CO_2Et-CH_3]^+$, 18.5 %), 116 ($[BnCN]^+$, 7.5 %), 78 ($[Ph+H]^+$, 4.5 %), 77 ($[Ph]^+$, 7.3 %), 29 ($[CH_2CH_3]^+$, 50.9 %).

5.4.3 Ethyl 3-amino-2-(4-methoxyphenyl)propionate hydrochloride 116[155]

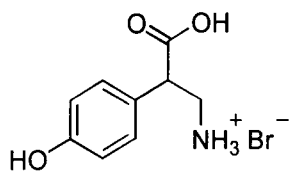


A solution of cyano-(4-methoxyphenyl)acetic acid ethyl ester **115** (814 mg, 3.72 mmol) in ethanol (40 ml) and chloroform (8.2 ml) was shaken under H_2 (2.5 atm) with platinum oxide (40 mg) for 18 h. The catalyst was removed by filtration and the solvent removed *in vacuo* to give a solid which was washed with diethyl ether (20 ml), collected by filtration and then dried to give the product as a white powder (916 mg, 95.0 %), mp *ca* 230°C (decomp.).

δ_H(200 MHz; D_2O) 1.06 (3H, t, J 7.2, CH_2CH_3), 3.25 (1H, dd, J_{vic} 7.8, J_{gem} 13.5, 3-H_B), 3.49 (1H, dd, J_{vic} 7.2, J_{gem} 13.3, 3-H_A), 3.70 (3H, s, OCH_3), 3.94 (1H, t, J 7.2, **CH**), 4.07 (2H, q, J 7.2, CH_2CH_3), 6.92 (2H, d, J 8.8, **Ph**), 7.17 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz;

D_2O) 13.0 ($CH_2\mathbf{C}H_3$), 40.9 ($\mathbf{C}H_2$), 47.8 (**C**H), 55.3 ($O\mathbf{C}H_3$), 62.8 ($\mathbf{C}H_2CH_3$), 114.9 (Ph **C**H), 126.4 (Ph **C**), 129.5 (Ph **C**H), 159.1 (Ph $\mathbf{C}OCH_3$), 173.0 (**C**O); υ_{max}(neat)/cm^{-1} 3100 (br NH_3^+), 2926, 2852, 1724 (CO), 1599, 1508, 1365, 1205, 825; m/z (EI) 224 ($[M+H]^+$, 1.2 %), 223 ($[M]^+$, 4.6 %), 195 ($[M+H-CH_2CH_3]^+$, 12.1 %), 194 ($[M-CH_2CH_3]^+$, 100.0 %), 150 ($[M-CO_2CH_2CH_3]^+$,19.3 %), 121 ($[Bn(OCH_3)]^+$, 46.2 %), 92 ($[BnH]^+$, 4.9 %), 91 ($[Bn]^+$, 21.3 %) 78 ($[PhH]^+$, 11.4 %), 77 ($[Ph]^+$, 29.5 %); *Anal.* Calcd for $C_{12}H_{18}NO_3Cl$: C, 55.49; H, 6.98; N, 5.39. Found: C 55.29, H 7.01, N 5.30.

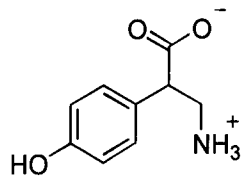
5.4.4 3-Amino-2-(4-hydoxyphenyl)propionate hydrobromide 112[155]



Ethyl 3-amino-2-(4-methoxyphenyl)propionate hydrochloride **116** (500 mg, 1.93 mmol) was stirred under reflux for 2 h in a solution of 48 % hydrogen bromide (5 ml) in acetic acid (5 ml). After cooling, evaporation *in vacuo* yielded the product as a pale brown solid (105 mg, 29.7 %), mp *ca* 230 °C (decomp.) (lit. 242-244 °C).[156]

δ_H(299.9 MHz; D_2O) 3.15 (1H, dd, J_{vic} 7.8, J_{gem} 12.8, 3-$\mathbf{H}_A$), 3.38 (1H, dd, J_{vic} 7.8, J_{gem} 12.8, 3-$\mathbf{H}_B$), 3.78 (1H, t, J 7.5, C**H**), 6.76 (2H, d, J 8.1, **Ph**), 7.07 (2H, d, J 8.7, **Ph**); δ_C(100.6 MHz; D_2O) 41.2 ($\mathbf{C}H_2$), 48.1 (**C**H), 116.1 (Ph **C**H), 126.4 (Ph **C**), 129.6 (Ph **C**H), 155.7 (Ph **C**OH), 175.4 (**C**O); υ_{max}(neat)/cm^{-1} 3455 (NH), 3020 (br, OH), 1722, 1592, 1479, 1214, 833.

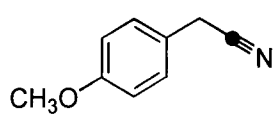
5.4.5 3-Amino-2-(4-hydoxyphenyl)propionate 112[155]



Ethyl 3-amino-2-(4-methoxyphenyl)propionate hydrochloride **116** (456 mg, 1.76 mmol) was stirred in dichloromethane (DCM) (13 ml) at -78 °C and 1M boron tribromide in DCM (4.5 ml, 4.50 mmol) was added. The solution was stirred overnight and allowed to warm to room temperature. 1M NaOH (5 ml) was added, the mixture stirred for 1 h and then neutralised. Evaporation *in vacuo* gave a brown solid, which was purified by ion-exchange chromatography over 50WX8-400 Dowex-H^+, using 1M NaOH as the eluent. Recrystallisation ($EtOH/H_2O$) gave the product as a white solid (139 mg, 36.4 %), mp *ca* 220 °C (decomp.) (lit. 242-244 °C).[156]

δ_H(200 MHz; D_2O) 2.82 (1H, dd, J_{vic} 7.4, J_{gem} 12.6, 3-$\mathbf{H_A}$), 3.06 (1H, dd, J_{vic} 7.6, J_{gem} 12.6, 3-$\mathbf{H_B}$), 3.40 (1H, t, J 7.6, C**H**), 6.62 (2H, d, J 8.4, **Ph**), 7.08 (2H, d, J 8.4, **Ph**); δ_C (50.3 MHz; D_2O) 41.1 (**C**H_2), 48.1 (**C**H), 115.2 (Ph **C**H), 126.7 (Ph **C**), 129.8 (Ph **C**H), 155.4 (Ph **C**OH), 173.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3450 (br), 2992, 2942, 2840, 1740, 1612, 1513, 1268, 834.

5.4.6 [1-^{13}C]-4-Methoxyphenylacetonitrile 114a[157]

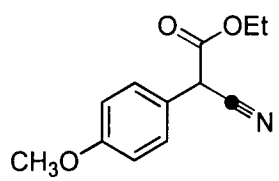


To a stirred solution of 4-methoxybenzyl chloride **113** (2.0 g, 12.8 mmol) in dry acetone (20 ml) was added finely ground and dried (105 °C) potassium [^{13}C]-cyanide (1.10 g, 16.7 mmol, 99 atom% ^{13}C) and sodium iodide (72 mg, 0.48 mmol). The mixture was heated under reflux for 12 h and then the solution was filtered to remove any unreacted cyanide. The acetone was reduced *in vacuo* to yield an orange oil which was purified

over silica gel (petroleum ether: ethyl acetate, 3:1) to give the product as a colourless oil (1.78 g, 94.1 %).

δ_H(499.8 MHz; $CDCl_3$) 3.69 (2H, d, J 11.0, $\mathbf{CH_2}$), 3.82 (3H, s, $OC\mathbf{H_3}$), 6.90 (2H, d, J 8.5, **Ph**), 7.23 (2H, d, J 8.6, **Ph**); δ_C(125.7 MHz; $CDCl_3$) 22.6 (d, J_{cc} 57.9, $\mathbf{C}H_2$), 55.3 (OCH_3), 114.5 (Ph **C**H), 118.2 (**C**N, enriched), 121.8 (Ph **C**), 129.1 (Ph **C**H), 159.3 (Ph **C**OCH_3); υ_{max}(neat)/cm^{-1} 3040, 2930, 2837, 2191 (CN), 1613, 1586, 1513, 1249, 812; m/z (EI) 149 ($[M+H]^+$, 9.5 %), 148 ($[M]^+$, 100.0 %), 147 ($[M-H]^+$, 54.6 %), 133 ($[M-CH_3]^+$, 47.7 %), 117 ($[M-OCH_3]^+$, 29.0 %), 107 ($[M-CH_2{}^{13}CN]^+$, 30.4 %), 77 ($[Ph]^+$, 50.0 %), 27 ($[{}^{13}CN]^+$, 3.3 %); Found 148.0718, C_9H_9NO (M^+) requires 148.0718.

5.4.7 [3-^{13}C]-Cyano–(4-methoxyphenyl)acetic acid ethyl ester 115a

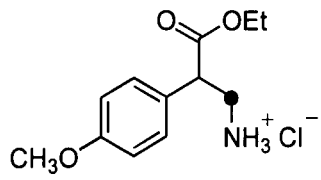


To a suspension of NaH (60 % in mineral oil, 169 mg, 4.4 mmol) in dry THF (5 ml) at 0 °C was added [1-^{13}C]-4-methoxyphenylacetonitrile **114a** (501 mg, 3.39 mmol) and the mixture stirred for 30 min. Diethyl carbonate (4.9 ml, 33.6 mmol) was added and the reaction mixture refluxed for 1 hour. The reaction was quenched with 1M HCl (10 ml) and the THF removed *in vacuo*. The aqueous solution was extracted into ethyl acetate (3x 15 ml), the organic layer was washed with brine (3x 10 ml) and dried ($MgSO_4$). Evaporation gave a residue, which was purified by kugelrohr distillation (~100 °C, 0.75 mmHg), to yield the product as a colourless oil (410 mg, 55.1 %).

δ_H(400 MHz; $CDCl_3$) 1.28 (3H, t, J 7.2, $CH_2C\mathbf{H_3}$), 3.82 (3H, s, $OC\mathbf{H_3}$), 4.24 (2H, 2x q, J 7.2, $C\mathbf{H_2}CH_3$), 4.65 (1H, d, J 10.4, C**H**), 6.93 (2H, d, J 8.8, **Ph**), 7.37 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 13.9 ($CH_2\mathbf{C}H_3$), 42.9 (d, J_{cc} 62.2, **C**H), 55.4 (OCH_3), 63.2 ($\mathbf{C}H_2CH_3$), 114.7 (Ph **C**H), 115.88 (**C**N, enriched), 118.4 (Ph **C**), 129.1 (Ph **C**H), 160.2 (Ph **C**OCH_3), 165.3 (**C**O); υ_{max}(neat)/cm^{-1} 3024, 2983, 2840, 2198 (CN), 1745 (CO), 1601, 1587, 1513, 1255, 836; m/z (EI) 220 ($[M]^+$, 17.3 %), 148 ($[M-CO_2CH_2CH_3]^+$,

27.5 %), 147 ([M-H-$CO_2CH_2CH_3$]$^+$, 100.0 %), 133 ([M+H-$CO_2CH_2CH_3$-CH_3]$^+$, 17.5 %), 91 ([Bn]$^+$, 8.2 %), 77 ([Ph]$^+$, 7.5 %), 29 ([CH_2CH_3]$^+$, 7.3 %); Found 220.0929, $C_{12}H_{13}NO$ (M^+) required 220.0929.

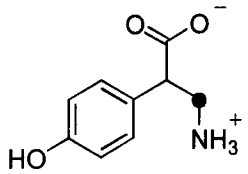
5.4.8 Ethyl [3-^{13}C]-3-amino-2-(4-methoxyphenyl)propionate hydrochloride 116a



A solution of [3-^{13}C]-cyano-(4-methoxyphenyl)acetic acid ethyl ester **115a** (663 mg, 3.01 mmol) in ethanol (33 ml) and chloroform (6.6 ml) was shaken under H_2 (2.5 atm) with platinum oxide (33 mg) for 64 h. The catalyst was then filtered off and the solvent removed *in vacuo* to give a solid which was triturated with diethyl ether, collected by filtration and dried to give the product as a white powder (734 mg, 93.5 %), mp *ca* 230°C (decomp.).

δ_H(200 MHz; D_2O) 1.04 (3H, t, J 7.2, CH_2CH_3), 3.27 (1H, ddd, J_{vic} 7.6, J_{gem} 13.3, J_{13C-H} 147.1, 3-H_A), 3.51 (1H, ddd, J_{vic}7.6, J_{gem} 13.3, J_{13C-H} 147.1, 3-H_B), 3.69 (3H, s, OCH_3), 3.87 (1H, m, **CH**), 4.06 (2H, q, J 7.2, CH_2CH_3), 6.90 (2H, d, J 8.4, **Ph**), 7.17 (2H, d, J 8.4, **Ph**); δ_C(100.6 MHz; D_2O) 12.7 (CH_2CH_3), 40.8 (CH_2, enriched), 47.8 (d, J_{CC} 40.1, **C**H), 55.1 (OCH_3), 62.5 (CH_2CH_3), 114.8 (Ph **C**H), 126.4 (Ph **C**), 129.6 (Ph **C**H), 158.9 (Ph **C**OCH_3), 173.1 (**C**O).

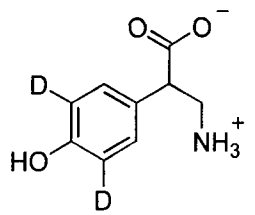
5.4.9 [3-^{13}C]-3-amino-2-(4-hydoxyphenyl)propionate 112a



Ethyl [3-^{13}C]-3-amino-2-(4-methoxyphenyl)propionate hydrochloride **116a** (100 mg, 0.38 mmol) was stirred in DCM (3 ml) at -78 °C and a solution of boron tribromide (1M in DCM, 1 ml, 1.00 mmol) added. The solution was stirred for 12 h and allowed to warm to room temperature. 1M NaOH (2 ml) was added and the mixture stirred for 1 h. The solution was then neutralised and evaporated *in vacuo* to give a brown solid, which after purification over Dowex-H^+ yielded the product as a white solid (35 mg, 41.7 %), mp *ca* 230 °C (decomp.).

δ_H(299.9 MHz; D_2O) 3.11 (1H, ddd, J_{vic} 7.5, J_{gem} 12.9, J_{13C-H} 145.4, 3-$\mathbf{H_A}$), 3.26 (1H, ddd, J_{vic} 7.5, J_{gem} 13.2, J_{13C-H} 145.7, 3-$\mathbf{H_B}$), 3.53 (1H, m, **CH**), 6.74 (2H, d, J 8.1, **Ph**), 7.04 (2H, d, J 7.5, **Ph**); δ_C(50.3 MHz; D_2O) 42.5 ($\mathbf{C}H_2NH_2$, enriched), 50.6 (d, J_{CC} 36.9, **C**H), 116.1 (Ph **C**H), 129.2 (Ph **C**), 129.6 (Ph **C**H), 155.2 (Ph **C**OH), 178.7 (**C**O); υ_{max}(KBr)/cm^{-1} 3404 (br), 3260, 2979, 2928, 1729, 1511, 1259, 816.

5.4.10 [3',5'-$^2H_2$]-3-amino-2-(4-hydoxyphenyl)propionate 112b



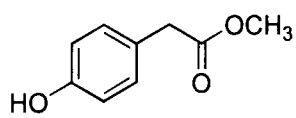
A solution of ethyl 3-amino-2-(4-methoxyphenyl)propionate **116** (99 mg, 0.46 mmol) in D_2O (2 ml) and 20% DCl/D_2O (4 ml) was placed in a Carius tube. After freezing with liquid nitrogen, the tube was evacuated and then allowed to warm to room temperature. This was repeated twice to ensure that all dissolved gases were removed. Finally the tube was cooled, evacuated again and then sealed before inserting into a strong, metal

pipe. This was then placed into the furnace. After 3 h at 80 °C, and once the furnace had cooled, the pipe was removed. The Carius tube was cooled with liquid nitrogen and then opened. Evaporation *in vacuo* yielded the product as a cream coloured solid (77 mg, 64.2 %). No further purification was attempted, mp *ca* 230 °C (decomp.).

δ_H(200 MHz; D_2O) 3.15 (1H, dd, J_{vic} 7.4, J_{gem} 13.2, 3-$\mathbf{H_A}$), 3.31 (1H, dd, J_{vic} 7.6, J_{gem} 13.2, 3-$\mathbf{H_B}$), 3.78 (1H, t, J 8.6, C**H**), 7.09 (2H, s, **Ph**); δ_C(100.6 MHz; D_2O) 41.0 (**C**H_2), 48.1 (**C**H), 116.1 (t, J 25.8, Ph **C**^{2}H), 126.5 (Ph **C**), 130.06 (Ph **C**H), 155.8 (Ph **C**OH), 175.8 (**C**O); υ_{max}(neat)/cm^{-1} 3403 (br NH & OH) 1724, 1588, 1473, 1217, 834.

5.5 Synthesis of 3-hydroxy-4-(hydroxy)phenylpropionic acid 118

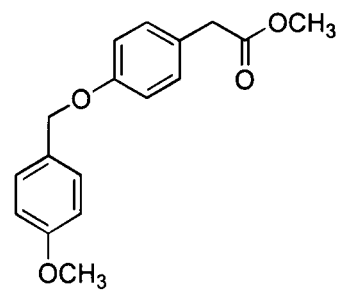
5.5.1 4-Hydroxyphenylacetic acid methyl ester 138[158]



A few drops of H_2SO_4 were added to a solution of 4-hydroxyphenylacetic acid **137** (2.0 g, 13 mmol) in dry methanol (20 ml). The mixture was refluxed for 12 h and then reduced *in vacuo* to give a brown oil. The residue was dissolved in diethyl ether (50 ml) and washed with saturated Na_2CO_3 solution (3x 25 ml) to remove any unreacted acid. After drying ($MgSO_4$), reduction *in vacuo* gave a brown oil (1.64 g, 75.1 %).

δ_H(250 MHz; $CDCl_3$) 3.48 (2H, s, C$\mathbf{H_2}$), 3.62 (3H, s, C$\mathbf{H_3}$), 6.65 (2H, d, J 8.8, **Ph**), 7.0 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 40.2 (**C**H_2), 52.1 (O**C**H_3), 115.5 (Ph **C**H), 125.8 (Ph **C**), 130.4 (Ph **C**H), 154.8 (Ph **C**OH), 172.8 (**C**O); υ_{max}(neat)/cm^{-1} 3355 (br, OH), 3027, 2949, 1704 (CO), 1615, 1514, 1340, 1215, 1147, 850; m/z (EI) 167 ([M+H]$^+$, 2.2 %), 166 ([M]$^+$, 27.6 %), 108 ([Bn(OH)+H]$^+$, 6.3 %), 107 ([Bn(OH)]$^+$, 100.0 %), 77 ([Ph]$^+$, 30.2 %), 59 ([CO_2CH_3]$^+$, 7.6 %).

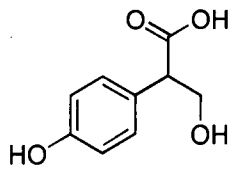
5.5.2 2-(4-(4-Methoxybenzyloxy)-phenyl)acetic acid methyl ester 139



A mixture of 4-hydroxyphenylacetic acid methyl ester **138** (1.0 g, 6.02 mmol), tetrabutylammonium iodide (2.43 g, 6.59 mmol), 4-methoxybenzyl chloride (1.09 g, 6.96 mmol) and potassium carbonate (1.81 g, 13.12 mmol) in dry acetone (50 ml) was stirred at 60 °C for 48 h. After this time, the reaction mixture was allowed to cool to room temperature and the acetone was removed *in vacuo*. Water (50 ml) was then added and the solution was extracted into diethyl ether (3x 50 ml). The combined organic extracts were washed with 1M NaOH (1x 20 ml) and brine (1x 20 ml) and then dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a yellow/brown solid, which was recrystallised from ethanol to generate the product as a white crystalline solid (0.98 g, 56.9 %), mp 60 °C.

δ_H(250 MHz; $CDCl_3$) 3.57 (2H, s, $\mathbf{CH_2}CO_2CH_3$), 3.68 (3H, s, $CO_2\mathbf{CH_3}$), 3.82 (3H, s, $ArO\mathbf{CH_3}$), 4.97 (2H, s, $\mathbf{CH_2}O$), 6.90 (2H, d, J 2.0, **Ph**), 6.93 (2H, d, J 2.0, **Ph**), 7.20 (2H, d, J 8.0, **Ph**), 7.33 (2H, d, J 8.0, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 40.3 ($\mathbf{C}H_2$), 52.0 ($CO_2\mathbf{C}H_3$), 55.3 ($ArO\mathbf{C}H_3$), 69.8 ($\mathbf{C}H_2O$), 114.0 (Ph **C**H), 114.9 (Ph **C**H), 126.2 (Ph **C**), 129.0 (Ph **C**), 129.2 (Ph **C**H), 130.3 (Ph **C**H), 157.5 (Ph **C**O), 159.4 (Ph **C**O), 172.3 (**C**O); υ_{max}(neat)/cm^{-1} 3004, 2951, 2836, 1738 (CO), 1611, 1511, 1241, 1152, 830; m/z (EI) 287 ($[M+H]^+$, 0.4 %), 286 ($[M]^+$, 1.8 %), 166 ($[M+H-Bn(OCH_3)]^+$, 4.0 %), 121 ($[Bn(OCH_3)]^+$, 100.0 %), 107 ($[Bn(OH)]^+$, 14.5 %), 78 ($[PhH]^+$, 5.8 %), 77 ($[Ph]^+$, 4.9 %), 59 ($[CO_2CH_3]^+$, 1.5 %).

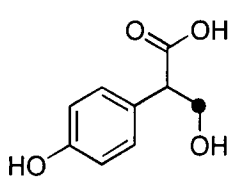
5.5.3 3-Hydroxy-2-(4-hydroxyphenyl)propionic acid 118



3-Amino-2-(4-hydroxyphenyl)propionic acid **112** (100 mg, 0.55 mmol) was stirred in water (1 ml) and cooled to 0 °C. 2M H_2SO_4 (0.5 ml) and 2M sodium nitrite solution (0.5 ml) were added and the mixture was stirred at 0 °C for 3 h and then at room temperature for 12 h. The reaction mixture was extracted into EtOAc (3x 10 ml), the combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to yield a brown oil. 1M NaOH (5 ml) was added and the solution stirred at room temperature for 1 h to hydrolyse the lactone formed and give the desired product as a brown oil (41 mg, 40.7 %).

δ_H(299.9 MHz; D_2O) 3.45 (1H, t, J 7.8, C**H**), 3.65 (1H, dd, J_{vic} 7.5, J_{gem} 10.8, 3-$\mathbf{H_B}$), 3.87 (1H, dd, J_{vic} 7.5, J_{gem} 10.8, 3-$\mathbf{H_A}$), 6.71 (2H, d, J 8.4, **Ph**), 7.07 (2H, d, J 8.4, **Ph**); δ_C(50.3 MHz; D_2O) 40.2 (**C**H), 63.1 (**C**H_2OH), 116.1 (Ph **C**H), 129.5 (Ph **C**), 129.7 (Ph **C**H), 160.1 (Ph **C**OH), 176.1 (**C**O).

5.5.4 [3-^{13}C]-3-hydroxy-2-(4-hydroxyphenyl)propionic acid 118a



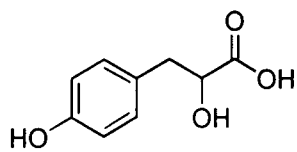
A solution of 3-[^{13}C]-amino-2-(4-hydroxyphenyl)propionic acid (52 mg, 0.29 mmol) in water (0.2 ml) was stirred and cooled to 0 °C. 2M H_2SO_4 (0.2 ml) and 2M sodium nitrite solution (0.2 ml) were added and the mixture was stirred at 0 °C for 3 h and then at room temperature for 12 h. After this time, the reaction mixture was extracted into EtOAc (3x 10 ml), the combined organic extracts were dried ($MgSO_4$) and the solvent

was removed *in vacuo* to yield a brown oil. 1M NaOH (3 ml) was added and the solution stirred for 1 h to hydrolyse the lactone formed and give the desired product as an orange/brown oil (23 mg, 44.0 %).

δ_H(299.9 MHz; D_2O) 3.63 (1H, t, J 7.4, C**H**), 3.85 (1H, m, J_{13C-H} 140.7, 3-$\mathbf{H_A}$), 4.02 (1H, m, J_{13C-H} 140.7, 3-$\mathbf{H_B}$), 6.90 (2H, d, J 8.4, **Ph**), 7.24 (2H, d, J 8.4, **Ph**); δ_C(50.3 MHz; D_2O) 39.7 (d, J_{CC} 25.3, **C**H), 63.1 (**C**H_2OH, enriched), 116.3 (Ph **C**H), 129.7 (Ph **C**), 129.8 (Ph **C**H), 157.9 (Ph **C**OH), 176.1 (**C**O).

5.6 Synthesis of lactic acids 249

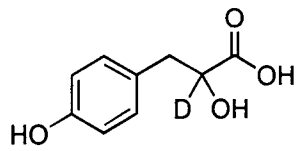
5.6.1 4-Hydroxyphenyllactic acid 249[71]



To a stirred solution of sodium borohydride ($NaBH_4$) (113 mg, 2.99 mmol) in water (7.5 ml) at 0 °C was added 4-hydroxyphenylpyruvic acid **248** (364 mg, 2.02 mmol) as an emulsion in water (15 ml). Stirring was continued at room temp for 1.5 h. Excess $NaBH_4$ was destroyed by the addition of 10 % aqueous HCl (5 ml). The aqueous solution was reduced *in vacuo* to 5 ml and extracted into ethyl acetate (3x 25 ml). The organic extracts were dried ($MgSO_4$) and the solvent was removed to produce an off-white solid (95 mg, 83.1 %).

δ_H(400 MHz; D_2O) 2.74 (1H, dd, J_{vic} 7.6, J_{gem} 14.0, 3-$\mathbf{H_A}$), 2.89 (1H, dd, J_{vic} 4.8, J_{gem} 14.0, 3-$\mathbf{H_B}$), 4.30 (1H, dd, J_{vic} 4.8, J_{vic} 7.6, C**H**), 6.67 (2H, d, J 8.4, **Ph**), 6.99 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz; D_2O) 38.8 (**C**H_2), 71.4 (**C**H), 115.5 (Ph **C**H), 128.7 (Ph **C**), 130.9 (Ph **C**H), 154.4 (Ph **C**OH), 177.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3476 (br, OH), 3219, 3026, 2952, 2927, 1723 (CO), 1615, 1599, 1513, 1233, 833;

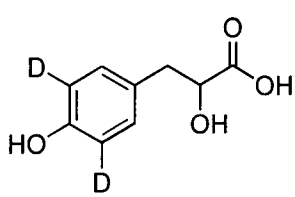
5.6.2 [2-^{2}H]-4-Hydroxyphenyllactic acid 249a[71]



[2-^{2}H]-4-Hydroxyphenyllactic acid was prepared *via* a method similar to 4-hydroxyphenyllactic acid **249** using hydroxyphenylpyruvic acid (362 mg, 2.01 mmol) in H_2O (20 ml) and sodium borodeuteride (147 mg, 3.52 mmol) in H_2O (10 ml). The product was recrystallised from ethyl acetate/chloroform to yield the product as a white powder (317 mg, 86.1 %), mp 120 °C.

δ_H(400 MHz; D_2O) 2.77 (1H, d, J 14.4, 3-$\mathbf{H}_A$), 2.92 (1H, d, J 14.4, 3-$\mathbf{H}_B$), 6.71 (2H, d, J 8.4, **Ph**), 7.03 (2H, d, J 8.4, **Ph**); δ_C(100.6 MHz; D_2O) 38.8 (**C**H_2), 71.5 (m, **C**^{2}H), 115.5 (Ph **C**H), 128.8 (Ph **C**), 130.9 (Ph **C**H), 154.4 (Ph **C**OH), 177.6 (**C**O); υ_{max}(KBr)/cm^{-1} 3474, 3256 (br), 3024, 2954, 2926, 1739 (CO), 1599, 1516, 1236, 834; m/z (EI) 183 ([M]$^+$, ^{2}H, 4.3 %), 182 ([M]$^+$, 0.2 %), 138 ([M-CO_2H]$^+$, ^{2}H, 3.2 %), 107 ([M-C^{2}H(OH)CO_2H]$^+$, 100.0 %), 77 ([Ph]$^+$, 10.1 %).

5.6.3 [3',5'-$^2H_2$]-4-Hydroxyphenyllactic acid 249b[71]

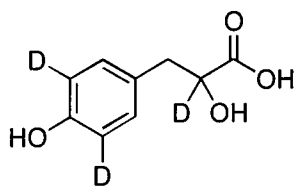


A solution of 4-hydroxyphenyllactic acid **249** (100 mg, 0.55 mmol) in 35 % DCl/D_2O (0.5 ml) and D_2O (0.5 ml) was placed in a Carius tube. The solution was treated in a similar manner to 3-amino-2-(4-hydroxyphenyl)propionate **112** (5.3.10) and then heated to 80 °C for 6 h. After cooling, the solution was saturated with NaCl and extracted into ethyl acetate (4x 10 ml). The organic extracts were dried ($MgSO_4$) and the solvent was

removed *in vacuo*. The product was recovered as a white solid (79 mg, 78.1 %). The material was found to be approximately 56 % deuterated as determined by ^{1}H NMR.

δ_H(400 MHz; D_2O) 2.76 (1H, dd, J_{gem} 7.2, J_{vic} 14.0, C**H**$_2$), 2.91 (1H, dd, J_{gem} 4.8, J_{vic} 14.4, C**H**$_2$), 4.32 (1H, dd, J_{vic} 4.8, J_{vic} 7.2, C**H**), 6.69 (44 %) (2H, d, J 8.8, **Ph**), 7.00 (56 %) (2H, s, **Ph**); δ_C(100.6 MHz; D_2O) 38.8 (**C**H$_2$), 71.4 (**C**H), 115.5 (m, Ph **C**H and Ph**C**^{2}H), 128.7 (Ph **C**), 130.8 (Ph **C**H), 154.3 (Ph **C**OH), 177.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3463 (br), 3246, 3032, 2956, 2928, 1738 (CO), 1580, 1508, 1237, 834; m/z (EI) 185 ([M+H]$^+$, $^2H_2$, 19.4 %), 184 ([M]$^+$, $^2H_2$, 10.3 %), 183 ([M]$^+$, ^{2}H, 3.7 %), 182 ([M]$^+$, 3.1 %), 167 ([M-OH]$^+$, $^2H_2$, 96.5 %), 151 ([M+H-(OH)$_2$]$^+$, $^2H_2$, 13.9 %), 139 ([M-CO_2H]$^+$, $^2H_2$, 100.0 %), 109 [M-CH(OH)CO_2H]$^+$, $^2H_2$, 38.7 %).

5.6.4 [2,3',5'-$^2H_3$]-4-Hydroxyphenyllactic acid 249c[71]

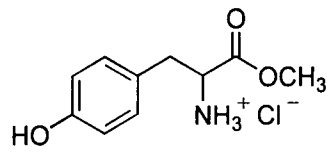


A solution of [2-^{2}H]-4-hydroxyphenyllactic acid **249a** (101 mg, 0.55 mmol) in 35 % DCl/D_2O (1 ml) and D_2O (1 ml) was placed in a Carius tube. The solution was treated in a similar manner to 3-amino-2-(4-hydroxy)phenylpropionic acid (5.4.10) and was then heated to 80 °C for 6 h. After cooling, the solution was saturated with NaCl and was extracted into ethyl acetate (4x 10 ml). The organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo*. The product was obtained as a white solid (101 mg, 98.9 %). Deuterium incorporation was 94 % in the *para* positions as determined by ^{1}H NMR.

δ_H(200 MHz; D_2O) 2.66 (1H, d, J_{gem} 14.0, C**H**$_2$), 2.83 (1H, d, J_{gem} 14.0, C**H**$_2$), 6.62 (6 %) (2H, d, J 8.2, **Ph**), 6.93 (94 %) (2H, s, **Ph**); δ_C(100.6 MHz; D_2O) 38.6 (**C**H$_2$), 71.2 (m, **C**H), 115.6 (m, Ph **C**H and Ph**C**^{2}H), 128.6 (Ph **C**), 130.8 (Ph **C**H), 154.4 (Ph **C**OH), 177.4 (**C**O); υ_{max}(KBr)/cm^{-1} 3474 (br), 3258, 3034, 2960, 2928, 1742 (CO), 1476, 1242, 1094, 832.

5.7 Synthesis of tetramic acid 177

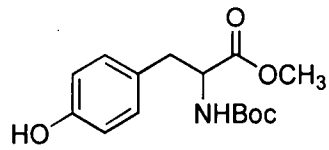
5.7.1 DL-Tyrosine methyl ester hydrochloride 216[159]



Thionyl chloride (2.2 ml, 30.4 mmol) was added dropwise to dry, distilled methanol (20 ml) and the solution was stirred rapidly at -5 °C. DL-Tyrosine **111** (5.0 g, 27.6 mmol) was added and the solution heated under reflux for 3 h. After cooling, the solution was diluted with diethyl ether (60 ml), resulting in the precipitation of the product. The white solid was collected by filtration, washed with diethyl ether (50 ml) and dried to yield the product as the hydrochloride salt (6.19 g, 97.0 %), mp 191 °C (decomp.) (lit. 189-190 °C)[129]

δ_H(400 MHz; D_2O) 3.06 (1H, dd, J_{vic} 7.6, J_{gem} 14.8, C**H**$_2$), 3.09 (1H, dd, J_{vic} 5.6, J_{gem} 14.8, C**H**$_2$), 3.70 (3H, s, OC**H**$_3$), 4.23 (1H, dd, J_{vic} 6.0, J_{vic} 7.2, C**H**), 6.75 (2H, d, J 8.4, **Ph**), 7.01 (2H, d, J 8.4, **Ph**); δ_C(100.6 MHz; D_2O) 34.9 (**C**H$_2$), 53.7 (O**C**H$_3$), 54.3 (**C**H), 116.1 (Ph **C**H), 125.5 (Ph **C**), 131.0 (Ph **C**H), 155.4 (Ph **C**OH), 170.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3303 (br, NH_2), 3002, 2877, 1746 (CO), 1613, 1590, 1517, 1240, 838; m/z (EI) 196 ([M+H]$^+$, 0.9 %), 195 ([M]$^+$, 7.5 %), 136 ([M-CO_2CH_3]$^+$, 31.7 %), 107 ([Bn(OH)]$^+$, 100.0 %), 91 ([Bn]$^+$, 16.0 %), 88 ([$CH(NH_2)CO_2CH_3$]$^+$, 35.3 %), 77 ([Ph]$^+$, 17.4 %).

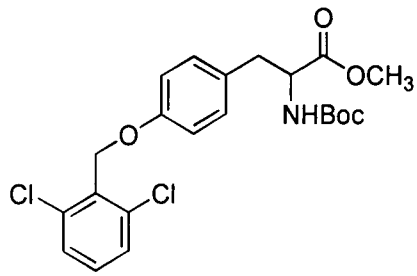
5.7.2 N-(*tert*-Butoxycarbonyl)-DL-tyrosine methyl ester 233[160]



A solution of tyrosine methyl ester hydrochloride **216** (2.0 g, 8.64 mmol) in water (4 ml) was mixed with a solution of K_2CO_3 (1.19 g, 8.64 mmol) in water (4 ml) and after 10 min the precipitated tyrosine methyl ester was collected by filtration and washed with water. A solution of *t*-butanol (4 ml) in ether (6 ml) was added, followed by dropwise addition with stirring of di-*tert*-butyl dicarbonate (1.93 ml, 8.40 mmol) over 15 min. Complete dissolution was evident after 10 min, but after a further 5 min precipitation occurred. The solution was diluted with ether (15 ml), washed with water (3x 15 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. The crystalline residue was redissolved in toluene (400 ml) and the solvent removed under reduced pressure. The solid material was dissolved in toluene (80 ml), hexane (20 ml) was added and the solid left to recrystallise at room temperature. After 1 h, the product was collected by filtration as a white powder (2.21 g, 92.2 %), mp 139-140 °C (lit. 143-144 °C).[161]

δ_H(400 MHz; CD_3OD) 1.39 (9H, s, $(\mathbf{CH_3})_3$), 2.80 (1H, dd, J_{vic} 8.4, J_{gem} 13.6, $\mathbf{CH_2}$), 2.97 (1H, dd, J_{vic} 5.6, J_{gem} 13.6, $\mathbf{CH_2}$), 3.67 (3H, s, $O\mathbf{CH_3}$), 6.70 (2H, d, J 8.4, **Ph**), 7.00 (2H, d, J 8.4, **Ph**); δ_C(100.6 MHz; CD_3OD) 28.7 ($C(\mathbf{CH_3})_3$), 38.0 ($\mathbf{CH_2}$), 52.5 ($\mathbf{CH_3}$), 58.8 (**C**H), 80.6 ($\mathbf{C}(CH_3)_3$), 116.2 (Ph **C**H), 128.9 (Ph **C**), 131.2 (Ph **C**H), 157.4 (Ph **C**OH), 157.8 (NH**C**O), 174.4 (**C**O); υ_{max}(KBr)/cm^{-1} 3370 (br, OH), 3359 (NH), 2983, 2956, 1734 (CO), 1686 (CO), 1517, 1228, 1062, 805; m/z (EI) 296 ($[M+H]^+$, 1.5 %), 295 ($[M]^+$, 0.5 %), 239 ($[M+H\text{-}C(CH_3)_3]^+$, 0.6 %), 195 ($[M+H\text{-}CO_2C(CH_3)_3]^+$, 2.6 %), 181 ($[(OH)PhCH_2CH(NH_2)CO_2H]^+$, 32.8 %), 108 ($[(OH)BnH]^+$, 98.0 %), 107 ($[Bn(OH)]^+$, 98.0 %), 77 ($[Ph]^+$, 9.6 %), 57 ($[C(CH_3)_3]^+$, 100.0 %).

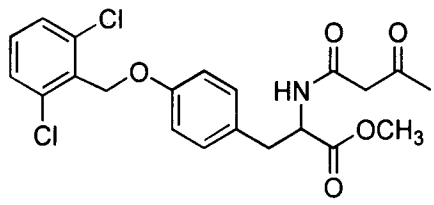
5.7.3 N-(*tert*-Butoxycarbonyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 227[162]



A mixture of N-(*tert*-butoxycarbonyl)-DL-tyrosine methyl ester **222** (1.50 g, 5.08 mmol), 2,6-dichlorobenzylchloride (1.19 g, 6.09 mmol) and K_2CO_3 (2.11 g, 15.3 mmol) in acetone (20 ml) were refluxed for 3 d. After cooling, water (15 ml) was added and the acetone was removed at the rotary evaporator. The aqueous phase was extracted with ethyl acetate (3x 20 ml), the combined organic extracts dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was then purified by column chromatography over silica gel (hexane: diethyl ether, 3:2) to yield a white powder (1.95 g, 84.5 %), mp 111.5-112.5 °C (lit. 108-109 °C).[161]

δ_H(200 MHz; $CDCl_3$) 1.37 (9H, s, C(CH_3)$_3$), 2.84 (1H, dd, J_{vic} 8.8, J_{gem} 13.8, CH_2), 3.05 (1H, dd, J_{vic} 5.7, J_{gem} 14.0, CH_2), 3.68 (3H, s, OCH_3), 4.33 (1H, dd, J_{vic} 5.8, J_{vic} 8.4, CH), 5.24 (2H, s, OCH_2), 6.94 (2H, d, J 8.6, **Ph**), 7.14 (2H, d, J 8.4, **Ph**), 7.28-7.45 (3H, m, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 28.3 (C(CH_3)$_3$), 37.4 (CH_2), 52.2 (OCH_3), 54.5 (CH), 65.1 (CH_2O), 79.9 (C(CH_3)$_3$), 115.0 (Ph CH), 128.4 (Ph CH), 128.7 (Ph C), 130.3 (Ph CH), 130.4 (Ph CH), 132.1 (Ph C), 137.0 (Ph C), 155.1 (Ph COR) 157.9 (NHCO), 172.4 (CO); υ_{max}(KBr)/cm^{-1} 3357 (NH), 2992, 2971, 2927, 1745 (CO), 1703 (CO), 1512, 1244, 1016, 827, 776, 768; m/z (EI) 456 ($[M]^+$, 2x ^{37}Cl, 0.03 %), 454 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.06 %), 452 ($[M]^+$, 2x ^{35}Cl, 0.05 %), 355 ([M-$CO_2C(CH_3)_3$], 2x ^{37}Cl, 0.6 %), 353 ([M-$CO_2C(CH_3)_3$], 1x ^{37}Cl, 1x ^{35}Cl, 0.8 %), 351 ([M-$CO_2C(CH_3)_3$], 2x ^{35}Cl, 0.4 %) 269 ([$BnOCH_2Ph(Cl)_2$, 2x ^{37}Cl, 3.0 %), 267 ([$BnOCH_2Ph(Cl)_2$], 1x ^{35}Cl, 1x ^{37}Cl, 18.7 %), 265 ([$BnOCH_2Ph(Cl)_2$, 2x ^{35}Cl, 29.1 %), 163 ([$Bn(Cl_2)$], 2x ^{37}Cl, 9.9 %), 161 ([$Bn(Cl_2)$], 1x ^{35}Cl, 1x ^{37}Cl, 58.5 %), 159 ([$Bn(Cl_2)$], 2x ^{35}Cl, 100.0 %), 107 ($[Bn(OH)]^+$, 11.6 %), 77 ($[Ph]^+$, 9.4 %) 57 ($[C(CH_3)_3]^+$, 0.7 %).

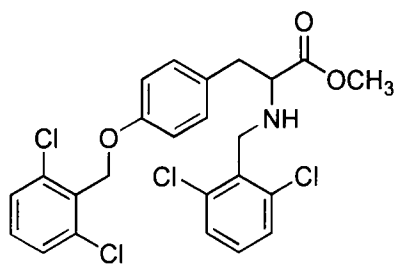
5.7.4 N-(3-Oxo-butyryl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 228



N-(*tert*-Butoxycarbonyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester **227** (100 mg, 0.22 mmol), pyridinium *para*-toluene sulfonate (PPTS) (60 mg, 0.24 mmol) and 2,2,6-trimethyl-4H-1,3-dioxin-4-one (34 mg, 0.24 mmol) were refluxed in toluene for 3.5 h. After cooling, the solvent was removed *in vacuo* and the product purified by column chromatography over silica gel (DCM:EtOAc, 5:2). Over 90 % of the *tert*-butoxycarbonyl group was found by ^{1}H NMR to have been cleaved.

δ_H(270 MHz; $CDCl_3$) 1.45 (7 %) (9H, s, $C(CH_3)_3$), 2.14 (3H, s, CH_3), 3.10 (1H, m, CH_2), 3.16 (1H, m, CH_2), 3.70 (3H, s, OCH_3), 4.83 (1H, m, CH), 5.21 (2H, s, CH_2O), 6.23 (1H, s, CH=C(OH)), 6.95 (2H, d, J 9.5, **Ph**), 7.19 (3H, m, **Ph**), 7.36 (2H, m, **Ph**) 10.21 (1H, s, COH).

5.7.5 N-(2,6-Dichlorobenzyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 229

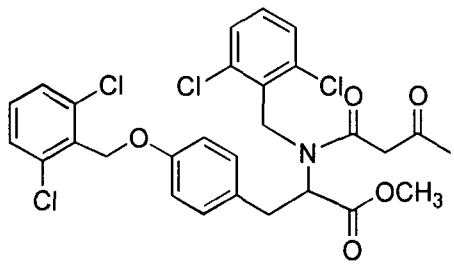


A suspension of DL-tyrosine methyl ester hydrochloride **216** (990 mg, 4.32 mmol), 2,6-dichlorobenzylchloride (1.86 g, 9.5 mmol) and K_2CO_3 (2.98 g, 21.6 mmol) in DMF (10 ml) were stirred at 60 °C for 12 h. The solvent was removed *in vacuo* and the residue dissolved in ethyl acetate (30 ml). The organic layer was washed with water (10x 15 ml) to remove any remaining DMF and then dried ($MgSO_4$). The solvent was removed

in vacuo and the product purified by column chromatography over silica gel (EtOAc:petroleum ether, 1:3). A white solid (1.32 g, 60.2 %) was obtained, mp 95-96 °C.

δ_H(400 MHz; $CDCl_3$) 1.98 (1H, s, N**H**), 2.77 (1H, dd, J_{vic} 8.0, J_{gem} 14.0, C$\mathbf{H}_2$), 2.90 (1H, dd, J_{vic} 6.0, J_{gem} 13.6, C$\mathbf{H}_2$), 3.47 (1H, dd, J_{vic} 6.0, J_{gem} 8.0, C**H**), 3.57 (3H, s, OC$\mathbf{H}_3$), 3.90 (1H, d, J 13.2, C$\mathbf{H}_2$N), 4.02 (1H, d, J 13.6, C$\mathbf{H}_2$N), 5.17 (2H, s, OC$\mathbf{H}_2$), 6.84 (2H, d, J 8.4, **Ph**), 7.01 (2H, d, J 8.4, **Ph**), 7.04 (1H, d, J 8.4, **Ph**), 7.18 (3H, m, **Ph**), 7.29 (2H, d, J 8.0, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 38.6 (**C**H_2), 47.0 (**C**H_2N), 51.8 (O**C**H_3), 61.9 (**C**H), 65.3 (**C**H_2O), 115.0 (Ph **C**H), 128.3 (Ph **C**H), 128.5 (Ph **C**H), 130.0 (Ph **C**H), 128.9 (Ph **C**H), 129.7-137.0 (Ph **C** & Ph **C**H), 157.7 (Ph **C**OH), 174.5 (**C**O); υ_{max}(KBr)/cm^{-1} 3340 (NH), 3074, 3038, 2949, 2896, 1736 (CO), 1610, 1511, 1239, 1018, 823, 767; m/z (EI) 515 ($[M]^+$, 2x ^{37}Cl, 2x ^{35}Cl, 0.2 %), 513 ($[M]^+$, 1x ^{37}Cl, 3x ^{35}Cl, 0.4 %), 511 ($[M]^+$, 4x ^{35}Cl, 0.4 %), 458 ($[M\text{-}CO_2CH_3]^+$, 3x ^{37}Cl, 1x ^{35}Cl, 1.0 %), 456 ($[M\text{-}CO_2CH_3]^+$, 2x ^{37}Cl, 2x ^{35}Cl, 4.0 %), 454 ($[M\text{-}CO_2CH_3]^+$, 1x ^{37}Cl, 3x ^{35}Cl, 8.0 %), 452 ($[M\text{-}CO_2CH_3]^+$, 4x ^{35}Cl, 6.1 %), 297 ($[M\text{-}CO_2CH_3\text{-}Bn(Cl)_2]^+$, 2x ^{37}Cl, 0.5 %), 295 ($[M\text{-}CO_2CH_3\text{-}Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 2.3 %), 293 ($[M\text{-}CO_2CH_3\text{-}Bn(Cl)_2]^+$, 2x ^{35}Cl, 3.3 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 0.9 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 2.4 %), 265 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 2.2 %), 250 ($[M\text{-}BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 7.8 %), 248 ($[M\text{-}BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 45.9 %), 246 ($[M\text{-}BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 70.7 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 10.5 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 63.9 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 100.0 %), 77 ($[Ph]^+$, 16.3 %).

5.7.6 *N-(2,6-Dichlorobenzyl)-(3-oxo-butyryl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 230*

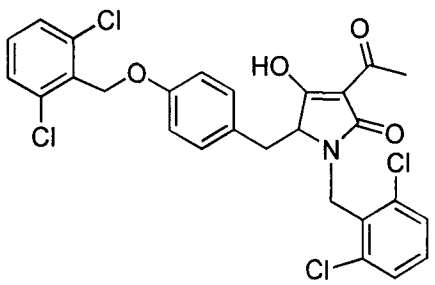


To a solution of 2,2,6-trimethyl-4H-1,3-dioxin-4-one (152 mg, 1.07 mmol) and N-(2,6-dichlorobenzyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester **229** (498 mg, 0.97 mmol) in toluene (7 ml) was added PPTS (243 mg, 0.97 mmol) and the mixture refluxed with stirring for 3 h. After cooling, the solvent was removed *in vacuo* and the product purified by column chromatography over silica gel (EtOAc; petroleum ether, 2:3) and recovered as a white solid (431 mg, 74.4 %), mp 99.5-100.0 °C. The NMR revealed a mixture of tautomers.

δ_H(400 MHz; $CDCl_3$) 3.17 (1H, dd, J_{vic} 8.8, J_{gem} 14.2, C**H**$_2$), 3.3 (1H, dd, J_{vic} 5.2, J_{gem} 14.2, C**H**$_2$), 3.63 (4H, m, OC**H**$_3$ and C**H**), 4.58 (1H, d, J 14.4, C**H**$_2$N), 4.73 (1H, d, J 14.4, C**H**$_2$N), 5.14 (2H, s, C**H**$_2$O), 6.73 (2H, d, J 8.4, **Ph**), 6.77 (2H, d, J 8.8, **Ph**), 7.21 (4H, m, **Ph**), 7.38 (2H, d, J 8.0, **Ph**), 14.34 (1H, s, O**H**); δ_C(100.6 MHz; $CDCl_3$) 30.3 (**C**H$_3$), 34.2 (**C**H$_2$), 49.3 (**C**H$_2$N), 51.0 (**C**H$_2$), 52.2 (O**C**H$_3$), 61.4 (**C**H), 65.0 (O**C**H$_2$), 114.6 (Ph **C**H), 128.5-132.2 (Ph **C** and Ph **C**H), 137.0 (Ph **C**H), 157.2 (Ph **C**OR), 167.8 (NH**C**O), 170.8 (**C**OCH$_3$), 202.1 (**C**O); υ_{max}(KBr)/cm^{-1} 3452 (br), 3074, 3002, 2954, 2848, 1741 (CO), 1640 (CO), 1583, 1510, 1437, 1231, 770; m/z (EI) 603 ([M]$^+$, 4x ^{37}Cl, 0.01 %), 601 ([M]$^+$, 3x ^{37}Cl, 1x ^{35}Cl, 0.05 %), 599 ([M]$^+$, 2x ^{37}Cl, 2x ^{35}Cl, 0.2 %), 597 ([M]$^+$, 1x ^{37}Cl, 3x ^{35}Cl, 0.4 %), 595 ([M]$^+$, 4x ^{35}Cl, 0.3 %), 458 ([M+H-CO_2CH_3-$COCH_2COCH_3$]$^+$, 3x ^{37}Cl, 1x ^{35}Cl, 0.7 %), 456 ([M+H-CO_2CH_3-$COCH_2COCH_3$]$^+$, 2x ^{37}Cl, 2x ^{35}Cl, 2.7 %), 454 ([M+H-CO_2CH_3-$COCH_2COCH_3$]$^+$, 1x ^{37}Cl, 3x ^{35}Cl, 5.5 %), 452 ([M+H-CO_2CH_3-$COCH_2COCH_3$]$^+$, 4x ^{35}Cl, 4.3 %), 340 ([M+H-$COCH_2COCH_3$-$OCH_2Ph(Cl)_2$]$^+$, 2x ^{37}Cl, 1.8 %), 338 ([M+H-$COCH_2COCH_3$-$OCH_2Ph(Cl)_2$]$^+$, 1x ^{37}Cl, 1x ^{35}Cl, 10.1 %), 336 ([M+H-$COCH_2COCH_3$-$OCH_2Ph(Cl)_2$]$^+$, 2x ^{35}Cl, 15.2 %), 250 ([M+H-$COCH_2COCH_3$-Bn$OCH_2Ph(Cl)_2$]$^+$, 2x ^{37}Cl, 5.8 %), 248 ([M+H-

$COCH_2COCH_3$-$BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 34.0 %), 246 ([M+H-$COCH_2COCH_3$-$BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 53.9 %), 163 ([$Bn(Cl)_2]^+$, 2x ^{37}Cl, 10.5 %), 161 ([$Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 62.6 %), 159 ([$Bn(Cl)_2]^+$, 2x ^{35}Cl, 100.0 %).

5.7.7 5-(4-(2,6-Dichlorobenzyloxy)-benzyl-1-(2,6-dichlorobenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 231

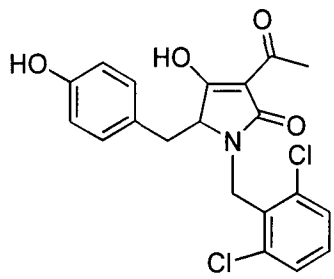


To a stirred solution of potassium *t*-butoxide (56 mg, 0.50 mmol) in *t*-butanol (10 ml) was added a solution of N-(2,6-dichlorobenzyl)-(3-oxo-butyryl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester **230** (152 mg, 0.25 mmol) in *t*-butanol (6 ml). After 30 min, the resultant yellow solution was quenched with 1M HCl (6 ml) and extracted into diethyl ether (2x 20 ml). The combined organic extracts were washed with water (1x 5 ml), dried ($MgSO_4$) and evaporated *in vacuo* to afford the product as an orange solid (141 mg, 97.4 %), mp 48 °C. Hexane was used to azeotropically remove final traces of *t*-butanol. Spectra were complicated by the presence of tautomers.

δ_H(400 MHz; $CDCl_3$) 2.28 (85 %) (3H, s, CH_3), 2.34 (15 %) (3H, s, CH_3), 2.9 (1H, dd, J_{vic} 5.2, J_{gem} 14.4, CH_2), 3.0 (1H, dd, J_{vic} 3.2, J_{gem} 14.4, CH_2), 3.4 (85 %) (1H, dd, J_{vic} 4.8, J_{vic} 5.2, CH), 3.6 (15 %) (1H, dd, J_{vic} 5.2, J_{vic} 5.6, CH), 4.70 (1H, d, J 14.8, CH_2N), 5.15 (2H, s, CH_2O), 5.27 (1H, d, J 14.8, CH_2N), 6.81 (2H, d, J 8.4, **Ph**), 6.97 (2H, d, J 8.4, **Ph**), 7.17 (2H, m, **Ph**), 7.29 (4H, m, **Ph**); δ_C(400 MHz; $CDCl_3$) 19.3 (CH_3), 19.8 (CH_3) 34.1 (CH_2), 39.6 (CH_2N), 64.6 (CH), 65.2 (CH_2O), 101.9 (C=C), 105.9 (C=C), 115.0 (Ph CH), 127.2-136.9 (Ph C & Ph CH), 157.8 (Ph COR), 172.9 (NRCO), 183.5 (CO), 194.1 (CO); υ_{max}(KBr)/cm^{-1} 3440 (br), 2942, 2928, 2856, 1716(CO), 1660 (CO), 1616, 1509, 1437, 1241, 1020, 770; m/z (EI) 571 ([M]$^+$, 4x ^{37}Cl, 0.1 %), 569 ([M]$^+$, 3x ^{37}Cl, 1x ^{35}Cl, 0.6 %), 567 ([M]$^+$, 2x ^{37}Cl, 2x ^{35}Cl, 2.2 %), 565 ([M]$^+$, 1x ^{37}Cl, 3x ^{35}Cl,

4.3 %), 563 ($[M]^+$, 4x ^{35}Cl, 3.3 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 3.7 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 21.3 %), 265 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 33.8 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 10.7 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 65.2 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 100.0 %), 107 ($[Bn(OH)]^+$, 6.1 %), 77 ($[Ph]^+$, 2.0 %).

5.7.8 5-(4-(Hydroxy)-benzyl-1-(2,6-dichlorobenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 232

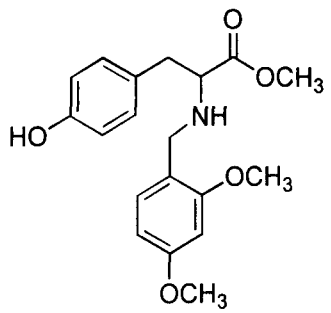


5-(4-(2,6-Dichlorobenzyloxy)-benzyl-1-(2,6-dichlorobenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **231** (30 mg, 0.05 mmol) was stirred in boron tribromide (1M solution in DCM) (0.27 ml, 0.27 mmol) at -78 °C and allowed to warm to room temperature over 24 h. The reaction was quenched with water (2 ml) and extracted into DCM (3x 10 ml). The organic extracts were combined and washed with 1M NaOH (3x 5 ml). The combined aqueous extracts were acidified and extracted into DCM (3x 10 ml). After drying ($MgSO_4$), the solvent was removed *in vacuo* to give the product as a red oil.

δ_H(400 MHz; $CDCl_3$) 2.28 (3H, bs, C**H**$_3$), 3.00 (2H, m, C**H**$_2$), 3.46 (72 %) (1H, m, C**H**), 3.61 (28 %) (1H, m, C**H**), 4.71 (1H, d, J 14.8, C**H**$_2$N), 5.28 (1H, d, J 14.8, C**H**$_2$N), 6.63 (1H, bs, O**H**), 6.89 (2H, m, **Ph**), 7.19 (3H, m, **Ph**), 7.21 (2H, d, J 7.6, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 29.7 (**C**H$_3$), 34.0 (**C**H$_2$), 39.6 (**C**H$_2$N), 64.6 (**C**H), 102.0 (**C**=C), 115.3 (Ph **C**H), 126.2-136.5 (Ph **C**, Ph **C**H & **C**=C(OH)), 154.5 (Ph **C**OH), 173.1 (NR**C**O), 183.7 (**C**O), 194.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3347 (br), 3058, 2974, 2930, 2852, 1710, 1614, 1515, 1265, 1090, 738; m/z (EI) 409 ($[M]^+$, 2x ^{37}Cl, 1.4 %), 407 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 7.4 %), 405 ($[M]^+$, 2x ^{35}Cl, 10.9 %), 303 ($[M+H-Bn(OH)]^+$, 2x ^{37}Cl, 9.7 %), 301 ($[M+H-Bn(OH)]^+$, 55.5 %), 299 ($[M+H-Bn(OH)]^+$, 2x ^{35}Cl, 87.7 %), 163

($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 11.0 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 61.2 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 95.7 %), 140 ($[M+H-Bn(Cl)_2-Bn(OH)]^+$, 15.0 %), 107 ($[Bn(OH)]^+$, 100.0 %), 77 ($[Ph]^+$, 21.6 %), 43 ($[CH_3CO]^+$, 49.7 %).

5.7.9 *N-2,4-Dimethoxybenzyl-DL-tyrosine methyl ester 221*

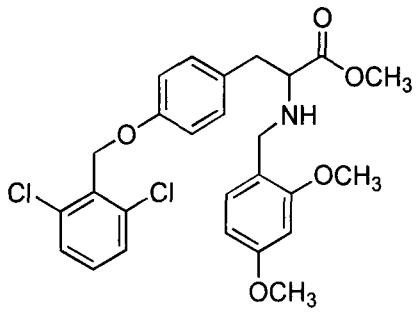


To a solution of DL-tyrosine methyl ester hydrochloride **216** (2.61 g, 11.3 mmol) in methanol (15 ml) was added 2,4-dimethoxybenzaldehyde (1.87 g, 11.3 mmol) and $MgSO_4$ (3.0 g). The mixture was stirred at room temp. for 24 h, filtered and then the solvent was removed *in vacuo*. The residue was redissolved in methanol (15 ml) and the solution cooled to 0 °C. $NaBH_4$ (854 mg, 22.6 mmol) was carefully added in small portions over 45 min and the mixture was left to stir for 1 h. The reaction was quenched with water (10 ml) and was then extracted into ethyl acetate (3x 50 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to yield a yellow solid which was purified over silica gel (EtOAc: petroleum ether, 3:2) to give a white solid (2.55 g, 65.6 %), mp 94.5-95.5 °C.

δ_H(400 MHz; $CDCl_3$) 2.85 (1H, dd, J_{vic} 8.0, J_{gem} 13.6, C**H**$_2$), 2.92 (1H, dd, J_{vic} 6.4, J_{gem} 13.6, C**H**$_2$), 3.47 (1H, dd, J_{vic} 6.4, J_{vic} 7.6, C**H**), 3.62 (3H, s, ArOC**H**$_3$), 3.62 (1H, d, J 13.6, C**H**$_2$N), 3.64 (3H, s, ArOC**H**$_3$), 3.75 (1H, d, J 13.6, C**H**$_2$N), 3.78 (3H, s, CO_2C**H**$_3$), 6.37 (2H, m, **Ph**), 6.67 (2H, d, J 8.4, **Ph**), 6.96 (2H, d, J 8.8, **Ph**), 7.03 (1H, d, J 8.0, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 38.5 (**C**H$_2$), 47.3 (**C**H$_2$N), 51.7 (CO_2**C**H$_3$), 55.1 and 55.3 (2x ArO**C**H$_3$), 61.9 (**C**H), 98.3 (Ph **C**H), 103.5 (Ph **C**H), 115.4 (Ph **C**H), 119.4 (Ph **C**), 128.6 (Ph **C**), 130.2 (Ph **C**H), 130.6 (Ph **C**H), 154.8 (Ph **C**OH), 158.6 (Ph **C**OCH$_3$), 160.3 (Ph **C**OCH$_3$), 174.7 (**C**O); υ_{max}(KBr)/cm^{-1} 3396 (br NH), 3304, 3006, 2950, 2836,

1735 (CO), 1615, 1516, 1208, 1033, 830; m/z (EI) 345 ($[M]^+$, 1.3 %), 286 ($[M-CO_2CH_3]^+$, 2.5 %), 238 ($[M-Bn(OH)]^+$, 18.9 %), 166 ($[NHBn(OCH_3)_2]^+$, 1.5 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 137 ($[Ph(OCH_3)_2]^+$, 2.0 %), 107 ($[Bn(OH)]^+$, 13.1 %), 77 ($[Ph]^+$, 6.4 %).

5.7.10 N-(2,4-Dimethoxy)benzyl-O-2,6-dichlorobenzyl-DL-tyrosine methyl ester 234

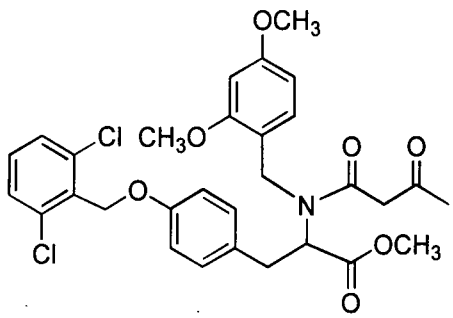


A mixture of N-(2,4-dimethoxy)benzyl-DL-tyrosine methyl ester **221** (4.80 g, 13.9 mmol), 2,6-dichlorobenzyl chloride (2.99 g, 15.3 mmol) and K_2CO_3 (4.22 g, 30.6 mmol) were stirred in DMF (40 ml) at 60 °C for 24 h. After cooling, water (20 ml) was added and the mixture extracted with ethyl acetate (3x 50 ml). The combined organic extracts were washed with water (10x 20 ml) to remove any remaining DMF, dried ($MgSO_4$) and the solvent was removed *in vacuo*. After purification over silica gel (EtOAc: petroleum ether, 2:3), the product was recovered as a white solid (5.67 g, 80.9 %), mp 87-87.5 °C.

δ_H(499.8 MHz; $CDCl_3$) 1.65 (1H, bs, N**H**), 2.86 (1H, dd, J_{vic} 8.5, J_{gem} 14.0, C$\mathbf{H}_2$), 2.96 (1H, dd, J_{vic} 6.0, J_{gem} 13.5, C$\mathbf{H}_2$), 3.47 (1H, dd, J_{vic} 6.5, J_{vic} 8.0, C**H**), 3.60 (1H, d, J 13.5, C$\mathbf{H}_2$N), 3.65 (6H, s, 2x ArOC$\mathbf{H}_3$), 3.75 (1H, d, J 13.5, C$\mathbf{H}_2$N), 3.79 (3H, s, OC$\mathbf{H}_3$), 5.24 (2H, s, C$\mathbf{H}_2$O), 6.38 (2H, m, **Ph**), 6.94 (2H, d, J 9.0, **Ph**), 7.03 (1H, d, J 8.0, **Ph**), 7.09 (2H, d, J 8.5, **Ph**), 7.26 (1H, m, **Ph**), 7.37 (2H, d, J 7.5, **Ph**); δ_C(125.7 MHz; $CDCl_3$) 38.6 (**C**H_2), 47.3 (**C**H_2N), 51.7 (CO_2**C**H_3), 55.1 & 55.3 (2x ArO**C**H_3), 61.9 (**C**H), 65.2 (O**C**H_2), 98.3 (Ph **C**H), 103.4 (Ph **C**H), 114.8 (Ph **C**H), 120.0 (Ph **C**), 128.5 (Ph **C**), 130.1 (Ph **C**H), 130.4 (Ph **C**H), 132.2 (Ph **C**), 137.0 (Ph **C**H), 157.6 (Ph **C**OR), 158.6 (Ph **C**OCH_3), 160.1 (Ph **C**OCH_3), 175.0 ($\mathbf{C}O_2CH_3$); υ_{max}(KBr)/cm^{-1} 3355 (NH), 3048,

2994, 2949, 2931, 2833, 1741 (CO), 1611, 1587, 1509, 1241, 832, 788; m/z (EI) 505 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.04 %), 503 ($[M]^+$, 2x ^{35}Cl, 0.1 %), 448 ($[M-CO_2CH_3]^+$, 2x ^{37}Cl, 0.01 %), 446 ($[M-CO_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.1 %), 444 ($[M-CO_2CH_3]^+$, 2x ^{35}Cl, 0.2 %), 238 ($[M-BnOCH_2Ph(Cl)_2]^+$, 1.4 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 0.4 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 2.1 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 3.2 %).

5.7.11 N-(2,4-Dimethoxybenzyl)-(3-oxo-butyryl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 235

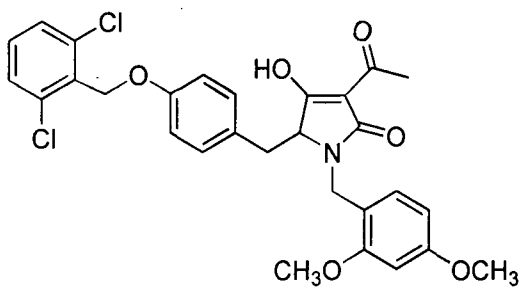


To a solution of 2,2,6-trimethyl-4H-1,3-dioxin-4-one **215** (343 mg, 2.42 mmol) and N-(2,4-dimethoxy)benzyl-O-2,6-dichloro-benzyl-DL-tyrosine methyl ester **234** (1.06 g, 2.10 mmol) in toluene (15 ml) was added PPTS (528 mg, 2.10 mmol) and the mixture refluxed with stirring for 3 h. After cooling, the solvent was removed *in vacuo* and the product was purified by column chromatography over silica gel (DCM; EtOAc, 6:1). The product was recovered as a white solid (980 mg, 79.2 %), mp 98-99 °C.

δ_H(499.8 MHz; $CDCl_3$) 2.27 (3H, s, C**H**$_3$), 3.05 (1H, dd, J_{vic} 9.0, J_{gem} 14.0, C**H**$_2$), 3.35 (1H, d, J_{vic} 6.0, J_{gem} 14.5, C**H**$_2$), 3.63 (3H, s, OC**H**$_3$), 3.75 (3H, s, OC**H**$_3$), 3.80 (3H, s, OC**H**$_3$), 4.00 (84 %) (1H, d, J 15.5, C**H**$_2$N), 4.05 (16 %) (1H, d, J 17.5, C**H**$_2$N), 4.21 (1H, dd, J_{vic} 6.0, J_{gem} 9.0, C**H**), 4.25 (84 %) (1H, d, J 16.0, C**H**$_2$N), 4.33 (16 %) (1H, d, J 17.0, C**H**$_2$N), 5.25 (2H, s, C**H**$_2$O), 6.39 (2H, m, **Ph**), 6.89 (2H, d, J 8.5, **Ph**), 6.93 (1H, d, J 8.5, **Ph**), 7.00 (2H, d, J 8.5, **Ph**), 7.26 (1H, d, J 8.5, **Ph**), 7.37 (2H, d, J 8.0, **Ph**); δ_C(125.7 MHz; $CDCl_3$) 30.4 (**C**H$_3$), 34.6 (**C**H$_2$), 49.0 (**C**H$_2$N), 50.4 (**C**H$_2$), 52.3 (CO_2**C**H$_3$), 55.3 (ArO**C**H$_3$), 55.7 (ArO**C**H$_3$), 60.7 (**C**H), 65.5 (**C**H$_2$O), 98.8 (Ph **C**H), 104.0 (Ph **C**H), 115.1 (Ph **C**H), 116.0 (Ph **C**H), 128.7 -137.2 (Ph **C** & Ph **C**H), 157.7

(Ph **C**OR), 158.9 (Ph **C**OCH_3), 161.3 (Ph **C**OCH_3), 167.8 (NR**C**O), 171.1 (**C**O), 202.8 (**C**O); υ_{max}(KBr)/cm^{-1} 3431, 3020, 2949, 2820, 1740 (CO), 1637, 1587, 1509, 1238, 1209, 833, 760; m/z (EI) 591 ($[M]^+$, 2x ^{37}Cl, 0.1 %), 589 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.2 %), 587 ($[M]^+$, 2x ^{35}Cl, 0.2 %), 506 ($[M\text{-}COCH_2COCH_3]^+$, 2x ^{37}Cl, 0.7 %), 504 ($[M\text{-}COCH_2COCH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 1.8 %), 502 ($[M\text{-}COCH_2COCH_3]^+$, 2x ^{35}Cl, 1.6 %), 448 ($[M\text{-}COCH_2COCH_3\text{-}CO_2CH_3]^+$, 2x ^{37}Cl, 0.2 %), 446 ($[M\text{-}COCH_2COCH_3\text{-}CO_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 1.0 %), 444 ($[M\text{-}COCH_2COCH_3\text{-}CO_2CH_3]^+$, 2x ^{35}Cl, 1.4 %), 336 ($[M\text{-}PhOCH_2Ph(Cl)_2]^+$, 7.5 %), 251 ($[(OCH_3)_2BnNHCOCH_2COCH_3]^+$, 26.3 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 9.2 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 53.8 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 77.8 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 78 ($[PhH]^+$, 14.7 %), 77 ($[Ph]^+$, 11.9 %).

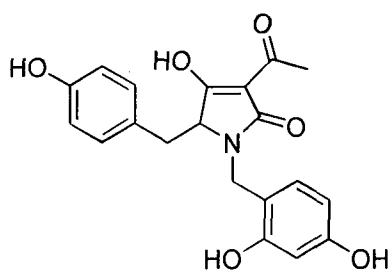
5.7.12 5-(4-(2,6-dichlorobenzyloxy)-benzyl-1-(2,4-dimethoxybenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 236



To a stirred solution of potassium *tert*-butoxide (190 mg, 1.70 mmol) in *t*-butanol (20 ml) was added a solution of DL-N-(2,4-dimethoxybenzyl)-(3-oxo-butyryl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester **235** (500 mg, 0.85 mmol) in *t*-butanol (10 ml). After 30 min, the resultant yellow solution was quenched with 1M HCl (6 ml) and extracted into diethyl ether (2x 25 ml). The combined organic extracts were washed with water (1x 10 ml), dried ($MgSO_4$) and evaporated *in vacuo* to afford an orange solid (461 mg, 97.5 %), mp 48 °C (decomp.). Hexane was used to azeotropically remove final traces of *t*-butanol. ^1H and ^{13}C spectra were complicated by the presence of tautomers.

δ_H(400 MHz; $CDCl_3$) 2.29 (88 %) (3H, s, CH_3), 2.35 (12 %) (3H, s, CH_3), 3.05 (88 %) (2H, d, J 4.8, CH_2), 3.07 (12 %) (2H, d, J 4.4, CH_2), 3.73 (3H, s, $ArOCH_3$), 3.74 (3H, s, $ArOCH_3$), 3.78 (1H, t, J 4.8, CH), 4.14 (88 %) (1H, d, J 14.8, CH_2N), 4.17 (12 %) (1H, d, J 14.4, CH_2N), 4.91 (88 %) (1H, d, J 14.8, CH_2N), 4.98 (12 %) (1H, d, J 14.4, CH_2N), 5.17 (2H, s, CH_2O), 6.37 (2H, m, **Ph**), 6.82 (2H, d, J 8.4, **Ph**), 6.97 (2H, d, J 8.8, **Ph**), 7.01 (1H, d, J 8.8, **Ph**), 7.16 (1H, d, J 8.8, **Ph**), 7.29 (2H, d, J, 8.0, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 19.5 (CH_3), 34.1 (CH_2), 38.3 (CH_2N), 55.3 (Ar OCH_3), 55.4 (Ar OCH_3), 62.3 (CH), 65.3 (CH_2O), 98.4 (Ph CH), 102 (C=C), 104.3 (Ph CH), 115.0 (Ph CH), 116.0-137.0 (Ph C & Ph CH), 157.7 (Ph COR), 158.5 (Ar $COCH_3$), 160.9 (Ar $COCH_3$), 173.2 (NRCO), 183.5 (C=COH), 194.7 (CO); m/z (EI) 559 ($[M]^+$, 2x ^{37}Cl, 1.9 %), 557 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 9.2 %), 555 ($[M]^+$, 2x ^{35}Cl, 13.4 %), 397 ($[M+H-Bn(Cl)_2]^+$, 1.3 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 4.5 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 29.4 %), 265 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 45.0 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 5.7 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 33.5 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 52.0 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 78 ($[PhH]^+$, 4.5 %), 77 ($[Ph]^+$, 6.8 %), 43 ($[CH_3CO]^+$, 26.5 %); Found 556.1289, $C_{29}H_{27}NO_6Cl_2$ (M^+) requires 556.1293.

5.7.13 5-(4-(Hydroxy)-benzyl-1-(2,4-dihydroxybenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 237

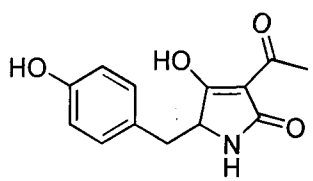


5-(4-(2,6-Dichlorobenzyloxy)-benzyl-1-(2,4-dimethoxybenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **236** (526 mg, 0.95 mmol) was stirred in a solution of BBr_3 (1M in DCM, 6.6 ml, 6.62 mmol) at -78 °C and allowed to warm to room temperature over 6 h. 1M HCl (20 ml) was added and extracted with diethyl ether (3x 20 ml). The combined organic extracts were washed with 1M NaOH (2x 10 ml), the aqueous

extracts were acidified and were then extracted with diethyl ether (3x 15 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to give an orange/brown solid (352 mg, 98.6 %), mp 88 °C (decomp.). ^1H NMR indicated that approximately 70 % of the benzyl methyl ether groups had also been removed.

δ_H(299.9 MHz, CD_3OD) 2.29 (3H, s, C**H**$_3$), 3.05 (2H, m, C**H**$_2$), 3.78 (3H, m, C**H**$_2$N and OC**H**$_3$ (30 %)), 4.24 (1H, t, J 15.6, C**H**), 4.89 (1H, m, C**H**$_2$N), 6.32 (2H, m, **Ph**), 6.62 (2H, d, J 6.6, **Ph**), 6.87 (2H, d, J 7.8, **Ph**), 6.9 (1H, t, J 8.4, **Ph**); δ_C(75.4 MHz, CD_3OD) 20.4 (**C**H$_3$), 35.5 (**C**H$_2$), 40.4 (**C**H$_2$N), 56.6 (**C**H), 100.7-133.6 (Ph **C**, Ph **C**H & **C**=C), 158.2-161.0 (Ph **C**OH and **C**O); υ_{max}(neat)/cm^{-1} 3347 (br), 3034, 2974, 2932, 1617, 1515, 1241, 846; m/z (EI) 397 ([M-2H+CH_3+CH_3]$^+$, 1.4 %), 383 ([M-H+CH_3]$^+$, 25.2 %), 370 ([M+H]$^+$, 16.9 %), 369 ([M]$^+$, 57.6 %), 327 ([M+H-$COCH_3$]$^+$, 4.5 %), 277 ([M+H-Ph(OH)]$^+$, 48.9 %), 263 ([M+H-Bn(OH)]$^+$, 61.6 %), 247 ([M+H-Bn$(OH)_2$]$^+$, 60.9 %), 123 ([Bn$(OH)_2$]$^+$, 89.0 %), 108 ([BnH(OH)]$^+$, 68.8 %), 107 ([Bn(OH)]$^+$, 100.0 %), 78 ([PhH]$^+$, 30.4 %), 77 ([Ph]$^+$, 68.1 %), 43 ([CH_3CO]$^+$, 68.2 %).

5.7.14 5-(4-(Hydroxy)-benzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 213[163]

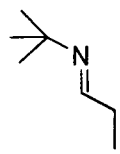


5-(4-(Hydroxy)-benzyl-1-(2,4-dihydroxybenzyl)-3-(acetyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **237** (150 mg, 0.41 mmol) was stirred in trifluoroacetic acid (TFA) (6 ml) at room temperature for 30 min. The reaction was then quenched with ice and extracted into DCM (2x 10 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to give an orange solid, mp 150 °C (decomp.).

δ_H(299.9 MHz; CD_3OD) 2.33 (3H, s, $COCH_3$), 2.85 (1H, dd, J_{vic} 6.0, J_{gem} 14.1, C**H**$_2$), 2.98 (1H, dd, J_{vic} 4.2, J_{gem} 13.8, C**H**$_2$), 4.07 (1H, t, J 5.4, C**H**), 6.65 (2H, d, J 8.4, **Ph**),

6.97 (2H, d, J 8.4, **Ph**); δ_C(75.4 MHz; CD_3OD) 20.2 (**C**H_3), 37.4 (**C**H_2), 64.0 (**C**H), 103.6 (**C**=C), 116.1 (Ph **C**H), 127.6 (Ph **C**), 129.4 (C=**C**), 131.8 (Ph **C**H), 157.3 (Ph **C**OH), 187.5 (**C**O), 198.2 (**C**O); υ_{max}(neat)/cm^{-1} 3404 (br), 3238 (br), 3058, 2962, 2928, 2856, 1699 (CO), 1641 (NHCO), 1601, 1515, 1267, 802; m/z (EI) 248 ($[M+H]^+$, 1.4 %), 247 ($[M]^+$, 8.2 %), 141 ($[M+H\text{-}BnOH]^+$, 13.7 %), 107 ($[Bn(OH)]^+$, 100.0 %), 77 ($[Ph]^+$, 8.7 %), 43 ($[CH_3CO]^+$, 12.8 %).

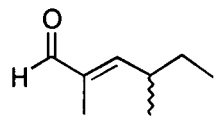
5.7.15 (1,1-Dimethyl-N-propylidene) ethylamine 240[164]



Propionaldehyde **239** (14.50 g, 0.25 mol) was added dropwise over 2 h to *t*-butylamine **238** (18.25 g, 0.25 mol) stirring at 0 °C. When addition was complete, stirring was stopped and potassium hydroxide pellets (10 g) were added. The mixture was left at -20 °C for 12 h and was then distilled twice at atmospheric pressure (bp 95-97 °C, 760 mmHg). The distillate was collected under nitrogen as a clear, colourless oil (13.7 g, 48.5 %). The product was stored over molecular sieves at -20 °C.

δ_H(400 MHz; $CDCl_3$) 0.92 (3H, 2x t, J 7.6, CH_2CH_3), 1.02 (9H, s, $C(CH_3)_3$), 2.09 (2H, m, CH_2CH_3), 7.44 (1H, dd, J_{vic} 5.2, J_{vic} 8.8, C**H**); δ_C(100.6 MHz; $CDCl_3$) 10.4 (CH_2CH_3), 29.54 ($C(CH_3)_3$), 56.0 (CH_2CH_3), 77.3 ($C(CH_3)_3$), 159.5 (**C**H=N); υ_{max}(neat)/cm^{-1} 2970, 2934, 2908, 2874, 1676 (C=N), 1476, 1462, 1222, 1095, 888; m/z (EI) 114 ($[M+H]^+$, 1.3 %), 113 ($[M+H]^+$, 4.1 %), 98 ($[M\text{-}CH_3]^+$, 50.0 %), 57 ($[C(CH_3)_3]^+$, 100.0 %), 41 ($[N=CHCH_2]^+$, 77.5 %), 29 ($[CH_2CH_3]^+$, 21.0 %).

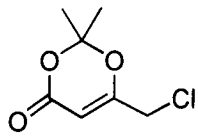
5.7.16 *E*-2,4-Dimethyl-2-hexenal 242[149]



Butyllithium (1.6 M in hexanes, 34.5 ml, 55 mmol) was added dropwise to a solution of *freshly distilled* (1,1-dimethyl-N-propylidene) ethylamine **240** (5.65 g, 50 mmol) in THF (30 ml) (under nitrogen) at -78 °C *via* a dry nitrogen flushed syringe over 10 min. The solution was warmed to 0 °C, stirred for 20 min and then cooled to -78 °C. 2-Methylbutyraldehyde **241** (3.88 g, 45 mmol) was added dropwise and then the solution was allowed to warm to 0 °C with continuous stirring for 4 h at this temperature. An aqueous slurry of oxalic acid (30 g in 110 ml H_2O) was added and the biphasic mixture was stirred vigorously for 72 h. The mixture was then filtered and extracted into DCM (3x 75 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent evaporated *in vacuo* to yield a colourless oil (3.43 g, 60.3 %). The oil was purified over silica gel (DCM), kept at -20 °C and repurified by column chromatography directly before use.

δ_H(200 MHz; $CDCl_3$) 0.88 (3H, t, J 7.4, CH_2CH_3), 1.06 (3H, d, J 6.6, $CHCH_3$), 1.41 (2H, m, CH_2CH_3), 1.75 (3H, s, $HC=CCH_3$), 2.62 (1H, m, $CH(CH_3)$), 6.25 (1H, d, J 9.8, CH=C), 9.40 (1H, s, CHO); δ_C(50.3 MHz; $CDCl_3$) 9.7 (CH_2CH_3), 12.2 ($CH(CH_3)$), 19.9 ($C(CH_3)$), 29.9 (CH_2CH_3), 35.6 ($CH(CH_3)$), 138.6 ($(CH_3)C=CH$), 160.8 ($CH=C(CH_3)$), 196.0 (CO); υ_{max}(neat)/cm^{-1} 2966, 2930, 2880, 2822, 2708, 1691 (CO), 1642, 1459, 1119, 1005; m/z (EI) 126 ($[M]^+$, 10.6 %), 111 ($[M-CH_3]^+$, 20.8 %), 97 ($[M-CH_2CH_3]^+$, 68.1 %), 69 ($[M-C_4H_9]^+$, 44.6 %), 55 ($[M+H-C_4H_9-CH_3]^+$, 64.0 %), 41 ($[C_3H_5]^+$, 100.0 %), 29 ($[C_2H_5]^+$, 77.3 %).

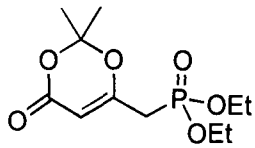
5.7.17 2,2-Dimethyl-6-chloromethyl-1,3-dioxin-4-one 243[150]



Freshly distilled 2,2,6-trimethyl-4H-1,3-dioxin-4-one **215** (5.0 g, 35.2 mmol, bp 59.5 °C, 1.5 mmHg) in THF (20 ml) was added dropwise to a solution of lithium diisopropylamide (LDA) (2M solution in THF, 24.0 ml, 48.0 mmol) in THF (100 ml) at -78 °C. The resultant bright yellow suspension was stirred for 30 min and then transferred *via* cannula to a stirred solution of hexachloroethane (12.5 g, 52.8 mmol) in THF (100 ml) (under nitrogen) at -55 °C. The resultant red solution was stirred for 1 h and allowed to warm to -20 °C over this time. The reaction was then quenched with 1M HCl (30 ml) and extracted into diethyl ether (3x 100 ml). The combined organic extracts were dried and the solvent was removed *in vacuo* to yield a yellow oil which was purified by column chromatography over silica gel (DCM). The product was recovered as a colourless oil (4.30 g, 69.2 %).

δ_H(200 MHz; $CDCl_3$) 1.72 (6H, s, $C(\mathbf{CH_3})_2$), 4.02 (2H, s, $\mathbf{CH_2}Cl$), 5.56 (1H, s, **CH**); δ_C(75.4 MHz; $CDCl_3$) 25.2 ($C(\mathbf{CH_3})_2$), 41.3 ($\mathbf{CH_2}Cl$), 96.0 (**C**H=C), 107.9 ($\mathbf{C}(CH_3)_2$), 160.8 (**C**O), 164.8 (HC=**C**CH_2Cl); υ_{max}(neat)/cm^{-1} 3449, 3106, 3002, 2945, 1732 (CO), 1646, 1394, 1212, 1016, 741; m/z (EI) 178 ($[M]^+$, ^{37}Cl, 4.0 %), 176 ($[M]^+$, ^{35}Cl, 12.4 %), 163 ($[M\text{-}CH_3]^+$, ^{37}Cl, 0.6 %), 161 ($[M\text{-}CH_3]^+$, ^{35}Cl, 1.7 %), 142 ($[M+H\text{-}Cl]^+$, 0.9 %), 121 ($[M+H\text{-}(CH_3)_2CO]^+$, ^{37}Cl, 8.3 %), 119 ($[M+H\text{-}(CH_3)_2CO]^+$, ^{35}Cl, 26.2 %), 84 ($[M+H\text{-}(CH_3)_2CO\text{-}Cl]^+$, 3.4 %), 69 ($[M\text{-}(CH_3)_2CO\text{-}CH_2Cl]^+$, 61.9 %), 59 ($[C_3H_7O]^+$, 93.5 %), 58 ($[C_3H_6O]^+$, 12.8 %).

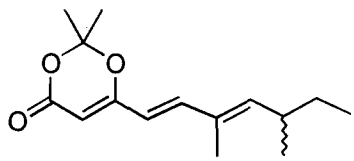
5.7.18 2,2-Dimethyl-6-(diethylphosphonomethyl)-1,3-dioxin-4-one 244[150]



To a stirred suspension of NaH (60 % in mineral oil, 636 mg, 15.9 mmol) in THF (30 ml) at 0 °C was added distilled diethyl phosphite (2.44 g, 17.7 mmol, bp 50 C, 2.0 mmHg). After stirring for 1h, 2,2-dimethyl-6-chloromethyl-1,3-dioxin-4-one **243** (1.03 g, 5.84 mmol) in THF (4 ml) was added dropwise over 10 min, and the resultant orange solution stirred at 0 °C for 90 min. 1M HCl (10 ml) was added to the reaction and the mixture extracted into diethyl ether (3x 50 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo*. The product was purified by column chromatography over silica gel (diethyl ether, then ethyl acetate), yielding a colourless oil (836 mg, 51.5 %). Diethyl phosphite was present in the early elution fractions.

δ_H(200 MHz; $CDCl_3$) 1.30 (6H, t, J 7.0, $(OCH_2C\mathbf{H}_3)_2$), 1.66 (6H, s, $C(C\mathbf{H}_3)_2$), 2.76 (2H, d, $^2J_{H\text{-}P}$ 22.2, $C\mathbf{H}_2P(O)(OCH_2CH_3)_2$), 4.10 (4H, 2x q, $^3J_{H\text{-}P}$ 7.2, $(OC\mathbf{H}_2CH_3)_2$), 5.34 (1H, d, $^4J_{H\text{-}P}$ 3.6, C**H**); δ_C(50.3 MHz; $CDCl_3$) 16.6 (d, $^3J_{C\text{-}P}$ 6.2, $(OCH_2\mathbf{C}H_3)_2$), 25.2 $(C(\mathbf{C}H_3)_2)$, 32.6 (d, $^1J_{C\text{-}P}$ 137.4, $\mathbf{C}H_2P(O)(OCH_2CH_3)_2$), 62.9 (d, $^2J_{C\text{-}P}$ 6.6, $(O\mathbf{C}H_2CH_3)_2$), 96.5 (d, $^3J_{C\text{-}P}$ 8.0, **C**H=C), 107.4 ($\mathbf{C}(CH_3)_2$), 160.8 (**C**O), 163.3 (d, $^2J_{C\text{-}P}$ 9.9, HC=**C**CH_2P); δ_P(81 MHz; $CDCl_3$) 21.58 ($CH_2\mathbf{P}(O)(OCH_2CH_3)_2$); υ_{max}(neat)/cm^{-1} 3468, 3097, 2987, 2911, 1731 (br, CO), 1634, 1392, 1260 (PO), 1023; m/z (EI) 279 ($[M+H]^+$, 0.1 %), 278 ($[M]^+$, 0.3 %), 220 ($[M\text{-}(CH_3)_2CO]^+$, 38.5 %), 192 ($M+H\text{-}(CH_3)_2CO\text{-}CH_2CH_3]^+$, 56.1 %), 164 ($M+H\text{-}(CH_3)_2CO\text{-}(CH_2CH_3)_2+H]^+$, 19.0 %), 152 ($[CH_3P(O)(OCH_2CH_3)_2]^+$, 32.3 %), 137 ($[P(O)(OCH_2CH_3)_2]^+$, 13.6 %), 109 ($[P(O)(OH)(OCH_2CH_3)]^+$, 19.7 %), 81 ($[P(O)(OH)_2]^+$, 21.5 %), 59 ($[C_3H_7O]^+$, 9.6 %), 58 ($[C_3H_6O]^+$, 23.4 %), 43 ($[C_2H_3O]^+$, 100.0 %).

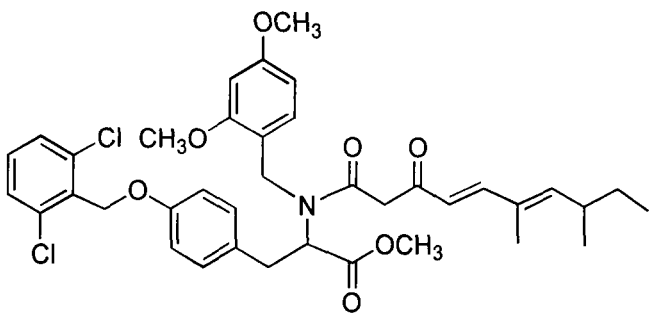
5.7.19 2,2-Dimethyl-6-(*E*,*E*-3,5,dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one 174[80]



Butyllithium (2 M solution in hexanes, 1.89 ml, 3.78 mmol) was added dropwise to a stirred solution of 1,1,1,3,3,3-hexamethyldisilazane (0.8 ml, 3.78 mmol) in THF (30 ml) under nitrogen at 0 °C. After 10 min, a solution of 2,2-dimethyl-6-(diethylphosphono-methyl)-1,3-dioxin-4-one **244** (1.01 g, 3.63 mmol) in THF (8 ml) was added. The resultant deep red solution was stirred for 20 min at 0 °C and then cooled to -78 °C. Freshly purified *E*-2,4-dimethyl-2-hexenal **242** (454 mg, 3.60 mmol) was added dropwise and the reaction allowed to warm to room temperature over 4 h, followed by stirring for a further 8 h at this temperature. The solvent was removed *in vacuo*, the residue dissolved in DCM and the solution filtered to remove any insolubles. The filtrate was reduced *in vacuo* and the residue purified by column chromatography over silica gel (DCM) to give the product as a colourless oil (386 mg, 42.9 %).

δ_H(200 MHz; $CDCl_3$) 0.83 (3H, t, J 7.4, CH_2CH_3), 0.97 (3H, d, J 6.6, $CH(CH_3)$), 1.33 (2H, m, CH_2CH_3), 1.70 (6H, s, $C(CH_3)_2$), 1.78 (3H, s, $C=C(CH_3)$), 2.44 (1H, m, $CH(CH_3)$), 5.29 (1H, s, CH=COR), 5.63 (1H, d, J 9.8, $C=CHCH(CH_3)CH_2CH_3$), 5.92 (1H, d, J 15.6, $CH=CHC(CH_3)$) 6.95 (1H, d, J 15.6, $CH=CHC(CH_3)$); δ_C(50.3 MHz; $CDCl_3$) 12.2 (CH_2CH_3), 12.6 ($CH(CH_3)$), 20.5 ($C(CH_3)$), 25.3 ($C(CH_3)_2$), 30.3 (CH_2CH_3), 35.3 ($CH(CH_3)$), 93.9 (CH=COR), 106.4 ($C(CH_3)_2$), 117.4 ($CH=C(CH_3)CH_2CH_3$), 132.1 ($C(CH_3)=CH$), 143.7 ($HC=CHC(CH_3)$), 148.3 ($HC=CHC(CH_3)$), 162.4 (HC=COR), 164.5 (CO); υ_{max}(neat)/cm^{-1} 3055, 2995, 2961, 2926, 2871, 1726 (br, CO), 1624, 1388, 1272, 1017; m/z (EI) 251 ($[M+H]^+$, 8.9 %), 250 ($[M]^+$, 33.8 %), 235 ($[M-CH_3]^+$, 1.0 %), 229 ($[M-CH_2CH_3]^+$, 0.7 %), 192 ($[M-(CH_3)_2CO]^+$, 14.6 %), 177 ($[M-(CH_3)_2CO-CH_3]^+$, 16.6 %), 163 ($[M-(CH_3)_2CO-CH_2CH_3]^+$, 37.1 %), 135 ($[M-(CH_3)_2CO-CH(CH_3)CH_2CH_3)]^+$, 81.5 %), 123 ($[CH=CHC(CH_3)CH=CH(CH_3)CH_2CH_3]^+$, 100.0 %), 57 ($[CH(CH_3)CH_2CH_3]^+$, 21.9 %).

5.7.20 N-(2,4-Dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester 245

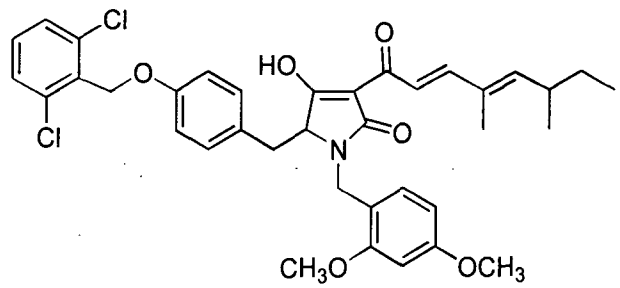


PPTS (77 mg, 0.31 mmol) was added to a solution of 2,2-dimethyl-6-(*E*,*E*-3,5-dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one **174** (70 mg, 0.28 mmol) and N-(2,4-dimethoxy)benzyl-O-2,6-dichlorobenzyl-DL-tyrosine methyl ester **234** (155 g, 0.31 mmol) in toluene (2 ml) and the mixture refluxed with stirring for 3.5 h. After cooling, the solvent was removed *in vacuo* and the product purified by column chromatography over silica gel (DCM, then DCM:diethyl ether, 20:1). A pale yellow solid (174 mg, 89.3 %) was produced, mp 41-62 °C (undefined). The NMR revealed a mixture of tautomers.

δ_H(299.9 MHz; $CDCl_3$) 0.75 & 0.77 (3H, 2x t, J 7.5, CH_2CH_3), 0.88 & 0.91 (3H, 2x d, J 6.9, $CH(CH_3)$), 1.30 (2H, m, CH_2CH_3), 1.67 & 1.74 (3H, 2x s, $C(CH_3)$), 2.36 (1H, m, $CH(CH_3)$), 3.04 (1H, m, CH_2), 3.26 (1H, dd, J_{vic} 6.0, J_{gem} 14.1, CH_2), 3.52 & 3.55 (3H, 2x s, OCH_3), 3.67 & 3.68 (3H, 2x s, OCH_3), 3.71 & 3.72 (3H, 2x s, OCH_3), 4.03 (1H, m, CH), 4.28 (1H, m, CH_2N), 5.11 (1H, s, COCH=COH), 5.16 (2H, s, CH_2O), 5.48 (1H, d, J 9.6, C=CHCH(CH_3)CH_2CH_3), 5.70 (1H, m, CH_2COCH=CH), 6.17 (1H, d, J 15.6, CH_2COCH=CH), 6.30 (2H, m, **Ph**), 6.81 (2H, m, **Ph**), 6.96 (3H, m, **Ph**), 7.17 (1H, d, J 7.2, **Ph**), 7.27 (2H, d, J 8.1, **Ph**); δ_C(75.4 MHz; $CDCl_3$) 12.2 (CH_2CH_3), 12.8 ($CH(CH_3)$), 20.4 & 20.7 ($C(CH_3)$), 30.3 & 30.5 (CH_2CH_3), 34.7 & 35.0 ($CH(CH_3)$), 35.5 (CH_2), 47.1 & 48.2 (CH_2N), 52.3 & 52.5 (OCH_3), 55.4 ($ArOCH_3$), 55.7 ($ArOCH_3$), 61.1 (CH), 65.6 (CH_2O), 89.8 (COC=COH), 98.5 (Ph CH), 104.0 (Ph C), 115.2 (Ph CH), 116.3-137.3 (Ph C, Ph CH & CH), 141.5-151.0 (CH), 157.7 & 157.8 (PhCOR), 158.1 & 159 0 (Ph $COCH_3$), 160.7 & 161.3 (Ph $COCH_3$), 168.0 (NRCO), 170.5 & 171.2 (CO_2CH_3) 173.3 (C=COH), 194.2 (CO); υ_{max}(neat)/cm^{-1} 3001, 2960, 2934, 2870, 2838, 1741 (CO), 1632, 1584, 1510, 1238, 819, 733; m/z (EI) 699 ($[M]^+$,

2x ^{37}Cl, 0.1 %), 697 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.2 %), 695 ($[M]^+$, 2x ^{35}Cl, 0.5 %), 506 ($[M\text{-}R]^+$, 2x ^{37}Cl, 0.2%), 504 ($[M\text{-}R]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.8 %), 502 ($[M\text{-}R]^+$, 2x ^{35}Cl, 0.9 %), 448 ($[M+H\text{-}R\text{-}CO_2CH_3]^+$, 2x ^{37}Cl, 0.1 %), 446 ($[M+H\text{-}R\text{-}CO_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 0.7 %), 444 ($[M+H\text{-}CO_2CH_3]^+$, 2x ^{35}Cl, 0.9 %), 238 ($[(CO_2CH_3)CHNCH_2Ph(OCH_3)_2]^+$, 10.0 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 77 ($[Ph]^+$, 7.0 %), where R = $COCH_2COCH{=}CHC(CH_3){=}CHCH(CH_3)CH_2CH_3$. Found 696.2495, $C_{38}H_{43}NO_7Cl_2$ (M^+) requires 696.2495.

5.7.21 5-(4-(2,6-Dichlorobenzyloxy)-benzyl)-1-(2,4-dimethoxybenzyl)-3-(E,E-4,6-dimethyl-octa-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 246

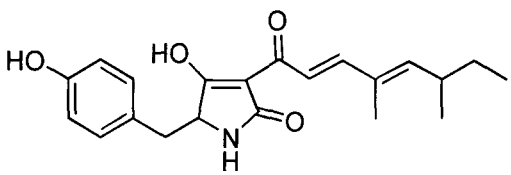


A solution of N-(2,4-dimethoxybenzyl)-N-(*E*,*E*-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-O-(2,6-dichlorobenzyl)-DL-tyrosine methyl ester **245** (90 mg, 0.13 mmol) in *t*-butanol (3 ml) was added to a stirred solution of potassium *t*-butoxide (29 mg, 0.26 mmol) in *t*-butanol (5 ml). After 30 min, the reaction was quenched with 1M HCl (3 ml) and then extracted with diethyl ether (1x 20 ml). The organic extract was washed with water (1x 3 ml), dried ($MgSO_4$) and evaporated *in vacuo* to yield a brown oil. Excess *t*-butanol was azeotropically removed with hexane to give the product as an orange/brown solid (67 mg, 78.6 %).

δ_H(400 MHz; $CDCl_3$) 0.77 (3H, 2x t, J 7.2, CH_3), 0.92 (3H, d, J 6.8, CH_3), 1.25 (2H, m, CH_2), 1.82 (3H, s, CH_3), 2.42 (1H, m, CH), 3.05 (2H, d, J 4.4, CH_2), 3.71 (3H, s, $ArOCH_3$), 3.72 (3H, s, $ArOCH_3$), 3.77 (82 %) (1H, dd, J_{vic} 4.0, J_{vic} 4.8, CH), 3.92 (18 %) (1H, t, J 4.4, CH), 4.11 (82 %) (1H, d, J 14.8, CH_2N), 4.18 (18 %) (1H, d, J 15.2, CH_2N), 4.92 (82 %) (1H, d, 14.8, CH_2N), 5.01 (18 %), (1H, d, J 14.8, CH_2N), 5.14 (1H, s, COCH=COH), 5.15 (2H, s, CH_2O), 5.73 (1H, d, J 9.6, CH), 6.36 (2H, m, **Ph**), 6.80-

7.18 (7H, m, **Ph** & C**H**), 7.27 (2H, d, J 8.0, **Ph**), 7.38 (1H, d, J 15.6, C**H**); δ_H(400 MHz; $CDCl_3$) 12.3 ($CH_2\mathbf{C}H_3$), 12.8 ($CH(\mathbf{C}H_3)$), 20.4 ($C(\mathbf{C}H_3)$), 30.3 ($\mathbf{C}H_2CH_3$), 34.7 ($\mathbf{C}H(CH_3)$), 35.6 ($\mathbf{C}H_2N$), 38.8 ($\mathbf{C}H_2$), 55.72 (2x $ArOCH_3$), 65.3 (**C**H), 65.3 ($\mathbf{C}H_2O$), 98.7 (Ph **C**H), 100.7 (CO**C**=COH), 104.6 (Ph **C**), 115.3 (Ph **C**H), 116.5-137.3 (Ph **C**, Ph **C**H, **C** & **C**H), 149.9 (**C**H), 151.8 (**C**H), 158.0 (Ph $\mathbf{C}OCH_3$), 158.9 (Ph $\mathbf{C}OCH_3$), 161 (Ph**C**OR), 174.2 &174.8 (NR**C**O & C=**C**OH), 194.8 (**C**O); υ_{max}(KBr)/cm^{-1} 3068, 2958, 2926, 2868, 1696 (CO), 1615, 1579, 1507, 1238, 1036, 772, 820; m/z (EI) 667 ($[M]^+$, 2x ^{37}Cl, 4.6 %), 665 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 18.3 %), 663 ($[M]^+$, 2x ^{35}Cl, 23.9 %), 610 ($[M\text{-}CH(CH_3)CH_2CH_3]^+$, 2x ^{37}Cl, 0.2 %), 608 ($[M\text{-}CH(CH_3)CH_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 2.0 %), 606 ($[M\text{-}CH(CH_3)CH_2CH_3]^+$, 2x ^{35}Cl, 2.8 %), 398 ($[M\text{-}BnOCH_2Ph(Cl)_2]^+$, 3.5 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 1.8 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 7.1 %), 265 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 10.8 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 3.7 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 29.7 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 35.2 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 107 ($[Bn(OH)]^+$, 5.4 %), 77 ($[Ph]^+$, 4.9 %). Found 664.2222 $C_{37}H_{39}NO_6Cl_2$ (M^+) requires 664.2232.

5.7.22 5-(4-hydroxybenzyl)-3-(E,E-4,6-dimethyl-octa-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 177



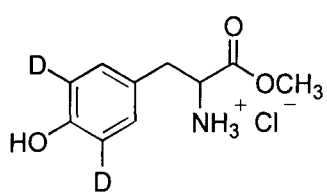
5-(4-(2,6-dichlorobenzyloxy)-benzyl-1-(2,4-dimethoxybenzyl)-3-(*E*,*E*-4,6-dimethyl-octa-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **246** (60 mg, 0.09 mmol) was stirred in 1M BBr_3 (1M solution in DCM) (0.63 ml, 0.63 mmol) at -78 °C for 6h. The reaction was quenched with water (5 ml) and extracted with diethyl ether (3x 10 ml). The organic extracts were extracted with 1M NaOH (3x 10 ml), then the combined aqueous extracts acidified and extracted with diethyl ether (3x 10 ml). The solvent was removed *in vacuo* to yield an orange oil. ^1H NMR showed that the 2,6-dichlorobenzyl had been cleaved.

The orange residue was dissolved in TFA (2 ml) and stirred under nitrogen at room temperature for 1 h. The reaction was quenched with ice and extracted with DCM (3x 10 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was isolated as an orange oil (285 mg, 89.1 %).

δ_H(499.8 MHz; CD_3OD) 0.77 (3H, t, J 7.0, CH_2CH_3), 0.92 (3H, d, J 6.4, $CH(CH_3)$), 1.38 (2H, m, CH_2CH_3), 1.79 (3H, s, $C(CH_3)$), 2.45 (1H, m, $CH(CH_3)$), 2.85 (2H, m, CH_2), 4.00 (1H, bs, **CH**), 5.62 (18 %) (1H, d, J 10.2, **CH**), 5.78 (82 %) (1H, d, J 10.4, **CH**), 6.59 (2H, d, J 6.6, **Ph**), 6.90 (2H, d, J 6.8, **Ph**), 6.97 (1H, d, J 15.8, **CH**), 7.42 (83 %) (1H, d, J 15.5, **CH**), 7.83 (17 %) (1H, d, J 15.5, **CH**); δ_{2H}(46.0 MHz; CH_3OH) 6.67 (Ph C^2H); δ_C(125.7 MHz; CD_3OD) 12.0 & 12.4 (CH_2CH_3), 12.5 ($C(CH_3)$), 20.4 ($CH(CH_3)$), 29.9 & 31.0 (CH_2CH_3), 35.5 & 36.5 ($CH(CH_3)$), 37.6 & 40.3 (CH_2), 64.5 (**C**H), 115.8 (t, J 23.0, Ph C^2H), 116.1 (Ph **C**H), 116.6 (**C**H), 127.7 (Ph **C**), 131.6 (Ph **C**H), 134.4 (**C**H), 151.1 (**C**H), 153.2 (**C**H), 157.2 (Ph **C**OH & NH**C**O), 175.9 (**C**O); υ_{max}(KBr)/cm^{-1} 3378, 3205, 3042, 2978, 2866, 1685, 1637, 1601, 1515, 1228, 821; m/z (EI) 356 ($[M+H]^+$, 6.5 %), 355 ($[M]^+$, 22.1 %), 298 ($[M-CH(CH_3)CH_2CH_3]^+$, 53.1 %), 249 ($[M+H-BnOH]^+$, 21.3 %), 232 ($[M-R]^+$, 5.6 %), 192 ($[M-CH(CH_3)CH_2CH_3$-$Bn(OH)]^+$, 18.1 %), 126 ($[M+H-BnOH-R]^+$, 18.2 %), 107 ($[Bn(OH)]^+$, 100.0 %), 77 ($[Ph]^+$, 20.3 %), where R= $CH{=}CHC(CH_3){=}CHCH(CH_3)CH_2CH_3$.

5.8 Synthesis of labelled tetramic acid 177

5.8.1 [3',5'-$^2H_2$]-Tyrosine methyl ester hydrochloride 216a[165]

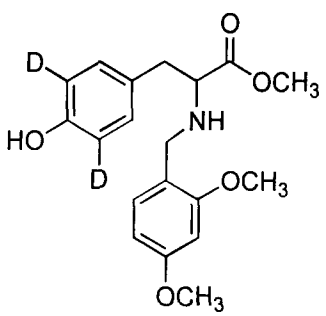


Thionyl chloride (1.4 ml, 12.0 mmol) was added dropwise to dry, distilled methanol (8 ml) and stirred rapidly at -5 °C. DL-Tyrosine **111** (2.0 g, 10.9 mmol) was added and the solution heated under reflux for 3 h. After cooling, the solution was diluted with diethyl

ether (35 ml), resulting in the precipitation of the product. The white solid was collected by filtration, washed with diethyl ether and dried to yield the product as the hydrochloride salt (2.55 g, 99.9 %), mp 190 °C (decomp.). The product was found by ^{1}H NMR to be 86 % deuterated.

δ_H(299.9 MHz; CD_3OD) 3.10 (1H, dd, J_{vic} 7.2, J_{gem} 14.4, C**H**$_2$), 3.17 (1H, dd, J_{vic} 6.3, J_{gem} 14.7, C**H**$_2$), 3.79 (3H, s, OC**H**$_3$), 4.25 (1H, dd, J_{vic} 6.3, J_{vic} 6.9, C**H**), 6.79 (14 %) (2H, d, J 9.0, **Ph**), 7.07 (2H, s, **Ph**); δ_H(75.4 MHz; CD_3OD) 37.4 (**C**H$_2$), 54.4 (O**C**H$_3$), 56.3 (**C**H), 117.4 (t, J 24.3, Ph **C**^{2}H), 117.7 (Ph **C**H), 126.4 (Ph **C**), 132.2 (86 %) (Ph **C**H), 132.4 (14 %) (Ph **C**H), 159.0 (Ph **C**OH), 171.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3378 (NH), 3341 (NH), 3100 (br), 3010, 2950, 2480, 1741 (CO), 1602, 1497, 1476, 1246, 1057, 771; m/z (EI) 197 ([M]$^+$, $^2H_2$, 7.1 %), 196 ([M]$^+$, $^2H_1$, 2.2 %), 195 ([M]$^+$, 0.3 %), 138 ([M-CO_2CH_3]$^+$, $^2H_2$, 28.1 %), 137 ([M-CO_2CH_3]$^+$, $^2H_1$, 12.9 %), 136 ([M-CO_2CH_3]$^+$, 3.8 %), 109 ([Bn(OH), $^2H_2$, 100.0 %), 108 ([Bn(OH)]$^+$, $^2H_1$, 31.2 %), 107 ([Bn(OH)]$^+$, 3.8 %), 88 ([M-Bn(OH)]$^+$, 43.1 %), 79 ([Ph]$^+$, $^2H_2$, 8.5 %), 78 ([Ph]$^+$, $^2H_1$, 8.1 %), 77 ([Ph]$^+$, 2.3 %).

5.8.2 N-2,4-Dimethoxybenzyl [3',5'-$^2H_2$]-tyrosine methyl ester 221a

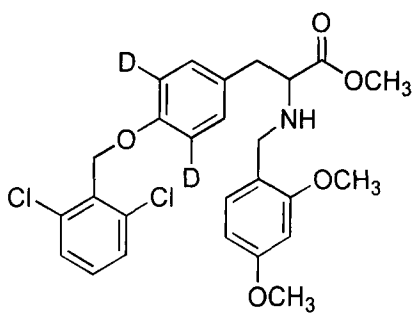


To a solution of [3',5'-$^2H_2$]-tyrosine methyl ester hydrochloride **216a** (1.21 g, 5.14 mmol) in methanol (20 ml) was added 2,4-dimethoxybenzaldehyde (896 mg, 5.40 mmol) and $MgSO_4$ (3.0 g). The mixture was stirred at room temperature for 24 h, filtered and then the solvent removed *in vacuo*. The residue was redissolved in methanol (15 ml) and cooled to 0 °C. $NaBH_4$ (408 mg, 10.8 mmol) was carefully added in small portions over 45 min and the mixture left to stir for 12 h. The reaction was

quenched with water (10 ml) and then extracted into ethyl acetate (3x 50 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to yield an orange oil which was purified over silica gel (EtOAc: petroleum ether, 3:2) to give a white solid (955 mg, 53.1 %), mp 95-96 °C.

δ_H(299.9 MHz; $CDCl_3$) 2.84 (1H, dd, J_{vic} 8.4, J_{gem} 13.8, C**H**$_2$), 2.92 (1H, dd, J_{vic} 6.0, J_{gem} 13.8, C**H**$_2$), 3.47 (1H, t, J 6.3, C**H**), 3.55 (3H, s, ArOC**H**$_3$), 3.58 (3H, s, ArOC**H**$_3$), 3.63 (1H, d, J 13.5, C**H**$_2$N), 3.71 (3H, s, COOC**H**$_3$), 3.76 (1H, d, J 13.5, C**H**$_2$N), 6.33 (2H, m, **Ph**), 6.68 (14 %) (2H, d, J 8.7, **Ph**), 6.90 (2H, s, **Ph**), 6.97 (1H, d, J 9.0, **Ph**); δ_C(75.4 MHz; $CDCl_3$) 38.4 (**C**H$_2$), 47.4 (**C**H$_2$N), 51.8 (O**C**H$_3$), 55.0 (ArO**C**H$_3$), 55.3 (ArO**C**H$_3$), 61.7 (**C**H), 98.4 (Ph **C**H), 103.7 (Ph **C**H), 115.6 (m, Ph **C**^{2}H), 115.8 (Ph **C**H), 118.8 (Ph **C**H), 127.5 (Ph **C**), 130.0 (84 %) (Ph **C**H), 130.1 (16 %) (Ph **C**H), 130.9 (Ph **C**H), 155.7 (Ph **C**OH), 158.7 (Ph **C**OCH$_3$), 160.5 (Ph **C**OCH$_3$), 174.5 (**C**O); υ_{max}(KBr)/cm^{-1} 3280 (NH), 3100 (Br), 2998, 2947, 2836, 1726 (CO), 1615, 1512, 1206, 1035, 816; m/z (EI) 347 ([M]$^+$, $^2H_2$, 0.26 %), 346 ([M]$^+$, $^2H_1$, 0.28 %), 345 ([M]$^+$, 0.12 %), 288 ([M-CO_2CH_3]$^+$, $^2H_2$, 1.4 %), 287 ([M-CO_2CH_3]$^+$, $^2H_1$, 0.5 %), 286 ([M-CO_2CH_3]$^+$, 0.17 %), 238 ([M-Bn(OH)]$^+$, 13.5 %), 151 ([Bn(OCH_3)$_2$]$^+$, 100.0 %), 137 ([Ph(OCH_3)$_2$]$^+$, 1.4 %), 109 ([Bn(OH)]$^+$, $^2H_2$, 7.3 %), 108 ([Bn(OH)]$^+$, $^2H_1$, 4.0 %), 107 ([Bn(OH)]$^+$, 0.9 %), 79 ([Ph]$^+$, $^2H_2$, 2.0 %), 78 ([Ph]$^+$, $^2H_1$, 3.8 %), 77 ([Ph]$^+$, 3.4 %).

5.8.3 *N-(2,4-dimethoxy)benzyl-O-2,6-dichlorobenzyl [3',5'-$^2H_2$]-tyrosine methyl ester 234a*

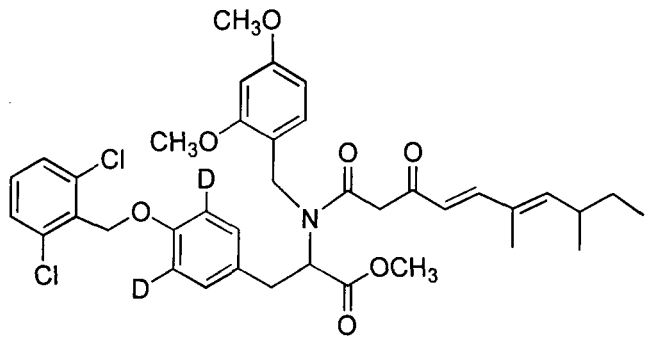


N-(2,4-dimethoxy)benzyl [3',5'-$^2H_2$]-tyrosine methyl ester **221a** (950 mg, 2.74 mmol), 2,6-dichlorobenzyl chloride (589 mg, 3.01 mmol) and K_2CO_3 (831 g, 6.02 mmol) were

stirred in DMF (15 ml) at 60 °C for 24 h. After cooling, water (10 ml) was added and the mixture extracted with ethyl acetate (3x 30 ml). The combined organic extracts were washed with water (10x 20 ml) to remove any remaining DMF, dried ($MgSO_4$) and the solvent removed *in vacuo*. After purification over silica gel (EtOAc: petroleum ether, 2:3), a white solid (955 mg, 69.0 %), mp 85.5-86.5 °C, was produced.

δ_H(299.9 MHz; $CDCl_3$) 2.87 (1H, dd, J_{vic} 7.8, J_{gem} 13.8, C**H**$_2$), 2.97 (1H, dd, J_{vic} 6.0, J_{gem} 13.5, C**H**$_2$), 3.49 (1H, dd, J_{vic} 6.3, J_{vic} 7.8, C**H**), 3.62 (1H, d, J 13.8, C**H**$_2$N), 3.65 (6H, s, 2x ArOC**H**$_3$), 3.78 (3H, s, C**H**$_3$), 3.79 (1H, d, J 13.8, C**H**$_2$N), 5.24 (2H, s, C**H**$_2$O), 6.39 (2H, m, **Ph**), 6.94 (14 %) (2H, d, J 9.0, **Ph**), 7.05 (1H, d, J 7.8, **Ph**), 7.10 (2H, s, **Ph**), 7.22 (1H, m, **Ph**), 7.35 (2H, d, J 8.1, **Ph**); δ_C(75.4 MHz; $CDCl_3$) 38.9 (**C**H$_2$), 47.5 (**C**H$_2$NH), 51.8 (O**C**H$_3$), 55.2 (ArO**C**H$_3$), 55.5 (ArO**C**H$_3$), 62.1 (**C**H), 65.3 (**C**H$_2$O), 98.5 (Ph **C**H), 103.7 (Ph **C**H), 114.7 (t, J 23.5, Ph **C**^{2}H), 115.0 (Ph **C**H), 120.1 (Ph **C**), 128.6 (Ph **C**H), 130.2 (Ph **C**H), 132.3 (Ph **C**), 137.1 (Ph **C**H), 157.7 (Ph **C**OH), 158.7 (Ph **C**OCH$_3$), 160.3 (Ph **C**OCH$_3$), 175.1 (**C**O); υ_{max}(KBr)/cm^{-1} 3446 (br), 3356 (NH), 3084, 2996, 2954, 2938, 2832, 1740, 1612, 1500, 1208, 830; m/z (EI) 509 ([M]$^+$, 2x ^{37}Cl, $^2H_2$, 0.05 %), 508 ([M]$^+$, 2x ^{37}Cl, $^2H_1$, 0.1 %), 507 ([M]$^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 0.21 %), 506 ([M]$^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 0.23 %), 505 ([M]$^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 0.34 %), 504 ([M]$^+$, 2x ^{35}Cl, $^2H_1$, 0.25 %), 503 ([M]$^+$, 2x ^{35}Cl, 0.19 %), 450 ([M-CO_2CH_3]$^+$, 2x ^{37}Cl, $^2H_2$, 0.17 %), 449 ([M-CO_2CH_3]$^+$, 2x ^{37}Cl, $^2H_1$, 0.23 %), 448 ([M-CO_2CH_3]$^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 0.60 %), 447 ([M-CO_2CH_3]$^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 0.52 %), 446 ([M-CO_2CH_3]$^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 1.1 %), 445 ([M-CO_2CH_3]$^+$, 2x ^{35}Cl, $^2H_1$, 0.37 %), 444 ([M-CO_2CH_3]$^+$, 2x ^{35}Cl, 0.22 %), 271 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 2x ^{37}Cl, $^2H_2$, 0.41 %), 270 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 2x ^{37}Cl, $^2H_1$, 0.54 %), 269 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 1.9 %), 268 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 1.2 %), 267 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 2.9 %), 266 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 2x ^{35}Cl, $^2H_1$, 0.92 %), 265 ([BnOCH$_2$Ph(Cl)$_2$]$^+$, 2x ^{35}Cl, 0.13 %), 238 ([(CHCO$_2$CH$_3$)NHBn(OCH$_3$)$_2$]$^+$, 13.2 %), 163 ([Bn(Cl)$_2$]$^+$, 2x ^{37}Cl, 1.5 %), 161 ([Bn(Cl)$_2$]$^+$, 1x ^{37}Cl, 1x ^{35}Cl, 8.6 %), 159 ([Bn(Cl)$_2$]$^+$, 2x ^{35}Cl, 13.4 %), 79 ([Ph]$^+$, $^2H_2$, 0.89 %), 78 ([Ph]$^+$, $^2H_1$, 1.6 %), 77 ([Ph]$^+$, 1.6 %), Found 506.1463, $C_{26}H_{25}D_2NO_5Cl_2$ (M$^+$) requires 506.1470.

5.8.4 *DL-N-(2,4-dimethoxybenzyl)-N-(E,E-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-O-(2,6-dichlorobenzyl) [3',5'-$^2H_2$]-tyrosine methyl ester 245a*

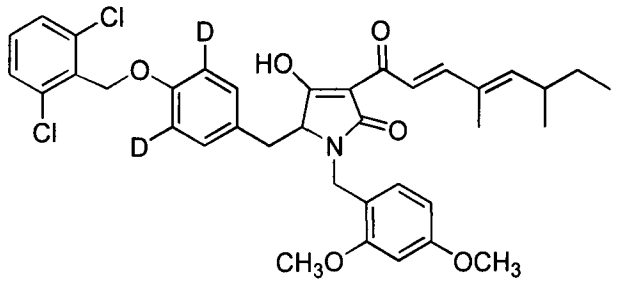


To a solution of 2,2-dimethyl-6-(*E*,*E*-3,5,dimethylhepta-1,3-dienyl)-1,3-dioxin-4-one **174** (415 mg, 1.66 mmol) and N-(2,4-dimethoxy)benzyl-O-2,6-dichlorobenzyl [3',5'-$^2H_2$]-tyrosine methyl ester **234** (800 g, 1.58 mmol) in toluene (2 ml) was added PPTS (417 mg, 1.66 mmol) and the mixture refluxed with stirring for 1.5 h. After cooling the solvent was removed *in vacuo* and the product purified by column chromatography over silica gel (DCM, then DCM:diethyl ether, 20:1). A pale yellow solid (508 mg, 46.0 %) was produced, mp 38 °C (decomp.).

δ_H(299.9 MHz; $CDCl_3$) 0.83 & 0.85 (3H, 2x t, J 7.0, CH_2CH_3), 1.00 (3H, 2x d, J 6.5, $CH(CH_3)$), 1.35 (2H, m, CH_2CH_3), 1.76 & 1.83 (3H, 2x s, $C(CH_3)$), 2.44 (1H, m, $CH(CH_3)$), 3.10 (1H, dd, J_{vic} 9.0, J_{gem} 14.5, CH_2), 3.35 (1H, dd, J_{vic} 6.0, J_{gem} 14.0, CH_2), 3.61 & 3.64 (3H, 2x s, OCH_3), 3.75 & 3.77 (3H, 2x s, OCH_3), 3.79 & 3.80 (3H, 2x s, OCH_3), 3.94 (43 %), (1H, dd, J_{vic} 3.5, J_{gem} 15.5, CH), 4.08 (1H, m, CH_2N), 4.36 (1H, m, CH_2N), 4.50 (57 %) (1H, m, CH), 5.20 (43 %) (1H, s, COCH=COH), 5.25 (2H, s, CH_2O), 5.57 (43 %) (1H, d, J 9.5, CH), 5.76 (57 %) (1H, d, J 16.0, CH), 5.79 (43 %) (1H, d, J 10.0, CH), 6.26 (33 %) (1H, d, J 15.5, CH), 6.39 (2H, m, **Ph**), 7.02 (3H, m, **Ph** & CH), 7.09 (57 %) (1H, d, J 15.0, CH), 7.24 (2H, m, **Ph** & CH), 7.36 (3H, m, **Ph** & CH); δ_C(75.6 MHz; $CDCl_3$) 12.2 (CH_2CH_3), 12.8, ($CH(CH_3)$), 20.4 & 20.7 ($C(CH_3)$), 30.2 & 30.4 (CH_2CH_3), 34.7 & 35.4 ($CH(CH_3)$), 35.0 & 35.1 (CH_2), 47.0 & 48.1 (CH_2N), 49.2 (CH), 52.3 & 52.4 (OCH_3), 55.4 & 55.5 (OCH_3), 55.6 (OCH_3), 60.7 (CH), 61.0 (CH_2N), 65.5 (CH_2O), 89.8 (COCH=COH), 98.5 & 98.8 (Ph CH & CH), 104.0 (Ph C), 114.9 (t, J 27.9, Ph C^2H), 115.2 (Ph CH), 116.3 (Ph C), 116.9 (Ph C), 120.7 (CH), 123.9 (CH), 128.7 (Ph CH), 1130.0-132.5 (Ph C, Ph CH & CH), 137.2 (Ph

C), 141.5 (**C**H), 145.9 (**C**H), 150.2 & 150.9 (**C**H), 157.7 (Ph **C**OR), 158.1 (Ph **C**OCH_3), 159.0 (Ph **C**OCH_3), 160.7 & 161.3 (NR**C**O), 170.5 & 171.1 (O**C**H_3), 171.7 & 173.3 (C=**C**-OH), 194.1 (**C**O); υ_{max}(KBr)/cm^{-1} 3446 (br), 2958, 2926, 2872, 1741 (CO), 1635, 1583, 1507, 1463, 1237, 834, 767; m/z (EI) 701 ($[M]^+$, 2x ^{37}Cl, $^2H_2$, 0.07 %), 700 ($[M]^+$, 2x ^{37}Cl, $^2H_1$, 0.14 %), 699 ($[M]^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 0.34 %), 698 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 0.27 %), 697 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 0.46 %), 696 ($[M]^+$, 2x ^{35}Cl, $^2H_1$, 0.12 %), 672 ($[M\text{-}CH_2CH_3]^+$, 2x ^{37}Cl, $^2H_2$, 0.35 %), 671 ($[M\text{-}CH_2CH_3]^+$, 2x ^{37}Cl, $^2H_1$, 0.31 %), 670 ($[M\text{-}CH_2CH_3]^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 1.2 %), 669 ($[M\text{-}CH_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 0.43 %), 668 ($[M\text{-}CH_2CH_3]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 1.7 %), 667 ($[M\text{-}CH_2CH_3]^+$, 2x ^{35}Cl, $^2H_1$, 0.29 %), 666 ($[M\text{-}CH_2CH_3]^+$, 2x ^{35}Cl, 1.2 %), 508 ($[M\text{-}R]^+$, 2x ^{37}Cl, $^2H_2$, 0.37 %), 507 ($[M\text{-}R]^+$, 2x ^{37}Cl, $^2H_1$, 0.32 %), 506 ($[M\text{-}R]^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 0.61 %), 505 ($[M\text{-}R]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 0.52 %), 504 ($[M\text{-}R]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 0.79 %), 503 ($[M\text{-}R]^+$, 2x ^{35}Cl, $^2H_1$, 0.31 %), 359 ($[NHRBn(OCH_3)_2]^+$, 2.9 %), 271 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, $^2H_2$, 0.53 %), 270 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, $^2H_1$, 0.63 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 2.2 %), 268 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 1.4 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 3.4 %), 266 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 1.1 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 79 ($[Ph]^+$, $^2H_2$, 4.9 %), 78 ($[Ph]^+$, $^2H_1$, 2.8 %), 77 ($[Ph]^+$, 5.7 %); Found 698.2611, $C_{38}H_{41}D_2NO_7Cl_2$ (M^+) requires 698.2620.

5.8.5 5-(4-(2,6-dichlorobenzyloxy)-[3',5'-$^2H_2$]-benzyl-1-(2,4-dimethoxybenzyl)-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 246a

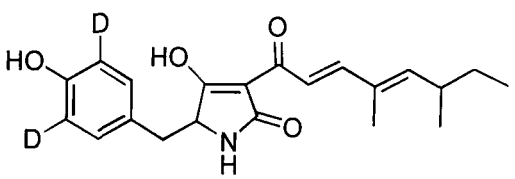


To a stirred solution of potassium *t*-butoxide (173 mg, 1.55 mmol) in *t*-butanol (10 ml) was added a solution of N-(2,4-dimethoxybenzyl)-N-(*E*,*E*-3-oxo-6,8-dimethyldeca-4,6-dienoyl)-O-(2,6-dichlorobenzyl) [3',5'-$^2H_2$]-tyrosine methyl ester **245** (540 mg, 0.77 mmol) in *t*-butanol (5 ml). After 30 min, the reaction was quenched with 1M HCl (5 ml) and extracted with diethyl ether (1x 20 ml). The organic portion was washed with water (1x 3 ml), dried ($MgSO_4$) and evaporated *in vacuo* to yield an orange solid (465 mg, 90.1 %), mp 56 °C (decomp.). Excess *t*-butanol was azeotropically removed with hexane.

δ_H(299.9 MHz; $CDCl_3$) 0.76 (3H, t, J 7.5, CH_2CH_3), 0.91 (3H, d, J 6.6, $CH(CH_3)$), 1.25 (2H, m, CH_2CH_3), 1.80 (3H, s, $C(CH_3)$), 2.40 (1H, m, $CH(CH_3)$), 3.04 (2H, d, J 4.5, CH_2), 3.70 (6H, s, 2x $ArOCH_3$), 3.76 (83 %) (1H, t, J 4.8, CH), 3.91 (17 %) (1H, t, J 4.5, CH), 4.11 (83 %) (1H, d, 14.7, CH_2N), 4.16 (17 %) (1H, d, 15.0, CH_2N), 4.92 (83 %) (1H, d, J 15.0, CH_2N), 5.01 (17 %) (1H, d, J 14.7, CH_2N), 5.14 (2H, s, CH_2O), 5.55 (7 %) (1H, d, J 10.8, CH), 5.72 (93 %) (1H, d, J 9.9, CH), 6.35 (2H, m, **Ph**), 6.80 (14 %) (2H, d, J , **Ph**), 6.97 (3H, m, **Ph** & CH), 7.13 (2H, m, **Ph** & CH), 7.25 (2H, d, J 7.5, **Ph**), 7.37 (1H, d, J 15.6, CH); δ_C(75.4 MHz; $CDCl_3$) 12.2 (CH_2CH_3), 12.7 & 12.8 ($CH(CH_3)$), 20.4 ($C(CH_3)$), 29.4 & 30.3 (CH_2CH_3), 34.2 & 34.6 (CH_2), 35.6 ($CH(CH_3)$), 38.0 & 38.7 (CH_2N), 55.6 & 55.7 (OCH_3), 63.1 (CH), 65.3 & 65.5 (CH_2O), 98.7 (Ph CH), 100.7 (CH), 104.6 (Ph CH), 115.3 (t, J 29.1, Ph C^2H), 116.3 (Ph CH), 116.0-133.7 (Ph C, Ph CH & CH), 137.3 (Ph C), 149.8 & 150.6 (CH), 151.7 & 152.1 (CH), 157.8 (Ph COR), 158.7 & 158.8 (Ph $COCH_3$), 160.8 & 161.1 (NRCO), 174.1 & 175.0 (C=COH), 194.7 (CO), 203.2 (CO); υ_{max}(KBr)/cm^{-1} 3446, (br OH), 3070, 2959, 2930, 2868, 2832, 1700 (CO), 1653 (CO), 1616, 1574, 1507, 1458, 1239, 844, 768; m/z

(EI) 669 ($[M]^+$, 2x ^{37}Cl, $^2H_2$, 0.74 %), 668 ($[M]^+$, 2x ^{37}Cl, $^2H_1$, 3.3 %), 667 ($[M]^+$, 2x ^{37}Cl & 1x ^{37}Cl, 1x ^{35}Cl, $^2H_2$, 0.79 %), 666 ($[[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 2.8 %), 665 ($[M]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x ^{35}Cl, $^2H_2$, 1.7 %), 664 ($[M]^+$, 2x ^{35}Cl, $^2H_1$, 1.1 %), 663 ($[M]^+$, 2x ^{35}Cl, 0.86 %), 271 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, $^2H_2$, 0.58 %), 270 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, $^2H_1$, 0.60 %), 269 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{37}Cl, 1x ^{37}Cl & 1x ^{35}Cl, $^2H_2$, 2.3 %), 268 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, $^2H_1$, 1.3 %), 267 ($[BnOCH_2Ph(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl & 2x^{35}Cl, $^2H_2$, 3.3 %), 266 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, $^2H_1$, 1.24 %), 265 ($[BnOCH_2Ph(Cl)_2]^+$, 2x ^{35}Cl, 0.23 %), 163 ($[Bn(Cl)_2]^+$, 2x ^{37}Cl, 1.6 %), 161 ($[Bn(Cl)_2]^+$, 1x ^{37}Cl, 1x ^{35}Cl, 5.1 %), 159 ($[Bn(Cl)_2]^+$, 2x ^{35}Cl, 7.5 %), 151 ($[Bn(OCH_3)_2]^+$, 100.0 %), 109 ($[Bn(OH)]^+$, $^2H_2$, 8.9 %), 108 ($[Bn(OH)]^+$, $^2H_1$, 3.1 %), 107 ($[Bn(OH)]^+$, 1.1 %), 79 ($[Ph]^+$, $^2H_2$, 2.7 %), 78 ($[Ph]^+$, $^2H_1$, 2.8 %), 77 ($[Ph]^+$, 3.4 %), 57 ($[CH(CH_3)CH_2CH_3]^+$, 5.9 %); Found 666.2352, $C_{37}H_{37}D_2NO_6Cl_2$ (M^+) requires 666.2358.

5.8.6 5-(4-(hydroxy)-[3',5'-$^2H_2$]-benzyl-3-(E,E-4,6-dimethylocta-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one 177a



5-(4-(2,6-dichlorobenzyloxy)-[3',5'-$^2H_2$]-benzyl-1-(2,4-dimethoxybenzyl)-3-(*E*,*E*-4,6-dimethylocta-2,4-dienoyl)-4-hydroxy-1,5-dihydro-pyrrol-2-one **246** (715 mg, 1.07 mmol) was stirred in 1M BBr_3 (1M solution in DCM) (7.51 ml, 7.51 mmol) at -78 °C for 6h. The reaction was quenched with water (10 ml) and extracted with diethyl ether (2x 30 ml). The organic extracts were extracted with 1M NaOH (2x 20 ml), then the combined aqueous extracts acidified and extracted with diethyl ether (3x 30 ml). The solvent was removed *in vacuo* to yield an orange oil. ^1H NMR showed that the 2,6-dichlorobenzyl had been cleaved.

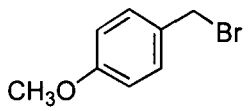
The orange residue was dissolved in TFA (20 ml) and stirred under nitrogen at room temperature for 1 h. The reaction was quenched with ice and extracted with diethyl

ether (3x 30 ml). The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo*. The product was afforded as an orange oil (338 mg, 85.8 %).

δ_H(499.8 MHz; CD_3OD) 0.85 (3H, 2x t, J 7.0, $CH_2C\mathbf{H}_3$), 0.99 (91 %) (3H, d, J 6.5, $CH(C\mathbf{H}_3)$), 1.07 (9 %) (3H, d, J 6.5, $CH(C\mathbf{H}_3)$), 1.38 (2H, m, $C\mathbf{H}_2CH_3$), 1.86 (91 %) (3H, s, $C(\mathbf{C}H_3)$), 1.93 (9 %), (3H, s, $C(\mathbf{C}H_3)$), 2.52 (87 %) (1H, m, $C\mathbf{H}(CH_3)$), 2.65 (13 %), (1H, m, $C\mathbf{H}(CH_3)$), 2.83 (1H, dd, J_{vic} 5.5, J_{gem} 14.0, $C\mathbf{H}_2$), 2.98 (1H, dd, J_{vic} 3.5, J_{gem} 14.0, $C\mathbf{H}_2$), 4.04 (1H, bs, C**H**), 5.69 (88 %) (1H, d, J 10.5, C**H**), 5.84 (12 %) (1H, d, J 9.5, C**H**), 6.66 (16 %) (2H, d, J 8.5, **Ph**), 6.98 (2H, s, **Ph**), 7.06 (83 %) (1H, d, J 16.0, C**H**), 7.14 (17 %) (1H, d, J 15.5, C**H**), 7.48 (89 %) (1H, d, J 15.5, C**H**), 7.90 (11 %) (1H, d, J 15.5, C**H**); δ_{2H}(46.0 MHz; CH_3OH) 6.67 (Ph $C^2\mathbf{H}$); δ_C(125.7 MHz; CD_3OD) 12.0 & 12.4 ($CH_2\mathbf{C}H_3$), 12.5 ($CH(\mathbf{C}H_3)$), 20.4 ($C(\mathbf{C}H_3)$), 29.9 & 31.0 ($\mathbf{C}H_2CH_3$), 35.5 & 36.5 ($\mathbf{C}H(CH_3)$), 37.6 & 40.3 ($\mathbf{C}H_2$), 64.5 (**C**H), 115.8 (t, J 23.0, Ph $\mathbf{C}^2$H), 116.1 (Ph **C**H), 116.6 (**C**H), 127.7 (Ph **C**), 131.6 (Ph **C**H), 134.4 (**C**H), 151.1 (**C**H), 153.2 (**C**H), 157.2 (Ph **C**OH & NH**C**O), 175.9 (**C**O); υ_{max}(KBr)/cm^{-1} 3382 (br), 3072, 3022, 2961, 2925, 2871, 1684, 1653, 1609, 1561, 1429, 1251, 858; m/z (EI) 358 ($[M+H]^+$, $^2H_2$, 10.1 %), 357 ($[M]^+$, $^2H_2$, 35.4 %), 356 ($[M]^+$, $^2H_1$, 13.2 %), 355 ($[M]^+$, 2.6 %), 300 ($[M-CH(CH_3)CH_2CH_3]^+$, $^2H_2$, 71.3 %), 299 (($[M-CH(CH_3)CH_2CH_3]^+$, $^2H_1$, 26.8 %), 298 (($[M-CH(CH_3)CH_2CH_3]^+$, 4.7 %), 249 ($[M+H-Bn(OH)]^+$, 39.1 %), 151 ($[R]^+$, 12.7 %), 124 ($[R+H-CO]^+$, 53.2 %), 109 ([Bn(OH), $^2H_2$, 100.0 %), 108 ($[Bn(OH)]^+$, $^2H_1$, 52.1 %), 107 ($[Bn(OH)]^+$, 29.5 %), 95 ($[R+H-CH(CH_3)CH_2CH_3]^+$, 53.0 %), 79 ($[Ph]^+$, $^2H_2$, 33.7 %), 78 ($[Ph]^+$, $^2H_1$, 11.1 %), 77 ($[Ph]^+$, 38.2 %), where R = $COCH{=}CH(CH_3){=}CHCH(CH_3)CH_2CH_3$. Found 358.1991 $C_{21}H_{23}D_2NO_4$ [M^+] requires 358.1987.

5.9 Synthesis of [2-^{2}H]-tyrosine 111b

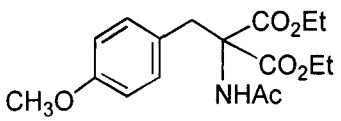
5.9.1 *p*-Methoxybenzyl bromide 251[166]



To a solution of *p*-methoxybenzyl alcohol **250** (710 mg, 5.1 mmol) and pyridine (0.4 ml, 5.1 mmol) in Et_2O (10 ml) at 0 °C was added a solution of phosphorus tribromide (PBr_3) (0.19 ml, 2.0 mmol) in Et_2O (10 ml). The mixture was stirred for 2 h at room temperature and then quenched with ice-water (10 ml). The organic layer was separated, washed with ice-cold 1M HCl (5 ml) and dried ($MgSO_4$). Evaporation of the solvent *in vacuo* yielded the crude bromide (902 mg, 87 %) as a pale yellow oil which was used directly in the next step without further purification.

δ_H(400 MHz; $CDCl_3$) 3.81 (3H, s, OC**H**$_3$), 4.51 (2H, s, C**H**$_2$), 6.87 (2H, d, J 8.4, **Ph**), 7.32 (2H, d, J 8.8, **Ph**); δ_C(100.6 MHz; $CDCl_3$) 34.0 (**C**H_2), 55.3 (O**C**H_3), 114.8 (Ph **C**H), 129.9 (Ph **C**), 130.4 (Ph **C**H), 159.6 (Ph **C**OCH_3); υ_{max}(neat)/cm^{-1} 3308, 3006, 2958, 1609, 1513, 831, 741, 721; m/z (EI) 202 ([M]$^+$, ^{81}Br, 2.3 %), 200 ([M]$^+$, ^{79}Br, 2.3 %), 121 ([M-Br]$^+$, 100 %), 106 ([M-Br-CH_3]$^+$, 3.6 %), 91 ([$C_6H_5CH_2$]$^+$, 6.0 %), 78 ([C_6H_6]$^+$, 27.8 %).

5.9.2 Diethyl 2-acetamido-2-*p*-methoxybenzylmalonate 252[153]

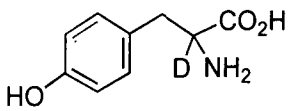


To a solution of sodium ethoxide in ethanol prepared from sodium (27 mg, 1.17 mmol) and ethanol (3.25 ml), was added diethyl acetamidomalonate (250 mg, 1.15 mmol). The solution was cooled to 0 °C and *p*-methoxybenzyl bromide **251** (230 mg, 1.15 mmol) slowly added. After stirring for 5 h, water (12 ml) was added to precipitate the product

and then the mixture stored in the refrigerator for 1 h before the product was filtered to give shiny, white crystals (350 g, 91.0 %), mp 96-96.5 °C (lit. 96-98 °C).[153]

δ_H(499.8 MHz; $CDCl_3$) 1.29 (6H, t, J 7.5, $(CH_2\mathbf{CH_3})_2$), 2.03 ($NCOC\mathbf{H_3}$), 3.58 (2H, s, $\mathbf{CH_2}$), 3.77 (3H, s, $OC\mathbf{H_3}$), 4.26 (4H, 2x q, J 7.0, $(\mathbf{CH_2}CH_3)_2$), 6.53 (1H, s, **NH**), 6.78 (2H, d, J 8.5, **Ph**), 6.92 (2H, d, J 9.0, **Ph**); δ_C(125.7 MHz; $CDCl_3$) 14.3 ($(CH_2\mathbf{CH_3})_2$), 23.3 ($CO\mathbf{CH_3}$), 37.2 ($\mathbf{CH_2}$), 55.4 ($O\mathbf{CH_3}$), 62.9 (**C**), 67.6 ($(\mathbf{CH_2}CH_3)_2$, 114.0 (Ph **C**H), 127.3 (Ph **C**), 131.1 (Ph **C**H), 159.0 (Ph $\mathbf{C}OCH_3$), 167.9 (**C**O), 169.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3423 (br, NH), 2949, 2858, 2809, 2759, 2544, 1617, 1457, 1351, 1081, 810; m/z (EI) 337 ($[M]^+$, 5.5 %), 292 ($[M\text{-}OC_2H_5]^+$, 2.5 %), 278 ($[M+H\text{-}OC_2H_5\text{-}CH_3]^+$, 73.8 %), 222 ($[M+H\text{-}(OC_2H_5)_2\text{-}CH_3]$, 9.9 %), 174 ($[NH_2C(CO_2C_2H_5)_2]$, 2.5 %), 121 ($[CH_3OBn]^+$, 100.0 %), 78 ($[C_6H_6]^+$, 4.9 %), 43 ($[COCH_3]$, 14.8 %).

5.9.3 DL-[2-$^2H_1$]-Tyrosine 111b[153]



To deuterium oxide (9.5 ml) at 0 C was added PBr_3 (2.9 ml) and the mixture allowed to warm to room temperature and stirring continued until the solution was homogenous. Diethyl 2-acetamido-2-*p*-methoxybenzylmalonate **252** (1.80 g, 5.34 mmol) was addede and the solution refluxed for 2 h. The pale yellow solution was concentrated *in vacuo* and then water (5 ml) added. This solution was decolourised with activated carbon and filtered through Celite. The pH was adjusted to 5 with concentrated ammonium hydroxide, the mixture cooled and the resulting precipitate filtered and dried to give a white powder (832 mg, 84.7 %), mp 220 °C (decomp.). The ^{1}H NMR spectrum showed the product to be deuterated at the 2-position and also at the positions *ortho* to the phenolic hydroxyl.

δ_H(200 MHz; D_2O) 2.95 (1H, d, J 14.6, $\mathbf{CH_2}$), 3.07 (1H, d, J 14.8, $\mathbf{CH_2}$), 6.98 (2H, s, Ph); δ_C(75.4 MHz; CD_3OD/TFA) 35.3 ($\mathbf{CH_2}$), 54.1 (t, J 22.7, **C**-^{2}H), 115.7 (Ph **C**H), 124.7 (Ph **C**), 130.3 (Ph **C**H), 157.0 (Ph **C**OH), 170.2 (**C**O).

To remove the aromatic deuterium atoms, the product (525 mg, 2.85 mmol) was refluxed in 2N HCl (12 ml) for 17 h. The solution was then concentrated and the pH adjusted to 6 with ammonium hydroxide. The precipitate formed was filtered and dried to give a white powder (164 mg, 31.6 %).

δ_H(200 MHz; CD_3OD/TFA) 3.07 (1H, d, J 14.7, C**H**$_2$), 3.24 (1H, d, J 14.7, C**H**$_2$), 6.81 (2H, d, J 8.4, **Ph**), 7.13 (2H, d, J 8.1, **Ph**); δ_C(75.4 MHz; CD_3OD/TFA) 37.3 (**C**H_2), 52.3 (t, J 22.7, **C**^{2}H), 117.8 (Ph **C**H), 126.8 (Ph **C**), 132 (Ph **C**H), 159.1 (Ph **C**OH), 172.3 (**C**O); υ_{max}(KBr)/cm^{-1} 3404 (br, NH), 3205, 3048, 2958, 1586, 1512, 1401 1245, 1109, 838.

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Conferences and meetings attended

RSC Stereochemistry, Sheffield, December 1997.

RSC National Congress and Young Researchers' Meeting, Durham, April 1998

RSC Heterocycle Symposium, Sunderland, May 1998

RSC Polyketides, Cambridge, December 1998

RSC Perkin Division - Bioorganic Group, Leicester, January 1999

RSC Annual Conference, Edinburgh, September 1999 (Poster presentation)

RSC Stereochemistry, Sheffield, December 1999

RSC Perkin Division-Bioorganic Group, Oxford, December 1999 (Poster presentation)

RSC International Fluorine Meeting, Durham, July 2000

Postgraduate Symposium, Durham, June 2000 (Oral presentation)